Br. J. Nutr. (1969), **23**, 343

Dietary depletion of vitamin B_{12} and the excretion of methylmalonic acid in the rat

By D. L. WILLIAMS and G. H. SPRAY

Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford

and G. E. NEWMAN and J. R. P. O'BRIEN

Nuffield Department of Clinical Biochemistry, Oxford

(*Received* 4 *October* 1968—*Accepted* 6 *December* 1968)

1. A semi-synthetic vitamin B_{12}-deficient diet, based on soya flour, is described. When supplemented with cyanocobalamin the diet appeared to be adequate for growth and reproduction in the rat.

2. Compared with their litter-mates on the supplemented diet, rats fed on the deficient diet from weaning showed reduced levels of vitamin B_{12} activity in the plasma and tissues, but their growth rates were unaffected unless they were bred from mothers that had been given the deficient diet since mating.

3. When they were reared on the deficient diet, rats bred from mothers on the deficient diet since mating excreted much more methylmalonic acid in the urine than their litter-mates on the supplemented diet. There was wide variation in the level of excretion, both between different animals and from day to day in the same animal.

4. Starvation for more than 16 h caused a marked depression in the amount of methylmalonic acid excreted by rats on the deficient diet.

5. Intraperitoneal injection of sodium propionate into deficient animals after starvation for 24 h caused increased excretion of methylmalonic acid during the following 16 h of continued starvation. Isoleucine had a similar but smaller effect.

6. Tested in the starved animal, sodium propionate and valine given either by intraperitoneal injection or by mouth, and isoleucine given intraperitoneally, caused increases in the excretion of methylmalonic acid. In contrast, methionine had no effect and threonine only a slight effect.

The excretion of excessive amounts of methylmalonic acid in the urine in vitamin B_{12} deficiency has been reported in human patients (Cox & White, 1962; Brozović, Hoffbrand, Dimitriadou & Mollin, 1967) and rats (Barness, Young & Nocho, 1963; Armstrong, 1967). Administration of propionate, valine, isoleucine or thymine has been found to enhance the excretion in vitamin B_{12}-deficient patients or rats, or both, but the reports have sometimes been conflicting. Kahn, Williams, Barness, Young, Shafer, Vivacqua & Beaupre (1965) were unable to show increased excretion after giving propionate to two vitamin B_{12}-deficient patients, whereas Gompertz, Hywel Jones & Knowles (1967) found increased excretion in two out of three such patients who received propionate. Barnabei, Valyasevi, Barness & György (1957) showed that valine added to a necrosis-producing diet increased the excretion of methylmalonic acid by rats, but Barness, Flaks, Young, Tedesco & Nocho (1963) reported that valine was not converted into methylmalonic acid in vitamin B_{12}-deficient rats.

In this paper we present observations on the effects of dietary deprivation of vitamin B_{12} in rats, mainly with reference to the excretion of methylmalonic acid and to some factors which influence it, including the administration of possible metabolic precursors.

EXPERIMENTAL

Animals and their management. Weanling albino rats of the Wistar strain were obtained from Allington Farm, Porton Down, Salisbury, Wilts., or were bred in the Department from adults from the same source. They were kept in Perspex cages with aluminium mesh floors and tops and were managed in a similar way to the iron-deficient rats studied by McCall, Newman, O'Brien, Valberg & Witts (1962). For breeding, pregnant does were kept in a darkened room in individual plastic breeding boxes containing sawdust and cotton wool. For the collection of urine, individual animals were kept in glass metabolic cages with wide mesh aluminium floors and devices for separating urine and faeces; they were given water *ad lib.* but were fed outside the cages for two periods of 1 h each day. Little urine was lost during the feeding periods. All other animals received food and water *ad lib.*

Diet. The diet was prepared by mixing 600 g soya flour (Soyolk; Soya Foods Ltd, London EC3), 5 g choline dihydrogen citrate, 205 g sucrose, 179 g sugar–salt mixture, 1 ml of a mixture of fat-soluble vitamins and 10 g of a mixture of water-soluble vitamins. The vitamin mixtures were of the same composition as those used by McCall *et al.* (1962) except for the omission of cyanocobalamin from the deficient diet. The sugar–salt mixture was prepared by mixing 636·4 g lactose, 11·2 g NaCl, 61·5 g $CaHPO_4$, 122·9 g calcium lactate, 167·6 g salt mixture C (McCall *et al.* 1962, diet 2) and 0·04 g riboflavine.

Determination of vitamin B_{12}. Rats were anaesthetized with ether and killed by bleeding from the aorta; the blood was heparinized and centrifuged, and the plasma was removed. Organs were homogenized in water. Extracts were prepared from plasma in the same way as from rat serum and from organs in the same way as for liver (Booth & Spray, 1960). Whole 3-day-old rats which had been killed with ether, or samples of diet, were extracted in the same way as tissues. Vitamin B_{12} activity in the extracts was determined by microbiological assay with *Lactobacillus leichmannii* (Spray, 1955), both the total and the alkali-stable activity being measured in plasma. The alkali-labile activity is taken to represent the true vitamin B_{12} level; none of the other materials studied contained appreciable amounts of alkali-stable activity.

Determination of methylmalonic acid. Methylmalonic acid was determined in urine by a modification of the colorimetric method of Giorgio & Plaut (1965). Urine (2 ml from rats receiving the deficient diet, or 5 ml from those on the supplemented diet) was adjusted to pH 6·5 (glass electrode) and was run through a column (1 cm diameter ×3 cm long) of Dowex analytical grade anion exchange resin, AG 3– ×4, 200–400 mesh chloride form (Bio-Rad Laboratories, USA). The columns were washed with 100 ml water and the methylmalonic acid was eluted with two 20 ml portions of 0·1 N-HCl. Portions (0·5 or 2 ml) of the eluates were mixed with 3 ml M-acetate buffer, pH 4·3, and cooled in ice. Cold diazotized *p*-nitro-aniline solution (3 ml, prepared by adding four volumes of cold 0·5% (w/v) $NaNO_2$ solution slowly to 15 volumes cold 0·075% (w/v) *p*-nitro-aniline solution in 0·2 N-HCl, followed by four volumes of cold 0·2 M-sodium acetate solution) was added, the tubes were heated at 100° for 3 min, 2 ml cold 4 N-NaOH solution was added and oxygen-free nitrogen

was passed through the solutions for 10 sec. The tubes were stoppered, cooled in ice, and after 15 min the optical density of the green colour was read at 620 nm. The amount of methylmalonic acid was determined by comparison with the densities produced by known amounts (0–100 μg) of the pure substance in solution in 0·1 N-HCl, treated in a similar way.

RESULTS

Vitamin B_{12} content of diets. No vitamin B_{12} activity was detected in six separate tests on the deficient diet. Results from the supplemented diet were between 14 and 18 μg/kg, in good agreement with the 15 μg cyanocobalamin/kg added.

Adequacy of the vitamin B_{12}-supplemented diet for growth and reproduction. In the first experiment eleven female weanling rats from Allington Farm were fed on the deficient diet, eleven on the supplemented diet, seven on the iron-supplemented diet of McCall *et al.* (1962) and seven on standard rat cake (modified diet 41 B; Herbert C. Styles (Bewdley) Ltd). The mean weight of each group increased from 60 to 160 g in 4–5 weeks.

Table 1. *Vitamin B_{12} activity in the plasma and tissues of rats fed on the vitamin B_{12}-deficient (−) or supplemented (+) diet*

Rat no. and diet	Time on diet (weeks)	Vitamin B_{12} activity (ng/ml or ng/g) in					
		Plasma	Liver	Kidneys	Brain	Muscle	Heart
Weanlings bought commercially, first experiment							
85−	8	0·02	12	86	9	—	—
87+		0·50	77	670	37	—	—
6−	11	0·00	17	130	10	—	—
7+		0·60	95	1100	52	—	—
23−	19	0·08	24	89	9	—	—
24+		0·40	64	1200	33	—	—
30−	33	0·10	14	95	7	3	24
33+		0·44	64	780	28	9	94
26−	64	0·09	10	75	7	2	18
27+		0·28	73	2200	56	13	200
Weanlings from vitamin B_{12}-depleted mothers, first experiment							
121−	5	—	23	32	11	8	—
122+		1·3	67	300	25	8	—
105−	8	0·02	21	120	15	4	10
108+		0·66	76	1200	63	13	200
91−	17	0·02	8	65	6	1	16
92+		0·35	58	890	23	4	130
93−	28	0·10	28	210	8	5	27
94+		—	100	1900	71	15	200

Three weanling rats of each sex were reared on the supplemented diet and were mated; the females produced ten, ten and seven young respectively, 90% surviving to weaning. The young were reared on the same diet and a pair from each litter were mated, producing nine, seven and five young with a mean survival of 90%. The programme was repeated for another generation, yielding four, seven and eight young

with a mean survival of 87%. The mean weights of the young at weaning were: 1st generation 38, 35 and 35 g; 2nd generation 35, 38 and 32 g; 3rd generation 45, 36 and 40 g.

Over 85% of female rats produced litters when fed either on the supplemented diet, on rat cake or on rat cake until mating and then on the deficient diet ('vitamin B_{12}-depleted' mothers). The mean numbers of young surviving to weaning in these three groups were: mothers fed on the supplemented diet, six (twenty-seven litters); mothers fed on rat cake, eight (ninety-eight litters); vitamin B_{12}-depleted mothers, seven (forty-eight litters). In contrast, only about 50% of does fed on the deficient diet from weaning produced litters and on average only four young per litter from twenty-four litters survived to weaning.

Effects of the deficient diet on growth rates and vitamin B_{12} levels

Preliminary observations. In the first experiment marked reductions were found in the levels of vitamin B_{12} activity in the plasma and tissues of the animals receiving the deficient diet as compared with those on the supplemented diet (Table 1), but, as already mentioned, there were no differences between their growth rates. A second experiment with similar animals confirmed these findings.

Table 2. *Total vitamin B_{12} content of 3-day-old rats from mothers on different diets*

Diet of mothers	No. of observations	Total vitamin B_{12} content (ng) of young rats	
		Mean	Range
Vitamin B_{12}-deficient	9	39	20–55
Vitamin B_{12}-supplemented	7	134	72–190
Modified diet 41 B	5	170	150–190
Modified diet 41 B until mating, then vitamin B_{12}-deficient ('vitamin B_{12}-depleted')	9	69	58–85

Effect of mothers' diet on the vitamin B_{12} content of young rats. Since the early experiments failed to reveal differences in growth rates due to the deficient diet, offspring of mothers fed on the deficient diet before parturition were studied. On average, 3-day-old rats from mothers that had received the deficient diet since weaning contained over three times less vitamin B_{12} than those from mothers on the supplemented diet (Table 2); the offspring of vitamin B_{12}-depleted mothers also contained considerably less vitamin B_{12} than those of supplemented mothers. Weanlings from vitamin B_{12}-depleted mothers were therefore used in subsequent studies, in order to reduce the level of vitamin B_{12} in the young without reducing the numbers of offspring produced.

Studies on weanling rats from vitamin B_{12}-depleted mothers. Twelve weanlings were fed on the deficient diet and their litter-mates received the supplemented diet. The mean weight of the supplemented group increased from 60 to 160 g in 4–5 weeks,

but in the deficient group this increase took over 13 weeks. The contents of vitamin B_{12} in the plasma and tissues were similar to those found previously (Table 1). A second experiment with six pairs of rats also showed a difference in growth rates, though this was smaller than in the first experiment.

Urinary excretion of methylmalonic acid

Method of estimation. Our modifications to the method of Giorgio & Plaut (1965), though minor, were found necessary in order to ensure valid results. Tests with a fraction collector showed that 40 ml 0·1 N-HCl were required for maximum elution of methylmalonic acid from the columns. To obtain maximum colour and a linear relationship between the amount of methylmalonic acid and optical density, it was necessary to carry out the diazotization below 4° and to cool the tubes rapidly after completing the reaction. The green colour faded unless nitrogen was passed through the solutions.

Under the conditions described, 82–92% (mean 86%) of methylmalonic acid (250–1000 μg) added in aqueous solution to the columns was recovered. When 100–600 μg methylmalonic acid were added to pooled samples (5 ml) of rat urine the recovery in five separate tests was 85–95% (mean 89%). No reduction was found in the amount of methylmalonic acid in fourteen different urine samples after storage at −20° for 2 weeks. On paper chromatography, ether extracts of those urines that gave the green colour with diazotised *p*-nitro-aniline produced a spot corresponding in R_F to methylmalonic acid; this spot was much smaller or absent from samples that did not produce the green colour.

Excretion of methylmalonic acid by rats receiving the vitamin B_{12}-deficient and vitamin B_{12}-supplemented diets. Female rats bred from vitamin B_{12}-depleted mothers were studied. Animals on the deficient diet were 'screened' by measuring their excretion for 1 or 2 days, 6–10 weeks after weaning. The mean value of fifty-six such observations, comprising single estimates on twenty-two animals and two measurements on each of seventeen others, was 16·3 mg/day (range 1·4–70, standard deviation 12·6). The litter-mates, receiving the supplemented diet, of twenty-seven of the deficient animals were studied. The mean of forty-five results, including single estimates on nine rats and two observations on each of eighteen others, was 1·3 mg/day (range 0·2–7·2, standard deviation 1·1). Except for the figure of 7·2 mg/day in one animal that, on re-examination, excreted only 2·8 mg/day, the highest value was 3·0 mg/day.

After 'screening', rats which excreted relatively large amounts of methylmalonic acid were selected for further study. Except for the preliminary experiments on the effect of giving different substances and the second experiment with methionine, all subsequent measurements were made on some or all of the same group of fourteen selected rats and their litter-mate controls on the supplemented diet, where indicated. Sequential observations on some of these animals showed that there was wide day-to-day variation in the results for each animal (Table 3).

Effect of starvation. It has been suggested that one source of the methylmalonic acid found in the urine in vitamin B_{12} deficiency in omnivorous species may be propionate formed in the caecum (Marston, Allen & Smith, 1961). The effect of starvation for

48 h on the excretion of methylmalonic acid was therefore studied. In a preliminary experiment, nine rats on the deficient diet excreted a mean of 12·5 mg (range 4·5–33) methylmalonic acid during the 1st day's starvation, compared with a mean of 1·1 mg (range 0·6–1·6) for their litter-mates receiving the supplemented diet. During the 2nd day the corresponding values were 5·5 mg (range 0·4–22) and 0·7 mg (range 0·2–1·4); the results for all but two of the deficient animals during the 2nd day were similar to those for their supplemented litter-mates. To explore this further, urine was collected from twelve rats on the deficient diet for the six periods of 8 h during 2 days' starvation. Most of the methylmalonic acid was excreted during the first 16 h (Table 4). The raised excretion during the 2nd day, found in two rats in the preliminary experiment, was not noted here; during the 2nd day the values were reduced almost to those found in unstarved supplemented animals.

Table 3. *Day-to-day variation in the urinary excretion of methylmalonic acid by litter pairs of rats receiving the vitamin B_{12}-deficient (−) or supplemented (+) diet*

Rat no. and diet	No. of observations	Methylmalonic acid in urine (mg/day) Mean	Range
45−	6	43·2	26–62
46+	7	1·2	0·9–1·6
47−	7	19·3	12–31
48+	7	0·9	0·5–1·1
49−	7	12·2	2·7–19
50+	7	0·9	0·4–1·5
51−	7	23·4	12–37
52+	7	0·9	0·4–1·3
59−	6	22·0	15–32
60+	6	0·7	0·2–1·5
61−	6	21·8	9·2–33
62+	7	0·8	0·2–1·1

Table 4. *Effect of starvation on the excretion of methylmalonic acid by twelve selected rats receiving the vitamin B_{12}-deficient diet*

Period of starvation (h)	Methylmalonic acid in urine (mg) Mean	Range
0–8	31·4	12–49
8–16	13·3	2·5–45
16–24	2·7	0·5–12
24–32	2·3	0·9–4·5
32–40	0·9	0·3–1·7
40–48	0·7	0·1–1·2

Effect of the administration of different substances. Initially, sodium propionate (0·5 or 1 m-mole), valine (1 m-mole), or thymine (0·25 m-mole) was injected intraperitoneally into deficient rats, which were fed twice daily. The results indicated that injection of 1 m-mole of propionate was followed by a marked increase in the excretion of methylmalonic acid, while the valine had less effect and the thymine little or none (Williams, 1967). Owing to the day-to-day variation in basal excretions (Table 3),

however, it was impossible to assess the increased excretion quantitatively. None of the substances had any apparent effect on the levels of excretion in the rats' litter-mates receiving the supplemented diet.

It seemed possible that more useful information might be obtained by giving the substances after the animals had been starved for 24 h and measuring the excretion during a 2nd day's starvation, when the basal excretion should be greatly reduced (Table 4). Two experiments were carried out to test this possibility. First, urine was collected for a day from six rats on the deficient diet and from their litter-mates on the supplemented diet while they were being fed twice daily. They were then given 1 m-mole sodium propionate intraperitoneally and the collections were continued for a second day. Later, the rats were starved for 48 h and 1 m-mole sodium propionate was given after the first 24 h. Propionate provoked increased excretion in the deficient animals even when they were starved, but had little or no effect in the supplemented ones (Table 5).

Table 5. *Excretion of methylmalonic acid by rats on the vitamin B_{12}-deficient and supplemented diets, before and after intraperitoneal injections of 1 m-mole sodium propionate during feeding or starvation (six rats in each group)*

	Methylmalonic acid in urine (mg/day)							
	During feeding				During starvation			
	Basal		After propionate		Basal		After propionate	
Diet	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Deficient	19·4	9·2–38	69·9	46–105	21·9	11–42	48·9	26–92
Supplemented	0·9	0·7–1·2	2·5	1·9–3·2	1·0	0·5–1·8	1·8	1·4–2·2

Table 6. *Excretion of methylmalonic acid by twelve vitamin B_{12}-deficient rats starved for 24 h and then given sodium propionate (1 m-mole) or isoleucine (1 m-mole) by intraperitoneal injection*

Period of starvation (h)	Methylmalonic acid in urine (mg)			
	Mean	Range	Mean	Range
0–8	22·3	5·6–43	18·6	4·4–36
8–16	4·5	0·2–14	1·8	0·5–4·2
16–24	1·3	0·1–3·0	0·8	0·3–1·6
	Sodium propionate given		Isoleucine given	
24–32	33·8	14–68	12·0	1·0–28
32–40	7·3	0·5–35	2·6	1·0–7·1
40–48	1·1	0·2–2·2	0·6	0·02–1·3

Secondly, propionate and isoleucine were given intraperitoneally to twelve rats, including the six used in the first experiment, after starvation for 24 h. Urine was collected for periods of 8 h. The results confirmed the reduction in excretion after starvation for 16 h and most of the methylmalonic acid excreted in response to the injections appeared during the first 16 h after administration (Table 6). Isoleucine caused a small increase in excretion.

These results suggested that the starved animal was suitable for testing the effects of different substances on the excretion of methylmalonic acid. Therefore, the sub-

stances listed in Table 7 were given either by intraperitoneal injection or by mouth in the order shown. Table 7 summarizes the results obtained in the ten rats surviving from the original twelve until the end of the experiment; the results for these ten animals after the first dose of propionate and after isoleucine are among those shown in Table 6. Two further doses of propionate were given intraperitoneally at the end of the experiment to see whether the response was maintained, but most of the rats excreted less methylmalonic acid after these doses than they did at first. Similarly, the amounts excreted during the initial periods of starvation fell as the experiment progressed. Therefore the results do not provide a quantitative comparison of the responses to the different substances.

Table 7. *Effect of various substances on the urinary excretion of methylmalonic acid in ten rats on the vitamin B_{12}-deficient diet*

(Urine was collected during the 1st day of starvation, then 1 m-mole of each substance was given and urine was collected during a 2nd day's starvation. The rats received food and water *ad lib.* for at least 5 days between each period of starvation)

Substance and route of administration	Methylmalonic acid in urine (mg)					
	0–24 h of starvation			24–48 h of starvation		
	Mean	SD	Range	Mean	SD	Range
Sodium propionate, intraperitoneal	30·7	17·0	6·7–60	45·8	23·6	21–104
Isoleucine, intraperitoneal	24·2	9·8	8·2–38	16·8	9·6	5·3–36
Valine, intraperitoneal	13·9*	7·9	5·9–30	10·7*	9·7	1·2–28
Sodium propionate, by mouth	13·2*	8·2	3·5–26	10·2†	5·6	2·3–19
Valine, by mouth	12·2	10·3	5·4–40	9·2	6·6	1·6–20
L-methionine, intraperitoneal	10·8	6·1	3·5–20	0·5	0·6	0·1–2·1
L-threonine, intraperitoneal	15·5	13·2	3·1–48	9·6	14·9	1·6–52
Sodium propionate, intraperitoneal	18·7	18·0	1·1–50	22·4	10·4	4·5–38
Sodium propionate, intraperitoneal	16·9	11·4	3·5–40	23·8	9·8	6·8–38

* Results for nine animals. † Results for eight animals.

Nevertheless, it is clear that methionine had no effect and that threonine had very little effect except in one animal. Propionate provoked higher levels of excretion when given intraperitoneally than when given by mouth; with valine the results were comparable after oral and intraperitoneal dosing. Isoleucine given intraperitoneally appeared to provoke a greater response than valine, but, since it was the second in the series, after the sodium propionate which caused the high levels of excretion, the apparent difference may be fortuitous. To confirm the lack of effect with methionine, the tests were repeated with five other deficient rats. Their mean excretion during the 1st day's starvation was 31·5 mg (range 6·3–61); during the 2nd day, after receiving 1 m-mole methionine by intraperitoneal injection, it was 4·4 mg (range 1·6–14).

DISCUSSION

Numerous diets have been described for inducing vitamin B_{12} deficiency in the rat (e.g. Cuthbertson & Thornton, 1952; Ericson, Harper, Williams & Elvehjem, 1956; Wagle, Mehta & Johnson, 1958; Fatterpaker, Lavate, Mulgaonkar, Noronha, Rege, Tipnis & Sreenivasan, 1959; Chang, Hsu, Davis & Chow, 1961; Dryden & Hartman, 1966). When the present work was begun in 1961 it proved difficult to decide which recipe to choose. The known nutritional requirements of the rat were therefore reviewed and the diet was designed in an attempt to provide all essential nutrients except vitamin B_{12}. The diet presumably satisfied this criterion because, when supplemented with cyanocobalamin, it appeared to support growth and reproduction in the rat as effectively as a standard commercial diet. In contrast, the breeding performance of female rats fed on the diet without cyanocobalamin was poor. The levels of vitamin B_{12} in the plasma and tissues of weanling rats fed on the deficient diet for a few weeks were greatly reduced, but the growth rates were unaffected unless the animals were partially depleted of vitamin B_{12} before weaning, by breeding from does given the deficient diet from the time of mating. Earlier workers often used second-generation rats for studies of vitamin B_{12} deficiency (Cuthbertson & Thornton, 1952; Chang *et al.* 1961; Dryden & Hartman, 1966). However, this initial depletion did not alter the levels of vitamin B_{12} found in the plasma and tissues later; nor did the levels decrease in proportion to the time the animals were kept on the diets.

These observations suggest either that the rat can retain small amounts of vitamin B_{12} tenaciously, or that the animals were able to obtain some of the vitamin, perhaps by coprophagy, by ingestion of faecal material during preening, or by absorbing a proportion of the vitamin B_{12} synthesized by intestinal organisms. Haematological studies failed to reveal any effect due to depletion of vitamin B_{12}. Thus, under our conditions, dietary depletion of vitamin B_{12} produced only minimal effects in the rat. Nevertheless, the depletion was sufficient for the urinary excretion of methylmalonic acid to be raised. This defect has been described in vitamin B_{12}-deficient human patients (e.g. Cox & White, 1962; Brozović *et al.* 1967) and rats (e.g. Armstrong, 1967). The rapid reduction in excretion during starvation supports the suggestion that in vitamin B_{12} deficiency in omnivorous species methylmalonic acid arises from the abnormal metabolism of some substance, possibly propionate (Marston *et al.* 1961), produced in the gastro-intestinal tract.

Of the various substances that have been reported to increase the excretion of methylmalonic acid when given to vitamin B_{12}-deficient patients or rats, sodium propionate given by intraperitoneal injection was the most effective under our conditions, with valine and isoleucine having less effect and methionine and threonine practically none. When given orally to human subjects, valine produced a greater response than isoleucine and sodium propionate had less effect than either amino acid (Gompertz *et al.* 1967); in our rats the responses to propionate and valine given by mouth were similar. The poor responses to methionine and threonine are difficult to understand in view of published information about their metabolic breakdown. It is perhaps conceivable that, since one of the pathways for the biosynthesis of methionine

is dependent on vitamin B_{12} (Woods, Foster & Guest, 1965), rats on the vitamin B_{12}-deficient diet might become deficient in methionine and might therefore utilize exogenous methionine anabolically. On the other hand, data supplied by the manufacturers on the amino acid composition of the soya flour suggest that the diet contained adequate amounts of methionine.

The present results do not show whether the methylmalonic acid excreted after the administration of various substances arises directly from their catabolism or whether it is the result of some indirect effect. In some preliminary experiments using [^{14}C]-sodium propionate (not described here), it was suggested that the urinary methylmalonic acid contained a large proportion of the administered isotope, so that it was presumably the result of the metabolism of the propionate. Further results using labelled precursors will be reported later.

The work on methylmalonic acid was completed during the tenure of a grant to G. H. S. from the Medical Research Council, whose support is gratefully acknowledged.

REFERENCES

Armstrong, B. K. (1967). *Br. J. Nutr.* **21**, 309.
Barnabei, O., Valyasevi, A., Barness, L. A. & György, P. (1957). *Archs Biochem. Biophys.* **69**, 259.
Barness, L., Flaks, J., Young, D., Tedesco, T. & Nocho, R. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22**, 259.
Barness, L. A., Young, D. G. & Nocho, R. (1963). *Science, N.Y.* **140**, 76.
Booth, M. A. & Spray, G. H. (1960). *Br. J. Haemat.* **6**, 288.
Brozović, M., Hoffbrand, A. V., Dimitriadou, A. & Mollin, D. L. (1967). *Br. J. Haemat.* **13**, 1021.
Chang, C., Hsu, J. M., Davis, R. L. & Chow, B. F. (1961). *Archs Biochem. Biophys.* **93**, 251.
Cox, E. V. & White, A. M. (1962). *Lancet* ii, 853.
Cuthbertson, W. F. J. & Thornton, D. M. (1952). *Br. J. Nutr.* **6**, 170.
Dryden, L. P. & Hartman, A. M. (1966). *J. Nutr.* **90**, 377.
Ericson, L.-E., Harper, A. E., Williams, J. N. Jr & Elvehjem, C. A. (1956). *J. biol. Chem.* **219**, 59.
Fatterpaker, P., Lavate, W. V., Mulgaonkar, A. G., Noronha, J. M., Rege, D. V., Tipnis, H. P. & Sreenivasan. A. (1959). *Br. J. Nutr.* **13**, 439.
Giorgio, A. J. & Plaut, G. W. E. (1965). *J. Lab. clin. Med.* **66**, 667.
Gompertz, D., Hywel Jones, J. & Knowles, J. P. (1967). *Clinica chim. Acta* **18**, 197.
Kahn, S. B., Williams, W. J., Barness, L. A., Young, D., Shafer, B., Vivacqua, R. J. & Beaupre, E. M. (1965). *J. Lab. clin. Med.* **66**, 75.
McCall, M. G., Newman, G. E., O'Brien, J. R. P., Valberg, L. S. & Witts, L. J. (1962). *Br. J. Nutr.* **16**, 297.
Marston, H. R., Allen, S. H. & Smith, R. M. (1961). *Nature, Lond.* **190**, 1085.
Spray, G. H. (1955). *Clin. Sci.* **14**, 661.
Wagle, S. R., Mehta, R. & Johnson, B. C. (1958). *J. biol. Chem.* **230**, 137.
Williams, D. L. (1967). The distribution of vitamin B_{12} in animals and the effects of dietary deficiency. D.Phil. Thesis, University of Oxford.
Woods, D. D., Foster, M. A. & Guest, J. R. (1965). In *Transmethylation and Methionine Biosynthesis*, p. 138. [S. K. Shapiro and F. Schlenk, editors.] Chicago & London: The University of Chicago Press.

Printed in Great Britain