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The effect of antibiotics on rats receiving a vitamin B_{12}-deficient diet

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1. Rats fed on a vitamin B_{12}-deficient or -supplemented diet were given either neomycin or a mixture of streptomycin and erythromycin by mouth for between 7 and 15 d. The urinary excretion of methylmalonic acid and the levels of vitamin B_{12} in plasma and tissues and of acetic and propionic acids in caecal contents were measured.

2. Both treatments caused prompt reduction of methylmalonate excretion in the deficient rats. This was apparently due to depression of the production of some precursor of methylmalonic acid, probably propionate, rather than to an immediate effect on vitamin B_{12} nutrition.

3. After withdrawal of the antibiotics, neomycin-treated vitamin B_{12}-deficient rats appeared to become partly repleted in vitamin B_{12}, but the change in the vitamin B_{12} status of those which had received streptomycin and erythromycin was much smaller.

In rats fed on a vitamin B_{12}-deficient diet the urinary excretion of methylmalonic acid was reduced after starvation for more than 16 h (Williams, Spray, Newman & O'Brien, 1969). This observation supports the suggestion that in omnivorous species urinary methylmalonate arises from the abnormal metabolism of some substance, possibly propionate, formed in the alimentary tract (Marston, Allen & Smith, 1961). Armstrong & Curnow (1967) showed that rats fed on a vitamin B_{12}-deficient diet excreted more methylmalonate than similar animals receiving a mixture of streptomycin and erythromycin in the drinking water. The difference was attributed to an effect of the antibiotics on vitamin B_{12} nutrition, but the levels of vitamin B_{12} in the rats' livers were similar. An alternative explanation might be that the antibiotics depressed the production of precursors of methylmalonate. To explore this problem further, we have studied the effects of neomycin and of a mixture of streptomycin and erythromycin on methylmalonate excretion in rats on vitamin B_{12}-deficient and vitamin B_{12}-supplemented diets.

EXPERIMENTAL

Material and methods

Animals and their management. Rats fed on a vitamin B_{12}-deficient diet and littermate controls of the same sex on the same diet supplemented with cyanocobalamin were bred, reared and managed as described by Williams *et al.* (1969). Animals on the deficient diet were selected for further study on the basis of their urinary excretion of methylmalonic acid, as described previously. During the urine collections, the rats in Expt 1 were fed outside the metabolism cages for two periods of 1 h each day and in the other experiments they were starved.

Determination of fatty acids in caecal contents. The contents of each caecum were homogenized and 0·5 g was treated with 0·1 ml 20% (w/v) trichloroacetic acid and 0·4 ml water. The solids were removed by centrifugation and suitable amounts (0·25–

1 μl) of the supernatant solutions were applied to a column with a liquid phase of FFAP (Free Fatty Acid Phase, a reaction product between Carbowax 20 M and 2-nitroterephthalic acid; Baker, 1966). The column was operated at 140° in a Perkin-Elmer model F 11 gas chromatograph with a flame ionization detector, using oxygen-free nitrogen as the carrier gas. The heights of the peaks for acetic and propionic acids were measured and compared with those produced by suitable standard solutions containing mixtures of acetic and propionic acids in 2% aqueous trichloroacetic acid.

Other methods. Methylmalonic acid was determined in urine, and vitamin B_{12} in plasma and tissues, by the methods used previously (Williams *et al.* 1969). Absorption of cyanocobalamin was assessed by giving each rat 0·5 μCi [^{58}Co]cyanocobalamin (Radiochemical Centre, Amersham; specific activity 40 μCi/μg) in aqueous solution by stomach tube. The fraction of the dose absorbed was determined by counting the radioactivity in each animal soon after dosing and 7 d later, in a plastic phosphor well counter (Warner & Oliver, 1962).

Experimental plan

Expt 1. Six deficient female rats were studied. After two urine collections, sufficient neomycin sulphate to give 0·5% (w/w) neomycin was added to the diet for 15 d. Sodium propionate (1 m-mole) was given to each rat by intraperitoneal injection 1 d before the neomycin was withdrawn. Three further collections were made during the following 16 d, after which three rats were killed and vitamin B_{12} was determined in their blood plasma, liver, kidneys and brain.

Expt 2. Five deficient male rats and their litter-mate controls were studied. After two urine collections, each rat was given a single dose of 10 mg streptomycin (as the sulphate) and 2 mg erythromycin (as the glucoheptonate) in aqueous solution by stomach tube. They then received a solution containing sufficient streptomycin sulphate and erythromycin glucoheptonate to give 0·15% (w/v) streptomycin and 0·0025% (w/v) erythromycin (Armstrong & Curnow, 1967) instead of drinking water, for 8 d. Propionate was given on the 13th d and further urine collections were made on the 16th, 22nd and 36th d.

Expt 3. Twenty litter pairs of male rats on the deficient or supplemented diets, from the same batch of litters, were assigned randomly to two equal groups. Urine was collected at the times shown in Table 2. All the rats received 1 m-mole sodium propionate by intraperitoneal injection on the 7th, 21st and 39th d and those that were given streptomycin and erythromycin received a further dose of propionate on the 57th d. One group of deficient rats and their controls were offered a solution of neomycin sulphate containing 0·5% (w/v) neomycin in place of drinking water from the 13th to the 21st d. During the same period the other groups received streptomycin and erythromycin in the same doses as in Expt 2. Blood for the determination of vitamin B_{12} in plasma was obtained from the tail veins at the times shown in Table 3. Shortly before the end of the experiments the absorption of cyanocobalamin was measured in the deficient groups. The rats were then killed, blood and livers were removed for the measurement of vitamin B_{12} and the caecal contents of the deficient rats were retained for the determination of fatty acids.

Expt 4. To obtain information on hepatic vitamin B_{12} and caecal fatty acid levels immediately after treatment with antibiotics, fifteen male rats of approximately the same age that were receiving the deficient diet were assigned randomly to three groups of five. Five control rats on the supplemented diet, from the same batch of litters, were also studied. Urine was collected on alternate days from all the animals. One deficient group was given neomycin and another was given streptomycin and erythromycin, in the same dosages as in the earlier experiments, from the 5th to the 11th d. One deficient group and the supplemented group did not receive antibiotics. All the animals were killed on the 11th d and the livers and caecal contents were removed.

RESULTS

Recovery of added methylmalonic acid from solutions of the antibiotics. To see whether the antibiotics would affect the determination of methylmalonic acid, this acid (2 mg) was added to 1 ml and 10 ml portions of the antibiotic solutions that were given to the rats in place of drinking water. The volumes were made up to 15 ml with water and the solutions were applied to columns of the Dowex resin, which were eluted in the usual way. Aqueous solutions containing methylmalonic acid (2 mg) in 15 ml were treated similarly. Compared with those from the aqueous solutions, the recoveries of methylmalonate from the antibiotic solutions were between 107 and 108%.

Accuracy of the fatty acid determinations. Since no attempt was made to concentrate the volatile fatty acids from the caecal contents, it was necessary to run the gas chromatograph at almost maximum sensitivity, and the accuracy of the measurements under these conditions was assessed in various ways. The relationships between peak heights and acid concentration for the standard solutions containing acetic and propionic acids were linear. Ten separate portions (1 μl) of a solution containing acetic acid (29·4 mM), propionic acid (7·6 mM) and n-butyric acid (9·5 mM) were estimated in sequence. The mean peak heights were: for acetic acid $13{\cdot}5 \pm 0{\cdot}18$ (standard error of mean) cm; for propionic acid $7{\cdot}1 \pm 0{\cdot}12$ cm; for n-butyric acid $10{\cdot}4 \pm 0{\cdot}26$ cm. Suitable amounts (0·2 and 0·05 ml respectively) of acetic acid solution (0·14 M as determined by gas chromatography) and propionic acid solution (0·24 M) were added to 0·5 g caecal contents from a number of rats. The mean percentage recovery of acetic acid was $105 \pm 4{\cdot}2$ in twenty-two tests and of propionic acid, $114 \pm 4{\cdot}0$ in twenty-three tests. Extracts of the contents of twenty-one separate caecums were assayed in duplicate; the difference between each pair of results was expressed as a percentage of their mean. For acetic acid the differences were between 0 and 13% (mean 4·1) and for propionic acid, 0–7·5% (mean 3·6).

Expt 1. In this preliminary experiment the mean methylmalonate excretion decreased over threefold between the 1st and 8th d of treatment with neomycin, but there was considerable variation in the response of individual rats. Injection of propionate at the end of the treatment provoked increases in all the animals. After withdrawing neomycin the excretion continued low until the end of the experiment except for a high value from one rat on one day. All the vitamin B_{12} levels in two of the rats at the end of the experiment, and that in the liver of the third, were similar to those found previously in animals on the vitamin B_{12}-supplemented diet (Williams *et al.* 1969).

Expt 2. There was a large decrease in the mean excretion of methylmalonate by the deficient rats during treatment with the antibiotics (Table 1), with little change in the control group. Propionate provoked marked increases in the deficient rats on the 13th d but had almost no effect in the controls. By the 22nd d, 9 d after discontinuing the antibiotics, the excretion had risen to levels approaching those before treatment. This increase was maintained 14 d later.

Table 1. *Expt* 2. *Effect of a mixture of streptomycin and erythromycin given by mouth on the excretion of methylmalonic acid by rats on the vitamin B_{12}-deficient and -supplemented diets*

(Five rats in each group)

Day	Treatment	Methylmalonic acid in urine (mg/d)			
		Deficient diet		Supplemented diet	
		Mean	SE	Mean	SE
1	None	43·2	14·42	1·4	0·23
5	None	43·7*	16·82	1·4	0·21
6	Streptomycin+erythromycin (see p. 406)	—	—	—	—
7		4·5	1·04	1·4	0·16
9		2·0	0·91	0·9	0·13
13	Sodium propionate, 1 m-mole intraperitoneal	28·8	6·96	1·7	0·24
16	None	4·8	1·69	2·0	0·29
22	None	32·2	16·35	—	—
36	None	34·5	10·74	—	—

* Results for four rats.

Expt 3. Before starting antibiotics, the rats on the deficient diet excreted much more methylmalonate than those on the supplemented diet (Table 2) and showed increased excretion after propionate. On the 15th d (after 3 d on the antibiotics) the excretion by the deficient groups was depressed, but it increased when propionate was given on the 21st d. When the antibiotics were withdrawn, the values for the neomycin-treated deficient group continued low and propionate did not cause any increase on the 39th d. On the other hand, the deficient group treated with the mixture of antibiotics excreted more methylmalonate than their controls on days 32 and 35, although the increase was less marked than that noted at a similar time after treatment in Expt 2. The deficient group also responded to propionate on the 39th and 57th d but their basal excretion was low on days 46 and 53. The results for the supplemented groups were low throughout and changed little in response to antibiotics or propionate.

The mean absorption of [^{58}Co]cyanocobalamin by the deficient groups shortly before the end of the experiment was $54{\cdot}6 \pm 5{\cdot}55$ % of the dose for the neomycin-treated group and $56{\cdot}8 \pm 2{\cdot}81$ % for the other group. The vitamin B_{12} activity in the plasma of the deficient groups was much lower than in the control groups throughout (Table 3). There was no significant difference between the mean concentrations of vitamin B_{12} in the livers of the deficient and supplemented neomycin-treated groups; the samples were re-assayed 28 d later and the similarity of the mean values was confirmed. In contrast, the mean value for the livers of the deficient group that had

received the mixture of antibiotics was significantly lower ($P < 0.001$) than both that in the corresponding control group and that in the neomycin-treated deficient group.

In the neomycin-treated deficient group the mean values for acetic acid in the caecal contents were 121 ± 14.1 μmoles/g or 210 ± 24.1 μmoles/100 g body-weight and for propionic acid, 47 ± 6.5 μmoles/g or 83 ± 11.6 μmoles/100 g body-weight. In the deficient group that received the mixture of antibiotics the results for acetic acid were 126 ± 10.8 μmoles/g or 245 ± 33.3 μmoles/100 g body-weight and for propionic acid, 43 ± 3.0 μmoles/g or 80 ± 9.1 μmoles/100 g body-weight.

Table 2. *Expt* 3. *Effect of antibiotics given by mouth on the excretion of methylmalonic acid by rats on the vitamin B_{12}-deficient and -supplemented diets*

(Ten rats in each group)

		Antibiotic							
		Neomycin				Streptomycin + erythromycin			
		Diet				Diet			
		Deficient		Supplemented		Deficient		Supplemented	
		Methylmalonic acid in urine (mg/d)							
Day	Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	None	25·8	3·99	1·8*	0·17	51·2	5·29	1·6	0·20
3	None	20·8	2·65	2·8	0·38	30·9	3·61	1·5	0·13
5	None	25·2	3·18	2·0	0·26	28·0	2·88	1·6	0·14
7	Sodium propionate, 1 m-mole intraperitoneal	58·0	5·49	4·6	0·53	72·4	6·87	3·9	0·48
9	None	17·6	3·20	2·0	0·32	19·9	2·65	1·2	0·19
11	None	18·8	2·11	2·2	0·27	17·8	5·02	2·2*	0·32
13	Antibiotics (see p. 406)	17·5	3·27	1·7	0·20	18·7*	2·70	1·6	0·22
15		3·7	0·85	0·8	0·12	3·6	1·25	0·8	0·08
18		4·0	1·05	1·1	0·25	1·2*	0·24	1·2	0·21
21	Propionate, as on day 7	24·8	3·73	1·4*	0·18	26·8*	3·74	3·0	0·31
24	None	8·5*	1·16	1·7	0·15	2·6†	0·46	1·6	0·12
32	None	1·9	0·31	2·1	0·15	8·9*	1·61	2·4	0·47
35	None	4·1	0·70	4·6	0·35	8·8*	2·05	2·4	0·24
39	Propionate, as on day 7	5·3	0·50	3·2	0·25	21·1*	4·67	3·7	0·30
46	None	2·8	0·28	1·5	0·09	3·7*	0·71	1·7	0·15
53	None	—	—	—	—	3·4‡	1·45	1·6	0·32
57	Propionate, as on day 7, to groups on mixture of antibiotics	—	—	—	—	18·6*	3·14	3·4	0·27

* Results for nine rats. One rat in the deficient group receiving streptomycin and erythromycin died between days 18 and 21.

† Results for seven rats.

‡ Results for eight rats.

Expt 4. As in the earlier experiments, the excretion of methylmalonate by the deficient rats that received antibiotics was reduced (Table 4), although two of the neomycin-treated animals excreted more on the 5th d of treatment than on the 3rd d. There were no significant differences between the vitamin B_{12} concentrations in the livers of the three deficient groups. The antibiotics depressed the concentration of propionate and acetate in the caecal contents and the level of propionate was also depressed

by the mixture of antibiotics when expressed on a body-weight basis. However, there were no apparent differences between the results for the neomycin-treated and the untreated deficient rats when the results were calculated on the basis of body-weight. The levels of both acids were higher in the vitamin B_{12}-supplemented rats than in the untreated deficient group.

Table 3. *Expt* 3. *Vitamin B_{12} activity in the plasma and livers of rats given short courses of antibiotics by mouth while on vitamin B_{12}-deficient and -supplemented diets*

(Ten rats in each group)

		Vitamin B_{12} activity (ng/ml or ng/g)			
		Deficient diet		Supplemented diet	
Day	Material studied	Mean	SE	Mean	SE
	Rats given neomycin				
12	Plasma	0·095	0·009	0·532	0·014
44	Plasma	0·241	0·037	0·470*	0·054
58	Plasma	0·181	0·024	0·513	0·020
	Liver	78·7	6·99	85·5	8·83
	Rats given streptomycin and erythromycin				
12	Plasma	0·115	0·014	0·456*	0·036
43	Plasma	0·163*	0·024	0·446	0·030
61	Plasma	0·083*	0·014	0·394	0·014
	Liver	41·9*	3·74	85·0	6·10

* Results for nine rats.

Table 4. *Expt* 4. *Urinary methylmalonate excretion, liver vitamin B_{12} concentration and caecal fatty acid levels in rats receiving antibiotics while on vitamin B_{12}-deficient or -supplemented diets*

(Five rats in each group)

		Deficient diet						Supplemented diet	
		Treatment							
		None		Neomycin days 5–11		Streptomycin + erythromycin days 5–11		None	
Day	Measurement	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	Urinary methylmalonate excretion (mg/d)	17·9	3·54	27·3	9·37	16·6	4·06	2·8	0·37
3		18·9	4·08	23·1	4·50	16·7	2·53	2·5	0·21
5		23·1	3·44	21·5	4·74	15·9	4·11	1·9	0·26
7		28·1	6·06	4·9	1·17	3·8	1·02	2·3	0·23
9		27·7	6·35	7·8	3·02	3·5	1·33	1·8	0·07
11	Liver vitamin B_{12} (μg/g)	19·0	2·85	25·4	1·94	22·8	3·71	52·8	4·54
11	Caecal propionate: μmoles/g	27·2	3·99	11·2	2·44	2·6	0·40	65·2	10·82
	Caecal propionate: μmoles/100 g body-weight	74·0	10·14	78·0	21·34	13·0	2·39	135	17·43
11	Caecal acetate: μmoles/g	112	6·58	43·0	3·95	55·2	5·24	166	8·99
	Caecal acetate: μmoles/100 g body-weight	305	18·18	290	32·81	269	24·26	383	32·4

DISCUSSION

Although rats fed on the vitamin B_{12}-deficient diet excreted much more methylmalonic acid than those given the supplemented diet, the values are probably lower than those that would have been obtained if the animals had been fed during the collection of urine (Williams *et al.* 1969). Methylmalonic acid was recovered quantitatively from solutions of the antibiotics and increased excretion was found after propionate while the rats were receiving antibiotics. Hence the reduced basal excretion found during treatment with antibiotics is unlikely to have been due to interference by the antibiotics with the determination of methylmalonate. Despite the reduced excretion, the rats were still vitamin B_{12}-deficient after treatment, as judged by the response to propionate in the earlier experiments and by the levels of vitamin B_{12} in the livers in Expt 4 (Table 4). The irregular results for the rats in Expt 1, when neomycin was added to the food, may have been due to differences in individual food consumption.

The observations suggest that initially the antibiotics depressed the production of precursors of methylmalonate in a way similar to the apparent effect of starvation (Williams *et al.* 1969), rather than that they influenced vitamin B_{12} nutrition as suggested by Armstrong & Curnow (1967). No facilities were available for measuring fatty acids during the earlier work. In the present study, therefore, five vitamin B_{12}-deficient rats were killed after starving for 48 h and propionic and acetic acids were measured in their caecal contents. The mean levels were: for propionic acid $9{\cdot}8 \pm 0{\cdot}98$ μmoles/g caecal contents or $8{\cdot}8 \pm 1{\cdot}69$ μmoles/100 g body-weight; for acetic acid, $41{\cdot}2 \pm 2{\cdot}89$ μmoles/g caecal contents or $37{\cdot}4 \pm 6{\cdot}17$ μmoles/100 g body-weight. These results, when compared with those for untreated deficient rats during feeding (Table 4), show that the level of propionate in the caecum decreased after starvation.

The amount of propionate in the caecal contents of vitamin B_{12}-deficient rats was reduced by treatment with streptomycin and erythromycin (Table 4). The results with neomycin are more difficult to interpret; the concentration of propionate in the caecal contents was reduced but, since the antibiotic caused enlargement of the caecum, the levels per 100 g body-weight were as high as those in the untreated deficient group. Neomycin had no immediate effect on the level of vitamin B_{12} in the livers (Table 4), so that the reduced methylmalonate excretion following neomycin is unlikely to have been due to a change in vitamin B_{12} status.

The results for the neomycin-treated rats after the end of the treatments suggest that their reserves of vitamin B_{12} increased, but this did not happen to the same extent in the animals that received the mixture of antibiotics. Thus in Expt 3 the methylmalonate excretion of the neomycin-treated deficient group continued at levels similar to those in the supplemented group and propionate did not cause increased excretion 18 d after the drug was withdrawn. The excretion by the deficient group that had received streptomycin and erythromycin also decreased towards the end of the experiment but the response to propionate was maintained. The levels of propionate in the caecal contents of the two groups at the end of the experiment were comparable and were similar to those in the untreated deficient group in Expt 4 (Table 4). Although

the caecal contents were collected several days after the final methylmalonate measurements, the results suggest that the continued low excretion was unlikely to have been due to a reduced supply of precursors. The observations in the group that received the mixture of antibiotics could be explained by a slight increase in vitamin B_{12} stores, sufficient to depress basal methylmalonate excretion but too small to prevent the abnormal metabolism of a large dose of propionate. The higher liver vitamin B_{12} levels (Table 3) as compared with those of the deficient rats in Expt 4 (Table 4) are in agreement with this suggestion.

Most of the values for vitamin B_{12} in the plasma and tissues of the rats in Expt 1 were higher than those usually found in rats on the deficient diet. In Expt 3, the concentrations of vitamin B_{12} in the livers of the deficient and supplemented neomycin-treated groups were not significantly different, but there was a highly significant difference for the rats that had received streptomycin and erythromycin. In spite of the similarity of the results for the livers of the neomycin-treated groups, the results for the plasma of the deficient group were lower than those for the controls. After total gastrectomy the level of vitamin B_{12} in the serum of rats fell more rapidly than that in the liver (Booth & Spray, 1960). This is the opposite of the present situation, but it seems possible that the concentration of vitamin B_{12} in serum or plasma may increase more slowly than that in liver during repletion.

The apparent tendency for neomycin-treated deficient rats to gain vitamin B_{12} might have been due to an effect of neomycin on intestinal absorption of vitamin B_{12} or to a decrease in the population of vitamin B_{12}-requiring organisms in the intestine. However, there was little difference between the values for the absorption of cyanocobalamin in the deficient groups in Expt 3, and the results were similar to those obtained by Biggs & Witts (1962) for rats on various diets.

In conclusion, it seems that alterations in basal methylmalonate excretion in vitamin B_{12} deficiency do not always reflect changes in vitamin B_{12} status. The findings must be interpreted in the light of other factors, such as starvation or treatment with antibiotics, that may influence the excretion. However, methylmalonate excretion in response to the administration of precursors such as propionate does seem to reflect vitamin B_{12} status.

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