Vitamin B_{12} deficiency and the excretion of ether-soluble acids in the rat

By B. K. ARMSTRONG

Department of Biochemistry, Royal Perth Hospital, Perth, Western Australia

(*Received* 7 *July* 1966—*Accepted* 5 *December* 1966)

1. Urinary excretion of total ether-soluble acids and of methylmalonic acid was studied in rats on vitamin B_{12}-deficient diets with and without a vitamin B_{12} supplement.

2. It was shown that urinary excretion of total ether-soluble acids and methylmalonic acid was increased in vitamin B_{12}-deficient rats and that this increase was somewhat variable between individual animals, males and females, and rats from different litters.

3. The increased excretion of these acids could readily be reversed by supplementing the diet with vitamin B_{12}.

Methylmalonic acid was first found in rat's urine by Boyland & Levi (1936). Later, excretion of total urinary ether-soluble acids (TUESA) and the proportion of methylmalonic acid were found to be elevated in rats fed on a diet deficient in vitamins E and B_{12} (Forbes, Barness, Moeksi & György, 1953; Barness, Moeksi & György, 1956). The levels of TUESA and methylmalonic acid excretion were unrelated to vitamin E deficiency. The relationship of vitamin B_{12} deficiency to increased TUESA and methylmalonic acid excretion in the rat was not realized until later (Barness, Young & Nocho, 1963).

Until now, studies of vitamin B_{12} nutrition in rats have used growth depression and anaemia (Wagle, Mehta & Johnson, 1958) as signs of deficiency. Anaemia rarely occurs and growth depression, although frequent, is not consistent (Schultze, 1950) unless thyroid powder or iodinated protein is included in the diet (Register, Ruegamer & Elvehjem, 1949; Frost, Fricke & Spruth, 1949). This investigation assesses the use of TUESA and methylmalonic acid excretion as indices of vitamin B_{12} deficiency in the rat.

EXPERIMENTAL

Male and female Wistar albino rats were used in all experiments.

Expt 1. *To compare TUESA and methylmalonic acid excretion and growth in vitamin B_{12}-deficient rats and those given a vitamin B_{12} supplement*

The rats were derived from two litters, A and B, and divided into two groups: (1) two males and two females from each of litters A and B; (2) two males and two females from litter A, two males and one female from litter B. Each group was housed in a single large, screen-floor cage and given *ad lib.* from weaning the vitamin B_{12}-deficient diet described in Table 1. Both groups received 1 mg/100 ml cobalt chloride ($CoCl_2.6H_2O$) in the drinking water and group 1 received 1 μg/100 ml vitamin B_{12}, as hydroxocobalamin, in the water. The rats were weighed at regular intervals during

6 weeks from weaning, and the TUESA and methylmalonic acid excretion was measured over 24 h at the end of this period.

Expt 2. To compare TUESA and methylmalonic acid excretion of individual vitamin B_{12}-deficient rats of different sexes and from different litters

Rats were derived from two litters: litter C consisting of five males and six females; litter D containing four males and five females. Litters C and D were born on the same day of mothers housed under similar conditions and fed on stock diet. From weaning the rats were housed in individual screen-floor cages and each was given 10 g/day of the vitamin B_{12}-deficient diet (Table 1). TUESA and methylmalonic acid excretions were measured and the rats were weighed 15 weeks after weaning.

Table 1. *The vitamin B_{12}-deficient diet*

Constituent	Proportion by weight
Basic diet:	
Soya flour	62
Sucrose	29
Salt mixture*	4
Agar	1·5
Hydrogenated vegetable oil	1
Choline chloride	0·1
Vitamin supplement:	
Dry mixture†	480 mg/kg basic diet
ABDEC‡	10 ml/kg basic diet

* Jones & Foster (1942), omitting $CoCl_2.6H_2O$.
† Frost, Fricke & Spruth (1953).
‡ Parke Davis and Company, Sydney, Australia.

Expt 3. To show the effect of different levels of vitamin B_{12} supplementation on TUESA and methylmalonic acid excretion by vitamin B_{12}-deficient rats

Rats of similar weight were drawn from three 12-week-old litters fed *ad lib.* on the vitamin B_{12}-deficient diet from weaning. They were housed in individual, screen-floor cages and given 10 g/day of the vitamin B_{12}-deficient diet. TUESA and methylmalonic acid excretions were measured and then each rat was given a different amount (see Fig. 1) of hydroxocobalamin added daily to the diet. The rats were weighed at regular intervals and TUESA and methylmalonic acid excretion was determined until relatively constant levels were reached.

Expts 1–3. Determination of TUESA and methylmalonic acid excretion

Urine was collected, under toluene, from rats housed for 24 h in individual metabolism cages. The 24 h urine collection was filtered, acidified to pH 1 with 10 N-H_2SO_4, saturated with $(NH_4)_2SO_4$ and extracted with ether continuously for 48 h. This method is similar to that of Bray, Neale & Thorpe (1946). The ether was evaporated, the last traces were removed under reduced pressure, and the extract was taken up in 5 ml of absolute ethanol. A sample (10 μl) of this solution was used for thin-layer chromatography. Water (about 10 ml) was added to the remaining ethanol solution

and the resulting solution titrated against 0·1 N-NaOH to pH 8·5, a glass electrode being used. The result was expressed as m-equiv. ether-soluble acid per 24 h per 100 g body-weight.

Thin-layer chromatography was performed on 20 × 20 cm glass plates, each carrying a 0·25 mm layer of Silica Gel G (Merck) which had been activated by heating at 80° overnight (Stahl, 1962). The 10 μl sample spots were applied at intervals of not less than 1·5 cm along a line parallel to, and 1·5 cm from, the edge of the plate. A standard spot containing 50 μg methylmalonic acid was included on each plate. Plates were run 15 cm in the solvent (Hinterberger, Bashir & Jones, 1965): amyl acetate 60 vol., glacial acetic acid 10 vol. and water 0·5 vol.

Table 2. *Key to semi-quantitative estimation (see below) of amount of methylmalonic acid in spots on thin-layer chromatograms*

Semi-quantitative representation	μg of methylmalonic acid in spot
0	< 5
Trace	5–20
+	20–60
++	60–100
+++	100–160
++++	> 160

After completion of the chromatography, the plates were dried for 1 h at 80° and allowed to cool; the spots were shown up with brom-cresol green. The brom-cresol green was prepared at 0·1% (w/v) in ethanol, diluted 1 part in 4 with acetone and sprayed on the plate. A semi-quantitative estimate of the amount of methylmalonic acid in the sample was made from the size and density of the methylmalonic acid spot (R_F 0·34 ± 0·03). Representations of approximate amounts of methylmalonic acid are given in Table 2.

The statistical significance of the differences in means of results obtained in Expts 1 and 2 was estimated by Student's *t* test.

RESULTS

The results are given in detail in Tables 3 and 4 and Fig. 1. Chromatography of the urinary extracts from rats in Expt 1 indicated that the increases in TUESA excretion (Table 3) were due to increases in excretion of methylmalonic acid. Expt 3 included, as well as those represented in Fig. 1, rats which were given 0·5, 1·0 and 2·0 μg hydroxocobalamin/day; the graphs for these rats were similar to that shown for rats given 0·2 μg hydroxocobalamin/day.

The results of Expt 1 (Table 3) showed that vitamin B_{12}-deficient rats excrete more TUESA and methylmalonic acid than normal rats. The difference in mean TUESA excretion between groups 1 and 2 was highly significant ($P < 0{\cdot}005$). Also the depression in growth of the deficient rats as compared with those on the supplemented diet was highly significant.

Expt 2 (Table 4) showed that there is considerable variability in TUESA and

Table 3. *Expt* 1. *Urinary excretion of ether-soluble acids after* 6 *weeks and mean increase in weight over* 6 *weeks of weanling rats maintained on vitamin* B_{12}*-supplemented or vitamin* B_{12}*-deficient diets*

(Mean values with their standard deviations)

Group	No. of rats	Mean excretion of total urinary ether-soluble acids after 6 weeks (m-equiv./100 g 24 h)	Mean increase in weight over 6 weeks (g)
(1) Vitamin B_{12}-supplemented	8	0·32 ± 0·06	117 ± 18
(2) Vitamin B_{12}-deficient	7	0·92 ± 0·23	82 ± 16

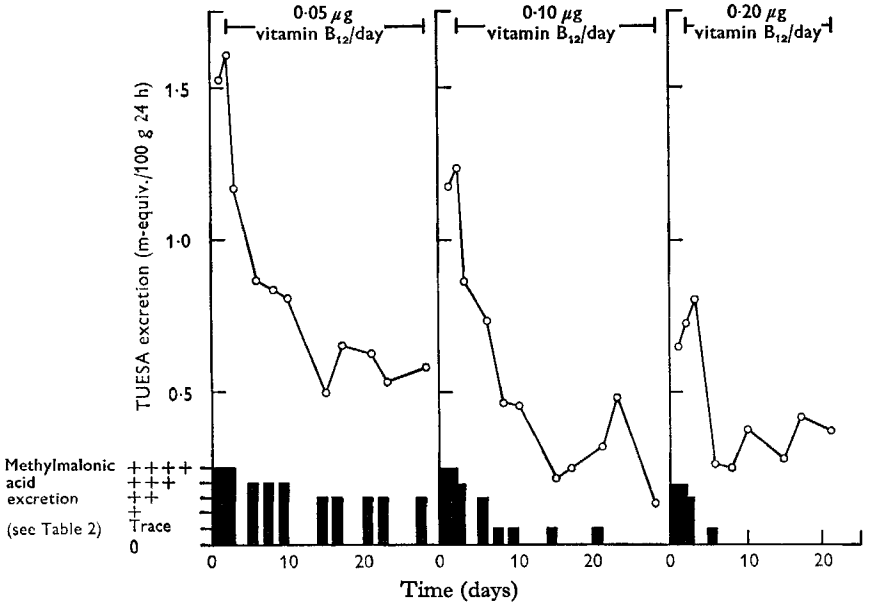

Fig 1. Expt 3. Urinary excretion of total ether-soluble acids and methylmalonic acid in three vitamin B_{12}-deficient rats supplemented with different levels of vitamin B_{12}.

methylmalonic acid excretion in rats of the same age given a vitamin B_{12}-deficient diet from weaning. The difference in mean TUESA excretion by male and female rats was not significant ($P > 0{\cdot}10$) in litter C, but it was significant in litter D ($P < 0{\cdot}05$). The difference between mean values for the two litters was highly significant. Some of the TUESA excretion levels of individual vitamin B_{12}-deficient rats were of the same order as those of the rats given the vitamin B_{12} supplement (Table 3), but methylmalonic acid was usually detectable, whereas it was not so with the rats given the supplement.

Table 4. *Expt 2. Excretion of total urinary ether-soluble acids and methylmalonic acid in two litters of rats, 15 weeks after weaning on to a vitamin B_{12}-deficient diet*

Sex	Total urinary ether-soluble acid excretion after 15 weeks (m-equiv./100 g 24 h)	Semi-quantitative estimate of methylmalonic acid excretion*
Litter C		
♀	0·46	+
♀	0·76	++
♀	0·38	Trace
♀	0·53	++
♀	0·49	Trace
♀	0·82	+++
Mean for females†	0·59±0·16	—
♂	0·08	0
♂	0·31	Trace
♂	0·86	+++
♂	0·29	Trace
♂	0·48	+
Mean for males†	0·40±0·26	—
Mean for litter†	0·50±0·23	—
Litter D		
♀	1·67	++++
♀	0·61	++
♀	0·44	Trace
♀	0·49	Trace
♀	0·45	Trace
Mean for females†	0·73±0·47	—
♂	1·85	++++
♂	0·66	++
♂	2·78	++++
♂	1·80	++++
Mean for males†	1·77±0·75	—
Mean for litter†	1·19±0·80	—

* See Table 2.
† Mean value with its standard deviation.

DISCUSSION

Increased TUESA excretion by rats maintained on a vitamin B_{12}-deficient diet, under the conditions of Expt 2, was not consistent between individuals. This situation is similar to that seen in growth studies. This lack of consistency may be associated with variations in the amount of vitamin B_{12} obtained from faeces through coprophagy (Barnes & Fiala, 1958; Morgan, Gregory, Kon & Porter, 1964) or through absorption direct from the large bowel (Merzbach & Grossowicz, 1965). Also the 15-week depletion period may have allowed refection to occur in some of the rats (Fredericia, Freudenthal, Gudjonnsson, Johansen & Schoubye, 1928). The rats depleted for only 6 weeks in Expt 1 showed a much more consistent increase of TUESA excretion.

Expt 3 (Fig. 1) showed that the increased TUESA and methylmalonic acid excretion by vitamin B_{12}-deficient rats was readily corrected by supplementing the diet with

vitamin B_{12}. The amount required to reduce methylmalonic acid excretion to '0' levels (Table 2) lies between 0·05 and 0·10 μg/day. This is of the same order as other estimates of the vitamin B_{12} requirement of rats (Henry & Porter, 1958).

Since methylmalonic acid is the main acid excreted in greater amounts in vitamin B_{12} deficiency, it is desirable to measure the excretion as precisely as possible. This was attempted by elution from the chromatogram, but was unsuccessful. Although exact measurement of methylmalonic acid excretion could probably be achieved by using different techniques (White, 1962; Giorgio & Plaut, 1965), the results reported here suggest that measurement of total urinary excretion of ether-soluble acids, with associated semi-quantitative estimates of methylmalonic acid from the chromatograms, is an adequate indication of vitamin B_{12} deficiency in the rat. Although it varies in a manner similar to growth depression it is probably a better criterion, since it is less likely to be affected by factors other than vitamin B_{12} deficiency; it can be assessed at any time, and does not require continuous observation over the growth period.

The author gratefully acknowledges the advice and assistance of Dr D. H. Curnow in whose Department this work was carried out. Thanks are due also to Mr A. Softly and staff of the Royal Perth Hospital Animal House. The work was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

Barnes, R. H. & Fiala, G. (1958). *J. Nutr.* **65**, 103.
Barness, L. A., Moeksi, H. & György, P. (1956). *J. biol. Chem.* **221**, 93.
Barness, L. A., Young, D. G. & Nocho, R. (1963). *Science, N.Y.* **140**, 76.
Boyland, E. & Levi, A. A. (1936). *Biochem. J.* **30**, 2007.
Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946). *Biochem. J.* **40**, 134.
Forbes, M., Barness, L. A., Moeksi, H. & György, P. (1953). *Proc. Soc. exp. Biol. Med.* **84**, 162.
Fredericia, L. S., Freudenthal, P., Gudjonnsson, S., Johansen, G. & Schoubye, N. (1928). *J. Hyg., Camb.* **27**, 70.
Frost, D. V., Fricke, H. H. & Spruth, H. C. (1949). *Proc. Soc. exp. Biol. Med.* **72**, 102.
Frost, D. V., Fricke, H. H. & Spruth, H. C. (1953). *J. Nutr.* **49**, 107.
Giorgio, A. J. & Plaut, G. W. E. (1965). *J. Lab. clin. Med.* **66**, 667.
Henry, K. M. & Porter, J. W. G. (1958). *Proc. Nutr. Soc.* **17**, vii.
Hinterberger, H., Bashir, H. & Jones, P. (1965). *Proc. Aust. Ass. clin. Biochem.* **1**, 143.
Jones, J. H. & Foster, C. (1942). *J. Nutr.* **24**, 245.
Merzbach, D. & Grossowicz, N. (1965). *J. Nutr.* **87**, 41.
Morgan, T. B., Gregory, M. E., Kon, S. K. & Porter, J. W. G. (1964). *Br. J. Nutr.* **18**, 595.
Register, U. D., Ruegamer, W. R. & Elvehjem, C. A. (1949). *J. biol. Chem.* **177**, 129.
Schultze, M. O. (1950). *J. Nutr.* **41**, 103.
Stahl, E. (editor) (1962). *Thin Layer Chromatography*. Berlin: Springer Verlag.
Wagle, S. R., Mehta, R. & Johnson, B. C. (1958). *J. biol. Chem.* **230**, 137.
White, A. M. (1962). *Biochem. J.* **84**, 41 P.

Printed in Great Britain