THE EXCRETION OF METHYLMALONIC AND FORMIMINOGLUTAMIC ACIDS DURING THE INDUCTION AND REMISSION OF VITAMIN B_{12} DEFICIENCY IN SHEEP

By J. M. GAWTHORNE*

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Summary

The excretion of methylmalonic acid (MMA) in the urine of severely vitamin B_{12}-deficient sheep was 5–12 times greater, and that of formiminoglutamic acid (FIGLU) was more than 30 times greater, than that of pair-fed, vitamin B_{12}-injected controls.

Urinary MMA excretion was significantly increased only when sheep were severely affected by vitamin B_{12} deficiency. In the early stages of the deficiency, sheep were able to utilize the amounts of methylmalonyl CoA arising from propionate absorbed from the alimentary canal.

Urinary FIGLU excretion was markedly elevated even in the early stages of the deficiency syndrome and increased progressively as vitamin B_{12} depletion continued.

The elevated MMA and FIGLU excretions of severely vitamin B_{12}-deficient sheep were restored to normal within 3 weeks by the intramuscular injection of cyanocobalamin.

The degree of impairment of metabolic reactions involving MMA and FIGLU during the induction of vitamin B_{12} deficiency in sheep is discussed.

I. INTRODUCTION

The urinary excretion of methylmalonic acid (MMA) increases during vitamin B_{12} deficiency in humans (Cox and White 1962; White 1962; Barness *et al.* 1963) and in rats (Barness, Young, and Nocho 1963; Armstrong 1967). This increased excretion is probably due to a decrease in the activity of methylmalonyl CoA isomerase, a vitamin B_{12}-requiring enzyme that catalyses the conversion of methylmalonyl CoA to succinyl CoA (Flavin and Ochoa 1957; Gurani, Mistry, and Johnson 1960).

Deficiencies of either vitamin B_{12} or folic acid can cause an increase in the excretion of formiminoglutamic acid (FIGLU) in humans (Baker *et al.* 1959; Luhby, Cooperman, and Teller 1959; Chanarin, Bennett, and Berry 1962; Knowles and Prankerd 1962), in rats (Silverman and Pitney 1958; Rabinowitz and Tabor 1958; Stokstad, Webb, and Shah 1966), and in chicks (Spivey Fox and Ludwig 1961). Herbert and Zalusky (1962) and Buchanan (1964) suggest that vitamin B_{12}, through participation in the methyltetrahydrofolate–homocysteine transmethylase reaction, regulates the availability of tetrahydrofolic acid and thereby indirectly affects FIGLU catabolism.

* Institute of Agriculture, University of Western Australia, Nedlands, W.A. 6009.

No information on the excretion of MMA or FIGLU in ruminants has appeared.

The present paper describes changes in the urinary excretion of MMA and FIGLU during the induction and remission of vitamin B_{12} deficiency in sheep. The excretion of both substances by severely vitamin B_{12}-deficient sheep is compared with that of pair-fed, vitamin B_{12}-injected controls. From these investigations some conclusions have been drawn regarding the degree of impairment of metabolic reactions involving MMA and FIGLU at certain stages in the induction of vitamin B_{12} deficiency.

II. Experimental

(*a*) *Experiment 1*

(i) *Animals.*—Eight adult Merino wethers were separately confined in all-polythene pens fitted with plastic feed and water containers. De-ionized water was available for drinking at all times.

(ii) *Experimental Design and Feed Materials.*—The animals were divided into two groups of four. A cobalt-deficient ration (0·03 μg cobalt/g) consisting of 700 g chopped wheaten hay, 50 g starch, 25 g wheat gluten, 12 g urea, and 26 g of a mineral mix was presented daily to each sheep in the first group (sheep 71, 72, 73, and 74). Preferential selection of particular components of the ration by the sheep was minimal. Animals in the second group (sheep 75, 76, 77, and 78) were fed a ration of similar percentage composition to that presented to the animals in the first group. However, the amounts fed to sheep 75, 76, 77, and 78 were equalized with the daily feed intakes of sheep 71, 72, 73, and 74 respectively (dry matter basis). In addition, animals in the second group received intramuscular injections of cyanocobalamin (50 μg each fourth day).

(iii) *Urine Collection.*—After consuming the cobalt-deficient rations for 8 months the sheep in the first group were severely vitamin B_{12}-deficient. This was apparent from marked decreases in voluntary feed intakes, body weights, blood haemoglobin concentrations, and plasma vitamin B_{12} concentrations. At this stage urine was collected from all animals in the experiment. Each animal was placed for 48 hr in a steel crate fitted with a polythene urine collection funnel. Urine was received into polythene bottles containing 15 ml of concentrated hydrochloric acid. For each sheep, the volume of the 48-hr urine output was recorded and a 100-ml sample of urine was stored at 4°C for MMA and FIGLU analyses.

The sheep were weighed at the end of the urine collection period.

(*b*) *Experiment 2*

(i) *Animals and Feed Materials.*—Four adult Merino wethers, sheep 25, 28, 32, and 33, were held in pens under conditions similar to the animals in experiment 1. Deionized water was available *ad libitum*. A cobalt-deficient ration (813 g) identical in composition with that used for experiment 1 was presented to each animal daily. The daily dry feed intake of each animal was recorded.

(ii) *Urine Collection.*—The sheep were weighed, and urine was collected from them at the following stages in the induction and remission of vitamin B_{12} deficiency:

Stage 1: No clinical signs of deficiency—feed intake 813 g/day.

Stage 2: Feed intake voluntarily reduced to approximately 500 g/day.

Stage 3: Signs of severe vitamin B_{12} deficiency apparent—feed intake approximately 200 g/day.

Stage 4: Within 3 weeks of commencing vitamin B_{12} injections—feed intake approximately 400 g/day. The vitamin B_{12} injections consisted of 25, 50, 100, and 200 μg cyanocobalamin injected intramuscularly each fourth day into sheep 28, 25, 32, and 33 respectively.

Stage 5: Clinical signs of vitamin B_{12} deficiency remitted—feed intake 813 g/day.

The method of urine collection was the same as that in experiment 1.

(c) *Analytical Methods*

MMA and FIGLU concentrations in urine were determined by the methods of Giorgio and Plaut (1965) and Chanarin and Bennett (1962) respectively.

(d) *Statistical Analysis*

The difference between means was tested for statistical significance by the Student's t-test (Steel and Torrie 1960).

III. Results

(a) *Methylmalonic Acid Excretion*

The urinary MMA excretion (measured as μmoles/kg body weight/24 hr) of the severely vitamin B_{12}-deficient sheep in experiment 1 was 5–12 times greater than that of the pair-fed control animals which had received intramuscular injections of 50 μg cyanocobalamin each fourth day, as the following tabulation shows:

	B_{12}-deficient				Controls			
Sheep No.	71	72	73	74	75	76	77	78
MMA excretion	164	187	146	84	26	18	13	16

In experiment 2 there was no significant increase in urinary MMA excretion when the animals were mildly vitamin B_{12} deficient (stage 2, Table 1). Urinary MMA excretion was increased only when the animals were profoundly affected by vitamin B_{12} deficiency (stage 3, Table 1). Parenteral injections of cyanocobalamin restored the elevated MMA excretion of severely deficient animals to normal. Within 3 weeks

Table 1

Changes in the urinary excretion of methylmalonic and formiminoglutamic acids during the induction and remission of vitamin B_{12} deficiency in sheep

Sheep Number	Stage 1*	Stage 2*	Stage 3*	Stage 4*	Stage 5*
	Urinary MMA Excretion (μmoles/kg body weight/24 hr)				
28	13	10	Dead†	—	—
25	18	19	45	17	11
32	15	24	66	21	21
33	18	12	70	12	16
Mean	16	16	60		
	n.s.	$P < 0{\cdot}01$			
	Urinary FIGLU Excretion (μmoles/kg body weight/24 hr)				
28	13	73	Dead†	—	—
25	9	65	80	24	< 1
32	20	88	218	11	< 1
33	3	25	84	1	< 1
Mean	11	33	127		
	$P < 0{\cdot}05$	n.s.			

* See Section II(*b*).

† Autopsy revealed signs of severe vitamin B_{12} deficiency.

of the commencement of vitamin B_{12} therapy, MMA excretion had declined to pre-deficiency levels (stage 4, Table 1) and remained at these levels during the period of remission of clinical signs of deficiency (stage 5, Table 1). Less than 200 μg cyanocobalamin administered intramuscularly as 50 μg doses each fourth day was sufficient to restore the MMA excretion of severely vitamin B_{12}-deficient sheep to normal.

(*b*) *Formiminoglutamic Acid Excretion*

The urinary excretion of FIGLU (measured as μmoles/kg body weight/24 hr) by severely vitamin B_{12}-deficient sheep in experiment 1 was more than 30 times as great as that of pair-fed control animals which had been injected with 50 μg cyanocobalamin each fourth day, as the following tabulation shows:

	B_{12}-deficient				Controls			
Sheep No.	71	72	73	74	75	76	77	78
FIGLU excretion	89	331	50	38	<3	<3	<1	<1

In Experiment 2, urinary FIGLU excretion was markedly elevated even in the early stages of the deficiency (stage 2, Table 1) and increased progressively as vitamin B_{12} depletion continued (stage 3, Table 1). Within 3 weeks of the commencement of vitamin B_{12} injections, urinary FIGLU excretion had declined substantially (stage 4, Table 1). For each animal the extent of reduction was dependent upon the amount of vitamin B_{12} administered. Nine weeks after beginning vitamin B_{12} injections, all visible signs of deficiency in the animals had been remitted. At this stage (stage 5), FIGLU could not be detected in the urine.

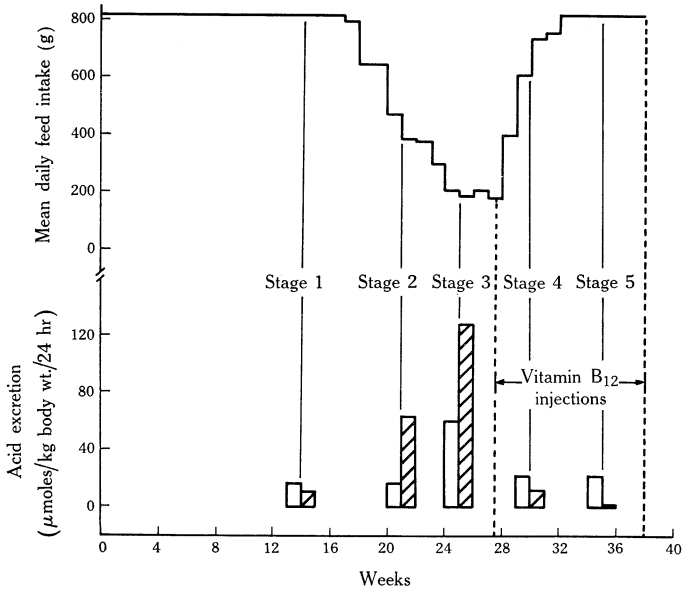

Fig. 1.—Typical patterns of excretion of methylmalonic acid (open rectangles) and formiminoglutamic acid (shaded rectangles) in the urine of sheep, shown in relation to changes in voluntary daily feed intake during the induction of cobalt deficiency and its remission with intramuscular injections of vitamin B_{12} (100 μg cyanocobalamin each fourth day).

(*c*) *Time Course of Acid Excretion*

The time course of changes in MMA and FIGLU excretion during the induction and remission of cobalt deficiency is shown in Figure 1.

IV. Discussion

A complete analysis of the vitamin B_{12} deficiency syndrome in sheep should include information on the metabolic reactions that are impaired by a deficiency of vitamin B_{12}, the order in which these reactions are impaired during the induction of the deficiency, and whether an impairment in these reactions accounts for all the clinical and other signs of the deficiency.

The present experiments provide evidence that reactions involving MMA and FIGLU are impaired during vitamin B_{12} deficiency. In experiment 1 the MMA and FIGLU excretion of severely vitamin B_{12}-deficient sheep was compared with that of sheep given comparable amounts of feed, but maintained at an adequate vitamin B_{12} status. The elevated excretion of MMA and FIGLU by the severely deficient animals was therefore due to an inadequate supply of vitamin B_{12} *per se* and was not a consequence of the decreased voluntary feed intakes that are a part of the deficiency syndrome.

The increase in MMA excretion is evidence that there is a decrease in the rate of isomerization of methylmalonyl CoA *in vivo*. Previous evidence has been obtained from liver-homogenate studies (Marston, Allen, and Smith 1961). From the results of experiment 2 it can be inferred that the rate of isomerization of methylmalonyl CoA is not significantly reduced until a state of severe vitamin B_{12} deficiency is reached (stage 3, Table 1). However, an early impairment in methylmalonyl CoA isomerization cannot be excluded. The quantity of methylmalonyl CoA presented for metabolism within each animal can be altered by changes in the amount of propionate absorbed from the alimentary canal. The formation of methylmalonyl CoA from propionyl CoA is not affected by vitamin B_{12} deficiency (Marston, Allen, and Smith 1961). Potential accumulation of methylmalonyl CoA and augmented excretion of MMA in the initial stages of the deficiency may therefore be offset by a progressive decrease in the amount of propionate presented for metabolism within the animal as daily feed intake declines. The elevated excretion of MMA in the later stages of the deficiency (stage 3, Table 1) suggests that methylmalonyl CoA isomerization became so severely impaired by vitamin B_{12} depletion that the sheep were no longer able to catabolize the quantities of propionate produced by rumen fermentation of their daily feed intakes. Acute failure in methylmalonyl CoA isomerization is therefore a late-developing feature of the deficiency syndrome.

The excretion of FIGLU by animals has been used as an indication of the levels of tetrahydrofolic acid in their tissues (Buchanan 1964; Sen and McGreer 1966). Although the changes in FIGLU excretion observed in experiments 1 and 2 are compatible with the hypothesis suggested by Herbert and Zalusky (1962) and Buchanan (1964), further research is required before the increased excretion of FIGLU in vitamin B_{12}-deficient sheep can definitely be ascribed to a decrease in the activity of methyltetrahydrofolate–homocysteine transmethylase.

Dawbarn, Hine, and Smith (1958) found that in sheep liver the concentration of folic acid and citrovorum factor (5-formyltetrahydrofolic acid) falls precipitately when the vitamin B_{12} concentration decreases below $0 \cdot 19$ μg/g. This fall was not due to the reduced feed intake of the deficient animals. It is not known if the concentration of

5-methyltetrahydrofolic acid is similarly affected by vitamin B_{12} deficiency. According to the hypothesis of Herbert and Zalusky (1962) and Buchanan (1964) the concentration of 5-methyltetrahydrofolic acid in liver should remain constant, or increase, during vitamin B_{12} deficiency.

Regardless of the nature of the biochemical lesion, increased FIGLU excretion appears to be a sensitive indicator of the severity of vitamin B_{12} deficiency in sheep. The marked increases in FIGLU excretion in the initial stages of the deficiency (stage 2, Table 1) suggest that the metabolic reactions involving this compound are impaired relatively early in the development of the deficiency syndrome.

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