

## The effects of dietary histidine, methionine and homocystine on vitamin B<sub>12</sub> and folate levels in rat liver

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1. L-histidine (20 g/kg) added to vitamin B<sub>12</sub>-deficient and cyanocobalamin-supplemented diets based on soya-bean flour reduced the growth of rats given the vitamin B<sub>12</sub>-deficient diet but stimulated growth of rats given the cyanocobalamin-supplemented diet. Liver weight (g/kg body-weight) increased, but the protein content of the livers decreased, in rats given histidine supplements. The histidine was associated with significantly higher folate concentrations in the livers of cyanocobalamin-supplemented rats.

2. Vitamin B<sub>12</sub>-deficient and cyanocobalamin-supplemented rats were given diets based on a mixture of amino acids that was balanced apart from methionine, which was added in various amounts, and with the addition of homocystine. The only vitamin B<sub>12</sub>-deficient rats which had reasonable gains in weight were those receiving a diet containing 8 g L-methionine/kg. The remainder, particularly those given diets containing only homocystine, had little or no increase in weight. All the cyanocobalamin-supplemented rats gained weight; those given diets containing 2 and 8 g L-methionine/kg, or 8 g homocystine/kg, had the highest gains.

3. There was a tendency for a higher concentration of either methionine or homocystine in the diet to be associated with higher concentrations of both folate and vitamin B<sub>12</sub> in the livers.

4. In vitamin B<sub>12</sub> deficiency methionine appeared to increase the accumulation of folate in the liver, affecting mainly the amounts of polyglutamate derivatives.

This work was begun to study the influence of vitamin B<sub>12</sub> deficiency on some of the enzymes involved in folate metabolism. For various reasons the results for the enzymes cannot be reported. However, the study produced some interesting results on the effects of different diets on folate and vitamin B<sub>12</sub> levels in liver, on liver weight and protein content, and on the growth of the animals. These results are reported in this paper.

The first part of the study involved the addition of supplementary histidine to the vitamin B<sub>12</sub>-deficient and cyanocobalamin-supplemented diets described previously (Williams, Spray, Newman & O'Brien, 1969). It seemed possible that extra tetrahydrofolic acid might be required to react with the formiminoglutamic acid formed during the metabolic breakdown of histidine; folate balance might therefore be affected.

Methionine influences folate metabolism in vitamin B<sub>12</sub> deficiency (Silverman & Pitney, 1958; Kutzbach, Galloway & Stokstad, 1967; Smith, Osborne-White & Gawthorne, 1974). It was therefore of interest to study the effects of vitamin B<sub>12</sub> deficiency on folate metabolism in rats maintained on synthetic diets containing a mixture of amino acids instead of protein. With these diets, individual amino acids can be eliminated, and their relative proportions varied with certainty; this is virtually impossible with diets containing proteins. Kutzbach *et al.* (1967) used a mixture of this type to provide a 'labile-methyl'-free diet.

## EXPERIMENTAL

*Amino acid diets.* These were based on agar-gel diets (Rogers & Harper, 1965) and contained (/kg): 100 g maize oil, 50 g salt mixture, 2 g choline dihydrogen citrate, 185 g L-amino acid mixture, 436 g starch, 216 g sucrose, 10 g water-soluble vitamin mixture, 1 ml fat-soluble vitamin mixture. The salt mixture was the same as that used by Rogers & Harper (1965; p. 268, footnote 3). The amino acid mixture was that recommended for diet no. 1 of Rogers & Harper (1965; Table 2), including asparagine, arginine HCl, and all amino acids listed in footnote 2 of the Table except methionine. The vitamin mixtures had the same composition as those used in the soya-bean-flour (soya) diets described by Williams *et al.* (1969) except that the amount of cyanocobalamin (when included) was increased to 75 µg/kg diet. When L-methionine or DL-homocystine were added, the corresponding amounts of starch were omitted.

*Animals and their management.* Male and female weanling albino rats of the Wistar strain (Scientific Products Farm Division, Charles River UK Ltd, Manston Road, Margate, Kent) were fed on a stock diet (modified diet 41b; Herbert C. Styles (Bewdley) Ltd, Severn Side Mills, Bewdley, Worcestershire; supplied by Oxoid Ltd, Southwark Bridge Road, London SE 1) until they were mated. After mating, the females were given the soya diets (Williams *et al.* 1969) containing either no added cyanocobalamin or 75 µg cyanocobalamin/kg diet; they received the same diets throughout pregnancy and lactation. The male offspring were weaned onto and reared on the same diets as their dams until they weighed 100–150 g. They were then randomly assigned to dietary groups within each experimental group.

The rats were housed in groups of ten (Expt 1), eight (Expt 2), eight or ten (Expt 3), or six, seven or eight (Expt 4), in plastic cages with tops and bases constructed of stainless-steel wire. The risk of coprophagy was minimal because the spaces between the bars of the floors were wide enough for faecal pellets to fall through. Food and water were supplied *ad lib*. No attempts were made to measure food intake because there was considerable spillage of food. The animals were weighed weekly. After the appropriate periods on the experimental diets they were killed.

*Expt 1.* Four groups, each of ten male rats weighing about 100 g, were fed on the vitamin B<sub>12</sub>-deficient soya diets with or without 20 g supplementary L-histidine/kg, or the corresponding cyanocobalamin-supplemented diets with and without histidine. After 7 weeks the survivors were killed by bleeding from the aorta under diethyl ether anaesthesia. The livers were removed, weighed and stored at -19° until required for the assays.

*Expt 2.* Two groups, each consisting of eight male rats (body-weight approximately 150 g), were fed on the vitamin B<sub>12</sub>-deficient amino acid diet with 8 g DL-homocystine added for 3 or 6 weeks, after which they were killed as described for Expt 1. Two further groups were given the cyanocobalamin-supplemented amino acid diet with the same level of homocystine for the same periods, and two received vitamin B<sub>12</sub>-deficient or cyanocobalamin-supplemented amino acid diets containing 8 g L-methionine/kg. The latter groups were killed after 6 weeks. A seventh group

continued to receive the vitamin B<sub>12</sub>-deficient soya diet, and was killed after 3 weeks.

*Expt 3.* Six groups of male rats that had been reared on the vitamin B<sub>12</sub>-deficient soya diet and six groups reared on the cyanocobalamin-supplemented soya diet were given vitamin B<sub>12</sub>-deficient or -supplemented amino acid diets containing various proportions of methionine or homocystine, or both (Table 3). Another group continued to receive the cyanocobalamin-supplemented soya diet. Each group contained eight or ten rats, each weighing about 100 g at the start of the experiment. All the rats that were given amino acid diets were killed after 4 weeks on the diets as described for Expt 1; those that continued on the soya diet were killed at the same time.

*Expt 4.* Seven male rats were given the vitamin B<sub>12</sub>-deficient amino acid diet containing 2 g L-methionine/kg, and eight received a similar diet with 8 g L-methionine/kg. Two other groups, of six and seven rats respectively, were given cyanocobalamin-supplemented diets with the same supplements of methionine. Each animal weighed about 150 g when the experiment began; all were killed after they had been given the amino acid diets for 10 weeks.

In this experiment the rats were killed by cervical dislocation. The livers were rapidly removed and freeze-clamped between aluminium discs that had been cooled in liquid nitrogen (Wollenberger, Ristau & Schoffa, 1960).

*Determination of total folate content.* Weighed portions of each liver were processed by the method of Bennett, Berry, Chanarin & Ardeman (1964). In Expt 4 the frozen livers were ground to powder in mortars that had been cooled in liquid N<sub>2</sub>. Samples of the powders were treated as described by Osborne-White & Smith (1973), to avoid autolysis and possible breakdown of conjugated folates, to enable pteroylmono-, di- and triglutamate derivatives to be determined. Folate was determined by microbiological assay with *Lactobacillus casei* as the test organism, as described by Spray (1964).

*Other methods.* Vitamin B<sub>12</sub> in liver was determined by the method of Booth & Spray (1960). Liver protein was estimated in diluted liver homogenates by the method of Lowry, Rosebrough, Farr & Randall (1951). All probabilities were calculated using Student's *t* test.

#### RESULTS AND DISCUSSION

*Expt 1.* Addition of L-histidine (20 g/kg diet) to the vitamin B<sub>12</sub>-deficient soya diet reduced the growth of rats significantly (Table 1). Although rats fed on cyanocobalamin-supplemented diets with supplementary histidine had a mean weight gain of 93 g in 7 weeks compared with only 66 g for rats not receiving histidine, the difference was not quite significant ( $P > 0.05$ ). There is no obvious explanation for these effects; if the rats' food intakes could have been measured, the results might have helped to resolve the problem.

The hepatic enlargement due to vitamin B<sub>12</sub> deficiency (Dryden & Hartman, 1966; Williams & Spray, 1970) was again evident, whether histidine was added to the diet or not. Histidine caused significant further growth of the liver, although the effect of

Table 1. *Expt 1.*\* *The effects of supplementary histidine (20 g/kg diet) and cyanocobalamin (75 µg/kg diet) on growth, and on liver weight, protein, vitamin B<sub>12</sub> and folate in groups of ten rats receiving soya-bean flour diets† for 7 weeks*

(Mean values with their standard errors; no. of determinations in parentheses)

Group no.	Dietary supplement	Liver									
		Body-wt gain (g)		Wt (g/kg body-wt)		Protein (mg/g)		Vitamin B <sub>12</sub> (ng/g)		Folate (µg/g)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	None	50 (9)	3.1	43 (9)	2.5	199 (9)	8	33 (9)	2.2	2.8 (9)	0.33
2	Histidine	29 (8)	6.8	73 (8)	0.4	156 (8)	11	28 (8)	5.1	3.3 (8)	0.21
Statistical significance of differences between groups 1 and 2: <i>P</i>		< 0.02		< 0.001		< 0.01		< 0.4		< 0.3	
3	Cyanocobalamin	66 (9)	9.9	32 (9)	1.1	179 (9)	8	136 (9)	10	5.0 (7)	0.34
4	Cyanocobalamin, histidine	93 (9)	7.8	43 (10)	1.1	149 (10)	7	103 (10)	14	6.2 (10)	0.15
Statistical significance of differences between groups 3 and 4: <i>P</i>		< 0.10		< 0.001		< 0.02		< 0.10		< 0.01	

\* For details of experiment, see p. 300.

† For details of diets, see p. 300 and Williams, Spray, Newman & O'Brien (1969).

vitamin B<sub>12</sub> depletion was still present. Histidine significantly decreased the concentrations of protein in the livers of the rats receiving both the vitamin B<sub>12</sub>-deficient and cyanocobalamin-supplemented diets; this suggests that the enlargement was due to some factor other than protein synthesis.

The histidine supplement did not affect the vitamin B<sub>12</sub> content of the liver in the vitamin B<sub>12</sub>-deficient or in the cyanocobalamin-supplemented rats, nor did it affect liver folate in the vitamin B<sub>12</sub>-deficient groups. However, with cyanocobalamin supplementation, histidine gave a significant increase in liver folate content. This might have been due to accumulation of 5-formiminotetrahydrofolate in cyanocobalamin-supplemented rats, due to the further breakdown of formiminoglutamic acid formed from histidine. In vitamin B<sub>12</sub> deficiency this acid would be excreted in the urine.

*Expt 2.* Rats given vitamin B<sub>12</sub>-deficient amino acid diets containing 8 g homocystine/kg for 3 or 6 weeks grew poorly or lost weight (Table 2), whereas those given similar diets supplemented with cyanocobalamin grew reasonably well. Rats receiving diets containing 8 g methionine/kg had moderate weight gains, although growth was reduced by vitamin B<sub>12</sub> deficiency.

Relative to body-weight, rats fed for 3 weeks on vitamin B<sub>12</sub>-deficient diets containing homocystine had smaller livers than those that received the same diet for 6 weeks ( $P < 0.005$ ) or whose diets contained methionine ( $P < 0.02$ ). In rats given

Table 2. *Expt 2\**. The effects of supplementary homocystine (HC) (8 g/kg diet), methionine (Met) (8 g/kg diet) and cyanocobalamin (75 µg/kg diet) on growth, and on liver weight, protein, vitamin B<sub>12</sub> and folate in groups of eight rats receiving amino acid (AA) diets† for 3 or 6 weeks, and in a control group receiving an unsupplemented soya-bean-flour (soya) diet† for 3 weeks

(Mean values with their standard errors; no. of determinations in parentheses)

Group no.	Diet	Dietary supplement	Period on diet (weeks)	Liver									
				Body-wt gain (g)		Wt (g/kg body-wt)		Protein (mg/g)		Vitamin B <sub>12</sub> (ng/g)		Folate (µg/g)	
				Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	AA	HC	3	3.1	3.9	31	0.9	214	6.1	20	2.0	2.5	0.24
				(8)		(8)		(7)		(8)		(6)	
2	AA	HC	6	-1.4	4.8	37	1.6	151	4.7	13	1.9	1.4	0.04
				(8)		(8)		(7)		(8)		(7)	
3	AA	Met	6	7.4	6.6	36	1.5	198	3.9	10	1.2	2.2	0.14
				(8)		(8)		(8)		(8)		(8)	
4	Soya	None	3	—		40	2.1	216	7.5	26	2.3	5.8	0.98
						(6)		(6)		(6)		(6)	
5	AA	HC, cyanocobalamin	3	7.8	9.6	35	1.1	203	7.6	52	7.8	3.8	0.30
				(8)		(8)		(8)		(8)		(8)	
6	AA	HC, cyanocobalamin	6	12.2	9.9	38	1.1	177	7.3	30	4.0	1.6	0.19
				(8)		(8)		(8)		(8)		(8)	
7	AA	Met, cyanocobalamin	6	14.2	6.9	27	0.8	144	11	75	8.8	2.4	0.22
				(8)		(8)		(7)		(8)		(6)	

\* For details of experiment, see p. 300.

† For details of diets, see p. 300 and Williams, Spray, Newman & O'Brien (1969).

diets containing homocystine and cyanocobalamin for 6 weeks the livers were on average similar in weight to those of animals given the corresponding diets without vitamin B<sub>12</sub>. The smallest livers were those from the animals given diets containing both cyanocobalamin and methionine.

The longer that rats were maintained on diets containing homocystine, with or without added cyanocobalamin, the lower were the concentrations of vitamin B<sub>12</sub> and folate in the livers. Compared with homocystine, the addition of methionine to the vitamin B<sub>12</sub>-deficient diet resulted in a slightly reduced liver level of vitamin B<sub>12</sub> but an increased concentration of folate. In the corresponding cyanocobalamin-supplemented groups methionine had a similar effect on liver folate, but in contrast there was an increase in liver vitamin B<sub>12</sub>. All animals given the amino acid diets had less folate in their livers than those that were given the soya diet.

The various dietary treatments did not greatly affect the concentration of protein in the livers, except in the homocystine-supplemented, vitamin B<sub>12</sub>-deficient group and the methionine-supplemented, cyanocobalamin-supplemented group.

*Expt 3.* All the rats given the vitamin B<sub>12</sub>-deficient diets grew poorly except those given the diet containing 8 g methionine/kg (Table 3). The higher and the lower levels

Table 3. *Expt 3\**. The effects of supplementary methionine (*Met*), homocystine (*HC*) and cyanocobalamin on growth, and on liver vitamin B<sub>12</sub> and folate in groups of rats receiving amino acid (*AA*) diets† for 4 weeks, and in a control group receiving a cyanocobalamin-supplemented soya-bean-flour (*soya*) diet† for 4 weeks

(Mean values with their standard errors; no. of determinations in parentheses)

Group no.	No. of rats	Diet	Dietary supplement			Body-wt gain (g)		Wt (g/kg body-wt)		<i>P</i> ‡	Liver					
			Met (g/kg)	HC (g/kg)	Cyanocobalamin (µg/kg)	Mean	SE	Mean	SE		Vitamin B <sub>12</sub> (ng/g)		Total folate (µg/g)			
											Mean	SE	Mean	SE		
1	10	AA	2	—	—	22	3.4	42	1.4	< 0.10	4.5	0.26	1.9	0.21		
						(8)		(8)			(8)		(7)			
2	10	AA	8	—	—	71	9.9	45	1.9	< 0.001	13	3.2	4.7	0.44		
						(7)		(7)			(7)		(7)			
3	10	AA	28	—	—	29	8.0	47	1.9	< 0.001	9.3	1.5	6.7	0.27		
						(7)		(7)			(7)		(7)			
4	8	AA	8	8	—	22	5.8	50	0.9	0.2	6.6	0.97	5.3	0.57		
						(7)		(7)			(7)		(7)			
5	10	AA	—	8	—	-4	3.8	43	1.7	< 0.01	11	2.0	3.2	0.54		
						(5)		(5)			(5)		(4)			
6	10	AA	—	16	—	8.2	8.4	55	5.2	< 0.01	28	4.4	3.2	0.35		
						(4)		(4)			(4)		(4)			
7	10	AA	2	—	75	82	5.2	45	1.0	—	19	2.3	3.8	0.36		
						(8)		(8)			(8)		(8)			
8	8	AA	8	—	75	86	7.2	34	1.7	—	70	9.8	3.9	0.34		
						(7)		(7)			(7)		(6)			
9	8	AA	28	—	75	58	2.8	38	0.9	—	88	7.3	7.6	0.40		
						(7)		(7)			(7)		(6)			
10	8	AA	8	8	75	56	6.6	47	2.3	—	34	3.4	7.3	0.67		
						(8)		(8)			(8)		(8)			
11	8	AA	—	8	75	82	9.6	37	0.7	—	50	5.0	3.3	0.29		
						(8)		(8)			(8)		(7)			
12	8	AA	—	16	75	41	3.8	40	1.1	—	97	4.2	5.6	0.65		
						(8)		(8)			(8)		(8)			
13	8	Soya	—	—	75	100	1.9	40	0.7	—	97	6.6	10	0.67		
						(8)		(8)			(8)		(8)			

\* For details of experiment, see p. 301.

† For details of diets, see p. 300 and Williams, Spray, Newman & O'Brien (1969).

‡ Statistical significance of differences between the vitamin B<sub>12</sub>-deficient diet and the corresponding cyanocobalamin-supplemented diet.

of methionine gave poor growth. Substitution of homocystine for methionine resulted in almost no growth or even loss of weight. Five rats died from the group that received 8 g homocystine/kg diet, as did six rats from the group whose diet contained 16 g homocystine/kg. Diets containing a mixture of 8 g methionine and 8 g homocystine/kg gave poorer growth than did those containing 8 g methionine/kg alone. Although a similar reduction was obtained when both amino acids were added to cyanocobalamin-supplemented diets, growth was adequate when 8 g homocystine/kg was given alone.

All groups fed on cyanocobalamin-supplemented diets gained weight, though some of the increases were small. However, none of the rats grew as well as those fed on the cyanocobalamin-supplemented soya diet.

These results, and those of Expt 2, confirm the well-known ability of homocystine to replace methionine in the growth of rats when adequate amounts of vitamin B<sub>12</sub> are present (Blakley, 1969). This evidently applies in animals fed on diets containing very little protein. Even then, high levels of homocystine inhibited growth. The results of our experiments do not elucidate the mechanism of this inhibition. The fact that homocystine at the 8 g/kg level replaces methionine when there is adequate cyanocobalamin discounts the possibility that this amount of homocystine caused loss of appetite. Higher levels of either methionine or homocystine, or the mixture of 8 g methionine and 8 g homocystine/kg diet, might have had this effect.

Although in some groups growth was poor, in four of the six vitamin B<sub>12</sub>-deficient groups the livers were significantly larger in proportion to body-weight than those of the corresponding animals given the cyanocobalamin-supplemented diets. The exceptions were with the diet containing 2 g methionine/kg and that with 8 g methionine and 8 g homocystine/kg.

Increased amounts of methionine in the food resulted in increased liver folate levels in vitamin B<sub>12</sub>-deficient rats (Table 3), in agreement with the report of Kutzbach *et al.* (1967). The high liver folate levels in the cyanocobalamin-supplemented rats fed on the soya diet (Table 3) were probably due to a high intake of folate from the soya-bean flour.

At 8 g/kg diet, methionine seemed to increase significantly the amounts of vitamin B<sub>12</sub> ( $P < 0.02$ ) and folate ( $P < 0.001$ ) available to the animals compared to the diet with 2 g methionine/kg (Table 3), assuming that the levels in liver reflect availability. A higher concentration of methionine in the diet produced a significant further increase in liver folate ( $P < 0.01$ ) but a slight though insignificant decrease in vitamin B<sub>12</sub> ( $P < 0.3$ ). If the effects on vitamin B<sub>12</sub> and folate were the only factors influencing growth, rats given the diet with 28 g methionine/kg should have grown as well as those given the diet containing 8 g/kg. Possibly the high level of methionine made the diet unpalatable, so that food intake was inadequate. Alternatively, the high level may have upset the amino acid balance in the diet.

A feature of some interest is the association between the higher concentration of homocystine in the diet and increased liver vitamin B<sub>12</sub> (Table 3). This occurred both with vitamin B<sub>12</sub>-deficient ( $P < 0.01$ ) and cyanocobalamin-supplemented ( $P < 0.001$ ) diets. A possible explanation may be that excess homocystine might induce the production of increased quantities of homocystine:tetrahydropteroylglutamate methyltransferase (EC 2.1.1.13), which requires vitamin B<sub>12</sub> as a co-factor.

*Expt 4.* In view of the influence of methionine on liver folate found in Expt 3, we studied the effect of two dietary levels on the distribution of 'free' (unconjugated) and polyglutamyl folates in the livers of vitamin B<sub>12</sub>-deficient and cyanocobalamin-supplemented rats. The total folate concentration in the livers of the rats fed on vitamin B<sub>12</sub>-deficient diets was lower than that in cyanocobalamin-supplemented rats, particularly when the methionine intake was also low (Table 4). Increasing the amount

Table 4. *Expt 4\**. Vitamin B<sub>12</sub> and 'free'† and total folate contents of livers from rats receiving amino acid diets‡ supplemented with 2 or 8 g methionine (Met)/kg, with or without cyanocobalamin (75 µg/kg) for 10 weeks

(Mean values with their standard errors; no. of determinations in parentheses)

Group no.	No. of rats	Dietary supplement (g/kg)	Liver folate							
			Liver vitamin B <sub>12</sub> (ng/g)		'Free' (µg/g)		Total (µg/g)		'Free' / Total × 100	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	7	2 Met	10 (7)	1.3	0.17 (7)	0.02	3.2 (7)	0.47 (7)	5.7 (7)	1.0
2	8	8 Met	20 (8)	3.1	0.42 (8)	0.12	6.2 (7)	0.44 (7)	7.6 (7)	2.4
3	6	2 Met, cyanocobalamin	130 (6)	18	1.6 (6)	0.43	11 (6)	0.85 (6)	18 (6)	4.7
4	7	8 Met, cyanocobalamin	130 (7)	16	1.1 (6)	0.21	10 (7)	0.77 (7)	12 (6)	2.9

\* For details of experiment, see p. 301.

† Unconjugated folates.

‡ For details of diets, see p. 300.

of methionine in the vitamin B<sub>12</sub>-deficient diet from 2 to 8 g/kg nearly doubled the mean values for both 'free' and total liver folate, but had no such effect with animals given the cyanocobalamin-supplemented diet. The 'free' folate fractions were lower as a proportion of the total folate in the vitamin B<sub>12</sub>-deficient than in the cyanocobalamin-supplemented rats.

These results suggested that methionine partially restored the reduced liver folate levels caused by vitamin B<sub>12</sub> deficiency. The increase was mainly in the polyglutamate fraction, as found by Thenen & Stokstad (1973). Additional folate accumulated, with a smaller proportion as polyglutamates, when the diet contained adequate vitamin B<sub>12</sub>. Vitamin B<sub>12</sub> or methionine may have caused increased food intake, as suggested by the improved weight gains of the rats whose diets were supplemented with cyanocobalamin or 8 g methionine/kg, or both (Tables 2 and 3). The extra food could have contributed additional folate for storage.

The concentration of vitamin B<sub>12</sub> in the livers of the rats given the vitamin B<sub>12</sub>-deficient diet with 8 g methionine/kg was higher than in those receiving the diet containing 2 g methionine/kg ( $P < 0.02$ ; cf. Expt 3). There was no effect in cyanocobalamin-supplemented rats, in contrast to the results in Expt 3. This difference may be because in Expt 4 the rats were kept on the diets for 10 weeks, compared with only 4 weeks in Expt 3. In the extra time the livers would have taken up more vitamin B<sub>12</sub> than those of the rats in Expt 3 and the levels might have reached plateaux. Any effect of methionine might therefore be masked. The higher liver vitamin B<sub>12</sub> concentrations in Expt 4 support this.

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