Folic Acid Metabolism in Vitamin B₁₂-Deficient Sheep

DEPLETION OF LIVER FOLATES

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1. Metabolism of folate was studied in six ewes in an advanced state of vitamin B₁₂ deficiency as judged by voluntary food intake and in their pair-fed controls receiving vitamin B₁₂. A group of four animals that were maintained throughout the experiment at pasture was also studied. 2. After 34–40 weeks on the cobalt-deficient diet urinary excretion of formiminoglutamate by four deficient animals was about 3.2 mmol/day and this was not significantly decreased by injection of three of them with about 4.5 µg of [2-¹⁴C]folate/kg body weight per day for 5 days. Three days after the last injection retention of [2-¹⁴C]folate by the livers of the deficient animals (5.5% of the dose) was lower than that of their pair-fed controls (26% of the dose) but there was no evidence of net retention of injected folate in the livers of either group. Urinary excretion of ¹⁴C indicated that renal clearance of folate may have been impaired in very severe vitamin B₁₂ deficiency. 3. As estimated by microbiological assays total folates in the livers of animals at pasture (12.9 µg/g) included about 24% of 5-methyltetrahydrofolate as compared with about 72% of a total of 12.5 µg/g in three further ewes fed on a stock diet of wheaten hay-chaff and lucerne-chaff. Liver folates of vitamin B₁₂-deficient animals (0.5 µg/g) included about 88% of 5-methyltetrahydrofolate as compared with about 51% of a total of 5.2 µg/g in pair-fed animals treated with vitamin B₁₂. 4. Chromatography of liver folates of the pair-fed animals permitted quantitative estimates of the pteroylglutamates present. The results showed that the vitamin B₁₂-deficient livers were more severely depleted of tetrahydrofolates and formyltetrahydrofolates than of methyltetrahydrofolates and that as the deficiency developed they were more severely depleted of the higher polyglutamates than of the monoglutamate within each of these classes. Results from animals injected with [2-¹⁴C]folate indicated an impairment of the exchange between pteroylmonoglutamates and pteroylpolyglutamates in the livers of deficient animals. 5. In vitamin B₁₂-deficient animals with food intakes below 200 g/day some of the liver folates were not completely reduced and some degradation of pteroylpolyglutamates was detected. The latter condition may have been associated with fatty liver. 6. The results are discussed in relation to current theories of vitamin B₁₂-folate interactions.

A deficiency of vitamin B₁₂ is accompanied in many species by symptoms of folic acid deficiency but the mechanism by which vitamin B₁₂ ensures normal folate metabolism remains uncertain. Thus a temporary haematological response may be brought about in human pernicious anaemia by treatment with large

doses of PteGlu* but complete remission requires treatment with vitamin B₁₂ (Mollin *et al.*, 1962). Excessive urinary excretion of formiminoglutamic acid accompanies impairment of the activity of *N*-formimino-L-glutamate-tetrahydrofolate 5-formimino transferase (EC 2.1.2.5). This condition may be brought about by a dietary deficiency either of folate or of vitamin B₁₂ and in either case a dietary supplement of methionine causes urinary excretion of formiminoglutamate to decrease (Silverman & Pitney, 1958).

Vitamin B₁₂ deficiency has been shown to cause depletion of intracellular folate concentrations in sheep liver (Dawbarn *et al.*, 1958), in human erythrocytes (Cooper & Lowenstein, 1964) and in rat liver (Kutzbach *et al.*, 1967). The depletion of

* Abbreviations: PteGlu, pteroyl-L-glutamic acid; H₂PteGlu and H₄PteGlu, 7,8-dihydro- and 5,6,7,8-tetrahydro-pteroyl-L-glutamic acid; 5-CH₃-H₄PteGlu, 5-methyltetrahydropteroyl-L-glutamic acid; 5 (and 10)-HCO-H₄PteGlu, 5 (and 10)-formyltetrahydropteroyl-L-glutamic acid. Forms with additional conjugated γ-glutamyl groups are numbered with a subscript indicating the total number of glutamate residues in the molecule as set out in IUPAC-IUB tentative rules, *Biochem. J.* (1967) 102, 19–20.

folates in erythrocytes (Jeejeebhoy *et al.*, 1965) and in rat liver (Kutzbach *et al.*, 1967) was shown to be largely due to a loss of pteroylpolylglutamates. Kutzbach *et al.* (1967) showed that the depleted liver folates in vitamin B₁₂-deficient rats could be restored to normal by dietary treatment with either vitamin B₁₂ or methionine.

Both vitamin B₁₂ and folic acid are involved in the synthesis of methionine from homocysteine and donors such as serine or formate and it seems likely that the effects of vitamin B₁₂ on retention of folate are mediated via methionine. Gawthorne & Stokstad (1971) showed that the effect of methionine in promoting the retention of folate by the livers of vitamin B₁₂-deficient rats occurs within a few hours of treatment.

Several hypotheses have been proposed to explain the failure of folate metabolism in vitamin B₁₂ deficiency. All of them involve the assumption that there is a decreased intracellular concentration of the essential coenzyme, H₄PteGlu, but this has not been directly shown. The present work was undertaken to define the changes that occur in tissue folates in vitamin B₁₂-deficient sheep to permit a better assessment of the nature of the vitamin B₁₂-folate interaction.

Materials and Methods

Animals

From a group of 23 Australian Merino ewes taken from pasture at 3 years of age (mean body weight 53.4 kg) four were removed at random and returned to pasture where they remained throughout the experiment. The experiment began in early December (early summer) and was concluded in the following September (early spring). The sown pasture was of mixed grasses and legumes and provided abundant feed. It was dry in summer and green during winter and spring. The remaining nineteen animals were used to produce individually pair-fed vitamin B₁₂-deficient and vitamin B₁₂-treated sheep on the diet and under the conditions described by Smith & Marston (1970) except that animals treated with vitamin B₁₂ received a single weekly dose of 250 µg of cyanocobalamin injected intramuscularly in 0.5 ml of water. The cobalt-deficient wheaten hay-chaff contained 0.02 µg of Co/g dry wt. The duration of the experiment for any pair for animals depended on the rapidity of development of symptoms in the deficient sheep (loss of appetite and loss of body weight) and varied from 38 to 43 weeks. Control animals were pair-fed and received vitamin B₁₂ for the final 12–16 weeks. The three pen-fed animals on a diet of equal parts of wheaten hay-chaff and dried lucerne-chaff were not from the homogeneous experimental group but were of similar age and were

drawn from the same flock. The treatment of these animals is described by Osborne-White & Smith (1973).

Throughout the experiment body weights were measured weekly except for animals at pasture which were weighed at six-weekly intervals. After animals were paired, daily food intakes were measured.

Animals were killed and liver extracts prepared as described by Osborne-White & Smith (1973). Extracts of whole blood for folate assay were prepared as described by Bird *et al.* (1965).

Separation and estimation of folates

Microbiological assays, column chromatography of liver extracts, determination of radioactivity and identification of separated folates were as described by Osborne-White & Smith (1973). In addition to the categories of folates detected in control animals, however, chromatograms of extracts of some vitamin B₁₂-deficient livers showed elution profiles in which activity for *Streptococcus faecalis* was more than twice that for *Pediococcus cerevisiae* (assayed at pH 6.3) and in some regions substantially more than that for *Lactobacillus casei*. Where this occurred it was no longer appropriate to apply the equations for differentiation of folates derived by Osborne-White & Smith (1973) and the following modifications were introduced.

1. Where activity for *S. faecalis* exceeded twice the activity for *P. cerevisiae* but did not exceed activity for *L. casei*

$$\left. \begin{aligned} (1) \text{ H}_4\text{PteGlu} &= 2 \text{ P. cerevisiae} \\ (2) \text{ HCO-H}_4\text{PteGlu} &= \text{P. cerevisiae} \\ (3) \text{ Incompletely reduced folate} &= \end{aligned} \right\} \begin{aligned} & \\ & \\ & (\text{S. faecalis} - 2 \text{ P. cerevisiae}) \end{aligned} \quad]$$

(Y series)

2. In those parts of the elution profiles where *S. faecalis* exceeded *L. casei*, eqns. (1) and (2) above were again used but the quantity (*S. faecalis* - 2 *P. cerevisiae*) was regarded as a minimum measure of degraded folates and is referred to as the Z series. The Y series consisted of five components that appeared with some regularity as distinct peaks, particularly the two peaks around fractions 65 and 55. The Z series occurred in only some vitamin B₁₂-deficient livers and showed general activity for *S. faecalis* over a broad area with little apparent structure. Estimates of tetrahydrofolates and of the formyl derivatives are maxima in the regions involved and conversely estimates of incompletely reduced folates and degraded folates are minima, but the quantities present were very small and these distinctions have not been made in reporting results. In calculating areas of overlapping peaks the individual peaks were calculated by an iterative process.

Estimation of vitamin B₁₂

Vitamin B₁₂ in liver and blood serum was estimated with *Lactobacillus leichmanii* as described by Hine & Dawbarn (1954).

Chemical determinations

Formiminoglutamate in urine was measured by the method of Tabor & Wyngarden (1958). Cobalt in the feed was measured by the method of Marston & Dewey (1940).

Materials

[2-¹⁴C]PteGlu was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was used at a specific radioactivity of 125 nCi/μg. Other materials were as described by Osborne-White & Smith (1973) or by Smith & Marston (1970).

Results*Vitamin B₁₂ status of animals*

The vitamin B₁₂ status of the experimental animals is shown in Table 1. Deficient sheep were all severely depleted of vitamin B₁₂ as judged by the concentrations in liver and serum and by the low voluntary food intakes, but except for one animal that

collapsed (no. 218) body weights were not hazardously low. With the possible exception of the latter animal, all the vitamin B₁₂-deficient sheep would probably have responded quickly to treatment with vitamin B₁₂ (Smith & Marston, 1970). Four of the deficient animals had food intakes of less than 200 g/day when killed; in three of these the livers were enlarged (Table 1) and appeared fatty (yellowish and friable). Body weights of vitamin B₁₂-deficient and pair-fed vitamin B₁₂-treated sheep were similar and were about half those of the animals at pasture. Concentrations of vitamin B₁₂ in the livers of the latter animals were slightly higher than those of the animals fed on the vitamin B₁₂-deficient diet and injected with vitamin B₁₂.

Excretion of formiminoglutamate in urine

In pair nos. 1, 2, 3 and 5 urinary formiminoglutamate was measured over the final 6 weeks of the experiment. The results for the first five weeks of these periods are shown in Fig. 1 together with mean food intakes and body weights. Despite a progressive fall in food intake during the period, mean daily excretion of formiminoglutamate in vitamin B₁₂-deficient animals remained at about 3 mmol/day. During this time the pair-fed animals receiving vitamin B₁₂

Table 1. *Vitamin B₁₂-status of experimental animals*

Animals were taken from a group of three-year-old ewes and either fed a cobalt-deficient diet for 38–43 weeks, or returned to pasture for 41 weeks. Pair-fed animals were treated with vitamin B₁₂ (250 μg/week) and restricted in their food intakes to those of individual deficient animals for the final 12–16 weeks. The degree of vitamin B₁₂-deficiency is indicated by low voluntary food intakes (initially 1060 g/day), loss of body weight (initially a mean of 53.4 kg) and low vitamin B₁₂ concentrations in liver and blood serum.

Animal no.	Pair no.	Treatment	Body wt. (kg)	Food intake for last 3 days (g/day)	Wt. of liver (g)	Vitamin B ₁₂ in liver (μg/g)	Vitamin B ₁₂ in serum (ng/ml)
003	1	Cobalt-deficient diet	30.0	295	376	0.03	0.15
230	2		28.2	226	340	0.04	0.19
340	3		32.8	174	553*	0.05	0.21
243	4		31.2	159	355	0.03	0.14
044	5		33.8	155	491*	0.03	0.18
218	6		25.1	99	511*	0.04	0.25
059	1	Pair-fed with corresponding animal on cobalt-deficient diet and injected with 250 μg of vitamin B ₁₂ per week	31.8	295	338	0.99	3.4
339	2		30.2	226	378	0.90	5.8
323	3		32.9	174	338	0.77	4.6
375	4		36.2	159	398	1.10	11.6
042	5		33.2	155	341	0.92	7.9
397	6		29.5	99	322	1.25	11.7
005	—	Maintained at pasture	64	<i>Ad libitum</i>	944	1.2	4.2
006	—		63	<i>Ad libitum</i>	1024	1.2	2.9
021	—		63	<i>Ad libitum</i>	1032	1.1	2.8
047	—		67	<i>Ad libitum</i>	941	1.3	3.2

* Macroscopically fatty liver.

excreted negligible amounts of formiminoglutamate (mean $0.037 \pm \text{S.E.M. } 0.007 \text{ mmol/day}$, 20 observations).

During the sixth week pair nos. 1, 2 and 3 were injected with $[2-^{14}\text{C}]\text{PteGlu}$ ($86.6 \mu\text{Ci}$ in $693 \mu\text{g}$ over 5 days, or about $4.5 \mu\text{g}$ of folate/kg body wt. per day). In one vitamin B_{12} -deficient animal (no. 340) formiminoglutamate excretion fell from 3.70 mmol/day to 2.08 mmol/day during the period of treatment but there was no change with either of the other deficient animals and the effect was not statistically significant (mean excretion of the four deficient animals during the final week was $2.94 \pm 0.29 \text{ mmol/day}$).

Fig. 1. Excretion of formiminoglutamate in urine of vitamin B_{12} -deficient and pair-fed vitamin B_{12} -treated sheep

Daily excretion of formiminoglutamate was measured for the final 6 weeks of the experiment in four of the six pairs of animals (animals no. 243 and no. 375 were measured for 1 week only and no measurements were made for animals no. 218 and no. 379). The figure shows the results for the first five weeks of these intervals together with mean body weights and mean food intakes. $\circ$, Vitamin B_{12} -deficient animals; $\bullet$, vitamin B_{12} -treated animals. The vertical bars represent S.E.M. for the values shown. In the sixth week three of the pairs of animals were injected with $[2-^{14}\text{C}]\text{PteGlu}$ but without significant effect on excretion of formiminoglutamate (see the text).

Retention of $[2-^{14}\text{C}]\text{PteGlu}$

The three pairs of animals that received labelled folate were injected with $8.66 \mu\text{Ci}$ of $[2-^{14}\text{C}]\text{PteGlu}$ twice daily for five days and killed 68 h after the final dose. A fourth pair on a similar regime were killed on the third day after only two days of treatment with $[2-^{14}\text{C}]\text{PteGlu}$ because the vitamin B_{12} -deficient member of the pair collapsed. Progressive urinary excretion of ^{14}C in the eight animals is shown in Fig. 2. In animals that received vitamin B_{12} (Fig. 2b) the course of urinary excretion of labelled folate was fairly uniform and amounted to about $22 \mu\text{Ci}$ over the 7 day period. In two of the vitamin B_{12} -deficient

Fig. 2. Urinary excretion of injected $[2-^{14}\text{C}]\text{PteGlu}$

The graphs show the cumulative urinary excretion of ^{14}C in vitamin B_{12} -deficient (a) and pair-fed vitamin B_{12} -treated (b) sheep injected daily with $17.3 \mu\text{Ci}$ ($138 \mu\text{g}$) of $[2-^{14}\text{C}]\text{PteGlu}$. The animals used were pair no. 1 ($\circ$), pair no. 2 ($\bullet$), pair no. 3 (Δ) and pair no. 6 ($\blacktriangle$) (see Table 1). The latter pair were treated for only 2 days and killed on the third day, but pair nos. 1, 2 and 3 were treated for 5 days and killed 3 days after the last injection.

animals about 35 μ Ci of ¹⁴C appeared in the urine over the same period and in the third animal about 23 μ Ci was excreted in this time. The rate of excretion of ¹⁴C in the animal that collapsed was less than half that of any other animal over the 2 day period for which it was studied, although urine volumes were not depressed.

Faecal excretion of injected [2-¹⁴C]PteGlu was small. Over the 7 day period an average of 1.1 μ Ci was excreted via this route by the three vitamin B₁₂-deficient animals and 0.9 μ Ci by the animals treated with vitamin B₁₂ (Table 2).

Retention of [2-¹⁴C]PteGlu by liver is shown in Table 2 together with the ¹⁴C content and the folate activity of whole blood determined by microbiological assay without conjugase treatment. Three days after the last injection the livers of animals treated with vitamin B₁₂ retained an average of 25.9% of the injected ¹⁴C whereas livers of vitamin B₁₂-deficient animals retained only about 5.5%. The concentrations both of ¹⁴C and of folate active for *L. casei* in blood, however, was higher in vitamin B₁₂-deficient animals than in their pair-fed controls receiving vitamin B₁₂. These differences were also apparent in the pair of animals killed after 2 days of treatment. Despite very high amounts both of radioactivity and of *L. casei* folate activity in the blood of the vitamin B₁₂-deficient animal, liver retention of labelled folate was low.

A further difference between vitamin B₁₂-deficient and control animals that was particularly pronounced with the terminally deficient animal was the comparative activities for *L. casei* and *S. faecalis* in blood. In vitamin B₁₂-deficient animals activity for *L. casei* was higher whereas that for *S. faecalis* was lower than in the corresponding pair-fed treated animals. In blood from vitamin B₁₂-treated animals activity for *S. faecalis* exceeded that for *L. casei* but this was true only for that vitamin B₁₂-deficient animal with the highest food intake; in the most severely affected animal activity for *L. casei* in blood greatly exceeded that for *S. faecalis* and indicated the presence of a high concentration of 5-CH₃-H₄PteGlu.

Folate activity of whole liver extracts

In Table 3 the folate activity for the three assay organisms measured before and after treatment of liver extracts with conjugase (γ -glutamylcarboxypeptidase) is shown for individual pair-fed animals, for the group of four animals kept at pasture and for a group of three animals kept in pens and fed on a stock diet of wheaten hay-chaff and lucerne-chaff. The pair-fed animals have been subdivided in the table into two categories with food intakes respectively above and below 200g/day. The results for all three assays show large effects of treatment, with the highest activity for each assay in the pasture-fed

Table 2. Retention of labelled folate by pair-fed animals

Animal no.	Pair no.	Vitamin treatment	Total [2- ¹⁴ C]PteGlu injected (μ Ci)	Specific radioactivity			Folate activity in blood			Distribution of administered [¹⁴ C]folate (%)			
				Liver (nCi/g)	Blood (nCi/ml)		<i>L. casei</i> (ng/ml)	<i>S. faecalis</i> (ng/ml)		Liver	Blood*	Urine	Faeces counted for
003†	1	-B ₁₂	86.6	11.8	0.083		1.66	2.32		5.1	0.3	40.1	1.2
059†	1	+B ₁₂	86.6	52.6	0.068		1.15	4.01		20.6	0.3	25.1	1.2
230†	2	-B ₁₂	86.6	15.7	0.091		2.84	1.06		6.2	0.3	41.1	0.8
339†	2	+B ₁₂	86.6	68.6	0.039		1.00	3.10		29.9	0.1	24.9	0.9
340†	3	-B ₁₂	86.6	8.1	0.111		1.77	1.34		5.2	0.4	26.9	1.7
323†	3	+B ₁₂	86.6	69.7	0.039		1.19	3.38		27.1	0.1	27.8	1.0
218†	6	-B ₁₂	34.6	11.2	0.898		18.67	0.54		16.5	6.5	5.1	0.5
397†	6	+B ₁₂	34.6	25.1	0.053		1.67	3.56		23.3	0.5	13.5	0.5
													Not ac- counted for
													53.3
													52.8
													51.6
													44.2
													65.8
													71.4
													62.2

* Blood volume was assumed to be 10% of body weight.

† Injected with [2-¹⁴C]PteGlu twice daily over 5 days and killed 68 h after last injection.

‡ Injected with [2-¹⁴C]PteGlu twice daily over 2 days and killed 18 h after last injection.

Table 3. *Folate activity in whole liver extracts*

Activities for the three assay organisms before and after treatment of liver extracts with hog kidney conjugase are shown for vitamin B₁₂-deficient sheep and their pair-fed controls receiving vitamin B₁₂ and for two other groups of animals. Results for the pair-fed sheep are shown individually and are presented in two categories with food intakes respectively above and below 200 g/day. Mean results only are shown for a control group of animals kept at pasture during the experiment and for three further animals fed on a stock diet of wheaten hay-chaff and dried lucerne-chaff. Means and S.E.M. are given with the numbers of observations in parentheses.

		Folate activity (ng/g)					
Assay organism ...		<i>L. casei</i>		<i>S. faecalis</i>		<i>P. cerevisiae</i>	
Animal no.	Treatment	No conjugase	With conjugase	No conjugase	With conjugase	No conjugase	With conjugase
003*	Vitamin B ₁₂ -deficient	0.043	1.06	0.007	0.103	0.002	0.055
230*	Food intake >200 g/day	0.054	0.69	0.013	0.099	0.003	0.094
	Mean ± S.E.M.	0.049 ± .006	0.88 ± .19	0.010 ± .003	0.101 ± .002	0.003 ± .001	0.075 ± .020
		(2)	(2)	(2)	(2)	(2)	(2)
340*	Vitamin B ₁₂ -deficient	0.050	0.29	0.010	0.041	<0.002	0.033
243	Food intake <200 g/day	0.053	0.26	0.014	0.049	<0.002	0.039
044		0.049	0.23	0.011	0.019	<0.002	0.026
	Mean ± S.E.M.	0.051 ± .001	0.26 ± .02	0.012 ± .001	0.036 ± .009	<0.002	0.033 ± .004
		(3)	(3)	(3)	(3)	(3)	(3)
059*	Pair-fed vitamin B ₁₂ -	0.59	2.98	0.176	2.05	0.071	1.01
339*	treated. Food intake	0.30	6.38	0.052	3.53	0.030	3.30
	>200 g/day						
	Mean ± S.E.M.	0.45 ± .15	4.68 ± 1.70	0.114 ± .062	2.97 ± .74	0.051 ± .021	2.16 ± 1.15
		(2)	(2)	(2)	(2)	(2)	(2)
323*	Pair-fed vitamin B ₁₂ -	0.53	4.21	0.137	1.14	0.059	0.91
375	treated. Food intake	0.13	8.13	0.113	4.89	0.052	1.32
042	<200 g/day	0.27	4.39	0.066	1.16	0.011	1.06
	Mean ± S.E.M.	0.31 ± .12	5.58 ± 1.28	0.105 ± .021	2.40 ± 1.25	0.041 ± .015	1.10 ± .12
		(3)	(3)	(3)	(3)	(3)	(3)
	Pasture fed						
	Mean ± S.E.M.	0.41 ± .04	12.95 ± .37	0.102 ± .019	9.85 ± 1.15	0.071 ± .015	7.51 ± .52
		(4)	(4)	(4)	(4)	(4)	(4)
	Pen-fed stock diet†						
	Mean ± S.E.M.	0.34 ± .08	12.52 ± 1.21	0.048 ± .004	3.55 ± .20	0.018 ± .003	3.13 ± .57
		(3)	(3)	(3)	(3)	(3)	(3)

* Injected with 693 µg of [2-¹⁴C]PteGlu over 5 days and killed 3 days later.

† Values from Osborne-White & Smith (1973).

animals and the lowest activity in the vitamin B₁₂-deficient animals with food intakes of less than 200 g/day. Although the total liver folates of vitamin B₁₂-deficient animals eating less than 200 g/day were consistently lower than those eating more than 200 g/day there were no comparable differences due to food intake in the pair-fed animals treated with vitamin B₁₂.

The results contain no evidence of an increase of liver folates in either vitamin B₁₂-deficient or treated sheep as a result of injection with [2-¹⁴C]-

folate, even though in the deficient animals the amount of folate injected was from 2–4 times the total liver load. Retention of [2-¹⁴C]folate in the livers of both vitamin B₁₂-deficient and -treated sheep may therefore represent exchange between existing stable folate pools and the injected material.

A further difference between treatments is apparent in the fraction of total folate activity available for assay without conjugase treatment. For animals at pasture or fed on a diet of hay-chaff and lucerne-chaff about 3% of the total activity for *L. casei* was

available without treatment with conjugase. In animals receiving vitamin B₁₂ and restricted amounts of food this fraction was about 7% but the concentration of 'unconjugated' material was about the same as that in the former two groups. In vitamin B₁₂-deficient animals with food intakes greater than 200 g/day, 'unconjugated' folates amounted to about 6% of total folates but when food intake was less than 200 g/day this fraction increased to almost 20%. A similar trend was found in *S. faecalis* activity, but with *P. cerevisiae* the tendency was less pronounced.

A characteristic feature of each of the treatment groups was the fraction of total folate (active for *L. casei*) that was active for *P. cerevisiae*. This fraction was highest in the pasture-fed animals (58%) and lowest in the vitamin B₁₂-deficient animals (about 10%). Intermediate values were obtained for paired animals receiving vitamin B₁₂ (29%) and for the pen-fed animals (25%).

In all treatment groups activity for *S. faecalis* was more closely related to that for *P. cerevisiae* than to that for *L. casei*. In most cases the value of the ratio *S. faecalis*/*P. cerevisiae* lay within the range 1.1 to 1.3 (the mean value of 1.7 for animals treated with vitamin B₁₂ was higher because of the result for animal no. 375).

The basis of the differences in whole liver folates between vitamin B₁₂-deficient and vitamin B₁₂-treated sheep was established by chromatographic separation of the folates followed by differential microbiological assay.

Chromatography of liver folates

Results of chromatograms of liver extracts of two pairs of animals that were treated with [2-¹⁴C]PteGlu are shown in Figs. 3 and 4. In Fig. 3 (animals no. 230 and no. 239) the results for the vitamin B₁₂-deficient animal are similar to those for the other deficient animal with food intake above 200 g/day (animal no. 003). Only to a small extent in an occasional fraction did activity for *S. faecalis* exceed twice that for *P. cerevisiae* to indicate the presence of incompletely reduced folates. At no point was activity for *S. faecalis* greater than that for *L. casei*. For both vitamin B₁₂-deficient and -treated animals radioactivity was fairly closely representative of activity for *L. casei* except for the presence of a disproportionately high peak of radioactivity around fraction 65 in the profile for the deficient animal.

Although activity for *L. casei* was much smaller in the vitamin B₁₂-deficient animal than in the paired control the general profiles of activity were similar and the various separated fractions were represented in the deficient animal approximately in the proportions found in animals treated with vitamin B₁₂. Profiles of activity for *S. faecalis* and *P. cerevisiae*

were smaller relative to the control animal than was the case for *L. casei* and the proportions of the profiles are more difficult to discern in Fig. 3 on the scale used. Nevertheless activity for *P. cerevisiae* and *S. faecalis* emerged in definite peaks at positions of the major peaks of those activities in control animals. The results for assays both before and after conjugase treatment indicate a more pronounced depletion of all components active for *S. faecalis* and *P. cerevisiae* than of those active only for *L. casei* but show no major distortion in the profiles representing the proportions of the polyglutamate forms.

In Fig. 4 (animals no. 340 and no. 323) the results for the vitamin B₁₂-deficient member are representative of the three deficient animals with food intakes below 200 g/day and show several distinctive features. First, the profile of *L. casei* activity was no longer a miniature of that of the vitamin B₁₂-treated animals but showed excessive depletion of higher polyglutamates and a tendency for unconjugated folates to accumulate. Among the animals in this category only this pair was given [2-¹⁴C]PteGlu but it is clear that the radioactivity profile did not follow the activity of *L. casei* as closely as with animals receiving vitamin B₁₂ or as with deficient animals with food intakes above 200 g/day (see Table 4). The isolated peak of radioactivity around fraction 65 was accompanied by microbiological activity for *L. casei* and *S. faecalis* that was available for assay without conjugase treatment. The presence of this microbiologically active material however could not be attributed to treatment with [2-¹⁴C]PteGlu as it occurred in all three vitamin B₁₂-deficient animals with food intakes below 200 g/day. Thus the second characteristic feature of chromatograms of liver folates of deficient animals in this category was the appearance below fraction 100 of activity for *S. faecalis* in excess of twice the activity for *P. cerevisiae* but not exceeding the activity for *L. casei*. Such activity is consistent with the presence of incompletely reduced folates. The third distinctive feature relates to the appearance beyond fraction 110 of activity for *S. faecalis* that was not accompanied by activity for either *L. casei* or *P. cerevisiae* and that appeared only after treatment with conjugase. From Fig. 4 the latter material did not exchange with injected labelled folate.

Table 4 shows that the separated folates (active for *L. casei*) in the livers of vitamin B₁₂-treated animals that were injected with [2-¹⁴C]PteGlu were uniformly labelled in comparison with those from vitamin B₁₂-deficient livers. The latter contained material of relatively high specific radioactivity around fraction 24 as well as around fraction 65 and in animal no. 340 material with very low specific radioactivity emerged around fractions 55 and 85.

In all vitamin B₁₂-deficient animals the most profound effect was the severe depletion of all classes of folate that were active for *P. cerevisiae*.

Fig. 3. Liver folates in pair-fed vitamin B_{12} -deficient and vitamin B_{12} -treated sheep with food intakes greater than 200g/day (pair no. 2)

Results of measurements of radioactivity and microbiological activity for the three assay organisms after chromatographic separation of liver folates in pair-fed animals. Results for the vitamin B_{12} -deficient extract are identified by the symbol [-] (no. 230, extract equivalent to 9.34g of liver chromatographed) and for the vitamin B_{12} -sufficient extract by the symbol [+] (no. 339, extract equivalent to 9.29g of liver chromatographed). Where microbiological activity was below the limits of detection this is indicated by a broken line. Fractions were all of 10ml. The elution profiles for vitamin B_{12} -deficient and -treated extracts did not coincide in the positions of the various peaks. These emerged up to 20 fractions earlier in the case of the deficient extract. This effect was presumably due to some variation in operating conditions and is not regarded as significant.

Distribution of liver folates

Table 5 shows the distribution of liver folates in five pairs of animals estimated as previously described (Osborne-White & Smith, 1973) or by the

modified method given in the Materials and Methods section. Table 6 shows individual results for the additional categories of 'folates' indicated by the presence of activity for *S. faecalis* in excess of twice

Fig. 4. Liver folates in pair-fed vitamin B₁₂-deficient and vitamin B₁₂-treated sheep with food intakes below 200 g/day (pair no. 3)

The results are to be compared with those of Fig. 3 but it should be noted that the scales are different. The significance of the symbols is the same as in Fig. 3. Vitamin B₁₂-deficient animal [-] (no. 340, extract equivalent to 9.47 g of liver chromatographed); vitamin B₁₂-treated animal [+] (no. 323, extract equivalent to 9.61 g of liver chromatographed).

the activity for *P. cerevisiae* (Y and Z series). The estimation of these substances and the effects of their presence on estimation of reduced folates is described in the Materials and Methods section.

All distinguishable classes of folates with the exception of the Y series and the Z series were severely depleted in vitamin B₁₂-deficient livers but no folate was completely absent and there was no overwhelming preponderance of any single class of folates in the

deficient livers. (The relatively high value for 5-CH₃-H₄PteGlu in vitamin B₁₂-deficient animals was partly due to a very high content of this substance in the liver of animal no. 044.) In Fig. 5 the mean concentration in vitamin B₁₂-deficient livers of all unambiguously distinguishable folates is expressed as a fraction of the mean concentration of the same substance in the pair-fed animals treated with vitamin B₁₂. Omitting the result for 5-CH₃-H₄PteGlu

Table 4. *Specific radioactivity of separated folates*

The specific radioactivity of discernible radioactive peaks in chromatograms of liver extracts of pair-fed vitamin B₁₂-deficient and vitamin B₁₂-treated sheep like those shown in Figs. 3 and 4 are given. The results show means and s.e.m. for the numbers of observations shown in parentheses. Concentrations of folate were determined by microbiological assay with *L. casei* after treatment with hog-kidney conjugase.

Animal no. ... Pair no. Radioactive peak around fraction no.	Specific radioactivity (nCi/ μ g)					
	Vitamin B ₁₂ -deficient sheep			Pair-fed, vitamin B ₁₂ -treated sheep		
	003 1	230 2	340 3	059 1	339 2	323 3
24	57 $\pm$ 18 (2)	39 $\pm$ 5 (2)	27 $\pm$ 6 (2)	17 $\pm$ 1 (2)	34 $\pm$ 17 (2)	19 $\pm$ 3 (2)
40	50 $\pm$ 5 (2)	34 $\pm$ 8 (2)	15 $\pm$ 1 (2)	12 $\pm$ 4 (2)	12 $\pm$ 1 (2)	16 $\pm$ 1 (2)
55	29 $\pm$ 2 (2)	24 $\pm$ 3 (2)	3 $\pm$ 1 (3)	12 $\pm$ 1 (3)	12 $\pm$ 2 (3)	11 $\pm$ 1 (3)
65	70 $\pm$ 12 (4)	125 $\pm$ 13 (3)	10 $\pm$ 1 (3)	15 $\pm$ 2 (3)	25 $\pm$ 1 (4)	16 $\pm$ 2 (3)
86	56 $\pm$ 11 (3)	41 $\pm$ 2 (3)	1 $\pm$ 1 (3)	15 $\pm$ 2 (3)	14 $\pm$ 2 (3)	16 $\pm$ 1 (3)
119	32 $\pm$ 2 (3)	25 $\pm$ 2 (6)	8 $\pm$ 1 (4)	14 $\pm$ 1 (3)	9 $\pm$ 1 (5)	13 $\pm$ 1 (4)
158	25 $\pm$ 2 (5)	19 $\pm$ 1 (5)	31 $\pm$ 6 (11)	16 $\pm$ 1 (5)	10 $\pm$ 1 (8)	11 $\pm$ 1 (6)
212	30 $\pm$ 1 (8)	22 $\pm$ 1 (10)	38 $\pm$ 1 (8)	14 $\pm$ 1 (7)	8 $\pm$ 1 (11)	13 $\pm$ 1 (7)
290	35 $\pm$ 3 (7)	14 $\pm$ 1 (11)	37 $\pm$ 6 (8)	14 $\pm$ 1 (9)	6 $\pm$ 1 (11)	13 $\pm$ 1 (7)

Fig. 5. *Relative concentrations of folates in vitamin B₁₂-deficient livers as compared with their pair-fed controls receiving vitamin B₁₂*

The data are taken from Table 5 and show the extent of depletion of liver folates as a function of polyglutamate chain length. Only those folates believed to be unambiguously identified have been included (see the text). ○, 5-Methyltetrahydropteroylglutamates; ●, tetrahydropteroylglutamates; Δ, 10-formyltetrahydropteroylglutamates; ▲, 5-formyltetrahydropteroylglutamates.

in animal no. 044 the vitamin B₁₂-deficient livers contained about 18% of the concentration of 5-CH₃-H₄PteGlu found in the pair-fed controls. With more highly conjugated methyltetrahydrofolates this frac-

tion declined to reach a minimum of about 8% for 5-CH₃H₄PteGlu₇. Depletion of tetrahydrofolate was more severe. Deficient livers contained about 8% of the concentration of H₄PteGlu in the livers of the pair-fed controls and this fraction fell to 2% for H₄PteGlu₇. Values for the concentrations of formyltetrahydrofolates were smaller and more variable; because of the incomplete resolution of the 5 and 10 series only those chromatographic peaks regarded as single substances have been included in Fig. 5. The results indicate that the two formyltetrahydrofolate series resembled the tetrahydrofolate series rather than the 5-methyltetrahydrofolate series.

Because of the wide range of values for many of the separated folates within treatment groups Table 5 reports only means and ranges. The statistical analysis shown for Table 5 refers to the differences between individual values for pair-fed animals as determined by the Wilcoxon (1945) signed rank test. There were no significant differences between pairs of animals eating more than 200g/day and those eating less in the extent to which vitamin B₁₂-treated animals differed from vitamin B₁₂-deficient animals. This permitted combination of sufficient results for each of the folates listed (five observations per treatment) to make the test viable. The difference between vitamin B₁₂-deficient and vitamin B₁₂-treated animals was significant ($P < 0.05$) for all the folates listed except 5-CH₃-H₄PteGlu and the unidentified components X1 and X4.

In Table 6 are given the results for both the 'incompletely reduced' folates below fraction 110 (Y series)

Table 5. *Concentration of liver folates (means and ranges)*

Means and ranges are given for the estimated concentrations of liver folates in five pairs of vitamin B₁₂-deficient and vitamin B₁₂-treated animals. The Wilcoxon (1945) signed rank test was used to determine whether the differences between the values for individual pair-fed animals were significantly greater than zero.

Component no.	Identity*	Vitamin B ₁₂ -deficient (5) (ng/g)	Vitamin B ₁₂ -treated pair-fed (5) (ng/g)
M1	5-CH ₃ -H ₄ PteGlu	57 (8.6–219)	80 (45–132)†
M2	5-CH ₃ -H ₄ PteGlu ₂	7.7 (3.1–11.5)	55 (17–158)
M3	5-CH ₃ -H ₄ PteGlu ₃	9.0 (1.3–21.6)	64 (14–122)
M4	5-CH ₃ -H ₄ PteGlu ₄	18 (4.1–28)	133 (30–233)
M5	5-CH ₃ -H ₄ PteGlu ₅	49 (21–81)	356 (184–562)
M6	5-CH ₃ -H ₄ PteGlu ₆	134 (31–312)	1380 (840–1780)
M7	5-CH ₃ -H ₄ PteGlu ₇	61 (21–178)	820 (430–1380)
H1	H ₄ PteGlu	5.3 (0.5–18.9)	62 (11–155)
H2	H ₄ PteGlu ₂	2.2 (0.2–7.1)	34 (3.8–63)
H3	H ₄ PteGlu ₃	1.8 (0–5.1)	70 (10–127)
H4	H ₄ PteGlu ₄	5.1 (0.7–9.9)	131 (53–222)
H5	H ₄ PteGlu ₅	11 (0.6–16)	217 (41–389)
H6	H ₄ PteGlu ₆	18 (0.4–28)	708 (99–1630)
H7	H ₄ PteGlu ₇	4.9 (0–13)	262 (52–457)
F1	10-HCO-H ₄ PteGlu	1.0 (0–4.3)	17 (10–37)
F2	10-HCO-H ₄ PteGlu ₂	1.7 (0–5.2)	14 (6.1–28)
F3	10-HCO-H ₄ PteGlu ₃ + 5-HCO-H ₄ PteGlu	1.1 (0.1–3.6)	12 (0–30)
F4	5-HCO-H ₄ PteGlu ₂	0.3 (0–1.0)	6.6 (1.2–22)
F5	10-HCO-H ₄ PteGlu ₄	0.2 (0–0.6)	9.4 (0–22)
F6	5-HCO-H ₄ PteGlu ₃	0.4 (0–1.4)	9.4 (2.7–17)
F7	10-HCO-H ₄ PteGlu ₅	2.0 (0–4.9)	38 (9.0–58)
F8	5-HCO-H ₄ PteGlu ₄	1.0 (0–2.9)	45 (20–74)
F9	10-HCO-H ₄ PteGlu ₆ + 5-HCO-H ₄ PteGlu ₅	3.3 (1.4–4.9)	101 (16–260)
F10	10-HCO-H ₄ PteGlu ₇ + 5-HCO-H ₄ PteGlu ₆	9.4 (6.1–14)	434 (173–1000)
F11	5-HCO-H ₄ PteGlu ₇	9.0 (6.6–13)	199 (84–406)
X1	Unknown <i>L. casei</i> activity around fraction 7	0.6 (0–2.0)	3.3 (0–6.0)†
X2	Unknown <i>L. casei</i> activity around fraction 23	3.7 (0.2–7.0)	17 (8.0–26)
X3	Unknown <i>L. casei</i> activity around fraction 38	6.1 (1.9–11)	51 (12–121)
X4	Unknown <i>L. casei</i> activity around fraction 56	8.8 (0–26)	41 (0–153)†

* According to the scheme proposed by Osborne-White & Smith (1973).

† Difference between pair-fed animals not significant. In all other cases $P < 0.05$.

and for the 'degraded folates' that eluted beyond fraction 110 (Z series). The results show that incompletely reduced folates appeared in both vitamin B₁₂-deficient and -treated animals at food intakes below 200g/day. The major component emerged around fraction 65 and was present in higher concentration in the livers of deficient animals than in their pair-fed controls. This component was probably either H₂PteGlu or PteGlu. Degraded folates appeared only in vitamin B₁₂-deficient animals and only at food intakes below 200g/day [the values listed are for the quantity (*S. faecalis*–2 *P. cerevisiae*) activity whether or not *S. faecalis* exceeded *L. casei*].

No regularity of profile was evident for the Z components which have been arbitrarily divided into five parts. The presence of this material was not apparent in the assays of whole liver folates in these animals.

Discussion

Although one of the effects of a deficiency of vitamin B₁₂ was to cause an increase in the proportion of methyltetrahydrofolates in liver and a corresponding decrease in the proportion of other reduced folates, the relative amounts of these substances were influenced by dietary factors other than vitamin

Table 6. *Estimated concentrations of incompletely reduced or degraded folates in livers of vitamin B₁₂-deficient and pair-fed vitamin B₁₂-treated sheep*
 The results are supplementary to Table 5 and show the estimated concentrations of a number of unidentified components detected in vitamin B₁₂-deficient livers. The criteria used to define the presence of these substances are described in the text.

Com- ponent no.	Identity	Folate concn. (ng/g)											
		Vitamin B ₁₂ -deficient sheep						Pair-fed vitamin B ₁₂ -treated sheep					
		Animal no.	...	003	230	340	243	044	059	339	323	375	042
		Pair no.	...	1	2	3	4	5	1	2	3	4	5
Y1	Incompletely reduced folate, fractions 29-44			0	0.3	2.6	4.8	0	0	0	0	0	17
Y2	Incompletely reduced folate, fractions 45-59			0.1	1.4	5.4	6.8	7.0	0	0	24	11	7.3
Y3	Incompletely reduced folate, fractions 60-75			0	0.3	24	6.4	86	0	0	3.3	3.0	12
Y4	Incompletely reduced folate, fractions 76-89			0	1.3	4.8	4.7	1.7	0	0	0	0	0
Y5	Incompletely reduced folate, fractions 90-110			0.3	0.8	6.9	3.4	10	0	0	0	0	0
Z1	Degraded folate, fractions 111-152			0.3	0	13	10	91	0	7.9	0	0	11
Z2	Degraded folate, fractions 153-194			0	3.3	42	10	45	0	0	0	0	0
Z3	Degraded folate, fractions 195-236			0	3.2	48	11	28	0	0	0	0	61
Z4	Degraded folate, fractions 237-278			0.4	4.2	25	8.5	28	0	0	0	59	0
Z5	Degraded folate, fractions 279-320			0.4	4.1	35	16	28	0	33	0	0	23

B₁₂. From the data of Table 3 it may be calculated that methyltetrahydrofolate (material active only for *L. casei*) comprised about 72% of the total liver folates of sheep fed on a stock diet of cereal hay-chaff and lucerne-chaff, but only 24% of almost identical concentrations of total liver folates in pasture-fed animals. The former diet contained more than 0.2 µg of Co/g (dry wt.) and so provided adequate cobalt for the vitamin B₁₂ needs of the animal (Marston, 1970) and the livers of the pasture-fed sheep were shown to contain adequate concentrations of vitamin B₁₂. The livers of vitamin B₁₂-treated sheep contained concentrations of vitamin B₁₂ approaching those of the pasture-fed animals but total folate concentrations less than half those of the latter. Methyltetrahydrofolates accounted for 51% of total liver folates in the vitamin B₁₂-treated group and 87% of the very low concentrations in the livers of the vitamin B₁₂-deficient animals. Thus proportions of methyltetrahydrofolate ranging from 24% to 72% were all associated with diets that ensured adequacy of vitamin B₁₂ status and the two extreme values both referred to equally high concentrations of total liver folates.

The specific failure of the vitamin B₁₂-deficient livers to retain dietary folate is shown both by the large differences in liver folates of the pair-fed animals and by the failure of the deficient livers to take up, other than by way of exchange, any of the substantial amounts of labelled folate that were injected. The failure of this dose to augment the pool of metabolically active folate in vitamin B₁₂-deficient sheep is also indicated by the absence of a significant effect on excretion of formiminoglutamate in the urine.

The specific radioactivity of liver folates in vitamin B₁₂-deficient animals injected with [2-¹⁴C]PteGlu (mean 21 µCi/µg of *L. casei* folate) was somewhat higher than that in pair-fed animals receiving vitamin B₁₂ (mean 15 µCi/µg) presumably because the tissues of deficient animals contained less folate. If this difference is taken into account then the rate of urinary excretion of ¹⁴C by vitamin B₁₂-deficient animals no. 003 and no. 230 represented a rate of elimination of total folate about the same as that for the vitamin B₁₂-treated sheep. By similar reasoning the rate of urinary excretion of injected folate by the severely vitamin B₁₂-deficient animal no. 340 was less, and by the terminally deficient animal no. 218 was much less, than in their respective pair-fed controls. Decreased rates of urinary excretion of injected folates by vitamin B₁₂-deficient patients have been recorded by Mollin *et al.* (1962) and by Chanarin & Perry (1968) but Nixon *et al.* (1969) and Nixon & Bertino (1972) report more rapid urinary excretion of injected folate by such patients. The present findings indicate a considerable variation between animals in the excretion of injected folates

by vitamin B₁₂-deficient sheep and suggest that urinary excretion may become abnormally low only when the deficiency is very severe. The blood of the terminally vitamin B₁₂-deficient animal no. 218 contained very high concentrations of methyltetrahydrofolate at an apparent specific radioactivity (48 nCi/µg) about 40% that of the injected [2-¹⁴C]-PteGlu. Assuming circulating blood volume to have been 10% of body weight then about 6.5% of the injected dose of ¹⁴C was present in the bloodstream of animal no. 218 at slaughter as compared with 0.5% in its pair-fed control.

The depleted state of liver folates in advanced vitamin B₁₂-deficiency was characterized by specific relationships in the proportions of the residual folates (Fig. 5). Although depletion of methyltetrahydrofolates was severe, depletion of tetrahydrofolates and the parallel loss of the formyl derivatives was even more drastic. Throughout each of these series the deficit of higher polyglutamates was more pronounced than that of monoglutamates but this effect became apparent only at food intakes below 200 g/day.

The increased ratio of methyltetrahydrofolates to non-methylated forms presumably reflects a failure of deficient tissues to carry out the vitamin B₁₂-dependent conversion of methyltetrahydrofolate into methionine plus tetrahydrofolate at the normal rate (Dickerman *et al.*, 1964). An increased proportion of 5-CH₃-H₄PteGlu among the pteroylmonoglutamates of vitamin B₁₂-deficient rat liver and an increased concentration of 5-CH₃-H₄PteGlu in the serum of some patients with pernicious anaemia led Noronha & Silverman (1962) and Herbert & Zalusky (1962) respectively to propose that the folates in vitamin B₁₂-deficient tissues became 'trapped' and unavailable as methyltetrahydrofolates. It is not apparent, however, that this effect alone could have led to depletion of methyltetrahydrofolates in liver.

The results of Kutzbach *et al.* (1967) show that treatment of vitamin B₁₂-deficient rats with dietary methionine restored to control values the concentrations of total liver folates including those active only for *L. casei*. Kutzbach & Stokestad (1967) found that activity of 5,10-methylenetetrahydrofolate reductase, the enzyme that leads to formation of 5-CH₃-H₄PteGlu, is inhibited by *S*-adenosylmethionine, a product of methionine metabolism. They suggested that the action of methionine in relieving the deficiency of folate was to inhibit formation of 5-CH₃-H₄PteGlu by this means, so decreasing the ratio of methylated to non-methylated folates, and leading to release of tetrahydrofolate for other metabolic functions. This hypothesis seems to conflict with the finding of Kutzbach *et al.* (1967) that methionine increases the concentration of 5-CH₃-H₄PteGlu in the livers of vitamin B₁₂-deficient

rats. Thus although the distortion in relative proportions of specific liver folates in vitamin B₁₂ deficiency upon which the 'methyltetrahydrofolate trap' hypothesis was based, is confirmed by the present results, the hypothesis itself does not appear to constitute a sufficient explanation of the condition. The scarcity of H₄PteGlu in vitamin B₁₂-deficient livers shown in the present work may be attributed more directly to the general failure of the liver to retain dietary folate of any kind than to the increased ratio of methylated to non-methylated forms. High proportions of methylated folates occurred on a normal stock diet without any symptoms of folate deficiency and without any depletion of total liver folates.

As the deficiency of vitamin B₁₂ became very severe and food intakes fell below 200g/day, certain further changes in liver folates took place. The most pronounced of these were the appearance of a peak of incompletely reduced folate (probably H₂PteGlu or PteGlu) around fraction 65 and the emergence of what may have been degraded forms of pteroyl-polyglutamates in later fractions. These two kinds of material did not appear to be related; whereas the incompletely reduced folate was labelled by injected [2-¹⁴C]PteGlu and occurred to some extent in control animals on low food intakes, the degraded material appeared only in deficient sheep and was not labelled. The latter material occurred in greater amounts in enlarged livers that were macroscopically fatty.

A further feature of the labelling of liver folates in vitamin B₁₂-deficient sheep that was more pronounced in an animal with low food intake was the degree of variation in specific radioactivity between the separated peaks. Thus in the severely deficient animal no. 340 the specific radioactivity of major peaks of *L. casei* activity ranged from 3.1nCi/μg around fraction 55 to 37.9nCi/μg around fraction 212, but all three vitamin B₁₂-deficient livers that contained label showed increased specific radioactivities around fraction 65. The activity for *L. casei* around fraction 55 in animal no. 340 represented a large peak of 5-CH₃-H₄PteGlu that was not in isotopic equilibrium with the higher polyglutamates and was presumably of recent dietary origin. An even larger peak of 5-CH₃-H₄PteGlu was found in the liver of a second vitamin B₁₂-deficient animal on low food intake (no. 044, not injected with labelled folate). The material with high specific radioactivity around fraction 65 occurred in all vitamin B₁₂-deficient animals given labelled folate but was accompanied by microbiological activity indicating the presence of folate or dihydrofolate only in the animal with food intake below 200g/day. However a peak of incompletely reduced folate at the same position was present in the chromatograms of the two animals with low food intake that had not received labelled folate.

The appearance in the severely vitamin B₁₂-deficient liver of incompletely reduced folate of high specific radioactivity together with unconjugated methyltetrahydrofolate of low specific radioactivity suggests the presence in the body of a pool of un-reduced folate that did not equilibrate quickly with 5-CH₃-H₄PteGlu of dietary origin. Much of the labelled folate injected into both deficient and control animals was not accounted for in liver, blood and excretory products (Table 2). The fraction unaccounted for was larger in vitamin B₁₂-deficient animals at low food intakes. Kidney cells have been reported to retain injected folate in incompletely reduced form (active for *L. casei* and *S. faecalis* but not for *P. cerevisiae*) for a few days (Grossowicz *et al.*, 1963; Goresky *et al.*, 1963).

The results suggest several possibilities of specific failures of folate metabolism in vitamin B₁₂-deficient sheep that do not appear to be related to the higher proportion of methyltetrahydrofolates. First, the very severe depletion of polyglutamate forms of all the reduced folates in all the deficient animals suggests a general failure to synthesize pteroyl-polyglutamates from monoglutamates. As the vitamin B₁₂ deficiency became more severe and food intake fell the depletion of higher polyglutamates of all three classes became even more pronounced than that of the monoglutamates. A failure of polyglutamate synthesis was suggested by Jeejeebhoy *et al.* (1965) to explain the depletion of pteroyl-polyglutamates in erythrocytes of vitamin B₁₂-deficient patients (Cooper & Lowenstein, 1964). This possibility is also consistent with the failure of label to equilibrate between monoglutamates and polyglutamates in the livers of the deficient sheep.

Secondly, the appearance of relatively large amounts of incompletely reduced folates in severe vitamin B₁₂ deficiency raises the possibility that retention of folates is dependent on prior reduction and that failure of the latter process may have been responsible for the observed depletion. However, in the two vitamin B₁₂-deficient animals with food intakes above 200g/day extensive depletion of liver folates had occurred in the absence of detectable amounts of incompletely reduced folates.

Thirdly, a failure to transport folate into the vitamin B₁₂-deficient cell might explain the inability to retain adequate amounts from the diet. Das & Hoffbrand (1970) have reported that phytohaemagglutinin-stimulated lymphocytes from patients with pernicious anaemia take up labelled methyltetrahydrofolate more slowly than lymphocytes from normal patients, although labelled PteGlu is taken up at the normal rate. However, the rate of clearance of substantial doses (5μg/kg) of injected folates (including methyltetrahydrofolate) from the serum of pernicious anaemia patients is generally reported to be more rapid than normal (Mollin *et al.*, 1962;

Chanarin & Perry, 1968, but see Nixon & Bertino, 1972), and in the present study more than half of a dose of about 4.5 µg of folate/kg per day, given to vitamin B₁₂-deficient sheep for five days was retained in tissues other than liver and blood. An efficient scavenging process, presumably involving transport, appeared to have operated, at least for PteGlu. Further, although in general the ratio 5-CH₃-H₄PteGlu in liver/*L. casei* activity in blood was greater in vitamin B₁₂-treated sheep (mean of five values, 73) than in vitamin B₁₂-deficient sheep (mean of five values, 18) this ratio tended to become higher in the more severely vitamin B₁₂-deficient animals.

The rapid response to large doses of methionine in the retention of folate by the livers of vitamin B₁₂-deficient rats (Gawthorne & Stokstad, 1971) again emphasizes the central role of methionine in folate homeostasis. Methionine synthesis is dependent on both vitamin B₁₂ and folate and the mechanism by which vitamin B₁₂ promotes retention of folate seems to be mediated in some way through methionine. A system in which an essential metabolic product promotes the means of its own synthesis is unusual and seems hazardous to the organism.

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