Folic Acid Metabolism in Vitamin B₁₂-Deficient Sheep

EFFECTS OF INJECTED METHIONINE ON LIVER CONSTITUENTS ASSOCIATED WITH FOLATE METABOLISM

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1. The effects of injected L-methionine (2g every second day for 28 days) on liver folates and other constituents of liver associated with folate metabolism were studied in vitamin B₁₂-deficient ewes and their pair-fed controls receiving vitamin B₁₂. The dose rate of methionine used was sufficient to restore almost to normal the elevated excretion in the urine of formiminoglutamate in the deficient animals. 2. Liver folates active for *Lactobacillus casei*, *Streptococcus faecalis* R and *Pediococcus cerevisiae* were severely depressed in deficient livers and were partly restored by methionine. Analysis of the folates after ion-exchange chromatography showed that the major effect of methionine was to increase the concentrations of tetrahydrofolates and formyltetrahydrofolates. Methyltetrahydrofolates were also increased, but there was no effect of methionine on the small amounts of incompletely reduced folates present in deficient livers. The folates present were predominantly penta-, hexa- and hepta-glutamates whether or not animals received vitamin B₁₂ or methionine. 3. Concentrations of ATP, NAD⁺, NADH and NADPH were lower in freeze-clamped liver from vitamin B₁₂-deficient sheep than in liver from pair-fed, vitamin B₁₂-treated sheep. These changes were not affected by methionine which was also without effect on the elevated K⁺/Na⁺ ratios found in deficient livers. 4. The livers of vitamin B₁₂-deficient animals contained lower concentrations of choline and higher concentrations of lipid than their pair-fed controls. These effects were reversed by methionine.

Many of the metabolic effects of vitamin B₁₂ deficiency in humans and experimental animals are due to a secondary deficiency of folate brought about by the lack of vitamin B₁₂. Depletion of liver folates in vitamin B₁₂ deficiency has been shown in sheep (Dawbarn *et al.*, 1958; Smith & Osborne-White, 1973) and rats (Kutzbach *et al.*, 1967; Gawthorne & Stokstad, 1971).

The effect of administered methionine on folate metabolism was first shown by Silverman & Pitney (1958), who found that methionine decreased the elevated excretion in the urine of formiminoglutamate in rats deficient either in vitamin B₁₂ or in folate. Subsequent studies have shown that the depleted liver folates in vitamin B₁₂-deficient rats receiving adequate dietary folate may be increased within a few hours by giving relatively large amounts of methionine (Kutzbach *et al.*, 1967; Gawthorne & Stokstad, 1971; Thenen & Stokstad, 1973). The known function of vitamin B₁₂ in the remethylation of homocysteine by 5-CH₃-H₄PteGlu* to reform methionine suggests that the effect of vitamin B₁₂ is exerted via methionine, but no satisfactory explanation of the effect of methionine itself has been made. The present experiments use vitamin B₁₂-deficient sheep to examine

this problem. The present paper describes the nature of the changes in liver folates and other liver constituents when vitamin B₁₂-deficient sheep are treated with methionine. An accompanying paper (Gawthorne & Smith, 1974) describes the associated changes in the activities of certain liver enzymes involved in folate metabolism.

Materials and Methods

Animals

Pair-fed vitamin B₁₂-deficient and vitamin B₁₂-treated sheep were produced as previously described (Smith & Marston, 1970) except that only one-half

* 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.

of the animals were transferred into cages at the time pair-feeding began. The remaining animals continued to be housed in pens where they were treated similarly to the caged pairs except that urine was not collected for estimation of formiminoglutamate.

The 16 experimental animals were drawn from a uniform group of 20 Australian Merino ewes at 2 years of age (mean body wt. 36.3 kg). The periods for which the experimental diet was fed and for which control animals were pair-fed and injected with vitamin B₁₂ (250 µg weekly) differed between animals and are shown in Table 1. Body weights were measured weekly and, after pair-feeding began, food intakes were measured daily.

During the final 4 weeks one-half of the pairs (two pairs in cages and two pairs in pens) were injected intravenously every second day with 2 g of L-methionine in 40 ml of sterile iso-osmotic saline (9 g of NaCl/litre) at pH 7. The remaining pairs were injected with sterile saline alone at a similar rate. Two of the latter pairs (those in pens) received the saline for less than 4 weeks before they were killed because of sharply falling food intakes (see Table 1).

Extraction and measurement of liver folates

Animals were killed and ice-cold homogenates of pre-chilled liver prepared in final concentrations of 1% ascorbate (pH 6.0) as previously described (Osborne-White & Smith, 1973). In the present work, however, homogenates were heated very rapidly to 80°C to prevent the action of endogenous conjugase (γ -glutamylcarboxypeptidase) on the pteroylpolyglutamates present. Samples (10 ml) of ice-cold homogenate were added dropwise with continuous stirring into glass tubes containing 10 ml of 1% ascorbate held in a water bath at 95°C. Addition of homogenate occupied 70 s and temperature during extraction remained above 80°C. Other procedures of assay, ion-exchange chromatography, treatment of samples with conjugase and identification and estimation of folates were as previously described (Osborne-White & Smith, 1973).

Determination of adenine nucleotides and nicotinamide nucleotides

Samples of liver up to 5 mm thick were freeze-clamped *in situ* with Wollenberger tongs pre-chilled in liquid N₂. This tissue was then ground in liquid N₂ before extraction by homogenization either in 5% (w/v) HClO₄ in aqueous ethanol 20% (v/v) (for measurement of AMP, ADP, ATP, NAD⁺ and NADP⁺) or in 1 M-KOH in 20% (v/v) aqueous ethanol containing 0.5 mM-cysteine hydrochloride for estimation of NADH and NADPH. Great care was taken during neutralization of extracts (at 0°C) not to proceed beyond pH 7 (glass electrode).

NAD⁺, NADH, NADP⁺ and NADPH were determined by the methods of Klingenberg (1963). ATP was measured by the method of Lamprecht & Trautshold (1963). ADP and AMP were measured by the method of Adam (1963). All enzymic analyses were performed with a Zeiss PMQ II recording spectrophotometer (Carl Zeiss, Oberkochen, W. Germany).

Clamping times for the normal animals ranged from 8–19 s and for the experimental animals from 9–27 s from the time of firing the captive-bolt pistol that killed the animal. Statistical analysis of the results showed no significant effects of clamping time within this range.

Determination of liver electrolytes and total P

A representative sample of about 20 g of chilled liver pieces (Osborne-White & Smith, 1973) was extruded through a 16 mesh stainless-steel gauze supported in a syringe barrel in order to remove connective tissue and to permit adequate sampling. Duplicate weighed samples (0.5 g) of the liver mash were heated with HNO₃ and HClO₄ to destroy organic matter, the HNO₃ was boiled off and the solution was diluted for determination of Ca²⁺, Mg²⁺, Na⁺ and total P. Further duplicate weighed samples (0.5 g) were extracted by homogenization in final volumes of 10 ml of 5% (w/v) trichloroacetic acid for determination of K⁺ and Cl⁻ and to repeat the determination of Na⁺. Ca²⁺ and Mg²⁺ were determined by atomic absorption spectrophotometry in the presence of 1% (w/v) La³⁺ by using a Techtron model AA4 instrument from Varian Techtron Pty. Ltd., Melbourne, Australia. Na⁺ and K⁺ were determined by using the flame photometer model I.L.143 from Instrumentation Laboratory Inc., Boston, Mass., U.S.A. Cl⁻ was determined with a Buchler-Cotlowe chloridometer (Buchler Instruments Inc., Fortlee, N.J., U.S.A.). Total P was determined by the method of Chen *et al.* (1956). All determinations were performed in duplicate.

Other constituents of liver

Duplicate samples of the liver mash described above were used for all the following determinations. The water content was estimated by oven drying 0.5 g samples for 24 h at 105°C. Choline was determined by the method of Fritz (1965) after extraction and hydrolysis by the method of Glick (1944). Total lipid was determined by the method of Folch *et al.* (1957). Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as the standard. The haemoglobin content of liver was determined by the method of Andersen & Shoemaker (1965) and converted into blood volumes from

Table 1. *Treatment and final vitamin B₁₂ status of animals*

The Table summarizes the periods of treatment and final condition of the experimental animals. Animals were treated with vitamin B₁₂ and pair-fed on an individual basis for the periods shown. Animals that received methionine were injected intravenously with 2g of L-methionine every second day over the 28 days immediately before they were killed. The remaining animals received comparable injections of iso-osmotic saline except that the two deficient animals in pens lost appetite rapidly during this period and were killed after receiving saline for only 2 weeks and 1 week respectively. The vitamin B₁₂-treated animals were scheduled similarly.

Pair no.	Animal no. [cage (C) or pen (P)]	B ₁₂ and methionine status	Treatment			Final body weight (kg)	Mean food intake over last 7 days (g/day)	Liver weight (g)	Vitamin B ₁₂ in liver (μ g/g)	Haematocrit (%)	Formimino- glutamate in urine (last 7 days) (mmol/day)
			Period fed on deficient diet (weeks)	Period pair-fed and B ₁₂ - treated (weeks)	Period injected with methionine (M) or saline (S) (weeks)						
1	006 (C)	-B ₁₂ - Met (fed <i>ad lib.</i>)	28	—	4 (S)	23.0	447	394	0.05	26.1	2.68
2	032 (C)		31	—	4 (S)	22.8	243	352	0.03	25.1	0.86
3	022 (P)		40	—	2 (S)	26.8	191	527	0.05	13.3	—
4	004 (P)		41	—	1 (S)	26.2	286	354	0.04	28.5	—
1	007 (C)	+B ₁₂ - Met (pair-fed)	28	11	4 (S)	25.5	447	312	1.14	29.1	0.00
2	024 (C)		31	9	4 (S)	24.9	243	286	1.21	35.6	0.07
3	034 (P)		40	18	2 (S)	27.6	176	334	1.05	31.3	—
4	010 (P)		41	19	1 (S)	27.6	286	331	1.25	30.6	—
5	016 (C)	-B ₁₂ + Met (fed <i>ad lib.</i>)	26	—	4 (M)	27.4	439	435	0.06	23.3	0.18
6	013 (C)		29	—	4 (M)	24.4	196	410	0.03	30.0	0.23
7	030 (P)		39	—	4 (M)	28.8	259	389	0.05	33.0	—
8	005 (P)		45	—	4 (M)	27.8	663	459	0.05	29.1	—
5	042 (C)	+B ₁₂ + Met (pair-fed)	26	8	4 (M)	28.5	439	383	1.26	30.0	0.03
6	026 (C)		29	9	4 (M)	25.0	196	272	1.26	31.1	0.00
7	008 (P)		39	12	4 (M)	28.2	259	399	1.18	27.2	—
8	031 (P)		45	22	4 (M)	32.4	663	439	1.13	35.3	—

the haematocrit values of Table 1 and the haemoglobin content of sheep erythrocytes. In calculating the contribution of Na^+ and K^+ from blood in the liver samples, normal values for sheep blood ($140\mu\text{g}$ -atoms of Na^+ /ml and $4\mu\text{g}$ -atoms of K^+ /ml) were assumed to apply to all the bloods.

Formiminoglutamate in urine

This was determined by the method of Tabor & Wyngarden (1958).

Vitamin B_{12} in liver

This was assayed with *Lactobacillus leichmanii* as described by Hine & Dawbarn (1954).

Materials

L-Methionine was obtained from the Sigma Chemical Co. Enzymes and substrates for determination of adenine nucleotides and nicotinamide nucleotides were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Other

materials were as described by Smith & Osborne-White (1973) or were the best commercially available grades.

Results

Status of vitamin B_{12} in the animals

The degree of vitamin B_{12} deficiency obtained in the untreated animals is indicated in Table 1 by the concentration of vitamin B_{12} in the livers and by the extent of depression of voluntary food intakes (initially 1060g/day). With the exception of animal no. 022 (whose average food intake fell by 170g/day during the final week) the deficient animals without methionine all consumed more than 200g/day and were not in immediate danger of collapse. The rapid decline in food intake of animal no. 022 was associated with a low haematocrit and with the enlarged liver frequently found in terminally deficient animals (Smith & Osborne-White, 1973). The remaining untreated animals did not show these effects which were also absent from deficient animals that were treated with methionine (food intakes 196 – 663g/day).

Fig. 1. Effect of injected methionine on formiminoglutamate in urine in vitamin B_{12} -deficient sheep

Formiminoglutamate in urine was measured over the final 5 weeks in two vitamin B_{12} -deficient animals ($-\text{B}_{12}$) and their pair-fed controls receiving vitamin B_{12} ($+\text{B}_{12}$). One pair of animals [(a) no. 6 pair from Table 1] was injected every second day during the final 28 days with 2g of L-methionine in 40ml of saline (9g of NaCl/litre). The second pair [(b), no. 2 pair from Table 1] received comparable injections of saline.

Table 2. *Effects of methionine on liver folates in vitamin B₁₂-deficient sheep*
Individual results are given for the four animals in each of the treatment groups. Four assay methods were used each with and without prior treatment of the samples with conjugase (γ -glutamylcarboxypeptidase). Because of wide variations within treatments, statistical analysis was based partly on ranking methods. Statistical results are given in the text.

Animal no. [cage (C) or pen (P)]	B ₁₂ and methionine status	Liver folate concentrations							
		<i>L. casei</i>		<i>S. faecalis</i>		<i>P. cerevisiae</i> (pH5.3)		<i>P. cerevisiae</i> (pH6.3)	
		No conjugase (ng/g)	+ conjugase (ng/g)	No conjugase (ng/g)	+ conjugase (ng/g)	No conjugase (ng/g)	+ conjugase (ng/g)	No conjugase (ng/g)	+ conjugase (ng/g)
006 (C)	-B ₁₂ - Met (fed <i>ad lib.</i>)	12.6	749	9.8	283	<2.6	105	<2.6	44.5
032 (C)		7.5	210	<6.4	35	<2.6	40	<2.6	14.5
022 (P)		14.1	325	9.7	42	<2.6	37	<2.6	14.9
004 (P)		14.5	1205	<6.6	397	<2.6	184	<2.6	81.6
007 (C)	+B ₁₂ - Met (pair-fed)	53.4	5863	11.6	3795	4.9	1104	<2.6	627
024 (C)		77.1	6894	17.7	1748	10.4	1344	<2.6	731
034 (P)		41.6	5417	11.9	1825	2.7	619	<2.6	525
010 (P)		103.3	4498	18.5	2150	11.7	784	<2.6	462
016 (C)	-B ₁₂ + Met (fed <i>ad lib.</i>)	29.8	2975	7.2	567	7.3	642	<2.6	417
013 (C)		11.4	504	<6.5	260	4.3	197	<2.6	111
030 (P)		21.5	1050	9.8	794	7.8	229	<2.6	134
005 (P)		26.4	1665	14.3	1426	5.3	261	<2.6	149
042 (C)	+B ₁₂ + Met (pair-fed)	70.3	4569	13.3	2922	2.7	762	<2.6	527
026 (C)		91.9	5181	15.7	4949	7.3	3178	<2.6	1662
008 (P)		86.3	4104	12.7	1468	4.2	523	<2.6	266
031 (P)		85.2	3242	11.7	1492	5.5	635	<2.6	263

Formiminoglutamate in urine

The effect of the methionine treatment on excretion of formiminoglutamate in the urine of the animals kept in cages is shown in Fig. 1 and Table 1. Injection of 2g of methionine every second day into a deficient animal previously excreting about 2mmol of formiminoglutamate/day was accompanied by progressive decline in formiminoglutamate in the urine to about 0.2mmol/day in 14 days, whereas injection of saline

was without effect. The 2-day cycle of formiminoglutamate excretion that occurred during part of the treatment period with methionine (Fig. 1) reflects the treatment schedule and indicates both the rapid response to methionine and the equally rapid relapse in its absence.

Table 1 shows the excretion of formiminoglutamate in urine of all caged animals during the final 7 days. Animals receiving vitamin B₁₂ excreted negligible amounts, in agreement with previous work.

Fig. 2. Effects of vitamin B₁₂ and methionine on liver folates

Chromatographic separation on DEAE-Sephadex of extracted liver folates of vitamin B₁₂-deficient (-B₁₂) and pair-fed vitamin B₁₂-treated (+B₁₂) sheep with (+Met) and without (-Met) supplementary methionine was followed by microbiological assay of the separate fractions. The fractions were treated with conjugase and assayed with *L. casei* (I), *S. faecalis* (II), *P. cerevisiae* at pH 6.3 (III) and *P. cerevisiae* at pH 5.3 (IV). The insets show the effects of treating the liver extracts with an excess of conjugase before they were chromatographed. The insets are marked with the elution positions of authentic pteroylglutamates: (1) 10-HCO-H₄PteGlu; (2) 5-HCO-H₄PteGlu; (3) 5-CH₃-H₄PteGlu; (4) H₄PteGlu; (5) H₂PteGlu; (6) PteGlu. All chromatograms represent the folates present in mixtures of two liver extracts. Such mixtures were prepared by combining equal amounts of *L. casei* activity from the two original extracts according to the assays shown in Table 2. (a) -B₁₂-Met, animals nos. 006 and 032; (b) -B₁₂-Met, animals nos. 022 and 004; (c) +B₁₂-Met, animals nos. 007 and 024; (d) +B₁₂-Met, animals nos. 034 and 010; (e) -B₁₂+Met, animals nos. 016 and 013; (f) -B₁₂+Met, animals nos. 030 and 005; (g) +B₁₂+Met, animals nos. 042 and 026; (h) +B₁₂+Met, animals nos. 008 and 031.

Liver folates

The severe depletion in liver folates of all kinds in vitamin B₁₂-deficient livers is shown in Table 2, which gives individual values for the four assay procedures both with and without prior treatment of samples with conjugase. Statistical analysis by procedures based on ranks (Koch, 1970) showed the effect of vitamin B₁₂ to be significant ($P < 0.01$) for all four assays after conjugase treatment and for *L. casei* ($P < 0.01$) and *S. faecalis* ($P < 0.05$) before conjugase treatment. No significant effects of the different food intakes or of housing in pens versus cages were found.

Analysis of the effects of methionine was carried out on the post-conjugase assay results by the *t* test after conversion of the values into logarithms. Significant increases of liver folate concentrations due to methionine in the absence of vitamin B₁₂ were found for *S. faecalis* ($P < 0.05$), *P. cerevisiae* (pH 5.3) ($P < 0.05$) and *P. cerevisiae* (pH 6.3) ($P < 0.01$). Mean activity for *L. casei* was 150% higher in deficient animals that were treated with methionine than in those that were not, but the effect was not statistically significant.

The distribution of folates among the forms present in liver is shown in Fig. 2. Chromatographic separation without prior conjugase treatment showed that for all treatment groups most of the folates emerged in peaks of activity between fractions 130 and 330. The positions and differential microbiological activities of peaks (Osborne-White & Smith, 1973) were characteristic of 5-CH₃-H₄PteGlu₅₋₇, H₄PteGlu₅₋₇, 5-HCO-H₄PteGlu₅₋₇ and 10-HCO-H₄PteGlu₅₋₇ with a preponderance of the hexa- and hepta-glutamates.

These profiles differ somewhat from those previously obtained for sheep liver (Osborne-White & Smith, 1973) in the very small quantities that were present of any folates with less than five glutamate residues. This may be attributed to the precautions taken to heat the extracts very rapidly to 80°C to avoid hydrolysis of polyglutamates by endogenous conjugase activity. The fractions of the major folates that were present as penta- to hepta-glutamates are shown in Table 3. There were no significant effects of either vitamin B₁₂ or methionine on the distribution of polyglutamate forms; in all cases most of the folates were higher polyglutamates despite the large differences in total concentrations.

A feature of liver folates of vitamin B₁₂-deficient animals with food intakes less than 200g/day described previously was the occurrence of relatively high concentrations both of 5-CH₃-H₄PteGlu and of H₂PteGlu (or PteGlu) seen respectively as peaks of activity for *L. casei* alone around fraction 55 and for *S. faecalis* and *L. casei* together between fractions 60 and 70 (Smith & Osborne-White, 1973). The presence of these folates in the liver of animal no. 022 (food intake 191g/day) is suggested by their occurrence in the combined extract with that of animal no. 004 (Fig. 2b). Similar material was present in the chromatogram that contained the liver extract of the pair-fed control to animal no. 022 (no. 034, Fig. 2d) but was absent from all other extracts.

Conversion of the polyglutamates into mono-glutamates before chromatography allowed treatment effects to be demonstrated in terms of specific classes of folate. These measurements were confined to the caged animals because food intakes with and

Table 3. Percentage of methyl-, formyl- or unsubstituted tetrahydrofolate occurring in liver as polyglutamates with five or more glutamate residues

The values (means $\pm$ S.E.M.) were calculated from the results shown in Fig. 2 by the method of Osborne-White & Smith (1973).

Treatment	Methyltetrahydrofolates (%)	Tetrahydrofolates (%)	Formyltetrahydrofolates (%)
-B ₁₂ - Met	98.4 $\pm$ 0.4	89.3 $\pm$ 1.6	75.2 $\pm$ 13.2
+B ₁₂ - Met	99.1 $\pm$ 0.4	81.3 $\pm$ 4.3	90.4 $\pm$ 7.7
-B ₁₂ + Met	98.0 $\pm$ 0.7	86.9 $\pm$ 5.7	81.1 $\pm$ 1.7
+B ₁₂ + Met	94.8 $\pm$ 0.5	94.2 $\pm$ 2.1	85.8 $\pm$ 9.0

Table 4. Effects of vitamin B₁₂ and methionine on concentrations of folates in liver

The values are derived from the insets to Fig. 2 by the method of Osborne-White & Smith (1973).

Treatment	5-CH ₃ -H ₄ PteGlu _n (μ g/g)	H ₄ PteGlu _n (μ g/g)	10-HCO-H ₄ PteGlu _n (μ g/g)	5-HCO-H ₄ PteGlu _n (μ g/g)	H ₂ PteGlu + PteGlu (μ g/g)
-B ₁₂ - Met	0.25	0.014	0.015	0.005	0.007
+B ₁₂ - Met	3.30	0.37	0.25	0.42	0.000
-B ₁₂ + Met	0.64	0.07	0.11	0.067	0.028
+B ₁₂ + Met	1.51	0.52	0.23	0.39	0.008

Table 5. *Effects of vitamin B₁₂ and methionine on adenine nucleotides and nicotinamide nucleotides in freeze-clamped liver*

The Table shows means $\pm$ s.e.m. for five normal animals followed by means for the four animals in each of the four treatment groups. Each standard error applies to each of the treatment means and has been calculated from the residual error mean square of a split-plot analysis of variance of the data (Cochran & Cox, 1957). A split-plot analysis was used because whereas the effects of vitamin B₁₂ were determined within pair-fed samples, the effects of methionine were determined between couples of pair-fed animals. The standard errors appropriate to the four treatment groups are followed in turn by the results of an analysis of variance showing the degree of significance of the effects attributable to the treatments. n.s., not significant.

Treatment	ATP (μ mol/g of liver)	ADP (μ mol/g of liver)	AMP (μ mol/g of liver)	Total adenine nucleotides (μ mol/g of liver)	Energy charge ATP+ $\frac{1}{2}$ ADP (ATP+ADP +AMP)	NAD ⁺ (μ mol/g of liver)	NADH (μ mol/g of liver)	NADH (NAD ⁺ + NADH)	NADP ⁺ (μ mol/g of liver)	NADPH (μ mol/g of liver)	NADPH (NADP ⁺ + NADPH)
Normal animals (5) (mean $\pm$ s.e.m.)	3.36 $\pm$ 0.043	0.59 $\pm$ 0.060	0.12 $\pm$ 0.017	4.06 $\pm$ 0.073	0.90 $\pm$ 0.009	1.17 $\pm$ 0.039	0.105 $\pm$ 0.007	0.08 $\pm$ 0.004	0.142 $\pm$ 0.006	0.285 $\pm$ 0.011	0.67 $\pm$ 0.014
-B ₁₂ - Met	2.08	0.84	0.20	3.12	0.80	0.53	0.025	0.05	0.091	0.105	0.52
+B ₁₂ - Met	2.78	0.79	0.19	3.75	0.84	0.75	0.056	0.07	0.113	0.208	0.64
-B ₁₂ + Met	2.17	0.81	0.20	3.18	0.81	0.60	0.041	0.06	0.106	0.128	0.55
+B ₁₂ + Met	2.41	0.77	0.22	3.39	0.82	0.76	0.058	0.07	0.094	0.161	0.63
Standard error	0.180	0.090	0.039	0.170	0.023	0.046	0.008	0.010	0.013	0.019	0.047
Significance of B ₁₂ effect	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	$P < 0.01$	$P < 0.05$	n.s.	n.s.	$P < 0.02$	n.s.
Significance of Met effect	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Significance of B ₁₂ /Met interaction	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 6. *Concentrations of ionic components of the livers of vitamin B₁₂-deficient and pair-fed vitamin B₁₂-treated sheep with and without methionine*

Results are means for the four animals on each treatment. Explanation of statistical analysis is as for Table 5. Statistical significance was established by analysis of variance. n.s., not significant.

Treatment	Ca ²⁺ (μ g/g of protein)	Mg ²⁺ (μ g/g of protein)	Cl ⁻ (μ g-atom/g of water)	Na ⁺ (μ g-atom/ g of water)	K ⁺ (μ g-atom/ g of water)	K ⁺ /Na ⁺	Blood content (ml of blood/g of liver)
-B ₁₂ - Met	280	1410	78.8	47.6	105	2.20	0.015
+B ₁₂ - Met	275	1370	79.9	53.0	96	1.82	0.013
-B ₁₂ + Met	282	1410	69.9	47.4	104	2.24	0.020
+B ₁₂ + Met	283	1290	75.8	51.4	95	1.85	0.014
Standard error	15.6	33.0	3.6	1.9	3.6	0.12	0.002
Significance of B ₁₂ effect	n.s.	$P < 0.05$	n.s.	$P = 0.05$	$P < 0.1$	$P < 0.02$	n.s.
Significance of Met effect	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	n.s.	n.s.
Significance of B ₁₂ /Met interaction	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

without methionine were comparable only in this set. The results are shown in Table 4 and in the insets in Fig. 2. The vitamin B₁₂-deficient animals showed severe depletion of all the fully reduced folates but particularly of tetrahydrofolate and its formylated derivatives. Treatment with methionine led to increases in all the reduced folates but particularly in tetrahydrofolate and the formyltetrahydrofolates. The occurrence of a small amount of incompletely reduced folate in the deficient livers was not prevented by methionine. Similar quantities of this material were present in the livers of some animals that received vitamin B₁₂.

The low concentration of 5-CH₃-H₄PteGlu_n in the vitamin B₁₂-treated methionine-treated animals seen in Table 4 and Fig. 2(g) deserves comment. This effect is attributable to animal no. 026 which showed unusually high liver concentrations of folates active for *P. cerevisiae* and *S. faecalis* (Table 2). This liver, like those of pasture-fed animals (Smith & Osborne-White, 1973) contained quite a high concentration of H₄PteGlu_n. The relationship of this effect to the concentration of S-adenosylmethionine is discussed by Gawthorne & Smith (1974).

Concentrations of adenine nucleotides and nicotinamide nucleotides in liver

Conversion of folate into dihydrofolate and tetrahydrofolate involves reduction by reduced nicotinamide nucleotides (Kaufman & Gardiner, 1966), and conversion of pteroylmonoglutamates into polyglutamates requires ATP (Gawthorne & Smith, 1973). The depletion of liver folates in vitamin B₁₂ deficiency could be brought about by failure of either of these processes (Smith & Osborne-White, 1973) and this prompted us to examine the concentrations of nicotinamide nucleotides and adenine nucleotides in the livers of the experimental sheep. The results are given in Table 5, which includes for comparison values for the livers of normal animals.

As compared with their pair-fed controls vitamin B₁₂-deficient animals showed a significant depletion in concentrations of ATP and total adenine nucleotides and in NAD⁺, NADH and NADPH, but there was no significant effect of methionine to restore any of these concentrations.

The effects of vitamin B₁₂ deficiency to diminish both the energy charge and the extent of reduction of NADP⁺, although not statistically significant was, nevertheless, perceptible particularly by comparison with the normal animals.

Ionic components of liver

Both dihydrofolate reductase and pteroylpolyglutamate synthetase are activated by relatively high concentrations of inorganic ions. Thus dihydrofolate reductase acquires maximum activity in the presence of about 800mM-Cl⁻ (Reyes & Huennekens, 1967) and pteroylpolyglutamatesynthetase shows maximum activity at 150mM-K⁺ (Gawthorne & Smith, 1973). The possibility that changes in ionic composition of liver cells may have been responsible for the failure of vitamin B₁₂-deficient livers to retain folates led to an examination of the concentrations of a number of cations and anions in the livers of the experimental animals (Table 6).

Livers deficient in vitamin B₁₂ showed a significant increase in Mg²⁺ content expressed on a protein basis, but there was no effect of methionine. Ca²⁺ concentrations were unaffected by any of the treatments. Livers in deficient animals contained lower concentrations of Na⁺ and higher concentrations of K⁺ than livers from animals receiving vitamin B₁₂, and although these effects were not individually significant they each contributed to the significantly higher K⁺/Na⁺ ratio. This effect cannot have been due to differential contamination with blood plasma since the content of residual blood was slightly higher in the livers from deficient animals than in those from control animals and in either case was small. There

Table 7. *Liver composition of vitamin B₁₂-deficient and pair-fed vitamin B₁₂-treated sheep with and without methionine*

Results are means for the four animals on each treatment. Explanation of statistical analysis is as for Table 5. The significance of the effects of vitamin B₁₂ or methionine or the interaction between them was established by analysis of variance. n.s., not significant.

Treatment	Water (g/g of liver)	Protein (g/g of liver)	Choline (mg/g of protein)	Lipid (g/g of protein)	Total P (mg/g of protein)
-B ₁₂ - Met	0.709	0.139	26.7	0.837	22.8
+B ₁₂ - Met	0.722	0.153	33.9	0.451	23.3
-B ₁₂ + Met	0.753	0.142	30.5	0.376	22.7
+B ₁₂ + Met	0.736	0.147	31.3	0.329	22.0
Standard error	0.00718	0.00432	1.15	0.080	0.38
Significance of B ₁₂ effect	n.s.	P<0.1	P<0.01	P<0.05	n.s.
Significance of Met effect	P<0.05	n.s.	P<0.1	P<0.001	n.s.
Significance of B ₁₂ /Met interaction	n.s.	n.s.	P<0.05	n.s.	n.s.

was no effect of methionine on the K⁺/Na⁺ ratio. The only remaining significant effect was a slight decrease in the Cl⁻ concentration due to methionine, but this probably has no importance for folate retention.

Lipid, protein and choline content of livers

The gross composition of the livers of the experimental animals together with the choline and total P contents are shown in Table 7. A highly significant effect of methionine was found in depressing the elevated lipid content of livers of deficient animals. A similar but less pronounced effect of vitamin B₁₂ occurred. The occurrence of fatty liver in the deficient animals and its remission by either vitamin B₁₂ or methionine was confirmed histologically after staining of sections with Oil Red O. The elevated lipid content of the livers of deficient animals was accompanied by depressed concentrations of total choline. These concentrations were increased by either vitamin B₁₂ or methionine.

No significant effects of either vitamin B₁₂ or methionine were found on total P or protein contents of deficient livers, but methionine significantly increased the water content presumably owing to replacement of lipid.

Discussion

Vitamin B₁₂ deficiency in sheep is accompanied by severe depletion of all fully reduced folates and in particular of tetrahydrofolates and the formyl-tetrahydrofolates. In very severe deficiency, when voluntary food intakes have fallen below 200g/day and death of the animal is imminent, several further effects are seen. Under these conditions higher polyglutamates become relatively more depleted than the corresponding monoglutamates, isotopic exchange between 5-CH₃-H₄PteGlu and its polyglutamate forms is hindered, perceptible accumulation of 5-CH₃-H₄PteGlu and of H₂PteGlu (or PteGlu) occurs and some degradation of folates may take place (Smith & Osborne-White, 1973). In the present work most of these secondary effects were avoided by working with animals whose food intakes were near or above 200g/day. Apart from small effects in animals nos. 022 and 034 none of the livers showed serious distortions in the profiles of their folates owing to the terminal effects mentioned. Thus although the livers from deficient sheep contained a much lower proportion of non-methylated folates than those from sheep supplemented with vitamin B₁₂, the elution patterns indicated a uniform depletion of conjugated and unconjugated forms within these classes and a small but uniform restoration of both conjugated and unconjugated forms on treatment with methionine (Fig. 2). This is borne out by the absence of any

significant effect of treatment on the fraction of the folates present as penta- to hepta-glutamates (Table 3).

Thenen & Stokstad (1973) have carried out a chromatographic analysis of the liver folates of vitamin B₁₂-deficient and -treated rats with and without methionine. They interpret their results to indicate that the concentrations of the pteroylmonoglutamates remained relatively unchanged in vitamin B₁₂ deficiency and that the effects of vitamin B₁₂ or methionine to increase total folates were confined to the conjugated forms. However, in the present work and in a previous study (Smith & Osborne-White, 1973) we have found that this does not occur in sheep. Treatment with either vitamin B₁₂ or methionine increased the concentrations of all forms of folate in the livers of vitamin B₁₂-deficient sheep, except those that accumulated owing to the terminal effects.

Thenen & Stokstad (1973) view their results as evidence in support of the 'methyltetrahydrofolate-trap' hypothesis. The hypothesis attributes the loss of availability of the essential coenzyme tetrahydrofolate in vitamin B₁₂ deficiency to a failure to reconvert 5-CH₃-H₄PteGlu into H₄PteGlu at the normal rate. This effect is attributed to loss of activity of the vitamin B₁₂ enzyme, methyltetrahydrofolate-homocysteine methyltransferase, the enzyme responsible for recycling methionine. The effect of methionine in alleviating the secondary deficiency of folate is explained as being due to inhibition of the enzyme responsible for forming 5-CH₃-H₄PteGlu, methylene tetrahydrofolate reductase. The reductase is known to be inhibited by S-adenosylmethionine, a product of methionine metabolism (Kutzbach & Stokstad, 1967; Gawthorne & Smith, 1974). Thus, according to the hypothesis, methyltetrahydrofolate should accumulate in vitamin B₁₂ deficiency and the accumulation should be reversed on treatment with vitamin B₁₂ or methionine. The present findings and, indeed, those of Thenen & Stokstad (1973), show the reverse effect in liver. Methyltetrahydrofolate, like other fully reduced folates, was depleted by vitamin B₁₂ deficiency and the depletion was reversed by either vitamin B₁₂ or methionine. The observed increase in the concentrations of non-methylated folates on treatment with methionine (Fig. 2) did not take place solely at the expense of the methylated forms as would be required by the 'methyltetrahydrofolate-trap' hypothesis. The high proportion of methylated folates in the deficient livers may properly be attributed to the low activity of the methyltransferase (Gawthorne & Smith, 1974), but this does not explain the general depletion of folates. Whereas it is true that plasma folate concentrations are increased during vitamin B₁₂ deficiency in rats as would be predicted by the 'methyltetrahydrofolate-trap' hypothesis (Williams & Spray, 1970; Thenen & Stokstad,

1973), the total amount of folate contained in the plasma of normal rats is less than 1% of the folate contained in the liver. Consequently, in formulating a general hypothesis about the interaction of vitamin B₁₂ and folate, much more cognizance should be taken of liver folate changes than of plasma folate changes.

The development of fatty liver in the vitamin B₁₂-deficient sheep and its complete prevention or remission by either methionine or vitamin B₁₂ demonstrates both that a deficiency of methionine occurred in the vitamin B₁₂-deficient animals and that the amounts of methionine given were adequate to make up the deficit, at least for lipid metabolism. A similar conclusion may be drawn from the effects of the treatments on liver choline concentrations. The limited response of total liver folates to methionine suggests a need for larger amounts of methionine to maintain liver folates than to maintain normal lipid metabolism. Gawthorne & Stokstad (1971) used rates of methionine administration approaching 500 mg/kg body wt. per day (0.7% of the diet) to restore normal liver folate amounts in vitamin B₁₂-deficient rats. Such amounts are substantially in excess of the normal requirements of rats for methionine (Young *et al.*, 1956) and are more than ten times higher than the dose rates used here for sheep. If methionine or one of its metabolites is indeed responsible for the effects of vitamin B₁₂ on retention of folate then vitamin B₁₂ must very substantially increase the effectiveness of small amounts of dietary methionine. Although this is not inconsistent with the known involvement of vitamin B₁₂ in remethylating homocysteine, the apparently much higher requirements of supplementary methionine for folate homeostasis than for maximum growth rate or the lipotropic effect requires explanation and is discussed below.

Several previously unreported effects of vitamin B₁₂ deficiency were discovered that could have affected folate retention, but none of them responded to methionine and so their involvement as primary agents cannot reasonably be inferred. These include the lower concentrations of ATP, NADH and particularly of NADPH in livers of deficient animals as compared with their pair-fed controls, and the surprisingly high K⁺/Na⁺ ratio in the livers of vitamin B₁₂-deficient animals. The former effects may be partly due to a general impairment of purine synthesis accompanying the deficiency of folate, but they suggest a respiratory derangement as well. Such an effect, if caused by a partial uncoupling of mitochondrial oxidative phosphorylation, would be consistent with the higher energy expenditure in starvation of vitamin B₁₂-deficient sheep as compared with their pair-fed controls (Smith & Marston, 1970).

The high K⁺/Na⁺ ratio was unexpected but might indicate the failure of a transport process. Transport of methionine (or glycine) into mouse ascites-tumour

cells is accompanied by discharge of the K⁺ and Na⁺ gradients across the cell membrane (Reid & Eddy, 1971). These gradients are normally maintained by the Na⁺ pump at the expense of ATP. Inhibition or failure of active transport processes of this kind without failure of the Na⁺ pump could lead to a steady state with high K⁺/Na⁺ ratios like those found in the livers of vitamin B₁₂-deficient animals. Methionine plays a unique role in initiating synthesis of at least some proteins in mammalian cells (Smith & Marcker, 1970; Jackson & Hunter, 1970) and it may be speculated that a breakdown of the methionine-transport system in vitamin B₁₂ deficiency could lead to a requirement for large doses of methionine to promote the synthesis of enzymes or other proteins involved in folate retention.

Even without the limitations on methionine metabolism imposed by vitamin B₁₂ deficiency there is evidence that the initiation of protein synthesis requires the presence of particularly high concentrations of methionine. McGown *et al.* (1973) have put forward evidence that the maintenance of polyribosome integrity in perfused rat liver requires large amounts specifically of methionine and similar conclusions have been reached for intact animals by Park *et al.* (1973). The higher methionine requirements for retention of folate than for the lipotropic effect may reflect a specific need of the former for methionine-initiated protein synthesis, but it is clear that in our experiments the methionine provided by the feed alone was sufficient for all these functions when the vitamin B₁₂ status was satisfactory.

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