Folic Acid Metabolism in Vitamin B₁₂-Deficient Sheep

EFFECTS OF INJECTED METHIONINE ON METHOTREXATE TRANSPORT AND THE ACTIVITY OF ENZYMES ASSOCIATED WITH FOLATE METABOLISM IN LIVER

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1. A study was made of the effects of injected L-methionine on the activity of several enzymes of folate metabolism, and on the transport of methotrexate in liver preparations from vitamin B₁₂-deficient ewes and their pair-fed controls receiving vitamin B₁₂. 2. The activities of dihydrofolate reductase (EC 1.5.1.3) and 5-methyltetrahydrofolate-homocysteine transmethylase were significantly decreased in the liver of vitamin B₁₂-deficient animals, but were unaffected by L-methionine. 3. The concentration of S-adenosyl-L-methionine in the liver of deficient animals was about one-half of that in normal animals, and was restored to normal by either vitamin B₁₂ or L-methionine. 4. Methylenetetrahydrofolate reductase (EC 1.1.1.68) from sheep liver was inhibited by S-adenosyl-L-methionine *in vitro*, but not by concentrations of S-adenosyl-L-methionine found in the liver of vitamin B₁₂-deficient animals after injection of physiological amounts of L-methionine. 5. Pteroylpolyglutamate synthetase activity was significantly increased in the liver of vitamin B₁₂-deficient animals, and was decreased by intravenous injections of L-methionine. 6. L-Methionine injections increased the initial rate of uptake of methotrexate in liver slices from deficient animals and acted synergistically with vitamin B₁₂ to increase the quantity taken up in 40 min. The failure of folate metabolism in vitamin B₁₂ deficiency can be satisfactorily explained if L-methionine similarly affects the membrane transport of naturally occurring folates. 7. Further details of the results have been deposited as Supplementary Publication SUP 50028 (4 pages) at the British Library (Lending Division), (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms given in *Biochem. J.* (1973) 131, 5.

Experimental evidence of lowered H₄PteGlu* concentrations in vitamin B₁₂-deficient animals was obtained by Smith & Osborne-White (1973). These workers pointed out that the scarcity of H₄PteGlu was more directly related to a general depletion of all types of folate in B₁₂-deficient liver, rather than to an increased proportion of folates being 'trapped' in the form of 5-CH₃-H₄PteGlu, as was previously thought. Their findings have since been extended to include the effect of methionine, which increases the

concentration of H₄PteGlu and other folates in the liver of vitamin B₁₂-deficient animals (Smith *et al.*, 1974).

Of the many enzymes involved in folate metabolism, there are four that could be directly implicated in these changes: dihydrofolate reductase (EC 1.5.1.3), pteroylpolyglutamate synthetase, 5-methyltetrahydrofolate-homocysteine transmethylase, and methylenetetrahydrofolate reductase (EC 1.1.1.68).

Dihydrofolate reductase catalyses the conversion of H₂PteGlu and other less reduced folates into metabolically active tetrahydrofolate forms. If the retention of folates by liver is dependent on prior reduction, then a failure in this process during vitamin B₁₂ deficiency could lead to the observed fall in liver folate concentrations.

Polyglutamate forms of reduced folates are severely depleted in vitamin B₁₂-deficient liver (Kutzbach *et al.*, 1967; Smith & Osborne-White, 1973) and in erythrocytes from patients with pernicious anaemia

* 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.

(Jeejeebhoy *et al.*, 1965), suggesting that the activity of pteroylpolyglutamate synthetase may be impaired.

Several workers have discussed the possibility that a metabolic deficiency of $H_4PteGlu$ exists in vitamin B_{12} deficiency as a consequence of a decrease in the rate of conversion of $5-CH_3-H_4PteGlu$ into $H_4PteGlu$, a reaction catalysed by the vitamin B_{12} -dependent enzyme 5-methyltetrahydrofolate-homocysteine transmethylase (Blakley, 1969). Because the formation of $5-CH_3-H_4PteGlu$, catalysed by methylenetetrahydrofolate reductase, is essentially irreversible *in vitro* (Katzen & Buchanan, 1965), it has been claimed that metabolically active folates become 'trapped' and unavailable as methyltetrahydrofolates. Although impaired transmethylase activity may contribute to the altered ratio of methylated to non-methylated folates observed in vitamin B_{12} -deficient liver, it cannot be the sole reason for the associated decrease in total liver folates, nor for the increase in methyltetrahydrofolate concentrations caused by methionine (Smith *et al.*, 1974). Kutzbach & Stokstad (1967) demonstrated that *S*-adenosyl-L-methionine inhibits the activity of methylenetetrahydrofolate reductase *in vitro* and attempted to explain part of the effect of methionine on folate metabolism by postulating that inhibition of this enzyme *in vivo* prevents the continued accumulation of folates as $5-CH_3-H_4PteGlu$. However, it is not known if amounts of methionine just sufficient to raise folate concentrations in the liver of vitamin B_{12} -deficient animals are also enough to increase the concentration of *S*-adenosyl-L-methionine to amounts that significantly inhibit methylenetetrahydrofolate reductase.

A specific failure in the membrane transport of folates during vitamin B_{12} deficiency might also account for the decreased concentration of folates in liver. True influx rates of the naturally occurring derivatives are difficult to measure because significant efflux and metabolism of these compounds occurs within seconds of their entry into the cell (Goldman, 1971*a*). Methotrexate (amethopterin-4-amino-10-methylpteroylglutamic acid), a structural analogue of folic acid appears to share the same transport system as natural folates, is strongly bound to dihydrofolate reductase within the cell, and is not further metabolized (Goldman, 1971*b*). This compound may therefore be used to study the effect of vitamin B_{12} or methionine on the unidirectional influx of folate in liver cells.

In the present paper we report changes in the activities of the above enzymes, and in the concentration of *S*-adenosyl-L-methionine in the liver of sheep during vitamin B_{12} deficiency, and after administration of methionine. Methotrexate transport was measured in liver slices, and the results are discussed in relation to current concepts of the role of vitamin B_{12} and methionine in folate metabolism. The concentrations of folate coenzymes, adenine nucleotides,

nicotinamide-adenine dinucleotides and other constituents in the livers of the same experimental animals are reported in the preceding paper (Smith *et al.*, 1974).

Experimental

Animals and diets

The experimental details have been previously described (Smith *et al.*, 1974). In brief, 16 Australian Merino ewes were divided equally into four treatment groups: $-B_{12}-Met$, $+B_{12}-Met$, $-B_{12}+Met$, $+B_{12}+Met$, and were fed a cobalt-deficient diet (Smith & Marston, 1970). Vitamin B_{12} -treated animals were pair-fed with vitamin B_{12} -deficient animals. There were eight pairs (nos. 006-007, 032-024, 022-034, 004-010, 016-042, 013-026, 030-008, 005-031). Vitamin B_{12} supplements were injected intramuscularly ($250\mu g$ weekly) and methionine intravenously (2g every second day). Conclusions regarding specific effects of vitamin B_{12} or methionine were drawn from the results obtained with this group of experimental animals. In addition, six normal sheep of similar age were fed *ad libitum* a stock diet consisting of equal parts of wheat hay chaff and dried lucerne chaff. Some parameters were measured in the liver of these animals, and the results have been included for general comparison with the experimental group.

Liver cytosol

Animals were killed by captive-bolt pistol and homogenates of pre-chilled liver (10g of liver mash plus 40ml of 0.25M-sucrose) were prepared as described by Smith *et al.* (1965). Each homogenate was centrifuged at $2^\circ C$ and 6600 *g* for 10min in a Spinco model L preparative ultracentrifuge and the sediment was discarded. The supernatant was re-centrifuged in the same machine at $2^\circ C$ and 105 500 *g* for 60min. The clear supernatant (liver cytosol) was removed and used in enzyme assays on the same day.

Enzyme assays

The activity of the following enzymes was assayed in liver cytosol. Enzyme activities were calculated on a mg of protein basis. There were no significant differences between the experimental groups in the mg of protein/g of liver (Smith *et al.*, 1974; Table 7).

Dihydrofolate reductase activity was measured by the procedure of Kaufman & Gardiner (1966) both with and without 1M-KCl (Reyes & Huennekens, 1967). $H_2PteGlu$ used in the assay was prepared by the method of Blakley (1960) and was stored at $2^\circ C$ as a suspension in 0.01M-HCl as recommended by Kaufman & Gardiner (1966).

The method used to determine pteroylpolyglutamate synthetase activity has been described previously (Gawthorne & Smith, 1973).

5-Methyltetrahydrofolate-homocysteine trans-methylase activity was determined by the method of Brot *et al.* (1966). Cyanocobalamin was omitted from the incubation medium.

The activity of methylenetetrahydrofolate reductase was measured in the forward direction by following the rate of conversion of 5,10-[¹⁴C]CH₂-H₄PteGlu into 5-[¹⁴C]CH₃-H₄PteGlu. Each incubation contained 100nmol of 5,10-[¹⁴C]CH₂-H₄PteGlu, 40nmol of FAD, 50nmol of NADH or NADPH, 29nmol of potassium ascorbate, 68nmol of potassium phosphate buffer, pH7.5, and 20μl of liver cytosol in a total volume of 0.5ml. 5,10-[¹⁴C]CH₂-H₄PteGlu was freshly prepared from [¹⁴C]formaldehyde (sp. radioactivity 0.53mCi/mmol) and H₄PteGlu by the method of Osborn *et al.* (1960) and was maintained under O₂-low N₂.

The buffer, ascorbate, NADH or NADPH and FAD were combined in small glass tubes and maintained at 0°C.

First, 5,10-[¹⁴C]CH₂-H₄PteGlu and then liver cytosol was dispensed from Agla microsyringes into the contents of each tube, under a stream of O₂-low N₂. The tubes were immediately stoppered and serially incubated at 37°C for 10, 20, 30 or 40min. A strict schedule was adhered to, so that all tubes had similar gassing times and accurate incubation periods. The reaction was terminated by plunging the tubes into a water bath at 95°C for 30s. Formaldehyde solution (20μl; approx 1%, w/v) was added to each tube, followed by 1ml of dimedone solution (10mg/ml in 0.1M-sodium acetate buffer, pH4.5). Each tube was heated for 10min at 95°C, cooled, centrifuged and 0.25ml of supernatant was applied to a column (0.5cm×3cm) of aluminium oxide type H. 5-[¹⁴C]CH₃-H₄PteGlu produced by the action of methylenetetrahydrofolate reductase was adsorbed on to the column, whereas other radioactive compounds were washed through with 5ml of acetic acid-acetone-methanol-benzene (1:1:4:14, by vol.). The 5-[¹⁴C]CH₃-H₄PteGlu was then eluted with 0.4M-HCl until exactly 3ml had emerged from the column. A portion (1ml) of this eluate was counted for radioactivity with 5ml of Triton X-100-toluene (6:7, v/v) scintillant (Patterson & Greene, 1965). The rate of reaction was calculated from the linear slope of the accumulation of radioactive counts during the incubation.

The activity of methylenetetrahydrofolate reductase was measured in the reverse direction also, with menadione (sodium bisulphite salt) as electron acceptor (Kutzbach *et al.*, 1967).

S-Adenosyl-L-methionine concentrations in liver

S-Adenosyl-L-methionine was measured in trichloroacetic acid extracts prepared without delay from pre-chilled liver mash by the procedures

described by Baldessarini (1966) and Baldessarini & Kopin (1963). S-Adenosyl-L-methionine used for standardization was purified by the ion-exchange treatment of Shapiro & Ehninger (1966) and was isolated from the 3M-H₂SO₄ eluate by the procedure of Schlenk & De Palma (1957).

S-Adenosyl-L-[methyl-¹⁴C]methionine was purified by chromatography on sheets of Whatman no. 1 filter paper with the solvent systems ethanol-water-acetic acid (65:34:1, by vol.) and propan-2-ol-1M-HCl-water (65:17:18, by vol.).

The hydroxyindole O-methyltransferase used in the assay was prepared from fresh bovine pineal glands (Axelrod & Weissbach, 1961).

Methotrexate transport in liver slices

[3',5'-³H]Methotrexate (sodium salt, sp. radioactivity 13Ci/mmol) and non-radioactive methotrexate, were purified by chromatography on DEAE-Sephadex with a linear concentration gradient of ammonium acetate (Gawthorne & Smith, 1973).

Methotrexate transport was studied in slices of liver taken from the eight penned animals (Smith *et al.*, 1974; Table 1). Liver slices, approx. 0.30mm thick and 1cm square, were hand-cut in a cold room (2°C) from blocks of liver that had been chilled in ice-cold 0.25M-sucrose within 1min of slaughtering the animals. The slices were rinsed in cold 0.85% (w/v) NaCl, blotted on Whatman no. 50 filter paper to remove excess of fluid, and then transferred into flat-bottomed glass tubes (one slice per tube) containing 1.9ml of cold Krebs-Ringer phosphate buffer (Rubin *et al.*, 1967) and 10μmol of D-glucose. Ten slices were used from each sheep, two for each incubation time. The tubes were removed from the cold room, placed in a water bath at 37°C and gently shaken for 2min to reach temperature equilibrium. The uptake of methotrexate was commenced by adding 0.1ml of [³H]methotrexate solution (4.0nmol, sp. radioactivity 0.241Ci/mmol) to each tube, and was terminated at 5, 10, 15, 20 and 40min in respective pairs of tubes by adding 10ml of ice-cold 0.85% (w/v) NaCl per tube and removing the tubes into an ice bath. The diluted medium was aspirated, and the slices were rinsed twice with 10ml portions of ice-cold 0.85% (w/v) NaCl in the cold room. After the final rinse, the slices were carefully blotted dry on Whatman no. 50 filter paper and were transferred to pre-weighed glass bottles. The slices were weighed and then heated with 0.5ml of 1M-KOH in a water bath for 45min at 80°C. Each digested slice was diluted to 10ml with water, and 1ml of this solution was mixed with 15ml of Triton X-100-toluene (6:7, v/v) scintillant (Patterson & Greene, 1965). The radioactivity was measured after 24h in the dark, when chemiluminescence had subsided.

Radioactive counting

Radioactive isotopes were counted in a Packard model 3375 scintillation counter. Quenching corrections were made by the external-standardization method.

Protein

This was measured 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.

Materials

Menadione (sodium bisulphite salt), dimedone, FAD, *S*-adenosyl-L-methionine and PteGlu were purchased from Sigma Chemical Co. Ascorbic acid and formaldehyde (sp. gravity 1.09 at 20°C) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., NADH and NADPH from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, methotrexate from K and K Laboratories Inc., Plainview, N.Y., U.S.A. and aluminium oxide (type H) from L. Light and Co. Ltd., Colnbrook, Bucks., U.K. [¹⁴C]Formaldehyde (sp. radioactivity 15mCi/mmol), [³H]acetic anhydride (sp. radioactivity 100mCi/mmol), *S*-adenosyl-L-[methyl-¹⁴C]methionine and [3',5'-³H]methotrexate (sp. radioactivity 13 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Other materials were as described by Gawthorne & Smith (1973) or were the best commercially available grades.

Results

The vitamin B₁₂ status and liver folate concentrations of the experimental animals have been reported (Smith *et al.*, 1974).

Dihydrofolate reductase

The effect of vitamin B₁₂ and methionine on the dihydrofolate reductase activity in the livers of the sheep is shown in Table 1. Reductase activity was maximally stimulated fourfold when 1M-KCl was included in the assay medium. The activity of dihydrofolate reductase in the liver of vitamin B₁₂-deficient sheep was only about 60% of that in pair-fed controls. This was true whether the assay was carried out in the absence ($P < 0.05$) or presence ($P < 0.001$) of 1M-KCl.

Methionine injections did not alter the lowered dihydrofolate reductase activity of deficient animals, and there was no significant interaction between methionine and vitamin B₁₂.

Pteroylpolyglutamate synthetase

Pteroylpolyglutamate synthetase activity tended to increase in the livers of vitamin B₁₂-deficient animals, and when 5-CH₃-H₄PteGlu was the substrate this effect was statistically significant ($P < 0.01$, Table 1).

Methionine did not alter the rate of polyglutamate synthesis from 5-CH₃-H₄PteGlu, but significantly decreased the rate of synthesis from 5-HCO-H₄PteGlu ($P < 0.05$, Table 1).

Table 1. Effect of vitamin B₁₂ and methionine on the activity of dihydrofolate reductase, pteroylpolyglutamate synthetase, and 5-methyltetrahydrofolate-homocysteine transmethylase in sheep liver cytosol

Each value is the mean $\pm$ s.e.m. for four animals. The results were statistically analysed by analysis of variance. Dihydrofolate reductase assays were performed in the presence or absence of 1M-KCl (see the text), and pteroylpolyglutamate synthetase activity was measured with either 5-CH₃-H₄PteGlu or 5-HCO-H₄PteGlu as substrate (Gawthorne & Smith, 1973). 5-Methyltetrahydrofolate-homocysteine transmethylase activity was determined in the absence of additional cyanocobalamin by the method of Brot *et al.* (1966). n.s., not significant. An expanded table, showing individual sheep values, is available as Supplementary Publication SUP 50028.

Treatment	Reductase activity (nmol/min per mg of protein)		Pteroylpolyglutamate synthetase activity (pmol/min per mg of protein)		Transmethylase activity (pmol/min per mg of protein)
	-KCL	+KCL	5-CH ₃ -H ₄ PteGlu	5-HCO-H ₄ PteGlu	
-B ₁₂ -Met	0.61 $\pm$ 0.04	2.26 $\pm$ 0.34	12.7 $\pm$ 0.9	5.0 $\pm$ 0.6	10.4 $\pm$ 2.2
+B ₁₂ -Met (pair-fed)	1.04 $\pm$ 0.06	4.57 $\pm$ 0.36	7.6 $\pm$ 0.7	3.4 $\pm$ 0.2	57.3 $\pm$ 8.6
-B ₁₂ +Met	0.66 $\pm$ 0.10	3.19 $\pm$ 0.35	10.1 $\pm$ 1.1	2.8 $\pm$ 0.7	16.3 $\pm$ 5.2
+B ₁₂ +Met (pair-fed)	0.91 $\pm$ 0.14	4.32 $\pm$ 0.29	7.4 $\pm$ 1.2	2.6 $\pm$ 0.4	46.9 $\pm$ 14.0
Significance of B ₁₂ effect	$P < 0.05$	$P < 0.001$	$P < 0.01$	n.s.	$P < 0.01$
Significance of Met effect	n.s.	n.s.	n.s.	$P < 0.05$	n.s.

5-Methyltetrahydrofolate-homocysteine transmethy-lase

Vitamin B₁₂ deficiency resulted in an 80% decrease in liver transmethy-lase activity ($P < 0.01$, Table 1).

Methionine had no significant effect on the activity of this enzyme.

5,10-Methylenetetrahydrofolate reductase

The methylenetetrahydrofolate reductase activity of normal and experimental sheep is shown in Table 2. There was no significant effect of vitamin B₁₂ or methionine, and this was true when either NADH or NADPH was used as the electron donor, or even when the enzyme was assayed in the reverse direction with menadione as electron acceptor. The methylene-tetrahydrofolate reductase activity in the livers of the experimental sheep was approximately twice that found in animals fed on a stock diet (Table 2).

S-Adenosyl-L-methionine concentrations in liver

The concentration of *S*-adenosyl-L-methionine in the livers of normal and experimental sheep at slaughter is shown in Table 2. Liver *S*-adenosyl-L-methionine concentrations were decreased 50% by vitamin B₁₂ deficiency ($P < 0.01$), and were restored to normal by either vitamin B₁₂ or methionine. With the exceptions of sheep no. 013 and no. 026 the *S*-adenosyl-L-methionine concentration in the liver of all animals given vitamin B₁₂ or methionine was not significantly greater than normal.

Inhibition of 5,10-methylenetetrahydrofolate reductase by S-adenosyl-L-methionine

The degree of inhibition of methylenetetrahydro-folate reductase from sheep liver caused by various concentrations of *S*-adenosyl-L-methionine *in vitro* is illustrated in Fig. 1.

By comparing the concentrations of *S*-adenosyl-L-methionine in liver with the results in Fig. 1 it was clear that for only one pair of animals (nos. 013 and 026) did *S*-adenosyl-L-methionine concentrations *in vivo* (43 nmol/ml and 100 nmol/ml respectively) persist at amounts sufficient to cause significant inhibition of methylenetetrahydrofolate reductase activity *in vitro*.

Methotrexate transport in liver slices

Methotrexate is strongly bound to dihydrofolate reductase within the liver cell and is not further metabolized (Blakley, 1969). If the quantity of methotrexate transported into the cell is less than the binding capacity of the dihydrofolate reductase present, the intracellular concentration of unbound methotrexate is negligible, and a linear rate of influx is observed (Goldman *et al.*, 1968). Linear rates of uptake were obtained in our experiments during the first 20 min of incubation. After this time the rate of uptake declined in slices of liver from all animals except those in the vitamin B₁₂-treated methionine-treated group (Fig. 2).

Intravenous injections of methionine increased the initial rate of transport of methotrexate in liver slices

Table 2. Effect of vitamin B₁₂ and methionine on the activity of methylenetetrahydrofolate reductase and the concentration of *S*-adenosyl-L-methionine in sheep liver

The activity of methylenetetrahydrofolate reductase in liver cytosol was measured in the forward direction for individual sheep as described in the text, and in the reverse direction with pooled cytosol (equal volume from each animal on a given treatment) and menadione as electron acceptor. Neither vitamin B₁₂, nor methionine significantly affected the activity of the enzyme. *S*-Adenosyl-L-methionine was measured in trichloroacetic acid extracts of liver by methods described by Baldessarini (1966) and Baldessarini & Kopin (1963). Each value is the mean $\pm$ S.E.M. for four animals. An expanded table, showing individual sheep values, is available as Supplementary Publication SUP 50028.

Treatment	Methylenetetrahydrofolate reductase activity (pmol/min per mg of protein)			<i>S</i> -Adenosyl-L-methionine	
	Forward direction		Reverse direction with menadione		
	with NADH	with NADPH		(nmol/g of liver)	(nmol/g of cell water)
-B ₁₂ -Met	422 $\pm$ 24	1179 $\pm$ 245	487	10.24 $\pm$ 0.60	14.49 $\pm$ 1.04
+B ₁₂ -Met (pair-fed)	558 $\pm$ 129	667 $\pm$ 57	487	20.77 $\pm$ 1.27	28.74 $\pm$ 1.62
-B ₁₂ +Met	549 $\pm$ 65	1021 $\pm$ 174	493	19.67 $\pm$ 4.97	25.92 $\pm$ 6.37
+B ₁₂ +Met (pair-fed)	709 $\pm$ 115	1005 $\pm$ 133	425	34.28 $\pm$ 13.7	46.28 $\pm$ 18.23
				20.71 $\pm$ 2.9*	28.24 $\pm$ 3.76*
Normal animals (fed <i>ad lib.</i>)	220 $\pm$ 21	202 $\pm$ 35	359	17.16 $\pm$ 0.91	23.20 $\pm$ 1.23

* Omitting sheep no. 026.

from deficient animals (Table 3, $P<0.05$), and acted synergistically with vitamin B₁₂ to increase the amount of methotrexate transported in 40min

($P<0.01$). Although vitamin B₁₂ did not significantly affect the rate of methotrexate transport in the first 20min, it did increase the quantity taken up in

Fig. 1. Inhibition of sheep liver methylenetetrahydrofolate reductase by S-adenosyl-L-methionine

The activity of methylenetetrahydrofolate in reductase the liver of normal sheep was measured *in vitro* in the presence of various concentrations of S-adenosyl-L-methionine. Assays were conducted with either NADH (○) or NADPH (●) as electron donors, and the reductase activity is expressed as d.p.m. of 5-[¹⁴C]CH₃-H₄PteGlu formed/min per mg of protein. Each value is the mean of four determinations.

Fig. 2. Effect of vitamin B₁₂ and methionine on methotrexate uptake in sheep liver slices

The figure shows the accumulation of methotrexate with time in liver slices incubated *in vitro*. Slices were taken from the liver of animals on the following treatments: -B₁₂-Met (○); +B₁₂-Met (●); -B₁₂+Met (Δ); +B₁₂+Met (▲). Values are means of duplicate determinations of each of two animals.

Table 3. Effect of vitamin B₁₂ and methionine on the uptake of methotrexate in slices of sheep liver

The initial uptake of methotrexate was calculated from the linear rate of uptake in the first 20 min of incubation (see the text). The 40 min uptake represents the cumulative amount of methotrexate taken up by the liver slices in the first 40 min of incubation. The results were statistically analysed by analysis of variance. The effect of methionine on the initial rate of uptake was significant ($P<0.05$) and there was a highly significant ($P<0.01$) interaction between methionine and vitamin B₁₂ in the 40min uptake.

		Methotrexate uptake	
Animal no.	Treatment	Initial rate (pmol/min per mg of slice)	40 min uptake (pmol/mg of slice)
022	-B ₁₂ -Met	0.035	1.119
004		0.047	1.167
		Mean	1.143
034	+B ₁₂ -Met (pair-fed)	0.066	1.550
010		0.057	1.425
		Mean	1.488
030	-B ₁₂ +Met	0.052	2.128
005		0.043	1.732
		Mean	1.930
008	+B ₁₂ +Met (pair-fed)	0.124	3.764
031		0.082	3.700
		Mean	3.732
5	Normal animals (fed <i>ad lib.</i>)	0.125	3.732
6		0.086	3.045
		Mean	3.389

40min. The size of the increase varied, and was greater if methionine had also been administered (Table 3, $P < 0.01$).

Discussion

The decrease in activity of dihydrofolate reductase and 5-methyltetrahydrofolate-homocysteine transmethylase probably contributed to the fall in concentration of H₄PteGlu and the increased ratio of methylated to non-methylated tetrahydrofolates (Smith *et al.*, 1974) observed in the liver of vitamin B₁₂-deficient animals. Because vitamin B₁₂-treated animals were pair-fed with vitamin B₁₂-deficient animals, it may be concluded that the impaired function of each enzyme was a specific result of depleted vitamin B₁₂ reserves and was not a secondary effect of the loss of appetite that accompanies this deficiency in sheep. Decreased transmethylation activity has previously been observed in the liver of vitamin B₁₂-deficient chickens (Dickerman *et al.*, 1964) and rats (Kutzbach *et al.*, 1967); decreased dihydrofolate reductase activity in vitamin B₁₂-deficient chicken liver has been noted by Pasquali *et al.* (1968).

In contrast with vitamin B₁₂, methionine did not significantly alter the activity of either enzyme, although it increased liver folate concentrations, including that of H₄PteGlu, by more than 100%; and partially restored the ratio of methylated to non-methylated forms (Smith *et al.*, 1974). It has been suggested that methionine influences folate metabolism by raising the concentration of *S*-adenosyl-L-methionine in liver thereby inhibiting methylenetetrahydrofolate reductase activity and the continued production of 5-CH₃-H₄PteGlu (Oace *et al.*, 1968). Decreased concentrations of 5-CH₃-H₄PteGlu_n and increased concentrations of H₄PteGlu_n and HCO-H₄PteGlu_n in liver would be expected as a result. In only one animal (no. 026) did methionine injections increase the concentration of *S*-adenosyl-L-methionine in liver markedly above normal. This was accompanied by depletion of methylated folates and accumulation of non-methylated folates (Smith *et al.*, 1974). This is consistent with significant inhibition of methylenetetrahydrofolate reductase activity *in vivo*. However, the concentration of *S*-adenosyl-L-methionine in the livers of the seven other animals treated with methionine was similar to that found in normal sheep fed on a stock diet (Table 2). Sheep liver methylenetetrahydrofolate reductase is inhibited by *S*-adenosyl-L-methionine *in vitro* (Fig. 1), but the degree of inhibition is less than that reported for the rat enzyme by Kutzbach & Stokstad (1967). Concentrations of *S*-adenosyl-L-methionine within the normal range of 20–30nmol/ml did not appreciably alter the activity of the sheep enzyme *in vitro*. Further, there was no significant correlation between the concentration of *S*-adenosyl-L-methionine in liver and

the relative proportion of non-methylated folates, indicating that these two factors were not closely linked. We conclude that a degree of inhibition of methylenetetrahydrofolate reductase activity sufficient to cause a significant change in the ratio of methylated to non-methylated folates in liver is only likely at concentrations of *S*-adenosyl-L-methionine outside the range sustained by injections of physiological amounts of methionine.

The effect of methionine in increasing the concentration of liver folates overshadowed any changes in the relative proportions of the coenzyme forms. This is also true for rats (Kutzbach *et al.*, 1967; Thenen & Stokstad, 1973). Such increases in folate retention cannot be accounted for by changes in the activities of the above enzymes because none of them responded to methionine. Although allosteric effects of methionine or a metabolic product of methionine other than *S*-adenosyl-L-methionine cannot be entirely ruled out, there are no known effects that can be invoked. Neither can a hypothesis of promotion of pteroylpolyglutamate synthesis by vitamin B₁₂ or methionine be sustained. First, pteroylpolyglutamates were not depleted to any greater extent than were pteroylmonoglutamates in liver from vitamin B₁₂-deficient animals (Smith *et al.*, 1974). Secondly, if changes in the activity of pteroylpolyglutamate synthetase were primarily responsible for the decrease in liver folate concentrations during vitamin B₁₂ deficiency and for the restoration of these concentrations by vitamin B₁₂ or methionine, then the activity of this enzyme would be expected to decrease in deficient animals and increase after administration of vitamin B₁₂ or methionine. The opposite occurred (Table 1). The increase in pteroylpolyglutamate synthetase activity during vitamin B₁₂ deficiency may represent a mechanism for conserving folates as supplies of pteroylmonoglutamates diminish.

The changes in concentration of folates that we have observed in sheep liver (Smith *et al.*, 1974) could be explained if vitamin B₁₂ or methionine were to regulate intracellular supplies of pteroylmonoglutamates by altering the rate of transport of these substances into the liver cell. The effect of intravenous administration of methionine on methotrexate transport in liver slices (Table 3) is evidence of such a role. Methotrexate is structurally similar to folic acid, and appears to share the same membrane transport system as 5-CH₃-H₄PteGlu and 5-HCO-H₄PteGlu in L1210 leukemia cells, Ehrlich ascites-tumour cells and rabbit reticulocytes (Goldman, 1971*b*). The binding of methotrexate to dihydrofolate reductase and the absence of further metabolism, makes it possible to measure the unidirectional inflow of this compound, rather than the net difference between inflow and outflow as is the case when the naturally occurring folates are used (Goldman, 1971*a*). The fact that methionine increases methotrexate uptake suggests that it also

stimulates the transport of folates from the plasma, into the liver. Vitamin B₁₂ may promote this action indirectly by increasing the activity of 5-methyltetrahydrofolate-homocysteine transmethylase (Table 1), thereby raising the intracellular concentration of methionine, or methionine derivatives such as *S*-adenosyl-L-methionine (Table 2).

In conclusion, our results indicate that folate metabolism fails in vitamin B₁₂-deficient sheep because of an impairment in the rate of transport of folates into the liver. Intracellular supplies of tetrahydropteroylmonoglutamates diminish and pteroylpolyglutamate stores are drawn on to make up for this deficit. Supplies of the essential coenzyme H₄PteGlu are further decreased by the decreased activity of 5-methyltetrahydrofolate-homocysteine transmethylase and dihydrofolate reductase. Methionine injections stimulate the transport of folates into the cell and relieve the failing folate metabolism by increasing the supply of tetrahydropteroylmonoglutamates. When comparatively large amounts of methionine are injected, the liver concentration of *S*-adenosyl-L-methionine may become unphysiologically high, and inhibition of methylenetetrahydrofolate reductase activity may increase the supply of non-methylated folates while decreasing the concentration of methylated forms.

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