

Effect of methionine on the metabolic fate of liver folates in vitamin B₁₂-deficient rats

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1. Hepatocytes isolated from vitamin B₁₂-deficient and vitamin B₁₂-supplemented rats were maintained in primary culture and were used to study the effect of methionine on the metabolism of [³H]folic acid and [5-¹⁴C]methyltetrahydrofolic acid.

2. Vitamin B₁₂ levels were reduced by approximately 75 % in the hepatocytes from the deficient animals. Total folate and methyltetrahydrofolic acid concentrations were also significantly reduced.

3. There was no significant difference in the uptake and retention of added [³H]folic acid and [5-¹⁴C]-methyltetrahydrofolic acid between the hepatocytes of the two groups. The incorporation of ¹⁴C into phospholipids was reduced by approximately 60 % in the vitamin B₁₂-deficient hepatocytes (*P* < 0.001).

4. The addition of methionine to the culture medium doubled the uptake and retention of ³H in both groups, but it did not change the amount of water-soluble ¹⁴C compounds. In the vitamin B₁₂-deficient hepatocytes mainly methylated folate increased, whereas non-methylated folate increased in the hepatocytes of the control animals. A tenfold increase of ¹⁴C incorporated into phospholipids was found in both groups after methionine was added.

5. Demethylation of methyltetrahydrofolic acid, the intracellular retention of folate and the utilization of liberated methyl groups, for example in the methylation of phospholipids, were highest in the presence of both methionine and vitamin B₁₂, suggesting an intimate co-ordination between these two substances in the regulation of folate metabolism.

The metabolic relationship between folate, vitamin B₁₂ and methionine has been a subject of several investigations and reviews in recent years (Vidal & Stokstad, 1974; Herbert & Das, 1976; Krebs *et al.* 1976). Both vitamins are required as cofactors in the biosynthesis of methionine from homocysteine (Fig. 1). The methyl group in methyltetrahydrofolic acid is transferred via vitamin B₁₂ and tetrahydropteroylglutamate methyltransferase (*EC* 2.1.1.13) to homocysteine. Methyltetrahydrofolic acid is produced from 5,10-methylene-tetrahydrofolic acid by an irreversible reaction, and the methylation of homocysteine is the only known way in which methyltetrahydrofolic acid may lose its methyl group (Katzen & Buchanan, 1965).

In vitamin B₁₂ deficiency, the methyltransferase activity is depressed; and owing to the irreversibility of the reaction forming methyltetrahydrofolic acid, this compound accumulates. Consequently, much of the available folate becomes trapped and unavailable (Herbert & Zalusky, 1961, 1962; Noronha & Silverman, 1962).

In the presence of methionine, the formation of methyltetrahydrofolic acid is depressed, whereas without methionine and vitamin B₁₂ methyltetrahydrofolic acid is trapped owing to a lack of inhibition of its synthesis by S-adenosylmethionine. Thus, methionine is a key factor in the regulation of the methyltetrahydrofolic acid concentration (Chiao & Stokstad, 1977).

It has also been reported that vitamin B₁₂ affects the cellular uptake of methyltetrahydrofolic acid (Lavoie *et al.* 1974) and is involved in the intracellular synthesis of pteroyl-polyglutamates (Chanarin *et al.* 1974).

The present investigation was designed to illustrate the interdependence of vitamin B₁₂,

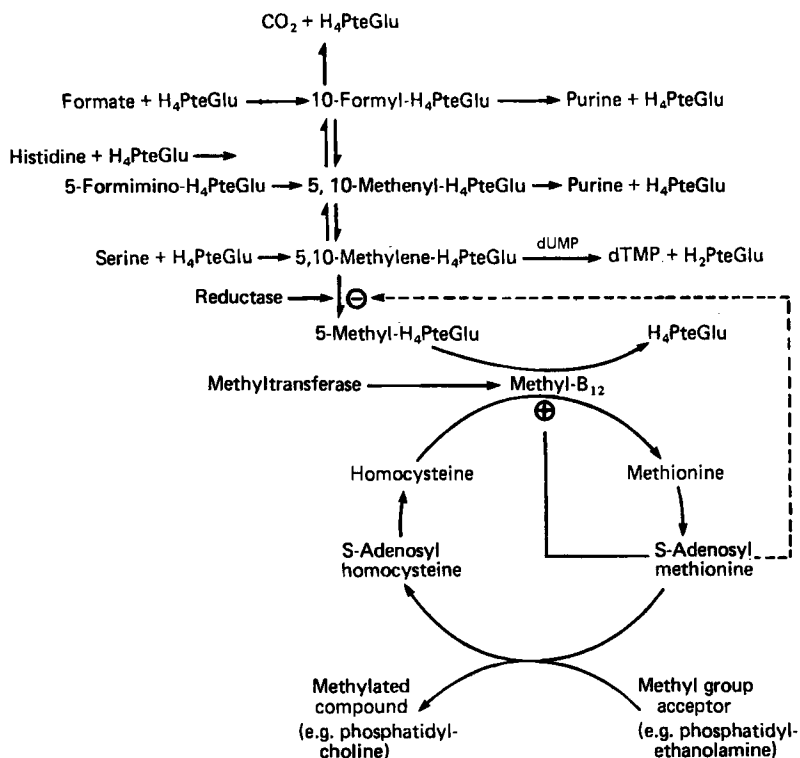


Fig. 1. Metabolic scheme showing the pathway for transfer of one carbon unit by folate derivatives with the involvement of vitamin B₁₂ and methionine. (Glu, glutamic acid; PteGlu, pteroylglutamic acid; H₄, tetrahydro; H₂, dihydro; dUMP, deoxyuridylylate; dTMP; deoxythymidylylate.)

folate and methionine at the cellular level by using cultured hepatocytes from vitamin B₁₂-deficient rats. The results obtained are discussed in relation to similar studies performed *in vivo*.

EXPERIMENTAL PROCEDURES

Animals

Wistar strain, male rats were placed on a vitamin B₁₂-deficient diet from 4 weeks of age, and the experiments were performed on 13-month-old animals. The control animals were supplemented with vitamin B₁₂ in the drinking-water (20 µg/l). The rats were housed in stainless-steel cages with elevated wire-mesh floors as described by Fehling *et al.* (1978).

Diet

The diet has been described previously (Fehling *et al.* 1978). Its gross composition was (g/kg diet): sugars 330, soya-bean protein 571, soya-bean oil 48, vitamin and salt mixtures 55. It also contained (/kg) 5.5 g L-methionine, 1 mg folic acid and less than 2 µg vitamin B₁₂.

Isotopes

[5-¹⁴C]methyltetrahydrofolic acid, barium salt (58 mCi/mmol), 3',5',9'[³H]folic acid, potassium salt (500 mCi/mmol) and [³H]ethanolamine (3.8 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks, UK.

In vivo experiments

Vitamin B₁₂-deficient rats and control rats were injected intraperitoneally with 70 μ Ci [³H]-ethanolamine and 1 mmol L-methionine/kg body-weight 4 h before slaughter. The rats were fasted for 24 h before death.

Preparation of hepatocytes

After the control and vitamin B₁₂-deficient rats had fasted overnight, hepatocytes were prepared as previously described (Åkesson, 1977). The hepatocytes were transferred in 2.5 ml medium to 60 mm plastic petri dishes coated with collagen (Lin & Snodgrass, 1975). The medium used was L-15 tissue-culture medium lacking methionine, choline, inositol and folic acid and supplemented with 28 mM-Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid), 1 mM-sodium succinate, insulin (0.5 μ g/ml), penicillin (100 μ g/ml), gentamycin (50 μ g/ml) and foetal calf serum (20 g/l) (Åkesson, 1977). After 1 h the medium in the petri dishes was changed to the same medium but with the addition of [³H]folic acid (2 μ Ci, 2 μ g), [⁵-¹⁴C]methyltetrahydrofolic acid (0.5 μ Ci, 5.7 μ g) or methionine (0.5 mM), or both.

After 20 h the medium was removed and the hepatocytes were washed with 2 \times 1 ml ice-cold isotonic sodium chloride (9 NaCl/l). The cells were transferred with 3 \times 1 ml 0.1 M-sodium phosphate buffer, pH 6.1, containing freshly added ascorbic acid (150 mg/100 ml buffer), into tubes, which were stored at -20° until assay.

Analytical procedures

Folate was liberated from the harvested hepatocytes by boiling them for 10 min in 0.1 M-sodium phosphate buffer, pH 6.1, containing freshly added ascorbic acid (150 mg/100 ml buffer). Folate concentration and radioactivity were determined in the supernatant fraction after centrifugation. Lipids were extracted from the pellet as described previously (Åkesson, 1977). Lipids from whole livers were extracted and separated by thin-layer chromatography (Åkesson *et al.* 1979). The phospholipid was regarded as representing the amount of hepatocytes present in each culture.

Methods for microbiological analyses of folates and vitamin B₁₂ have been described earlier (Fehling & Jägerstad, 1978).

RESULTS

Experiments in cultured hepatocytes

Effect of vitamin B₁₂ status on folate metabolism. The hepatocytes obtained from vitamin B₁₂-deficient rats contained 0.02-0.04 nmol of vitamin B₁₂ per μ mol P (P = lipid phosphorus as a measure of cellular mass). The corresponding value for the control hepatocytes was 0.15-0.19 nmol per μ mol P. The deficient hepatocytes contained lower concentrations of total folates and methylated tetrahydrofolates, although the proportion of methylated tetrahydrofolates was not significantly different (Table 1).

To investigate the influence of vitamin B₁₂ status on the incorporation of folates, [³H]folic acid and [⁵-¹⁴C]methyltetrahydrofolic acid were added to the culture medium separately or in combination. No difference between the groups could be demonstrated regarding the uptake of ³H radioactivity and ¹⁴C radioactivity after incubation for 20 h (Table 2). The added folates retained in the cells accounted for approximately half the total folate in deficient hepatocytes and a quarter to a half in the controls (Table 1).

Addition of [⁵-¹⁴C]methyltetrahydrofolic acid increased total folate and methylated folates in both groups ($P < 0.001$). The proportion of methylated folate rose from 52 to 71 % in the vitamin B₁₂-deficient and from 70 to 79 % in the vitamin B₁₂-supplemented group (Table 1).

Table 1. Comparison between cultured hepatocytes deprived or supplemented with vitamin B₁₂ as regards concentrations of total folate (A, nmol/μmol P), methyltetrahydrofolate (B, nmol/μmol P) and non-methyltetrahydrofolate (C, nmol/μmol P); methyltetrahydrofolate acid calculated as the difference between Lactobacillus casei-active folate (total folate) and Streptococcus faecalis-active folate after enzymic hydrolysis of pteroylpolylglutamates

Group	[P]folate	Methionine	[5- ¹⁴ C]-MTHF	(Mean values with their standard errors for eight observations)									
				A			B			C			
				Vitamin B ₁₂ -		Vitamin B ₁₂ -		Vitamin B ₁₂ -		Deficient		Supplemented	
				Deficient	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
I	+	-	-	0.25***	0.01	0.44	0.03	0.13***	0.02	0.31	0.02	0.12 ^{NS}	0.02
II	+	+	-	0.38*	0.04	0.53	0.04	0.25 ^{NS}	0.03	0.32	0.03	0.13*	0.02
III	+	-	+	0.49*	0.04	0.63	0.04	0.35**	0.04	0.50	0.02	0.14 ^{NS}	0.02
IV	+	+	+	0.70 ^{NS}	0.12	0.91	0.06	0.51 ^{NS}	0.11	0.55	0.09	0.19**	0.01

+, Present; -, absent.

P, phospholipids as a measure of cellular mass; MTHF, methyltetrahydrofolate; NS, not significant (Student's *t*-test, vitamin B₁₂-supplemented v vitamin B₁₂-deficient).

* *P* < 0.05, ** 0.001 < *P* < 0.01, *** *P* < 0.001.

Table 2. The uptake and retention of [³H] activity from [³H] folic acid and [¹⁴C] activity from [^{5-¹⁴C}]MTHF (percentage of administered dose/ μ mol phospholipids (P) in hepatocytes cultured with and without methionine and the folate isotopes for 20 h

(Mean values with their standard errors for eight observations)

[³ H]-folic acid	Methio-nine	[^{5-¹⁴C}]MTHF	[³ H] activity				[¹⁴ C] activity			
			B ₁₂ -deficient		B ₁₂ -supplemented		B ₁₂ -deficient		B ₁₂ -supplemented	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
+	-	-	2.52 ^{NS}	0.09	2.62	0.14
+	+	-	4.40 ^{NS}	0.43	4.86	0.52
+	-	+	2.63 ^{NS}	0.25	2.82	0.20	2.22 ^{NS}	0.16	2.60	0.45
+	+	+	3.79 ^{NS}	0.29	4.38	0.41	2.57 ^{NS}	0.25	3.65	0.69

+, Present; -, absent.

MTHF, methyltetrahydrofolic acid; P, phospholipids as a measure of cellular mass; NS, not significant (Student's *t*-test, different from B₁₂-supplemented rats).

Table 3. Effect of methionine on methylation of phospholipids (P; as a measure of cellular mass) from [^{5-¹⁴C}]methyltetrahydrofolic acid in cultured hepatocytes from rats deprived of or supplemented with vitamin B₁₂

(Mean values with their standard errors for eight observations; [¹⁴C] activity is expressed as a percentage of administered doses/ μ mol P; P = lipid phosphorus which was used as a measure of cellular mass)

	Vitamin B ₁₂ -deficient		Vitamin B ₁₂ -supplemented	
	Mean	SE	Mean	SE
With methionine	0.76 ^{***}	0.24	1.72 ^{***}	0.49
Without methionine	0.06	0.02	0.16	0.05

*** $P < 0.001$ (Student's *t*-test, effect of methionine).

The incorporation of ¹⁴C from [^{5-¹⁴C}]methyltetrahydrofolic acid into lipids was significantly lower ($P < 0.001$) in the vitamin B₁₂-deficient hepatocytes (Table 3). The radioactivity was found almost exclusively in phosphatidylcholine (81–93%), indicating that it was incorporated by phospholipid methylation.

Effect of methionine. When methionine was added to the culture medium the metabolism of ³H folate was affected in several respects (Table 1, group II versus group I). The total amount of folate increased in both groups but significantly ($P < 0.05$) only in the vitamin B₁₂-deficient group. Furthermore, in the vitamin B₁₂-deficient group the methylated pool was nearly doubled ($0.001 < P < 0.01$), whereas in the vitamin B₁₂-supplemented group only the non-methylated folates increased to a comparable extent ($0.001 < P < 0.01$). Addition of methionine to the medium in the presence of both [³H]folic acid and [^{5-¹⁴C}]methyltetrahydrofolic (Table 1, group IV versus group III) also increased methylated folate in the vitamin B₁₂-deficient group ($0.001 < P < 0.01$), whereas in the supplemented group only non-methylated folate increased significantly ($0.001 < P < 0.01$). The uptake of ³H from [³H]folic acid was nearly doubled in both groups when methionine was added to hepatocyte cultures (Table 2). The incubation period for the uptake of [³H]folic acid is shown in Fig. 2. Methionine administration caused a steady increase of ³H with time in hepatocytes from both vitamin B₁₂-deficient and supplemented rats.

The addition of methionine did not affect the amount of cellular water-soluble ¹⁴C

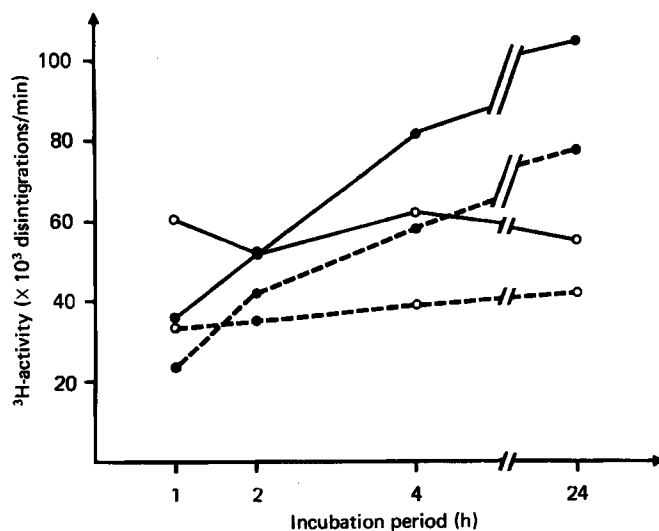


Fig. 2. Effect of methionine on the uptake and retention of [^3H]folic acid in cultured hepatocytes from vitamin B_{12} -deficient rats and controls. Controls with methionine (●—●), without methionine (○—○); vitamin B_{12} -deficient with methionine (●---●), without methionine (○---○).

Table 4. Effect of methionine on phospholipid methylation in liver of rats deprived of or supplemented with vitamin B_{12} determined after injection of [^3H]ethanolamine

	Vitamin B_{12} -deficient				Vitamin B_{12} -supplemented	
	Mean	SE	Mean	SE	Mean	SE
Liver uptake of [^3H]ethanolamine injected intraperitoneally ($\times 10^{-3}$ /g wet tissue)	174***	22	241*	27	331	13
^3H in phosphatidylcholine (%) \ddagger	12	1.4	14.5***	0.8	19.9	0.7
No. of rats	5		4		6	

† 1 mmol methionine/kg body-weight was injected intraperitoneally 4 h before slaughter.

‡ (^3H in phosphatidylcholine) $100/^3\text{H}$ in phosphatidylcholine plus phosphatidylethanolamine.

Statistical significance of difference between vitamin B_{12} -deficient and vitamin B_{12} -supplemented animals calculated using Student's t -test; * $P < 0.05$, *** $P < 0.001$.

compounds after incubation with [$5\text{-}^{14}\text{C}$]methyltetrahydrofolic acid (Table 2), but increased the incorporation of ^{14}C into phospholipids tenfold in both groups regardless of vitamin B_{12} status (Table 3).

Experiments in vivo

The vitamin B_{12} -deficient rats weighed less than the control rats, and their plasma levels of vitamin B_{12} were approximately 57 pmol/l versus 916 pmol/l in the control rats. Corresponding figures for the liver vitamin B_{12} were 11 nmol/g versus 79 nmol/g.

The effect of methionine on phospholipid methylation was studied after injection of [^3H]ethanolamine. The uptake of [^3H]ethanolamine into liver phospholipids was significantly lower in vitamin B_{12} -deficient rats (Table 4). The rate of phospholipid methylation expressed as a percentage of phospholipid radioactivity in phosphatidylcholine was decreased in vitamin B_{12} -deficient rats. The increase observed after injection of methionine was not significant.

DISCUSSION

The depressed concentrations of folate found in vitamin B₁₂-deficient hepatocytes (Table 1) are in accordance with observations *in vivo* (Smith & Osborne-White, 1973; Thenen & Stokstad, 1973), where rats were kept on vitamin B₁₂-free diets also low in methionine. It has been demonstrated by Krebs *et al.* (1976) that hepatocytes may lose as much as 90 % of their methionine content during cell preparation. Therefore, both the vitamin B₁₂-deficiency and control hepatocytes might be depleted of methionine in analogy to the dietary studies cited above.

The most probable explanation of the depressed liver folate secondary to the vitamin B₁₂-deficient is that the synthesis of polyglutamic folates is disturbed. (Buehring *et al.* 1972; Chiao & Stokstad, 1977). These folates are the dominating forms intracellularly and tetrahydrofolic acid the preferred substrate in their synthesis (Lavoie *et al.* 1974; Chiao & Stokstad, 1977). Furthermore, the synthesis of polyglutamic folate is a relatively slow process with maximal radioactivity 24 h after administration of labelled folate (Chiao & Stokstad, 1977). In the presence of methionine, the uptake and retention of [³H]folic acid is greatly increased (Vidal & Stokstad, 1974; Williams & Spray, 1976; Chiao & Stokstad, 1977), as was also demonstrated *in vitro* in the present study. This effect of methionine has been found to be associated with increased synthesis of polyglutamic folate. (Buehring *et al.* 1972; Chiao & Stokstad, 1977).

The suggestion of a direct involvement of vitamin B₁₂ in the cellular uptake of folates has not received much support in recent studies (Shane *et al.* 1977; Horne & Briggs, 1980). The vitamin B₁₂ state of the cultured hepatocytes in the present study did not affect the uptake and retention of exogenous labelled folate derivatives ([³H]folic acid and [5-¹⁴C]-methyltetrahydrofolic acid). This is not consistent with *in vivo* findings (Kutzbach *et al.* 1967; Gawthorne & Stokstad, 1971; Thenen & Stokstad, 1973; Fehling & Jägerstad, 1978), where vitamin B₁₂-deficient animals usually retain smaller amounts of exogenous labelled folate derivatives. One explanation for this discrepancy in the results of *in vivo* and *in vitro* experiments could be differences in the methionine concentrations with consequences on the formation of polyglutamic folates. Both vitamin B₁₂-deficient and supplemented *in vitro* hepatocytes may be more, and equally, depleted in methionine (Krebs *et al.* 1976) than livers of living rats. *In vivo*, control rats probably have higher tissue concentrations of methionine when compared with the vitamin B₁₂-deficient animals because the vitamin B₁₂-dependent conversion of homocysteine to methionine may occur fully only in the control group (Fig. 1).

According to the methyl folate trap theory (Noronha & Silverman, 1962; Herbert & Zalusky, 1962), 5-methyltetrahydrofolic acid accumulates during vitamin B₁₂-deficiency. Such an accumulation of 5-methyltetrahydrofolic acid has been demonstrated in livers of vitamin B₁₂-deficient rats when radioactivity from administered labelled folates have been studied (Buehring *et al.* 1972; Thenen & Stokstad, 1973; Davidson *et al.* 1975). After equilibration of added labelled folate and the endogenous folate pool, microbiological assay of folate does not lend support to an intracellular accumulation of methylated folates in vitamin B₁₂-deficiency (Davidson *et al.* 1975; Fehling & Jägerstad, 1978). Neither could an accumulation of 5-methyltetrahydrofolic acid be shown in the cultured hepatocytes where methylated folate constituted approximately 50 % of the total folate in the vitamin B₁₂-deficient hepatocytes compared to 70 % in the vitamin B₁₂-supplemented cells.

The levels of 5-methyltetrahydrofolic acid appear to be controlled by two enzymes. The first one, 5,10-methylenetetrahydrofolate reductase, catalyses the conversion of 5,10-methyltetrahydrofolic acid to 5-methyltetrahydrofolic acid, and this conversion is most likely irreversible under physiological conditions (Katzen & Buchanan, 1965). The second enzyme

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controlling the level of 5-methyltetrahydrofolic acid is the vitamin B₁₂-dependent 5-methyltetrahydrofolate:homocysteine methyltransferase (Fig. 1). In vitamin B₁₂-deficiency this methyltransferase is depressed (Dickerman *et al.* 1964; Kutzbach *et al.* 1967), and consequently 5-methyltetrahydrofolic acid should accumulate according to the methyl folate trap theory. A combined deprivation of vitamin B₁₂ and of methionine also should result in an accumulation of 5-methyltetrahydrofolic acid, because S-adenosylmethionine formed from methionine inhibits 5,10-methylenetetrahydrofolate reductase (Kutzbach & Stokstad, 1971). Thus, in the absence of methionine, this inhibitory effect fails to appear.

Addition of methionine to the culture medium altered the proportions of 5-methyltetrahydrofolic acid, mainly because of an increase of methylated folate in the vitamin B₁₂-deficient cells from 50 to 70 %, whereas non-methylated folate increased in the control cells altering the proportion of methylated folate from 70 to 60 %. These different effects of the methionine supply may seem inconsistent but are possible to explain on the basis of the methyl folate trap theory.

According to Fig. 1 the added methionine may be converted to S-adenosylmethionine. That S-adenosylmethionine actually is formed is supported by the enhanced methylation of phosphatidylethanolamine to phosphatidyl choline (Table 3). Consequently, the levels of S-adenosylhomocysteine may increase, too, and the inhibitory effect of S-adenosylmethionine on the 5,10-methylenetetrahydrofolate reductase has been shown to be partially reversed by S-adenosylhomocysteine (Kutzbach & Stokstad, 1971). Therefore, formation of 5-methyltetrahydrofolic acid is possible in the presence of methionine. In the presence of adequate amounts of vitamin B₁₂, as in the control group, a demethylation of methylated folates may rapidly occur resulting in a net increase of non-methylated folates, whereas a lack of vitamin B₁₂ could depress the demethylation by inhibition of methyltransferase resulting in an accumulation of 5-methyltetrahydrofolic acid (Fig. 1).

A study performed in cultured bone marrow cells obtained from vitamin B₁₂-deficient rats reports similar effects of added methionine on the levels of methylated folate (Cheng *et al.* 1975).

The present study also confirms a previous report (Åkesson *et al.* 1978), where vitamin B₁₂-deficient rats were shown to have reduced ability to methylate phospholipids in their livers. This is consistent with a depressed activity of the vitamin B₁₂-dependent methyltransferase reaction producing less methionine for methyl group transfer (Fig. 1).

In the vitamin B₁₂-depleted cultured hepatocytes, the phospholipids incorporated only approximately half of ¹⁴C-labelled methyl groups from [5-¹⁴C]methyltetrahydrofolic acid as in the control hepatocytes. The addition of methionine increased this incorporation tenfold in both groups. In living animals, however, the effect of methionine was less pronounced (Table 4). The methionine concentration may be of great importance for this effect and was probably lower in the *in vivo* experiment.

The use of cultured hepatocytes is promising for further research to elucidate the complex relationship between methionine, vitamin B₁₂ and folate metabolism. Several of the previous results obtained *in vivo* have been confirmed in the hepatocyte model used in the present study. On the other hand, divergent results here and in other studies may be due to unphysiologically high concentrations in the culture medium of the substances studied.

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