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# Effect of Vitamin $B_{12}$ deficiency on phosphatidylethanolamine methylation in rat liver

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1. In vitamin $B_{12}$ deficiency the activity of tetrahydropteroylglutamate methyltransferase (*EC* 2.1.1.13) is depressed and the synthesis of methionine is reduced. Because the methyl group of methionine is largely utilized for the methylation of phosphatidylethanolamine, we investigated the effects of vitamin $B_{12}$ deficiency on phosphatidylcholine synthesis.

2. The incorporation of injected [$^{14}$C]formaldehyde into liver phosphatidylcholine was reduced by approximately 50% in vitamin $B_{12}$-deficient rats. Also the corresponding incorporation of 5-[$^{14}$C]methyltetrahydrofolic acid tended to decrease. The findings are consistent with a lower conversion of these precursors to methionine.

3. The effect of the deficient methyl-group supply on phosphatidylcholine synthesis was also investigated by the injection of [$^{14}$C]ethanolamine. The amount (%) of lipid-$^{14}$C recovered in phosphatidylcholine was significantly reduced in vitamin $B_{12}$ deficiency.

4. Chemical analysis of liver phospholipids showed that the vitamin $B_{12}$-deficient rats had a higher proportion of phosphatidylethanolamine and a lower proportion of phosphatidylcholine, indicating that the impaired synthesis of phosphatidylcholine by methylation leads to changes in membrane phospholipid composition.

Several components of the diet are important for the supply and turnover of one-carbon fragments in the body, such as methionine, choline, vitamin $B_{12}$ and folate. The effects on phospholipid composition and metabolism have been studied primarily for the two former nutrients. Choline is incorporated into phosphatidylcholine via CDP-choline (Kennedy, 1957) and the methyl group of methionine is used for the methylation of phosphatidylethanolamine to phosphatidylcholine (Bremer, Figard & Greenberg, 1960) (Fig. 1). In rats fed on a diet deficient in methionine and choline the proportion of phosphatidylethanolamine in the liver increases at the expense of phosphatidylcholine (Blumenstein, 1964; Haines, 1966). In the same condition an increased activity of phosphatidylethanolamine methyltransferase (*EC* 2.1.1.17) has been reported (Fallon, Gertman & Kemp, 1969; Turkki & Silvestre, 1970; Glenn & Austin, 1971; Acheampong-Mensah & Feuer, 1974). Folate plus vitamin $B_{12}$ in the diet may also influence phospholipid levels (Blumenstein, 1964) but hitherto the effect of isolated vitamin $B_{12}$ deficiency has not been studied. Methionine is synthesized by methylation of homocysteine by tetrahydropteroylglutamate methyltransferase (*EC* 2.1.1.13), a vitamin $B_{12}$-dependent enzyme (Loughlin, Elford & Buchanan, 1964). Part of the methionine is converted to S-adenosyl-L-methionine which donates methyl groups to several acceptors, above all to phosphatidylethanolamine (Fig. 1). In vitamin $B_{12}$ deficiency, the amounts of methionine available for phosphatidylcholine synthesis may therefore be reduced. Preliminary experiments showed no changes in liver phospholipid composition in vitamin $B_{12}$ deficiency (Fehling, Jägerstad & Arvidson, 1978). In this investigation, rats with a more severe deficiency were used for a detailed study of phosphatidylethanolamine methylation. A lower methylation of phosphatidylethanolamine to phosphatidylcholine was found in vitamin $B_{12}$ deficiency, leading to a larger proportion of phosphatidylethanolamine in the liver phospholipids.

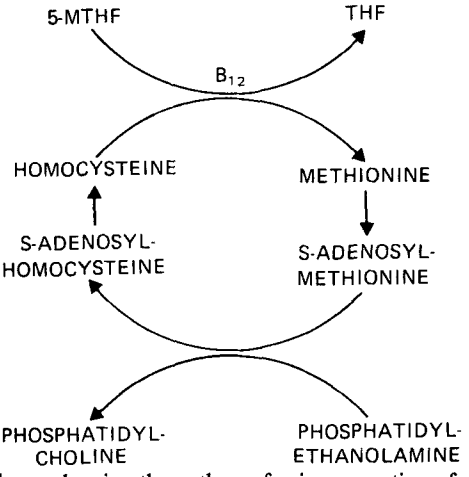


Fig. 1. Metabolic scheme showing the pathway for incorporation of methyl groups into phosphatidylcholine. THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate.

## EXPERIMENTAL PROCEDURE

### *Animals*

Male and female Wistar rats were reared on a vitamin $B_{12}$-deficient diet as described elsewhere (Fehling, Jägerstad, Åkesson, Axelsson & Brun, 1978). The content in the diet of choline chloride was 760 mg/kg, folic acid 0·95 mg/kg, vitamin $B_{12}$ less than 2 $\mu$g/kg and L-methionine 11·4 g/kg (Expts A–C) or 6·7 g/kg (Expt D). The control animals received in addition cyanocobalamin, 40 $\mu$g/l in the drinking-water. Information on vitamin $B_{12}$ levels, growth characteristics and neurological symptoms in these rats have been described elsewhere (Fehling, Jägerstad, Åkesson, *et al.* 1978). In short, the vitamin $B_{12}$ concentration in plasma was 28–101 pg/ml after 12–15 months of deficiency, the supplemented rats having a concentration of more than 550 pg/ml. Vitamin $B_{12}$ in liver was 1–15 ng/g wet weight in vitamin $B_{12}$-deficient rats and 51–91 ng/g wet weight in supplemented rats. Neurological symptoms were detected after 7 months of age.

### *Isotope injection experiments*

[$^{14}$C]formaldehyde (15·4 mCi/mmol), 5-[$^{14}$C]methyltetrahydrofolic acid (53 mCi/mmol) and [$^{14}$C]ethanolamine (55 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks., UK. The labelled compounds were dissolved in saline (9 mg sodium chloride/ml) and 1 ml was injected intraperitoneally into rats.

*Expt A.* [$^{14}$C] formaldehyde (20 $\mu$ Ci) was injected into female rats, 15 months old.

*Expt B.* 5-[$^{14}$C]methyltetrahydrofolic acid (5 $\mu$Ci, 0·1 $\mu$mol) was dissolved in 1 ml 0·1 M-sodium phosphate buffer (pH 7·0), containing 1 $\mu$l mercaptoethanol/ml, and injected into female rats, 13 months old.

*Expt C.* [$^{14}$C]ethanolamine (2 $\mu$Ci) was injected into male rats, 12 months old. The day before the experiment these rats were fasted.

*Expt D.* [$^{14}$C]ethanolamine (2 $\mu$Ci) was injected into 8-month-old female rats.

The livers were taken out 1 h after [$^{14}$C]formaldehyde injection, 24 h after [$^{14}$C]methyltetrahydrofolic acid injection and 2 h after [$^{14}$C]ethanolamine injection. They were immediately immersed in liquid nitrogen (Fehling & Jägerstad, 1978). Lipid extraction and analysis was performed as described previously (Fehling, Jägerstad, Åkesson *et al.* 1978), except that the amounts of individual phospholipids were determined according to Belfrage, Wiebe & Lundquist (1970).

Table 1. *Expts A and B.* Distribution of $^{14}C$ among rat liver lipids (% lipid $^{14}C$) after injection of [$^{14}C$]formaldehyde (Expt A) and [5-$^{14}C$]methyltetrahydrofolic acid (Expt B) into rats deprived of or supplemented with vitamin $B_{12}$*

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

| | Vitamin $B_{12}$-deficient (3) | | Vitamin $B_{12}$-supplemented (3) | |
|---|---|---|---|---|
| Expt A | Mean | SE | Mean | SE |
| Lipids in solvent front | 10·0 | 1·9 | 6·4 | 0·6 |
| Phosphatidylethanolamine | 6·3 | 0·3 | 3·2 | 0·8 |
| Phosphatidylcholine | 81·2 | 2·3 | 85·3 | 1·4 |
| Sphingomyelin + lysophosphatidylcholine | 2·5 | 0·9 | 5·2 | 1·4 |
| | Vitamin $B_{12}$-deficient (2) | | Vitamin $B_{12}$-supplemented (2) | |
| Expt B | | | | |
| Lipids in solvent front | 2·0 | | 0·0 | |
| Phosphatidylethanolamine | 1·6 | | 0·5 | |
| Phosphatidylcholine | 86·1 | | 90·1 | |
| Sphingomyelin + lysophosphatidylcholine | 11·3 | | 9·4 | |

* For details of experiments, see p. 522.

Table 2. *Expts A and B.† Incorporation of [$^{14}C$]formaldehyde (Expt A) and [5-$^{14}C$]methyltetrahydrofolic acid (Expt B) into liver phosphatidylcholine of rats deprived of or supplemented with vitamin $B_{12}$ (% injected dose/g tissue wet weight)*

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

| | Vitamin $B_{12}$-deficient (6) | | Vitamin $B_{12}$-supplemented (5) | |
|---|---|---|---|---|
| Expt A | Mean | SE | Mean | SE |
| $^{14}C$ in liver lipids | 0·018 | 0·002* | 0·036 | 0·003 |
| Lipid phosphorus ($\mu$mol/g) | 40·4 | 1·2*** | 38·6 | 1·1 |
| | Vitamin $B_{12}$-deficient (4) | | Vitamin $B_{12}$-supplemented (5) | |
| Expt B | Mean | SE | Mean | SE |
| $^{14}C$ in liver lipids | 0·28 | 0·05*** | 0·40 | 0·08 |

Statistical significance of difference between vitamin $B_{12}$-deficient and supplemented animals calculated using Student's $t$ test: * $0{\cdot}001 < P < 0{\cdot}01$, *** $P > 0{\cdot}05$.

† For details of experiments, see p. 522.

## RESULTS

To investigate the consequences of vitamin $B_{12}$ deficiency for phosphatidylcholine synthesis, two precursors of the methyl group in methionine were administered and the appearance of radioactivity in liver lipids was measured. With both precursors, most of the lipid radioactivity was located in phosphatidylcholine which is consistent with the isotope being incorporated via methylation of phosphatidylethanolamine (Table 1). After [$^{14}C$]formaldehyde injection, $^{14}C$ in liver lipids in vitamin $B_{12}$-deficient rats was only half that in controls (Table 2). Also after the injection of labelled methyltetrahydrofolic acid, there was a tendency towards a decreased incorporation but in this instance the variation between animals was larger and no statistically significant difference was observed. The results

Table 3. *Expts C and D.† Methylation of phosphatidylethanolamine to phosphatidylcholine (PC) 2 h after the injection of 2 uCi [$^{14}$C]ethanolamine into rats deprived of or supplemented with vitamin $B_{12}$*

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

| | Vitamin $B_{12}$-deficient (6) | | Vitamin $B_{12}$-supplemented (3) | |
|---|---|---|---|---|
| Expt C | Mean | SE | Mean | SE |
| $^{14}$C in liver lipids ($10^{-3}$ × counts/min per g) | 76·8 | 9·9*** | 66·1 | 4·8 |
| $^{14}$C in PC‡ (%) | 11·0 | 0·9* | 16·6 | 0·7 |
| | Vitamin $B_{12}$-deficient (6) | | Vitamin $B_{12}$-supplemented (5) | |
| Expt D | Mean | SE | Mean | SE |
| $^{14}$C in liver lipids ($10^{-3}$ × counts/min per g) | 88·1 | 17·0** | 134·8 | 9·4 |
| $^{14}$C in PC‡ (%) | 15·8 | 1·6** | 21·4 | 1·4 |

Statistical significance of difference between vitamin $B_{12}$-deficient and supplemented animals calculated using Student's *t* test: * $0{\cdot}001 < P < 0{\cdot}01$, ** $0{\cdot}01 < P < 0{\cdot}05$, *** $P > 0{\cdot}05$.

† For details of experiments, see p. 522.

‡ ($^{14}$C in phosphatidylcholine) 100/$^{14}$C in phosphatidylcholine plus phosphatidylethanolamine.

Table 4. *Expt D.† Phospholipid composition (% lipid P) in livers of rats deprived of or supplemented with vitamin $B_{12}$*

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

| | Vitamin $B_{12}$-deficient (6) | | Vitamin $B_{12}$-supplemented (5) | |
|---|---|---|---|---|
| Main compounds of phospholipid fractions | Mean | SE | Mean | SE |
| Solvent front + cardiolipin | 6·5 | 0·6*** | 5·8 | 0·1 |
| Phosphatidylethanolamine | 27·7 | 0·9** | 24·2 | 1·1 |
| Phosphatidylcholine | 60·0 | 3·8** | 65·4 | 1·1 |
| Sphingomyelin + lysophosphatidylcholine | 4·8 | 0·4*** | 4·6 | 0·3 |

Statistical significance of difference between vitamin $B_{12}$-deficient and supplemented animals calculated using Student's *t* test: ** $0{\cdot}01 < P < 0{\cdot}05$, *** $P > 0{\cdot}05$.

† For details of experiments, see p. 522.

indicate that a reduced activity of tetrahydropteroylglutamate methyltransferase is reflected in the lower incorporation of 1C units into phosphatidylcholine.

Another approach for measuring phosphatidylcholine synthesis via methylation was to study the conversion of phosphatidylethanolamine to phosphatidylcholine after administration of labelled ethanolamine. In isolated hepatocytes this conversion is markedly stimulated by the addition of methionine (Sundler & Åkesson, 1975). In Expts C and D [$^{14}$C]ethanolamine was injected intraperitoneally, and the conversion of phosphatidylethanolamine to phosphatidylcholine was measured (Table 3). The appearance of $^{14}$C in liver lipids was similar in the vitamin $B_{12}$-deficient and supplemented groups in Expt C, whereas in Expt D the uptake was somewhat higher in the vitamin $B_{12}$-supplemented group. In both experiments the conversion (%) to phosphatidylcholine was significantly lower in the vitamin $B_{12}$-deficient rats. The conversion rates were higher in both vitamin $B_{12}$-deficient and supplemented rats in Expt D (females) than in Expt C (males). A higher methylation

rate in female rats has been observed previously (Natori, 1963; Bjørnstad & Bremer, 1966). The reduced conversion of phosphatidylethanolamine to phosphatidylcholine in vitamin $B_{12}$ deficiency correlates well with the decreased incorporation of methyl group precursors into phosphatidylcholine (Table 2).

The changes in phospholipid synthesis during vitamin $B_{12}$ deficiency also changed the phospholipid composition (Table 4). The vitamin $B_{12}$-deficient rats had a higher level of phosphatidylethanolamine and a lower level of phosphatidylcholine, which can be ascribed to the decreased methylation rate. No change in the total phospholipid per g liver was noted (Table 2).

## DISCUSSION

This study demonstrated derangements in liver phospholipid metabolism and composition in vitamin $B_{12}$ deficiency. The low incorporation of [5-$^{14}$C]methyltetrahydrofolic acid and its precursor [$^{14}$C]formaldehyde into liver phosphatidylcholine of vitamin $B_{12}$-deficient rats is in accordance with the depressed activity of tetrahydropteroylglutamate methyltransferase in this condition (Dickerman, Redfield & Weissbach, 1964). The slower phospholipid synthesis is in line with the finding that the incorporation of [$^{14}$C]formate into choline and methionine is reduced in vitamin $B_{12}$ deficiency (Arnstein, 1959).

$^{14}$C from [5-$^{14}$C]methyltetrahydrofolic acid is less readily retained or taken up or both in the livers of vitamin $B_{12}$-deficient rats than in their controls after 24 h under certain circumstances (Fehling & Jägerstad, 1978). In the present study $^{14}$C activity in whole liver was not significantly lower in the vitamin $B_{12}$-deficient rats. Also, the proportion of endogenous 5-methyltetrahydrofolate was normal. The lower incorporation of $^{14}$C in phosphatidylcholine therefore most probably reflects the relative inhibition of tetrahydropteroylglutamate methyltransferase occurring in vitamin $B_{12}$ deficiency (Dickerman *et al.* 1964).

The lower incorporation of [$^{14}$C]formaldehyde into liver phosphatidylcholine could not be due to changes in formate oxidation because this process is normal in vitamin $B_{12}$ deficiency, provided the diet is adequate in methionine and folate (Brothers, O'neill Rowley & Gerritsen, 1975). Furthermore, if the dietary methyl-group supply were low the incorporation of [$^{14}$C]formaldehyde into liver phosphatidylcholine would rather be increased (Acheampong-Mensah & Feuer, 1974). This may reflect increased activities both in tetrahydropteroylglutamate methyltransferase (Finkelstein, Kyle & Harris, 1971) and phosphatidylethanolamine methyltransferase (Fallon *et al.* 1969; Turkki & Silvestre, 1970; Glenn & Austin, 1971; Acheampong-Mensah & Feuer, 1974).

A large part of methionine in liver is converted to S-adenosylmethionine, most of which is used for phosphatidylethanolamine methylation (Bjørnstad & Bremer, 1966). Regulation of the methionine concentration involves primarily inhibition of methylenetetrahydrofolate reductase (*EC* 1.1.1.68) by S-adenosylmethionine and stimulation of formyltetrahydrofolate dehydrogenase (*EC* 1.5.1.7) by methionine (Krebs, Hems & Tyler, 1976). In spite of these regulatory mechanisms the liver concentration of S-adenosylmethionine is decreased in vitamin $B_{12}$-deficient rats (Vidal & Stokstad, 1974) and sheep (Gawthorne & Smith, 1974). The levels are normalized after the administration of methionine.

The changes in phosphatidylethanolamine methylation in rats on methyl-group-deficient diets have been studied by several investigators. The activity of phosphatidylethanolamine methyltransferase increases in choline deficiency and the administration of choline to rats decreases the activity (Skurdal & Cornatzer, 1975). Phosphatidylethanolamine methylation may also be assessed by following the appearance of radioactivity in phosphatidylcholine after the injection of labelled ethanolamine. Since the total incorporation of ethanolamine into liver phospholipids may vary, it is most relevant to express this factor as the percentage of total phospholipid radioactivity in phosphatidylcholine. In the present study, a decreased

incorporation of ethanolamine into total phospholipids was observed only in vitamin $B_{12}$-deficient female rats (Table 3). More important, in both female and male rats the conversion of labelled phosphatidylethanolamine to phosphatidylcholine was depressed in vitamin $B_{12}$ deficiency (Table 3). This indicates that in spite of the adequate dietary supply of methionine, there was a decreased availability of methionine in the liver.

Vitamin $B_{12}$ deficiency increased the proportion of phosphatidylethanolamine and decreased the proportion of phosphatidylcholine (Table 4). Changes in the same direction have been observed in livers from rats reared on a diet deficient in choline, methionine or both (Blumenstein, 1964; Haines, 1966; Thompson, MacDonald & Mookerjea, 1969).

No changes in total phospholipid (Table 2) or total lipid (Fehling, Jägerstad, Åkesson *et al.* 1978) were observed. This is compatible with the previous finding that dietary choline or methionine are much more effective than vitamin $B_{12}$ in preventing fatty liver in animals fed on a diet deficient in choline, methionine and vitamin $B_{12}$ (Tuma, Barak & Sorrel, 1975).

Normally, the relative proportion of different liver phospholipids are kept within close limits. The viscosity of different membranes may be affected by a change in the value for phosphatidylethanolamine:phosphatidylcholine, as recently demonstrated in a strain of mouse fibroblasts (LM cells) (Esko, Gilmore & Glaser, 1977). Phospholipid composition can also be modified in primary hepatocyte culture by base substitution (Åkesson, 1977) or methionine availability (Åkesson, unpublished results) and therefore this experimental system represents a useful model for future studies on the functional role of exogenously-induced alterations in membrane phospholipid composition.

It is difficult to evaluate the functional significance of such membrane changes in the liver. They could conceivably have remote effects on other organs, such as the brain or spinal cord, the specialized functions of which are even more membrane dependent. Work is in progress to explore this possibility, which is at present purely speculative.

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## REFERENCES

Acheampong-Mensah, D. & Feuer, G. (1974). *Nutr. Rep. int.* **10**, 297.
Åkesson, B. (1977). *Biochim. Biophys. Res. Commun.* **76**, 93.
Arnstein, H.R.V. (1959). *Biochem. J.* **73**, 23P.
Belfrage, P., Wiebe, T. & Lundquist, A. (1970). *Scand. J. clin. Lab. Invest.* **26**, 53.
Bjørnstad, P. & Bremer, J. (1966). *J. Lipid Res.* **7**, 38.
Blumenstein, J. (1964). *Can. J. Biochem. Physiol.* **42**, 1183.
Bremer, J., Figard, P. H. & Greenberg, D. M. (1960). *Biochim. biophys. Acta* **43**, 477.
Brothers, V., O'neill Rowley, B. & Gerritsen, T. (1975). *Archs Biophys. Biochem.* **166**, 475.
Dickerman, H., Redfield, B. G. & Weissbach, H. (1964). *J. biol. Chem.* **239**, 2545.
Esko, J. D., Gilmore, J. R. & Glaser, M. (1977). *Biochemistry, Easton* **16**, 1881.
Fallon, H. J., Gertman, P. M. & Kemp, E. L. (1969). *Biochim. biophys. Acta* **187**, 94.
Fehling, C. & Jägerstad, M. (1978). *Nutr. Metabl.* **22**, 90.
Fehling, C., Jägerstad, M., Åkesson, B., Axelsson, J. & Brun, A. (1978). *Br. J. Nutr.* **39**, 501.
Fehling, C., Jägerstad, M. & Arvidson, G. (1978). *Nutr. Metabl.* **22**, 82.
Finkelstein, J. D., Kyle, W. E. & Harris, B. J. (1971). *Archs Biochem. Biophys.* **146**, 84.
Gawthorne, J. M. & Smith, R. M. (1974). *Biochem. J.* **142**, 119.
Glenn, J. L. & Austin, W. (1971). *Biochim. biophys. Acta* **231**, 153.
Haines, D. S. M. (1966). *Can. J. Biochem. Physiol.* **44**, 45.
Kennedy, E. P. (1957). *A. Rev. Biochem.* **26**, 119.
Krebs, H. A., Hems, R. & Tyler, B. (1976). *Biochem. J.* **158**, 341.

Loughlin, R. E., Elford, H. L. & Buchanan, J. M. (1964). *J. biol. Chem.* **239**, 2888.
Natori, Y. (1963). *J. biol. Chem.* **238**, 2075.
Skurdal, D. N. & Cornatzer, W. E. (1975). *Int. J. Biochem.* **6**, 579.
Sundler, R. & Åkesson, B. (1975). *J. biol. Chem.* **250**, 3359.
Thompson, W., MacDonald, G. & Mookerjea, S. (1969). *Biochim. biophys. Acta* **176**, 306.
Tuma, D. J., Barak, A. J. & Sorrel, M. F. (1975). *Biochem. Pharmac.* **24**, 1327.
Turkki, P. R. & Silvestre, M. T. G. (1970). *Nutr. Rep. int.* **2**, 141.
Vidal, A. J. & Stokstad, E. L. R. (1974). *Biochim. biophys. Acta* **362**, 245.

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