# DEFECTIVE METABOLISM OF VITAMIN $B_{12}$ IN FIBROBLASTS FROM CHILDREN WITH METHYLMALONICACIDURIA

Maurice J. Mahoney and Leon E. Rosenberg
Division of Medical Genetics, Yale University School of Medicine
New Haven, Connecticut 06510

S. Harvey Mudd and B. William Uhlendorf
National Institute of Mental Health and
Laboratory of Viral Immunology, Division of Biologics Standards
National Institutes of Health, Bethesda, Maryland 20014

Received May 24, 1971

SUMMARY. Fibroblasts from a patient with $B_{12}$ responsive methylmalonicaciduria and from a second patient with methylmalonicaciduria plus abnormal sulfur amino acid metabolism were examined for their ability to accumulate the two $B_{12}$ coenzymes, deoxyadenosyl-$B_{12}$* and methyl-$B_{12}$, and for $N^5$-methyltetrahydrofolate homocysteine methyltransferase activity. Cells from the first patient accumulated very little deoxyadenosyl-$B_{12}$ but accumulated methyl-$B_{12}$ normally. His cells had normal methyltransferase activity when assayed with and without added methyl-$B_{12}$ coenzyme. In contrast, cells from the second patient accumulated neither coenzyme. Methyltransferase activity was very low when assayed without added methyl-$B_{12}$ but increased markedly when coenzyme was added. We conclude that both patients have primary defects in vitamin $B_{12}$ metabolism and that these defects are biochemically distinct.

Two mammalian enzymes are known to require a $B_{12}$ coenzyme (1). One is methylmalonyl-CoA mutase which catalyses the isomerization of L-methylmalonyl-CoA to succinyl-CoA and requires as coenzyme, deoxyadenosyl-$B_{12}$. The second $B_{12}$ requiring enzyme is $N^5$-methyltetrahydrofolate homocysteine methyltransferase (methyl$FH_4$-methyltransferase) which catalyses the methylation of homocysteine to methionine while regenerating tetrahydrofolate from methyltetrahydrofolate. Isolation of methyl-$B_{12}$ from the methyl$FH_4$-methyltransferase of porcine kidney (2) as well as from the enzyme of *E. coli* (3,4) strongly supports methyl-$B_{12}$ as the cofactor for the methyltransferase.

Recent investigations of two children with methylmalonicaciduria have suggested that abnormalities in the metabolism of vitamin $B_{12}$ underlie their disease states (5,6). One of these children, R.P., had $B_{12}$ responsive methylmalonicaciduria (7). In culture his fibroblasts were shown to have a normal methylmalonyl-CoA mutase apoenzyme but to contain only a small fraction of the normal amount of deoxyadenosyl-$B_{12}$ (8). Treating the child with huge

*$B_{12}$ is used in place of cobalamin, i.e. cyano-$B_{12}$ for cyanocobalamin, etc.

doses of vitamin $B_{12}$ (1000 μg/day) led to 70% reduction in methylmalonate excretion, and growing his fibroblasts in a culture medium supplemented with large amounts of cyano-$B_{12}$ or hydroxo-$B_{12}$ (1μg/ml) corrected the deficient mutase activity in the cells and restored the amount of intracellular deoxyadenosyl-$B_{12}$ to normal. No abnormality of sulfur amino acid metabolism was found in this child.

In the second child with methylmalonicaciduria, E.M., both $B_{12}$ requiring reactions were blocked. Post-mortem examination showed a very low deoxyadenosyl-$B_{12}$ content of liver and kidney (5) and studies with cultured skin fibroblasts had findings compatible with deficient methylmalonyl-CoA mutase activity which was corrected by adding 1 μg/ml of hydroxo-$B_{12}$ to the culture medium (9). In contrast to the first child, E.M. also had abnormalities of sulfur amino acid metabolism (5,10). Decreased methionine and increased homocystine and cystathionine were found in plasma and tissues. Methyl$FH_4$-methyltransferase activity in fibroblast extracts was barely detectable when assayed without added methyl-$B_{12}$ whereas activity increased greatly with methyl-$B_{12}$ in the assay medium (9).

Both of these children appear to have defects in the biosynthesis of $B_{12}$ coenzymes. The problem is different in the two, for R.P.'s defect involves only the deoxyadenosyl-$B_{12}$ dependent reaction, while E.M.'s defect blocks both the deoxyadenosyl-$B_{12}$ and the methyl-$B_{12}$ dependent reactions. In this report direct evidence for the hypothesis of abnormal $B_{12}$ metabolism, with a different abnormality in each child, has been obtained by measuring the accumulation of newly synthesized $B_{12}$ coenzymes in cultured fibroblasts, and by performing studies of methyl$FH_4$-methyltransferase activity in R.P.'s fibroblasts similar to those carried out previously in E.M. (9).

## MATERIALS AND METHODS

Fibroblasts were grown from skin biopsies from R.P., the 3 year old boy with $B_{12}$ responsive methylmalonicaciduria; from E.M., the 7 week old infant with methylmalonicaciduria plus abnormal sulfur amino acid metabolism; and from several controls, ages 1 week to 35 years. Cells were grown in tissue culture as previously described (9,11). The cultures were maintained in absolute darkness except for occasional exposure to dim light during examination or manipulation. Methods for assay of methyl$FH_4$-methyltransferase activity and protein concentration (9,10) and for measuring the accumulation of $B_{12}$ coenzymes (11) have been published. A sample of propyl-$B_{12}$ was provided through the kindness of Mr. C. Spears and Dr. H. Weissbach.

## RESULTS AND DISCUSSION

$N^5$-Methyltetrahydrofolate homocysteine methyltransferase. The results of assays of methyl$FH_4$-methyltransferase activity in cell free, gel filtered extracts of control cells and cells from E.M. and R.P. are summarized in Table 1. The methyltransferase activity in extracts of R.P. cells was within the control range, whether assayed with or without added methyl-$B_{12}$, and whether the cells had been grown in basal medium or in medium to which hydroxo-$B_{12}$ had been added in a concentration of 1 μg/ml. In contrast, as has been shown previously (9), the methyltransferase activities in E.M. cells differed from control activities in several ways. Extracts of cells grown in basal medium had no significant methyltransferase activity if the assays were done without an added $B_{12}$ compound. Addition of methyl-$B_{12}$ restored activity to 57% of the mean control value. Extracts of cells grown in medium with added hydroxo-$B_{12}$ contained only 6% of mean control methyltransferase activity when assayed without added $B_{12}$. Addition of methyl-$B_{12}$ restored activity to 31% of control.

By analogy with the $B_{12}$ dependent methyltransferase from E. coli we have interpreted the methyltransferase activity in the absence of added $B_{12}$ as a measure of methyltransferase holoenzyme (apoenzyme complexed to suitable $B_{12}$ coenzyme) and the activity in the presence of methyl-$B_{12}$ as a measure of total enzyme (holoenzyme plus apoenzyme). For the E. coli enzyme, methyl-$B_{12}$ is specifically able to convert apoenzyme to holoenzyme, whereas propyl-$B_{12}$ is not (12). We tested the effect of propyl-$B_{12}$ (13.6 μg) in our standard assay. No significant methyltransferase activity was observed in extracts of E.M. cells grown in basal medium and assayed in the presence of propyl-$B_{12}$. In control cell extracts, propyl-$B_{12}$ did not increase enzyme activity above the level observed in an assay without added $B_{12}$. This result supports the previous interpretation that the $\frac{\text{activity with no added } B_{12}}{\text{activity with methyl-}B_{12}}$ ratio is a measure of the fraction of total enzyme present as holoenzyme. Control cells grown in basal medium have about half their methyltransferase present as holoenzyme. Cells grown with added hydroxo-$B_{12}$ show 84% of total enzyme as holoenzyme. R.P. cell extracts are normal in this regard. In E.M. cells, however, only a small fraction of total enzyme is present as holoenzyme, even when the cells are grown in the presence of hydroxo-$B_{12}$. Almost all of this active methyltransferase protein is apoenzyme. We believe the striking deficiency of methyltransferase holoenzyme in E.M. cells occurs because of a failure of the cells to supply the $B_{12}$ coenzyme required for activity.

Accumulation of $B_{12}$ coenzymes in fibroblasts. The data describing the accumulation of total $B_{12}$, deoxyadenosyl-$B_{12}$, and methyl-$B_{12}$, by fibroblasts

TABLE 1: $N^5$-Methyltetrahydrofolate Homocysteine Methyltransferase Activity in Extracts of Control Cells and Cells from Patients with Methylmalonicaciduria

| Conditions | Activity (nmole methionine formed/mg protein/hr) | | |
|---|---|---|---|
| | Controls | Patients with methylmalonicaciduria | |
| | | E.M. | R.P. |
| **Cells grown in basal medium** | | | |
| No added $B_{12}$ | 4.2 (2.9-7.3) | 0.03 (0-0.08) | 4.6 (5.0, 4.3) |
| With methyl $B_{12}$, $5 \times 10^{-6}$M | 7.9 (6.1-11.3) | 4.5 (3.5-5.7) | 7.8 (7.8, 7.9) |
| Unsupplemented activity, % | 53 (41-65) | 0.7 (0-2) | 59 (64, 54) |
| **Cells grown in basal medium + hydroxo-$B_{12}$ (1 μg/ml)** | | | |
| No added $B_{12}$ | 15.6 (11.5-18.7) | 0.9 (0.6-1.4) | 12.9 (10.9-15.4) |
| Methyl-$B_{12}$, $5 \times 10^{-6}$M | 18.8 (14.3-23.4) | 5.9 (4.6-7.1) | 15.6 (14.4-18.1) |
| Unsupplemented activity, % | 84 (73-98) | 15 (11-25) | 82 (76-86) |

Cell free extracts were prepared and subjected to gel filtration before assay without added $B_{12}$ or with an optimal concentration of methyl-$B_{12}$. Cells were studied from 15 control subjects, comprised of 6 normal volunteers, 4 patients, or relatives of patients, with diseases not known to involve abnormalities of amino or organic acid metabolism, and 5 patients with homocystinuria due to cystathionine synthase deficiency. There was no systematic difference in the methyltransferase activities of these groups. The values listed are mean specific enzyme activities with the range of observed values in parentheses. For the control subjects, the ranges indicate variation among values for individual cell lines. For the patients with methylmalonic-aciduria, the ranges indicate the variation among repeated measurements on separate samples of cells grown from one or more biopsies of the patient in question. The numbers of cell samples assayed follows. (a) Cells grown in basal medium: control cells, 11; E.M., 8; R.P., 2. (b) Cells grown in basal medium + hydroxo-$B_{12}$: control cells, 15: E.M., 4; R.P., 3. The unsupplemented activity, as percent, was calculated by: $\frac{\text{activity with no added } B_{12}}{\text{activity with methyl-}B_{12}} \times 100$.

Values for control cells and E.M. represent current means and ranges and are extensions of previously published values (9).

grown in culture are summarized in Table 2. It should be emphasized that the experimental method measured labelled coenzymes synthesized from $^{57}$Co-

TABLE 2: Accumulation of $B_{12}$ Coenzymes by Cultured Fibroblasts from Control Subjects and Patients with [1]Methylmalonicaciduria

| Subjects Studied | Cobalamin Accumulation (pg/mg wet weight) | | |
|---|---|---|---|
| | Deoxyadenosyl-$B_{12}$ | Methyl-$B_{12}$ | Total $B_{12}$ accumulation |
| Controls | 0.26 ± 0.09 (19) | 0.38 ± 0.12 (15) | 1.81 ± 0.46 (38) |
| R.P. | 0.01 (10) (0-0.03) | 0.52 ± 0.23 (7) | 2.04 ± 0.58 (23) |
| E.M. | 0.005 (6) (0-0.016) | 0.013 (6) (0-0.055) | 0.56 ± 0.12 (16) |

Skin fibroblasts were grown in culture medium containing $^{57}$Co-hydroxo-$B_{12}$ as precursor vitamin (140-200 pg/ml). At confluence (3-5 days), the cells were harvested and the cobalamins extracted and separated by thin layer chromatography. The newly synthesized $^{57}$Co-labelled coenzymes, deoxyadenosyl-$B_{12}$ and methyl-$B_{12}$, were quantitated radioisotopically with a gamma counter. [1]The quantitation of total labelled $B_{12}$ accumulation by the cells assumed that the $^{57}$Co remained in cobalamins. [1]Data are given as mean ± SD or mean and range plus number of observations in parentheses. Control cells came from biopsies of 8 subjects, both infants and young adults.

hydroxo-$B_{12}$. Fetal calf serum, a necessary part of the growth medium, provided an unlabelled cobalamin pool which also contributed to coenzyme synthesis (11).

R.P. cells contained similar amounts of total labelled cobalamin as control cells and they had accumulated as much, and perhaps more, methyl-$B_{12}$. However, there was almost no accumulation of deoxyadenosyl-$B_{12}$ by R.P. cells. This was true for cells grown to confluence (72-120 hr) or for shorter periods (12, 24 or 48 hr). Previous data had shown a normal methylmalonyl-CoA mutase apoenzyme in R.P. cells but very low deoxyadenosyl-$B_{12}$ content unless the cells were grown in medium supplemented with a high concentration of hydroxo-$B_{12}$ (8). The new finding, that R.P. cells accumulate methyl-$B_{12}$ but do not accumulate significant deoxyadenosyl-$B_{12}$ when grown in medium with physiologic amounts of precursor vitamin, provides strong evidence for a defect in the synthesis of deoxyadenosyl-$B_{12}$. This defect would lead to deficient mutase activity despite a normal apoenzyme since there would be a deficiency of holoenzyme.

E.M. cells differed from control cells and from R.P. cells. There was virtually no accumulation of deoxyadenosyl-$B_{12}$ or methyl-$B_{12}$ either at

confluence or at shorter times. In addition, the total amount of labelled cobalamin attached to or within E.M. cells was only one-third the amount in control cells after 4 days in culture. Failure to accumulate deoxyadenosyl-$B_{12}$ explains the deficient methylmalonyl-CoA mutase activity in these cells and failure to accumulate methyl-$B_{12}$ accounts for the deficient methyl$FH_4$-methyltransferase activity. These observations explain the clinical observations in E.M.: methylmalonicaciduria caused by deficient mutase activity, and abnormal sulfur amino acid metabolism caused by deficient methyltransferase activity.

Recent work has begun to elucidate the synthetic pathways for the $B_{12}$ coenzymes. Walker, et al. have shown in Clostridium tetanomorphum that hydroxo-$B_{12}$ ($Co^{+++}$) is reduced in two enzymatic steps, first to $B_{12r}$ ($Co^{++}$) and then to $B_{12s}$ ($Co^{+}$) before adenosylation from ATP yields deoxyadenosyl-$B_{12}$ (13). The adenosylation of reduced $B_{12}$ has also been demonstrated in mammalian cells (14). Since methyl-$B_{12}$, like deoxyadenosyl-$B_{12}$, also contains $Co^{+}$, there must be reduction steps at some point in methyl-$B_{12}$ synthesis. Further details of methyl-$B_{12}$ formation are not known at present and it is not clear whether reduction steps are shared in the synthesis of the two coenzymes.

An attractive hypothesis for the defect in R.P. cells would be a failure of adenosylation of $B_{12s}$. E.M. cells accumulate neither coenzyme, nor do they appear to accumulate precursor vitamin normally. These findings could result from a defective membrane binding or transport of the $B_{12}$ molecule into the cell, or from a block in an early intracellular step common to the synthesis of both coenzymes. Excessive loss of coenzymes by degradation or efflux from the cell offers another alternative explanation. Work is in progress to investigate these possibilities.

Acknowledgement. This work was supported by research grants from the USPHS (AM 12579) and the John A. Hartford Foundation; by training grant HD 00198 (M.J.M.); and by research career development award AM 28987 (L.E.R.). We thank Dr. Robert H. Abeles for his continuing advice throughout these investigations.

## REFERENCES

1. Silber, R. and Moldow, C.F., Amer. J. Med. 48, 549 (1970).
2. Burke, G.T., Mangum, J.H., and Brodie, J.D., Biochemistry 9, 4297 (1970).
3. Taylor, R.T. and Weissbach, H., Arch. Biochem. Biophys. 123, 109 (1968).
4. Rudiger, H. and Jaenicke, L., Eur. J. Biochem. 10, 557 (1969).
5. Mudd, S.H., Levy, H.L., and Abeles, R.H., Biochem. Biophys. Res. Commun. 35, 121 (1969).
6. Mahoney, M.J. and Rosenberg, L.E., Amer. J. Med. 48, 584 (1970).

7. Rosenberg, L.E., Lilljeqvist, A.-Ch., and Hsia, Y.E., Science 162, 805 (1968).
8. Rosenberg, L.E., Lilljeqvist, A.-Ch., Hsia, Y.E., and Rosenbloom, F.M., Biochem. Biophys. Res. Commun. 37, 607 (1969).
9. Mudd, S.H., Uhlendorf, B.W., Hinds, K.R., and Levy, H.L., Biochem. Med. 4, 215 (1970).
10. Mudd, S.H., Levy, H.L., Morrow, G. III, Biochem. Med. 4, 193 (1970).
11. Mahoney, M.J. and Rosenberg, L.E., J. Lab. Clin. Med., in press.
12. Weissbach, H., Redfield, B., and Dickerman, H., Biochem. Biophys. Res. Commun. 17, 17 (1964).
13. Walker, G.A., Murphy, S., and Huennekens, F.M., Arch. Biochm. Biophys. 134, 95 (1969).
14. Kerwar, S.S., Spears, C., McAuslan, B., and Weissbach, H., Arch. Biochem. Biophys. 142, 231 (1971).