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Regulation of 5-Methyltetrahydrofolate:Homocysteine Methyltransferase Activity by Methionine, Vitamin B_{12}, and Folate in Cultured Baby Hamster Kidney Cells

(derepression/cell culture)

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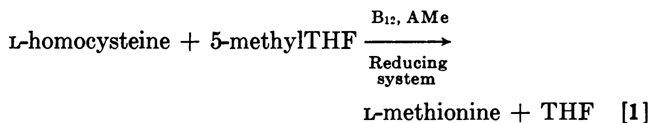
ABSTRACT **Rapid growth of BHK cells in methionine-deficient medium required supplementation with homocysteine, B_{12}, and over 40-fold greater levels of folic acid than growth in methionine-supplemented medium. The activity of the B_{12}-dependent 5-methyltetrahydrofolate: homocysteine methyltransferase was studied in extracts of BHK cells grown in media containing various concentrations of the components of the enzyme reaction. The methyltransferase activity increased over 4-fold when B_{12}-deficient medium was supplemented with optimal levels of B_{12}; this increase was not prevented by puromycin. Addition of homocysteine to growth medium containing methionine, B_{12}, and folic acid was without effect. However, methyltransferase activity increased 2.5- to 4.0-fold further beyond the highest levels obtained in the presence of methionine, B_{12}, and folic acid when homocysteine was substituted for methionine in the growth medium. This increase was blocked by puromycin and was not due to removal of feedback inhibition of activity by the product methionine. These results suggest that methyltransferase activity may be regulated in part by derepression of the enzyme's synthesis on substitution of the substrate homocysteine for the product methionine.**

Studies of mammalian cells grown in tissue culture have identified relatively few enzymes whose synthesis is either derepressed or induced in response to changes in the concentrations of metabolically related substances (1–3). Such enzymes, however, are of great interest for the understanding of regulation in mammalian cells. In animal tissues, two pathways of methionine biosynthesis have been identified. In the first, the terminal reaction is catalyzed by 5-methyltetrahydrofolate:homocysteine methyltransferase (reaction 1). First



$$\text{L-homocysteine} + \text{5-methylTHF} \xrightarrow[\text{Reducing system}]{B_{12},\ \text{AMe}} \text{L-methionine} + \text{THF} \quad [1]$$

identified in extracts of hog (4–6) and chick (7) livers, the reaction requires *S*-adenosyl-L-methionine (AMe) (5), a reducing system (8), and B_{12} (7–9). Although studied in mammals in less detail, the B_{12}-dependent methyltransferase in liver appears to be similar to the corresponding enzyme in *Escherichia coli* that has been extensively characterized (10). Analogous methyltransferase activity is widely distributed in rat tissues (11) and has been detected in various cultured mammalian cells (12–15). Studies of the regulation of 5-methylTHF:homocysteine methyltransferase have shown that supplementation of a deficient diet with B_{12} elevates the methyltransferase activity in chicken (7) and rat (16) liver and that this activity increases still further when methionine is removed from a B_{12}-containing diet (7, 11, 16).

In the second methionine biosynthetic pathway, the terminal reaction is catalyzed by betaine-homocysteine methyltransferase (EC 2.1.1.5) (reaction 2). This activity has no

$$\text{L-homocysteine} + \text{betaine} \rightarrow \text{L-methionine} + N,N\text{-dimethylglycine.} \quad [2]$$

cofactor requirements (18), uses preformed dietary methyl groups, and is detectable in only a few mammalian tissues (11). No activity was detected in the single reported study of cultured cells (14).

Studies of 5-methylTHF:homocysteine methyltransferase in cultured mammalian cells have focused on the effects of B_{12}. BHK cells transferred from a B_{12}-deficient to a B_{12}-supplemented medium showed a rapid, 10- to 20-fold increase in methyltransferase activity when assayed with B_{12} omitted from the assay mixture (13). The increase was interpreted as resulting from conversion of inactive apomethyltransferase to active B_{12}-containing holomethyltransferase. Similar results were obtained in cultured HeLa cells (15). Diploid human-skin fibroblasts showed a 2- to 4-fold increase in apo- and holomethyltransferase activities when grown in B_{12}-supplemented media (14).

The present studies demonstrate that cultured BHK cells can be used to analyze in detail the relative effects of folic acid, B_{12}, methionine, and homocysteine on the activity of B_{12}-dependent 5-methylTHF:homocysteine methyltransferase.

MATERIALS AND METHODS

Growth of BHK Cells. BHK cells were originally obtained from Dr. M. Stoker and have been maintained in this laboratory for several years by standard culture methods. Cells were grown in roller bottles (Bellco) in an atmosphere of 5% CO_2–95% air. The basal medium consisted of Eagle's minimal essential medium (which contains no B_{12}) lacking methionine

Abbreviations: BHK, baby hamster kidney; AMe, *S*-adenosyl-L-methionine; 5-methylTHF, 5-methyltetrahydropteroylglutamic acid. B_{12} is used as the generic name for several cobalamin compounds; where specified, cyano-B_{12} is α-(5,6-dimethylbenzimidazolyl)cobamide cyanide and hydroxo-B_{12} is α-(5,6-dimethylbenzimidazolyl)-hydroxocobamide.

TABLE 1. *Requirements for methionine synthesis in BHK cell extracts*

Reaction system	Methionine formed nmol/mg of protein
Complete	5.1
− Homocysteine	0.5
− AMe	1.8
− B_{12}	0
− 2-Mercaptoethanol	1.7

The cells were grown in Eagle's minimal essential medium containing 4% fetal-calf serum. Extracts were prepared and methyltransferase activity was assayed as described in *Methods* except that, where indicated, single components were omitted from the assay reaction mixtures.

and folic acid (GIBCO). To this were added, where indicated, 0.1 mM DL-homocysteine-thiolactone-HCl (Sigma), 0.1 mM folic acid (Calbiochem), and/or 1.5 μM hydroxo-B_{12} (Schwartz). All basal and supplemented media contained 4% fetal-calf serum (Gray Industries, Inc.) which contributes trace amounts of folic acid, B_{12}, and methionine, bringing the final concentration of folic acid in the basal medium to 5 nM (19), of B_{12} to 50 pM (Beck, W. S., personal communication), and of methionine to 0.2 μM (20).

Preparation of Cell Extracts. Cells were released from the surface by exposure to 0.025% trypsin (GIBCO) for 5–10 min at 37°. Trypsinization was stopped by addition of a 5-fold excess of serum-containing medium. An aliquot was removed for enumeration in a Coulter counter. The cells were washed twice in phosphate-buffered saline (pH 7.2), suspended in 0.25 M sucrose solution, and disrupted for 2 min in a sonic oscillator (Raytheon model DF 101). The debris was removed by centrifugation at 50,000 × *g* for 15 min, and the supernatant was assayed for methyltransferase activity (see below) and for protein by the method of Lowry *et al.* (21). Cells used for enzyme assay were always harvested before the confluent phase of growth since the methyltransferase activity increased variably at confluence (up to 2-fold).

Methyltransferase Activity Was Determined by measurement of the formation of [^{14}C]methionine from [5-^{14}C]methyl-THF (22). A standard reaction mixture contained in a total volume of 0.200 ml: 100 mM Na phosphate buffer (pH 7.4); 125 mM 2-mercaptoethanol; 250 μM AMe (Calbiochem); 50 μM cyano-B_{12} (Sigma); 250 μM DL-homocysteine (prepared just before use from the thiolactone derivative); 600 μM [5-^{14}C]methylTHF (Amersham–Searle); and 30–90 μl of cell extract. The reaction mixtures were incubated for 1 hr at 37°, and the reaction was terminated by addition of 0.8 ml of ice-cold water. The mixtures were passed through a Dowex 1-Cl column (Biorad), which retains the [5-^{14}C]-methylTHF, and after the column was washed with an additional 1.0 ml of water, the [^{14}C]methionine formed was measured in the pooled effluent by counting an aliquot in a toluene–Triton X-100–PPO–POPOP solution with a liquid scintillation spectrometer (Beckman). Specific activities are expressed in nmol of methionine formed per mg protein per hr at 37°, and are the average of three determinations varying less than 10%.

RESULTS

Requirements for Growth of BHK Cells. BHK cells ceased growing and detached from the surface when transferred to medium deficient in methionine, folic acid, B_{12}, and homocysteine. Similarly the cells did not grow when this deficient medium was supplemented with 7.2 μM choline, a betaine precursor, and 0.1 mM homocysteine. In basal medium supplemented with methionine, cells grew rapidly (6.3 generations in 84 hr) at a folic acid concentration of 2.3 μM. Higher levels of folic acid did not change this rate, while reduction of the folic acid concentration to 5 nM decreased growth (3.4 generations in 84 hr). In contrast, rapid growth (6.4 generations in 84 hr) in medium lacking methionine but supplemented with B_{12} and homocysteine required an over 40-fold higher level of folic acid, 0.1 mM. Thus, rapid growth required supplementation of the medium with either (*1*) methionine and 2.3 μM folic acid, or (*2*) homocysteine, B_{12}, and 0.1 mM folic acid.

In order to test the relative effects of folic acid, B_{12}, and homocysteine on methyltransferase activity, it was necessary

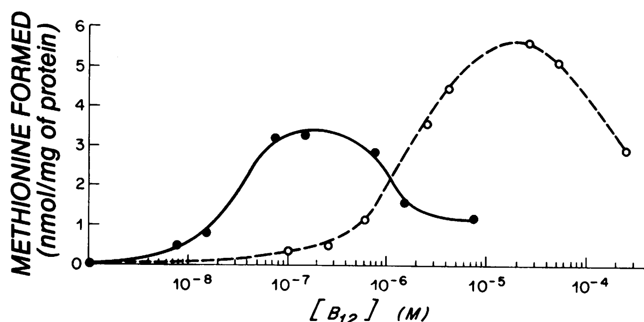


FIG. 1. Effect of *in vitro* B_{12} concentration on methyltransferase activity. Cells were grown in basal medium supplemented with 0.1 mM L-methionine, extracts were prepared, and methyltransferase was assayed. The final concentration of B_{12} added to the assay reaction mixture is indicated. ●, hydroxo-B_{12}; ○, cyano-B_{12}.

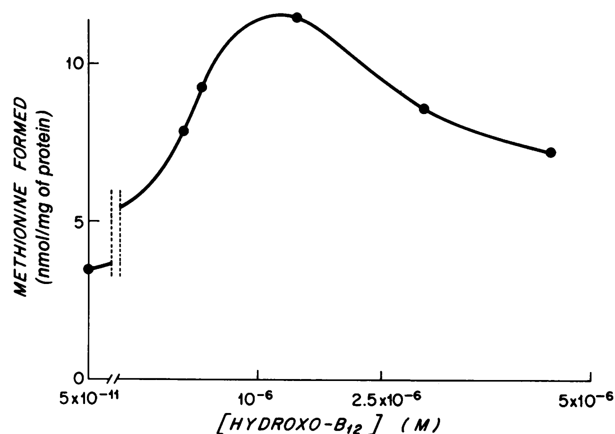


FIG. 2. Effect of B_{12} content of the growth medium on methyltransferase activity in cell extracts. Cells were grown in basal medium supplemented with 0.1 mM L-methionine, 2.3 μM folic acid, and various concentrations of hydroxo-B_{12}.

to add one or more of these components to medium containing sufficient methionine to support normal rates of growth.

Methyltransferase Activity in Extracts of BHK Cells. When cells were grown in basal medium supplemented with 0.1 mM L-methionine, methyltransferase showed substrate and cofactor requirements similar to those of *E. coli* B_{12}-dependent methyltransferase. The results of a representative experiment are shown in Table 1. Activity was markedly reduced in the absence of homocysteine and undetectable in the absence of B_{12}. AMe was partially required. Omission of 2-mercaptoethanol reduced the activity to one-third of control. In the presence of 125 mM 2-mercaptoethanol, methyltransferase activities assayed aerobically were essentially the same as those obtained under a nitrogen atmosphere. The observed methyltransferase activity varied with the amount and type of B_{12} derivative added to the assay reaction mixture (Fig. 1). While the optima for both cyano-B_{12} and hydroxo-B_{12} are rather broad, the midpoint of the activity curve with hydroxo-B_{12} is about two orders of magnitude lower than that obtained with cyano-B_{12}. At the optimal concentrations, however, activity with cyano-B_{12} was nearly double that obtained with hydroxo-B_{12}.

In contrast to the optima observed with B_{12}, addition of folic acid in concentrations as high as 1 mM to extracts of cells grown in low or high levels of folic acid in methionine-supplemented medium did not affect methyltransferase activity.

Absence of an Effect of Folic Acid. Methyltransferase activity was constant in BHK cells grown in methionine-containing medium over a wide range of folic acid concentrations. The specific activity of the methyltransferase was 3.7 ± 0.04 (mean ± 2 SD) when cells were grown in medium containing folic acid in concentrations ranging from 2.3 μM to 1 mM. When the medium contained less than 2 μM folic acid, the growth rate of the cells decreased and methyltransferase activity was unreliable.

Effect of B_{12}. Addition of hydroxo-B_{12} to methionine-supplemented basal medium resulted in an increase in methyltransferase activity (Fig. 2). When the B_{12} concentration was increased from that of the basal medium (5×10^{-11} M) to 1.5×10^{-6} M, the methyltransferase activity increased 3- to 6-fold in repeated experiments, averaging 4-fold. The maximal activity was consistently observed at a hydroxo-B_{12} concentration of about 1.5×10^{-6} M and decreased at higher levels of B_{12}. Methyltransferase activity reached a half-maximal level 12 hr after addition of B_{12} and the maximal level by 24 hr, remaining constant thereafter for at least 72 hr (Fig. 3). In order to determine whether this increase required protein synthesis or resulted from activation of preformed methyltransferase molecules, the time course was repeated in the presence of puromycin (Fig. 4). After addition of both hydroxo-B_{12} and puromycin, methyltransferase activity increased about 4-fold in 9–12 hr, decreasing thereafter. In contrast, when puromycin alone was added, methyltransferase activity decayed exponentially from the basal level.

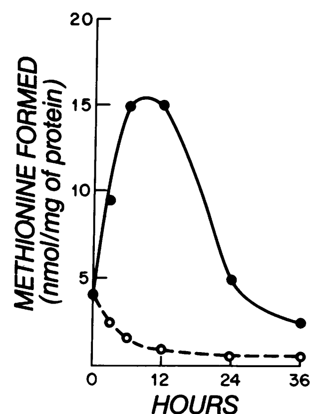


FIG. 4. Effect of puromycin in the presence and absence of B_{12} on methyltransferase activity in cell extracts. Cells were grown in basal medium supplemented with 0.1 mM L-methionine, 2.3 μM folic acid, 10 μg/ml of puromycin, and 1.5 μM hydroxo-B_{12} (●), or with 0.1 mM L-methionine, 2.3 μM folic acid, and 10 μg/ml of puromycin (○).

Effect of Substituting Homocysteine for Methionine in Growth Medium Containing B_{12} and Folic Acid. Addition of 0.1 mM homocysteine to medium containing folic acid, B_{12}, and methionine did not alter the methyltransferase activity. In contrast, decreasing the methionine concentration of the medium containing homocysteine, folic acid, and B_{12} resulted in a progressive increase in methyltransferase activity (Fig. 5). As the methionine concentration was decreased from 10^{-4} M

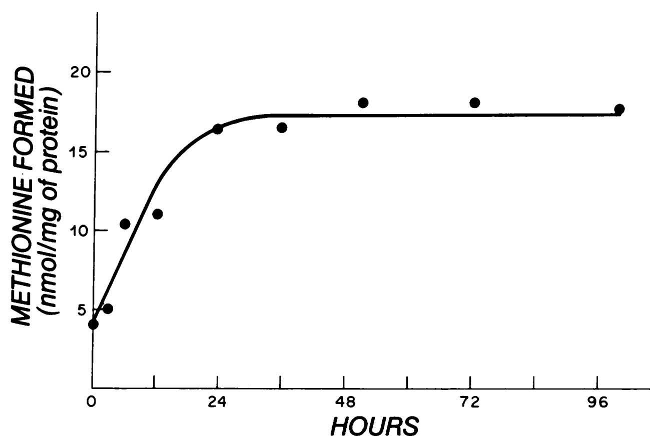


FIG. 3. Methyltransferase activity in cell extracts after addition of 1.5 μM hydroxo-B_{12} to basal medium supplemented with 0.1 mM L-methionine and 2.3 μM folic acid.

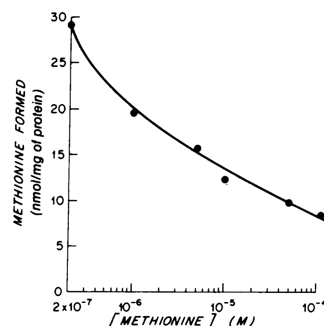


FIG. 5. Effect of methionine concentration of the growth medium on methyltransferase activity in cell extracts. Cells were grown in basal medium supplemented with 0.1 mM folic acid, 0.1 mM DL-homocysteine, 1.5 μM hydroxo-B_{12}, and various concentrations of L-methionine.

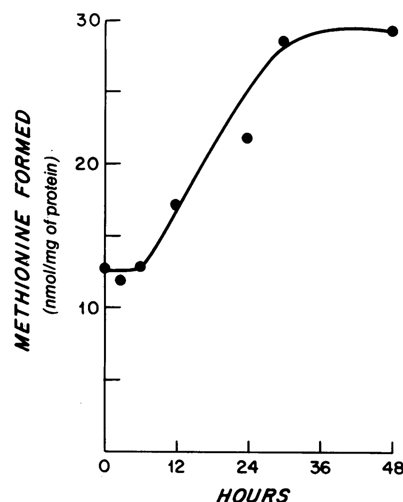


FIG. 6. Methyltransferase activity in cell extracts after refeeding with methionine-deficient medium. Cells were grown in basal medium supplemented with 0.1 mM folic acid, 0.1 mM DL-homocysteine, and 1.5 μM hydroxo-B_{12}.

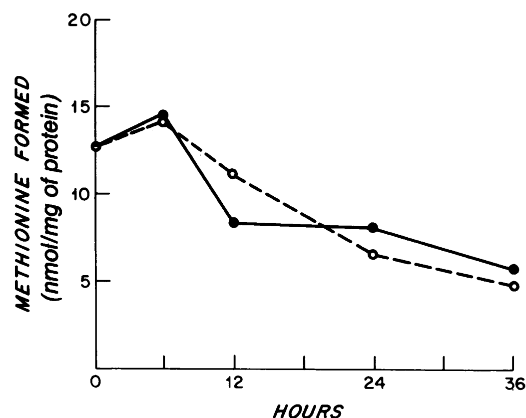


FIG. 7. Effect of puromycin in the presence and absence of methionine on methyltransferase activity in cell extracts. Cells were grown in basal medium supplemented with 0.1 mM folic acid, 1.5 μM hydroxo-B_{12}, 0.1 mM DL-homocysteine, 10 μg/ml of puromycin, and 0.1 mM L-methionine (○), or with 0.1 mM folic acid, 1.5 μM hydroxo-B_{12}, 0.1 mM DL-homocysteine, and 10 μg/ml of puromycin (●).

to 10^{-7} M, methyltransferase activity increased about 3.5-fold, from a specific activity of 8 to 29. The time course of this increase in methyltransferase activity is shown in Fig. 6. Upon removal of methionine from the growth medium, methyltransferase activity remained constant for about 6 hr, then increased over the next 18 hr, reaching a maximal value of 30 after about 30 hr in the methionine-deficient medium and remaining constant for the subsequent 24 hr. This maximal activity is thus 2.5- to 4.0-fold greater than that in cells grown in medium containing methionine and B_{12}, and 9-fold greater than in cells grown in basal medium supplemented only with methionine. In contrast to the increase observed when B_{12} was added to the growth medium, the rise in methyltransferase activity upon removal of methionine did not occur when protein synthesis was blocked by puromycin (Fig. 7). Upon addition of puromycin, methyltransferase activities were comparable in extracts of cells grown in the presence and absence of methionine, remained essentially constant during the first 6 hr, and then decreased in parallel over the next 30 hr.

In order to rule out the possibility that the increased methyl transferase activity resulted from the removal of feedback inhibition of activity rather than derepression of methyltransferase activity, methionine was added directly to the assay reaction mixtures. Methyltransferase activity was not significantly changed at *in vitro* methionine concentrations ranging from 10^{-7} M to 10^{-3} M, the latter concentration being 10-fold greater than the corresponding concentration in methionine-supplemented media.

DISCUSSION

The results of the present studies confirm the previous observations (12, 13) that survival and growth of cultured aneuploid mammalian cells in methionine-deficient media require the presence of homocysteine and B_{12}, and demonstrate, in addition, that the requirement for folic acid depends in part on the methionine content of the medium. Previous studies of the relationship of methyltransferase activity to growth conditions have used medium containing 2.3 μM folic acid, the level originally defined by Eagle as adequate for the growth of several mammalian cell lines (23). Our results, however, indicated that cell growth depends, in part, on the methionine content of the medium. Cell growth decreases in the presence of 0.1 mM methionine if the folic acid content of the medium is reduced from 2.3 μM to 5 nM. In contrast, in methionine-deficient medium supplemented with homocysteine and B_{12}, higher levels of folic acid are required. The growth rate under the latter conditions is maximal at a folic acid concentration of 0.1 mM, over 40-fold higher than the concentration in Eagle's minimal essential medium (23). Thus, supplementation with methionine markedly reduced the level of exogenous folic acid required by cultured cells, consistent with a similar effect of methionine observed in whole animals (16, 17).

BHK cells were unable to grow when choline, a precursor of betaine, and homocysteine replaced methionine in the culture medium. This finding is consistent with the inability to detect betaine-homocysteine methyltransferase activity in the single reported study where this activity was sought in cultured mammalian cells (14).

Methyltransferase activity in extracts of cells grown in basal medium supplemented with methionine manifests substrate and cofactor requirements similar to the B_{12}-dependent methyltransferase from *E. coli* (10). Extracts of cells grown in this B_{12}-deficient medium showed a complete dependence on B_{12} added to the assay reaction mixture. Thus, there appears to be little holoenzyme present in these B_{12}-deficient cells. Although the optimal concentrations differ, higher activities were obtained with cyano-B_{12} than with hydroxo-B_{12}, as has been observed in extracts of cultured human fibroblasts (14). Since methyl-B_{12} is presumed to be the B_{12} derivative that is the cofactor for the methyltransferase (summarized in ref. 24), the BHK extracts are apparently able to convert cyano-B_{12} and hydroxo-B_{12} to methyl-B_{12}.

While growth requires supplementation with folic acid at levels that depend upon whether supplemental methionine is also present, variation of the folic acid concentration over wide ranges in excess of these minimal folate levels both *in vivo* and *in vitro* does not alter the methyltransferase activity

of the cell extracts. To obtain maximal rates of growth, it thus appears necessary to provide a level of folate sufficient for methyltransferase activity, suggesting a critical role of the folate- and B_{12}-dependent methyltransferase in cellular function.

In contrast, variation of the B_{12} content of methionine-supplemented medium resulted in a substantial change in methyltransferase activity. Previous studies have shown that an increasing proportion of methyltransferase activity was present as holoenzyme as the B_{12} content of the medium was raised but the total methyltransferase activity was unchanged (13, 15). In contrast, our studies, in which cyano-B_{12} was added to all assay reaction mixtures, indicate that total methyltransferase increases as the B_{12} concentration of the medium is raised from 5×10^{-11} M to 1.5×10^{-6} M, in agreement with similar findings in cultured human fibroblasts (14).

The present results demonstrate for the first time that the increase in methyltransferase activity produced by B_{12} is not dependent on concomitant protein synthesis, being insensitive to puromycin, but do not provide a specific explanation for the molecular basis of the increased activity. As one possibility, stabilization of the methyltransferase by the B_{12} cofactor might be expected to retard the rate of turnover of the enzyme. Purification of the methyltransferase would be required to test this hypothesis. Alternatively, the possibility that B_{12} might activate preformed methyltransferase by some other mechanism is under investigation.

The most striking present result is the further increase in methyltransferase activity under conditions requiring increased methionine synthesis. Addition of homocysteine to basal medium supplemented with folic acid, B_{12}, and methionine is without effect. However, removal of methionine from this culture medium results in a further 2.5- to 4.0-fold increase in methyltransferase activity. Increased activity resulting from removal of the reaction product in the presence of adequate B_{12} has not been demonstrated in cultured cells, although a similar change has been observed in whole animal studies (7, 11, 16). Variation over a wide range in the concentration of methionine added to the extracts *in vitro* does not alter the methyltransferase activity, indicating that the increased activity in growing cells is not due to removal of feedback inhibition of activity. Further, the rise in methyltransferase activity *in vivo* requires concomitant protein synthesis, being completely blocked in the presence of puromycin. This increase in activity may be due to derepression of methyltransferase synthesis, although the present studies do not distinguish changes at the level of messenger RNA transcription from those at the level of translation.

Thus, the level of methyltransferase activity is dependent upon the concentration of both cofactor and product, and these affect the enzyme levels by different mechanisms. It appears that 5-methylTHF:homocysteine methyltransferase can be added to the still small number of enzymes (2, 3) that can be used productively to study regulation of enzyme activity in cultured mammalian cells.

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1. Schimke, R. T. (1964) *J. Biol. Chem.* **239**, 136–145.
2. Schimke, R. T. & Doyle, D. (1970) *Ann. Rev. Biochem.* **39**, 929–976.
3. Littlefield, J. W. (1970) in *Genetic Concepts and Neoplasia* (Williams and Wilkins Co., Baltimore, Md.), pp. 439–451.
4. Sakami, W. & Ukstins, I. (1961) *J. Biol. Chem.* **236**, PC50–PC51.
5. Mangum, J. H. & Scrimgeour, K. G. (1962) *Fed. Proc.* **21**, 242.
6. Loughlin, R. E., Elford, H. L. & Buchanan, J. M. (1964) *J. Biol. Chem.* **239**, 2888–2895.
7. Dickerman, H. W., Redfield, B. G., Bieri, J. G. & Weissbach, H. (1964) *J. Biol. Chem.* **239**, 2545–2552.
8. Buchanan, J. M., Elford, H. L., Loughlin, R. E., McDougall, B. M. & Rosenthal, S. (1964) *Ann. N.Y. Acad. Sci.* **112**, 756–773.
9. Dickerman, H. W., Redfield, B. G., Bieri, J. G. & Weissbach, H. (1964) *Ann. N.Y. Acad. Sci.* **112**, 791–798.
10. Weissbach, H. & Taylor, R. T. (1970) *Vitam. Horm.* (New York) **28**, 415–440.
11. Finkelstein, J. D., Kyle, W. E. & Harris, B. J. (1971) *Arch. Biochem. Biophys.* **146**, 84–92.
12. Mangum, J. H. & North, J. A. (1968) *Biochem. Biophys. Res. Commun.* **32**, 105–110.
13. Mangum, J. H., Murray, B. K. & North, J. A. (1969) *Biochemistry* **8**, 3496–3499.
14. Mudd, S. H., Uhlendorf, B. W., Hinds, K. R. & Levy, H. L. (1970) *Biochem. Med.* **4**, 215–239.
15. Kerwar, S. S., Spears, C., McAuslan, B. & Weissbach, H. (1971) *Arch. Biochem. Biophys.* **142**, 231–237.
16. Kutzbach, C., Galloway, E. & Stokstad, E. L. R. (1967) *Proc. Soc. Exp. Biol. Med.* **124**, 801–805.
17. Vitale, J. J. & Hegsted, D. M. (1969) *Brit. J. Haematol.* **17**, 467–475.
18. Finkelstein, J. D. & Mudd, S. H. (1967) *J. Biol. Chem.* **242**, 873–880.
19. Goulian, M. & Beck, W. S. (1966) *Amer. J. Clin. Pathol.* **46**, 390–391.
20. Efron, M. L. (1966) *Proc. Technicon Symposium on Automation in Analytical Chemistry*, New York, N.Y., pp. 637–642.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
22. Weissbach, H., Peterkofsky, A., Redfield, B. & Dickerman, H. (1963) *J. Biol. Chem.* **238**, 3318–3324.
23. Eagle, H. (1955) *Science* **122**, 501–504.
24. Blakeley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines* (American Elsevier Publishing Co., New York), pp. 349–351.