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Vitamin B_{12} Dependent Methionine Biosynthesis in Cultured Mammalian Cells*

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ABSTRACT: Several established mammalian cell lines were cultured in such a way that they appeared to be dependent upon vitamin B_{12}. Methionine was replaced by homocysteine in the growth medium and the cells survived and proliferated only when vitamin B_{12} was added. The level of 5-methyltetrahydrofolate–homocysteine transmethylase activity was elevated some 10–20-fold in these cells. Elevated enzyme levels were also observed in cells cultured in medium containing methionine. However, this only occurred when vitamin B_{12} was also included in the medium. The cofactor requirements for the transmethylase obtained from cultured mammalian cells were found to be similar to those observed for mammalian tissues.

The terminal reaction in methionine biosynthesis involves the transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine (Larrabee *et al.*, 1963; Sakami and Ukstins, 1961). Enzyme systems catalyzing this reaction have been

$$N^5\text{-methyltetrahydrofolate} + \text{homocysteine} \longrightarrow \text{methionine} + \text{tetrahydrofolate}$$

partially purified from *Escherichia coli* (Stravrianopoulos and Jaenicke, 1967; Taylor and Weissbach, 1967) and from mammalian (Buchanan *et al.*, 1964; Kerwar *et al.*, 1966) and avian liver (Dickerman *et al.*, 1964). The cofactors which have been implicated in methionine biosynthesis include $FADH_2$ (Hatch *et al.*, 1959) and *S*-adenosylmethionine (Mangum and Scrimgeour, 1962). In addition, 5-methyltetrahydrofolate–homocysteine transmethylase isolated from *E. coli* has been shown to contain a bound form of vitamin B_{12} (Takeyama *et al.*, 1961). There is also evidence that methionine biosyntheis in animal tissues requires a vitamin B_{12} prosthetic group. The dietary requirement of rats for methionine can be replaced by homocysteine and vitamin B_{12} (du Vigneaud *et al.*, 1950). A nutritional deficiency of vitamin B_{12} in young chicks resulted in a marked reduction of the transmethylase activity (Dickerman *et al.*, 1964). A partial restoration of the activity occurred when the animals were subsequently provided vitamin B_{12}.

It has been shown that cultured mammalian cells require methionine but not vitamin B_{12}. However, vitamin B_{12} increased the rate of proliferation of a particular strain of L cells cultured in a medium deficient in deoxycytidine and thymine (Sanford and Dupree, 1964).

It was recently reported (Mangum and North, 1968) that HEp-2 cells cultured in a growth medium where methionine was replaced by homocysteine appeared to have an absolute dependency upon vitamin B_{12}. This dependency resulted in a marked elevation of 5-methyltetrahydrofolate–homocysteine transmethylase activity. The present study provides a more detailed examination of vitamin B_{12} dependent methionine synthesis in cultured mammalian cells. Conditions are also described for obtaining elevated levels of the transmethylase in cells that are not dependent upon *de novo* methyl group formation.

Materials and Methods

Minimum essential medium (Eagle, 1955) both with and without methionine and choline was purchased from Grand Island Biological Co. Chemicals were obtained from the following sources: vitamin B_{12} and FAD from Sigma Chemical Co.; [^{14}C]formaldehyde from New England Nuclear Corp; *S*-adenosylmethionine, folic acid, and Dowex 1-Cl^- from California Corp. for Biochemical Research; and DL-homocysteine from Nutritional Biochemical Corp.

Tetrahydrofolate was prepared by the catalytic hydrogenation of folic acid (Hatefi *et al.*, 1960) and [*methyl*-^{14}C]N^5-methyltetrahydrofolate was chemically synthesized by reduc-

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† U. S. Public Health Service Research Career Development Awardee K3-GM-10,215 of the National Institute of General Medical Sciences.

ing a mixture of [^{14}C]HCHO and tetrahydrofolate with potassium borohydride (Keresztesy and Donaldson, 1961). The desired product was purified by chromatography on DEAE-cellulose.

$FADH_2$ was prepared by the catalytic reduction of FAD (Loughlin *et al.*, 1964). Vitamin B_{12} (cyanocobalamin) was converted into vitamin B_{12a} (hydroxocobalamin) by the procedure described by Vitols *et al.* (1966). The latter form of the vitamin was used exclusively in this investigation.

The following cell lines were used in these experiments: HEp-2, human larynx carcinoma cells; HeLa, human cervix carcinoma cells; PS, porcine-stable kidney cells; HaK, hamster (Syrian) kidney cells; and BHK-21 (C-13), baby hamster (Syrian) kidney cells. Monolayer cultures of the respective cell lines were employed. The cells were grown in one of the the following media supplemented with 10% calf serum: (1) Eagle's minimum essential medium (MEM); (2) MEM plus vitamin B_{12} (0.5 mg/l.); (3) MEM plus homocysteine (15 mg/l.) and vitamin B_{12} (0.5 mg/l.); or (4) MEM which lacked methionine and choline, but contained homocysteine (15 mg/l.) and vitamin B_{12} (0.5 mg/l).

The cells were allowed to grow for 96 hr and then harvested. The cell sheet was washed three times with balanced salt solution (Hanks and Wallace, 1949), and then scraped from the glass. The cells were weighed and suspended in two volumes of 0.03 M phosphate buffer (pH 6.5). The cells were ruptured in a Teflon–glass homogenizer and then centrifuged at 27,000*g* for 10 min at 2°. An aliquot of the supernatant fraction was used in the analysis for 5-methyltetrahydrofolate–homocysteine transmethylase activity.

The assay system for methionine synthesis consisted of the following components present in a total volume of 1 ml: [*methyl*-^{14}C]N^5-methyltetrahydrofolate, 0.25 μmole; *S*-adenosylmethionine, 0.10 μmole; homocysteine, 2.5 μmoles; $FADH_2$, 0.08 μmole; phosphate buffer (pH 7.4), 50 μmoles; and from 0.5 to 1.0 mg of protein. Incubations were carried out in 5-ml wide-mouthed stoppered serum bottles at 37° for 30 min under a hydrogen atmosphere.

A 0.2-ml aliquot of the reaction mixture was placed on a small Dowex 1-Cl^- column (6 × 30 mm) which retained the N^5-methyltetrahydrofolate but not the methionine (Weissbach *et al.*, 1963). The column was washed with 1.8 ml of water and after the addition of 10 ml of a naphthalene–dioxane scintillation fluid (Bray, 1960) the radioactive solution was counted with a Nuclear-Chicago Unilux I liquid scintillation counting system.

All values are reported in terms of specific activity which is defined as the millimicromoles of methionine synthesized per hour per milligram of protein.

Protein concentrations were determined by the method of Warburg and Christian (1941).

Results

The various established cell lines readily adapted to the methionine-deficient medium supplemented with homocysteine and vitamin B_{12}. Essentially no difference could be observed in the rate of proliferation in the above medium and in regular MEM. However, when homocysteine and vitamin B_{12} were omitted from the methionine-deficient medium, the cells failed to grow.

The 5-methyltetrahydrofolate–homocysteine transmethylase levels of cells cultured in regular MEM are shown in Table I. The specific activity ranged from a low of 2.0 for HaK cells to a high of 12.5 mμmoles of methionine/hr per mg of protein for BHK-21 cells. PS cells, HEp-2 cells, and HeLa cells were found to have activities of from 3.6 to 5.5. When these cell lines were grown in methionine-deficient MEM containing the homocysteine and vitamin B_{12}, a marked increase in the transmethylase activity was observed as shown in Table II. The enzyme activity was elevated some 20-fold in HaK and HEp-2 cells and at least 10-fold in PS, HeLa, and BHK-21 cells.

TABLE I: 5-Methyltetrahydrofolate–Homocysteine Transmethylase Levels in Cultured Mammalian Cells Grown in MEM.[a]

Cell Type	Transmethylase Act. (mμmoles/hr mg of protein)
HAK	2.0
HeLa	3.6
PS	4.2
HEp-2	5.5
BHK-21	12.5

[a] All incubations were carried out as described in the Materials and Methods section.

5-Methyltetrahydrofolate–homocysteine transmethylase levels were also determined in HEp-2 cells and BHK-21 cells which were cultured in MEM supplemented with either vitamin B_{12} or vitamin B_{12} and homocysteine. Elevated levels (Table III) were again observed and there was no difference in the specific activity found in cells provided vitamin B_{12} and those which were given vitamin B_{12} and homocysteine. All that was necessary to obtain elevated levels of the transmethylase in cultured mammalian cells was to supplement the medium with vitamin B_{12}. The homocysteine apparently had very little or no effect under conditions where the cells were provided with exogenous methionine.

It was not necessary to include vitamin B_{12} in the growth medium over the entire proliferation period in order to obtain

TABLE II: 5-Methyltetrahydrofolate–Homocysteine Transmethylase Levels in Methionine- and Choline-Deficient MEM Supplemented with Homocysteine and Vitamin B_{12}.[a]

Cell Type	Transmethylase Act. (mμmoles/hr mg of protein)
HaK	51
HeLa	42
PS	55
HEp-2	143
BHK-21	138

[a] All incubations were carried out as described in the Materials and Methods section.

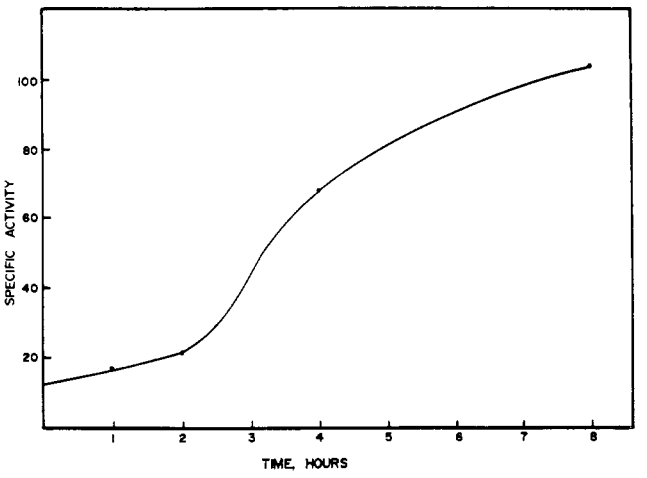

FIGURE 1: Time-dependent appearance of 5-methyltetrahydrofolate–homocysteine transmethylase activity after providing HEp-2 cells with vitamin B_{12}. HEp-2 cells were cultured initially in MEM in the absence of vitamin B_{12} for 72 hr and then the MEM was replaced with MEM which contained vitamin B_{12}. The cells were then harvested at the indicated time intervals and assayed for transmethylase activity as described in the Materials and Methods section.

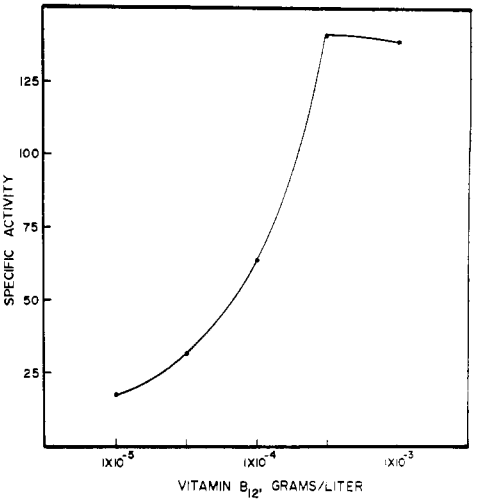

FIGURE 2: The dependency of 5-methyltetrahydrofolate–homocysteine transmethylase activity upon the concentration of vitamin B_{12} in the culture medium. HEp-2 cells were cultured initially in MEM in the absence of vitamin B_{12} for 72 hr and then the MEM was replaced with MEM which contained vitamin B_{12} at the indicated concentrations. The cells were then harvested 8 hr after the addition of vitamin B_{12} and assayed for transmethylase activity as described in the Materials and Methods section.

elevated levels of 5-methyltetrahydrofolate–homocysteine transmethylase. HEp-2 cells were initially cultured in MEM in the absence of vitamin B_{12} for 72 hr and then this medium was replaced with MEM containing vitamin B_{12}. The cells were then harvested at various time intervals and the transmethylase activity determined. From Figure 1 it can be seen that there is a time lag of approximately 2 hr in the appearance of the transmethylase activity. A very rapid increase in enzyme activity occurred from 2 to 8 hr. After 8 hr the level approached that found in cells cultured continuously in vitamin B_{12} for the 96 hr.

Utilizing the procedure just described, the optimal vitamin B_{12} concentration for 5-methyltetrahydrofolate–homocysteine transmethylase formation was determined (Figure 2). HEp-2 cells were grown in regular MEM and then after 72 hr a fresh overlay of MEM and vitamin B_{12} at the indicated concentrations was added. After 8 hr the cells were harvested and assayed for the transmethylase activity. A vitamin B_{12} concentration of 5×10^{-4} g/l. of medium resulted in the maximum enzyme production.

The cofactors necessary for methionine biosynthesis in both a cell-free extract obtained form HEp-2 cells and a 0–50% ammonium sulfate fraction of this extract are described in Table IV. The ammonium sulfate fraction was also passed

TABLE III: 5-Methyltetrahydrofolate–Homocysteine Transmethylase Levels in Cultured Mammalian Cells Grown in MEM Supplemented with either Vitamin B_{12} or Homocysteine and Vitamin B_{12}.[a]

Cell Type	Addn To Medium	Transmethylase Act. (mμmoles/hr mg of protein)
HEp-2	Homocysteine + vitamin B_{12}	73
HEp-2	Vitamin B_{12}	67
BKH-21	Homocysteine + vitamin B_{12}	105
BKH-21	Vitamin B_{12}	127

[a] All incubations were carried out as described in the Materials and Methods section.

TABLE IV: Substrate and Cofactor Requirements for 5-Methyltetrahydrofolate–Homocysteine Transmethylase Obtained from HEp-2 Cells.[a]

Omission	Source of Enzyme: Crude Fraction Transmethylase Act (mμmoles/hr mg of protein)	Source of Enzyme: $(NH_4)_2SO_4$ Fraction Transmethylase Act (mμmoles/hr mg of protein)
None	138	63
Enzyme		3
Homocysteine	53	5
S-Adenosyl-methionine	55	39
$FADH_2$	4	8

[a] All incubations were carried out as described in the Materials and Methods section, except where indicated one of the components of the incubation mixture was omitted. The cells were cultured in methionine- and choline-free MEM supplemented with homocysteine (15 mg/l.) and vitamin B_{12} (0.5 mg/l.).

through a column of Bio-Gel P-10. The ammonium sulfate fractionation and the treatment with the polyacrylamide gel were desighed to remove both low molecular weight cofactors and the endogenous substrate homocysteine. Only a partial requirement for *S*-adenosylmethionine could be demonstrated in the crude extract; however, an absolute dependency existed for $FADH_2$.

The ammonium sulfate fractionation and gel treatment did not alter the cofactor requirements. However, this procedure removed endogenous homocysteine since the ammonium sulfate fraction was totally dependent upon homocysteine and only a partial requirement for the substrate was observed with the crude extract.

Discussion

Cells grown in methionine- and choline-free MEM supplemented with vitamin B_{12} and homocysteine were completely dependent upon *de novo* methyl group formation. Cells failed to proliferate in the absence of vitamin B_{12}. This dependency upon *in vivo* methionine biosynthesis usually resulted in a 10–20-fold elevation of 5-methyltetrahydrofolate–homocysteine transmethylase activity. Occasionally cell preparations were obtained which had the capability of synthesizing over 200 mμmoles of methionine/hr per mg of protein. This represented a 40-fold increase in the specific activity of the enzyme. Dickerman *et al.* (1964) reported that young chicks maintained on a vitamin B_{12} deficient diet had a marked reduction of transmethylase activity and a tenfold increase in the enzyme was observed when vitamin B_{12} was restored to the diet of the animals. However, the elevated level of transmethylase activity reported for young chicks represented only 5% of that found for cultured cells.

Elevated levels of 5-methyltetrahydrofolate–homocysteine transmethylase were also found in cells cultured in MEM supplemented with B_{12}. This finding was quite unexpected since these cells had adequate amounts of methionine in the growth medium to allow for the optimal proliferation of the cells.

Cells which were not dependent upon *de novo* methyl group formation still carried the potential to synthesize methionine. All that was needed to realize this potential was to provide the cells with vitamin B_{12}. Since similar elevated levels of 5-methyltetrahydrofolate–homocysteine transmethylase activity were observed in cells provided vitamin B_{12} during their entire culture period and in those which received the vitamin only during the last 8 hr, cell proliferation was independent of the enzyme. The rapid increase in enzyme activity after the addition of vitamin B_{12} would suggest that the cells already contained the apoenzyme and the addition of vitamin B_{12} simply resulted in the *in vivo* conversion of the apoenzyme to the holoenzyme.

Takeyama *et al.* (1961) similarily found that cells of strain 113-3 of *E. coli* which were cultured on methionine contained 5-methyltetrahydrofolate–homocysteine transmethylase apoenzyme. The apoenzyme was readily converted into the holoenzyme when incubated *in vitro* with vitamin B_{12}.

The cofactor requirements for the enzyme obtained from the cultured cells were found to be similar to those observed for 5-methyltetrahydrofolate–homocysteine transmethylase of kidney tissue (J. H. Mangum and J. A. North, unpublished data). This correspondence of cofactor requirements would suggest that the enzyme found in cultured mammalian cells was identical with or very similar to the enzyme of mammalian tissues.

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