# Uptake and Utilization of DL-5-[*methyl*-$^{14}$C] Tetrahydropteroylmonoglutamate by Cultured Cytotrophoblasts Associated with Neural Tube Defects (43571)

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*Abstract.* **A significant advance in the primary prevention of neural tube defects (NTD) is the recent finding that the periconceptional supplementation with folate has a 72% preventive effect against recurrence of NTD. However, failure of folate supplements to prevent all recurrences supports the multifactorial causation hypothesis, with inherited components exerting their influence, possibly through defects of storage, transport, or metabolism of folate. We have assessed the kinetics of DL-5-[*methyl*-$^{14}$C]tetrahydropteroylmonoglutamate ([$^{14}$C]MTHF) uptake and incorporation into the nucleic acid and protein pools by NTD-associated and control trophoblasts cultured in a medium lacking thymidine and other DNA precursors. We report a significant initial "lag" in the rate of incorporation of $^{14}$C label into the nucleic acid pool in NTD-associated trophoblasts. This we attribute to a defect in the *de novo* pathway of folate metabolism and its associated pathways, including the pathway for methionine synthesis, although the rate of incorporation of $^{14}$C label into the protein pool was not significantly different from that of the control cells. We discuss the possible pathways involved in the transfer of the label from the methyl group of [$^{14}$C]MTHF to the nucleic acid pool, and argue that a slightly (but significantly) reduced rate of uptake into the NTD-associated cells is a reflection of the lag in incorporation into the nucleic acid pool. It is concluded that in the absence of thymidine, most of the NTD-associated trophoblasts require a longer period than controls to adjust to utilization of [$^{14}$C]MTHF for synthesis of DNA, a period that could be crucial for completion of neural tube embryogenesis. We suggest that these findings could offer a way to a marker for risk of NTD.** [P.S.E.B.M. 1993, Vol 203]

An early suggestion that folate may be involved in the causation of neural tube defects (NTD) (1) was followed by a number of clinical studies showing an association between reduction in recurrence of NTD and periconceptional use of folate, either alone (2, 3) or combined with other multivitamins (4–6). A recently published multinational randomized double-blind trial showed that periconceptional supplementation with folate alone had a 72% preventive effect against recurrence of NTD (7). However, until possible toxicity of large doses of folate, or the efficacy of lower doses, is ascertained, prophylactic folate supplementation cannot be extended to general populations to reduce first occurrences of NTD (7). An understanding of the mechanisms that link folate with NTD may lead to a marker for risk of NTD and hence to rationalization of steps to prevent first occurrences.

Folate deficiency in early pregnancy is known to occur in Western societies (8). Significantly lower than average red blood cell folate levels have been reported in women who subsequently were found to have NTD-affected fetuses (9), and in those with a history of one or more NTD pregnancies (10, 11). However, the cause of NTD is more complex than a maternal dietary deficiency (12), and this is supported by the fact that

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folate supplementation has not prevented all recurrences of NTD (7). The cause is almost certainly multifactorial, with inherited components playing a role (12). The genetic influence could be through metabolic defects of storage, transport, or metabolism of folate and other related vitamins by maternal and fetal cells. It has already been suggested that disturbances in the metabolism of folate (1, 11) and vitamin $B_{12}$ (13, 14) are implicated in the causation of NTD.

It is, therefore, important that the mechanisms by which a vitamin or a combination of vitamins act, and interact with the genetic component, are determined, so that the underlying pathogenetic process may be understood. Animal models of human congenital abnormalities are, in some ways, the most convenient for studying such mechanisms and interrelationships. The major disadvantage of animal models is that the findings are not necessarily applicable to humans. For instance, thalidomide has little effect on mice and rats but is severely teratogenic in humans. Gross deficiency of folate, in particular, is a well-known cause of central nervous system defects in the rat (15). However, when the guinea pig, in which the switchover from histiotrophic to hemotrophic nutrition occurs at a stage similar to humans (16), was subjected to folate and ascorbate deficiencies during neural tube embryogenesis, NTD did not occur (17). Thus, it is preferable to use human cells to study the relationship between vitamin deficiencies and NTD.

Nutritional factors, especially vitamins and their cofactors, are essential to many enzymatic activities involved in the normal metabolic processes of the cells of both mother and fetus. A deficiency of one or several of those factors would impair these vital pathways, and this is likely to be particularly devastating to the rapidly proliferating and differentiating cells of the embryo. Therefore, we investigated the kinetics of uptake and utilization (incorporation into nucleic acid and protein) of 5-methyltetrahydropteroylmonoglutamate ([MTHF] the main form of folate in human plasma [18]) by placental cytotrophoblasts isolated from mothers after the delivery of a conceptus with NTD (experimental samples) and compared the results with the same measurements on trophoblasts from mothers (matched for gestational and maternal age) who had delivered a normal child (control samples).

We have chosen placental trophoblasts because they are derived from the same embryonic tissue as the fetus and form the syncytiotrophoblasts, the basal selective membrane of the placenta for transport of nutrients to the fetus (19).

## Materials and Methods

**Culture Media and Other Materials.** Cell culture media were based on Ham's F10 nutrient mixture (20), supplemented with 100 μg/ml of bovine insulin, 90 μg/ml of human transferrin, 578 n*M* sodium selenate, 8.28 n*M* hydrocortisone, 50 μ*M* progesterone, 4 n*M* L-3,5,3′-triiodothyronine, 33 mg/ml of bovine albumin fraction V, 4 ng/ml of tissue-culture-grade epidermal growth factor, 50 units/ml of penicillin G, 50 μg/ml of streptomycin, and 3.2 μg/ml of amphotericin B. This was further supplemented with a Canadian-origin fetal calf serum (FCS) to a final concentration of 10% to produce the medium (GM) for optimal growth of the trophoblast cells. The trophoblast selective medium was a variant of GM in which L-valine was replaced by D-valine.

Two experimental media based on GM were used. The first (EM1) lacked hypoxanthine, thymidine, and pteroylglutamic acid (folate). The second (EM2) lacked FCS as well as hypoxanthine and thymidine, while pteroylglutamic acid (2.9 μ*M*) was replaced with 65 n*M* (2.2 GBq/mmol) DL-5-[*methyl*-$^{14}$C]tetrahydropteroylmonoglutamate ([$^{14}$C]MTHF), and 250 μ*M* 2,β-mercaptoethanol (ME) and 10 μ*M* sodium ascorbate were added as antioxidants for [$^{14}$C]MTHF.

The phosphate-buffered saline used (DPBS-A) was based on Dulbecco's formula (21), excluding calcium, magnesium, and sodium bicarbonate. The cell culture basic medium, all supplements, serum, buffered saline, trypsin, and versene solutions were purchased from Gibco Ltd. The basic medium purchased was Ham's F10 nutrient mixture (20), except that it lacked valine, glutamine, hypoxanthine, thymidine, and folic acid.

[$^{14}$C]MTHF was the barium salt of DL-5-[*methyl*-$^{14}$C]tetrahydropteroylmonoglutamic acid ($^{14}$C label forming the methyl group attached to position 5 of the substituted pteridine moeity) with a sp act of 3.33 MBq/mg and was purchased from Amersham International plc. Culture substrates were tissue-culture-treated 30- and 80-cm$^2$ Nunclon flasks that were further treated with Engelberth-Holm-Swarm mouse tumor laminin (Gibco Ltd) at 2 μg/cm$^2$.

The cell cultures were maintained in an environment of 36.5°C, 5% $CO_2$, 16% $O_2$, and 98% humidity within a Queue tissue culture incubator.

The monoclonal antibodies (mAb) used for cell characterization were the antihyaluronic acid $NDOG_1$, anti-mucin-like-glycoprotein $HMFG_1$ and the anti-human placental lactogen (anti-hPL). These were gifts from Dr. J. Bulmer, Academic Unit of Pathology, University of Leeds, Leeds, England. The peroxidase-conjugated immunoglobulins and the nonimmune serum were purchased from Dako Corp. The scintillation material comprised 20-ml low potassium borosilicate glass vials and a multipurpose high efficiency scintillation cocktail (Optiphase MP), which were purchased from LKB/Pharmacia. Radiation was counted in an LKB 1215 Rackbeta scintillation counter.

All the reagents (diaminobenzidine, hydrogen peroxide, Mayer's hematoxylin, dimethyl sulfoxide, unla-

beled MTHF [barium salt], Tris-HCl, perchloric acid, sodium hydroxide, calf thymus DNA, diphenylamine, protein standard [5.0 g% human albumin and 3% globulin], sodium dodecyl sulfate, Folin Ciocalteu's phenol reagent, and control sera) were obtained from Sigma Chemical Co.

**Source of Human Cells.** Heterogenous placental cell populations were first grown from three separate source samples. One was explants of chorionic villi from either placentae of midpregnancy terminations (about 18–20 weeks of gestation) after NTD diagnosis by ultrasound or placentae of two babies born with NTD. The second source samples were chorionic villi samples obtained by a transabdominal technique (22) at around 16–20 weeks of gestation; only those chorionic villi samples that were subsequently shown to be of normal karyotype and from normal babies were used as control samples. The third group comprised explants of normal term placentae, matched for maternal age with the two babies born with NTD.

**Trophoblast Isolation and Culture.** The procedures were based on those of Daniels-McQueen and colleagues (23). A detailed description will be published separately, but the relevant techniques are as follows: within 36 hr after they were obtained, chorionic villi explants were washed in DPBS-A and were cut into pieces of about 1 $mm^3$ with a pair of curved scissors. The pieces were suspended in the growth medium GM at a density of about 1 mg/ml, and every milliliter of this suspension was spread evenly on the surface of a 30-$cm^2$ flask treated previously with laminin. After overnight incubation, an additional 4 ml of the medium GM were added to each flask. Thereafter, the medium was renewed every 48 hr until cells had grown out of the explant to half confluency. This was achieved in about 14 days.

At this point, the growth culture medium GM was exchanged for the trophoblast selective medium, which contained D-valine instead of L-valine, in order to kill the fibroblastic mesenchyme cells. The adherent cells were grown in this medium for 10 to 12 days, with the media renewed every 48 hrs, when confluency was reached.

The medium was then aspirated and the cells were trypsinized off the 30-$cm^2$ substrate by covering the monolayer with 0.5 ml of a solution of 0.175% 1:125 trypsin and 166 $\mu M$ 1:5000 versene in modified Puck's saline A at 22°C. The cells detached from their substrate within 3 min of the treatment. They were immediately suspended in 12 ml of GM and their viability was assessed by trypan blue exclusion test using the hemocytometer (improved Neubauer) for cell counting.

This cell suspension, which comprised 0.5–1.0 × $10^6$ viable cells, was added to a 80-$cm^2$ tissue culture flask treated previously with laminin. This was allowed to grow for 6 to 7 days to near confluency (first subculture), and the medium was changed every 48 hr.

**Trophoblast Characterization.** The purity of the trophoblast population was assessed using the indirect immunoperoxidase staining technique of Bulmer and Sunderland (24). After trypsinization, as described above (at the conclusion of the first subculture), the cells were resuspended in GM and seeded onto tissue-culture-treated polymer slides in Leighton tubes (Costar) at a density of about 2.5 × $10^4$ cells/ml/tube, 12 tubes/sample. The tubes were incubated overnight to allow the cells to attach to the slide substrate, which were then subjected to the staining procedure.

Three mAb were used: $NDOG_1$, which is specific for all column cytotrophoblasts (25), and the anti-hPL, which confirms the presence of all intermediate trophoblasts (26), while the third mAb ($HMFG_1$) is specific to gland epithelial cells and reacts only with some chorionic laeve trophoblasts (25). Briefly, a Leighton tube slide with its attached cells was first washed in a solution of 0.15 $M$ NaCl and 0.05 $M$ Tris-HCl at pH 7.6 (tromethamine-buffered saline [TBS]), and was then overlain with an mAb (one mAb per slide) at the appropriate dilution (neat $NDOG_1$, 1/200 hPL, and 1/500 $HMFG_1$) for 45 min. After two 1-min washes in TBS, the slide was incubated with the appropriate immunoglobulin (rabbit anti-mouse IgM for $NDOG_1$, swine anti-rabbit IgG for hPL, and rabbit anti-mouse IgG for $HMFG_1$), diluted to 1/50 with TBS containing 10% nonimmune serum, for about 20 min. After two additional TBS washes, the dye reaction was developed with 50 mg% diaminobenzidine, containing 0.01% hydrogen peroxide, for 5 to 10 min. Finally, the slide was plunged into excess water and lightly counterstained with Mayer's hematoxylin. Two types of negative controls were employed that included the use of the fibroblast cell line BHK21 subjected to the above procedure and omission of the primary antisera in staining placental cells.

The Leighton tube preparations from each cell sample were stained in triplicate with each of the mAb, with the proportion of the stained cells on these slides representing the proportion of those cells in the sample that would react with the appropriate mAb. Stained cells following reaction with $NDOG_1$ and anti-hPL mAb were considered to be column and intermediate cytotrophoblasts, whereas the cells that stained after an application of $HMFG_1$ represented contaminating gland epithelial cells in the sample. Cell numbers on each slide were the average count in 20 fields of view (×100 objective).

A few of the cell samples were characterized, as above, subsequent to a second subculture which followed a period of preservation in liquid nitrogen (Table I).

**Trophoblast Preservation.** The remainder of the

**Table I.** Homogeneity of Trophoblast Cultures after Isolation

| mAb designates | Percentage of stained cells resulting from single mAb reactions | | | |
|---|---|---|---|---|
| | After 1st subculture[a] | | | After 2nd subculture[b] |
| | Control samples ($n = 4$) | Experimental samples ($n = 5$) | All samples ($n = 9$) | All samples ($n = 5$) |
| $NDOG_1$ | 67 ± 3 | 63 ± 3 | 65 ± 4 | 64 ± 5 |
| hPL | 9 ± 6 | 7 ± 4 | 9 ± 5 | 12 ± 4 |
| $HMFG_1$ | 7 ± 3 | 11 ± 4 | 9 ± 4 | 9 ± 5 |

[a] Results for the control and experimental samples, and those for the first subculture and second subculture, were compared using Wilcoxon's test. No significant differences were observed. Data are expressed as mean ± SD.
[b] Second subculture cells were in the experimental medium and had followed a period of storage in liquid nitrogen. Data are expressed as mean ± SD.

trypsinized first subculture cells were suspended in GM containing 9% dimethyl sulfoxide at a cell density of 2–3 × $10^6$ cells/ml, which was then aliquoted into 1.5-ml fractions in 1.8-ml cryogenic tubes (Nunclon). The vials were first frozen in liquid nitrogen vapor at a rate of about 1.5°C per min in a Taylor-Wharton freezing tray to about −160°C, and were then stored on canes under liquid nitrogen.

**Karyotype Analysis.** Chromosome spreads were prepared from cell samples at two stages of the culture process: first, immediately after outgrowth from the explants, and second, after an overnight culture of the frozen and defrosted cells. A hundred cell spreads for each sample were analyzed for transformation in terms of chromosome counts and Q-banding. The results from the two stages were compared to assess the possible effect of the culture and preservation processes on the chromosomal integrity.

**Experimental Culture Medium and Preservation of MTHF in Culture Conditions.** 2,β-Mercaptoethanol and sodium ascorbate (AA) were used as antioxidant of [$^{14}$C]MTHF in the experimental culture medium EM2. Initially, in a series of experiments, the concentration of 2,β-mercaptoethanol was varied between 100 n*M* and 2 μ*M*, and then the concentration of AA was varied between 10 and 500 μ*M* while MTHF concentration was kept constant at 100 n*M*. The media were changed every 18 hr and the media concentrations of MTHF were assessed with the high-performance liquid chromatography method of Lucock and colleagues (27) at 30-min intervals. Hence, the optimum concentrations of ME and AA, with little adverse effect on cell growth and a satisfactory protection against MTHF oxidation, were determined.

**Relation between Concentration and Uptake of [$^{14}$C]MTHF.** The cells were grown in the experimental medium EM2, while the concentration of [$^{14}$C]MTHF was varied between 10 and 100 n*M* for 66 hr (twice doubling time) and the cellular radioactivity was measured as described below. Thus, the required optimum concentration of [$^{14}$C]MTHF was assessed. The measurements were repeated in the presence of 100 μ*M* unlabeled MTHF to assess the degree of nonspecific binding of the radiolabeled material.

**Assessment of [$^{14}$C]MTHF Uptake and Utilization.** The frozen cells were thawed rapidly, suspended in the medium EM1 (which lacked MTHF but contained 10% FCS), and seeded into 12 × 80-$cm^2$ flasks (laminin treated) at a density of about 1.2 × $10^3$ cells/$cm^2$. After a 4-hr incubation to allow for cell adhesion, this medium was replaced with a similar medium that lacked FCS and MTHF, and the flasks were returned to the incubator for an additional 18 hr. This medium was then replaced with the experimental medium, EM2, while at every 18-hr interval, two flasks were removed for quantitation of cellular radioactivity. Hence, the cells adherent to the first two flasks were grown for 18 hr, and those in the last two flasks for 108 hr, in the presence of [$^{14}$C]MTHF.

**Cell Fractionation.** The medium EM2 was aspirated out, and the cells were trypsinized off their substrate and washed four times in DPBS-A by centrifugation at 4°C. A small portion of the final wash was removed to determine cell numbers using a hemocytometer, and the final cell pellet from each flask was fractionated into "total nucleic acid," "total protein," and the "cell remainder" using an adaptation of the method described in Leyva and Kelly (28).

Briefly, the cell pellet was resuspended in 200 μl of ice-cold extraction buffer (0.01 *M* Tris-HCl, pH 7.4) and lysed by rapid freezing and thawing in liquid nitrogen. The lysate was centrifuged at 11,000*g* for 20 min at 4°C and the supernatant was collected as part of the cell remainder. The resulting pellet was suspended in 800 μl of 0.2 *M* perchloric acid (PCA) and kept at 4°C for 30 min to precipitate protein and nucleic acids. This was then centrifuged at 11,000*g* for 10 min, the supernatant was removed as the final part of the cell remain-

der, and the hot-acid-soluble fraction of the pellet (nucleic acids) was extracted with 500 μl of M PCA at 70°C for 50 min. This hydrolysate was centrifuged at 11,000*g* for 20 min and the acid supernatant was used as the total nucleic acid. The final pellet comprised the total protein, which was dissolved in 100 μl of 2.5 *M* NaOH at 37°C overnight and neutralized with 150 μl of 2 *M* PCA.

To assess the efficiency of the fractionation method, all cell fractions were analyzed for DNA content using the diphenylamine colorimetric method of Leyva and Kelly (28), and the results were compared with those from DNA content of the nonfractionated cells following a one-step hot acid extract (29). Furthermore, all the cell fractions were analyzed for total protein (30), by comparing the results with the total protein content of the nonfractionated cells.

**Measurement of Radioactivity.** Each cell fraction was washed with 150 μl of 2 *M* PCA into a 20-ml glass scintillation vial, and 10 ml of the scintillation cocktail were added and mixed until the solution was clear. The vials were wiped with 70% ethanol and stored in the dark overnight, and the activity was measured with a four-channel scintillation counter (LKB 1215 Rackbeta) at efficiencies >85%. Quench correction and calculation of the counting efficiencies were obtained by external standard channel ratio technique.

The activities of the cell fractions were expressed as dpm/$10^6$ cells.

**Statistical Analysis.** Due to unequal variances within groups, statistical procedure was carried out on the log transformations of the original data. Control and experimental groups were compared using multivariate analysis of variance for repeated measures (SPSS), and where F ratios for between-group or group × time interaction were significant ($P < 0.01$), a test of simple main effect at individual repeated-measure levels was carried out using SAS PROC GLM with the repeated statement (31).

## Results

**Cell Culture.** A considerable number of red blood cells migrated out of the explants in the first 24 hr. These underwent rapid degradative changes and disappeared by the fifth day. The first cells to appear were spindle-shaped fibroblast-like cells which began growing out rapidly from the explants at about the seventh day. Polygonal and flattened epithelium-like cells began to appear in the outgrowth toward the end of the second week. Upon replacing L-valine for D-valine in the medium, the spindle-shaped cells began detaching from the substrate and disappeared from the culture within 9–10 days, leaving the culture consisting predominantly of epithelium-like cells with intercellular adherence appearing to result from short "dendritic" extensions.

Staining techniques showed that the trophoblast isolation procedure had produced similar trophoblast-enriched cell populations from the control and the experimental samples (Table I). Some 70% of the cell population was stained with $NDOG_1$, which is reactive with all trophoblastic cells except the proliferating villi cytotrophoblasts (25). About 10% of the cell population was stained with hPL, which reacts with all intermediate trophoblasts (26), and less than 10% were reactive with $HMFG_1$, which is indicative of nontrophoblast epithelial cells (25).

A second subculture and growth in the experimental medium, subsequent to a period of storage in liquid nitrogen, did not alter the cell population (Table I). This suggests that the 10–15% unstained cells were unlikely to be fibroblast-like cells. Such cells were likely to overgrow the epithelium cells in a medium without D-valine, hence altering cell population. The remaining unstained cells are, therefore, likely to be villi cytotrophoblasts which do not react with $NDOG_1$.

The karyotype of three control samples remained unaltered after three subcultures and a period of storage in liquid nitrogen.

A control sample was divided into two equal parts. One part was grown in the growth medium GM and the other portion was grown in the experimental medium EM2, while the remaining culture conditions remained unaltered. The results showed that, at the third subculture following a period of initial growth in GM and EM2 as described above, the fully defined experimental medium EM2 could support a growth response similar to that supported by the growth medium GM (Fig. 1). The doubling time at log phase was about 36 hr. It should be remembered that growth in EM2 medium had followed an initial period of some 30 days in the full GM medium and a weaning period of 4 hr in the EM1 (see Materials and Methods). EM2 would not have supported growth from the explants or cell adhesion to the substrate because it lacked FCS.

**Preservation of MTHF in Cell Culture Conditions.** Figures 2 and 3 illustrate the preservative effect of ME on the rate of degradation of MTHF in the experimental conditions. Figure 3 also shows that the rate of degradation is linear. Although a concentration of 500 μ*M* was effective in preserving MTHF (Figs. 2 and 3), this concentration of ME slowed cell proliferation significantly (data not shown). Therefore, the experimental medium ME2 was supplemented with ME at 250 μ*M*, which was sufficient to preserve more than 45% of MTHF in the medium after an 18-hr incubation (Fig. 2).

AA is an antioxidant and as such is normally used as a preservative for MTHF. However, in our experiments, concentrations greater than 10 μ*M* slowed cell proliferation (data not shown), perhaps by reducing the buffering capacity of the medium which lacked FCS.

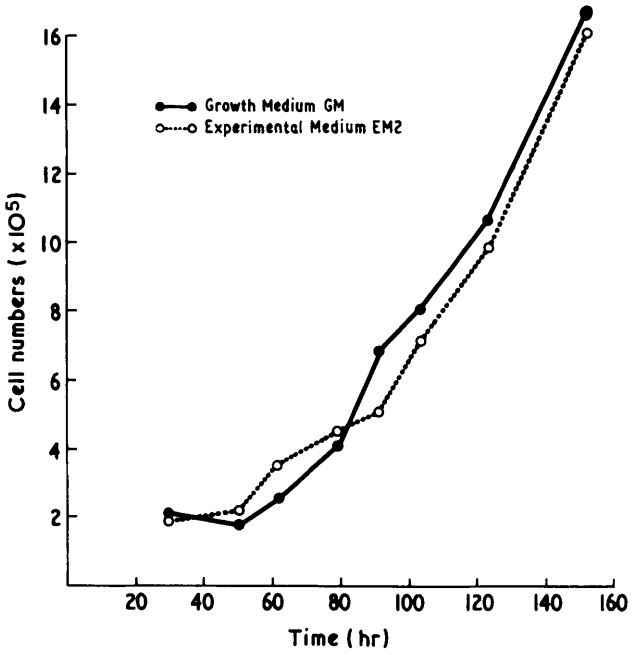


**Figure 1.** Rate of cell proliferation of a control sample following the third subculture on laminine substrate in the growth medium GM (closed circles) and in the experimental medium EM2 (open circles). Other culture conditions were the same and both media were based on Ham's F10. GM was F10 supplemented with 100 $\mu$g/ml of bovine insulin, 90 $\mu$g/ml of human transferrin, 578 n*M* sodium selenate, 8.28 n*M* hydrocortisone, 50 $\mu$*M* progesterone, 4 n*M* L-3,5,3-triiodothyronine, 33 mg/ml of bovine albumin fraction V, 4 ng/ml of epidermal growth factor, 50 units/ml of penicillin G, 50 $\mu$g/ml of streptomycin, 3.2 $\mu$g/ml of amphotericin B, and 10% FCS. EM2 was similar to GM, except that it lacked hypoxanthine, thymidine, and FCS, and folate was replaced with 65 n*M* [$^{14}$C]MTHF, 250 $\mu$*M* ME, and 10 $\mu$*M* sodium ascorbate. All values are means of duplicates.

**Characteristics of [$^{14}$C]MTHF Uptake and Utilization by Trophoblast Cells.** Figure 4 shows cellular radioactivity of a control sample after exposure to a range of medium concentrations of [$^{14}$C]MTHF at 37°C for 100 hr. The total cell radioactivity represents both specific uptake and utilization and nonspecific binding of [$^{14}$C]MTHF during the period 96 hr, which is three times the doubling time (Fig. 1). The nonspecific binding was achieved in the presence of excess (100 $\mu$*M*) unlabeled MTHF. It remained at about 5% of the total cell radioactivity throughout the range of extracellular [$^{14}$C]MTHF used (Fig. 4).

Within the physiologic range of MTHF (10–40 n*M*), total cell radioactivity was directly proportional to the extracellular concentration of [$^{14}$C]MTHF. Thereafter, a saturation phenomenon became evident.

**Uptake and Utilization of [$^{14}$C]MTHF by Control versus Experimental Samples.** The experimental trophoblasts incorporate $^{14}$C from [$^{14}$C]MTHF into total nucleic acid at an initially slower rate than controls (Fig. 5B). This difference in the rate of [$^{14}$C]MTHF utilization between the experimental and control samples was statistically significant for the first 54 hr, despite two of the experimental samples (one term and one at 18 weeks of gestation) utilizing [$^{14}$C]MTHF at a rate similar to those shown by the control samples.

There was no significant difference between groups in the rate of $^{14}$C incorporation into total cellular protein (Fig. 5C), and a difference in total cell content of $^{14}$C at the 36-hr level (Fig. 5A) may be a reflection of the difference in the level of activity within the nucleic acid components of these two groups.

The difference in the rate of [$^{14}$C]MTHF incorporation between the experimental and control groups is not reflected by changes in cell growth (Fig. 6).

## Discussion

The results show that the $^{14}$C label attached to the methyl group of [$^{14}$C]MTHF and taken up into the cell (Fig. 5A) is incorporated into the total protein (Fig. 5C) and total nucleic acid (Fig. 5B) components of the cells. Presumably, the methyl group carrying the label is passed onto homocysteine early in the *de novo* pathway of folate metabolism and the resulting labeled methionine contributes to the presence of activity in the protein component (32, 33). The $^{14}$C label can be incorporated in the cellular pool of nucleic acid (much of which is DNA) via at least two known pathways related to the *de novo* pathway. The first possibility is that the labeled methyl group, now part of methionine, is transferred to glycine via *S*-adenosylmethionine under the enzymic activity of glycine-*N*-methyl transferase (33, 34). The labeled sarcosine so formed gives its labeled one-carbon group to tetrahydrofolate to form methylene-tetrahydrofolate, which is involved in methylation of dUMP under the activity of thymidylate synthase (32). The second possibility is that the labeled one-carbon group of *S*-adenosylmethionine enters the *de novo* pathway as formate (32, 35) and, as such, will contribute to the synthesis of IMP as well as dUMP (35, 36). The labeled amino acids which can be formed in these pathways can also contribute to the level of radioactivity in the pool of cellular amino acids and, hence, to the protein component. Some posttranscription methylation of DNA may also be a contributory factor in the transfer of the label to the nucleic acid pool.

Figure 5 shows a significant difference between the groups in the rate of incorporation of the labeled material into the whole cell and into the nucleic acid pool. The difference in the incorporation of $^{14}$C into the protein pool did not reach conventional levels of statistical significance, and while the overall pattern was the same, larger numbers of samples would be required to investigate whether the difference is real. We believe, therefore, that the difference between the two groups in the uptake of $^{14}$C into the whole cell is largely a reflection of the difference in incorporation into the nucleic acid pool. We are fairly confident in this assumption

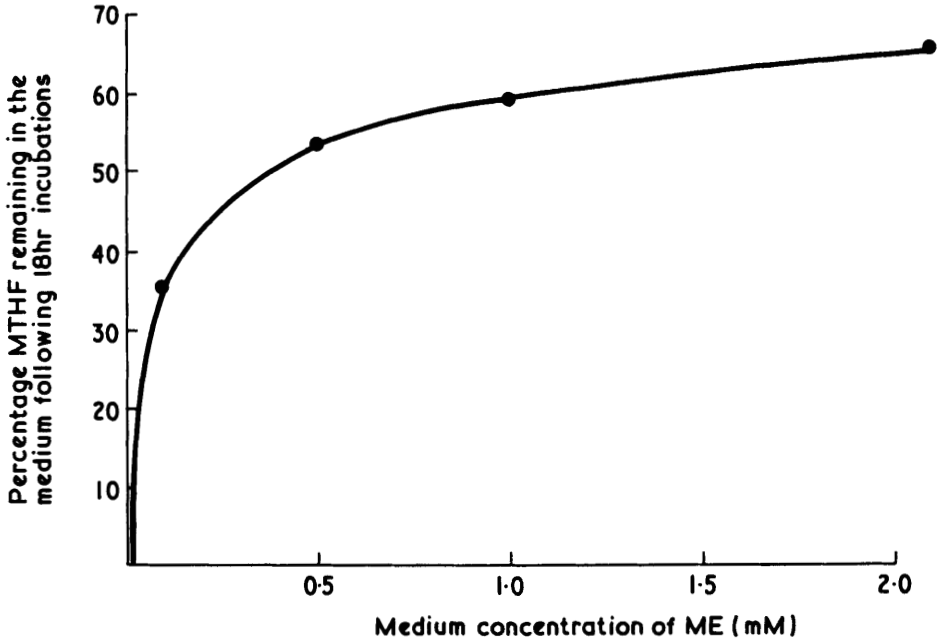


**Figure 2.** Illustrates preservative effect of ME against oxidative degradation of MTHF in the experimental medium (EM2) after incubation at 37°C for 18 hr. EM2 was based on Ham's F10 nutrient mixture, but it lacked hypoxanthine, thymidine, and folic acid, and was supplemented with 100 $\mu$g/ml of bovine insulin, 90 $\mu$g/ml of human transferrin, 578 n*M* sodium selenate, 8.28 n*M* hydrocortisone, 50 $\mu$*M* progesterone, 4 n*M* L-3,5,3-triiodothyronine, 33 mg/ml of bovine albumin fraction V, 4 ng/ml of epidermal growth factor, 50 units/ml penicillin G, 50 $\mu$g/ml of streptomycin, 3.2 $\mu$g/ml of amphotericin B, 65 n*M* [$^{14}$C]MTHF, 10 $\mu$*M* sodium ascorbate, and varying concentrations of ME. All values are means of duplicates.

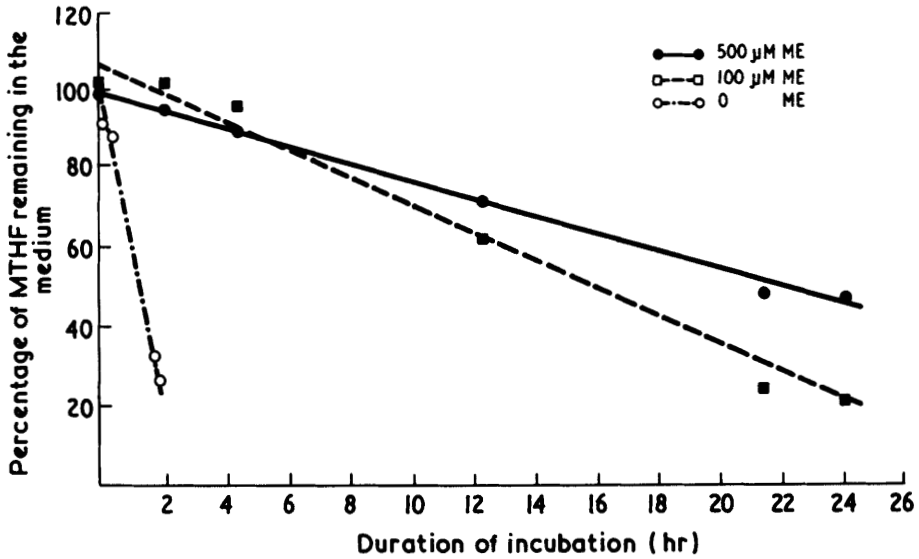


**Figure 3.** Rate of degradation of MTHF in experimental culture medium (EM2) at 37°C containing either (i) 500 $\mu$*M* or (ii) 100 $\mu$*M* ME as the preservation agent or (iii) lacking ME. EM2 composition appears in the legend of Figure 2 and in the text. All values are means of duplicates.

because in another set of experiments (to be published), we have shown that the kinetics of the [$^{14}$C]MTHF transport across the isolated placental microvillous membranes are similar in the two groups under observation.

This difference between the groups of cells in their ability to incorporate labeled one-carbon into the nucleic acid pool cannot be attributed to variations in cell populations, because the staining techniques have shown that all samples tested were of similar purity (Table I). It is also unlikely to be a result of transformation following culture procedures because no chromosomal transformation was ever detected in the control samples subjected to karyotype analysis before and after cell culture procedures. Furthermore, cells from the NTD-associated and from control samples seem to show similar growth patterns in the experimental medium (Fig. 6).

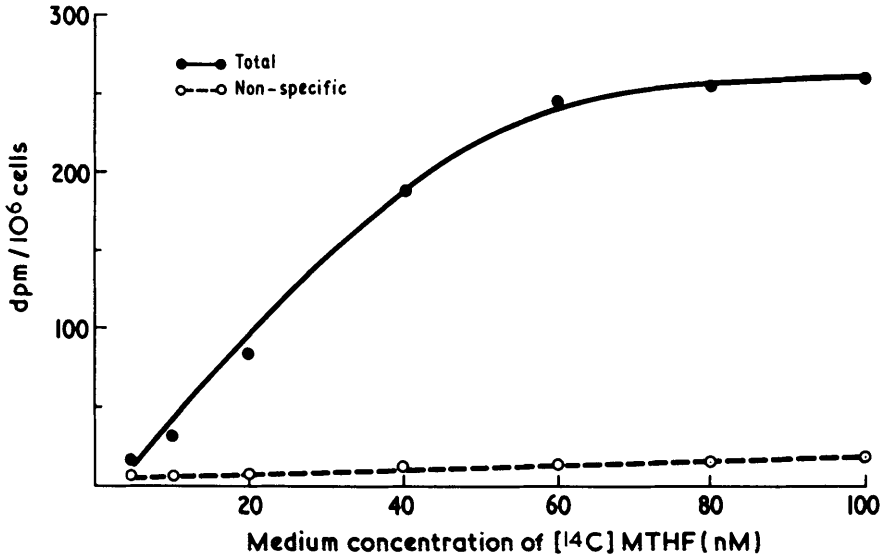


**Figure 4.** Total and nonspecific incorporation of [$^{14}$C]MTHF after incubation at 37°C of a control sample in experimental medium EM2 (see legend to Fig. 2 for details) with various concentrations of [$^{14}$C]MTHF (5–100 n*M*) either with excess unlabeled MTHF (100 $\mu$*M*) for nonspecific binding determination (open circles) or without unlabeled MTHF for determination of total incorporation of [$^{14}$C]MTHF (closed circles). All values are means of duplicates.

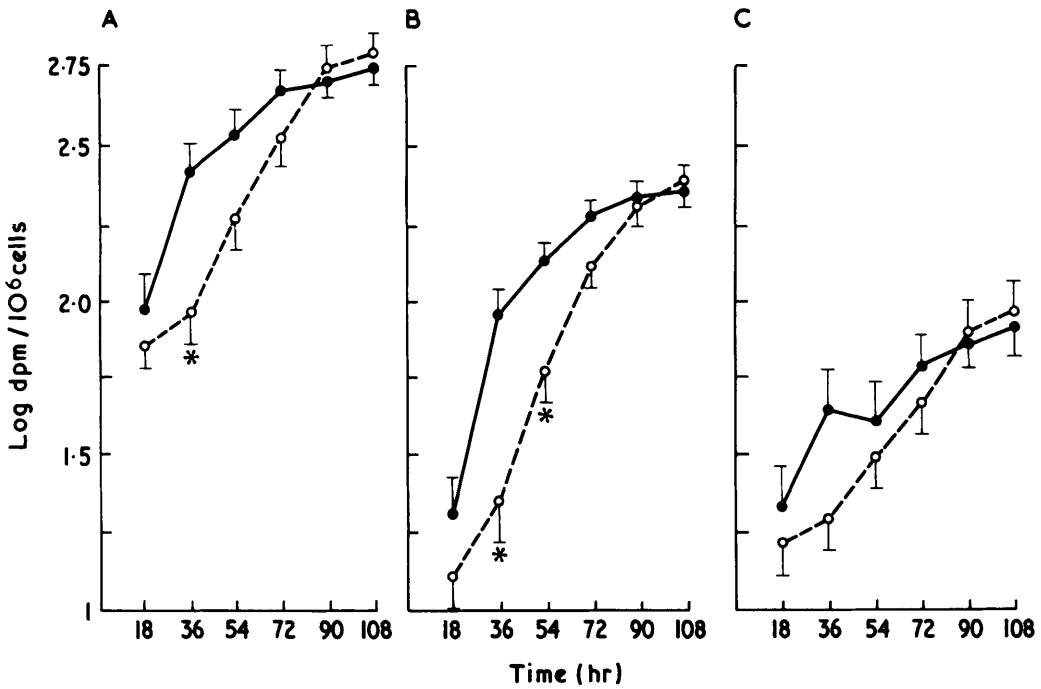


**Figure 5.** Rate of incorporation of $^{14}$C into (A) total cell substance, (B) total cell nucleic acid, and (C) total cell protein, after culture of control (closed circles: $n = 10$) and experimental (open circles: $n = 11$) samples in experimental medium EM2 (see legend to Fig. 2 for details of EM2) containing 65 n*M* [$^{14}$C]MTHF and lacking nucleotides. Samples were dispersed at 18-hr intervals, total nucleic acid was extracted by hot acid and the final precipitate represented total protein (see text for details). The groups were compared using multivariate analysis of variance for repeated measures on log of values (significance of between-group F ratios: A = 0.15, B = 0.02, C = 0.5; and significance of group × time interaction F ratios: A and B = 0.001, C = 0.002) followed by test of simple main effect at individual repeated-measure levels. Vertical bars represent SE of means. * Significantly different ($P < 0.01$) from the control value at the same time level.

Therefore, we suggest that the relatively slower initial rate of folate utilization exhibited by the NTD-associated cells is a reflection of their metabolic capability, probably resulting from a subtle defect within the enzyme system of the *de novo* pathway for folate metabolism and its associated pathways. If this inference is correct, the implication is that the NTD-associated trophoblasts require a longer period than the controls to adjust to the utilization of MTHF for synthesis of DNA. While a relatively small period of "lag"

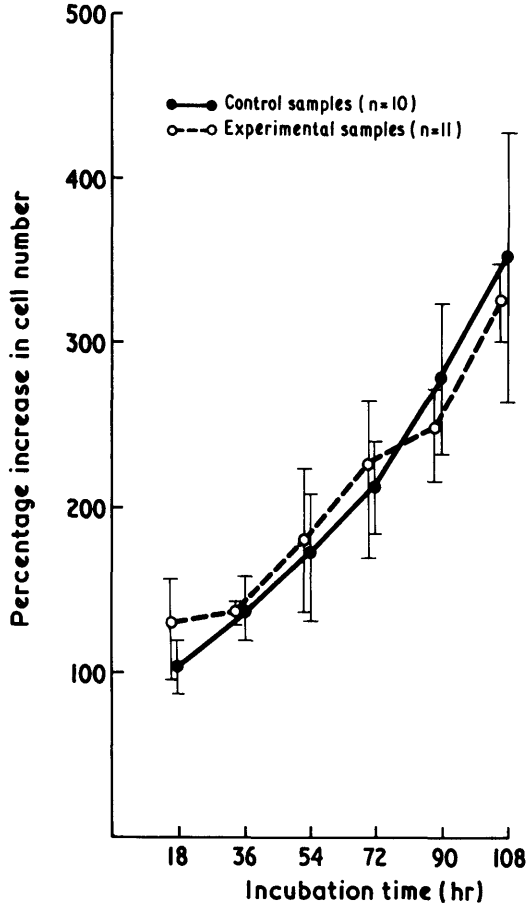


**Figure 6.** Rate of cell proliferation of the control samples (closed circles: $n = 10$) and the experimental samples (open circles: $n = 11$) in the experimental medium EM2 (see legend to Fig. 2 for details), with the medium renewed every 18 hr. Cell numbers for every sample were obtained by counting adherent cell nuclei in 18 fields of a ×40 phase contrast objective. Vertical bars represent SE of means.

in the cellular folate utilization may not affect the ability of the trophoblast to grow in culture (Fig. 6), it could be subtle enough to affect the structure of a less forgiving fetal tissue, such as the developing neural tube. This may be especially so when, at the time of closure, cells of the neural tube are relying on the diffusion of nutrients from maternal sources more distant than the medium in the tissue culture model used in this study.

As some clinical trials demonstrate, it is feasible that such a metabolic defect can be compensated for by larger intakes of folate than is provided by a normal diet (2, 5–7).

Finally, it should be remembered that two of the NTD-associated samples did not show a lag period in incorporating $^{14}C$ from [$^{14}C$]MTHF into the nucleic acid pool. This gives support to the multifactorial causation hypothesis, indicating that a minority of NTD may not be due to a metabolic defect of folate metabolism. Indeed intervention studies have suggested that 20% of NTD recurrences are not prevented by folate supplements. However, our results support the findings of the clinical trials by indicating that a significant majority of NTD are folate related. Hence, this discovery of a folate-related defect could also be a significant advance in the primary prevention of NTD, if it leads to a marker which could identify women at risk of an NTD-affected pregnancy before they conceive.

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1. Hibbard ED, Smithells RW. Folic acid metabolism and human embryopathy. Lancet **i**:1254, 1965.
2. Laurance KM, James N, Miller MH, Tennant JB, Campbell H. Double-blind control trials of folate treatment before conception to prevent recurrence of neural tube defects. Br Med J **282**:1509–1511, 1981.
3. Vergel RG, Sanchez LR, Heredero BL, Rodriguez PL, Martinez AJ. Primary prevention of neural tube defects with folic acid supplementation: Cuban experience. Prenat Diagn **10**:149–152, 1990.
4. Seller MJ. Periconceptional vitamin supplementation to prevent recurrence of neural tube defect. Lancet **i**:1392–1393, 1985.
5. Mulinare J, Cordero JF, Erickson JD, Berry RJ. Periconceptional use of multivitamins and the occurrence of neural tube defects. JAMA **260**:3141–3145, 1988.
6. Smithells RW, Sheppard S, Wild J, Schorah CJ. Prevention of neural tube defect recurrences in Yorkshire: Final report. Lancet **ii**:498–499, 1989.
7. MRC Vitamin Study Research Group. Prevention of neural tube defects: Results of the Medical Research Council vitamin study. Lancet **338**:131–137, 1991.
8. Rogozinski H, Ankers C, Lennon D, Wild J, Schorah C, Sheppard S, Smithells RW. Folate nutrition in early pregnancy. Hum Nutr Appl Nutr **37A**:357–364, 1983.
9. Smithells RW, Sheppard S, Schorah CJ. Vitamin levels and neural tube defect. Arch Dis Child **51**:944–949, 1976.
10. Schorah CJ, Wild J, Hartley R, Sheppard S, Smithells RW. The effect of periconceptional supplementation on blood vitamin concentrations in women at recurrence risk for neural tube defect. Br J Nutr **49**:203–211, 1983.
11. Yates JRW, Ferguson-Smith MA, Guzman-Rodriques R, White M, Clark BJ. Is disordered folate metabolism the basis for the genetic predisposition to neural tube defect? Clin Genet **31**:279–287, 1987.
12. Campbell LR, Dayton DH, Sohal GS. Neural tube defect: A review of human and animal studies on the etiology of neural tube defect. Teratology **34**:171–187, 1986.
13. Magnus P, Magnus EM, Berg K. Increased level of apotranscobalamins I and II in amniotic fluid from pregnant women with previous neural tube defect offspring. Clin Genet **30**:167–172, 1986.
14. Gardiki-Kouidou P, Seller MJ. Amniotic fluid folate, vitamin $B_{12}$ and transcobalamins in neural tube defect. Clin Genet **33**:441–448, 1988.
15. Kalter H. Teratology and pharmacogenetics. Ann NY Acad Sci **151**:997–1000, 1968.
16. Nishimura H, Tanimura T. Survey of embryology and teratology.

In: Wolstenholms GEW, O'Connor CM, Eds. Clinical Aspects of Teratogenicity of Drugs. London: J & A Churchill, pp1–29, 1976.
17. Habibzadeh N, Schorah CJ, Smithells RW. The effects of maternal folic acid and vitamin C nutrition in early pregnancy on reproductive performance in the guinea-pig. Br J Nutr **55**:23–35, 1986.
18. Steinberg SE. Mechanisms of folate homeostasis. Am J Physiol **246**:G319–G324, 1984.
19. Fox H. Placental structure. In: Macdonald RR, Ed. Scientific Basis of Obstetrics and Gynaecology. London: Churchill Livingstone, pp1–27, 1985.
20. Ham RG. An improved nutrient solution for diploid Chinese hamster and human cell lines. Exp Cell Res **29**:515–526, 1963.
21. Dulbecco R, Vogt M. Plaque formation and isolation of pure line with poliomyelitis virus. J Exp Med **99**:167–182, 1954.
22. Lilford RJ, Irving HC, Linton G, Mason MK. Transabdominal chorion villus biopsy: 100 consecutive cases. Lancet **i**:1415–1416, 1987.
23. Daniels-McQueen S, Krichevesky A, Boime I. Isolation and characterization of human cytotrophoblast cells. In: Miller RK, Thiede HA, Eds. Trophoblast Research, Vol 2. Cellular Biology and Pharmacology of the Placenta, Techniques and Applications. New York: Plenum Medical Book Co., pp423–445, 1987.
24. Bulmer JN, Sunderland CA. Immunohistological characteristics of lymphoid cell populations in the early human placental bed. Immunology **52**:349–357, 1984.
25. Bulmer JN, Johnson PM. Antigen expression by trophoblast population in the human placenta and their possible immunobiological relevance. Placenta **6**:127–140, 1985.
26. Kurman RJ, Main CS, Chen H-C. Intermediate trophoblast: A distinctive form of trophoblast with specific morphological, biochemical and functional features. Placenta **5**:349–370, 1984.
27. Lucock MD, Hartley R, Smithells RW. A rapid and specific HPLC-electrochemical method for the determination of endogenous 5-methyltetrahydrofolic acid in plasma using solid phase sample preparation with internal standardization. Biomed Chromatogr **3**:58–63, 1989.
28. Leyva A Jr, Kelly WN. Measurement of DNA in cultured human cells. Anal Biochem **62**:173–179, 1974.
29. Adams RLP. Cell culture for biochemists. In: Work TS, Burdon RH, Eds. Laboratory Techniques in Biochemistry and Molecular Biology. Oxford: Elsevier Science Publishers BV, pp266–267, 1983.
30. Markwell MAK, Haas SM, Bieber LL, Talbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87**:206–210, 1978.
31. Statistical Analysis System. SAS User's Guide. Cary, NC: Statistical Analysts Systems Institute, p153, 1982.
32. Shane B. Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. Vitam Horm **45**:263–335, 1989.
33. Anonymous. Methyl group deficiency: Effects on one-carbon unit and folate metabolism. Nutr Rev **47**:375–377, 1989.
34. Horne DW. Effect of nitrous oxide inactivation of vitamin $B_{12}$ and of methionine on folate coenzyme metabolism in rat liver, kidney, brain, small intestine and bone marrow. Biofactors **2**:65–68, 1989.
35. Chanarin I, Deacon R, Lumb M, Perry J. Cobalamin-folate interrelations. Blood Rev **3**:211–215, 1989.
36. Strong WB, Schrich V. In Vitro conversion of formate to serine: Effect of tetrahydropteroylpolyglutamates and serine hydroxymethyltransferase on the rate of 10-formyltetrahydrofolate synthetase. Biochemistry **28**:9430–9439, 1989.