ABSTRACT

Normal and Rous sarcoma virus-infected chicken fibroblasts proliferate maximally in a culture medium containing a physiological (10 ng/ml) concentration of 5-methyltetrahydrofolate or folinic acid (5-formyltetrahydrofolate acid), while their maximal proliferation requires a hyper-physiological (1000 ng/ml) concentration of folic acid. The normal and Rous-infected fibroblasts do not differ in their requirements for 5-methyltetrahydrofolate, folinic acid, or folic acid.

INTRODUCTION

Folic acid derivatives are important cofactors in thymidylate synthesis and are necessary for cell growth in culture, as well as in numerous other biosynthetic processes of importance (5). Because of their significant role in DNA synthesis, folates and folate antagonists have received much attention in experimental oncology and in cancer chemotherapy (5, 10).

5-Methyltetrahydrofolate acid is the principal form in which folic acid is present in the plasma and extracellular fluids of humans and chickens, this at concentrations of 5 to 15 ng/ml (1, 5, 12). Folate derivatives within cells, on the other hand, are largely present in the form of polyglutamates (5, 9). In spite of the fact that 5-methyltetrahydrofolate is the principal folate of the plasma and extracellular fluids, most cell culture studies on normal and neoplastic cells have been done in cell culture media containing folic acid itself or folinic acid (11, 13, 14). The experiments reported in the present publication were performed in order to study and compare the requirements of normal and Rous sarcoma virus-infected chicken fibroblasts for 5-methyltetrahydrofolate acid, folinic acid, and folic acid.

The present system was ideal for this study for two reasons: (a) It employs heat-defibrinogenated plasma, a reagent that we introduced for cell culture work (2, 4, 12, 13). We have shown that chicken serum, unlike heat-defibrinogenated chicken plasma, contains enough thymidine and purine to override a folate deficiency in the culture medium or a methotrexate block (3, 4, 12). Plasma-containing medium must, therefore, be used for cell culture studies of folate requirements; (b) 100% efficient infection of cells with tumor virus is obtained in the Rous sarcoma virus-chicken fibroblast system. This highly efficient infection yields populations of normal and neoplastic cells whose growth requirements can be compared with great experimental rigor.

MATERIALS AND METHODS

The basic materials and methods used in the experiments reported here were described previously (2-4, 12, 13).

Incubation Conditions. Cell cultures were incubated at 41.9° (chicken body temperature) in a humidified air-5% CO₂ atmosphere.

Culture Medium. The synthetic culture medium has been described (12); it includes methionine at 4 mg/liter, a physiological (plasma) concentration. Ninety-five parts of synthetic medium were combined with five parts of heat-defibrinogenated chicken plasma (see below) to yield the complete culture medium that was used for stock cultures and experiments. Although vitamin B₁₂ is not included in the synthetic medium, microbiological assays have indicated that it is present in the defibrinogenated chicken plasma at 1500 to 1700 pg/ml.

Preparation of Heat-defibrinogenated Chicken Plasma. This reagent was prepared as described (2-4, 12, 13). Cockerel blood was collected, chilled, and then freed of formed elements by centrifugation. This plasma was defibrinogenated by holding at 57° for 30 min followed by 2 cycles of freezing and thawing; the copious fibrinogen precipitate was removed by centrifugation.

Preparation of Stock and Experimental Cultures of Normal and Rous Sarcoma Virus-infected Chicken Fibroblasts. Primary cultures of normal fibroblasts were prepared by trypsinizing the pectoral muscles of 6-week-old male chickens. Secondary cultures were then prepared, and half of them infected with the Schmidt-Ruppin strain of Rous sarcoma virus. The normal and Rous secondary cultures were each passaged again, yielding tertiary cultures; these tertiary cultures were the sources of cells for the quaternary cultures used for the experiment. Replicate quaternary experimental cultures of, respectively, normal and Rous sarcoma virus-infected fibroblasts were prepared by seeding 60,000 cells per 35-mm Lux Contour dish.

Primary, secondary, and tertiary stock cultures were prepared, and experimental dishes were seeded with medium to which no folate had been added but which contained, instead, 10-9 M thymidine and 10-8 M hypoxanthine.
By substituting thymidine and hypoxanthine for folate in the medium used for growth of stock cultures and seeding of experimental cultures, our normal and neoplastic fibroblasts were introduced into experiments without significant folate pools. On the day following seeding, the experimental cultures were changed to test media of different folate compositions.

Chart 1, A to D, represents the trials in a single experiment which lasted for 6 days. Culture media were changed on Day 2 and on each day thereafter.

Construction of Experimental Media. For the folic acid-containing media (Chart 1A), a 100-ng/liter stock of folic acid was added to 95% synthetic medium-5% plasma to yield a final concentration of 1000 ng/ml (1 mg/liter). Serial log dilutions were then made, with 95% synthetic medium-5% plasma, to yield experimental media of folic acid concentrations of 100 and 10 ng/ml.

For the folinic acid-containing experimental media (Chart 1B), a 200-ng/liter stock of the racemic calcium salt, representing 100 mg of the biologically active L(-)-isomer per liter, was diluted into culture medium to yield a 1000-ng/ml (1 mg/liter) experimental medium, with further log dilutions to yield experimental media of L(-)-folinic acid concentrations of 100, 10, and 1.0 ng/ml.

DL-5-Methyltetrahydrofolic acid, sodium salt (Chart 1, C and D) was obtained from Sigma (St. Louis, Mo.). The supplier reports this preparation as being at least 90% pure. Because this compound has been reported to be unstable to oxidation under certain conditions, a 220-ng/liter (100%/90% x 200) stock of the racemic salt was prepared in glass-distilled water that had been gassed with nitrogen and the stock stored at -80°C. For the trial detailed in Chart 1C experimental media were prepared fresh, by dilution from aliquots of stock, for each medium change, i.e., on Days 0, 2, 3, 4, and 5. These dilutions yielded L(-)-methyltetrahydrofolate concentrations of 100, 10, and 1.0 ng/ml.

For the detailed trial in Chart 1D, enough culture medium for all of the medium changes was prepared on Day 0 and then stored at 4°C.

Cell Counts. Cell counts were done with a Coulter electronic cell counter. Each experimental point reported represents the mean ± S.E. of 2 culture dishes.

RESULTS

Proliferation in Folate-free Medium and Media of Physiological and Hyperphysiological Folic Acid Concentration (Chart 1A). After some initial "carry over" from the thymidine and hypoxanthine containing medium in which they had been grown out and seeded, neither normal nor Rous sarcoma virus-infected fibroblasts proliferated significantly in a culture medium composed of 95% synthetic medium, folate-free, and 5% defibrinogenated plasma.

In culture medium containing folic acid at 10 ng/ml, a physiological concentration, the normal and Rous-infected fibroblasts proliferated at slow, nearly identical, rates. In culture medium containing folic acid at 100 ng/ml, the normal and neoplastic fibroblasts proliferated at rapid, again nearly identical, rates. Only a slight increase in proliferative rate was observed in culture medium containing 1000 ng/ml (1 mg/liter) of folic acid.

The Day 4 to 6 "levelling off" of Rous-infected fibroblasts in 1000 ng/ml of folic acid medium is apparent, rather than real, being due to the well-known aggregation and detachment that these cells undergo at high densities.

Proliferation in Media of Physiological, Hyperphysiological, and Subphysiological L(-)-Folinic Acid Concentrations (Chart 1B). In culture medium containing a physiological (10 ng/ml) concentration of L(-)-folinic acid, the normal and Rous sarcoma virus-infected fibroblasts proliferated at rapid, essentially identical, rates. These rates were the same as those observed in 1000 ng/ml of folic acid and did not increase when the folinic acid concentration was raised to 100 or (data not shown) 1000 ng/ml. A reduced proliferative rate was observed with a subphysiological (1.0 ng/ml) concentration of folinic acid.

Maximal cell proliferation was observed, therefore, at a physiological concentration (10 ng/ml) of L(-)-folinic acid.

Proliferation in Media of Physiological, Hyperphysiological, and Subphysiological L(-)-5-Methyltetrahydrofolic Acid Concentration (Chart 1, C and D). The normal and Rous sarcoma virus-infected fibroblasts proliferated at maximal, again nearly identical, rates in medium containing a physiological concentration (10 ng/ml) of L(-)-5-methyltetrahydrofolate, the folate derivative that is present in the plasma and extracellular fluids of the body. This maximal rate of proliferation was observed regardless of whether the 10 ng/ml of L(-)-5-methyltetrahydrofolic acid was prepared fresh for each medium change (Chart 1C; Days 0, 2, 3, 4, and 5) or was prepared in batch at the beginning of the experiment (Chart 1D).

No increase in rate of proliferation was observed in media containing 5-methyltetrahydrofolic acid, added fresh for each medium change, at concentrations of 100 and (data not shown) 1000 ng/ml. A reduced proliferative rate was observed with a subphysiological (1.0 ng/ml) concentration of 5-methyltetrahydrofolic acid.

DISCUSSION

We have found that both normal and Rous sarcoma virus-
infected chicken fibroblasts proliferate at maximal and equivalent rates in culture medium containing a physiological concentration (10 ng/ml) of L(-)-5-methyltetrahydrofolic acid, the principal form in which folate is present in the human and avian plasma and extracellular fluid. Our findings confirm those of Chello and Bertino (6), who found that a physiological concentration of 5-methyltetrahydrofolate would support maximal proliferation of L-5178Y mouse leukemia cells in culture, provided that hydroxycobalamin and transcobalamin II were present in the serum component of the culture medium. (In our system, the defibrinogenated plasma provided vitamin B<sub>12</sub>, while the methionine present in the synthetic medium and plasma probably generated the homocysteine necessary for the operation of the 5-methyltetrahydrofolate methyltransferase system.) The fact that cultured cells proliferate maximally in the presence of a physiological concentration of 5-methyltetrahydrofollic acid suggests that cells in vivo are replete with folates, i.e., that the bioavailability of folate does not limit the rate of cell proliferation in the whole organism.

We have found that 5-methyltetrahydrofollic acid and folic acid are approximately 100 times as active as folic acid itself in supporting cell proliferation, again confirming the findings of other investigators (6, 7). The fact, however, that folic acid at 10 ng/ml and folic acid at 1000 ng/ml (1 mg/liter) did not support a proliferative rate in excess of that supported by a physiological concentration of the plasma form suggests that these two “nonphysiological” forms of folate can be used legitimately, i.e., without the introduction of artifact, in culture studies on control of cell proliferation. In culture studies dealing with folate metabolism per se, on the other hand, 5-methyltetrahydrofolate itself should probably be used. In this connection, it is significant that this compound appeared to have considerable stability in our culture system.

In our earlier publications, we demonstrated that normal and Rous sarcoma virus-infected fibroblasts have identical folic acid requirements for proliferation (4). Our present data suggest that this is the case for folinic acid and 5-methyltetrahydrofollic acid as well.

Halpern et al. (8) have published data indicating that cultured normal rat liver fibroblasts, rat liver epithelial cells, mouse liver fibroblasts, and human breast fibroblasts can be rescued from methotrexate toxicity by 5-methyltetrahydrofolic acid, while Walker-256 rat breast carcinosarcoma cells, L-1210 mouse lymphatic leukemia cells, Ehrlich’s mouse ascites cells, and J-111 human monocytic leukemia cells cannot be so rescued; Halpern et al. suggest that this difference is due to a lack, in the neoplastic cells that they studied, of vitamin B<sub>12</sub>-dependent 5-methyltetrahydrofolate methyltransferase, the enzyme that catalyzes transfer of the methyl group from 5-methyltetrahydrofolate to homocysteine to yield methionine and tetrahydrofolate. The tumor cell lines studied by Halpern et al. were not, however, the neoplastic counterparts of the normal cells in their study, but have very different histogenetic origins and culture histories. Indeed, Halpern et al. state in their publication that only “certain malignant cells” are distinguished from normal cells by their apparent lack of methyltransferase activity. These considerations, coupled with our current observation that Rous sarcoma virus-infected and normal fibroblasts proliferate at equivalent rates in 5-methyltetrahydrofolic acid, make it unlikely that conversion of cells to the neoplastic state results in diminished ability to metabolize this important compound.

REFERENCES

5-Methyltetrahydrofolic Acid, 5-Formyltetrahydrofolic Acid (Folinic Acid), and Folic Acid Requirements of Normal and Rous Sarcoma Virus-infected Chicken Fibroblasts

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