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The Role of Folic Acid Binding Proteins (FABP) in the Cellular Uptake of Folates[1] (38433)

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Recent studies have demonstrated the presence of specific folic acid binding proteins in cow's milk (1), human milk (2), leukocyte lysates of some patients with chronic myelocytic leukemia (3), sera obtained from patients with folate deficiency (4), taking oral contraceptive agents and during pregnancy (5), in some uremics (6) and in the brush border membrane of the rat small intestine (7). The folic acid binding proteins (FABP) from these varied sources and conditions share some characteristics (8), however, little is known concerning their possible physiologic significance. Therefore, we utilized folate replete and deficient HeLa cell monolayer cultures and human lymphocyte cell lines to test the role of FABP on the uptake of various labeled folates. An abstract of part of these studies has been presented (9).

Methods and Materials. ^{3}H pteroylglutamic acid (^{3}HPGA) 40 Ci/m*M* was purchased from Amersham Searle Corporation, Arlington Heights, Illinois. ^{3}H methyl-tetrahydrofolic acid (^{3}H methyl-THFA) 11 Ci/m*M* was provided by Dr. Peter Nixon and ^{14}C teropterin (pteroyltriglutamic acid) by Dr. Charles Baugh. The purity of these compounds varied from 90% to 95% as determined by descending paper chromatography in 0.1 *M* phosphate buffer pH 7.4. Serum was obtained from uremics and from patients with folic acid deficiency and was stored at −10°. Cow's milk (Carnation Instant Powdered Milk), crystallized beta lactoglobulin from cow's milk (Sigma, St. Louis, MO), human milk[2] and serum were assayed for folate level and FABP by radioassay (4). Hemoglobin-coated charcoal was used to separate bound from free folate (4).

HeLa cells were grown in monolayer cultures in MEM (Minimal Essential Media-Earle's Salts, Associated Biomedic Systems, Inc., Buffalo, NY) containing 10% fetal calf serum and 4 m*M* glutamine. HeLa cells were adapted to folate deficiency by passage through folic acid free MEM containing 10% dialyzed fetal calf serum and 4 m*M* glutamine. Dialyzed fetal calf serum contains < 1 ng/ml of folate. Human lymphocytes[3] were similarly adapted to folate deficiency in folate-free MEM suspension media. The deoxyuridine suppression of ^{3}H thymidine (^{3}HTdR) into DNA (10) and the growth curve were used as indicators of folate availability and were measured in both HeLa cells and lymphocytes conditioned in folic acid free media and complete media. The trypan blue exclusion test was used as an index of cell viability.

Uptake of ^{3}HPGA and ^{3}H methyl-THFA into folate replete and deficient HeLa cell monolayers was measured at 37 and 4° in Hank's Balanced Salt Solution (HBSS) as previously described (13). ^{3}HPGA or ^{3}H methyl-THFA (0.5 ng) was added to confluent HeLa monolayers (5×10^{6} cells) in 6 cm tissue culture plates containing 10 ml HBSS and incubated in a humidified 5% Co_2 in air atmosphere. At the end of the incubation time the cell layers were washed two times with HBSS. The cells were harvested, placed in test tubes and centrifuged. The resultant pellet was dissolved in 1.0 ml NCS reagent (Packard Instruments) prior to counting. Radioactivity recovered was calculated as percent uptake/10^{7} HeLa cells. This was compared to the uptake of ^{3}HPGA and ^{3}H methyl-THFA in

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[2] Mother's Milk Bank, Wilmington, DE.

[3] Line PGLC #H33 provided by Dr. Philip Glade, Mount Sinai School of Medicine, New York, NY.

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the presence of 5% normal human albumin, normal serum, folate-deficient and uremic serum with varying amounts of FABP, cow's milk, beta lactoglobulin and human milk. FABP was measured as previously reported (4) and represents the percentage of ^{3}HPGA (0.5 ng) bound/0.4 ml serum. These substances were dialyzed for 48 hours against 0.9% saline to remove endogenous folates and then incubated with 0.5 ng ^{3}HPGA or ^{3}H methyl-THFA for 30 minutes and placed into the HeLa cell monolayer plates for the uptake experiment. The effects of 1% ethanol and Dilantin (0.1 mg/ml) in phosphate buffer pH 7.4 on ^{3}HPGA uptake and efflux were similarly studied. The efflux of ^{3}HPGA was studied in HeLa cells pulsed with ^{3}HPGA (0.5 ng), washed, incubated with fresh HBSS, and measured by ^{3}HPGA recovered in the supernatant at 37 and 4° over a 90 min period. All experiments were done in triplicate.

Radioactivity was measured in 20 ml of scintillation mixture (toluene with 0.6% PPO, 0.03% dimethyl-POPOP and 25% ethanol), and was counted in a Beckman LS-250 liquid scintillation counter to a counting error of 1% or less.

Results. The uptake of ^{3}HPGA and ^{3}H methyl-THFA by folate replete HeLa cells increased for 3 hr at 37 but not at 4° and appeared to plateau after 1 hr. The uptake of ^{3}H methyl-THFA was 9.3% of the 0.5 ng ^{3}H methyl-THFA added while the uptake of ^{3}HPGA was 5.5% of the 0.5 ng ^{3}HPGA added. Dilantin (100 μg/ml) or ethanol (1%) did not inhibit the uptake of ^{3}HPGA (Fig. 1, Table I). HeLa cells conditioned in folate deficient media exhibited progressive deranged DNA synthesis (decreased dU suppression of ^{3}HTdR into DNA) which was corrected by the addition of PGA (50 μg) (Fig. 2). The uptake of ^{3}HPGA and ^{3}H methyl-THFA into folate deficient HeLa cells was greater (five and three times respectively) than that of folate replete HeLa cells (Fig. 3, Table I). There was no difference in the amount of ^{3}HPGA and ^{3}H methyl-THFA uptake into folate deficient HeLa cells. There was no significant uptake of ^{14}C teropterin in either normal or folate deficient HeLa cells (Table I).

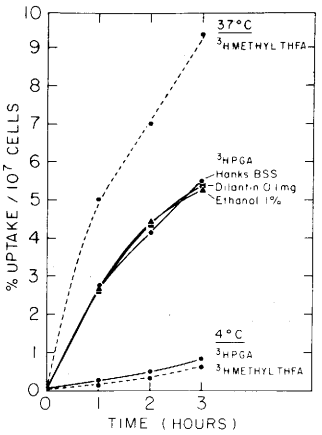

FIG. 1. Uptake of ^{3}HPGA (0.5 ng) and ^{3}H methyl-THFA (0.5 ng) into HeLa cells. Effect of Dilantin 0.1 mg/ml and ethanol 1%. (HeLa cells were preincubated with Dilantin or ethanol for 90 min at 37° prior to addition of the ^{3}H folates).

^{3}HPGA or ^{3}H methyl-THFA in the presence of FABP of normal serum, folate deficient serum, uremic serum (containing FABP), human or cow's milk or beta lactoglobulin (BLG), was less available for uptake into the HeLa cells as compared to their uptake either in buffer or in albumin (Table II). FABP (*i.e.*, BLG) added to the culture medium following a 1 hr pulse of ^{3}HPGA did not decrease the cellular uptake of ^{3}HPGA. The percent inhibition of uptake of ^{3}HPGA into HeLa cells was directly proportional to the amount of FABP in several sera tested (Fig. 4). An aliquot of the culture medium obtained from folate deficient HeLa cells incubated in HBSS for a 6 hr period, when assayed, did not contain FABP.

The efflux of ^{3}HPGA into HBSS from HeLa

TABLE I. Uptake of Folate[a] in Normal and Folate Deficient HeLa Cells.

	Normal		Folate deficient[b]	
	% uptake	pg per 10^7	% uptake	pg per 10^7
^{3}HPGA (0.5 ng)	5.5	29.9	27.9	139.5
^{3}H Methyl-THFA (0.5 ng)	9.3	43.9	28.9	144.5
^{14}C Teropterin (50.0 ng)	< 0.05		< 0.05	

[a] Uptake of folates at 3 hr at 37°. All experiments were done in triplicate and the average is presented.
[b] Two week folate deficient HeLa cells.

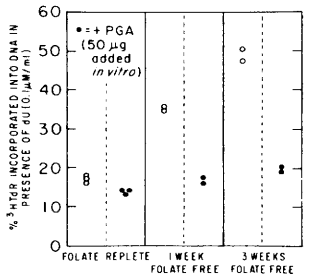

FIG. 2. Deoxyuridine suppression of ^{3}H thymidine in normal and folate deficient HeLa cells. dU 0.1 μM/ml and PGA 50 μg per ml were used. The growth rate of the folate deficient HeLa cells was 50% of that of the folate replete HeLa cell cultures.

cells previously pulsed with ^{3}HPGA for 2 hr was found to be greater at 37 than at 4° (Fig. 5).

Discussion. Specific folic acid binding proteins (FABP) sharing some characteristics (8) have been found in serum from normals, folate deficients (4), some uremics (6), some pregnant women (5), as well as in milk (2), and some chronic myelocytic leukemic leukocyte lysates (3). FABP has also been found in cow's milk (probably beta lactoglobulin), (1, 11), hog kidney (12), and the brush border membranes of rat small intestinal epithelial cells (7). The characteristics of FABP include a rapid association and slow dissociation rate for the binding of ^{3}HPGA, and a preference for the binding of oxidized folyl mono and polyglutamates as compared to reduced folates. FABP appears to represent two proteins, one eluting as a beta globulin with a molecular weight of approximately 50,000 and another larger protein excluded by G-200 Sephadex. Milk, an apocrine secretion, is much higher in FABP content than serum. For these reasons we have previously suggested that FABP represents a cellular, perhaps membrane derived protein, important in regulating the cellular uptake, distribution and storage of various folate coenzymes (2).

To further explore the physiology of FABP we utilized cultures of HeLa cells and human lymphocytes. The HeLa cell line was selected because it represents a well studied human line with a rapid doubling time. Thus, a larger population of cells are in DNA synthesis and the requirement for folate is greater than in a cell line with a slower doubling time. The uptake of ^{3}HPGA and ^{3}H methyl-THFA was significantly greater in HeLa cells than in human reticulocytes or normal bone marrow (13) or phytohemagglutinin (PHA) stimulated lymphocytes (14). As in human bone marrow (13), the uptake of ^{3}HPGA and ^{3}H methyl-THFA increased for 3 hr, was temperature dependent, greater for ^{3}H methyl-THFA than for ^{3}HPGA and was not influenced by Dilantin or ethanol. All of these features provide evidence for an energy dependent, active transport mechanism for both PGA and methyl-THFA, perhaps more operative in rapidly dividing cells. There was no significant uptake of teropterin, a folyl polyglutamate, consistent with a similar finding in PHA stimulated lymphocytes (15).

HeLa cells and human lymphocyte lines could be conditioned to a state of folate deficiency when grown in folic acid free media. This was evident within 1 week and became more severe with time. The metabolic consequence was a progressive derangement in a reduced folate-dependent step (methylation of deoxyuridylate to thymidylate) as measured by defective dU suppression of ^{3}HTdR into DNA which was correctable by the *in vitro* addition of PGA (10). This was associated with a decrease in the growth curve (50% reduction in 1 week of folate-deprived cells) followed by an increase in cell death after three weeks.

The uptake of both ^{3}HPGA and ^{3}H methyl-THFA increased (five- and threefold, respectively) in folate-deficient HeLa cells. There was no difference in the amount of uptake of ^{3}HPGA and ^{3}H methyl-THFA in the folate deficient HeLa cells. These data suggest that there is a limiting factor in folate uptake which could be explained by a carrier mediated uptake process.

^{3}HPGA and ^{3}H methyl-THFA bound to various sources of FABP (folate deficient serum, some uremic serum, human milk, powdered cow's milk or BLG) was less available for uptake into the HeLa cells as compared to their

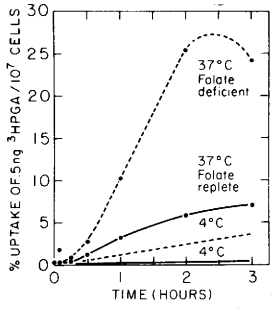

FIG. 3. The uptake of ^{3}HPGA in folate replete and folate deficient HeLa cells.

TABLE II. Effect of Various FABP on ^{3}HPGA and ^{3}H Methyl-THFA Uptake into HeLa Cells.

	% Inhibition[a]	
	^{3}HPGA	^{3}H Methyl THFA
Normal serum with 5% FABP	18.1	13.0
Folate deficient serum with 48% FABP	85.9	59.4
Uremic serum with 5% FABP	15.3	19.0
Uremic serum with 29% FABP	40.2	36.3
Cow's milk (10% Carnation, Instant Powder)	66.5	68.4
Human milk (average of three different samples)	80.2	81.3
BLG (1 mg/ml)	74.5	69.6
BLG (1 mg/ml) added following a hr ^{3}HPGA pulse	< 10%	
BLG (1 mg/ml) removed before ^{3}HPGA pulse	+60%	

[a] As compared to uptake of ^{3}HPGA of ^{3}H methyl-THFA in buffer or albumin.

[b] % FABP represents the percentage of 0.5 ng ^{3}HPGA bound by 0.4 ml of serum.

uptake in buffer or albumin. The addition of FABP following ^{3}HPGA uptake into the cell had no significant effect on the uptake of ^{3}HPGA. The percent inhibition of uptake of ^{3}HPGA into HeLa cells was directly proportional to the amount of FABP in several sera tested. Therefore, FABP in serum retards delivery of bound folates into the cell and as such proves not to be a serum delivery protein for folate. Serum FABP theoretically could provide a state of intracellular folate deficiency despite adequate serum folate levels. Such a condition has been suggested by Hines *et al.* (6) as a cause for the megaloblastic marrow maturation in some uremic patients whose serum contain FABP. Ford (17) recently reported that the uptake of folate into bacteria was severely depressed by the simultaneous addition of milk FABP. Thus, FABP in the mammary gland may act as a trapping mechanism to accumulate folate from blood plasma into milk and, in the gut, to facilitate their absorption by preventing folate uptake by intestinal microorganisms. Therefore, FABP might influence vitamin availability to the neonate as well as the ecology of the gut microflora.

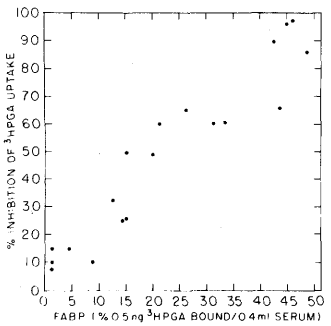

FIG. 4. The effect of varying concentrations of serum FABP on the uptake of ^{3}HPGA into HeLa cells.

The efflux of ^{3}HPGA from HeLa cells was temperature dependent and supports previous studies in L1210 leukemia cells (16) that this is an active, energy dependent process. The efflux process was not inhibited by the addition of various FABP to the culture medium.

FABP was not measurable in aliquots of culture medium obtained from the folate deficient HeLa cell cultures. However, FABP was recently reported to be actively synthesized and released into culture medium by chronic myelogenous leukemia cells (18). FABP has been found in extracts of membranes of the brush border of the rat small intestine (7) and human lymphocytes (8). Thus, it appears that FABP is a cellular derived protein and may be important in the intra-cellular accumulation of folate. The FABP in the membrane may be the rate limiting factor in folate uptake since it could act as a folate carrier during transport through the membrane and its availability being determined by the folate state of the cell.

Summary. HeLa cells and human lymphocytes were grown in complete media or conditioned in folate deficient media and were used to study the physiology of folic acid binding proteins (FABP). The uptake of tritiated folic acid (^{3}HPGA) and *N*-5-methyl tetrahydrofolic acid (^{3}H methyl-THFA) by folate replete HeLa cells increased for 3 hr, was temperature dependent, was greater for ^{3}H methyl-THFA than ^{3}HPGA and was not influenced by preincubation with Dilantin or ethanol. HeLa cells conditioned in folate deficient media after 1 week exhibited deranged DNA synthesis (abnormal de-

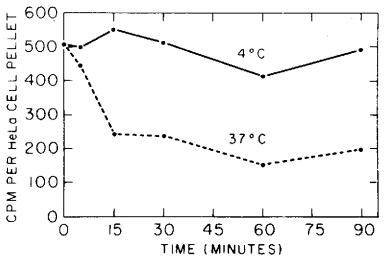

FIG. 5. The effect of temperature on the efflux of tritiated PGA from HeLa cells.

oxyuridine suppression of ^{3}H thymidine into DNA and a decreased growth curve) which was progressive with degree of folate deficiency. The uptake of ^{3}HPGA and ^{3}H methyl-THFA was greater (five and three times, respectively) in the folate deficient HeLa cells than in the folate replete HeLa cells. ^{3}HPGA or ^{3}H methyl-THFA bound to the FABP of folate deficient sera, some uremic sera, human or cow's milk was less available for the uptake by the HeLa cells. The percent uptake of ^{3}HPGA by HeLa cells was inversely related to the amount of FABP in several sera tested. FABP added to the culture medium following a 1 hr pulse of ^{3}HPGA did not decrease the cellular uptake of ^{3}HPGA. Evidence was found for an energy dependent, active mechanism for efflux of ^{3}HPGA from the HeLa cell.

1. Ghitis, J., Amer. J. Clin. Nutr. **20,** 1 (1967).

2. Waxman, S., and Schreiber, C., Blood **42,** 291 (1973).

3. Rothenberg, S. P., and daCosta, M., J. Clin. Invest. **50,** 719 (1971).

4. Waxman, S., and Schreiber, C., Blood **42,** 281 (1973).

5. daCosta, M., and Rothenberg, S. P., J. Lab. Clin. Med. **83,** 207 (1974).

6. Hines, J. D., Kamen, B., and Caston, D., Blood (Abstract) (1973).

7. Leslie, G. I., and Rowe, P. B., Biochemistry **11,** 1696 (1972).

8. Waxman, S., and Schreiber, C., Clin. Res. **11,** 571 (1973).

9. Waxman, S., and Schreiber, C., Blood (Abstract) (1972).

10. Metz, J., Kelly, A., Swett, V. C., Waxman, S., and Herbert, V., Brit. J. Haematol. **14,** 575 (1968).

11. Salter, D. N., Ford, J. E., Scott, K. J., and Andrews, P., FEBS Lett. **20,** 302 (1972).

12. Kamen, B., and Caston, J. D., J. Lab. Clin. Med. **83,** 164 (1974).

13. Corcino, J. J., Waxman, S., and Herbert, V., Brit. J. Haematol. **20,** 503 (1971).

14. Das, K. C., and Hoffbrand, A. V., Brit. J. Haematol. **19,** 203 (1970).

15. Hoffbrand, A. V., Tripp, E., Houlihan, C. M., and Scott, G. M., Blood **42,** 141 (1973).

16. Lichtenstein, N. S., Oliverio, V. T., and Goldman, I. D., Biochim. Biophys. Acta **193,** 456 (1969).

17. Ford, J. E., Brit. J. Nutr. **31,** 243 (1974).

18. daCosta, M., and Rothenberg, S. P., Clin. Res. **22,** 486 (1974).

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