Vol. 98, No. 4, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1981 Pages 983-989

THE EFFECT OF NITROUS OXIDE-INDUCED VITAMIN B_{12} DEFICIENCY ON IN VIVO FOLATE METABOLISM

S.E. Steinberg, C. Campbell and R.S. Hillman
Division of Hematology, University of Washington
Seattle, Washington 98195

Received December 31, 1980

SUMMARY

The effect of N_2O-induced vitamin B_{12} deficiency on in vivo folate metabolism was studied in an animal model previously developed for studies of the folate enterohepatic cycle,and in rats with localized, subcutaneous tumor nodules. While N_2O inhibited liver folate polyglutamate formation, it did not affect the absorption of $(^3H)PteGlu_1$ from the gut, its reduction, methylation, and transport to the liver, or the subsequent secretion of $CH_3H_4(^3H)PteGlu_1$ into bile--the folate enterohepatic cyle. In addition, N_2O did not impair folate polyglutamate formation in the fibrosarcoma tumor nodule suggesting that tumor tissue can either demethylate $CH_3H_4PteGlu_1$ by an alternate pathway or can utilize it as a substrate for polyglutamate formation without demethylation.

Vitamin B_{12} deficiency impairs folate polyglutamate formation and decreases the total cell content of folate (1,2,3,4,5,6,7,8). Two theories explaining these effects on folate metabolism have evolved. The first is the methyl trap hypothesis (9,10,11) which holds that the conversion of $CH_3H_4PteGlu_1$ to other biologically necessary folate congeners can only occur through the pathway involving vitamin B_{12} dependent methyltransferase (5 methyltetrahydrofolate homocysteine methyltransferase, EC 5.4.99.2). Since folate polyglutamate synthesis requires $H_4PteGlu$ and/or its formyl or methylene derivatives as substrate (8,10), decreased intracellular polyglutamate levels would be predicted by the methylfolate trap theory. It would also explain the fall in total cell folate content if the $CH_3H_4PteGlu_1$ which could not be demethylated and converted to polyglutamate leaves the cell. A second hypothesis has proposed that vitamin B_{12} is essential to membrane transport of folate (12,13,14). Conflicting data exists on this point. Past studies using in vitro cell cultures have demonstrated a subnormal uptake of $CH_3H_4PteGlu_1$ by vitamin B_{12} deficient

0006-291X/81/040983-07$01.00/0

cells (13,14) and in vivo animal studies have shown reduced uptake by the liver with an increased rate of loss of labeled folates in urine (5,7,15). However, a recent study by Horne and Briggs (16) using isolated hepatocytes failed to show an impairment of uptake of labeled $CH_3H_4PteGlu_1$.

To gain further information regarding these two theories an animal model previously developed for studies of the kinetics of the folate enterohepatic cycle (17) was used to examine in vivo folate kinetics following N_2O exposure (7,8,18). In addition, rats with subcutaneous fibrosarcoma tumor implants (19) were studied to determine the effects of N_2O-induced vitamin B_{12} deficiency on tumor cell folate polyglutamate synthesis.

MATERIALS AND METHODS

Folate compounds: (^{3}H)$PteGlu_1$ (specific activity 20-40 Ci/mM) was obtained from Amersham Corp, Arlington Heights, Illinois. Chromatography of a standard aliquot at the time of study revealed greater than 90% purity. Labeled and unlabeled folate standards for use as column markers were prepared as previously described (17).

Animals: Female Wistar-Furth rats weighing 150-250 g were used for all studies. Animals were maintained on standard Purina rat chow diet containing 30 mg/g of L. caseii active folate, and had serum folate levels in the range of 100-150 ng/ml. In selected animals an isolated 2-5 fibrosarcoma tumor nodule was produced by subcutaneous injection of a cell line infected with polyoma virus DW-7410. In the 18 h prior to the study, the animals were exposed to a 50:50 N_2O/O_2 air mixture. Then under N_2O anesthesia with Innovar supplementation, the abdominal wall was incised, the common bile duct isolated and cannulated and an in vivo gut loop created as previously described (17). 100 ng of (^{3}H)PteGlu was instilled into the gut loop and both ends clamped. At one hour, when greater than 90% of the isotope had been absorbed, the loop was unclamped, flushed with warm saline and returns collected for counting and chromatography. All bile and urine was collected for 6 h after which the animal was sacrificed, the liver and tumor removed and immediately processed for determination of total radioactivity per gram wet weight of tissue and for column chromatography. Control animals were treated in an identical fashion without N_2O exposure.

Chromatographic Identification of Folates: As soon as liver and tumor tissue were removed they were weighed and immediately homgenized in 30 ml, iced 1% ascorbate in phosphate buffer, pH 6.0. The homogenate was then boiled for 7 min and centrifuged to remove protein. An aliquot of the supernatent was then diluted in Aquasol to measure total radioactivity while the remainder was placed on a 0.9 x 100 cm column of Sephadex G15-120. The effluent was collected in 1.6 ml fractions at a flow rate of 15 ml/h and 1 ml of each fraction was pipetted into Aquasol for counting. To identify $CH_3H_4PteGlu_1$, an aliquot of eluate volumes 125-150 ml was co-chromatographed on Sephadex DEAE-A25. Selected markers were used for each column to identify $CH_3H_4PteGlu_1$, PABA, $PteGlu_1$, 5 $CHOH_4PteGlu_1$, and $CH_3H_4PteGlu_{5-7}$.

Total labeled folate in fluids and tissues was expressed as a percentage of

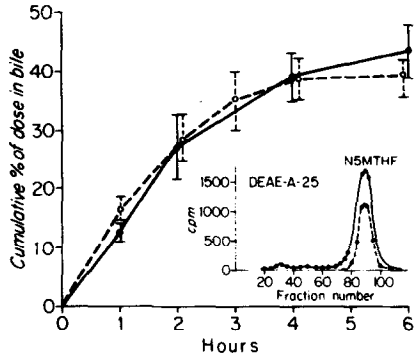

Figure 1: The cumulative percents of the enteric dose of (^{3}H)PteGlu$_1$ appearing in bile over 6 h for normal (solid dots) and N_2O treated animals (open circles) were the same. All of the (^{3}H) activity in bile was associated with a single chromatographic peak which eluted in the same position as a $^{14}CH_3H_4$PteGlu$_1$ marker on DEAE-A-25 Sephadex (broken line in insert).

total label absorbed. All data was expressed as means ± SEM and statistics performed by the student's t test.

RESULTS

Absorption of(^{3}H)PteGlu$_1$ by the gut of N_2O animals (91.6 ± 1%) was similar to normals (89 ± 1.7%). The rate of appearance of labeled folate in bile (CH_3H_4(^{3}H)PteGlu$_1$) over 6 h was also the same (Fig. 1). Furthermore, after 6 h, there was no apparent difference in the uptake of labeled folate by liver or tumor tissue when N_2O and normal animals were compared (Table 1). The slight reduction in the percent (^{3}H)PteGlu$_1$ per gram wet weight liver for N_2O animals ($p > 0.05$) was not apparent when calculated as total activity per liver.

N_2O exposure did have a dramatic effect on polyglutamate formation by the liver (Table 2). The percent of labeled folate present as polyglutamate at 6 h was less than one third that of normal liver. At the same time, N_2O did not affect polyglutamate formation by tumor cells. Approximately 41% of the CH_3H_4(^{3}H)PteGlu$_1$ taken up the tumor was converted to polyglutamate in both N_2O and normal animals. Another difference in the behavior of the two tissues was the form of the residual, non-polyglutamate folate. When this

Table 1: Distribution of Labeled Folates at 6 hours
(percent of $^{3}HPteGlu_{1}$ absorbed)

	Normal Percent/g (Total) N=4	N_2O Percent/g (Total) N=7	P
Liver [1]	1.34 ± .12 (8.6 ± .72)	1.03 ± .1 (8.5 ± 5.3)	> .05/NS
Tumor [1]	0.37 ± .05	0.39 ± .17	NS

[1] Results are mean ± SEM

was analyzed by chromatography on DEAE-A25 Sephadex, the residual labeled folate in liver consisted primarly of (^{3}H)$PteGlu_{1}$ which had not been reduced or methylated (Fig. 2).

DISCUSSION

Studies of the effect of vitamin B_{12} deficiency on the uptake of folate by cells have suggested that $CH_3H_4PteGlu_1$ uptake and/or storage by cells is abnormal. In marrow (14) and stimulated lymphocyte (13) cultures there appears to be a defect in the initial uptake by the cell which may result from the block in demethylation of intracellular $CH_3H_4PteGlu_1$ or a defect in membrane transport. A recent *in vitro* study by Horne and Briggs (16) using isolated hepatocytes demonstrated normal membrane transport for $CH_3H_4PteGlu_1$ although folate polyglutamate synthesis was clearly blocked by N_2O treatment. The present *in vivo* study demonstrates that all of

Table 2: Polyglutamate Formation (percent of total tissue activity)

	Normal N=4	N_2O N=7	P
Liver [1]	53.8 ± 8.8	15.8 ± 3.1	0.005
Tumor [1]	41.0 ± 1.7	41.5 ± 4.5	NS

[1] Results are mean ± SEM

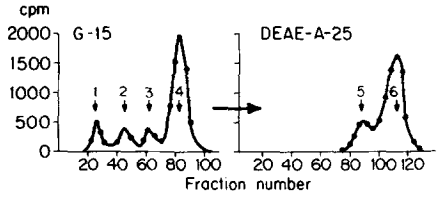

Figure 2: Chromatography of liver extract at 6 h on G-15 Sephadex demonstrated three minor and one major radioactive peak, associated with (1) $CH_3H_4PteGlu_{5-7}$. (2) p-aminobenzoylglutamate (3) 5-$CHOH_4PteGlu_1$ and (4) $PteGlu_1/CH_3H_4PteGlu_1$ markers, as shown. When the eluate from volumes 125-150 ml (fraction number 75-100) was co-chromatographed on DEAE-A-25 Sephadex most of the radioactivity eluted in the same position as (6) the $PteGlu_1$ marker and not (5) the $CH_4H_4PteGlu_1$ marker.

the steps involved in (^{3}H)$PteGlu_1$ absorption by the gut mucosa (uptake, reduction and methylation), transport to the liver and other tissues, and recycling of folates by the liver for secretion into bile (the enterohepatic cycle) (17) are unaffected by vitamin B_{12} deficiency. Thus, we have confirmed the findings of Horne and Briggs (16).

This does not, however, negate past reports of a decreased overall content of folate in the livers of vitamin B_{12} deficient animals (5, 7, 15). In fact, the absence of an effect on the folate enterohepatic cycle, in the face of a block in the demethylation of $CH_3H_4PteGlu_1$, would help explain why the folate content of liver falls. Since the liver normally secretes $CH_3H_4PteGlu_1$ into bile for recirculation to the gut, a block in demethylation would encourage clearance of folate from the liver and a depletion of total folate content with time. In the present study, the persistence of radioactivity in the liver during the first 6 h resulted from a delayed clearance of (^{3}H)$PteGlu_1$;liver content of CH_3H_4(^{3}H)$PteGlu_1$ and labeled polyglutamate was decreased.

The ability of N_2O-induced vitamin B_{12} deficiency to inhibit liver folate polyglutamate formation (5, 7, 8, 15, 16) was again demonstrated, although over 6 h up to one third of normal synthesis was observed. This is greater than that reported by other investigators (7, 8) and may suggest that a small amount of (^{3}H)$PteGlu_1$ which escapes methylation in the gut wall (17) can serve as a

substrate for polyglutamate formation by conversion to $H_4PteGlu_1$ or a formyl or methylene derivative of $H_4PteGlu_1$ (8). In vitro studies of PHA-stimulated, vitamin B_{12} deficient lymphocytes (10) have shown that $PteGlu_1$ is taken up and after 72 hours converted largely to pteroylpolyglutamate froms. Thus, if the present study had been extended to 12-24 hours or more, all of the $(^3H)PteGlu_1$ appearing in liver could possibly serve as substrate for polyglutamate formation. At 6 h, however, there was a partial block in the reduction and methylation of $(^3H)PteGlu_1$ by the hepatocytes of N_2O treated animals.

Finally, N_2O-induced vitamin B_{12} deficiency did not impair folate polyglutamate formation in tumor tissue. This finding is in sharp contrast to previous experiments on hepatocytes (16), other types of tumor cells (20), bone marrow cells (14), and cultured lymphocytes (10, 13). The explanation for this disparity is unclear. $(^3H)PteGlu_1$ administered enterically does not appear in the fibrosarcoma tumor nodules as $PteGlu_1$ (19), although a small portion of the absorbed folate may be converted to a formyl or methylene derivative of $H_4PteGlu_1$. However, it is unlikely that this could restore tumor polyglutamate formation to a normal level in the presence of a major block in the utilization of $CH_3H_4PteGlu_1$, the principle form of folate taken up by the tumor cells. The identical rates of polyglutamate formation in N_2O and normal animals would suggest that the conversion of $CH_3H_4PteGlu_1$ to polyglutamate was not affected, thereby raising two intriguing possibilities. Either this particular tumor tissue can demethylate $CH_3H_4PteGlu_1$ by an alternate pathway or it can utilize $CH_3H_4PteGlu_1$ as a substrate for polyglutamate formation without demethylation.

REFERENCES

1. Chanarin, I., Perry, J., and Lumb, M. (1974) Lancet i, 1251-1252.
2. Scott, J.M., O'Broin, J.D., and Weir, D.G. (1974) Lancet ii, 906.
3. Lavoie, A., Tripp, E., and Hoffbrand, A.V. (1974) Clin. Sci. Mol. Med. 47, 617-622.

4. Kutzbach, C.A., Galloway, E. and Stokstad, E.L.R. (1967) Proc. Soc. Exp. Biol. Med. 124, 801-805.
5. Smith, R.M. and Osborne-White, M.S. (1973) Biochem J. 136, 279-293.
6. Davidson, G.E., Weir, D.G. and Scott, J.M. (1975) Biochem. Biophys. Acta 392, 207-215.
7. McGuig, P., Reed, B., Wier, D.G. and Scott, J.M. (1978) Biochem. and Biophys. Res. Commun. 82, 540-546.
8. Perry, J., Chanarin, I., Deacon, R. and Lumb, M. (1979) Biochem. and Biophys. Res. Commun. 91, 678-684.
9. Herbert, V. and Zalusky, R. (1962) J. Clin. Invest. 41, 1263-1276.
10. Lavoie, A., Tripp, E. and Hoffbrand, A.V. (1974) Clin. Sci. Mol. Med. 47, 617-630.
11. Noronha, J.M. and Silverman, M. (1962) In: Vitamin B_{12} and Intrinsic Factor, Second Eur. Symp. (Heinrich, H.C., ed.) pp 728-736 (F. Enke, Stuttgart).
12. Gawthorne, J.M. and Smith, R.M. (1974) Biochem. J. 142, 119-126.
13. Das, K.C. and Hoffbrand, A.V. (1970) Brit. J. Haemat. 19, 203-221.
14. Tisman, G. and Herbert, V. (1973) Blood 41, 465-469.
15. Thenen, S.W. and Stokstad, E.L.R. (1973) J. Nutr. 103, 363-370.
16. Horne, D.W. and Briggs, W.T. (1980) J. Nutr. 110, 223-230.
17. Steinberg, S., Campbell, C. and Hillman, R.S. (1979) J. Clin. Invest. 64, 83-88.
18. Deacon, R., Lumb, M., Perry, J., Chanarin, I., Minty, B., Halsey, M.J., and Nunn, J.F. (1978) Lancet ii, 1023-1024.
19. Steinberg, S., Campbell, C., and Hillman, R.S. (1980) Clin. Res. 28, 601A
20. Chello, P.L. and Bertino, J.R. (1973) Cancer Res. 33, 1898-1904.