Vol. 91, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1979 Pages 678-684

THE SUBSTRATE FOR FOLATE POLYGLUTAMATE BIOSYNTHESIS IN THE VITAMIN B_{12}- INACTIVATED RAT

Janet Perry, I. Chanarin, Rosemary Deacon and M. Lumb
Section of Haematology, M.R.C. Clinical Research Centre, Harrow, U.K.

Received October 22,1979

SUMMARY

Rats were maintained for 24 hours in a chamber containing 50% N_2O in order to inactivate cobIalamin. Control animals breathed air. Folate analogues [0.01μmol] labelled with either ^3H or ^{14}C, were given IP and their hepatic uptake and conversion into folate polyglutamate measured. There was impaired hepatic uptake of folate by N_2O-treated animals varying from 21% of that in controls given H_4PteGlu to 69% with 10-CHO-H_4PteGlu. There was no detectable formation of folate polyglutamate in the N_2O-treated animal with H_4PteGlu and 5-CH_3-H_4PteGlu but 'normal' polyglutamate synthesis with 5-CHO-H_4PteGlu, 10-CHO-H_4PteGlu and 5,10-CH-H_4PteGlu.

Vitamin B_{12} deficiency in man is accompanied by a low level of folate polyglutamate in red blood cells [1,2,3]. In vitamin B_{12} deficiency induced in experimental animals there is a fall in the folate level in the liver [4,5] and impaired folate polyglutamate synthesis. An explanation offered in the methylfolate-trap hypothesis [6,7,8] is that the vitamin B_{12}-dependent methionine synthetase reaction (homocysteine to methionine) also converts 5-CH_3-H_4PteGlu into H_4PteGlu, the latter, it is postulated, being the necessary substrate for the action of the ligase that adds glutamic acid residues to form polyglutamate. Failure to donate the methyl group from 5-CH_3-H_4PteGlu to homocysteine in vitamin B_{12} deficiency leads to trapping of folate as the methyl analogue.

Exposure to the anaesthetic gas nitrous oxide (N_2O) leads to rapid oxidation of vitamin B_{12} from the active reduced cobIalamin form to

Abbreviations: nitrous oxide, N_2O; tetrahydropteroylglutamic acid, H_4PteGlu; 5-methyltetrahydropteroylglutamic acid, 5-CH_3-H_4PteGlu; 10-formyltetrahydropteroylglutamic acid, 10-CHO-H_4PteGlu; 5,10-methenyltetrahydropteroylglutamic acid, 5,10-CH-H_4PteGlu.

0006-291X/79/220678-07$01.00/0

the inactive oxidized cobIIIalamin form. This occurs both *in vitro* [9,10] and *in vivo* in man [11,12,13] and animals [14,15]. In man megaloblastic haemopoiesis is produced by N_2O inhalation [11,12] and the abnormal deoxyuridine suppression test using marrow aspirate is improved by the addition of vitamin B_{12} [12]. There is impaired folate polyglutamate synthesis from PteGlu in the N_2O-treated rat [14].

The purpose of this study was to determine the folate analogues, if any, used for polyglutamate synthesis by the B_{12}-inactivated rat and hence to clarify the role of B_{12} in this pathway.

MATERIALS AND METHODS

Folate compounds: [2-^{14}C] PteGlu (55 mCi/mmole) purchased from Radiochemical Centre, Amersham, U.K., was used to prepare [2-^{14}C]H_4PteGlu by the method of Davis [16]. 5-CH_3-H_4PteGlu, 10-CHO-H_4PteGlu and 5,10-CH-H_4PteGlu were prepared from [G-^{3}H]5-CHO-H_4PteGlu (1.1 Ci/mmole) as described elsewhere [17,18,19].

Animals: Male, Sprague-Dawley, 80 to 120g rats were given 0.01 µmole of a folate analogue intraperitoneally. Three animals were placed immediately in a chamber in which a mixture of N_2O (50%)/oxygen (50%) was passed and CO_2 and humidity controlled. Another 3 animals were left in air. After 24 h all 6 animals were killed by exsanguination, livers removed and labelled folate analogues separated by chromatography on DEAE cellulose as described [20]. Total folate retained in the liver and the proportion converted into folate polyglutamate was estimated.

Identification of folates: Fractions obtained following DEAE cellulose chromatography were assayed microbiologically with *L.casei* [ATCC 7469] and *P.cerevisiae* [ATCC 8081], and counted for radioactivity on an LKB-Wallac 8100 liquid scintillation spectrometer as described previously. Polyglutamyl folates were deconjugated to monoglutamates using human plasma γ-glutamylcarboxypeptidase. Folate compounds were identified on the basis of their activity for the assay organisms and position of elution from the column relative to standard marker compounds ([^{3}H]5-CH_3-H_4PteGlu$_{1-6}$ prepared from *L.casei* and kindly donated by Dr. K.U. Buehring; [G-^{3}H]5-CHO-H_4PteGlu; [2-^{14}C]H_4PteGlu]). Generally three peaks of radioactivity eluted in the first 100-175 ml (Fig.1). The first, at about 120 ml, had no microbiological activity with the 2 assay organisms and was probably a breakdown product. The second [approx 140-150 ml] co-chromatographed with 5-CHO-H_4PteGlu and had the same assay responses. It is uncertain how much of the formylfolate activity in this peak is due to interconversion of 10-CHO-H_4PteGlu and 5,10-CH-H_4PteGlu during the chromatographic and folate extraction steps. The third peak [160-170ml] was active with *L.casei* only and corresponded to 5-CH_3-H_4PteGlu. H_4PteGlu eluted at about 180 ml.

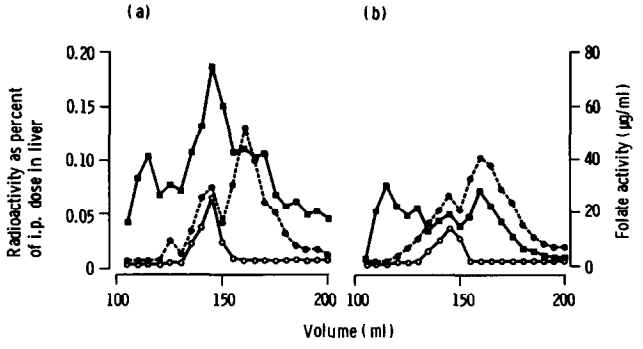

Figure 1. Separation of liver folates on DEAE cellulose after an intraperitoneal dose of 0.01μmol [2-^{14}C]H_4PteGlu. The eluate fractions were measured for radioactivity (■—■) and assayed microbiologically with Pediococcus cerevisiae (○—○) and Lactobacillus casei (●--●). Only fractions containing monoglutamate forms are shown.

a. Control animal breathing air. The radioactive peak eluting at about 120 ml. does not support the growth of the assay organisms (?2-amino-4-hydroxy-pteridine), the fractions at 140 to 150 ml. are active with P.cerevisiae and co-chromatograph with 10-formylH_4PteGlu and the fraction eluting at 170 ml. is active only with L.casei and co-chromatographs with authentic 5,CH_3H_4PteGlu.

b. N_2O-exposed animal. Note loss of labelled formylH_4PteGlu peak.

Table 1: Incorporation of labelled folate into rat liver

Folate ^{3}H or ^{14}C (0.01 μmole IP)	% Radioactivity in liver. Control (air)	N_2O	Control/N_2O %	% liver radioactivity as polyglutamate. Control (air)	N_2O
H_4PteGlu	3.8	0.7			
	3.3	0.5	21	55	0
	4.0	1.1			
	m.3.7	m.0.8			
5-CH_3-H_4PteGlu	6.0	1.2			
	4.3	1.5	38	42	0
	2.5	2.3			
	m.4.3	m.1.7			
10-CHO-H_4PteGlu	6.8	5.2			
	6.2	4.1	69	52	46
	7.2	4.6			
	m.6.7	m.4.6			
5,10-CH-H_4PteGlu	5.3	4.1			
	5.3	3.7	69	55	59
	4.3	2.8			
	m.5.0	m.3.5			
5-CHO-H_4PteGlu	5.7	3.2			
	4.4	2.9	56	52	49
	5.9	2.7			
	4.7	4.0			
	5.8	2.7			
	5.3	2.3			
	m.5.3	m.3.0			

RESULTS

Exposure to N_2O reduced the uptake of folate by rat liver [Table 1] to 21% of the control valve with H_4PteGlu, 38% with 5-CH_3-H_4PteGlu, 56% with 5-CHO-H_4PteGlu and 69% with both 10-CHO-H_4PteGlu and 5,10-CH-H_4PteGlu.

All the preparations that contained labelled folate polyglutamate also contained peaks corresponding to CHO-H_4PteGlu. In particular in figure 2 the control liver showed a marked formylfolate peak while the N_2O-treated animal showed none. With 5-CH_3-H_4PteGlu there is a small formylfolate peak on the ascending arm of the methylfolate peak

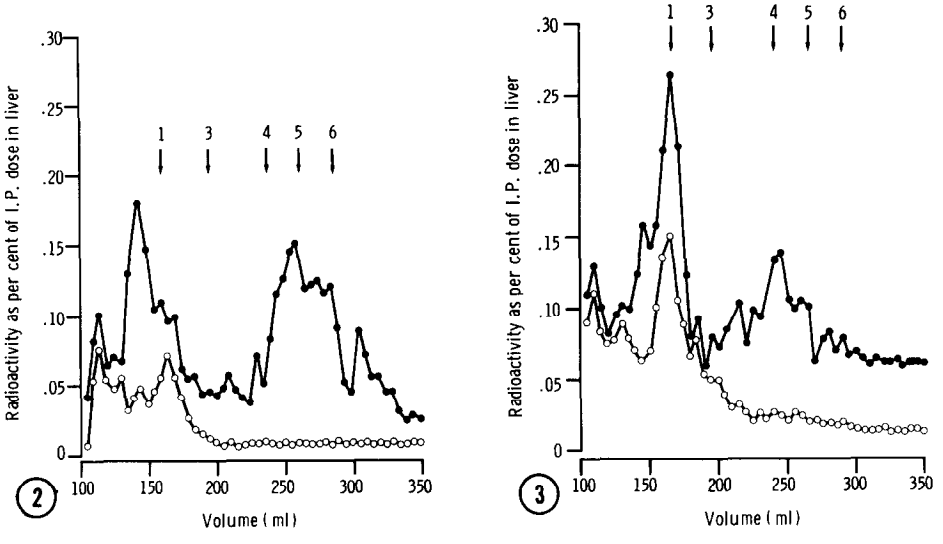

Figure 2. Separation of liver folates on DEAE cellulose after an intraperitoneal dose of 0.01μmol [2-^{14}C]H_4PteGlu. Animal (●—●) breathed air and animal (○—○) 50% N_2O/oxygen. The numbers above the arrows indicate the number of glutamic acid residues in folates eluting in the marked fractions. The liver of the animal breathing air shows a large peak labelled folate at 140ml (formylH_4PteGlu) and further peaks of folate polyglutamate eluting after the 200ml fractions. These 2 features are absent from the liver fractions of the N_2O-breathing animal.

Figure 3. Separation of liver folates on DEAE cellulose after an intraperitoneal dose of 0.01μmol. [G-^{3}H]5-CH_3-H_4PteGlu. Animal (●—●) breathed air and animal (○—○) 50% N_2O/oxygen. The numbers above the arrows indicate the number of glutamic acid residues in folates eluting in marked fractions. The liver of the animal breathing air shows 5-CH_3-H_4PteGlu eluting at 165ml with formylH_4PteGlu overlapping the ascending limb of this peak and further peaks of folate- polyglutamate eluting after the 200ml fractions. There is no formylfolate or folatepolyglutamate activity in the liver fractions from the N_2O-treated animal.

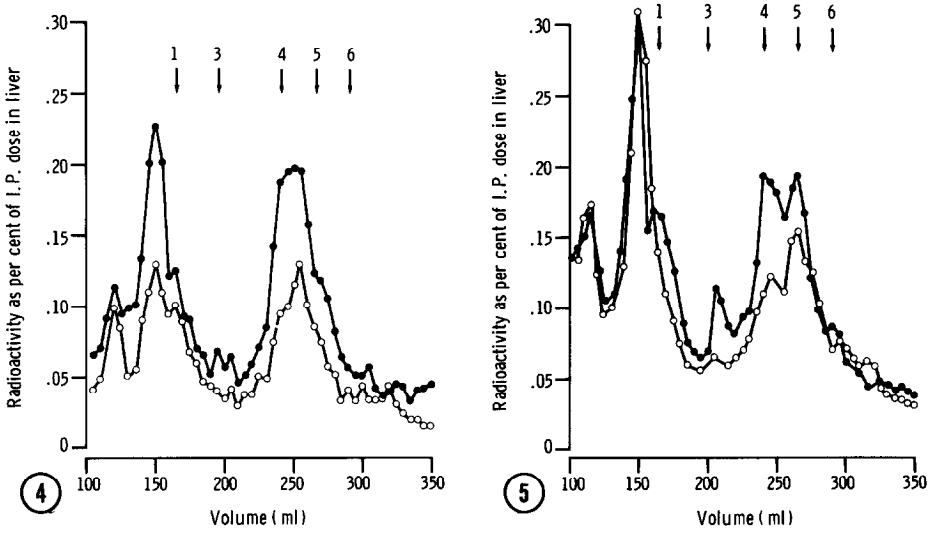

Figure 4. Separation of liver folates on DEAE cellulose after an intraperitoneal dose of 0.01μmol [G-^{3}H]5-CHO-H_4PteGlu. Animal (●—●) breathed air and animal (○—○) 50% N_2O/oxygen. The numbers above the arrows indicate the number of glutamic acid residues in folates eluting in the marked fractions. The livers of both animals show a large formylH_4PteGlu peak and substantial synthesis of folatepolyglutamates.

Figure 5. Separation of liver folates on DEAE cellulose after an intraperitoneal dose of 0.01μmol [G-^{3}H]10-CHO-H_4PteGlu. Animal (●—●) breathed air and animal (○—○) 50% N_2O/oxygen. The numbers above the arrows indicate the number of glutamic acid residues in folates eluting in the marked fractions. The livers of both animals show a large formylH_4PteGlu peak and substantial synthesis of folatepolyglutamates.

(Fig.3). In the studies shown in Figs. 4 and 5 both N_2O-treated and control preparations show CHO-H_4PteGlu at 150 ml.

Separation of folates on the basis of glutamic acid chain length showed that no polyglutamate was formed from either H_4PteGlu or from 5-CH_3-H_4PteGlu in the N_2O-treated animal [Table 1 and Fig.2 and 3]. With 5-CHO-, 10-CHO- and 5,10-CH-H_4PteGlu as substrates, the proportion converted into polyglutamate was the same as in the control animals [Table 1 and Figs. 4 and 5].

In vitro gassing of a solution of H_4PteGlu with 100% N_2O, both in the presence and absence of hydroxocobalamin produced no spectral changes in H_4PteGlu suggesting that N_2O or its products did not have any direct effect on the folate analogues.

DISCUSSION

Rats exposed to N_2O failed to form detectable amounts of folate polyglutamate from both H_4PteGlu and 5-CH_3-H_4PteGlu although the expected amounts were formed by rats left in air. The failure to utilize methylfolate can be attributed to loss of methionine synthetase activity and hence 'trapping' of folate in the methyl form, but this will not explain the failure to utilize H_4PteGlu for polyglutamate synthesis.

On the other hand when H_4folate with a substituent at the oxidation level of formate was given, the N_2O-treated rats converted the same proportion of folate into polyglutamate as did controls. The data suggest, firstly, that there is no impairment in folate polyglutamate ligase or synthetase in the N_2O-treated rat, secondly, that formyl-H_4folate may be the substrate for the action of this ligase and, thirdly, that vitamin B_{12} may be concerned, directly or indirectly, with the provision of formylfolate.

Some support for this interpretation is seen in Figure 2 which shows data following a dose of H_4PteGlu. The control animal which made polyglutamate had a substantial peak of radioactivity in the elution position of CHO-H_4PteGlu, whereas this was virtually lacking in the N_2O-treated rat which failed to make polyglutamate.

A recent study on the incorporation of [^{14}C]formate into serine by lymphocytes showed an abnormal result in all of 16 pernicious anaemia patients [21]. The result remained abnormal in pernicious anaemia patients treated with folic acid but became normal promptly after an injection of vitamin B_{12}.

Unlike micro-organisms there appears to be a single multifunctional enzyme in mammals concerned with formyl-methenyl-methylene interconversion [22,23] but hitherto a role for B_{12} in this system has not been identified.

References

1. Jeejeebhoy, K.N., Pathare, S.M. and Noronha, J.M. (1965) Blood, 26, 354-359.
2. Chanarin, I. Perry, J. and Lumb, M. (1974) Lancet, i, 1257-1252.
3. Scott, J.M., O'Broin, J.D. and Weir, D.G. (1974) Lancet, i, 906.
4. Smith, R.M. and Osborne-White, W.S. (1973) Biochem. J. 136, 279-293.
5. Kutzbach, C.A., Galloway, E. and Stokstad, E.L.R. (1967) Proc. Soc. Exp. Biol. Med. 124, 801-805.
6. Herbert, V. and Zalusky, R. (1962) J.Clin.Invest. 41, 1263-1276.
7. Noronha, J.M. and Silverman, M. (1962) in 2nd Eur. Symp. Vitamin B_{12} Intrinsic factor (Heinrich, H.C., ed.) pp728-736. (F. Enke, Stuttgart).
8. Lavoie, A., Tripp, E. and Hoffbrand, A.V. (1974) Clin.Sci.Mol.Med. 47, 617-630.
9. Blackburn, R., Kyaw, M. and Swallow, A.J. (1977) J.Chem.Soc.Farad. Trans. 73, 250-255.
10. Pratt, J.M. (1972) Inorganic Chemistry of Vitamin B_{12}, p.204 (Academic Press, London).
11. Lassen, H.C.A., Henricksen, E., Neukirch, F. and Kristensen, H.S. (1956) Lancet, i, 527-530.
12. Amess, J.A.L., Burman, J.F., Rees, G.M., Naucekievill, D.G. and Mollin, D.L. (1978) Lancet, ii, 339-342.
13. Layzer, R.B., Fishman, R.A. and Schafer, J.A. (1978) Neurology 28, 504-506.
14. McGing, P., Reed, B., Weir, D.G. and Scott, J.M. (1978) Biochem. Biophys. Res. Commun. 82, 540-546.
15. Deacon, R., Lumb, M., Perry, J., Chanarin, I., Minty, B., Halsey, M.J. and Nunn, J.F. (1978) Lancet ii, 1023-1024.
16. Davis, L. (1968) Anal. Biochem. 26, 459-460.
17. Perry, J. and Chanarin, I. (1973) Brit. Med. J. ii, 588-589.
18. Rabinowitz, J.C. (1963) Methods in Enzymology. (Colowick, S.P. and Kaplan, N.O. ed.), vol. 6, pp. 814-815. (Academic Press, New York).
19. Rowe, P.B. (1968) Anal. Biochem. 22, 166-168.
20. Perry, J., Lumb, M., van der Westhuyzen, J., Fernandes-Costa, F., Metz, J. and Chanarin, I. (1979) Biochim. Biophys. Acta. 586, 632-636.
21. Tikerpae, J. and Chanarin, I. (1978) Brit. J. Haematol. 38, 353-358.
22. MacKenzie, R.E. (1973) Biochem. Biophys. Res. Commun. 53, 1088-1095.
23. Paukert, J.L., Straus, L.D. and Rabinowitz, J.C. (1976) J.Biol. Chem. 251, 5104-5111.