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EVIDENCE THAT THE DECREASED LIVER FOLATE STATUS FOLLOWING VITAMIN B-12 INACTIVATION IN THE MOUSE IS DUE TO INCREASED LOSS RATHER THAN IMPAIRED UPTAKE

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Summary

Using N_2O to inactivate vitamin B-12, mouse liver polyglutamate distribution has been examined at short time intervals after injection of [^{3}H]pteroyglutamate. An increased monoglutamate pool is found initially which is lost by 24 h. This finding strongly supports the suggestion that the reduction in total liver folate in vitamin B_{12} deficiency is due to increased loss rather than decreased uptake.

The biochemical effects on folate metabolism of dietary vitamin B-12 deficiency are now well recognized, a reduction in liver folate, an altered ratio of the forms of the co-factor present [1,2] and an elevated excretion of formiminoglutamic acid [3] being the major ones. Until recently the proper investigation of the underlying biochemical factors causing these effects was hampered by the difficulties of producing total vitamin B-12 deficiency in experimental animals by dietary means. The occurrence of transient megaloblastic changes in patients receiving N_2O [4] suggested to us that the use of N_2O might represent a means of providing a model vitamin B-12 deficient state in experimental animals by specific inactivation of methylcobalamin-dependent methionine synthetase (EC 2.1.1.13). Banks et al. [5] had previously reported that N_2O oxidises the active form of vitamin B-12 ($B\text{-}12_s$) to the inactive form ($B\text{-}12_r$) and an in vitro study using N_2O to inhibit vitamin B-12-dependent ethanolamine ammonia lyase had been reported [6]. The finding of altered folate metabolism consistent with vitamin B-12 deficiency [7] was quickly followed by reports on

abnormal deoxyuridine suppression in rat bone-marrow [8,9] and neurological changes in monkeys [10,11], thus verifying the effectiveness of the method. These findings have been confirmed and extended by other groups [12—15].

While decreased liver folate has been consistently observed in vitamin B-12 deficiency produced by dietary means or by N_2O, opinion differs as to how this comes about. One theory is that it is caused by a restriction on transport of folate into the liver [16,17]. A second and more widely held view is that 5-methyltetrahydrofolate cannot itself be utilised for polyglutamate biosynthesis and that its conversion to tetrahydrofolate, the form that can be utilised, is blocked in vitamin B-12 deficiency. This inability to convert recently transported folate into the polyglutamate form might then lead to an inability of the cell to retain it [18]. A third theory to explain decreased liver folate in vitamin B-12 deficiency has recently been promulgated [19]. This suggests that by decreasing the supply of formate via methionine biosynthesis vitamin B-12 deficiency leads to a decrease in formyl folate derivatives, which it is suggested are the only substrates for polyglutamate biosynthesis. It would be expected that if the transport theory is the correct explanation vitamin B-12 deficiency would lead to a decreased folate uptake, while if either of the other two situations pertained, folate loss would be the cause of the reduced liver folate.

Although some early investigations on vitamin B-12 deficiency in man [20, 21] reported an altered liver folate polyglutamate status, the accuracy of the methods used and the reproducibility of the results have ben disputed [22]. Subsequently Perry and Chanarin [23], using similar techniques, reported an increased pool of 'free' folate (mono- to tri-glutamates) in a few vitamin B-12 deficient patients. These findings might suggest that folate loss rather than decreased uptake was more important in explaining the decreased folate status of liver, red blood cells and other tissues associated with lack of vitamin B-12.

Due to its rapid diffusion [24], together with its rapid and selective oxidation of vitamin $B\text{-}12_s$ [5] it may be assumed that administration of N_2O is followed extremely quickly by inactivation of vitamin B-12. In support of this it has been shown [12,25] that methionine synthetase activity in rat liver declines within 30 min of exposure to N_2O and is undetectable after 6 h. Thus nitrous oxide allows, for the first time, an investigation of the short term effects of vitamin B-12 deficiency. To date all investigations have examined folate incorporation only after 24 h or longer. We feel that if excess monoglutamate is being lost from the cells most of the excess would be lost by 24 h. The object of this study was to examine the liver monoglutamate status of N_2O-treated mice at shorter time intervals to determine if an increased monoglutamate pool can be detected.

The radiochemical pteroyglutamate, potassium salt, labelled in the 3', 5', 7, 9 positions (46 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks. To induce functional vitamin B-12 deficiency animals were placed in sealed chambers through which N_2O-O_2 (50 : 50, v/v) was flowing at a flow rate of 0.5 l/min [7]. Adult Laca random bred white mice (approx. 30 g) were injected intraperitoneally with 5 μCi [^{3}H]pteroyglutamate. One group of animals was placed in N_2O-O_2 (50 : 50, v/v) for 30 min before injection and returned to that environment until they were killed. The control mice were left in air for the same period. Food and water were supplied ad. lib. to both groups.

Animals were killed by cranial fracture and their livers removed immediately onto a chilled surface and weighed. Folates were extracted by the method of Scott and Reed [26] and a sample taken for radioactive counting. Tritium was measured as described previously [27]. Total counts incorporated were determined and significance was calculated using Student's *t*-test. Following extraction, samples from three or five mice were pooled and analysed for polyglutamates by the method of Reed and Scott [28] using Whatman DEAE-cellulose DE-52. Monoglutamate position was determined by addition to the column sample of authentic *para*-aminobenzoylglutamate. The monoglutamate pool size was calculated as a percentage of the total folate and significance was calculated.

At 24 h the total labelled liver folate of the vitamin B-12-inactivated mice was significantly less than that of the controls (Table I). However, although some livers from vitamin B-12-inactivated mice did have a larger than normal monoglutamate pool, overall there was no significant difference compared to control pools. The investigations at the shorter time intervals of 4, 6 and18 h showed that the same significant decrease in the total liver folate was accompanied by a significant increase in the proportion of liver folate in a monoglutamate pool. In addition, while total folate increases ($P < 0.05$) and the monoglutamate pool decreased ($P < 0.05$) in the controls between 4 and 6 h no significant differences were seen in the vitamin B-12-inactivated rats implying a difference in the metabolism of the respective pools.

The 'decreased uptake' theory is based on reports [16,17] of impaired up-

TABLE I

EFFECT OF VITAMIN B-12 INACTIVATION ON MOUSE LIVER INCORPORATION OF INJECTED [^{3}H]PTEROYLGLUTAMIC ACID

The figures represent mean values with their standard errors, the number of animals used is given in parenthesis. For incorporation into the monoglutamate pool, numbers in parenthesis represent the number of experiments for which livers from three or five mice were pooled. Time is the exposure time after injection. The controls are maintained in air from injection to time of death, otherwise animals were maintained in $N_2O : O_2$ (50 : 50, v/v) from injection to time of death. The significance values were calculated using the Student's *t*-test.

Total incorporation (μCi/g)

Time (h)	Controls	vitamin B-12-inactivated	Significance
4	0.16 ± 0.01 (12)	0.12 ± 0.01 (12)	$P < 0.02$
6	0.20 ± 0.01 (20)	0.10 ± 0.01 (16)	$P < 0.01$
18	0.29 ± 0.03 (19)	0.21 ± 0.02 (20)	$P < 0.02$
24	0.32 ± 0.03 (32)	0.23 ± 0.02 (25)	$P < 0.05$

Incorporation into monoglutamate pool (% of total folate)

Time (h)	Controls	Vitamin B-12-inactivated	Significance
4	29.2 ± 3.1 (4)	41.0 ± 4.0 (4)	$P < 0.05$
6	19.0 ± 1.8 (4)	39.0 ± 6.3 (4)	$P < 0.05$
18	5.5 ± 0.7 (4)	13.4 ± 2.3 (4)	$P < 0.02$
24	7.8 ± 2.1 (5)	10.7 ± 4.8 (5)	n.s.

take of 5-methyltetrahydropteroylglutamate by lymphocytes and bone marrow cells respectively from vitamin B-12 deficient patients. However, more recently Horne and Briggs [14] reported no impairment of the membrane transport of 5-methyltetrahydropteroylglutamate by isolated hepatocytes prepared from rats deficient in vitamin B-12 and methionine and from N_2O-treated rats compared to controls. We conclude that existing evidence does not support the concept of impaired folate transport in vitamin B-12 deficiency.

The hypothesis that reduced liver folate incorporation is due to decreased polyglutamate biosynthesis arising from non-availability of the correct substrate for such synthesis, whether this is tetrahydrofolate [18] or formyltetrahydrofolate [19], would be supported by this study (Table I). As one might expect with this hypothesis an increased monoglutamate pool appears to occur if sufficiently short time intervals are used. After longer time periods the radioactive monoglutamate pool is lost and consequently has not been detected in other studies.

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References

1 Herbert, V. and Zalusky, R. (1962) J. Clin. Invest. 41, 1263—1276
2 Kutzbach, C., Galloway, C. and Stokstad, E.L.R. (1967) Proc. Soc. Exp. Biol. Med. 124, 801—805
3 Silverman, M. and Pitney, A.J. (1958) J. Biol. Chem. 233, 1179—1182
4 Amess, J.A.L., Burman, J.F., Greary, M. and Naneckievill, D.G. (1977) Internal. Soc. Haematol., Istanbul, p. 614.
5 Banks, R.G.S., Henderson, R.J. and Pratt, J.M. (1967) Chem. Commun. 387—388
6 Schrauzer, G.N. and Stadlbauer, E.A. (1975) Bioinorg. Chem. 4, 185—198
7 McGing, P., Reed, B., Weir, D.G. and Scott, J.M. (1978) Biochem. Biophys. Res. Commun. 82, 540—546
8 Scott, J.M., Reed, B., McKenna, B., McGing, P., McCann, S., O'Sullivan, H., Wilson, P. and Weir, D.G. (1979) in Proceedings of the 6th International Symposium on the Chemistry and Biology of Pteridines, 1978, (Kisliuk, R.L. and Brown, G.M., eds.), pp. 335—340, Elsevier/North-Holland, New York
9 McKenna, B., Weir, D.G. and Scott, J.M. (1980) Biochim. Biophys. Acta 628, 314—321
10 Dinn, J.J., McCann, S., Wilson, P., Reed, B., Weir, D. and Scott, J. (1978) Lancet ii, 1154
11 Reed, B., Dinn, J., McCann, S., Wilson, P., O'Sullivan, H., Weir, D.G. and Scott, J.M. (1979) in Vitamin B-12. Proceedings of the 3rd European Symposium on Vitamin B-12 and Intrinsic Factor, 1979, (Zagalach, B. and Friedrich, W., eds.), pp. 1061—1064, Walter de Gruyter and Co., Berlin
12 Deacon, R., Lumb, M., Perry, J., Chanarin, I., Minty, B., Halsey, M.J. and Nunn, J.F. (1978) Lancet ii, 1023—1024
13 Linnell, J.C., Quadros, E.V., Matthews, D.M., Jackson, B. and Hoffbrand, A.V. (1978) Lancet ii, 1372
14 Horne, D.W. and Briggs, W.T. (1980) J. Nutr. 110, 223—230
15 Deacon, R., Lumb, M., Perry, J., Chanarin, I., Minty, B., Halsey, M. and Nunn, J. (1980) Eur. J. Biochem. 104, 419—422
16 Das, K.C. and Hoffbrand, A.V. (1970) Brit. J. Haematol. 19, 203—221
17 Tisman, G. and Herbert, V. (1973) Blood 41, 465—469
18 Lavoie, A., Tripp, E. and Hoffbrand, A.V. (1974) Clin. Sci. Mol. Med. 47, 617—630
19 Chanarin, I., Deacon, R., Lumb, M. and Perry, J. (1980) Lancet ii, 505—508
20 Jeejeebhoy, K.N., Pathare, S.M. and Noronha, J.M. (1965) Blood 26, 354—359
21 Chanarin, I., Perry, J. and Lumb, M. (1974) Lancet ii, 906
22 Scott, J.M., O'Broin, J.D. and Weir, D.G. (1974) Lancet ii, 906
23 Perry, J. and Chanarin, I. (1977) Brit. J. Haematol. 35, 397—402
24 Cullen, B.F. and Eger, E.I. (1972) Anesthesiology 36, 168—173
25 Lumb, M., Deacon, R., Perry, J., Chanarin, I., Minty, B., Halsey, M.J. and Nunn, J.F. (1980) Biochem. J. 186, 933—936
26 Scott, J.M. and Reed, B. (1980) in Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.), Vol. 66, pp. 638—641, Academic Press, London
27 McGing, P.G. and Scott, J.M. (1980) Br. J. Nutr. 43, 235—237
28 Reed, B. and Scott, J.M. (1980) in Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.), Vol. 66, pp. 501—507, Academic Press, London