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Reduction of folate levels in the rat: Difference in depletion between the central and the peripheral nervous system

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With 1 table

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The morphological identity of the megaloblastic anaemia of folate[1]) deficiency with that of vitamin B_{12} deficiency has led to speculation that there also exists a neuropathy caused by folate deficiency, similar to the well-known vitamin B_{12} deficiency neuropathy.

Several reports have appeared indicating a causal relation between folate deficiency and neurological disease (references, see 1). Folate deficiency is rather common in man (2). Poor nutrition, pregnancy, intestinal disease, medication, or alcoholism, occurring alone or in combinations are the usual causes. Some of these conditions can cause neuropathy by means other than a folate deficiency; therefore a simple folate-deficiency neuropathy is hard to prove in man.

The object of the present work was to induce folate depletion in rats in order to find signs of dysfunction of the central or the peripheral nervous system. The gross distribution of some folate forms was determined in the nervous system.

Materials and methods

Preliminary series

Eight male *Wistar* rats, weight approximately 120 g, were kept in individual cages and were given folate-deficient powdered food[2]). One per cent succinyl-

[1]) In this paper the term "folate" is used for all folate forms, oxidized as well as reduced, with or without methyl-, formyl-, or metyhlene groups, regardless of the number of glutamic acid residues.

[2]) *Basic composition* (g/kg): gelatin 80, glucose 458, corn oil 20, hydrogenated vegetable oil 188, vitamin-free casein 200. *Minerals* (g/kg): $CaCO_3$ 9.96, $Ca_3(PO_4)_2$ 28.28, $CuSO_4$ 0.01, $FeC_6H_5O_7$ 0.20, $MgSO_4$ 3.0, $MnSO_4$ 0.25, KCl 6.96, KI 0.01, NaCl 3.96, Na_2HPO_4 6.96, $ZnCO_3$ 0.13. *Vitamins* (mg/kg): retinol 2.0, ergocalciferol 0.1, alpha tocopherol 2.2, ascorbic acid 19.9, menaphthone 1.0, thiamin 0.4, riboflavin 0.4, nicotinic acid 2.0, pyridoxine 0.4, calcium pantothenate 1.3, choline 33.1, p-aminobenzoic acid 2.2, biotin 0.009, cyanocobalamin 0.0006.

sulfathiazol was added to the food 3 days a week to suppress the growth of folate-producing intestinal bacteria. Four of the rats received pteroylmonoglutamic acid in addition, 10 mg/kg diet.

Definite series

Twenty-four male *Wistar* rats, approximate weight 130 g, were randomly assigned to six cages, four in each. They were given free access to folate-deficient food[2]). Water was administered in black-painted bottles. A mixture of the vitamins of the B group was added to the water in the following concentrations (mg/l): Thiamin 4.2, riboflavin 2.1, nicotinic acid 2.1, pyridoxine 2.1, calcium pantothenate 4.2. Twelve of the rats also received 16.6 mg/l of water of pteroylmonoglutamic acid; they constituted the control group of the definite series.

Folate assay

Whole blood from the tip of the tail was haemolyzed in water and assayed with *Lactobacillus casei* (A.T.C.C. 7469) (3). The tissues were assayed both with *L. casei* and with *Streptococcus faecalis* (A.T.C.C. 8043) before and after treatment with lyophilized rat pancreatic juice, containing gammaglutamyl-carboxypeptidase (4). The difference between the *L. casei* and the *S. faecalis* values after the enzymatic treatment was taken to represent the amount of 5-methyltetrahydrofolate (5-CH_3-H_4 PteGlu) present. *L. casei* is stimulated quantitatively by pteroylmono-, di-, and triglutamate, somewthat less by tetraglutamic-folate and only slightly by pentaglutamic folates (5), which is in agreement with our experience (*Jägerstad* and *Westesson*, unpublished observations).

Neurological tests

Besides noting the gait and the general appearance of the animals, we carried out the following tests:

1. The rats were taken by the tail and somersaulted in the air: The way they came down was rated on a 4-point scale, 0 reflecting normal behaviour and 3 severely impaired righting reflexes.
2. The rats were placed on a horizontal steel rod. The way the animals clung to the rod and their ability to stay on it when it was briskly moved was rated on the same 4-point scale.
3. The rats were put on a steel platform which was tilted 90 degrees. The inclination at which the animal started to slide down was recorded.
4. A needle was pressed against the dorsal surface of the proximal phalange of the small toe and the lowest pressure at which the animal showed signs of discomfort was recorded.

All the tests were repeated several times on each occasion before the performance was assessed. The validity of the first three tests in measuring neurological dysfunction has been demonstrated in experimental methylmercury poisoning (6).

Measurement of motor nerve conduction velocities (MCV)

These were measured in both sciatic nerves in the animals of the preliminary series and on both sides of the tail in the definite series. Light anaesthesia was induced with a mixture of Halothane, nitrous oxide and oxygen. The MCV of the sciatic nerves were measured according to *Fullerton* and *Barnes* (7), but the temperature was not controlled. The MCV of the tails were measured according to *Miyoshi* and *Goto* (8). The temperature was measured with a needle probe inserted midway between the proximal and the distal electrodes.

Preparation of tissues and extracts

The animals were anaesthetized as described above. The sciatic nerve and the whole spinal cord were removed. The animals died when the thoracic medulla

was dissected. Then the brain, except the olfactory bulbs, was quickly removed and divided at the collicular level. Finally, the liver was removed. All tissues were frozen immediately in liquid nitrogen and transferred to separate small plastic bags which were sealed and kept in the dark at −17° C until analysis, which was done individually for all specimens, except the spinal cords and the sciatic nerves which were pooled. Extraction of folate was performed by homogenization in 1% ascorbic acid (pH 6.0) and the suspension was autoclaved at 118° C for 10 min. The supernatant, after centrifugation, was used for the microbiological assay directly and after enzymatic hydrolysis of the conjugated folates as follows: 1 ml of the supernatant was incubated with 0.25 mg of lyophilized rat pancreatic juice in a water bath at 37° C and shaken for 2 h. The hydrolysis was stopped by boiling the test tubes for 10 min.

Statistics

Student's t-test was used for the comparison between the folate values, and between the MCV in the deficient rats and the control rats in the definite series. Level of significance was chosen as 0.05.

Results

Preliminary series

After 2 months, the blood folate levels of the rats receiving the deficient diet had fallen to about 70 μg/l, whereas the blood folate of the controls was about 300 μg/l. During the next 4 months, the rats gained weight normally. Thereafter, all the rats began to have loose stools intermittently, and their weights decreased slowly. The blood folate concentration decreased in both groups of animals, and at the time of the determination of the MCV (12 months after the start of the experiment), it was about 120 μg/l in the controls and 21 μg/l in the deficient group. The weights of the controls ranged from 300 to 340 g; those of the deficient group from 220 to 270 g. Three animals died. Thus, the MCV were measured in three control rats (six sciatic nerves) and in two on the folate-deficient diet. It ranged from 42 to 53.5 m/s in the former group and from 38 to 40.5 m/s in the latter. The neurological tests were repeated several times during the 12 months. The results were normal on all occasions, no difference being found between the two groups.

Definite series

Losses: One animal from each group was lost by accident after 7 months. One non-supplemented rat died unexpectedly after 8 months, leaving eleven supplemented and ten non-supplemented rats for the final experiments.

Weights: The weights increased steadily during the 9.5 months of the experiment and ranged from 350 to 480 g at the time of sacrifice. There was no difference between the groups.

Neurological tests: There was no difference between the results of the two groups at any time.

Measurement of motor nerve conduction velocities (MCV). The mean MCV at 37° C in the non-supplemented group was 44.3 ± 3.4 (1 S.D.) m/s; in the control group, it was 44.5 ± 4.8 (1 S.D.) m/s. The MCV of each rat was corrected for tail temperature (8), which varied between 35° and 37.2° C at the time of measurement.

Blood folate concentrations (μg/l): These ranged from 243 to 452 at the start of the experiment with a mean of 350. After 14 weeks the mean value was 258 in the controls and 126 in the experimental group. The final analysis 4 weeks before sacrifice showed a mean blood folate concentration of 225 in the controls and 86 in the experimental group. There was no overlapping between the groups at any time after the experiment started.

Folate concentrations in other tissues (μg/g): Table 1 shows the salient results of the assays. L.C. + RPJ means analysis of extracts with *L. casei* after treatment with rat pancreatic juice, L.C. means analysis without that pretreatment. The difference between a and b is a rough estimate of the amount of pteroylpentaglutamate and pteroylpolyglutamates of higher order present in the extract.

The folate concentration in the livers of the supplemented animals was 15.9 while in the non-supplemented group it was 8.0. The brain folate levels were 16% lower in the latter group than in the former ($p < 0.001$). The difference between the spinal cords of the two groups was of the same order of magnitude as the difference between the brains. The decrease struck polyglutamates and 5-CH_3-H_4PteGlu to approximately the same extent. The folate concentration in the pooled sciatic nerves was 59% lower in the non-supplemented group. The proportion of

Tab. 1. Mean folate concentrations in tissues from rats on a folate-deficient diet with and without folic acid supplementation (values are given as μg/g tissue $\pm$ SEM n = number of animals)

Tissue	With supplementation n = 11	Without supplementation n = 10
Liver		
a) L.C.*) with RPJ**)	15.9 $\pm$ 1.0	8.0 $\pm$ 0.5
b) L.C.*) without RPJ**)	15.9 $\pm$ 0.8	7.7 $\pm$ 0.4
c) 5-CH_3-H_4PteGlu***)	11.4 $\pm$ 0.9	5.5 $\pm$ 0.4
Brain		
a) L.C.*) with RPJ**)	0.59 $\pm$ 0.01	0.49 $\pm$ 0.02
b) L.C.*) without RPJ**)	0.52 $\pm$ 0.01	0.46 $\pm$ 0.01
c) 5-CH_3-H_4PteGlu***)	0.31 $\pm$ 0.02	0.23 $\pm$ 0.02
Spinal cords (pooled)		
a) L.C.*) with RPJ**)	0.27	0.22
b) L.C.*) without RPJ**)	0.27	0.22
c) 5-CH_3-H_4PteGlu***)	0.15	0.13
Sciatic nerves (pooled)		
a) L.C.*) with RPJ**)	0.29	0.12
b) L.C.*) without RPJ**)	0.17	0.08
c) 5-CH_3-H_4PteGlu***)	0	0

*) Lactobacillus casei
**) Rat Pancreatic Juice
***) 5-methyltetrahydrofolate

polyglutamates seemed to be small in the brains and the cords but in the sciatic nerves it was about one third of the total folate. In this tissue no $5\text{-}CH_3\text{-}H_4PteGlu$ was found in contrast to all other examined tissues.

Discussion

Folate depletion in the preliminary series was more conspicuous than in the definite one. The animals' condition was worse and they had loose stools during the final months. They only received the standard amount of vitamins contained in the commercial diet and no "extra" supplement. Therefore, some of the difference in MCV between the groups may have been due to deficiency of vitamins other than folic acid which could have been more pronounced in the non-supplemented group. Thus it cannot be definitely stated that folate depletion was the cause of the slower MCV in this group of the preliminary series. This made the experiment with the definite series necessary.

In the definite series the control animals received comparatively large amounts of pteroylglutamic acid. It could be argued that the difference in the folate levels between the groups was due to an abnormally high content of folate in the supplemented group, and not to a relative folate depletion in the experimental group. However, there was a decline of the blood folate with time in both groups which was most marked in the experimental group. Even if it is accepted that blood folate levels normally fall with age in the rat – which is in accordance with our experience – it is hard to believe that the reduction to 25% of the initial value would be physiological. Furthermore, in other rats of the same age and the same strain, fed a standard rat diet (*Astra-Ewos*, Södertälje, Sweden) we have found the blood folate levels to lie between 180 and 270 μg/ml, being influenced very little by folate supplementation of the same magnitude as in the present experiment. Thus, the blood folate of the non-supplemented animals was depleted to 40% or less of the control value during the main part of the experiment. The latter value was not high.

The "extra" vitamin of the B-complex in the definite series was supplemented because of findings by others, indicating that thiamin-deficiency is induced in rats on a folate-deficient diet, and that there are intimate relations between folate, nicotinic acid, and riboflavin in the liver, deficiency of one vitamin leading to deficiency of the others (9, 10, 11). We have only measured folates, but we think that we have avoided secondary deficiency of the other vitamins by the extra supplement in the drinking water. This view is indirectly supported by the low death rate and the normal weight gain of the rats in the definite series.

Despite the long-standing reduction in blood-folate, brain folate was reduced by a mere 16%, spinal cord folate being reduced to a comparable degree. Small as it may seem, this reduction is none the less enough to induce changes in higher cerebral function, as reported by us elsewhere (12). *Allen* and *Klipstein* found no decrease in brain folate in moderately folate-deficient rats, but their range of normal values is remarkably wide (13). *Thomson* et al. did not find any difference in brain folate after 11 months between two groups of rats, one of which was on a folate-deficient

diet (11). Our results corroborate the earlier findings that the CNS is able to extract folate from the blood and retain most of it even if the blood levels are low.

Our figures of the absolute concentration of folate in the brain agree fairly well with those previously mentioned (11, 13). *Korevaar* et al. have published figures about the regional concentration of 5-CH_3-H_4PteGlu in the brain and the spinal cord of the rat in a preliminary report (14). The highest regional value in their study is only half of our value for 5-CH_3-H_4PteGlu in the whole brain. The difference is hard to explain without access to all details of their experiment. The difficulties in comparing the results of microbiological assays from one laboratory with those from another are well recognized.

Contrasting with the relatively unaffected folate levels of the CNS, the substantial reduction of peripheral nerve folate levels in the non-supplemented rats agrees with the reduction of blood folate levels. The peripheral nerves thus seem to lack the ability of the CNS to resist low systemic folate levels. None the less, no impairment of function was found, as judged by the neurophysiological measurements or the neurological tests. Possibly, a severe dficiency could have resulted in a difference between the groups, as indicated by the results in the preliminary series.

The proportion of folate polyglutamate found in the different tissues may not represent their true polyglutamate content. Both liver and brain contain folic acid conjugase (15). Freezing and thawing disrupt the cell membrane, and hydrolysis might start during the warming procedure (16). The true values are therefore probably higher than those calculated from the table. Because all tissues were treated alike, a difference in the estimated folate polyglutamate content may be due to a difference in folic acid conjugase activity. Table 1 indicates that a substantial part of the peripheral nerve folate is present as polyglutamate. This suggests that folate conjugase has a low activity in that tissue. The nerve is peculiar also in its lack of 5-CH_3-H_4PteGlu – the most common folic acid derivative in the serum. At present, no satisfactory explanations can be offered for these findings.

This investigation indicates that in systemic folate depletion folate seems to be retained in the CNS, whereas the peripheral nerves are depleted of it to the same extent as the blood. In this connexion it is interesting that human cerebrospinal fluid contains three times as much folate as serum, despite a much lower protein content. But preliminary studies in our laboratory show that such a gradient is not present between the cerebrospinal fluid and the serum in the rat. Nonetheless, a two-way folate "barrier" seems to exist between the CNS and the blood in the rat making a folate concentration gradient possible between these tissues (17). The presence of such a gradient does not exclude a continuous folate exchange over the "barrier".

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Summary

1. Rats were deprived of dietary folate for 12 and 9.5 months in two experiments; one with and one without succinylsulfathiazole added to the diet. Folate levels decreased faster in the former experiment and the final blood values were lower. Tissue folate was examined in the latter experiment only.

2. The animals were repeatedly examined with neurological tests and compared with a control group supplemented with folic acid. Every test proved normal. Measurement of motor nerve conduction velocities after 12 months in the experiment where succinylsulfathiazole was given revealed lower velocities in the non-supplemented group. There was no difference between the groups in the experiment without succinylsulfathiazole.

3. In the latter experiment brain folate was reduced by only 16% in the non-supplemented animals compared with the controls, whereas whole blood folate fell by 60%, liver folate by 50% and sciatic nerve folate by 59%.

4. The central nervous system is resistant to systemic folate depletion, whereas the peripheral nerves are depleted to the same degree as the extraneural tissues.

Zusammenfassung

1. In zwei Experimenten erhielten Ratten 12 und $9^1/_2$ Monate lang folsäurearme Nahrung, in dem einen mit und in dem anderen ohne Zusatz von Succinylsulfathiazol. Die Folsäureniveaus im Blut fielen im ersten Experiment schneller, und die Endwerte waren kleiner. Die Folsäure in den Geweben wurde nur im Experiment ohne Succinylsulfathiazol-Zusatz untersucht.

2. Die Tiere wurden wiederholt neurologisch untersucht und mit einer Kontrollgruppe verglichen, die einen Folsäure-Zusatz erhalten hatte. Unterschiede traten nicht auf. Bei der Bestimmung der Nervleitungsgeschwindigkeiten bei dem Experiment mit Succinylsulfathiazol wies die Gruppe, die keinen Folsäure-Zusatz erhalten hatte, geringere Geschwindigkeiten auf. Bei dem Experiment ohne Succinylsulfathiazol konnte zwischen den Gruppen kein Unterschied festgestellt werden.

3. Im letztgenannten Experiment war die Folsäure-Konzentration 16% geringer im Gehirn in der Gruppe ohne Folsäure-Zusatz, dagegen war die Konzentration im Blut 60%, in der Leber 50% und im Nervus ischiadicus 59% geringer als in der Kontrollgruppe.

4. Das zentrale Nervensystem ist gegen Folsäure-Entarmung resistent, dagegen wird das periphere Nervensystem im gleichen Grad entarmt wie anderes Gewebe.

References

1. *Fehling, C., M. Jägerstad, K. Lindstrand, D. Elmqvist,* Arch. Neurol. **30,** 263–265 (1974). – 2. *Hoffbrand, A. V., T. J. Peters,* Schweiz. Med. Wschr. **100,** 1954–1960 (1970). – 3. *Fehling, C., M. Jägerstad, K. Lindstrand, A.-K. Westesson,* Clin. Sci. *44,* 595–600 (1973). – 4. *Jägerstad, M., K. Lindstrand, A.-K. Westesson,* Scand. J. Gastroenterology **7,** 593–597 (1972). – 5. *Tamura, T., Y. Shin, M. Williams, E. Stokstad,* Analyt. Biochem. **49,** 517–521 (1972). – 6. *Fehling, C., M. Abdulla, A. Brun, M. Dictor, A. Schütz, S. Skerfving,* J. Toxicol. Appl. Pharmacol., in press (1975). – 7. *Fullerton, P. M., J. M. Barnes,* Brit. J. Industr. Med. **23,** 210–221 (1966). – 8. *Miyoshi, T., I Goto,* Electroenceph. Clin. Neurophysiol. **35,** 125–131 (1973). – 9. *Tamburro, C., O. Frank, A. D. Thomson, M. F. Sorrell, H. Baker,* Nutr. Rep. Internat. **4,** 185–189 (1971). – 10. *Thomson, A. D., H. Baker, C. M. Leevy,* Folate-Gastroenterology **60,** 756 (1971). – 11. *Thomson, A. D., O. Frank, B. De Angelis* and *H. Baker,* Nutr. Rep. Internat. **6,** 107–110 (1972). – 12. *Fehling, C., Z. Cesarec,* J. Internat. Res. Comm. **2,** 1498 (1974). – 13. *Allen, C. D.,*

F. A. Klipstein, Neurology (Minneap.) **4**, 403 (1970). – 14. *Korevaar, W. C., M. A. Geyer, S. Knapp, L. L. Hsu, A. J. Mandell*, Nature New Biol. **245**, 244–245 (1973). – 15. *Baugh, C. M., C. L. Krumdieck*, Lancet **1969/2**, 519–521 – 16. *Bird, O., V. McGlohon, J. Vatikus*, Analyt. Biochem. **12**, 18–35 (1965). – 17. *Hommes, O. R., E. A. M. T. Obbens, C. C. B. Wijffels*, J. Neurol. Sci. **19**, 63–71 (1973).

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