Effect of 3-Acetylpyridine on the Content of Myelin in the Rat Brain

Yoko Nakashima and Ryokuero Suzue

National Institute of Nutrition,
1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan
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Summary Ten-week old rats were fed an AIN-76 purified diet ad libitum and were given 3-acetylpyridine which is an antagonistic agent of nicotinic acid. The quantity and composition of myelin in the brain were analyzed after administration of 3-acetylpyridine for 40 days. Body weight, myelin yield, cerebroside level and specific activity of 2',3'-cyclic nucleotide-3'-phosphohydrolase in the brain decreased by administration of 3-acetylpyridine. Despite the decreased myelin yield, the proportion of protein and total lipid and the percentage composition of lipid in myelin did not change by administration of 3-acetylpyridine. Therefore, we have concluded that 3-acetylpyridine plays a significant role in the loss of myelin.

Key Words cerebroside, myelin, 3-acetylpyridine, CNP activity

Nicotinic acid has been demonstrated to be a preventive factor against pellagra, which is frequently accompanied by disturbances of the central nervous system, leading to dementia. Mental symptoms with nicotinic acid deficiency are various, depression, irritability and anxiety being the most common. In addition, administration of an antagonistic agent of nicotinic acid which is effective in animals causes a disease in animals characterized by many of the signs seen in nicotinic acid deficiency including mental deficiency (1, 2). This disease appeared very rapidly following administration of the drug. When a sufficient amount of nicotinic acid or nicotinamide was given, no sign of disease appeared.

Previous studies from our laboratory have shown that the content of myelin and cerebroside in the brain of rats fed a nicotinic acid-deficient diet was lower than in those on nicotinic acid-supplemented diet (3-6). Deposition of cerebroside in the brain of nicotinic acid-deficient rats was delayed due to the decline of biosynthesis of cerebroside which contains high levels of long chain fatty acids (4, 5). In these experiments, we used a nicotinic acid-free low-casein diet to which a small amount of both methionine and threonine was added and from which tryptophan was

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excluded (tryptophan-imbalanced diet) (7-10), because pyridine nucleotides are synthesized from tryptophan in the rat (11). Therefore, an inadequate amount of tryptophan in the tryptophan-imbalanced diet was considered to be implicated in the formation of myelin. In order to clarify only the influence of nicotinic acid, AIN-76 diet was used in this experiment. We measured the quantity and composition of myelin in the brain of rats fed an AIN-76 diet and successively given 3-acetylpyridine intraperitoneally, once a day.

**METHODS**

*Animals and diet.* The AIN-76 purified diet was prepared as described by Bieri (12, 13). The composition of the diet is shown in Table 1. Ten week-old male Sprague-Dawley rats weighing about 240 g were housed in individual wire cages. They were fed an AIN-76 purified diet *ad libitum* for 10 days prior to administration of 3-acetylpyridine. They were divided into three groups and were fed the same diet *ad libitum.* Group I was given 2 ml of 0.9% NaCl solution by intraperitoneal injection. Group II and Group III were given 3-acetylpyridine in an equal volume of saline solution for 40 days (Group II; 2.5 mg/100 g body weight/day and Group III; 5.0 mg/100 g body weight/day). They were weighed at least twice weekly during the experimental period.

| Ingredient       | %   |
|------------------|-----|
| Casein           | 20.0|
| pH-Methionine    | 0.3 |
| Cornstarch       | 65.0|
| Cellulose powder | 5.0 |
| Soybean oil      | 5.0 |
| Mineral mix.     | 3.5 |
| Vitamin mix.     | 1.0 |
| Choline bitartrate| 0.2 |

*Measurement of total lipid and cerebroside content in the brain.* After administration of 3-acetylpyridine for 40 days, animals were sacrificed by decapitation and the whole brains were removed. The brains were weighed and homogenized in 10 ml of ice-cold 0.32 M sucrose in a Potter-Elvehjem homogenizer. To 1.0 ml of the homogenate was added 19 volumes of chloroform–methanol 2:1 (v/v). The mixture was kept in a nitrogen atmosphere overnight at 4°C and then filtered. The filtrate was washed according to the method of Folch *et al.* (14). Total lipid was determined gravimetrically. Total lipid was fractionated by silicic acid (Wakogel Q-23, 100–200 mesh) column chromatography and purified by thin layer chromatography.
(TLC) on Silica-gel (Merk) as described by Norton and Poduslo (15). Bands corresponding to cerebroside standards were scraped from the plate, and cerebroside was determined as previously described (4).

**Preparation of myelin fraction.** The remaining homogenate of 0.32 M sucrose was used for myelin isolation. The procedure of myelin isolation was that of Norton (15) as described previously (5). For determination of dry weight, a myelin pellet was washed several times with distilled water and freeze-dried in a vacuum. The purity of myelin was checked for microsomal and mitochondrial contamination by marker-enzyme assay. NADP-cytochrome c reductase and succinate dehydrogenase were used as the microsomal and mitochondrial marker, respectively (16). The lyophilized myelin was weighed and analyzed for protein and lipid.

**Protein and lipid analysis.** Myelin protein was determined using the method of Lowry et al. (17). Lipid was extracted with 20 vol chloroform–methanol 2:1 (v/v) from the lyophilized myelin fraction in a nitrogen atmosphere using the method of Folch et al. (14). The lipid extract was taken to dryness under nitrogen at 60°C and placed in a vacuum desiccator at 4°C after lyophilization to measure the total lipid weight. The total lipid was fractionated by silicic acid column chromatography and purified by thin layer chromatography on Silica-gel (Merk) as described by Norton and Poduslo (15). The total lipid and purified lipid were analyzed for galactose (3), phosphorus (18) and cholesterol (19). The average molecular weight for mixed galactolipids was assumed to be 846, phospholipids 755, and cholesterol 387.

**Assay of 2',3'-cyclic nucleotide-3'-phosphohydrolase activity.** An aliquot of 0.32 M sucrose homogenates of the brain of rats which received 3-acetylpyridine and that of control rats were separately diluted with 50 mM of Tris-buffer (pH 7.4). These diluted homogenates were separately placed in a cellulose tube and dialyzed against the same buffer for 24 h. 2',3'-Cyclic nucleotide-3'-phosphohydrolase (CNP) activities of these dialysates were determined by the method of Drummond et al. (20). Protein of the dialysate and the homogenate of 0.32 M sucrose was assayed by the method of Lowry et al. (17).

**RESULTS**

**Effect of 3-acetylpyridine on body weight of rats**

After administration of 3-acetylpyridine (2.5 mg/100 g body weight/day) (Group II), the mean body weight of rats showed a slight decrease only during the first 10 days, but they grew satisfactorily after 10 days of administration of 3-acetylpyridine. Control rats showed a steady gain of body weight throughout the experimental period. Final body weights of rats which received 3-acetylpyridine for 40 days were significantly lower than controls (Table 2). When 5 mg of 3-acetylpyridine per 100 g body weight per day (Group III) was given, half of the animals of the group died within a few days.

In a few hours after administration of 3-acetylpyridine, the animals began to breathe quite rapidly. Within 2 days, almost complete paralysis of the hind legs
developed. At about the same time the rats appeared reddish and unkempt, and emaciation was usually prominent.

**Effect of 3-acetylpyridine on concentration of cerebrosides and total lipid in the brain**

The effects of 3-acetylpyridine on brain composition are described in Table 2. Brain weights of rats which received 3-acetylpyridine were much less than those of controls. The whole brain showed decreased myelin yields with 3-acetylpyridine treatment (Table 3). Whole brain content of cerebrosides and activity of CNP were assayed, since their levels have been shown to increase in parallel with myelination (21, 22). Cerebroside content in the brain of rats which received 3-acetylpyridine was markedly lower than that of control rats, but no significant difference in total lipid of rats which received 3-acetylpyridine was markedly lower than that of controls. The decrease in myelin and cerebroside content could not be accounted for by an increased water content (gross edema), because the protein concentrations of the whole brain were essentially unaltered (Table 2).

A separate experiment was performed to determine whether the reduced myelin yields by administration of 3-acetylpyridine was attributable to inadequate nutritional intake. Starved animals controlled for weight loss were not affected with regard to yield of myelin, concentration of cerebrosides and levels of CNP activity (yield of myelin: 46.9 ± 3 mg/brain, concentration of cerebrosides: 10.4 ± 0.5 mg/brain, levels of CNP activity: 3.4 ± 0.2 μmol product/mg protein/min). Therefore, inadequate nutritional intake which presumably accounts for the deficit in weight gain of 3-acetylpyridine-injected rats plays a significant role in loss of myelin.
Table 2. Effect of 3-acetylpyridine on concentration of total lipid, cerebroside and CNP activity in the brain of rats.

Group I (control group) was given 2 ml of 0.9% NaCl solution by intraperitoneal injection. Group II and Group III were given 3-acetylpyridine in an equal volume of saline solution for 40 days (Group II: 2.5 mg/100 g body weight/day and Group III: 5.0 mg/100 g body weight/day). The rats were sacrificed by decapitation and their brains were removed and homogenized in 10 ml of ice cold 0.32 M sucrose. Lipid in the brain homogenate was extracted according to the method of Folch et al. and assayed for total lipid and cerebroside as described under METHODS. CNP activities and protein concentration of the brain homogenates were determined as described in METHODS. All values represent mean ± SE.

| Group | Body weight (g) | Weight (g) | Protein (mg/g brain) | CNP (μmol product/mg prot./min) | Total lipid (mg/g brain) | Cerebroside (mg/g brain) | Cerebroside/Total lipid |
|-------|----------------|------------|----------------------|---------------------------------|-------------------------|-------------------------|--------------------------|
| I (6)* | 393 ± 10       | 2.2 ± 0.1  | 118 ± 4              | 3.6 ± 0.1                       | 112 ± 12                | 11.1 ± 0.1              | 0.099                    |
| II (7)*| 349 ± 21       | 1.9 ± 0.1  | 110 ± 8              | 2.8 ± 0.3                       | 103 ± 16                | 8.2 ± 1.0               | 0.080                    |
| III (3)*| 315 ± 20       | 1.8 ± 0.1  | 115 ± 5              | 2.5 ± 0.3                       | 98 ± 18                 | 7.9 ± 0.9               | 0.081                    |

* Number of observations.
Table 3. Effect of 3-acetylpyridine on myelin composition in the rat brain.

Group I (control group) was given 2 ml of 0.9% NaCl solution by intraperitoneal injection. Group II and Group III were given 3-acetylpyridine in an equal volume of saline solution for 40 days (Group II; 2.5 mg/100 g body weight/day and Group III; 5.0 mg/100 g body weight/day). Treatment of groups is described in the footnote to Table 1. Isolation and analysis of myelin in the brain was performed as described under METHODS. All values represent mean ± SE.

| Group      | I (6)*  | II (6)* | III (3)* |
|------------|---------|---------|----------|
|            | (mg/brain) | (%)     | (mg/brain) | (%)     | (mg/brain) | (%)     |
| Yield of myelin | 50.2 ± 3.8 | (27.1) | 38.1 ± 4.0 | (26.5) | 36.9 ± 4.5 | (27.3) |
| Myelin composition |         |         |         |         |         |         |
| Protein    | 13.6 ± 1.6 | (27.1) | 10.1 ± 0.9 | (26.5) | 10.1 ± 1.0 | (27.3) |
| Total lipid| 36.1 ± 2.5 | (71.9) | 27.5 ± 2.5 | (72.2) | 26.5 ± 2.7 | (71.8) |
| Lipid composition |         |         |         |         |         |         |
| Cholesterol| 9.4 ± 0.5 | (26.0) | 7.2 ± 0.6 | (26.2) | 6.8 ± 0.5 | (25.7) |
| Galactose  | 7.7 ± 0.4 | (21.3) | 5.5 ± 0.4 | (21.6) | 5.2 ± 0.5 | (19.5) |
| Phospholipid| 18.8 ± 0.8 | (52.1) | 14.2 ± 0.7 | (51.8) | 13.8 ± 0.9 | (52.1) |

*Number of observations.

Effect of 3-acetylpyridine on myelin composition in the rat brain

Table 3 shows the gross composition of myelin, proportion of protein and total lipid, and percentage composition of lipid. The content of isolatable myelin in the whole brain was decreased by the treatment with 3-acetylpyridine. Despite the decreased yields of myelin, the percentage distribution of protein and total lipid did not change by administration of 3-acetylpyridine. The proportion of protein and lipid in myelin is 27% and 72%, respectively.

The total amount of lipid in myelin (mg dry weight/brain) of rats which received 3-acetylpyridine was lower than that of control rats. Despite the difference in the total amount of lipid, the percentage composition of cholesterol, galactolipid and phospholipid in myelin was not altered by 3-acetylpyridine treatment.

DISCUSSION

The dose levels of 3-acetylpyridine used in the present study are 2.5 mg and 5.0 mg/100 g body weight/day. When 5 mg of 3-acetylpyridine was given, the animals exhibited rapid respiratory rates and died within a few days. Administration of 0.5 mg, 1.0 mg and 1.5 mg of 3-acetylpyridine per 100 g body weight did not produce any signs of the disease, significant deficit in brain weight, concentration of cerebrosides and myelin, and CNP activity in the brain (data not shown). However, administration of 2.0 mg and 2.5 mg resulted in poor weight gain for
several days after administration. Since there were no deaths with administration of 2.5 mg, it was selected as the dose level in this study.

The present study demonstrated that the amount of myelin in the whole brain of rats which received 3-acetylpyridine was significantly lower than that of controls. The deficit of myelin was not due to deficit in body weight gain after administration of 3-acetylpyridine, since starved animals controlled for this deficit in body weight gain were not affected with regard to the yield of myelin and levels of CNP activities. This suggests that 3-acetylpyridine plays an important role in the loss of myelin rather than undernourishment from restricted food intake. However, the possible interference by 3-acetylpyridine with the handling of some vitamins of trace elements at the blood-brain barrier cannot be excluded.

To determine whether 3-acetylpyridine readily penetrates the blood-brain barrier or not, the concentration of NAD and total pyridine nucleotide in the brain were measured at different times after administration of 3-acetylpyridine. The content of NAD in the brain was spectrophotometrically determined by the method of Racker using alcohol dehydrogenase (23). The total pyridine nucleotide content was determined by the cyanide method (24). The concentrations of NAD and total pyridine nucleotide in the brain reached a maximum level 6 h after administration of 3-acetylpyridine and then declined gradually with time. The NAD derivatives of 3-acetylpyridine are treated by alcohol dehydrogenase and the pyridine nucleotide derivatives of 3-acetylpyridine were assayed by the cyanide method. Therefore, a higher concentration of NAD and total pyridine nucleotide in the brain was observed 6 h after administration of 3-acetylpyridine. The increase in NAD level was 28% and that in total pyridine nucleotide was 39% 6 h after administration of 3-acetylpyridine (3 mg/100 g body weight).

3-Acetylpyridine inhibited the growth of Lactobacillus casei cultured in the nicotinic acid-free medium. When the microorganisms were cultured in the presence of brain extract of rats which received 3-acetylpyridine (the extract was prepared as described previously (25)), the growth of microorganisms was inhibited. Therefore, it is considered that 3-acetylpyridine readily penetrates the blood-brain barrier and is transported into the brain.

The yield of isolatable myelin was depressed in the brain of rats which received 3-acetylpyridine. There was also an accompanying decrease of brain cerebroside content and CNP specific activity. The whole brain cerebroside content and CNP specific activity of rats which received 3-acetylpyridine decreased in proportion to isolatable myelin, since much of the cerebroside and CNP is assumed to be in myelin. Therefore, the present results suggest that adult rats suffer from myelin deficits by administration of 3-acetylpyridine. Previous studies from our laboratory have shown that the content of myelin and cerebroside in the brain of weanling rats fed a nicotinic acid-deficient diet was lower than in those receiving a nicotinic acid-supplemented diet (3–6, 26). As for the cause, it is considered that nicotinic acid plays an important role in myelination associated with synthesis of cerebroside which contains high levels of long chain fatty acid. However, the immediate cause of
myelin deficit in adult rats is somewhat different from weanling rats. In contrast to the results in young animals, decrease of myelin observed following treatment of adults with 3-acetylpyridine is presumably due to the breakdown and removal of previously formed myelin, as myelin loss is fairly rapid (27).

Myelination is a complex biological process by which oligodendroglial cells in the central nervous system produce a multilayer membrane around the nerve fibers. The cerebroside concentration and CNP activity closely associated with myelin formation were shown to be decreased in rats which received 3-acetylpyridine. Therefore, 3-acetylpyridine was considered to affect the loss of myelin, but the mechanism of deficit of myelin is not yet understood.

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