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

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Summary Suckling rats were given 3-acetylpyridine, an antagonistic agent of nicotinic acid, on the 6th day after birth. The quantity of myelin and cerebroside, total lipid synthesis, and activities of fatty acid synthetase and fatty acyl-CoA elongating enzyme were measured in rats which received 3-acetylpyridine. At both 20 days and 30 days of age, rats which had received 3-acetylpyridine showed lower values in body weight, myelin yield, cerebroside level and specific activity of brain 2',3'-cyclic nucleotide-3-phosphohydrolase, when compared with those of the controls. Biosynthetic activity of lipid in brain slices of rats which had received 3-acetylpyridine was about 50% less than that of the controls. Moreover, activities of the microsomal palmitoyl-CoA, stearoyl-CoA and arachidyl-CoA elongating systems in the brain of rats which had received 3-acetylpyridine were about 80%, 50% and 50% those of the controls, respectively. These findings imply that 3-acetylpyridine affects the synthesis of long chain fatty acid in the developing rat brain. Therefore, nicotinic acid may play an important role in myelination associated with the synthesis of cerebrosides which contain high levels of long chain fatty acids.

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

Myelination is a complex biological process by which oligodendroglial cells in the central nervous system produce a multilayered membrane around nerve fibers, and is one of the major events during brain maturation (1). The current theory suggests that this process involves the transfer of myelin-specific lipids and proteins from the site of their synthesis to the site of myelin assembly (2). Myelin is composed of lipids and proteins in a ratio of approximately 3:1; cerebroside is a characteristic myelin lipid (3). Myelination in the rat cerebral hemisphere starts approximately at the 9th or 10th postnatal day. Nonaka and Kishimoto have indicated that cerebroside does not exist in the rat brain before myelination begins (4).
In the previous studies using developing and adult rat brain, we have shown that the content of myelin and cerebroside in the brain of rats fed on nicotinic acid-deficient diet is lower than in those on nicotinic acid-supplemented diet (5–8). 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 acid (6, 7). 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 excluded (tryptophan-imbalanced diet) (9–12), because pyridine nucleotides are synthesized from tryptophan in rats (13). Therefore, inadequate amounts 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 developing rats fed on AIN-76 diet, and consistently given 3-acetylpyridine intraperitoneally once a day.

METHODS

Animals and diet. AIN-76 purified diet was prepared as described by Bieri (14, 15). The composition of the diet is shown in Table 1.

Animals used were rats of the Sprague-Dawley strain. Dams of suckling animals were fed on AIN-76 purified diet from day 14 of gestation and throughout the lactational period ad libitum. Litters were reduced to ten each at birth. Pups of either sex were used for the experiment. On day 6 after birth, pups were divided into three groups. Group I was given 3-acetylpyridine (3 mg/100 g body weight, 0.1% solution) by intraperitoneal injection beginning at day 6 after birth and ending 1 day before death. Group II and Group III were given an equal volume of 0.9% NaCl solution. Rats were weighed daily and their general condition noted. To compensate for diminished weight gains of animals receiving 3-acetylpyridine, pair-fed rat litters were produced by limiting the length of time during the day that the dam was available to the pups (Group II). By this means, the average weight gain of the pair-

Table 1. Composition of diet.

| Ingredient          | %   |
|---------------------|-----|
| Casein              | 20.0|
| DL-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 |

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fed rats was adjusted to equal that of the rats receiving 3-acetylpyridine.

Measurement of total lipid and cerebroside content of the brain. After administration of 3-acetylpyridine for 14 or 24 days, animals were sacrificed by decapitation and the whole brain was removed. The brain was weighed and homogenized in 10 ml of ice-cold 0.32 M sucrose in a Potter-Elvehjem homogenizer. We added 19 volumes of chloroform–methanol 2:1 (v/v) to 1.0 ml of the homogenate. 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. (16). 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 as described by Norton and Poduslo (17). Bands corresponding to cerebroside standards were scraped from the plate, and cerebroside was determined as described previously (6).

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 and Poduslo (17) as described previously (7). For determination of dry weight, myelin pellet was checked for microsomal and mitochondrial contamination by marker-enzyme assay. NADPH-cytochrome c reductase and succinate dehydrogenase were respectively used as microsomal and mitochondrial markers (18).

Assay of 2', 3'-cyclic nucleotide-3'-phosphohydrolase activity. An aliquot of 0.32 M sucrose homogenates of the brain of rats which had received 3-acetylpyridine and an aliquot of control rats were separately diluted with 50 mM of Tris-buffer (pH 7.4). These diluted homogenates were separately placed in cellulose tubes and dialyzed against the same buffer for 24 h. 2',3'-Cyclic nucleotide-3'-phosphohydrolase (CNP) activities of these dialyzates were determined by the method of Drummond et al. (20).

Lipid synthesis studies. Six-day-old rat pups were divided into two groups. One group was given 3-acetylpyridine (3 mg/100 g body weight, 0.1% solution) by intraperitoneal injection for 14 days. The other group was given an equal volume of 0.9% NaCl solution. The rats were killed by decapitation, the brain then being removed and weighed. Slices of the whole brain (about 0.4 mm in thickness) were prepared using a Y. H. slicer (Hotta Rica S. S.). Slices of the whole brain were immediately transferred to a flask containing 5 ml of Krebs-Hanselte-Ringer bicarbonate buffer, pH 7.4 (115 mM NaCl, 25 mM KHCO₃, 5.9 mM KCl, 1.18 mM MgCl₂, 1.2 mM KH₂PO₄, 1.195 mM K₂SO₄, 2.5 mM CaCl₂). The buffer contained 10 mM [1-¹⁴C]acetate (1 µCi) and 10 mM glucose. The flasks were gassed out with O₂ + CO₂ (95:5) and were placed in a shaking water-bath at 37°C for 1 h. At the end of the incubation period, 0.5 ml of HClO₄ (36%, w/v) was injected into the medium to stop the reaction, and [¹³C]oleic acid was added as internal standard. The medium containing the tissue was mixed with chloroform–methanol (2:1, v/v) of 20 vol. (v/v) of the medium and left overnight. Lipids were extracted using the method of Folch et al. (16). An equal volume of 0.9% (w/v) NaCl solution was added to the solution of lipid extract, splitting it into a lower chloroform and an upper aqueous
phase. The lower chloroform layer was washed 9 times with 3 vol. (v/v) of saline in each wash. The top aqueous phase was frequently checked for any residual radioactivity from the precursors ([14C]acetate). The chloroform layer was washed with 3 vol. (v/v) of saline until the aqueous phase became free of contaminating [14C]acetate radioactivity. The washed chloroform layer was evaporated to dryness under N2 and then counted for 14C and 3H radioactivities in a Bray scintillation counter (21). Loss of lipids during extraction and washing was corrected from recovery of the internal standard [3H]oleic acid, which was added to each sample.

**Assay of fatty acid synthetase and fatty acid elongation enzyme.** Brains of twenty-day-old rats receiving 3-acetylpyridine for 14 days were homogenized in 6 vol. of chilled 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 0.15 M NaCl and 2 mM dithiothreitol. Brain homogenates were prepared in this buffer, using a motor-driven glass homogenizer with a Teflon pestle and subsequently centrifuged at 17,500 × g for 30 min. The supernatant was re-centrifuged at 17,500 × g for 30 min and the resulting supernatant was centrifuged at 105,000 × g for 60 min to yield a crude microsomal and soluble fraction. The supernatant was re-centrifuged and the final supernatant was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, 0.15 M NaCl and 2 mM dithiothreitol. The microsomal suspension was centrifuged at 105,000 × g for 60 min. The final microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mM dithiothreitol (1 ml/g wet weight of tissue) and stored in 1 ml aliquots at −70°C.

Fatty acid synthetase activity in the cytoplasmic fraction (105,000 × g supernatant) was measured by incorporation of [2-14C]malonyl-CoA into fatty acids as described by Goldberg et al. (22). Fatty acid elongation activities in the microsomal fractions were determined essentially as described by Brophy and Vance (23).

Protein was measured by the method of Lowry et al. with crystalline bovine serum albumin as the standard (19).

**RESULTS**

**Effect of 3-acetylpyridine on body and brain weight of developing rats**

The growth response curves of suckling rats given 3-acetylpyridine are shown in Fig. 1. After administration of 3-acetylpyridine, the body weight gain of the group (Group I) was much less than that of the control group (Group III) during the first 7 days only, but the Group I rats grew satisfactorily after 7 days of administration of 3-acetylpyridine. The control group showed a steady gain of body weight throughout the experimental period. Final body weights of rats which receiving 3-acetylpyridine for 14 and 24 days were significantly lower than those of the controls (Table 2). Body weight gain of the pair-fed group (Group II) was significantly lower compared to the control group. No remarkable difference in brain weight was observed between the group which had received 3-acetylpyridine, and controls (control and pair-fed group) at both 19 and 33 days of age (Table 2).

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Fig. 1. Effect of 3-acetylpyridine on growth of rats. Six days after birth, pups were divided into three groups. Group I (–△–) was given 3-acetylpyridine (3 mg/100 g body weight, 0.1% solution) by intraperitoneal injection for 24 days. Group II (–□–) and Group III (–●–) were given equal volumes of 0.9% NaCl solution. To compensate for diminished weight gains of rats which had received 3-acetylpyridine, pair-fed rat litters were produced by limiting the length of time during the day that the dam was available to the pups (Group II). Each plot represents mean value ± SE for twelve animals in each group.

Table 2. Effect of administration of 3-acetylpyridine on myelin yield in the rat brain. Treatment groups are explained in the footnote to Fig. 1. Isolation of myelin and assay of CNP activity were done as described under METHODS. Values are mean ± SE of six animals per group.

| Age at death (days) | Group | Brain | Myelin | CNP activity (μmol/mg protein) |
|---------------------|-------|-------|--------|-------------------------------|
|                     |       | Weight (g) | Protein (mg/g brain) | Yield (mg) | Protein (mg/g brain) |                                    |
| 20                  | I 3-AP | 35 ± 6 | 1.1 ± 0.1 | 106 ± 4 | 3.5 ± 0.7 | 1.1 ± 0.2 | 0.48 ± 0.05 |
|                     | II Pair-fed | 34 ± 3 | 1.1 ± 0.1 | 105 ± 5 | 5.8 ± 0.3 | 1.6 ± 0.2 | 0.65 ± 0.01 |
|                     | III Control | 54 ± 5 | 1.2 ± 0.1 | 101 ± 5 | 7.0 ± 0.5 | 1.8 ± 0.1 | 0.70 ± 0.03 |
| 30                  | I 3-AP | 72 ± 5 | 1.4 ± 0.1 | 107 ± 6 | 6.2 ± 0.7 | 2.1 ± 0.2 | 0.84 ± 0.05 |
|                     | II Pair-fed | 73 ± 1 | 1.4 ± 0.1 | 108 ± 5 | 9.7 ± 0.4 | 3.9 ± 0.2 | 1.28 ± 0.02 |
|                     | III Control | 90 ± 6 | 1.5 ± 0.1 | 110 ± 6 | 13.3 ± 0.6 | 4.5 ± 0.2 | 1.41 ± 0.03 |

Effect of 3-acetylpyridine on myelin yield of suckling rats

The effects of 3-acetylpyridine on brain composition of suckling rats are shown in Tables 2 and 3. There was no significant difference between the three groups in Vol. 30, No. 5, 1984
Table 3. Effect of 3-acetylpyridine on concentration of total lipid and cerebroside in brain of rats.
Treatment groups are explained in the footnote to Fig. 1. Analytical procedures were performed as described in METHODS. Values are mean ± SE of six animals per group.

| Age at death (days) | Group       | Total lipid (mg/g brain) | Cerebroside (mg/g brain) | Cerebroside Total lipid |
|--------------------|-------------|--------------------------|--------------------------|-------------------------|
| 20                 | I 3-AP      | 50 ± 6                   | 1.8 ± 0.3                | 0.036                   |
|                    | II Pair-fed | 52 ± 5                   | 2.3 ± 0.1                | 0.044                   |
|                    | III Control | 58 ± 5                   | 2.5 ± 0.3                | 0.043                   |
| 30                 | I 3-AP      | 61 ± 3                   | 2.5 ± 0.3                | 0.041                   |
|                    | II Pair-fed | 60 ± 6                   | 4.4 ± 0.1                | 0.073                   |
|                    | III Control | 66 ± 6                   | 5.1 ± 0.3                | 0.077                   |

both brain weight and concentration of protein in the brain. The whole brain showed decreased myelin yields and myelin protein with 3-acetylpyridine treatment for each age bracket studied. The whole-brain content of cerebroside and the activity of CNP were assayed, since their levels have been shown to increase in parallel with myelination (24, 25). Cerebroside content of the brain of rats which had received 3-acetylpyridine was markedly lower than that of the control and pair-fed rats, but no significant difference in total lipid concentration was observed between the three groups (Table 3). Therefore, the ratio of cerebroside to total lipid of rats which had received 3-acetylpyridine was markedly lower than that of the control or pair-fed rats. The decrease in myelin and cerebroside content might not owe to increased water content (gross edema), because the protein concentrations of the whole brain were essentially unaltered (Table 2).

The specific activity of CNP in the brain was depressed in rats which had received 3-acetylpyridine in each age bracket studied (Table 2). Therefore, the reduced myelin yield cannot be attributed to inadequate nutritional intake, as pair-fed rats produced neither myelin deficit nor decrease in brain cerebroside concentration and in CNP-specific activity. This suggests that 3-acetylpyridine plays an important role in the loss of myelin rather than undernourishment from restricted food intake.

**Effect of 3-acetylpyridine on biosynthesis of lipid and fatty acids**

During the development of nervous tissue, relatively large amounts of lipid are accumulated, particularly in the white matter of the central nervous system (28). Galactolipids are the only lipids characteristic of brain white matter. Experiments with radioactive isotopes have shown that cerebrosides and sulphatides are synthesized after birth in rats; galactolipids, especially cerebrosides, are only detectable in significant amounts at the onset of myelination and possibly are of key

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Table 4. Incorporation of [1-14C]acetate into lipids of brain slices of rats receiving 3-acetylpyridine for 14 days. Treatment groups are explained in METHODS. Lipid extractions and radioactivity counting were carried out by the procedures described in METHODS. Values are mean ± SE of six animals per group.

| Regimen | Radioactivity incorporated (dpm/brain) |
|---------|----------------------------------------|
| Control | 2,410 ± 300                             |
| 3-AP    | 1,190 ± 320                             |

Table 5. Effect of 3-acetylpyridine on fatty acid synthesis and fatty acyl-CoA elongation activities in 20-day-old rats. Fatty acid synthetase activities in the cytoplasmic fractions (105,000 × g supernatant) and fatty acyl-CoA elongation activities in the microsomal fractions were assayed as described under METHODS. Activities of these enzyme were expressed as nmol [2-14C]malonyl-CoA incorporated into fatty acid/h per mg of protein. Values are mean ± SE of six animals per group.

| Enzyme          | Substrate      | 3-Acetylpyridine |
|-----------------|----------------|------------------|
|                 |                | -    | +    |
|                 |                | (nmol/mg prot.) | (nmol/mg prot.) |
| Fatty acid      | Acetyl-CoA     | 15.6 ± 0.2       | 15.0 ± 2.2      |
| synthetase      | Palmitoyl-CoA  | 2.2 ± 0.2        | 1.8 ± 0.2       |
|                 | Stearoyl-CoA   | 0.7 ± 0.1        | 0.3 ± 0.2       |
|                 | Arachidyl-CoA  | 0.2 ± 0.03       | 0.1 ± 0.03      |
| acyl-CoA elongation | Arachidyl-CoA  | 0.2 ± 0.03       | 0.1 ± 0.03      |

Importance in this process (27, 28). Cerebrosides of the nervous system which are characterized by a relatively high content of long chain fatty acids dramatically increase at the onset of myelination (22, 29, 30). Biosynthesis of long chain fatty acids involves de novo synthesis by cytoplasmic enzyme producing mainly palmitic acid, and chain elongation by microsomal and mitochondrial enzyme (31–33). In order to clarify the causes of reduced myelin yield of rats receiving 3-acetylpyridine, we measured the synthesis of fatty acid using brain slices, and fatty acid synthetase and fatty acyl-CoA elongation activities in brain extracts.

The rates of total lipid synthesis were measured by using the incorporation of labeled acetate into lipids by the whole-brain slices (Table 4). In the rats which received 3-acetylpyridine for 14 days, incorporation of radioactivity into the lipid fraction of the brain slices was about 50% less than that of the control. Therefore,
biosynthesis of lipid in the brain was considered to decrease in rats receiving 3-acetylpyridine.

Effects of 3-acetylpyridine on activities of de novo fatty acid synthesis and of elongation of palmitoyl-CoA, stearoyl-CoA and arachidyl-CoA in extracts of brain are shown in Table 5.

The soluble fatty acid synthetase activity in the brain of rats which had received 3-acetylpyridine was of the same level as that of the control. The activities of the microsomal palmitoyl-CoA, stearoyl-CoA and arachidyl-CoA elongation systems in the brain of rats which had received 3-acetylpyridine were about 80%, 50% and 50% of the control, respectively. We therefore suggest that a defect in the elongation of C_{18}-CoA and C_{20}-CoA rather than of C_{16}-CoA is responsible for the remarkably reduced content of long chain fatty acids in brain lipids of rats receiving 3-acetylpyridine.

DISCUSSION

The dose level of 3-acetylpyridine used in the present study was 3 mg/100 g body weight/day. Within a few hours of the administration of 3-acetylpyridine, the animals used began to breathe quite rapidly. Within 2 days, almost complete paralysis of the hind legs had developed. No signs were observed on the administration of less than 2 mg of 3-acetylpyridine per 100 g body weight, but administration of 3 mg resulted in poor weight gain for several days after administration. Since no animals died after the administration of 3 mg, this dose level was selected for this study.

Preliminary experiments have been performed to determine whether or not 3-acetylpyridine readily penetrates the blood-brain barrier (34). As shown in the previous paper, the whole-brain content of NAD and total pyridine nucleotide derivatives of 3-acetylpyridine was increased 6 h after administration of 3-acetylpyridine. After culturing the microsomes in the presence of brain extract of rats which had received 3-acetylpyridine, the growth of microorganisms was inhibited. Therefore, it was considered that 3-acetylpyridine readily penetrates the blood-brain barrier and is transported into the brain.

The present study demonstrated that the yield of myelin in the brain of rats which had received 3-acetylpyridine was lower than that of control rats. Previous studies from our laboratory have shown that the amount of myelin in the whole brain of nicotinic acid-deficient rat was significantly lower than with animals fed on a nicotinic acid-supplemented diet. However, no significant difference was observed in the percentage of the gross composition of myelin: total lipids, protein and the ratio of cholesterol, galactolipids and phospholipids (7, 34). Galactolipids, especially cerebrosides, are only detectable in significant amounts at the onset of myelination and possibly are of high importance in this process (2, 3). The other lipid compounds of myelin have been shown to occur in other membranes and are already present in large quantities at the onset of myelination (26). Therefore, it has

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been suggested that the myelin synthesis depends on cerebroside production. Moreover, it has been considered that the marked reduction of myelin in the nicotinic acid-deficient rats and in rats receiving 3-acetylpyridine owes to the decreased synthesis of cerebroside.

Cerebrosides of the nervous system are characterized by a relatively high content of long chain fatty acids (29). The synthesis of long chain fatty acids dramatically increased at the onset of myelination (22–24). Biosynthesis of long chain fatty acids involves de novo synthesis by cytoplasmic enzyme producing mainly palmitic acid, and chain elongation by microsomal and mitochondrial enzymes (22, 23, 30). Since pyridine nucleotide is the preferred cofactor for these fatty acid elongating enzymes, it is supposed that the rate of elongation of fatty acid decreases in the brain of rats fed on nicotinic acid-deficient diet or of rats receiving 3-acetylpyridine.

As shown in a previous paper, a comparison of the fatty acid pattern in myelin lipids of rats fed on nicotinic acid-deficient diet with that in those of rats fed on nicotinic acid-supplemented diet confirmed the decreases to be proportional to long chain fatty acids in nicotinic acid-deficient rats. It therefore seems that in the normal maturation process, the appearance of fatty acid follows the appearance of cerebrosides. A deficiency of cerebrosides, which contain long chain fatty acids, may lead to the cessation of myelination as observed in nicotinic acid-deficient rats and in rats receiving 3-acetylpyridine. The key step may be the formation of these long chain fatty acids in association with cerebroside precursor through the action of chain length specific enzyme.

In the brain, almost all nicotinic acid is in the form of pyridine nucleotide (35). Pyridine nucleotide is the preferred cofactor for fatty acid synthetase, fatty acid elongating enzyme and fatty acid α-hydroxylating enzyme. Recent reports have suggested that cytochrome b₅ may play a physiological role in elongation of fatty acid (36). The results presented in our studies clearly show that nicotinic acid can influence the amount of long chain fatty acids incorporated in myelin in the developing rat brain. Because metabolism of cerebrosides which contain high levels of long chain fatty acid is associated with myelination, further study is necessary to clarify the role of nicotinic acid in myelin synthesis in the developing rat brain.

It therefore seems likely that a deficit of myelin in nicotinic acid-deficient rats may be one of the causes of the mental symptoms associated with nicotinic acid deficiency.

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