Effects of Pyridoxine Deficiency on the Metabolism of N-Nitrosodimethylamine in the Rat

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Summary The alteration in the metabolic activation of N-nitrosodimethylamine (NDMA) was investigated in the rat during dietary pyridoxine deficiency. The in vitro metabolism of NDMA by demethylase system was measured in both liver and kidney microsomes. The profile of the kidney enzyme appears similar to that of the liver indicating that at least two forms of isozymes with the low and the high $K_m$'s are present. Pyridoxine deficiency significantly increased the activity of NDMA-demethylase of both organs. The increase in the activity of NDMA-demethylase induced by dietary pyridoxine deficiency can be reversed by supplementation of pyridoxine (500 $\mu$g), i.p., daily for two consecutive days. The increase in the NADPH cytochrome c reductase activity was observed after 6 weeks on pyridoxine-deficient diet.

Key Words pyridoxine deficiency, vitamin B6 deficiency, N-nitrosodimethylamine metabolism, N-nitrosodimethylamine demethylase, microsomal mixed function oxidase, cytochrome P-450 isozymes

Pyridoxine or vitamin B6 deficiency is still prevalent in rural areas of many developing countries, particularly in children (1). This deficiency can occur rapidly within a period of a few weeks after insufficient intake of food containing the vitamin. Experimentally, it has been shown that pyridoxine which plays an important role in the intermediary metabolism as a cofactor of many enzymes is also involved in carcinogenesis and in the induction of cancer by other chemicals. Pyridoxine deficiency has been implicated in the production of hepatic and renal hyperplastic lesions in baboons and in rats (2,3). The synergistic effect of

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pyridoxine deficiency on chemical carcinogenesis by 2-acetylaminofluorene has also been reported (4).

The present study was undertaken to investigate the effects of pyridoxine deficiency on the metabolic activation of N-nitrosodimethylamine (NDMA) which is a carcinogen found in food and beverages and can also be formed in the human stomach from its precursor amine and nitrite (5). NDMA requires metabolic activation by microsomal cytochrome P-450 mixed function oxidase, namely NDMA-demethylase, for its toxic and carcinogenic action (6–8). Metabolism takes place mainly in the liver and to a lesser extent in the kidney (7). This enzymatic pathway is believed to be the major pathway in the metabolism of NDMA (8). It is thus conceivable that factors which modify or interfere with the metabolism of NDMA may eventually alter its carcinogenicity. Since the liver and the kidney are the two main target organs for NDMA carcinogenesis and also affected by pyridoxine deficiency, it is of interest to examine the capability of these organs to metabolize NDMA during pyridoxine deficiency.

MATERIALS AND METHODS

**Chemicals.** NDMA (CAS; 62-75-9; N-nitrosodimethylamine) was purchased from Sigma Chemical Co. (Milwaukee, Wis.); NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and semicarbazide hydrochloride were obtained from Sigma Chemical Co. (St. Louis, Mo.). All dietary ingredients, vitamins, and Rogers and Harper salt mixture were obtained from NBCo. Biochemicals (Cleveland, Ohio).

**Animals and diet.** Weanling, male inbred F344 rats, 21 days old, were housed individually in a stainless steel hanging cage, and food and water were provided ad libitum. Dietary pyridoxine deficiency was induced by feeding the semipurified diet as described earlier (9). Pyridoxine deficiency was assessed by the erythrocyte glutamate-oxaloacetate transaminase (EGOT) activity as described by Heller et al., 1973 (10). The activation coefficient (αGOT), which is the ratio of the increased activity with pyridoxal-5' phosphate to the original activity without pyridoxal-5'-phosphate, indicates pyridoxine deficiency when the value exceeds 1.66.

**Animal treatment.** All animals were fasted for 16 h prior to the experiment. They were killed by decapitation between 9.00 and 10.00 a.m. For supplementation study, a daily dose of pyridoxine HCl (500 μg) was given as a solution in 0.9% NaCl, i.p., for 2 days prior to sacrifice.

**Enzymes assays.** Microsomal NDMA demethylase activity was determined as described earlier (11). The amount of formaldehyde formed was determined by the method of Nash (12) as modified by Cochin and Axelrod (13). The activity of the enzyme was expressed as nanomoles formaldehyde formed per hour per milligram microsomal protein. The hepatic microsomal cytochrome P-450 and cytochrome b₅ contents were determined by the method of Omura and Sato (14), NADPH cytochrome c reductase activity was assayed at room temperature using cyto-
chrome c as an artificial electron acceptor \((15)\). Protein was determined by the method of Bradford \((16)\).

RESULTS

Alteration in the microsomal metabolic activation of NDMA during the development of pyridoxine deficiency

Changes in the microsomal NDMA demethylase activity and the components of the mixed function oxidase system during the development of pyridoxine deficiency are shown in Table 1. Development of pyridoxine deficiency was evidenced with a decrease in growth rate which became apparent after feeding the deficient diet for 4 weeks; a slight decrease in food intake was also observed during deficiency \((9)\).

The metabolic activation of NDMA was investigated by using liver microsomes incubated with NDMA at a low \((4\text{mM})\) and a high \((200\text{mM})\) concentration. These two concentrations were reported \((17)\) to be the optimum substrate concentrations of the low \(K_m\) NDMA-demethylase \((\text{NDMA-D I})\) and the high \(K_m\) NDMA-demethylase \((\text{NDMA-D II})\) although it is believed that the lower \(K_m\) isozymic form may be more relevant to the metabolism of NDMA in vivo \((18)\).

In this initial study, changes in the activity of the enzyme and the components of the mixed function oxidase system were monitored from the period when the animal began to develop deficiency until it showed clinical signs and symptoms which corresponded to the level of \(\alpha\text{EGOT}\) from 1.36 to 1.90 in the deficient groups. It was found that pyridoxine deficiency significantly increased the activity of NDMA-demethylase. This effect was observed as early as 3 weeks after the animals were fed the deficient diet and increased slightly further when deficiency progressed at 4 weeks. At this period on the dietary regimen, 81.8\% increase in the activity of the low \(K_m\) and 59.3\% increase in the activity of the high \(K_m\) NDMA demethylase were observed in the pyridoxine deficient group. However, after 4 weeks NDMA demethylase activity did not seem to further increase with the increasing severity of deficiency, which was monitored up to 8 weeks on the deficient diet. Cytochrome P-450 and \(b_5\) contents were not changed during pyridoxine deficiency while NADPH cytochrome c reductase was significantly increased when the rat was maintained on the deficient diet for 6 weeks or longer. \(\alpha\text{GOT}\), the biochemical assessment of the pyridoxine status indicated that the animals were moderately deprived of pyridoxine after feeding the deficient diet for 6 weeks \((\text{Table 1})\). The 6-week feeding period was therefore selected as the experimental condition for the subsequent experiments.

Effects of pyridoxine deficiency on the microsomal NDMA-demethylase system in rat liver and rat kidney

The effect of pyridoxine deficiency was further investigated in both the liver and the kidney which are major organs responsible for NDMA metabolism. They
Table 1. Effect of pyridoxine deficiency on NDMA-demethylase and components of the microsomal mixed function oxidase system.

| Parameter                        | Group     | Feeding period (weeks) |
|----------------------------------|-----------|------------------------|
|                                  |           | 3          | 4          | 6          | 8          |
| NDMA-D I activity (nmol HCHO/mg protein/h) | Control  | 72.6±6.4  | 76.3±7.7  | 61.3±9.0  | 67.7±4.2  |
|                                  | Deficient | 110.6±9.4* | 138.8±14.7* | 120.6±10.9* | 106.7±5.6*  |
| NDMA-D II activity (nmol HCHO/mg protein/h) | Control  | 109.0±8.5  | 149.3±18.0  | 147.5±4.0  | 142.0±22.4  |
|                                  | Deficient | 204.6±30.3* | 237.8±18.4* | 243.6±20.2* | 242.5±16.2*  |
| Cyt. P-450 (nmol/mg protein)     | Control  | 1.1±0.1   | 1.4±0.1   | 1.1±0.1   | 1.1±0.1  |
|                                  | Deficient | 1.3±0.2   | 1.3±0.1   | 1.1±0.0   | 1.0±0.1  |
| Cyt. b$_5$ (nmol/mg protein)     | Control  | 0.5±0.1   | 0.4±0.0   | 0.5±0.0   | 0.5±0.0  |
|                                  | Deficient | 0.4±0.1   | 0.5±0.1   | 0.5±0.0   | 0.5±0.0  |
| NADPH cyt. c reductase (nmol cyt. c reduced/mg protein/min) | Control  | 180.6±19.4 | 200.0±46.8 | 190.5±7.4 | 191.0±9.9  |
|                                  | Deficient | 174.8±8.5 | 227.7±17.3 | 241.9±8.6* | 215.6±7.6*  |
| $a$EGOT AC                      | Control  | 1.0±0.1   | 1.0±0.1   | 1.1±0.1   | 1.0±0.1  |
|                                  | Deficient | 1.4±0.2* | 1.6±0.2* | 1.7±0.4* | 1.9±0.4*  |

$\alpha$EGOT AC $= \frac{\text{activity of GOT with pyridoxal-5'-phosphate}}{\text{activity of GOT without pyridoxal-5'-phosphate}}$. Results are expressed as M±SE. Values in parentheses are the number of animals. *Significantly different from the control value ($p<0.05$).
are also susceptible to pathological effects of pyridoxine deficiency which has been reported to induce both hepatic and renal hyperplastic lesions (2–4).

The effects of pyridoxine deficiency on the hepatic and renal NDMA demethylases are shown in Fig. 1a and b, which demonstrate the Michaelis-Menten plots of substrate concentrations versus velocity of the liver and kidney microsomal

Fig. 1a. The Michaelis-Menten plots of the hepatic microsomal DMN-demethylation in the pyridoxine-deficient and control rats. prot., protein; □, control rats; ◆, pyridoxine deficient. The inset figure represents the plot at low substrate concentrations (up to 4 mM).

Fig. 1b. The Michaelis-Menten plots of the kidney microsomal DMN-demethylation in the pyridoxine-deficient and control rats. prot., protein; □, control rats; ◆, pyridoxine deficient. The inset figure represents the plot at low substrate concentrations (up to 4 mM).

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NDMA-demethylation, respectively. Generally, the renal NDMA-demethylase exhibits the specific activity much lower than that of the liver. Pyridoxine deficiency significantly enhances the activity of NDMA-demethylase in both organs throughout the whole range of substrate concentrations tested. For the liver, the graphs of velocity ($V$) against substrate concentrations ([S]) at low substrate concentration (0.0625-4.0mM) were fitted to the Michaelis-Menten equation using a non-linear least squares program ENZFITTER (19) to give best fit values of $K_m$ and $V_{max}$ directly. Both the curves for control and pyridoxine deficient gave a good fit with values of $K_m$ being $0.37\pm0.02$ and $0.33\pm0.07$ mM and of $V_{max}$ being $86.5\pm6.8$ and $106.9\pm6.4$ nmol formaldehyde/mg protein/h for the control and pyridoxine-deficient enzymes, respectively. In the kidney, the graph of $V$ against [S] (0.5–4 mM) was also fitted to the Michaelis-Menten equation as those of the liver enzyme. The best fit values of $K_m$ obtained were $0.49\pm0.04$ and $0.61\pm0.08$ mM and of $V_{max}$ were $6.32\pm0.09$ and $7.93\pm0.21$ nmol formaldehyde/mg protein/h for the control and pyridoxine deficient-enzymes, respectively. However, the initial velocity seems to reach plateau at high substrate concentrations in both liver and kidney. Pyridoxine deficiency seemed to increase the $V_{max}$ values of both liver and kidney NDMA-demethylase with no change in the $K_m$ values. The similar $K_m$ but different $V_{max}$ values may be interpreted as indicating an increase in the amount of enzyme present in the pyridoxine-deficient microsomes.

To confirm that the observed increase in NDMA-demethylase activity was due solely to pyridoxine deficiency and not complicated by the effect of reduction of food intake which occurred during the development of pyridoxine deficiency, the deficient rats were given 2 daily doses of pyridoxine-HCl prior to the experiment. Table 2 demonstrates that supplementation of pyridoxine to the deficient rats lowered the activity of the liver and kidney NDMA-demethylase to the control levels. Microsomal protein contents were unaffected by pyridoxine deficiency.

Table 2. Effects of pyridoxine deficiency and pyridoxine supplementation on the NDMA-demethylase activity of rat liver and kidney.

| Organ | Group       | Microsomal protein (mg protein/g tissue) | NDMA-D I (nmol HCHO/mg microsomal protein/h) |
|-------|-------------|-----------------------------------------|---------------------------------------------|
|       |             |                                         |                                             |
| Liver | Control     | 22.7±3.4 (6)                           | 83.4±12.6 (6)                                |
|       | Deficient   | 23.6±3.8 (7)                           | 127.7±31.2* (7)                              |
|       | Supplemented| 20.5±3.3 (7)                           | 75.9±18.5 (7)                                |
| Kidney| Control     | 11.4±0.7 (3)                           | 5.3±0.8 (3)                                  |
|       | Deficient   | 10.9±0.4 (3)                           | 9.1±1.7* (3)                                 |
|       | Supplemented| 12.8±1.0 (3)                           | 7.2±1.0 (3)                                  |

For liver, the number in parentheses is the number of animals. For kidney, the number in parenthesis is the number of determinations, each obtained from microsomes pooled from 10 rats. *Significantly different from the corresponding control value ($p<0.005$).
METABOLIC ACTIVATION OF NMDA

DISCUSSION

The present study demonstrated the influence of dietary pyridoxine deficiency on the N-demethylation pathway of NDMA metabolism in liver and kidney microsomes.

The study showed that pyridoxine deficiency significantly increased the specific activity of NDMA-demethylase which is believed to be responsible for activation of NDMA to the ultimate carcinogen (8). NDMA-demethylase is the cytochrome P-450 isozyme which exists in at least 2 isozymic forms of the low and high $K_m$ values (17). Pyridoxine deficiency increases the activity of both isozymic forms to about the same extent. The effect of pyridoxine deficiency seems to be different from those caused by the other members of the B vitamins such as riboflavin and thiamine which selectively stimulate either the low or the high $K_m$ NDMA-demethylase (20–24). The enhancement effect of pyridoxine deficiency is consistent in both the liver and kidney enzyme systems.

At present the mechanism by which pyridoxine deficiency exerts its effect on NDMA metabolism is not fully understood. The increase in NDMA-demethylase activity was not related to the gross P-450 content which remained unchanged during deficiency. However, this is not unexpected since it was earlier reported by Tu and Yang (18) that the increase in NDMA-demethylase activity did not correlate with the gross P-450 content and the NADPH cytochrome c reductase activity. Only the specific form of P450, namely cyt. P450 II E1, has been reported to increase under the condition which NDMA metabolism is stimulated. The result of this study serves as a preliminary finding that deficiency of this vitamin does alter the enzyme system involved with metabolism of the carcinogen. The increase in the metabolism of NDMA by the liver and kidney during pyridoxine deficiency suggests that pyridoxine deficiency may alter NDMA carcinogenesis. It remains to be determined whether the denitrosation pathway, DNA alkylation and carcinogenicity of NDMA would be affected if the animal is exposed to NDMA during pyridoxine deficiency.

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