EFFECT OF PYRIDOXINE DEFICIENCY ON 
CHOLESTEROGENESIS IN RATS FED 
DIFFERENT LEVELS OF PROTEIN

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Summary Hepatic cholesterol contents in rats fed a 70% or 20% 
casein diet with or without pyridoxine was determined. In the case of 
the 70% casein group, pyridoxine-deficient rats showed a higher content 
than the control. The increment was mainly due to the accumulation of 
an ester form of the cholesterol. On the other hand, pyridoxine-deficient 
rats in the 20% casein group showed a slightly lower content. The 
cholesterol content in liver microsomal fractions was lower in the 20%- 
casein pyridoxine-deficient group and serum cholesterol level was lower 
in the 70%-casein pyridoxine-deficient group than those in respective 
control groups. Incorporation of [14C]acetate into cholesterol was 
studied using liver slices, and significant stimulation was observed in 
pyridoxine-deficient rat fed a 20% or 70% casein diet. There was no 
difference in intestinal cholesterogenesis between the control and the 
deficient groups.

Since RINEHART and GREENBERG found artherosclerosis in Rhesus monkey 
fed a pyridoxine-deficient diet for almost one year (1), divergent observations have 
been reported in the literature with regard to cholesterol metabolism in pyridoxine 
deficiency. There are findings of hypercholesterolemia in pyridoxine-deficient 
rats (2), chicks (3) and rabbits (4) as well as no change (5) or even a decrease (6) 
in plasma sterol level of depleted rats. The liver cholesterol ester was reported 
to be reduced in pyridoxine-deficient rat (6), while both LUPZEN et al. (7-9) and 
SHAH et al. (10) reported no change in cholesterol contents and an increase of 
cholesterogenesis in the pyridoxine-deficient rat liver. These previous studies 
have not paid any special attention to the feeding condition of animals, although 
cholesterogenesis in liver is known to show a diurnal rhythm (11-13). Recently, 
we found that dietary protein levels influence lipid metabolism in pyridoxine-
deficient rats (14, 15). On being fed a high (70%) protein diet, the pyridoxine-

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deficient rats accumulated triglyceride and cholesterol ester in the liver. We have also obtained evidence of increased cholesterogenesis on rats fed a 70% casein pyridoxine-deficient diet (16). In the present paper, we describe the results of a more detailed study on cholesterol metabolism in pyridoxine-deficient rats fed a 20% or 70% protein diet.

EXPERIMENTAL

Chemicals. Sodium [1-\textsuperscript{14}C]acetate (54.9 mCi/mmole) was obtained from Daiichi Pure Chemicals Co., Ltd.

Treatment of animals. Male rats of the Wistar strain weighing about 50 g were fed a 70% or 20% casein diet with or without supplemented pyridoxine for 3 to 6 weeks. The diet used contained 70% or 20% vitamin-free casein (NBC), 7% or 57% corn starch, 10% sucrose, an 8% oil mixture (cod liver oil-soybean oil, 1:4), a 4% mineral mixture, a 1% vitamin mixture and 0.2% choline chloride as previously described (14). Unless otherwise specified, control animals received an amount of food equal to that eaten by the pyridoxine-deficient animals.

Preparation of tissue samples. A liver microsomal fraction was prepared as a 100,000×g pellet in an isotonic sucrose solution, the liver slices were prepared with a slicer devised by Stadie and Riggs (17). The small intestine was washed with chilled saline to remove the contents. A section of the jejunum (central 5 cm) and a section of the ileum (distal 5 cm) were cut to small pieces.

Incubation conditions. Incubation was carried out as described by Kandutsch and Saucier (11). Slices of liver or pieces of intestine (approximately 200 mg in wet weight) were put into a Warburg flask containing 2.5 ml of Krebs-Ringer bicarbonate buffer and 7.5 μmoles (2 μCi) of sodium [1-\textsuperscript{14}C]acetate. The side arm of the flask contained 0.3 ml of 9N H\textsubscript{2}SO\textsubscript{4}. This condition allowed an optimum rate of acetate incorporation into cholesterol for at least 3 hr. The flask was gassed with 95% O\textsubscript{2}-5% CO\textsubscript{2}, stoppered and incubated for 2 hr at 37°C in a shaking bath. At the end of the incubation period the contents of the flask was acidified by the addition of sulfuric acid from the side arm and 0.3 ml of Hyamine hydroxide solution was injected into the center well. The flask was shaken for an additional 30 min.

Determination of the reaction products. Radioactivity of CO\textsubscript{2}, absorbed in 0.1 ml Hyamine solution, was determined in 10 ml dioxane scintillator (18) with an Aloka liquid scintillation counter. After incubation, liver slices were taken up and homogenized with a mixture of 4 ml 1 N H\textsubscript{2}SO\textsubscript{4} and 15 ml CHCl\textsubscript{3}-methanol (1:2). The homogenate was centrifuged to obtain the supernatant and the resultant precipitate was washed with a mixture of 2 ml 1 N H\textsubscript{2}SO\textsubscript{4} and 7.5 ml CHCl\textsubscript{3}-methanol. The residue was used for the determination of protein content. The washing was combined with the original supernatant, then 6 ml water and 7.5 ml CHCl\textsubscript{3} were added to the mixture. After mixing, the two phases were
separated and the lower phase was used for the following lipid analyses. The solvent was removed by evaporation, the residue was dissolved in petroleum ether and washed several times with 0.1 M sodium acetate to remove the radioactive substrate completely. After concentrating this solution to a small volume, the lipid fraction was separated by thin-layer chromatography on Kiesel Gel with a solvent mixture of petroleum ether–ethyl ether–acetic acid (80: 30: 1). The lipid bands on the plate were detected by iodine vapor, scrapped off, extracted with CHCl₃–methanol (1: 1), and the radioactivities were determined in toluene-based scintillator.

**Analyses.** Protein was determined by the biuret reaction \( (19) \). Alanine aminotransferase \[ \text{E. C. 2.6.1.2} \] activity was determined by measuring pyruvate produced from L-alanine and 2-oxoglutarate as phenyl hydrazone \( (20) \). Total lipid in liver tissue was gravimetrically determined after extraction by the method of Folch et al. \( (21) \). Cholesterol was measured with an FeCl₃ reagent as described by Zak \( (22) \), and free and ester cholesterol was separated by a thin-layer chromatography prior to the chemical determination.

**RESULTS**

Hepatic alanine aminotransferase activity was assayed as a marker of pyridoxine deficiency. The activities observed in this experiment were as follow; control 11.58±0.55 µmole/mg/hr, deficient 2.02±0.28 µmole/mg/hr in the 70% casein-fed group and control 2.93±0.47 µmole/mg/hr, deficient 1.43±0.18 µmole/mg/hr in the 20% casein-fed group.

**Cholesterol contents in liver and serum**

Hepatic cholesterol was determined in rats fed a 70% or 20% casein diet (Table 1). In the case of rats fed a 70% casein diet, the total cholesterol was almost twice in the pyridoxine-deficient rat and this increase was mainly due to an increase in the ester form. On the other hand, the cholesterol content was decreased in the pyridoxine-deficient rat fed a 20% casein diet, especially in the ester form. Since microsomal cholesterol is known to influence the rate of cholesterol synthesis, the cholesterol content of the microsomes was determined. No difference in the cholesterol contents was observed between the control and the deficient group fed a 70% casein diet. The deficient rats fed a 20% casein diet showed a lower cholesterol contents level than the control animals. The serum cholesterol level of a 70%-casein pyridoxine-deficient group was lower than that of the control, while no difference was observed in 20% casein groups.

**Diurnal rhythm of hepatic cholesterol synthesis**

Incorporation of \[^{14}C\]acetate into cholesterol fraction in liver slices was determined at both midday and midnight, since a diurnal rhythm has been observed in cholesterol synthesis in the liver \( (11–13) \). Rats fed a 70% casein diet,
Table 1. Cholesterol content of liver and serum. Rats fed a 70% casein diet were maintained for 3 weeks and a 20% casein diet for 6 weeks. All rats were fasted overnight and killed at noon. All analytical methods were described in the text. Figures are mean ±s.d. and those in parentheses are numbers of rats.

|                     | 70% Casein (6) | 20% Casein (6) |
|---------------------|---------------|---------------|
|                     | Deficient     | Control       | Deficient     | Control       |
| Liver wt./100 g B.W.| 4.1±0.7       | 3.9±0.5       | 4.0±0.2       | 3.4±0.2       |
| Total lipid, liver |               |               |               |               |
| (% of wet wt.)      | 8.4±2.7*      | 4.4±0.2       | 5.2±0.6       | 6.0±0.4       |
| Cholesterol, liver  |               |               |               |               |
| (µmole/g)           | 9.80±3.19*    | 5.81±0.56     | 6.73±0.53*    | 7.88±0.55     |
| Free                | 6.20±0.41*    | 5.22±0.52     | 6.14±0.27*    | 6.73±0.35     |
| Ester               | 3.60±2.56*    | 0.59±0.00     | 0.58±0.38*    | 1.43±0.29     |
| Cholesterol, liver  |               |               |               |               |
| microsome (nmole/mg protein) | 77.5±8.5 | 66.7±6.7 | 53.8±5.1* | 71.3±9.3 |
| (nmole/mg lipid)    | 126.7±23.7    | 117.9±12.4    | 102.9±3.6*    | 139.1±20.6    |
| Cholesterol, serum  |               |               |               |               |
| (µmole/ml)          | 1.58±0.13*    | 2.16±0.10     | 1.91±0.32     | 2.04±0.25     |

* p<0.05

with and without supplemented pyridoxine, were used for this experiment. The pyridoxine supplemented group was divided into paired-control and ad libitum control groups to observe effects of feeding patterns. As shown in Table 2, the acetate incorporation into free cholesterol fraction at midnight gave similar high values in all groups. The midday activities were, however, different among three groups; ad libitum control group showed almost the same value with that at midnight, while the midday activities were decreased to 20% and 40% of the midnight activities in pair-fed control and the deficient groups, respectively. The incorporation into cholesteryl ester in various groups showed a similar trend with that into free cholesterol. Liberation of \(^{14}\)CO\(_2\) and triglyceride synthesis would serve as internal standards of the experiment. Triglyceride synthesis was active at midnight in all groups and decreased at midday in the order of ad libitum control, pair-fed control and deficient group. Liberation of \(^{14}\)CO\(_2\) was almost constant at any time tested in all groups.

**Cholesterol synthesis in the livers of fasted rats**

The results so far obtained indicate that the change in feeding conditions profoundly influences the rate of hepatic cholesterol synthesis. Therefore, rats fed a 20% or 70% casein diet were fasted overnight to set a constant condition in all groups and the cholesterol synthesis was measured in the liver slice system (Table 3). The deficient groups on both 20% and 70% casein diets showed significantly higher activities than the control groups. We thus conclude that hepatic cholesterol synthesis is enhanced in pyridoxine deficiency, not reflecting
Table 2. Incorporation of [14C]acetate into lipid components and liberation of \(^{14}\)CO\(_2\). Rats were divided into 3 groups, each consisting of 12 rats, and 70% casein diets were given for 3 weeks. One group was fed pyridoxine-free diet \textit{ad libitum} and other two groups were fed pyridoxine-supplemented diet as pair-fed control or \textit{ad libitum} control. Rats were killed at the time indicated. The liver slice system was incubated under the conditions described in the text. Values are expressed as pmoles/mg protein/hr of acetate incorporated into lipid or nmoles/mg protein/hr of acetate oxidized to CO\(_2\). Figures are means ± standard deviation.

|                        | Time killed | Deficient   | Control                     |
|------------------------|-------------|-------------|----------------------------|
|                        |             |             | Pair-fed | Ad libitum |
| Free cholesterol       | midday      | 200.1±49.2  | 108.8±13.0 | 465.2±83.9 |
|                        | midnight    | 498.1±117.7 | 479.0±240.9 | 520.7±50.0 |
| Ester cholesterol      | midday      | 21.8±7.5    | 13.2±0.7   | 24.7±1.5   |
|                        | midnight    | 45.6±12.2   | 61.1±24.9  | 61.8±4.5   |
| Triglyceride           | midday      | 187.9±49.0  | 337.1±27.9 | 747.1±76.4 |
|                        | midnight    | 594.5±259.8 | 859.0±142.4 | 1,346±175.4 |
| CO\(_2\)               | midday      | 11.63±0.98  | 14.24±0.07 | 14.02±2.50 |
|                        | midnight    | 13.12±0.41  | 11.33±1.13 | 13.37±1.30 |

Table 3. Metabolism of [14C]acetate in liver slices of rat fed a 20% or 70% casein diet. Rats fed a 70% casein diet were maintained for 3 weeks and a 20% casein diet for 6 weeks. The data were obtained from the same rats with used in Table 1. Other experimental conditions were described in the text. Values were expressed as pmoles/mg protein/hr of acetate incorporated into lipid fraction or nmoles/mg protein/hr of acetate oxidized. Figures are means ± standard deviation.

|                    | 70% Casein | 20% Casein |
|--------------------|------------|------------|
|                    | Deficient  | Control    | Deficient  | Control    |
| Free cholesterol   | 77.9±22.4  | 17.7±6.0   | 242.0±61.1 | 21.9±2.5   |
| Ester cholesterol  | 8.3±1.6    | 1.8±0.5    | 12.5±2.1   | 2.4±0.3    |
| Triglycerides      | 61.6±14.3  | 90.1±16.9  | 152.7±29.7 | 120.8±7.4  |
| CO\(_2\)           | 12.47±1.32 | 13.12±1.96 | 13.23±1.61 | 12.22±1.52 |

the change in the levels of cholesterol in the liver. Triglyceride synthesis and \(^{14}\)CO\(_2\) liberation were not significantly different between the control and the deficient groups.

\textit{Intestinal cholesterogenesis in pyridoxine-deficient rat}

Cholesterol synthesis was also measured in rat intestine which is considered to be the most important site of extrahepatic cholesterogenesis. Since cholesterol synthesis has been shown to be different in the central and distal portions of intestine (23), the incorporation of acetate was studied in both portions. As
shown in Table 4, the distal portion displayed a higher synthetic activity in the 20% casein group. But, in both levels of protein intake, pyridoxine deficiency exerted no effect on intestinal cholesterogenesis.

Table 4. Lipogenesis in intestinal segments. This experiment was carried out using the same rats as in Table 3. Experimental conditions were described in the text. Values were expressed as mean ± standard deviation.

| Cholesterol        | 70% Casein | 20% Casein |
|--------------------|------------|------------|
|                    | Deficient  | Control    | Deficient  | Control    |
| Central            | 80.0±32.6  | 113.2±36.0 | 51.3±0.5   | 31.6±10.3  |
| Distal             | 100.5±61.5 | 102.6±34.7 | 102.4±65.2 | 73.7±34.3  |
| Triglyceride       |            |            |            |
| Central            | 65.2±18.4  | 59.6±14.7  | 14.1±7.5   | 9.6±2.2    |
| Distal             | 58.3±18.6  | 57.5±25.2  | 23.3±5.8   | 35.4±14.7  |

DISCUSSION

Pyridoxine deficiency causes disturbances in lipid metabolism in the liver. We previously reported that the total liver lipid was elevated twice in the pyridoxine-deficient rats fed a 70% casein diet, although no such elevation was observed in the case of a below 30% casein diet (14, 15). The present study has shown that the content of cholesterol, particularly the ester form, is increased in the livers of pyridoxine-deficient rats fed a 70% casein diet and is decreased in pyridoxine-deficient rats fed a 20% casein diet. Thus the discrepancy in the literature with respect to the effect of pyridoxine deficiency on the hepatic cholesterol level can be attributed to the different protein contents in the diets employed by various investigators. The present study has shown that hepatic cholesterogenesis is increased in rats fed pyridoxine-deficient diet containing either a 20% or 70% casein. The rate of hepatic cholesterogenesis is known to be influenced by the level of cholesterol content in the liver.

JAKOI and QUARFORDT (24) have proposed that microsomal cholesterol content may regulate hepatic cholesterogenesis. In the present study, however, no direct relationship was found between the microsomal cholesterol content and the rate of cholesterol synthesis. Serum lipoprotein fraction has also been implicated to play a regulatory role in hepatic cholesterogenesis (25). We found that serum cholesterol level was somewhat decreased in the pyridoxine-deficient groups, but it is questionable if the increased cholesterogenesis by pyridoxine deficiency can be explained by this finding alone. LUPIEN et al. (7-9) and SHAH et al. (10) reported that cholesterol synthesis was stimulated by pyridoxine deficiency in livers of rats fed a 18-22% casein diet, although the hepatic cholesterol content was
unchanged. LUPIEN et al. attributed the stimulation of cholesterogenesis by pyridoxine deficiency to the activation of taurocholate conjugation in the liver since the conjugation reaction in the liver homogenate was inhibited by pyridoxal phosphate. This is not likely since cysteine metabolism in general and taurine formation in particular have been found to be decreased in pyridoxine deficiency (26). We have in fact observed a marked decrease in taurocholate conjugate in the bile of pyridoxine-deficient rat (27). Obviously further work is necessary to clarify cholesterol metabolism in pyridoxine deficiency. We are now investigating the level of 3-hydroxy-3-methylglutaryl CoA reductase activity and the rate of cholesterol turnover in pyridoxine-deficient rats.

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