METABOLISM OF FATTY ACIDS AND THE LEVELS OF KETONE BODIES IN THE LIVERS OF PYRIDOXINE-DEFICIENT RATS

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Summary Lipid metabolism was examined in rats fed a high-protein pyridoxine-deficient diet, and their livers were found to contain large amounts of lipids, mainly in the forms of triglycerides and cholesteryl ester. The contents of ketone bodies in the livers of pyridoxine-deficient and the control rats were similar. Their NAD*/NADH ratios, calculated from the amounts of ketone bodies, were also similar in pyridoxine-deficient and control groups when the animals were fed, but the ratio in pyridoxine-deficient rats was lower than that of control rats when the animals were starved. After injection of 14C-linoleic acid, the amounts of expired 14CO2 in pyridoxine-deficient and control rats were similar. The pattern of incorporations of 14C-linoleic acid into various lipid components of the livers were examined; incorporation into the phospholipid fraction was similar in control and deficient rats, but the incorporation into the triglyceride fraction was slower, and the incorporation into cholesterol was faster in deficient animals than in controls.

Vitamin B6 is known to be involved in lipid metabolism. Swell et al. (1), and Schiefer and Williams (2) reported that vitamin B6 is related to the conversion of linoleic acid to arachidonic acid, but other investigators (3, 4) failed to confirm this role of vitamin B6. Dussault and Lepage (5) suggested that vitamin B6 might be connected with catabolism of arachidonic acid. The lipid content of the livers has been reported to be lowered (1, 6, 7) or unchanged in pyridoxine deficiency (8). Recently we found that the dietary protein level influenced lipid metabolism in pyridoxine-deficient rats (9, 10). On feeding a high (70%) protein diet, deficient rats accumulated triglyceride and cholesteryl ester in the liver. Both Lupien et al. (11–13) and Shah et al. (14) observed no change in the cholesterol content but observed an increase in cholesterogenesis in the livers in pyridoxine-deficient rats. We also obtained evidence for increased cholesterogenesis.
from acetate in rats fed a 70%-casein pyridoxine-deficient diet (15). We are interested in the mechanism of fatty liver induced by a high-protein pyridoxine-deficiency. It is known that this fatty liver is not caused by a decreased synthesis of lipoprotein (16) or choline deficiency (10). In the present work we studied the incorporation of linoleic acid into various lipid components and fatty acid oxidation in pyridoxine-deficient rats in vivo. We also determined ketone bodies in their livers in relation to cholesterol metabolism.

MATERIALS AND METHODS

Male Wistar strain rats were purchased from Nishin Kikai Co. All dietary materials except vitamin-free casein (NBC) were obtained from Tanabe Amino Acid Research Foundation, and 3-hydroxybutyrate dehydrogenase [EC 1.1.1.30], NAD and NADH were from Boehringer Mannheim. 1-14C-Linoleic acid (35 mCi/mmole) was from Daiichi Pure Chemicals Co., Ltd.

Animals. Rats were maintained on a 20% casein diet for 3 days. Then rats weighing 50 to 60 g were fed a 70% casein diet for 3 to 4 weeks. The composition of the diet (in g) was the same as described previously (17): vitamin-free casein, 70; sucrose, 10; cornstarch, 7; oil mixture, 8; choline chloride, 0.2; salt mixture, 4; vitamin mixture with or without pyridoxine, 1. The rats were divided into two groups: the pyridoxine-deficient animals received the diet ad libitum and the control animals were pair-fed with the deficient animals on a pyridoxine-supplemented diet. Radioactive potassium linoleate was prepared by adding alcoholic KOH to dried 14C-linoleic acid, and emulsified by adding 0.9% NaCl and defatted bovine serum albumin (18). This emulsion contained 50 μCi/ml (1.42 μmole) and doses of 0.1 ml per 100 g body weight were injected into the tail vein of rats (19). Rats were usually sacrificed between 10:00 and 12:00 hr to determine incorporation of 14C-linoleic acid into their lipid fractions.

Preparation of liver powder. Animals were decapitated and their livers were rapidly removed and immersed in dry ice-acetone. The frozen liver was pulverized to fine powder in a Waring blender and samples of the powder were used to determine ketone bodies and lipids.

Determination of ketone bodies. Liver samples were prepared by the method of BERRY et al. (20): the frozen liver powder was homogenized in 30% HClO4 and the supernatant was neutralized with KOH; the mixture was centrifuged and samples of the supernatant were treated with florisil to remove flavin and analysed for β-hydroxybutyrate and acetoacetate enzymatically (21). NAD+/NADH ratios were calculated from the concentrations of these ketone bodies by the method of WILLIAMSON et al. (22).

Extraction and assay of liver lipids. Samples of liver were homogenized in 0.9% NaCl solution, and the lipids were extracted by the method of FOLCH et al. (23), and determined gravimetrically as described before (17). The lipid fraction was separated by thin-layer chromatography on a silica gel G plate with a
mixture of petroleum ether–ethyl ether–acetic acid (80:20:1, by vol.) as solvent. The bands of lipid on the plate were detected with iodine vapor and scrapped off. Free cholesterol, cholesteryl ester and triglyceride were extracted with chloroform–methanol (2:1, by vol.), and cholesterol (free and ester) and triglyceride were measured by the method of ZAK (24) and a modification of the method of VAN HANDEL (25), respectively. Phospholipid was either measured directly in the crude lipid fraction as inorganic phosphate by a slightly modification of the method of HOELMAYER-FRIED (26), or it was extracted from the thin-layer chromatogram with chloroform–methanol–acetic acid–water (50:39:1:10, by vol.) (27). The recoveries of cholesterol, cholesteryl ester and triglyceride were over 95%, and that of phospholipid was about 70%. The cholesteryl ester fraction was hydrolyzed by a modification of the method of FLINT (28); then the hydrolysate was rechromatographed, and the fractions corresponding to cholesterol and cholesteryl ester were eluted and their radioactivities were determined. The radioactivities of the fatty acid and cholesterol moiety were calculated as follows:

\[ C_{(e)} = CE \times \frac{C}{C+F} \]
\[ F_{(e)} = CE \times \frac{F}{C+F} \]

Where \( C_{(e)} \) is the activity of the cholesteryl moiety, \( F_{(e)} \) is that of esterified fatty acid, \( CE \) is that of cholesteryl ester before hydrolysis, \( C \) is that of cholesterol after hydrolysis and \( F = CE - (C + \text{radioactivity of cholesteryl ester remained after hydrolysis}) \). The rates of hydrolysis were more than 90% under the conditions used.

**Determination of expired \(^{14}\text{CO}_2\)**. The expired \(^{14}\text{CO}_2\) from a rat injected with \(^{14}\text{C}-\text{linoleic acid} \) was collected for 1 hour in ethanolamine–ethylene glycol monomethylether (1:2, by vol.) by application of a negative pressure of 7–8 mmHg (29, 30) and the radioactivity was determined.

**Measurement of radioactivities**. The radioactivities of lipid fractions and \(\text{CO}_2\) were measured in toluene-based scintillator and toluene–ethylene glycol monomethylether (2:1, by vol.) scintillator, respectively, with an Aloka liquid scintillation counter.

**RESULTS**

**Contents of liver lipids**

As described before (9, 10), the amounts of lipids, and especially triglyceride and cholesteryl ester in the liver were higher in pyridoxine-deficient rats than in the controls (Table 1).

**Levels of ketone bodies and the \(\text{NAD}^+ / \text{NADH} \) ratio**

The accumulation of cholesteryl ester in the liver of pyridoxine-deficient rats prompted us to test whether the synthesis of ketone bodies decreased in the deficient rats. Accordingly, we measured the amounts of \(\beta\)-hydroxybutyrate and acetoacetate in the livers of fed and starved (for 14 hours) rats. As shown in
Table 1. Analysis of liver lipid.

| Diet          | Total lipid (mg/g) | Phospholipid (µmole/g) | Triglyceride (µmole/g) | Free cholesterol (µmole/g) | Cholesteryl ester (µmole/g) |
|---------------|--------------------|------------------------|------------------------|---------------------------|-----------------------------|
| Deficient (10) | 71.3±21.1          | 47.2±8.76              | 21.4±14.8              | 4.53±0.70                 | 1.77±0.94                   |
| Control (10)  | 43.4±2.53          | 46.6±10.4              | 3.72±0.88              | 4.34±0.33                 | 0.76±0.17                   |

ns: not significant as compared to control. Feeding conditions are described in the text. Figures in parentheses are number of rats. Values are expressed as means±S.D.

Table 2, when the rats were fed, the amounts of both ß-hydroxybutyrate and acetoacetate were similar in the pyridoxine-deficient and control groups. In starved rats, the level of ß-hydroxybutyrate in the liver of pyridoxine-deficient rats tended to increase, but this increase was not significant (p>0.05). Thus the levels of total ketone bodies were similar in pyridoxine-deficient and control animals. Redox state in various compartment can be calculated by measuring the ratio of the concentrations of the oxidized and reduced metabolites of suitable NAD-linked dehydrogenase systems that are located in different cell compartments. WILLIAMSON et al. (22) showed that the use of ß-hydroxybutyrate-acetoacetate system is suitable for the assessment of the NAD+/NADH ratio within the rat liver mitochondria. The NAD+/NADH ratios were therefore calculated from the these levels of ketone bodies (Table 3); when the rats were fed, the NAD+/NADH ratios of pyridoxine deficient and control rats were similar, whereas when the rats were starved, the ratio was lower in the deficient rats due to increase in NADH.

Metabolism of linoleic acid in vivo

After injection of 14C-linoleic acid, the expired 14CO2 was collected for 1 hour and its radioactivity was determined. As shown in Table 4, there was no difference in the radioactivities in two groups. The incorporations of injected 14C-linoleic acid into various lipid fractions were also determined after various times; linoleic acid was incorporated into the phospholipid fraction of pyridoxine-deficient rats rather slowly but very similar pattern were observed in the two groups (Fig. 1). The incorporation of linoleic acid into triglycerides is shown in Fig. 2; slow incorporation and delayed removal of triglycerides were observed in the deficient group. Figure 3 shows the incorporation of linoleic acid into the free cholesterol fraction; the rate of incorporation was greatly increased in the deficient animals, but the rates of removal of radioactivity from the cholesterol fraction were similar in the two groups. The incorporations of linoleic acid into cholesteryl ester were initially similar in the two groups, but later the pyridoxine-deficient rats showed increased incorporation (Fig. 4). The radioactivities incorporated in the earlier period were found entirely in the fatty acid moiety of cholesteryl ester in both groups (Table 5).
Table 2. Levels of ketone bodies in the liver.

|                  | Fed (6)       | Starved (9)   |
|------------------|---------------|---------------|
| Pyruvate (mole/%) | 0.298±0.108   | 0.705±0.226   |
| Acetoacetate (mole/%) | 0.074±0.028 | 0.085±0.020   |
| Total (mole/kg)   | 0.373±0.099   | 0.468±0.360   |

Fed (6) and Starved (9) values are expressed as means±S.D.

Deficient vs. Control: ns; not significant as compared to control. Feeding conditions and analytical methods are described in the text. Values are expressed as means±S.D.
DISCUSSION

Pyridoxine deficiency is known to disturb lipid metabolism. We previously reported increases in the contents of triglyceride and cholesteryl ester in the liver of rats fed a high-protein pyridoxine-deficient diet (9, 31), and we confirmed these results in the present study (Table 1). Cholesterol and ketone bodies are

Table 3. Redox state of liver mitochondria.

|            | NAD⁺/NADH |
|------------|-----------|
|            | Fed (6)   | Starved (9) |
| β-Hydroxybutyrate/Acetoacetate |           |
| Deficient  | 4.86±2.38 | 8.36±2.41   |
| Control    | 4.88±2.85 | 4.30±2.02   |
| p          | ns        | <0.01       |

ns: not significant as compared to control.

NAD⁺/NADH was calculated from $\frac{[\text{Acetoacetate}][\text{NADH}]}{[\beta\text{-Hydroxybutyrate}][\text{NAD}^+]^+} = K$, $K=4.93 \times 10^{-2} \text{mM}$, obtained by WILLIAMSON et al. (22), was used in this calculation (38°C, pH 7.0 and I, 0.25).

Fig. 1. Incorporation of ¹⁴C-linoleic acid into phospholipid. ●, Deficient; ○, control. The same animals were used in the experiments shown in Fig. 1–4. Experimental conditions were described in the text. Vertical lines show the extents of standard deviations.

Fig. 2. Incorporation of ¹⁴C-linoleic acid into triglyceride. ●, Deficient; ○, control.
both synthesized via 3-hydroxy-3-methylglutaryl CoA (32, 33). Thus the accumulation of cholesteryl ester in the pyridoxine-deficient rats suggested that the syntheses of ketone bodies might be abnormal. But does not seen to be so,

![Fig. 3. Incorporation of 14C-linoleic acid into cholesterol. $\bullet$, Deficient; $\circ$, control.](image1)

![Fig. 4. Incorporation of 14C-linoleic acid into cholesteryl ester. $\bullet$, Deficient; $\circ$, control.](image2)

Table 4. Liberation of 14CO₂ from 14C-linoleic acid.

|                | cpm/100 g body weight x 10⁻⁴ |
|----------------|-----------------------------|
| Deficient (4)  | 102.00±6.55                 |
| Control (4)    | 135.00±55.60                |
| $p$            | ns                          |

ns: not significant as compared to control. Experimental conditions are described in the text. Values are expressed as means±S.D.

Table 5. Distribution of radioactivities in cholesteryl ester.

|                | min* | Deficient (4)   | Control (4)  | $p$   |
|----------------|------|-----------------|--------------|-------|
| Fatty acids (cpm/g) | 7.5  | 650±291         | 947±173      | ns    |
|                 | 15.0 | 975±251         | 767±134      | ns    |
| Cholesterol (cpm/g) | 7.5  | 81±54           | 79±1         | ns    |
|                 | 15.0 | 81±50           | 54±13        | ns    |

* Time after injection. ns: not significant as compared to control. This experiment was carried out using the same animals as in Fig. 1-4. Experimental conditions were described in the text. Values are expressed as means±S.D.
because we could not detect any appreciable difference the levels of ketone bodies in the livers of pyridoxine-deficient and control animals. Recently, CLINKENBEARD et al. (34) suggested that there might be separate pools of 3-hydroxy-3-methylglutaryl CoA. Fatty liver may also be caused by impaired oxidation of fatty acid: ARTOM (35) reported inhibition of oxidation of stearic acid in animals treated with DL-ethionine and CORREDOR (36) also described impaired oxidation of long chain fatty acid in choline-deficient animals. However, in this work we obtained no evidence for impaired oxidation of fatty acids in pyridoxine-deficient rats from either the mitochondrial NAD+/NADH ratio, calculated from the values for ketone bodies, or results on the oxidation of injected 14C-linoleic acid (Tables 3 and 4). We also observed no difference in the oxidations of acetate in liver slices from pyridoxine-deficient and control rats (31). Studies on the pool sizes of fatty acids in pyridoxine-deficient and control animals are necessary to clarify this problem.

In this work we also examined the incorporation of 14C-linoleic acid into phospholipids. The radioactivities in phospholipids may be largely due to that in phosphatidic acid, which has a more rapid turnover than other phospholipids (27, 37). The incorporation of 14C-linoleic acid into triglycerides in pyridoxine-deficient and control rats were different from 45 minutes after the times of injection (Fig. 2); the slower removal of radioactivity in deficient rats may be due to impaired oxidation, impaired secretion from the liver or increased influx from extrahepatic tissues. It could also be due to indirect utilization of linoleic acid from fatty ester other than triglyceride (38) or reutilization of acetate produced from linoleic acid. In the latter case the glycerol moiety of triglycerides may also contain radioactivity. Increased cholesterogenesis in the livers of rats fed a pyridoxine-deficient diet has been reported from this laboratory and by others (11–15, 31), and it was confirmed in the present study using 14C-linoleic acid (Fig. 3). As shown in Fig. 4, the rates of esterification of cholesterol in the deficient and control groups were very similar. Thus it seems likely that cholesteryl ester accumulates in the liver of pyridoxine-deficient rats because synthesis of the cholesterol moiety increases, rather than because the rate of esterification increases.

REFERENCES

1) SWELL, L., LAW, M. D., SCHOOLS, P. E., Jr., and TREADWELL, C. R. (1961): Tissue lipid fatty acid composition in pyridoxine-deficient rats. J. Nutr., 74, 148–156.
2) SCHEIER, G. E., and WILLIAMS, M. A. (1964): Sequential changes in liver and heart lipids after giving linoleate or linoleate plus pyridoxine to rats depleted of fat and pyridoxine. Biochem. J., 92, 422–429.
3) KURSCHMAN, J. C., and CONIGLIO, J. G. (1961): The role of pyridoxine in the metabolism of polyunsaturated fatty acids in rats. J. Biol. Chem., 236, 2200–2203.
4) SÖDERJUEL, L. (1962): Influence of pyridoxine and dietary fat on the distribution of serum fatty acids in dogs. J. Nutr., 78, 438–444.
5) DUSSAULT, P. E., and LEPAGE, M. (1975): Effects of pyridoxine deficiency on the com-
position of plasma and liver fatty acids in rats fed low and high fat diets. *J. Nutr.*, 105, 1371–1376.

6) Carter, C. W., and Phizackerley, J. R. (1951): The influence of pyridoxine on fat metabolism in the rat. *Biochem. J.*, 49, 227–232.

7) Audet, A., and Lupien, P. J. (1974): Triglyceride metabolism in pyridoxine-deficient rats. *J. Nutr.*, 104, 91–100.

8) Desikachar, H. S. R., and McHenry, E. W. (1954): Some effects of vitamin B₆ deficiency on fat metabolism in rats. *Biochem. J.*, 56, 544–547.

9) Okada, M., and Ochi, A. (1971): Effect of dietary composition on vitamin B₆ deficiency state (II). Fatty liver induced by high protein diet without B₆. *Vitamins* (in Japanese), 43, 241–244.

10) Suzuki, K., Nakamura, T., Fujita, M., Iwami, T., Abe, M., and Okada, M. (1976): Factors affecting liver lipid content in pyridoxine-deficient rats. I. Dietary protein levels. *J. Nutr. Sci. Vitaminol.*, 22, 291–298.

11) Lupien, P. J., Hinse, C. M., and Avery, M. (1969): Cholesterol metabolism and vitamin B₆. I. Hepatic cholesterogenesis and pyridoxine deficiency. *Can. J. Biochem.*, 47, 631–635.

12) Hinse, C. M., and Lupien, P. J. (1971): Cholesterol metabolism and vitamin B₆. III. The stimulation of hepatic cholesterogenesis in the vitamin B₆-deficient rat. *Can. J. Biochem.*, 49, 933–935.

13) Avery, M. D., and Lupien, P. J. (1971): Cholesterol metabolism and vitamin B₆. IV. Synthesis of cholic acid conjugates in vitamin B₆-deficient rats. *Can. J. Biochem.*, 49, 1026–1030.

14) Shah, S. N., Johnston, P. V., and Kummerow, F. A. (1960): The effect of pyridoxine on cholesterol metabolism. *J. Nutr.*, 72, 81–86.

15) Suzuki, K., and Okada, M. (1973): *Seikagaku* (in Japanese, Abstract of Japanese Biochemical Society), 45, 529.

16) Okada, M., and Suzuki, K. (1974): Amino acid metabolism in rats fed a high protein diet without pyridoxine. *J. Nutr.*, 104, 287–293.

17) Okada, M., and Ochi, A. (1971): The effect of dietary protein level on transaminase activities and fat deposition in vitamin B₆-depleted rat liver. *J. Biochem.*, 70, 581–585.

18) Garland, P. B., Newsom, E. A., and Randle, P. J. (1964): Regulation of glucose uptake by muscle. *Biochem. J.*, 93, 665–678.

19) Schotz, M. C., and Oliver, T. (1966): The effect of anesthesia on the fate of injected free fatty acid. *Biochim. Biophys. Acta*, 125, 174–175.

20) Berry, M. N., Williamson, D. H., and Wilson, M. B. (1965): Concentrations of acetocetate and d-(-)-3-hydroxybutyrate in rat liver and blood. *Biochem. J.*, 94, 17c–19c.

21) Williamson, D. H., Mellany, J., and Krebs, H. A. (1962): Enzymic determination of d-(-)-3-hydroxybutyric acid and acetocetic acid in blood. *Biochem. J.*, 82, 90–96.

22) Williamson, D. H., Lund, P., and Krebs, H. A. (1967): The redox state of free nicotinamide adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.*, 103, 514–527.

23) Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957): A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, 226, 497–509.

24) Zark, B. (1957): Simple rapid microtechnique for serum total cholesterol. *J. Clin. Path.*, 27, 583–588.

25) Kawai, M. (1966): *Rinsho-kagaku Bunseki*, Tokyo Kagaku Dojin, Vol. 3, p. 40.

26) Kuo, H., and Fukuji, I. (1967): Determination of serum phospholipids. The method of H-Eoffmeyr-Fried. *Rinsho Byori*, 15, 853–856.

27) Åkesson, B. (1970): Initial esterification and conversion of intraportally injected [1-¹⁴C]
linoleic acid in rat liver. Biochim. Biophys. Acta, 218, 57–70.

28) FLINT, A. P. F., and DENTON, R. M. (1969): Glucose metabolism in the superovulated rat ovary in vitro. Effects of luteinizing hormone and the role of glucose metabolism in steroidogenesis. Biochem. J., 112, 243–254.

29) ARTHUR, F. A., and VON SCHUCHING, S. L. (1970): Catabolism of ascorbic acid labeled with radioactive carbon to CO₂. Excretory pathway of ¹⁴CO₂ by respiratory exhalation, in Methods in Enzymology, ed. by Colowick, S. P. and Kaplan, N. O., Academic Press, New York and London, Vol. 18, pp. 34–46.

30) JEFFAY, H., and ALVAREZ, J. (1961): Liquid scintillation counting of carbon-14 use of ethanolamine-ethylene glycol monomethyl ether-toluene. Anal. Chem., 33, 612–615.

31) IWAMI, T., SUZUKI, K., and OKADA, M. (1975): Seikagaku (in Japanese, Abstract of Japanese Biochemical Society), 47, 501.

32) BARTH, C. A., HACKENSCHMIDT, H. J., WEIS, E. E., and DECHER, K. F. A. (1973): Influence of kynurenate on cholesterol and fatty acid synthesis in isolated perfused rat liver. J. Biol. Chem., 248, 738–739.

33) WILLIAMSON, D. H., BATES, M. W., and KREBS, H. A. (1968): Activity and intracellular distribution of enzymes of ketone-body metabolism in rat liver. Biochem. J., 108, 353–361.

34) CLINKENBEARD, K. D., REED, W. D., MOONEY, R. A., and LANE, M. D. (1975): Intracellular localization of the 3-hydroxy-3-methylglutaryl coenzyme A cycle enzymes in liver. J. Biol. Chem., 250, 3108–3116.

35) ARTOM, C. (1959): Fatty acid oxidation in the livers of rats receiving DL-ethionine. J. Biol. Chem., 234, 2259–2264.

36) CORREDOR, C., MANSBACH, C., and BRESSLER, R. (1967): Carnitine depletion in the choline-deficient state. Biochim. Biophys. Acta, 144, 366–374.

37) VAVRČEKA, M., MITCHELL, M. P., and HÜBNER, G. (1969): The effect of starvation on the incorporation of palmitate into glycerides and phospholipids of rat liver homogenates. Biochem. J., 115, 139–145.

38) BAKER, N., and SCHOTZ, M. C. (1967): Quantitative aspects of free fatty acid metabolism in the fasted rat. J. Lipid Res., 8, 646–660.