Effect of cadmium treatment on hepatic flavin metabolism

Debashis Bandyopadhyay, Ajay K. Chatterjee, and Asoke G. Datta

Indian Institute of Chemical Biology, Calcutta, India; and Department of Physiology, University of Calcutta, Calcutta, India

The effect of cadmium on levels of hepatic riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), as well as the activities of flavokinase and FMN-phosphatase were studied following injection of cadmium sulfate solution subcutaneously to male Sprague-Dawley rats at a dose of 0.44 mg/kg body weight, every alternate day for 15 days. The body weights and wet weights of liver were found to increase significantly following cadmium treatment ($P < 0.01$ and $P < 0.001$, respectively). The levels of hepatic-free riboflavin and FAD increased, while the FMN level remained unaltered ($P < 0.01$, $P < 0.001$, and $P > 0.05$, respectively). A significant decrease in activities of hepatic flavokinase and FMN-phosphatase were observed both in in vivo and in vitro experiments, although the degree of inhibition was found to be more pronounced in case of flavokinase than FMN-phosphatase ($P < 0.001$ and $P < 0.05$, respectively).

Keywords: liver; riboflavin; flavin mononucleotide; flavokinase; flavin mononucleotide phosphatase; cadmium

Introduction

The importance of riboflavin (vitamin B_2) in human nutrition has long been appreciated. The metabolic role of riboflavin largely depends on its conversion to two coenzymes, flavin mononucleotide (FMN) and flavin dinucleotide (FAD). The formation of such coenzymic forms depends on a number of enzymatic reactions that might be sensitive to metal ions. But, to the best of our knowledge, no attempt has yet been made to investigate the role of cadmium on the metabolism of these two coenzymes. The present investigation was undertaken to study the effect of cadmium on riboflavin metabolism. Accordingly, the present report deals with the measurements of hepatic levels of free riboflavin, FMN, and FAD, and the activities of two of the associated enzymes, flavokinase and FMN phosphatase.

Methods and materials

Materials

Cadmium sulfate ($3CdSO_4.8H_2O$) was purchased from E. Merck, Darmstadt, Germany. Riboflavin, adenosine triphosphate (ATP), and FMN were procured from Sigma Chemical Company (St. Louis, MO USA). Other chemicals used were of analytical grade.

Address reprint requests to Dr. Asoke Datta at the Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Calcutta 700032 India.
Received May 14, 1992; accepted March 2, 1993.

Animals and diet

Male Sprague-Dawley rats, I.I.C.B. strain, weighing approximately 150–160 g were used for the present investigation. They were housed individually in cages in a temperature-(22–24° C) and light-controlled room (12 hr/day). Water was fed ad libitum. All rats were allowed to acclimate for at least 7 days and were fed a diet containing 18% protein (casein) along with other ingredients, which were the same as reported elsewhere.[1–3] Thus, the other ingredients—carbohydrate (Amylum), fat (ground nut oil), and salt mixture—were kept at 71%, 7%, and 4%, respectively. The composition of the salt mixture used was as described by Hawk and Oser.[4] Fat-soluble and water-soluble vitamins were furnished according to Berg.[5] The amounts of fat-soluble vitamins added per 100 g of the diet were: cod liver oil concentrate (Adexolin, Glaxo, Bombay, India), 0.2 mg; (120 I.U. vitamin A and 20 I.U. vitamin D), α-tocopherol acetate (Viteolin, Glaxo), 3 mg; acetomenaphthone (Kapalin, Glaxo), 0.2 mg. Water-soluble vitamins together with inositol and choline chloride were also added per 100 g of the diet as follows: vitamin B_{12}, 0.005 mg; biotin, 0.06 mg; thiamin hydrochloride, 1.0 mg; folic acid, 1.1 mg; para-aminobenzoic acid, 2.0 mg; riboflavin, 2.0 mg; nicotinic acid, 4.0 mg; calcium pantothenate, 10.0 mg; inositol, 80.0 mg; and choline chloride 100 mg.

Treatment of animals with cadmium

Before commencement of the treatment, rats were divided into two groups of equal average body weight. The animals of one group were injected subcutaneously with a cadmium sulfate solution at a dose of 0.44 mg/kg body weight every alternate day for 15 days. This dose of cadmium and the period of treatment were close to those described elsewhere.[6] The

animals of the other group served as pair-fed controls. Under these experimental conditions, cadmium had no apparent toxicity and none of these animals died during this period. The body weight gain and food intake did not differ between cadmium-treated animals and their pair-fed controls. However, when the dose of cadmium was increased to 0.88 mg/kg body weight, five animals out of seven died in 24 hr.

Removal of liver tissue

The animals were fasted overnight before sacrifice. The rats were anesthetized with sodium pentobarbitone (50 mg/kg body weight) and livers were removed, chilled, blotted dry, and weighed. A portion of these livers were immediately used for estimation of tissue flavins and the remaining portions were deep frozen for studying the activities of flavokinase and FMN phosphatase.

Storage of liver tissue at frozen condition did not alter flavokinase and FMN phosphatase activities under our experimental conditions.

Estimation of flavins

The fresh liver tissue was homogenized in a glass homogenizer with 10 mL of ice-cold water per gram of tissue. Thirty percent of the cold suspension was mixed with an equal volume of ice-cold 11% TCA, and the mixture was kept undisturbed in cold (0–4° C) for 15 minutes. The precipitated proteins were removed by centrifugation at 5,000 rpm for 15 minutes in a Sorvall Model RC-5B refrigerated centrifuge. The aliquots of the supernatant thus obtained were employed for the fluorometric estimation of free riboflavin, flavin mononucleotide, and flavin dinucleotide according to the method of Burch.[7] Special care was taken to protect the samples from light because riboflavin and FMN are sensitive to destruction by light. The samples were kept in ice during the estimation procedure to prevent hydrolysis of FAD.

Preparation of supernatant for the assay of enzymes

A 20% homogenate of liver tissue was prepared in a glass-homogenizer. Sucrose[8] (0.25 mol/L) and ice-cold water[9] were used as homogenizing media for flavokinase and FMN phosphatase, respectively. All subsequent operations were performed at 0–4° C. The homogenates were centrifuged at 18,500*g* for 30 minutes in Sorvall Model RC-5B-refrigerated centrifuge. The supernatants thus obtained were used as an enzyme preparation.

Assay of flavokinase

Flavokinase was assayed according to the method of McCormick[10] with the exception that the final volume of incubation mixture was 1.0 mL instead of 5.0 mL. The incubation mixture, in a final volume of 1.0 mL, contained riboflavin (0.2 mmol/L), potassium phosphate buffer; pH 8.0 (75 mmol/L), adenosine triphosphate; pH 8.0, (1 mmol/L) zinc sulfate (0.1 mmol/L), and appropriate volume of enzyme solution. The reaction was started by the addition of ATP. Incubation was carried out in the dark at 37° C for 60 minutes in a shaker incubator. The reaction was terminated by the addition of 0.4 mL of 17.5% trichloroacetic acid (TCA). Each time a control was run by adding TCA before the addition of the enzyme, FMN, the product of the reaction was determined by the differential extraction method of Burch et al.,[11] as modified by Kearney and Englard.[12]

Assay of FMN phosphatase

FMN phosphatase was assayed by the method of McCormick and Russell[9] with some modification. Mixtures for determining phosphatase activity contained FMN (0.2 mmol/L); sodium acetate buffer, pH 5.0 (75 mmol/L); and different volumes of enzyme solution in a final volume of 2.0 mL. The reaction was started by the addition of the enzyme. The incubation was carried out in the dark at 37° C for 30 minutes in a shaker incubator. The reaction was terminated by the addition of 0.8 mL of 17.5% TCA. A control was run as described above. The precipitated protein was removed by centrifugation. Riboflavin formed during the enzymatic reaction was measured in an aliquot of neutralized filtrate by Kearney's adaptation[12] of the differential extraction method of Burch et al.[11] Liberation of inorganic phosphate during hydrolysis of FMN was determined spectrophotometrically at 820 nm in a Pye-Unicam SP-800 UV/VIS spectrophotometer by Ame's method.[13]

In vitro effect of cadmium on flavokinase and FMN phosphatase

A portion of liver from control animals was homogenized (20% homogenate) in 0.25 mol/L sucrose for flavokinase, and ice-cold water for FMN-phosphatase. The 18,000*g* supernatant obtained by centrifugation was used as the source of enzyme. The enzyme preparation was incubated in vitro for flavokinase and FMN phosphatase assays in presence or absence of different concentrations of the cadmium sulfate solution.

Protein analysis

The protein was determined by the method of Lowry et al.[14] using bovine serum albumin as standard.

Statistical analysis

A two-tail Student's *t* test by the difference method was employed for statistical analysis.

Results

The present investigation shows that subcutaneous administration of cadmium at a dose of 0.44 mg/kg body weight caused a significant increase in the body weight of animals, as well as a highly significant increase in the wet weight of livers *(Table 1)*. *Figure 1* shows that cadmium-treated rats maintained a steady gain in body weight during the study period. The average food intake of cadmium-treated rats per day during that period re-

Table 1 Effect of administration of cadmium on body weight and wet weight of liver of rat

Groups of animals	Body weight (g)	Liver weight (g)
Pair-fed controls	197.1 ± 13.6 (7)	5.71 ± 0.60 (7)
Treated	214.3 ± 8.4 (7)*	5.92 ± 0.46 (7)†

The values are means ± SD. The figures in the parentheses indicate the number of animals.
*$P < 0.01$ of respective controls.
†$P < 0.001$ of respective controls.

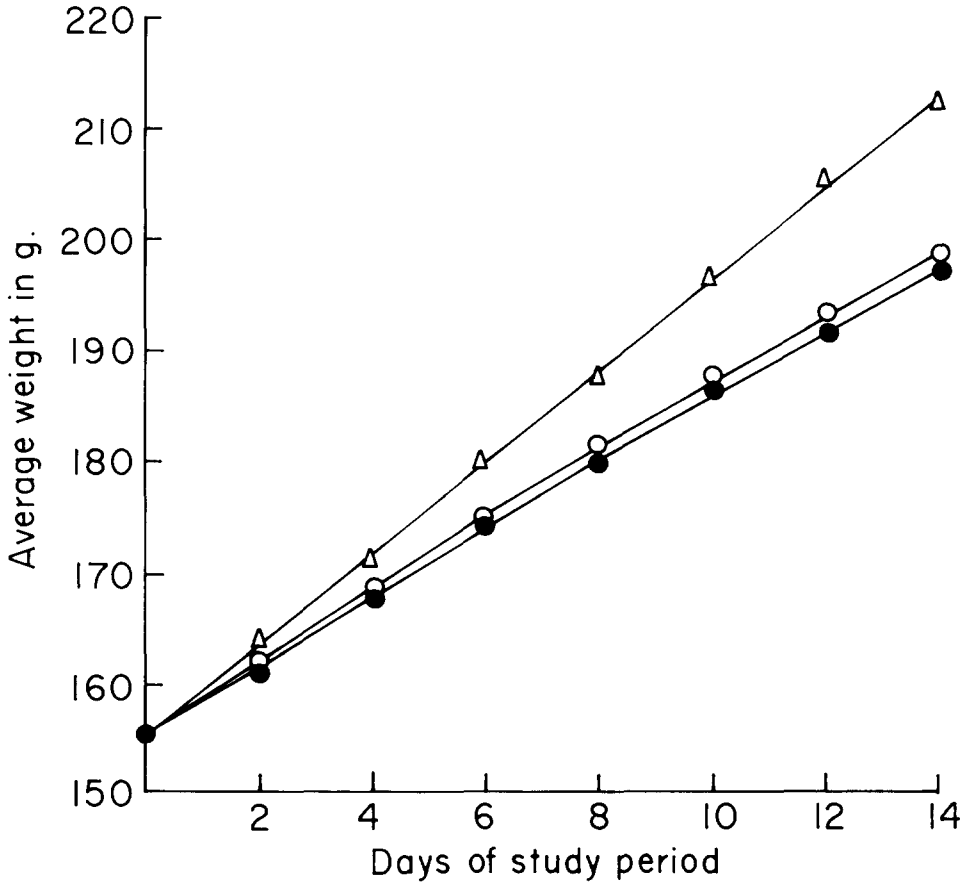

Figure 1 Body weight gain of rat during the study period. Ad libitum control o——o. Pair-fed control ●——●. Cadmium-treated △——△.

mained fairly constant *(Figure 2)*. *Table 2* shows that hepatic levels of free riboflavin and FAD increased, while FMN levels remained unaltered following such treatment. Further, a significant decrease in hepatic flavokinase *(Table 3)* and FMN-phosphatase *(Table 4)* activities were observed, and the decrease in flavokinase activity was found to be more pronounced compared with FMN-phosphatase activity.

It can be seen from *Table 5* that cadmium inhibited both flavokinase and FMN-phosphatase in vitro, and the inhibitions were dose dependent. Cadmium was more effective on flavokinase than on phosphatase in the in vitro experiment as well as the in vivo experiment.

Discussion

Studies on the impact of cadmium toxicity on vitamin metabolism are scarce.[15] A literature survey shows very few reports on the effect of cadmium on the metabolism of B-vitamins, and riboflavin in particular, at the cellular level. It is needless to state that the coenzymes derived from these vitamins are fundamental in controlling tissue metabolism.

Treatment of rats with the present dose of cadmium for a period of 15 days increased their body weights when compared with controls. A highly significant increase in the wet weight of livers of the treated rats were in conformity with an earlier report.[16] This increase in the wet weight of liver may be due to hypertrophy of the tissue as reported by Dudley et al.[17] Although histological examinations of body tissues and behavioral studies were not undertaken in the present investigation, Dudley et al.[18] reported histological studies on liver and kidney tissues of rats chronically exposed to cadmium. Prior to 4 weeks of cadmium treatment they did not find any cell injury or other morphological changes in liver or in kidney. The dose we used in the present investigation did not show any observable changes in behavioral pattern in animals injected with cadmium, although contradictory results have been reported on behavioral effects of cadmium.[19-21]

The significant increase in the hepatic level of free riboflavin in cadmium-treated rats was found to be accompanied by diminished activity of flavokinase. This indicates an impairment of further utilization of riboflavin.

The present investigation also revealed that cadmium treatment inhibited FMN-phosphatase activity significantly, but the inhibition is much less compared with flavokinase.

Although we have not studied the effect of cadmium in vivo on a dose-dependent manner, similar studies were undertaken in in vitro. These studies revealed increasing inhibition of both flavokinase and FMN-phosphatase with increasing concentration of cadmium *(Table 5)*. Like in vivo effects, the in vitro effects of cadmium on

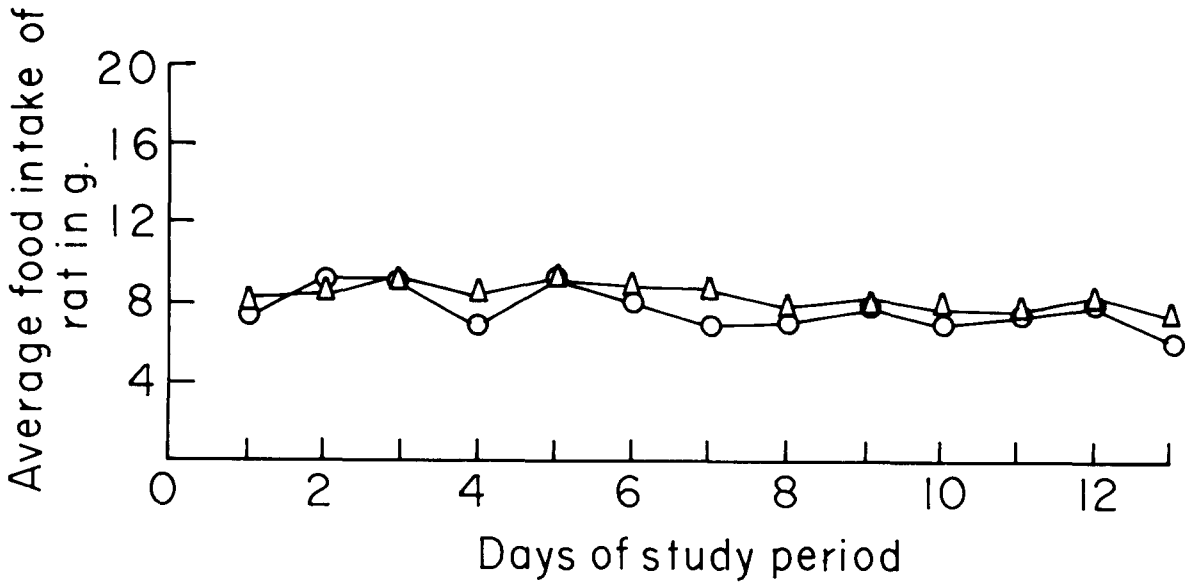

Figure 2 Food intake of rats during the study period. Cadmium-treated ○——○. Ad libitum control △——△.

Table 2 Effect of administration of cadmium on hepatic levels of free riboflavin, FMN, and FAD

Groups of animals	Riboflavin (μg/g liver)	FMN (μg/g liver)	FAD (μg/g liver)
Pair-fed control	5.92 ± 0.78 (6)	11.75 ± 1.86 (6)	35.62 ± 3.38 (6)
Treated	9.24 ± 1.59 (6)*	12.16 ± 2.1 (6)†	46.38 ± 4.1 (6)‡

The values are means ± SD. The figures in parentheses indicate the number of animals.
*$P < 0.01$ of respective controls.
†$P > 0.05$ of respective controls.
‡$P < 0.001$ of respective controls.

Table 3 Effect of administration of cadmium on hepatic flavokinase of rat

	Flavokinase activity			
Groups of animals	nmol FMN/g liver/hr	% Control	nmol FMN/mg protein/hr	% Control
Pair-fed controls	480.3 ± 89.5 (7)	100	4.02 ± 1.07 (7)	100
Treated	271.7 ± 77.6* (7)	55.6	2.19 ± 1.02* (7)	54.5

The values are mean ± SD. The figures in parentheses indicate the number of animals.
*$P < 0.001$ of respective controls.

Table 4 Effect of administration of cadmium on hepatic FMN phosphatase activity of rat

	Riboflavin				Inorganic phosphate			
Group of animals	μmol/g liver/hr	% Control	μmol/mg protein/hr	% Control	μmol/g liver/hr	% Control	μmol/mg protein/hr	% Control
Pair-fed controls	18.31 ± 3.2(3)	100.0	2.9 ± 0.63(4)	100.0	21.84 ± 5.8(3)	100.0	3.6 ± 0.8(3)	100.0
Treated	13.86 ± 2.34(3)*	75.7	2.13 ± 0.34(4)*	91.42	15.64 ± 3.54(3)*	71.61	2.6 ± 0.5(3)*	72.2

The values are mean ± SD. Figures in parentheses indicate number of animals.
*$P < 0.05$ of respective controls.

Table 5 Effect of cadmium on flavokinase and FMN phosphatase in vitro

Enzyme	Cadmium concentration (mol/L)	Product formed (nmol)	% inhibition
Flavokinase	None	7.0 ± 0.72	—
	10^{-5}	5.27 ± 0.25	24.7
	10^{-4}	4.39 ± 0.01	37.3
	10^{-3}	*3.53 ± 0.22	49.6
FMN-phosphatase	None	79.49 ± 1.27	—
	10^{-5}	76.47 ± 0.79	3.8
	10^{-4}	70.41 ± 2.97	11.4
	10^{-3}	†62.43 ± 1.70	21.5

For flavokinase assay, 2 mg enzyme protein was used, and FMN produced was measured. In phosphatase assay 0.05 mg protein was used and riboflavin formed was measured. Other conditions were as described in the Methods section. Values are mean ± SD ($n = 3$).
*$P < 0.01$ against none.
†$P < 0.01$ against none.

FMN-phosphatase was less pronounced compared with those on flavokinase. Because zinc inhibits FMN-phosphatase,[9] one may think that the cadmium effect on this enzyme is mediated through modulation of the level of zinc or any other metal in liver. The in vitro effects of cadmium on these two enzymes suggest that cadmium may have a direct effect on flavokinase and FMN-phosphatase, although they do not exclude the possibility that cadmium may compete with zinc in binding to FMN-phosphatase, and that zinc metabolism may therefore be a site of action of cadmium.

It is also observed that cadmium treatment leaves the hepatic FMN level unaltered in spite of diminished conversion of riboflavin to FMN. This unaltered FMN level might be due to either diminished degradation of FMN or increased conversion of FMN to FAD.

A significant increase in the hepatic level of FAD in cadmium-treated rats may be due to increased FAD-pyrophosphorylase, which is responsible for the formation of FAD from FMN, or decreased FAD pyrophosphatase, which catalyses the hydrolysis of FAD to FMN. We have not measured either of these two enzymes under our present experimental condition. For the same reason, it is difficult to explain the unchanged hepatic FMN level in cadmium-treated animals.

The tissue FMN and FAD levels were found to be regulated by the activity of one or more of the riboflavin-metabolizing enzymes in altered physiological situations.[22,23] The activities of flavokinase and/or FAD-pyrophosphorylase are sometimes reduced due to decreased availability of free riboflavin in the tissues.[22,23] However, in the present investigation, tissue riboflavin level was enhanced. It is therefore possible that the alterations in the levels of riboflavin and FAD in liver as noted in the present investigation may be due to the influence of cadmium on the enzyme activities. However, a definite conclusion can only be drawn by studying the rates of incorporation of radioactive riboflavin into various hepatic flavin fractions.

References

1 Chatterjee, A.K., Dutta, S.C., and Ghosh, B.B. (1972). Effects of emetine on the metabolism of ascorbic acid. *Brit. J. Pharmacol.* **44,** 810–813
2 Chatterjee, A.K. (1977). Studies on the involvement of testicular hormone in the regulation of pyridoxal phosphate formation by the kidney. *Endocrinology* **90,** 880–884
3 Chatterjee, A.K., Basu, J., Dutta, S.C., Sengupta, K., and Ghosh, B.B. (1976). Effects of L-lysine administration on certain aspects of ascorbic acid metabolism. *Internat. J. Vit. Nutr. Res.* **46,** 286–290
4 Hawk, P.B. and Oser, B.L. (1931). In *Hawk's Physiological Chemistry,* 14th ed. (B.L. Oser, ed.), p. 1376, McGraw-Hill Inc., New York, NY USA
5 Berg, B.N. (1967). Maintenance of pregnancy in protein-depleted rats by transitory protein supplements during early gestation. *J. Nutr.* **92,** 66–70
6 Sato, M. and Nagai, Y. (1980). Mode of existence of cadmium in rat liver and kidney after prolonged subcutaneous administration. *Toxicol. Appl. Pharmacol.* **54,** 90–99
7 Burch, H.B. (1957). Fluorometric assay of FAD, FMN and riboflavin. In *Methods in Enzymology,* (S.P. Colowick and N.O. Kaplan, eds.), p. 960–967, Academic Press, New York, NY USA
8 McCormick, D.B. (1962). The intracellular localization, partial purification and properties of flavokinase from rat liver. *J. Biol. Chem.* **237,** 959–962
9 McCormick, D.B. and Russel, M. (1962). Hydrolysis of flavin mononucleotide by acid phosphatases from animal tissues. *Comp. Biochem. Physiol.* **5,** 113–121
10 McCormick, D.B. (1971). Flavokinase from rat liver. In *Methods in Enzymology,* (D.B. McCormick and L.D. Wright, eds.), p. 544–548, Academic Press, New York, NY USA
11 Burch, H.B., Bessey, O.A., and Lowry, O.H. (1948). Fluorometric measurements of riboflavin and its natural derivatives in small quantities of blood, serum and cells. *J. Biol. Chem.* **175,** 457–470
12 Kearney, E.B. and Englard, S. (1951). The enzymatic phosphorylation of riboflavin. *J. Biol. Chem.* **193,** 821–834
13 Ames, B.N. (1966). Assay of inorganic phosphates, total phosphate and phosphatases. In *Methods in Enzymology,* (E.F. Newfeld and V. Ginsberg, eds.), p. 115–118, Academic Press, New York, NY USA
14 Lowry, O.H., Rosebrough, N.J., Parr, A.R., and Randall, R.J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193,** 265–275
15 Nath, R. (1984). Health effects. In *Environmental pollution of cadmium,* p. 50–53, Interprint, New Delhi, India
16 Nath, R. (1984). Health effects. In *Environmental pollution of cadmium,* p. 17–19, Interprint, New Delhi, India
17 Dudley, R.E., Svoboda, D.J., and Klassen, C.D. (1982). Acute exposure to cadmium causes severe liver injury in rats. *Toxicol. Appl. Pharmacol.* **65,** 302–313
18 Dudley, R.E., Cammal, I.M., and Klaassen, C.D. (1985). Cadmium-induced Hepatic and Renal injury in chronically exposed rats: Likely role of hepatic cadmium-metallothionein in nephrotoxicity. *Toxicol. Appl. Pharmacol.* **77,** 414–426
19 Hastings, L., Choudhury, H., Petering, H.G., and Cooper, G.P. (1978). Behavioral and biochemical effects of low-level prenatal cadmium exposure in rats. *Bull. Environ. Contam. Toxicol.* **20,** 96–101
20 Rastogi, R.B., Merali, Z., and Singhal, R.L. (1977). Cadmium alters behaviour and biosynthetic capacity for catecholamines and serotonin in neonatal rat brain. *J. Neurochem.* **28,** 789–794
21 Wong, K.L. and Klaassen, C.D. (1982). Neurotoxic effects of cadmium in young rats. *Toxicol. Appl. Pharmacol.* **63,** 330–337
22 Rivlin, R.S. (1970). Riboflavin metabolism. *New. Engl. J. Med.* **283,** 463–472
23 Fass, S. and Rivlin, R.S. (1969). Regulation of riboflavin metabolizing enzymes in riboflavin deficiency. *Am. J. Physiol.* **217,** 988–991