

Inhibition of Riboflavin Metabolism in Rat Tissues by Chlorpromazine, Imipramine, and Amitriptyline

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ABSTRACT Prompted by recognition of the similar structures of riboflavin (vitamin B₂), phenothiazine drugs, and tricyclic antidepressants, our studies sought to determine effects of drugs of these two types upon the conversion of riboflavin into its active coenzyme derivative, flavin adenine dinucleotide (FAD) in rat tissues. Chlorpromazine, a phenothiazine derivative, and imipramine and amitriptyline, both tricyclic antidepressants, each inhibited the incorporation of [¹⁴C]riboflavin into [¹⁴C]FAD in liver, cerebrum, cerebellum, and heart. A variety of psychoactive drugs structurally unrelated to riboflavin were ineffective. Chlorpromazine, imipramine, and amitriptyline in vitro inhibited hepatic flavokinase, the first of two enzymes in the conversion of riboflavin to FAD.

Evidence was obtained that chlorpromazine administration for a 3- or 7-wk period at doses comparable on a weight basis to those used clinically has significant effects upon riboflavin metabolism in the animal as a whole: (a) the activity coefficient of erythrocyte glutathione reductase, an FAD-containing enzyme used as an index of riboflavin status physiologically, was elevated, a finding compatible with a deficiency state, (b) the urinary excretion of riboflavin was more than twice that of age- and sex-matched pair-fed control rats, and (c) after administration of chlorpromazine for a 7-wk period, tissue levels of flavin mononucleotide and FAD were significantly lower than those of pair-fed littermates, despite consumption of a diet estimated to contain 30 times the recommended dietary allowance. The present study suggests that certain psychotropic drugs interfere with riboflavin metabolism at least in part by inhibiting the conversion of riboflavin to its coenzyme derivatives, and that as a consequence of such inhibition, the overall utilization of the vitamin is impaired.

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INTRODUCTION

It has not been widely appreciated that vitamin B₂ (riboflavin) is similar in structure to the phenothiazine derivatives that are clinically used as psychotropic drugs (Fig. 1). In a series of reports, Gabay and Harris (1-5) called attention to the structural analogies, and showed that phenothiazine drugs in vitro inhibit D-amino acid oxidase and NADPH-cytochrome *c* reductase, both of which contain flavin adenine dinucleotide (FAD),¹ the coenzyme derived from riboflavin. The biological activity of a series of phenothiazine derivatives in vivo could be correlated with their ability to inhibit these FAD-containing enzymes in vitro (6) perhaps because phenothiazine and the isoalloxazine ring of FAD form an electron donor-acceptor complex (7).

Prompted by these observations, we sought to determine whether chlorpromazine in doses comparable to those used clinically is a riboflavin antagonist and inhibits the formation of its active derivatives, flavin mononucleotide (FMN) and FAD. Initial studies from this laboratory revealed that large doses (20-25 mg/kg) of chlorpromazine markedly inhibit riboflavin metabolism both in vitro and in vivo, and impair thyroxine stimulation of riboflavin incorporation into FAD (8).

The present studies were designed to determine whether chlorpromazine inhibits riboflavin metabolism in the rat at doses comparable to those in clinical use, and whether such inhibition may be of physiological significance in terms of the riboflavin status of the animal as a whole. In addition, investigations were performed with two tricyclic antidepressants, imipramine and amitriptyline, to determine whether these compounds inhibit riboflavin metabolism, inasmuch as their structures also have a number of features in common with that of riboflavin (Fig. 1). To determine whether inhibition of flavin metabolism is

¹ Abbreviations used in this paper: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

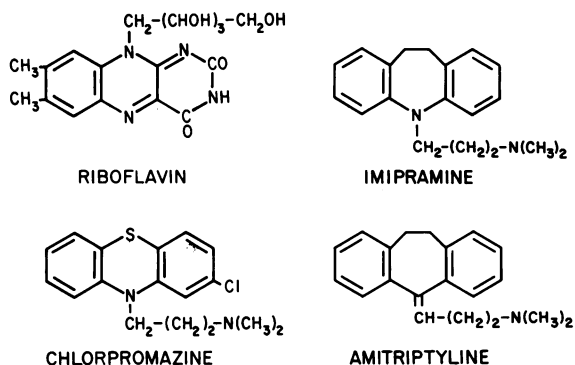


FIGURE 1 Formulae of riboflavin (vitamin B₂), chlorpromazine, a phenothiazine derivative, and imipramine and amitriptyline, both of which are tricyclic antidepressant drugs, illustrating the marked similarities among these compounds.

characteristic of this class of compounds, studies were performed using various psychoactive drugs structurally unrelated to riboflavin. Evidence has been obtained that both phenothiazine derivatives and tricyclic antidepressants impair riboflavin metabolism.

METHODS

Isotopes, chemicals, and diet. [¹⁴C]riboflavin, 28 mCi/mmol, was purchased from Amersham Corp., Arlington Heights, Ill. and the specific activity assayed in our laboratory before use. Nonradiolabeled riboflavin, riboflavin-5'-phosphate (flavin mononucleotide, FMN), FAD, and NADPH were purchased from Sigma Chemical Co., St. Louis, Mo. Chlorpromazine·HCl, imipramine·HCl and amitriptyline·HCl were gifts from Smith Kline & French Laboratories, Division of Smith Kline Corp., Philadelphia, Pa.; Pharmaceuticals Division, Ciba-Geigy Corp., Summit, N. J., and Merck & Co., Rahway, N. J., respectively. Phenytoin sodium injection was purchased from Parke-Davis & Co., Detroit, Mich.; haloperidol injection was purchased from McNeil Laboratories, Fort Washington, Pa; phenobarbital sodium injection was purchased from Winthrop Laboratories, New York; diazepam and chlordiazepoxide·HCl injectable from Hoffman-La Roche, Inc., Nutley, N. J. All other chemicals were of the highest grade commercially available. Animals were maintained on standard Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) determined in this laboratory to contain riboflavin at a concentration of 7.9 μg/g.

Animals. All experiments were performed on adult male rats, Holtzman Co., Madison, Wis., weighing 200–220 g. Unless otherwise stated, animals were maintained on an ad lib. regimen of tap water and pelleted food.

Drug treatments. In the first study, rats were distributed into four groups (seven to nine animals per group) designated as control, chlorpromazine-, imipramine-, and amitriptyline-treated, and were treated for 3 d with high doses (25 mg/kg body wt, i.p. twice daily) of the appropriate psychotropic agent dissolved in saline. Controls received an identical volume of isotonic saline. In the next experiment, a variety of psychoactive drugs were administered intraperitoneally twice daily for 3 d in the following dosages per kilogram: phenytoin (14 mg), haloperidol (0.17 mg), phenobarbital (9 mg), diazepam (0.6 mg), and chlordiazepoxide (1.7 mg). These

doses were arbitrarily selected as representing approximately four times the upper limit of the usual therapeutic range used clinically (9). In each experiment, food was removed from all cages 16 h before death; 1 h before death, each animal received an injection of [¹⁴C]riboflavin, 25 μCi/kg body wt, s.c., and was killed by decapitation. The liver, heart, cerebrum, and cerebellum were excised from each animal and stored at -20°C until assay for [¹⁴C]riboflavin incorporation into [¹⁴C]FAD. Samples could be stored for up to 30 d without detectable loss of activity.

To study the effects of chronic administration of psychotropic drugs upon flavin metabolism, investigations were conducted on animals treated for 3- and 7-wk periods with chlorpromazine (2 mg/kg body wt, i.p.). Chlorpromazine solutions were prepared fresh daily by reconstituting preweighed vials with normal saline at the time of injection (10, 11). Age- and sex-matched animals were housed in metabolic cages so that periodic 24-h urine specimens could be collected. Because treatment with chlorpromazine may induce hyperphagia (12), controls were pair-fed to drug-treated animals. Each animal consumed ~30 g/d of Purina Rat Chow, which on a weight basis, corresponds to a daily riboflavin intake of 790 μg/kg, or 30 times the recommended dietary allowance for the rat (13). At the end of 3 and 7 wk, animals were killed by decapitation and blood was collected for determination of glutathione reductase activity in erythrocyte hemolysates. Urine samples collected during metabolic studies were centrifuged at 800 g for 10 min and stored at -20°C until analysis for riboflavin and creatinine. In addition, liver, heart, and brain were excised and assayed for FAD, FMN, and riboflavin concentrations.

Analysis of [¹⁴C]FAD formation in tissues. Tissue analyses for [¹⁴C]FAD formation were performed using the reverse isotope dilution technique previously established in this laboratory (14).

Analysis of tissue FAD, FMN, and riboflavin concentrations. Concentrations of FAD, FMN, and riboflavin in liver, brain, and heart were determined by fluorometric methods as previously shown (15). Data were expressed as micrograms per gram tissue.

Analysis of urinary riboflavin. Riboflavin was measured in urine using internal flavin standards and measured in the absence and presence of sodium dithionite (16). Measurements were made with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.) equipped with a high pressure mercury-xenon lamp. Flavin excitation and emission were monitored at 450 and 525 nm, respectively, using 5-mm slit widths. The addition of chlorpromazine in concentrations of 0 to 200 mM had no effect upon urinary riboflavin fluorescence. This finding does not eliminate the possibility that metabolites of chlorpromazine in vivo may interfere with the riboflavin assay. The excretion of riboflavin was expressed as micrograms per milligram creatinine (17).

Flavokinase activity. Flavokinase (ATP: riboflavin-5'-phosphotransferase, EC 2.7.1.26) was isolated from rat liver by the method of McCormick (18, 19). Measurements of enzymic activity in vitro in the absence and presence of varying concentrations of each psychotropic agent were made both by a spectrophotometric method (18) and by a radioisotopic procedure (19). Both methodologies depend upon the differential solubility of the generated FMN in benzyl alcohol and water and were performed using reaction conditions similar to those described previously (18). Flavokinase activity was expressed as disintegrations per minute ([¹⁴C]-FMN formed) per hour.

Erythrocyte glutathione reductase activity. The preparation of the erythrocyte samples and determination of gluta-

thione reductase activity were carried out by slight modification of the method of Beutler (20).

Hemoglobin concentration of the hemolysate was determined by following the extent of oxidation of strontium peroxide-orthotolidine complex in the presence of hemoglobin (21). Enzyme activity was ultimately expressed as micromoles of NADPH oxidized per gram hemoglobin per minute. Activity coefficients were determined by expressing the ratio of the enzyme activity in the presence of exogenous FAD to that in the absence of FAD *in vitro*.

The addition of chlorpromazine in concentrations of 0 to 200 mM *in vitro* had no effect upon the activity of glutathione reductase in erythrocyte hemolysates.

RESULTS

Inhibition of formation of [¹⁴C]FAD in vivo. In animals treated for 3 d with twice daily injections of either chlorpromazine, imipramine, or amitriptyline (25 mg/kg body wt, *i.p.*), the incorporation of [¹⁴C]riboflavin into [¹⁴C]FAD was significantly diminished in all the organs studied (Table I). In control rats, the magnitude of incorporation in liver was the greatest of any of the organs studied, and very low levels were recorded in cerebrum and cerebellum, as in previous reports from

this laboratory (22). In each organ from drug-treated rats, the magnitude of incorporation into [¹⁴C]FAD was reduced approximately one-third below that of controls. At this dose level, the drugs were all about equally effective, and each of the differences from controls was statistically significant.

To obtain some indication of the specificity of the inhibition of FAD formation by psychoactive drugs that are structurally unrelated to riboflavin, a series of agents used for antipsychotic, antiemetic, or anticonvulsant therapy were investigated (9). Under the conditions utilized, phenytoin, haloperidol, phenobarbital, diazepam, and chlordiazepoxide were each ineffective in inhibiting FAD formation in liver or heart.

In vitro studies of hepatic flavokinase. To gain insight into possible mechanisms of inhibition of [¹⁴C]FAD formation, experiments were performed in which increasing concentrations of chlorpromazine, imipramine, and amitriptyline were incubated with hepatic flavokinase in the assay mixture, and [¹⁴C]FMN, the precursor of [¹⁴C]FAD, determined.

As indicated in Fig. 2, chlorpromazine inhibited flavokinase activity *in vitro*. Imipramine and amitrip-

TABLE I
Inhibition of Incorporation of [¹⁴C]Riboflavin into [¹⁴C]FAD 1 h after a Subcutaneous Injection in Liver, Cerebrum, Cerebellum, and Heart of Adult Male Rats Treated with Chlorpromazine, Imipramine, or Amitriptyline (25 mg/kg body wt *i.p.*) Twice Daily for 3 d*

Organ	Treatment group	[¹⁴ C]FAD	Significance of difference from control
		dpm/100 mg	P
Liver	Control	12,769±787†	
	Chlorpromazine§	9,994±499	<0.02
	Imipramine	10,389±632	<0.05
	Amitriptyline	10,022±683	<0.02
Cerebrum	Control	774±37	
	Chlorpromazine	499±43	<0.001
	Imipramine	503±24	<0.001
	Amitriptyline	510±32	<0.001
Cerebellum	Control	941±26	
	Chlorpromazine	608±36	<0.001
	Imipramine	676±38	<0.001
	Amitriptyline	698±52	<0.001
Heart	Control	4,932±362	
	Chlorpromazine	3,227±214	<0.01
	Imipramine	3,295±128	<0.01
	Amitriptyline	3,340±172	<0.01

* 25 μCi/kg body wt.

† Data are shown as mean±SEM with seven to nine animals per group.

§ Groups of animals received intraperitoneal injections of each drug; controls were age- and sex-matched and were injected with a similar volume of isotonic saline.

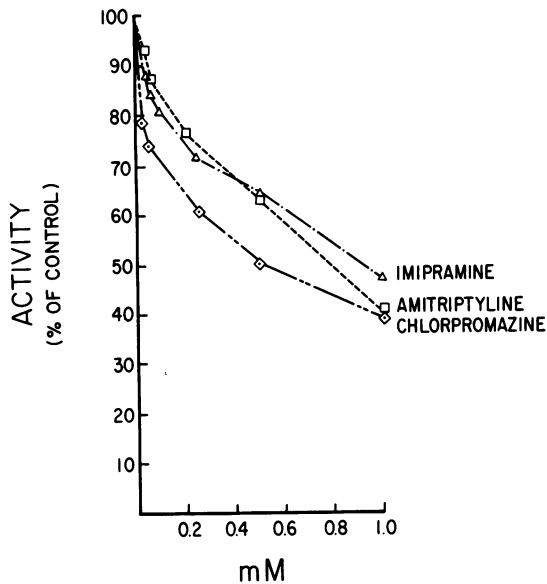


FIGURE 2 Effect of chlorpromazine, imipramine and amitriptyline in increasing concentrations upon inhibiting the enzymatic formation of [^{14}C]FMN from [^{14}C]riboflavin in vitro. Conditions are the same as for the flavokinase assay, with each drug incubated for 5 min prior to the start of the reaction.

tyline also inhibited flavokinase actively in vitro but to a lesser degree than chlorpromazine, particularly at lower concentration. These data suggest that all three drugs inhibit flavokinase, which is the first of two steps in the formation of FAD from riboflavin.

Erythrocyte glutathione reductase activity and urinary riboflavin excretion. In order to gain some

understanding of the possible physiological consequences of the inhibition of FAD formation by psychotropic drugs, experiments were performed in which rats received a relatively low dose of chlorpromazine, 2 mg/kg body wt, for a 3- and 7-wk period. At the end of each period, the animals were killed and measurements were made of glutathione reductase activity in erythrocyte hemolysates.

Results of the measurements of glutathione reductase at the end of each study period are shown in Table II. The basal activity of glutathione reductase differed slightly between both groups at both the 3- and the 7-wk period. A significant difference was noted between control and chlorpromazine-treated groups at both time periods in the activity determined after incubation with cofactor FAD in vitro, and in the activity coefficient, i.e., ratio of enzyme activity with to that without addition of FAD in vitro. The significant increase in the activity coefficient of glutathione reductase in the chlorpromazine-treated animals is in the same direction as that observed with dietary riboflavin deficiency (23, 24). Thus, the chronic administration of a relatively low dose of chlorpromazine produces changes in riboflavin homeostasis that are detectable by assays that are utilized to assess riboflavin deficiency clinically.

During the 3- and 7-wk experimental periods, measurements were made at intervals of the urinary riboflavin excretion, after the animals had been placed in metabolic cages. As shown in Table II, after 3 and 7 wk of treatment with chlorpromazine, the urinary riboflavin excretion was more than twice that of pair-fed controls.

Hepatic concentrations of FAD, FMN, and riboflavin.

TABLE II
*Erythrocyte Glutathione Reductase Activity and Urinary Riboflavin Excretion in Adult Male Rats Treated with Chlorpromazine, 2.0 mg/kg body wt, by Daily Intraperitoneal Injection for 3- and 7-wk Periods, and in Age- and Sex-matched Pair-fed Control Rats Treated with Saline**

Study period	Treatment group	Erythrocyte glutathione reductase activity†			Urinary riboflavin excretion‡
		Without FAD	FAD added	Activity coefficient	
<i>wk</i>		$\mu\text{mol NADPH oxidized/g Hb per min}$	$\mu\text{mol NADPH oxidized/g Hb per min}$		$\mu\text{g/mg creatinine}$
3	Control	7.06±0.92	7.88±1.03	1.13±0.02	3.92±0.35
	Chlorpromazine	9.73±1.00	12.24±1.07	1.28±0.04	9.13±0.48
	Significance of difference	NS	$P < 0.02$	$P < 0.005$	$P < 0.001$
7	Control	5.49±0.26	5.48±0.26	1.00±0.03	3.80±0.35
	Chlorpromazine	7.03±0.33	9.25±0.53	1.33±0.08	8.57±0.97
	Significance of difference	$P < 0.005$	$P < 0.001$	$P < 0.005$	$P < 0.001$

* Data are shown as mean±SEM with 8–13 animals per group.

† Urinary riboflavin excretions were obtained on the last day of the treatment period; erythrocyte glutathione reductase activities were measured in samples obtained at death.

To determine whether the inhibition of FAD formation demonstrated above leads to actual tissue depletion, the concentrations of FAD, FMN, and riboflavin were measured in liver at the end of the 7-wk period. Liver was selected for assay because this organ is the most sensitive one to dietary riboflavin deficiency (20). As shown in Table III, chlorpromazine treatment led to significant reductions in FAD and FMN concentrations compared to pair-fed controls. This depletion of FAD and FMN levels occurred despite a diet that was abundant in riboflavin.

DISCUSSION

Despite widespread usage of phenothiazine derivatives and tricyclic antidepressants, the mechanisms underlying the clinical actions of these drugs are complex and incompletely understood. With regard to the toxicities of psychotropic agents that have been reviewed elsewhere (25, 26), little attention has been paid to possible nutritional complications associated with their use. This series of investigations demonstrates that chlorpromazine, imipramine, and amitriptyline impair the incorporation of riboflavin into its active coenzyme form, flavin adenine dinucleotide. Diminished FAD formation is demonstrable in each of the organs studied, i.e., liver, cerebrum, cerebellum, and heart. A likely underlying mechanism is inhibition of the enzyme flavokinase, which catalyses the initial phosphorylation of riboflavin to form the coenzyme FMN, which then combines with a second molecule of ATP to yield FAD, the major biologically active flavin coenzyme, in a reaction catalyzed by FAD pyrophosphorylase. It is not known whether these psychoactive drugs also inhibit FAD pyrophosphorylase, or alter the activities of the enzymes that are involved in the degradation of FAD to riboflavin, namely FAD pyrophosphatase and FMN phosphatase. Such modulations of phosphatase activities have been demonstrated in thiamine metabolism. Chlorpromazine decreases thiamine triphosphatase activity and markedly increases thiamine diphosphatase activity (27).

TABLE III

*Hepatic Concentrations of FAD, FMN, and Riboflavin in Livers of Rats Treated with Chlorpromazine (2.0 mg/kg) Daily for 7 wk and in Pair-fed Controls Treated with Saline**

Treatment group	FAD	FMN	Riboflavin
	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$
Pair-fed control	63.7 \pm 1.2	10.7 \pm 0.6	1.6 \pm 0.1
Chlorpromazine	58.2 \pm 1.0	8.9 \pm 0.3	1.7 \pm 0.1
Significance of difference	$P < 0.005$	$P < 0.01$	NS

* Data shown as mean \pm SEM with eight animals per group.

The findings in the present report may be clinically relevant because the dose of chlorpromazine that inhibits FAD biosynthesis (2 mg/kg body wt) is well within the ordinary therapeutic range for psychiatric patients. In addition, it is of interest that riboflavin metabolism in the animal as a whole appears to be disturbed in animals treated with a low dose of chlorpromazine for a 3- or 7-wk period. The ratio of the activity of erythrocyte glutathione reductase after incubation with its cofactor FAD in vitro to the activity prior to incubation (activity coefficient) has been widely used as an index of riboflavin deficiency (20, 23, 24). Greater than normal augmentation of activity after incubation with FAD in vitro is thought to reflect undersaturation of the apoenzyme with its cofactor due to decreased riboflavin intake (20). In the present report, chlorpromazine-treated animals had significantly increased activity coefficients of glutathione reductase even though their dietary riboflavin intake was the same as in pair-fed control rats and was \sim 30 times the recommended dietary allowance (13). This finding certainly suggests that chlorpromazine produces an endogenous form of riboflavin deficiency, resulting from impaired formation of FAD from riboflavin, as we have previously observed in hypothyroidism (15). The present data do not exclude the possibility that chlorpromazine, which produces changes in the surface properties of membranes (28), may impede the transport of circulating riboflavin into the erythrocyte or alter the binding of FAD to erythrocyte glutathione reductase, as has been proposed for glucose-6-phosphate dehydrogenase deficiency (29).

In chlorpromazine-treated rats the basal activity of glutathione reductase was increased slightly compared to controls. Elevated glutathione reductase activity has been observed in a variety of diverse conditions, including iron deficiency (30), cobalt deficiency (31), glucose-6-phosphate dehydrogenase deficiency (29), cancer (32), uremia (23), and cirrhosis of the liver (33). It is the elevated activity coefficient, not the basal level, that appears to be the most reliable index of riboflavin deficiency (20, 24).

The observation that chlorpromazine treatment leads to a marked increase of urinary riboflavin excretion to more than twice that of pair-fed controls clearly indicates that riboflavin loss is a consequence of drug treatment. Continued over a prolonged period of time, drug treatment could result in significant depletion of vitamin stores. Evidence that vitamin stores are in fact depleted by chlorpromazine at low doses has been provided here. Tissue concentrations of FAD and FMN were reduced significantly below levels in pair-fed controls after 7 wk of treatment with chlorpromazine despite a diet abundant in riboflavin. It is of interest that under these conditions free ribo-

flavin concentrations in liver are not depleted. Since the block in riboflavin metabolism caused by the drug is presumably at the riboflavin to FMN step, or possibly also at the FMN to FAD step, the levels of the initial substrate, i.e., riboflavin, would not be expected to decrease. By contrast, in dietary riboflavin deficiency, concentrations of FAD, FMN, and particularly riboflavin are all depleted (22). The increased urinary excretion of riboflavin is postulated to be an overflow phenomenon, resulting directly from tissue blockade of utilization of riboflavin to form FAD.

The experiments in their entirety highlight previously unrecognized nutritional consequences of treatment with psychoactive drugs. The doses of chlorpromazine used in the rat are clearly comparable on a weight basis to those used clinically; doses of the tricyclic antidepressants appear to be generally higher than those which are clinically relevant, but this point needs to be pursued in more detail. Some psychiatric patients eat poorly and many are in a marginal nutritional state generally (34) that could be compounded by treatment with drugs that cause increased vitamin excretion. The present studies raise the possibility that drug-induced nutritional deficiency may be an unrecognized and undesirable result of antipsychotic drug therapy in patients, particularly when treatment is prolonged. It must be emphasized that riboflavin deficiency, even when marginal and subclinical, will result in significant deterioration of personality (35).

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