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CHLORPROMAZINE ANTAGONISM OF THYROXINE-INDUCED FLAVIN FORMATION*

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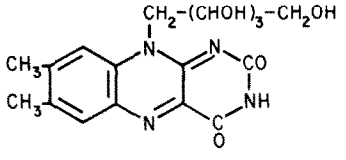
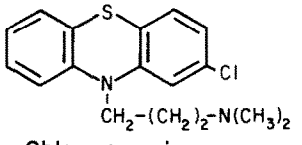
Abstract—Chlorpromazine inhibits the incorporation of riboflavin into flavin adenine dinucleotide and flavins bound covalently to proteins *in vivo* in rat liver. In addition, this drug markedly blunts thyroxine enhancement of riboflavin incorporation into flavins, both observations being made at concentrations of chlorpromazine commonly used clinically. These findings indicate that chlorpromazine is a riboflavin antagonist and inhibits effects of thyroxine upon riboflavin metabolism.

The antipsychotic drug chlorpromazine and riboflavin (vitamin B_2) have a number of structural analogues [1–5], as shown in Fig. 1. In addition to their similar planar and three dimensional structure, the phenothiazine ring of chlorpromazine and the isoalloxazine ring of riboflavin are known to form an electron donor–acceptor complex [6–9]; chlorpromazine *in vitro* inhibits several liver and brain enzymes containing flavin coenzymes, including D-amino acid oxidase (EC 1.4.3.3) [4] and NADPH-cytochrome *c* reductase (EC 1.6.2.4) [10, 11]. Riboflavin deficiency [12] and chlorpromazine treatment [13] cause similar patterns of congenital malformations, particularly of the skeletal system, and riboflavin treatment prevents malformations due to a phenothiazine drug related to chlorpromazine [14].

These considerations suggest that chlorpromazine may be a riboflavin antagonist, inhibiting the formation of flavin coenzymes from riboflavin. To explore this hypothesis, studies were performed in rats before and after the administration of thyroxine, a hormone which enhances flavin biosynthesis in rat liver *in vivo* [15, 16]. Our findings demonstrate that chlorpromazine is a potent inhibitor of flavin adenine dinucleotide (FAD) formation and of covalent binding of flavins to tissue proteins, and also inhibits the stimulatory effects of pharmacological doses of thyroxine on the formation of flavins.



Riboflavin



Chlorpromazine

Fig. 1. Formulae of riboflavin, composed of an *N*-substituted isoalloxazine ring, and chlorpromazine, composed of an *N*-substituted phenothiazine ring. By convention the side chain of riboflavin is represented above the ring and that of chlorpromazine below the ring.

MATERIALS AND METHODS

Chemicals. Thyroxine was obtained from the Nutritional Biochemicals Division of ICN Life Sciences Group, Cleveland, OH. D-[2-^{14}C] riboflavin, 28 mCi/m-mole, was purchased from the Amersham Corp., Arlington Heights, IL, and the specific activity was assayed in our laboratory prior to use. Non-radioactive riboflavin, riboflavin-5′-phosphate (flavin mononucleotide, FMN) and FAD were purchased from the Sigma Chemical Co., St. Louis, MO. Chlorpromazine HCl was a gift from the Smith Kline and French Laboratories, Philadelphia, PA. All other chemicals were of the highest grade commercially obtainable.

Experimental design. Adult male Holtzman rats (Holtzman Rat Company, Madison, WI) each received daily intraperitoneal injections of thyroxine, 100 μg/100 g body weight, a dose many times the daily secretory rate [17], for 8 days prior to death. Thyroxine was dissolved in 0.9% NaCl and titrated with 0.01 N NaOH until the pH was 10.0. The control rats received daily i.p. injections of isotonic saline of the same volume and pH as the thyroxine injections. For 3 days prior to killing, half the thyroxine- and saline-treated rats received, twice daily, intraperitoneal injections of chlorpromazine, 1.8 mg/100 g body weight, dissolved in isotonic saline. The remaining thyroxine- and saline-treated animals received intraperitoneal injections of isotonic saline comparable to those administered to the chlorpromazine-treated rats. Food was removed from all cages 16 hr prior to killing. One hr prior to decapitation, each rat received a subcutaneous injection of D-[2-^{14}C] riboflavin, 2.5 μCi/100 g body weight. The liver was removed from each animal, weighed and main-

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tained at −20° until assay. In other experiments, groups of adult male Holtzman rats received intraperitoneal injections, twice daily for 3 days, of chlorpromazine or imipramine, 0.2 or 2.0 mg/100 g body weight, dissolved in saline; the control animals for these experiments received saline of the same volume. The remainder of the procedures were the same as in the previous experiments.

Analysis of D-[2-^{14}C] *FAD.* To prepare liver samples for analysis of formation of FAD, 50- to 100-mg aliquots were homogenized for 3 min with 7.0 ml methanol and 1.0 ml each of freshly prepared riboflavin, FMN and FAD, 0.1 mg/ml. Incorporation of D-[2-^{14}C] riboflavin into hepatic D-[2-^{14}C] FAD was determined by reverse isotope dilution and anion exchange column chromatography [18] with DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). The procedure for extraction of flavins yielded 98 per cent of the total radioactivity in liver; the percentage variation from the mean of duplicate determinations was approximately 3–5 per cent.

Analysis of D-[2-^{14}C]*-covalently bound flavins.* To prepare liver samples for the analysis of covalently bound flavin formation, liver aliquots weighing 100–400 mg were homogenized for 3 min with 7 ml of an acidified methanol solution (1 ml of 6 N HCl/100 ml of 70% methanol) in a Potter–Elvehjem homogenizer equipped with a teflon pestle. Incorporation of D-[2-^{14}C] riboflavin into D-[2-^{14}C]-covalently bound flavins was determined by techniques described previously [19 20], which involved sonication of the tissue extract, successive washings with acid-methylethylketone, trichloroacetic acid, and water, followed by proteolytic digestion with trypsin and chymotrypsin to release covalently bound flavins from denatured proteins. After digestion was complete, the radioactivity of 1-ml aliquots in 10 ml Aquasol (New England Nuclear, Boston, MA) was determined in a Packard Tri-Carb model 3375 liquid scintillation spectrometer. The incorporation of D-[2-^{14}C]riboflavin into D-[2-^{14}C]FAD and D-[2-^{14}C]-covalently bound flavins was expressed as dis./min/100 mg of tissue; the percentage variation from the mean of duplicate determinations was approximately 4–5 per cent.

Flavokinase determination. Flavokinase (ATP: riboflavin 5′-phosphotransferase, EC 2.7.1.26) was purified by the method of McCormick [21] and was measured both by a spectrophotometric method [22] and by a radioisotopic procedure which measures the generation of D-[2-^{14}C] FMN from D-[2-^{14}C]riboflavin. Both methods depend upon the differential solubility of FMN in benzyl alcohol and water; the percentage variation from the mean of duplicate determinations was approximately 2 per cent.

RESULTS

Chlorpromazine treatment significantly ($P < 0.001$) depressed the incorporation of D-[2-^{14}C] riboflavin into D-[2-^{14}C]FAD (Fig. 2). As demonstrated in our previous report [23], thyroxine treatment significantly ($P < 0.001$) elevated D-[2-^{14}C]FAD formation. In thyroxine-treated rats, chlorpromazine inhibited markedly the stimulatory effects of thyroxine, and values in this group were nearly identical to those in the saline-treated controls. The magnitude of incorporation of D-[2-^{14}C]riboflavin into flavins bound covalently to tissue proteins represented a small percentage of the incorporation into FAD, as observed previously in this laboratory [20]. Chlorpromazine inhibited the formation of this tissue fraction ($P < 0.01$) as well (Fig. 2). Thyroxine enhanced the formation of covalently bound flavins ($P < 0.001$), an effect which was also inhibited markedly by chlorpromazine. Thus, values in rats treated with both thyroxine and chlorpromazine did not differ significantly ($P > 0.05$) from those in saline treated control rats.

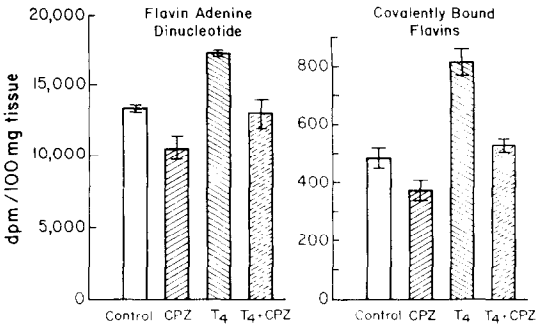

Fig. 2. Incorporation of a tracer dose of D-[2-^{14}C]riboflavin into FAD and flavins bound covalently to tissue proteins 1 hr after a single subcutaneous injection in saline control, chlorpromazine (CPZ), thyroxine (T_4), and CPZ + T_4-treated adult male rats. Doses of CPZ and thyroxine and the time course of injections are described in the text. Data are means ± S.E.M. with eight to fifteen rats per group.

To search for a possible mechanism of chlorpromazine inhibition of incorporation of riboflavin into FAD and covalently bound flavins, investigations were made of hepatic flavokinase, the enzyme which converts riboflavin to FMN, the first of two steps in the conversion of riboflavin to FAD [24, 25]. Both ATP and riboflavin are required to stabilize and maintain the activity of this enzyme [16]. The preincubation of flavokinase with increasing concentrations of chlorpromazine *in vitro* for 15 min prior to initiation of the enzyme assay with riboflavin markedly inhibited flavokinase activity. Twenty per cent of the flavokinase activity was lost at 0.02 mM chlorpromazine, with greater loss of activity as chlorpromazine concentration increased. At 0.05 mM chlorpromazine, 25 per cent of activity was lost; at 0.25 mM chlorpromazine 40 per cent of activity was lost; at 0.5 mM chlorpromazine 50 per cent was lost; and at 1.0 mM, 62 per cent was lost. If both ATP and riboflavin were omitted from the preincubation medium, 100 per cent of the flavokinase activity was lost.

Utilizing a fixed concentration of chlorpromazine (0.25 mM) *in vitro* during the incubation period and varying concentrations of riboflavin, a study of the kinetics of inhibition by chlorpromazine was made (Fig. 3). As shown by the double reciprocal plots, the addition of chlorpromazine increased the K_m of the flavokinase for riboflavin indicating competitive inhibition. The data shown in Fig. 3 were obtained with a protein concentration of 330 μg/3-ml cuvette.

To explore the specificity of the inhibitory effects of chlorpromazine upon FAD formation, chlorpromazine and imipramine at two dose levels were each administered to groups of adult rats. Both drugs inhibited the incorporation of D-[2-^{14}C]riboflavin into D-[2-^{14}C]

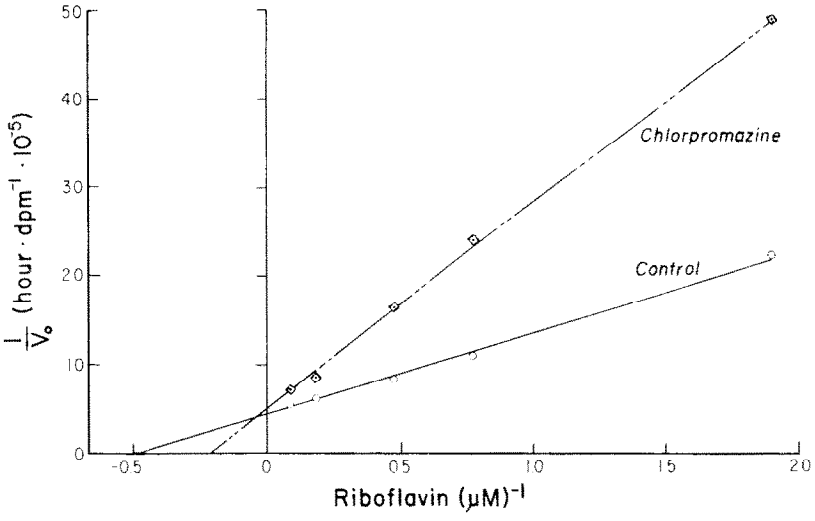

Fig. 3. Double-reciprocal plots of the velocity of D-[2-^{14}C]FMN formation from D-[2-^{14}C]riboflavin (flavokinase reaction) in the presence and absence of chlorpromazine (0.25 mM).

Table 1. Effects of intraperitoneal administration of chlorpromazine or imipramine twice daily for 3 days upon the incorporation of tracer doses of D-[2-^{14}C]riboflavin into FAD in livers of adult male rats, 1 hr after a subcutaneous injection*

Treatment group	D-[2-^{14}C]FAD (dis./min/100 mg)	Significance of difference from control
Control	13.218 ± 1.071	
Chlorpromazine		
2.0 mg/100 g body wt	10.009 ± 1.230	$P < 0.02$
0.2 mg/100 g body wt	10.668 ± 1.567	$P < 0.05$
Imipramine		
2.0 mg/100 g body wt	10.597 ± 594	$P < 0.02$
0.2 mg/100 g body wt	13.612 ± 1.655	NS†

* All data are means ± S.E.M., with three to nine rats per group.
† NS not significant.

FAD at a dose of 2.0 mg/100 g body weight, but only chlorpromazine was effective at 0.2 mg/100 g body wt (Table 1).

DISCUSSION

These investigations demonstrate that chlorpromazine inhibits flavin formation from riboflavin both *in vitro* and *in vivo*. We have shown previously that doses of thyroxine which are in the physiological range augment flavokinase activity *in vivo* [22]. In this study, chlorpromazine blocked the effects of pharmacological doses of thyroxine *in vivo*. Imipramine also inhibited FAD formation *in vivo*, but at a dose level higher than that of chlorpromazine.

The small fraction of flavin which is bound covalently to tissue proteins is a newly recognised entity, the physiological function of which is not entirely known. Four mammalian enzymes, succinic dehydrogenase (EC 1.3.99.1) [26] sarcosine dehydrogenase (EC 1.5.99.1) [19], L-gulono-γ-lactone oxidase (EC 1.1.3.8) [27], and monoamine oxidase (EC 1.4.3.4) [28], have been shown to contain flavin in the form of FAD attached to the apoenzyme in covalent linkage. The factors controlling the formation of this fraction as a whole have not been elucidated. Measurements of the time course of formation of this fraction suggest that covalent attachment to tissue proteins occurs subsequent to the formation of FAD [29, 30], and that in rats born of maternally riboflavin deficient dams, the incorporation of a tracer dose of radioactive riboflavin into this fraction is increased [31]. The present report provides evidence of endocrine control of the generation of the covalently bound flavin fraction (Fig. 2).

Others have observed previously [5] that chlorpromazine *in vitro* inhibits D-amino acid oxidase and NADPH-cytochrome *c* reductase, both FAD-requiring enzymes, by competing with available FAD for the apoenzyme. Other phenothiazine derivatives also inhibit these enzymes, with the degree of pharmacological efficacy as an antipsychotic agent *in vivo* commensur-

ate with the degree of enzyme inhibition *in vitro*. Recently, evidence has been presented to show that human platelet monoamine oxidase may be inhibited both *in vitro* and *in vivo* by doses of tricyclic drugs commonly used therapeutically [32]. Our studies demonstrate that chlorpromazine has an additional effect, that of impairing FAD biosynthesis *in vivo*, thereby limiting the availability of coenzymes which are required for enzyme stability. It is important to note that the established value of K_i for the interaction between phenothiazines and FAD-requiring enzymes, i.e. 10^{-5} [5], represents drug concentrations which may be encountered in patients during treatment [5, 33], and also that the dosages of chlorpromazine utilized in the present study in rats are comparable on a body weight basis to the dosage range currently used therapeutically in patients. These considerations raise the possibility that the inhibition of FAD biosynthesis by chlorpromazine may well be of clinical significance.

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