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EFFECT OF THE CATECHOLAMINE-DEPLETING AGENT 1-PHENYL-3-(2-THIAZOLYL)-2-THIOUREA (U-14,624) ON DRUG METABOLISM IN THE RAT*

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Abstract—Male rats injected with 1-phenyl-3-(2-thiazolyl)-2-thiourea (U-14,624) (25 mg/kg/day i.p.) for 3 days prior to induction of anesthesia with pentobarbital (40 mg/kg, i.p.) slept significantly ($P < 0.05$) longer than control animals. Plasma and brain half-lives of pentobarbital were also prolonged in the treated animals, but both control and treated groups awakened with similar brain levels of pentobarbital. In addition, the plasma half-life of antipyrine in treated animals was also prolonged significantly. Subacute administration of U-14,624 (50 mg/kg/day i.p.) to male rats for 5–7 days suppressed the activities of aminopyrine *N*-demethylase, aniline hydroxylase and *p*-nitroanisole *O*-demethylase enzymes *in vitro*; this effect could not be demonstrated at lower doses. Single doses of U-14,624 (100–200 mg/kg, i.p.) also suppressed the activities of the three oxidative enzymes. The suppression was positively correlated with reduced levels of hepatic microsomal cytochrome P-450. Levels of cytochrome b_5 and NADPH-cytochrome *c* reductase activity were not affected consistently by acute dosage with U-14,624. The inhibitory effects of single doses (100–400 mg/kg, i.p.) on all enzymatic systems were reversible, and recovery was complete within 48 hr. Whereas all three oxidative drug-metabolizing enzymes were inhibited in a mixed manner by *in vitro* exposure to U-14,624 (10^{-5}–10^{-2} M), neotetrazolium diaphorase was not inhibited by U-14,624 at concentrations as high as 5 mM. Inhibition of oxidative drug metabolism by U-14,624 is mechanistically related to depletion of cytochrome P-450, but inhibition of these enzymes *in vitro* indicates that a second inhibitory mechanism may also be operative.

1-Phenyl-3-(2-thiazolyl)-2-thiourea (U-14,624) has been used extensively as a pharmacological tool to deplete central norepinephrine (NE) levels as a consequence of its ability to inhibit the copper-containing enzyme, dopamine β-hydroxylase [1–4]. Effects associated with U-14,624 administration alone or prior to other drugs have usually been interpreted in terms of the central dopaminergic system without consideration of a possible peripheral contribution to pharmacological responses [5–22]. At least two effects of U-14,624, its ability to protect against the neurotoxicity of 6-hydroxydopamine and its ability to impair the diabetogenic action of alloxan, apparently are unrelated to its inhibition of dopamine β-hydroxylase [23]. Preliminary studies in this laboratory indicated that animals pretreated with U-14,624 were more sensitive to pentobarbital anesthesia than were control animals under similar experimental conditions. Potentially, this finding may be explained by a central and/or peripheral action of U-14,624. The present study was initiated to investigate the possibility that this highly lipophilic metal chelator may modify the functional status of the hepatic mixed-function oxidase system in the male rat.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Carworth Farms, New York, NY) weighing 150–260 g were used in all studies, except as noted. Purina lab chow and water were available *ad lib.* during a 12-hr, light–dark cycle (6:00 a.m.–6:00 p.m.). Six or seven animals were housed together in wire-mesh cages over SAN-I-CEL bedding (Paxton Processing Co., Inc., Whitehouse Station, NY) in rooms maintained at 22–25°.

Drugs and dosing. U-14,624 (Aldrich Chemical Co., Milwaukee, WI) was uniformly suspended in 0.9% saline solution containing 1% Tween 80 (Sigma Chemical Co., St. Louis, MO). The final concentration of the suspension was 25 mg U-14,624 per ml of suspension. The control suspending agent or suspensions of U-14,624 were administered intraperitoneally in all experiments. A minimum of 24 hr or a maximum of 48 hr intervened between final drug injections and the initiation of remaining experimental protocols.

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In vivo *procedures*. The LD_{50} (48-hr, i.p.) of U-14,624 was established in two weight ranges of male rats (184–208 g and 400–510 g) in order to define a useful, nontoxic dosage range for U-14,624. The LD_{50} was computed from the number of deaths at each dosage level by the moving average interpolation method of Weil [24]. Four animals were used in each dosage group.

Analysis of pentobarbital in plasma and brain. Pentobarbital elimination from plasma and brain tissues was assessed in animals pretreated with U-14,624 (25 mg/kg/day × 3, i.p.) and in control animals. Twenty-four hr after the last treatment, control and treated animals were divided into groups of five. Killing and tissue sampling were begun 10 min after an intraperitoneal injection of pentobarbital (40 mg/kg) and were terminated when a group of control and a group of treated animals awakened. Blood was drawn by aortic puncture under ether anesthesia and brains (minus cerebella) were removed following decapitation. Analysis of pentobarbital in tissues was performed according to the method of Brodie *et al.* [25] using a double extraction with the organic phase.

Plasma clearance of antipyrine. The plasma clearance of antipyrine was monitored in additional animals (N = 5) pretreated with U-14,624 as above. Under light ether anesthesia each animal was cannulated via the external jugular vein for bolus antipyrine injections (100 mg/kg) and via the femoral artery for blood collections (0.5 ml) at approximately 20, 40 and 60 min post-antipyrine injection. Plasma antipyrine was assayed according to the method of Brodie *et al.* [26], except that plasma protein was precipitated with 0.3 N barium hydroxide and 5% zinc sulfate.

Microsomal enzyme assays. After either subacute (50 mg/kg/day × 3–7 days) or acute (50–400 mg/kg) treatment with U-14,624, groups of control and treated animals were decapitated; their livers were excised and homogenized in 3 vol. (25%, w/v) of ice-cold 50 mM Tris–1.15% KCl buffer (pH 7.5). After centrifugation at 12,000 *g* for 10 min, 1.0-ml aliquots of the soluble fractions were assayed for aminopyrine *N*-demethylase [27], aniline hydroxylase [28] and *p*-nitroanisole *O*-demethylase [29, 30] activities. Oxidative drug metabolism was evaluated under the optimal incubation conditions as outlined by Fouts [31]. Microsomes were isolated from hepatic soluble fractions by centrifuging aliquots of the soluble fraction for 1 hr at 100,000 *g*. The resulting pellet was assayed for protein content by the method of Lowry *et al.* [32], using bovine serum albumin as the reference standard. The cytochrome P-450 and cytochrome b_5 contents of microsomal suspensions were determined with an Aminco DW-2 spectrophotometer as described by Omura and Sato [33, 34], using extinction coefficients of 91 $mM^{-1}cm^{-1}$ and 171 $mM^{-1}cm^{-1}$, respectively. NADPH-cytochrome *c* reductase activity of these suspensions was estimated according to the method of Phillips and Langdon [35] on the same instrument operated at 550 nm in the time base and split-beam modes. The reductive process was followed for 5 min at 25°; during this time the assay was linear. An extinction coefficient of 18.8 $mM^{-1}cm^{-1}$ was employed for determinations of NADPH-cytochrome *c* reductase activity. This extinction coefficient was calculated from the sodium dithionite reduction of oxidized cytochrome *c* (Sigma Type III).

In vitro *enzyme kinetics*. Lineweaver–Burk plots were used to ascertain the capacity of U-14,624 to inhibit three enzyme systems *in vitro*. In these kinetic studies the source of the oxidative enzyme systems was hepatic supernatant fraction (12,000 *g*) prepared from livers of naive male rats, whereas the source of reductive enzymes (neotetrazolium diaphorase) was microsomal suspension (7.5 mg protein/ml) from identical animals. U-14,624, dissolved in acetone (20 mg/ml), was added to incubation beakers, and the acetone was evaporated prior to initiation of incubations.

In all kinetic studies, hepatic fractions (12,000 *g* supernatant fractions or 100,000 *g* suspensions) were routinely incubated with U-14,624 for 10 min prior to initiating enzymatic reaction by substrate addition. Control reactions were run in the absence of U-14,614; however, the vehicle (acetone) was added to each and then evaporated. Oxidative enzyme activities were determined as mentioned previously; the reductive activity of neotetrazolium diaphorase was determined *in vitro* according to the method of Williams and Kiamin [36]. An extinction coefficient of 14 $mM^{-1}cm^{-1}$ was used to quantify neotetrazolium formazan formation. This reductive pathway was monitored in order to determine whether U-14,624 would inhibit a microsomal pathway which does not require cytochrome P-450.

Statistics. The Student's two-tailed *t*-test for independent samples was used to test for statistical significance ($P < 0.05$) between U-14,624 and control treatments. All curves represent best-fit regression curves.

RESULTS

The intraperitoneal LD_{50} (48-hr) of U-14,624 in male rats was found to vary with their age and size. In animals weighing from 184 to 208 g, the LD_{50} was found to be 519 mg/kg (95 per cent confidence interval = 436–617 mg/kg). As the animals aged and increased in weight (400–510 g), they became approximately three times more susceptible to the lethal effects of U-14,624, an effect evidenced as a reduction in the LD_{50} to 178 mg/kg (95 per cent confidence interval = 101–313 mg/kg).

As shown in Fig. 1, U-14,624 pretreatment (3 days at 25 mg/kg/day, i.p.) prolonged the time required for clearance of pentobarbital from both the plasma and brain. The $T_{1/2}$ of pentobarbital elimination from plasma was increased from a control value of 48 ± 0.3 min to 78 ± 0.4 min by U-14,624 pretreatment. Similarly, U-14,624 pretreatment increased the $T_{1/2}$ of pentobarbital elimination from brain from 53 ± 0.7 min to 90 ± 0.7 min. The pentobarbital sleep time of treated animals (106 ± 7 min) was significantly ($P < 0.05$) prolonged relative to that of controls (64 ± 2 min). At the termination of hypnosis, the mean plasma and brain levels of pentobarbital (± S.E.) in control animals were 14.2 ± 0.2 μg/ml and 20.5 ± 1.1 μg/g, respectively, while the corresponding levels of the treated animals were 15.3 ±

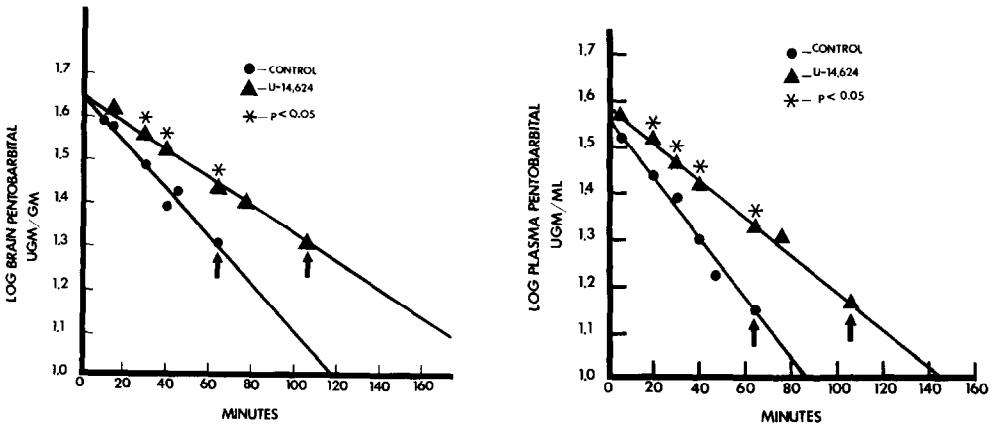

Fig. 1. Brain and plasma clearances of pentobarbital (40 mg/kg, i.p.) in male rats 24 hr after the cessation of three daily intraperitoneal injections of U-14,624 (25 mg/kg/day). Pentobarbital concentrations of either brain or plasma are shown on the ordinate; the abscissa represents time (min) from the loss of the righting reflex. Bold arrows denote the return of the righting reflex. At least five animals were used to establish each point. An asterisk (*) indicates $P < 0.05$.

0.8 μg/ml and 20.2 ± 0.8 μg/g. An identical dosage schedule also resulted in protracted elimination of antipyrine from the plasma of treated animals. U-14,624 pretreatment in this instance increased the plasma half-life of antipyrine to 152 ± 13 min, which was 49 per cent greater than the control half-life of 102 ± 7 min.

Although administration of U-14,624 at 25 mg/kg/day, i.p., for 1–4 days was associated with no significant change in the activities of aminopyrine *N*-demethylase, aniline hydroxylase or *p*-nitroanisole *O*-demethylase *in vitro*, it was possible to inhibit these hepatic drug-metabolizing enzymes by increasing the daily dose of U-14,624 to 50 mg/kg/day as shown in Table 1. A pattern of enzyme inhibition was seen with all three enzyme systems in treated animals with significant suppression ($P < 0.05$) of aminopyrine *N*-demethylase and aniline hydroxylase occurring within 5 days, while 7 days were required to inhibit significantly *p*-nitroanisole *O*-demethylase activity *in vitro*.

Single doses of U-14,624 (100–200 mg/kg, i.p.) produced linear, dose-dependent suppressions of aminopyrine *N*-demethylase, aniline hydroxylase and *p*-nitroanisole *O*-demethylase activities. As seen in Fig. 2, suppression of oxidative enzymatic activity is strongly correlated with reductions in microsomal cytochrome P-450 levels. Other components of the hepatic microsomal electron transport chain (NADPH-cytochrome *c* reductase and cytochrome b_5) were not modified consistently by U-14,624 treatments. Recovery from the acute inhibitory effects of U-14,624 was complete within 48 hr in all survivors.

Table 1. Activities of hepatic microsomal oxidative enzymes *in vitro* 24 hr after U-14,624 administration (50 mg/kg/day i.p.) to male rats

Days pretreatment with U-14,624 (days)	Treatment group	Aminopyrine *N*-demethylase*	Aniline hydroxylase†	*p*-Nitroanisole *O*-demethylase‡
3	C	96.8 ± 4.3 (6)	9.0 ± 1.2 (6)	15.8 ± 1.6 (6)
	T	86.7 ± 5.9 (6)	9.6 ± 1.0 (6)	19.8 ± 1.6 (6)
4	C	117.3 ± 6.3 (6)	11.6 ± 0.6 (6)	27.1 ± 2.7 (6)
	T	106.5 ± 7.6 (6)	8.6 ± 1.0 (6)	22.0 ± 2.1 (6)
5	C	108.6 ± 3.9 (6)	9.8 ± 0.7 (6)	16.2 ± 1.7 (6)
	T	78.8 ± 6.6§(6)	6.3 ± 0.7§(6)	11.6 ± 1.5 (6)
7	C	107.6 ± 5.2 (4)	12.5 ± 0.5 (4)	34.9 ± 1.2 (4)
	T	40.3 ± 6.0§(4)	3.6 ± 1.2§(4)	17.4 ± 2.8§(4)

* Values represent mean activities expressed as nmoles CH_2O formed per 30 min per mg microsomal protein ± S.E. The numbers of animals are in parentheses.

† Values represent mean activities expressed as nmoles *p*-aminophenol formed per 20 min per mg microsomal protein ± S.E. The numbers of animals are in parentheses.

‡ Values represent mean activities expressed as nmoles *p*-nitrophenol formed per 20 min per mg microsomal protein ± S.E. The numbers of animals are in parentheses.

§ $P \leq 0.05$.

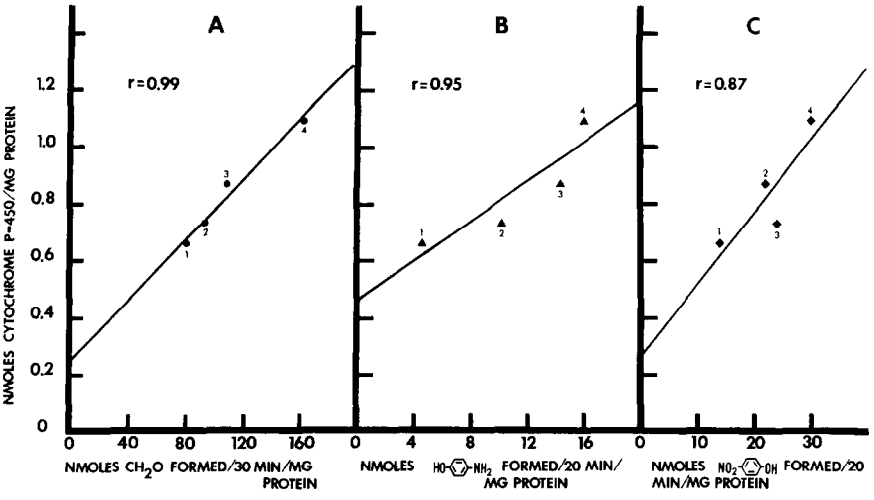

Fig. 2. Least squares regression curves and correlation coefficients demonstrating the relationship between hepatic microsomal cytochrome P-450 levels and oxidative enzymatic activities 24 hr after a single i.p. dose of U-14,624: (1) 200 mg/kg, N = 6; (2) 150 mg/kg, N = 6; (3) 100 mg/kg, N = 12; and (4) 50 mg/kg, N = 6. Key: (A) aminopyrine *N*-demethylase (●); (B) aniline hydroxylase (▲); and (C) *p*-nitroanisole *O*-demethylase (◆).

Repeated doses (> 25 mg/kg/day i.p.) and single doses of U-14,624 impeded normal body weight gain, but reduced initial body weights by less than 10 per cent in all instances. Liver weight to body weight ratios and total microsomal protein were not depressed consistently by doses of U-14,624.

Figure 3 demonstrates that U-14,624 (10^{-5}–10^{-2} M) is directly inhibitory when added to oxidative drug metabolism systems *in vitro*. The inhibition appears to be of a mixed type with an increased apparent K_m as well as reduced V_{max} for all three systems [37]. Although U-14,624 is inhibitory to hepatic microsomal aminopyrine *N*-demethylase, aniline hydroxylase and *p*-nitroanisole *O*-demethylase *in vitro*, it failed to inhibit microsomal neotetrazolium diaphorase activity *in vitro* at concentrations as great as 5×10^{-3} M.

DISCUSSION

In contrast to the work of Khalsa and Davis [18], we observed that the acute toxicity of U-14,624 (LD_{50} = 519 mg/kg) is not great in young, healthy male rats. However, as our animals aged and as respiratory difficulties appeared, the LD_{50} value was observed to decrease. We suspect that the apparent

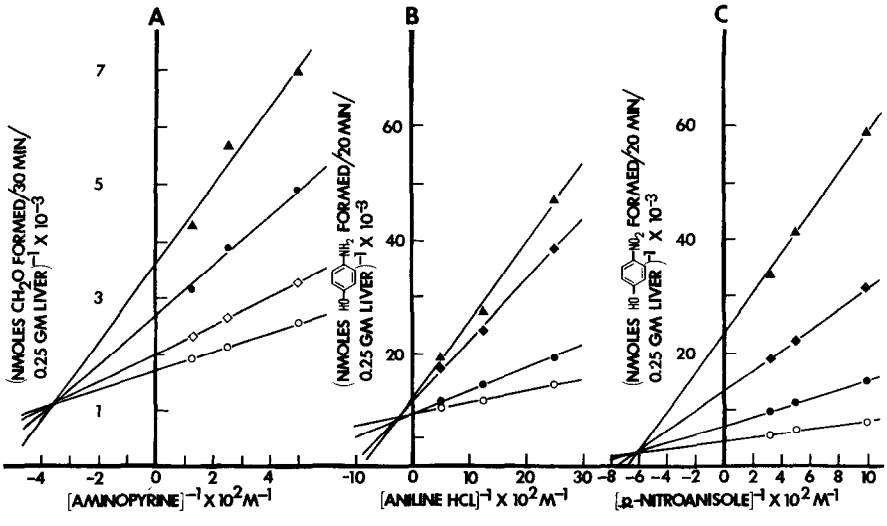

Fig. 3. Double reciprocal plots of oxidative drug metabolism in the absence (controls) and in the presence of U-14,624. (A) aminopyrine *N*-demethylase: 2, 4 and 8×10^{-3} M aminopyrine; (B) aniline hydroxylase: 4 and 8×10^{-4} M and 2×10^{-3} M aniline HCl; and (C) *p*-nitroanisole *O*-demethylase: 1, 2 and 3×10^{-3} M *p*-nitroanisole. Concentrations of U-14,624 used were: controls (○), 10^{-5} M (◇), 10^{-4} M (●), 10^{-3} M (◆) and 10^{-2} M (▲). The source of oxidative enzyme systems and glucose-6-phosphate dehydrogenase was hepatic soluble fraction (12,000 *g*) equivalent to 250 mg of liver (7.5 mg of microsomal protein).

discrepancy between the LD_{50} values is a function of the respiratory difficulties seen with chronically maintained rats together with the CNS depressant properties of U-14,624. Animals receiving either multiple (> 25 mg/kg/day, i.p.) or single doses of U-14,624 developed static body weight growth patterns. In the present experiments, U-14,624 depressed normal body weight increases, but did not cause the pronounced weight decrements noted by Khalsa and Davis [38]. Liver mass was not depressed by relatively toxic doses of U-14,624.

Small, nontoxic doses of U-14,624 significantly impaired the biotransformation of two type I substrates, pentobarbital and antipyrine, *in vivo*. In the case of pentobarbital, clearance of the drug was slowed and sleeping times were prolonged without any apparent increase in the sensitivity of the CNS to the anesthetic. In the absence of increased CNS sensitivity as indicated by identical plasma and brain pentobarbital levels at awakening, increased barbiturate sleeping times can be attributed to inhibition of barbiturate inactivation by microsomal enzymes [39]. Welch *et al.* [40] have demonstrated that the plasma clearance of antipyrine accurately reflects the functional status of several hepatic drug-metabolizing enzymes. Accordingly, the depressed hydroxylation of antipyrine found in the present experiments reinforces the hypothesis that U-14,624 is inhibitory to drug-metabolizing systems *in vivo*.

Although the activities of hepatic microsomal aminopyrine *N*-demethylase, aniline hydroxylase and *p*-nitroanisole *O*-demethylase were inhibited by multiple as well as single dosage regimens of U-14,624, the latter regimen is especially effective in inhibiting oxidative drug metabolism. This suppression of activity is correlated positively with reductions in hepatic microsomal cytochrome P-450 levels, but not with reductions in cytochrome b_5 levels or NADPH-cytochrome *c* reductase activity. The pattern of cytochrome P-450 depression with minimal effects on other components of the mixed-function oxidase system very closely simulates the interaction of disulfiram with these same components [41]. Furthermore, both U-14,624 and disulfiram produce reductions in the microsomal cytochrome P-450 content that are quantitatively similar to the reduced levels of cytochrome P-450 initiated by certain trace metal deficiencies [42, 43]. It appears from the work of Wagner and Tephly [44] and Johnson *et al.* [5] that both U-14,624 and disulfiram chelate copper and in this manner depress cytochrome P-450 levels by interfering with heme biosynthesis. In addition, both drugs, as chelators of heavy metal ions, may negatively influence heme synthesis by chelation of zinc ions which are required for δ-aminolevulinic acid dehydratase activity and by chelation of magnesium ions which are essential to the functioning of δ-aminolevulinic acid synthetase [43, 45]. Since the half-life of the porphyrin moiety in cytochrome P-450 is short-lived (22 hr) relative to that of cytochrome b_5 (45 hr) [46], the content of cytochrome P-450 would decrease prior to any change in the levels of cytochrome b_5 as observed in our experiments.

Mixed inhibition of aminopyrine *N*-demethylase, aniline hydroxylase and *p*-nitroanisole *O*-demethylase resulted when enzymatic preparations from naive animals were exposed to U-14,624 *in vitro*. Inhibition kinetics of this type are frequently interpreted in terms of two or more enzymes catalyzing the same reaction or of a single enzyme that is only partially inhibited [47]. Another explanation for the mixed inhibition is suggested by the work of Hunter and Neal [48] and DeMatteis [49]. These investigators have reported that certain sulfur-containing chemicals cause inhibition of drug metabolism and loss of cytochrome P-450 on incubation with microsomes *in vitro* or after their administration to rats *in vivo*. Among these chemicals are disulfiram, diethyldithiocarbamate and thionosulfur compounds of diverse structure. Metabolic activation by way of oxidative desulfuration has been implicated in the mechanism of toxicity of all of these chemicals. Activation of U-14,624 to a substance which is toxic to cytochrome P-450 could well be instrumental in producing the inhibition of drug metabolism observed in these studies. The evidence of such a mechanism must await further experimentation.

U-14,624 failed to inhibit the reduction of neotetrazolium to its formazan derivative *in vitro*. Since this reaction can proceed via an alternate pathway not involving cytochrome P-450 [50], this result was not unexpected. This observation supports the impression that U-14,624 acts specifically on cytochrome P-450 with minimal involvement of other MFO components.

Studies which employ U-14,624 to evaluate the pharmacologic manifestations of drugs after the establishment of catecholamine imbalance should be interpreted cautiously. The present data demonstrate that U-14,624 impairs the bioconversion of several drugs and prolongs the exposure of animals to these compounds. In the presence of U-14,624, pharmacologic responses to administered drugs may or may not relate directly to inhibition of dopamine β-hydroxylase.

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