The coprecipitates of Drug B with the salt of cholic acid increased toxicity, while coprecipitates with sodium glycocholate were the most toxic. Cholic acid and its salts may have increased the drug solubility by micellar effects and thus showed better absorption. This type of effect is well documented (17).

For Drug B, which was stable in alkaline medium, the coprecipitate with sodium glycocholate was more toxic than the physical admixture. This result indicates that a molecular dispersion was obtained and was absorbed more readily than the physical admixture. Thus, the coprecipitate formulation containing sodium glycocholate would be considered a formula of choice for further development work.

The excipients used in the formulations of both drugs administered individually had little effect on the LD_{50} values in the maximum amount used in the formulation and, therefore, did not contribute to the toxicity (Table VI).

CONCLUSION

The LD_{50} procedure can estimate relative absorption rate differences between formulations of drugs with very low solubility. The most pharmaceutically acceptable formulations in this study were the povidone coprecipitate with Drug A and the sodium glycocholate coprecipitate with Drug B.

In the design of future drug delivery systems, a formulation with an optimum availability rate would be necessary for an acceptable pharmaceutical product. The relatively inexpensive LD_{50} studies can be a practical rapid method of achieving comparative ratings of drug formulations.

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Bunolol Metabolism by Dogs: Identification of Basic Metabolites and Their Conjugates

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Abstract ◻ Female beagles dosed once with encapsulated ^{14}C-bunolol (10 mg/kg) excreted 61% of the isotope in urine in 24 hr. The pooled urine contained a minimum of 18 labeled compounds. Two previously unknown metabolites were purified and were identified by UV and mass spectral data; they were hydroxybunolol (10.1% of urinary radioactivity) and hydroxydihydrobunolol (9.8%). The urine also contained bunolol (0.7% of urinary carbon-14), dihydrobunolol (0.5%), conjugated dihydrobunolol (2.8%), β-(5-oxytetralonyl)lactic acid (16.3%), and (5-oxytetralonyl)acetic acid (7.1%).

Keyphrases ◻ Bunolol—metabolites and conjugates identified, dog urine ◻ Metabolism—bunolol in dogs, metabolites and conjugates identified in urine ◻ Antiadrenergic agents—bunolol, metabolism in dogs, metabolites and conjugates identified in urine

Bunolol [*dl*-5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride] (I) is a potent β-adrenoceptor blocking agent (1–3). Previous studies showed that I was absorbed rapidly by dogs (4) and biotransformed extensively to acidic metabolites (5). Additionally, I was reduced to a secondary alcohol by human cadaver liver (6), human and rat erythrocytes, and liver and extrahepatic tissues (7).

The secondary alcohol, dihydrobunolol (II), is an interesting metabolite because it is a β-adrenergic blocking agent with approximately the same potency as I (6). The present report describes the identification and quantification of II and previously unidentified basic metabolites in the urine of dogs dosed with ^{14}C-labeled bunolol.

EXPERIMENTAL

^{14}C-Labeled Bunolol—Bunolol labeled on the 1-carbonyl was synthesized (8). The preparation was 99.0% pure, both chemically and radiochemically, as judged by TLC; it had a specific activity of 4.90 mCi/g.

Radioactivity Counting—Quantitative assays for carbon-14 were performed using a liquid scintillation spectrometer[1]. The external standardization method was used for quench corrections.

TLC—Chromatograms for analytical purposes were run on 5 × 20-cm glass plates coated with 250 μm of silica gel G bound with calcium sulfate[2]. For preparative efforts, 20 × 20-cm plates were used; they were prewashed by one development in methanol, dried in air, and heated for 1 hr at 100°.

[1] Packard Tri-Carb model 3320.
[2] Analtech.

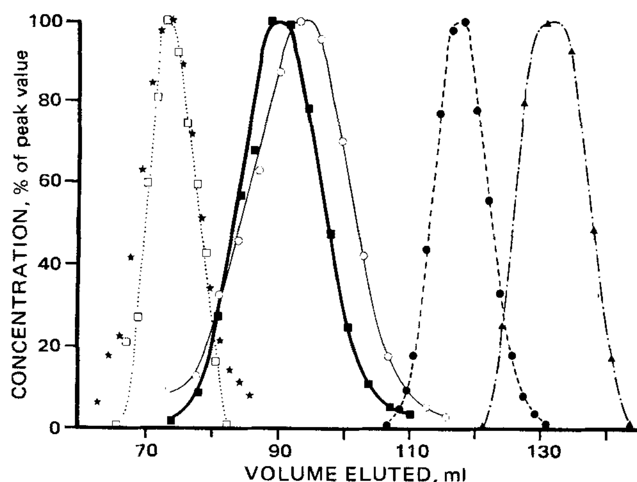


Figure 1—*Separation of bunolol and its metabolites and analogs on a dextran column (1.5 × 83 cm). Separate runs were made using the following amounts: 3 μmoles each of I (●), II (■), and V (▲); 3.6 μmoles of ^{14}C-labeled III (○); 1 μmole of ^{14}C-labeled IV from unconjugated fraction (□); and 2.3 μmoles of ^{14}C-labeled IV from conjugated fraction (★). The peak at 73 ml was drawn only for the values of unconjugated IV.*

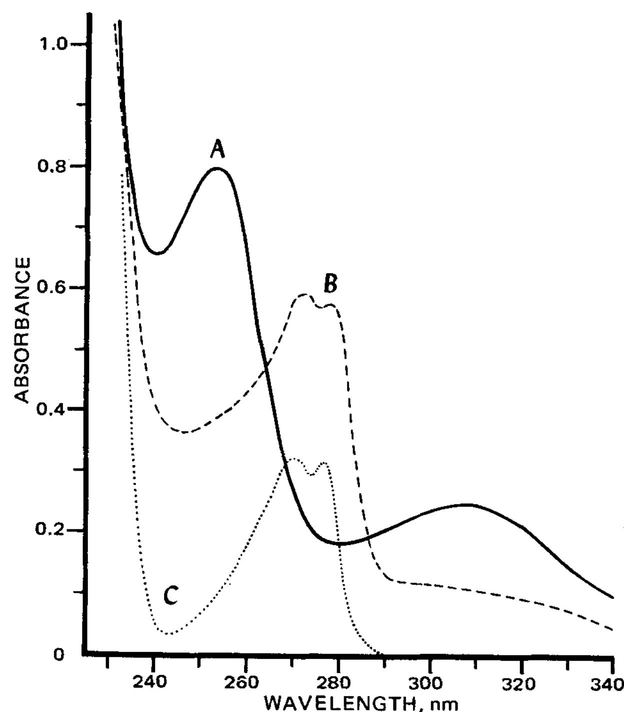


Figure 2—*UV absorption spectra of urinary III (A, 108 μM), urinary IV (B, 348 μM), and nadolol (C, 300 μM). Each compound was dissolved in methanol.*

The solvent systems used were: A, ethanol–15 *N* ammonium hydroxide–distilled water (12:1:7); B, 1-butanol–15 *N* ammonium hydroxide–distilled water (4:1:3, upper phase); C, 1-butanol–acetic acid–ether–distilled water (9:6:3:1); D, 1-butanol–acetic acid–ether–distilled water (6:3:9:1); E, chloroform–acetic acid–methanol (15:1:4); and F, ethyl acetate–15 *N* ammonium hydroxide–ethanol–distilled water (4:1:1:2, upper phase).

One-dimensional chromatograms were scanned for radioactivity with a radiochromatogram scanner[3]. The area under each peak was determined with a compensating polar planimeter[4]. In this manner, R_f values and relative amounts of each metabolite were determined. Radioactivity on preparative chromatograms was located with a two-dimensional scanner fitted with a dot printer[5]. Radioactive bands were scraped from the plates, and the metabolites were eluted with methanol. The estimation of the quantities of metabolites isolated is based on a calculated value of 1813 dpm/μg of administered ^{14}C-bunolol.

Phenolic compounds were detected on TLC plates by spraying with ferricyanide–ferric chloride solution (9); this test permits detection of 0.1 μg of 8-hydroxybunolol.

Mass and UV Spectrometry—Mass spectra were obtained with a double-focusing mass spectrometer using a heated direct insertion probe[6]. The accelerating potential was 8 kv, the ionizing voltage was 60 v, and the trap current was 100 μamp. Hydroxybunolol (III) was run at a probe temperature of 75°; 120° was used for hydroxydihydrobunolol (IV). Exact mass determination was done by the peak matching technique using perfluorokerosene as a reference. UV spectra were recorded[7]. The solvent for all compounds was methanol.

Protocol—Twenty-milligram portions of ^{14}C-bunolol were weighed into gelatin capsules, and enough carrier bunolol was added to provide 10-mg/kg doses. The capsules were administered to female beagles weighing 11.5 (Dog 1), 11.5 (Dog 2), 13.0 (Dog 3), and 10.0 (Dog 4) kg. The dogs had free access to food and water. Urine collected from 0 to 24 hr from each dog was kept frozen until needed. The 0–24-hr urine samples from Dogs 1–3 were pooled before investigation and were identical with those used to determine the extent of bunolol excretion (4).

Dog 4 was given two additional identical doses of ^{14}C-bunolol 8 and 15 days after the first dose. Urine was collected for 24 hr following each dose. The three urine collections contained 47.6, 58.7, and 59.4% of the radioactivity of the respective doses. These urine collections, totaling 1050 ml, were pooled and stored frozen.

Recovery of Reference I–IV from Urine of Untreated Dogs—To determine the stability of bunolol and its basic metabolites during isolation, 50 μg of each radioactive compound in 50 μl of methanol was added to a separate 10-ml portion of urine from an untreated dog. After adding 1 ml of ether to each solution, air was passed over the surface for 4 hr to simulate the operation designed to free urine of residual ether.

Each urine specimen was extracted six times with ether, and the extracts were concentrated. Comparative TLC in Solvent E was performed on the ether concentrates and on solutions of the four reference compounds. Radiochromatogram scanning showed that no decomposition resulted from exposure to air under these experimental conditions.

Fractionation of Unconjugated Labeled Bases—The pooled urine (1050 ml) from Dog 4, containing 165-mg equivalents of bunolol, was adjusted to pH 2 and extracted 10 times with 500-ml volumes of ether. The pH of the aqueous phase was brought to 10, and 12 extractions with 500 ml each of ether were performed. The pooled ether extract obtained at pH 10 contained 30-mg equivalents of bunolol.

After the volume was reduced to 2 ml, the solution was chromatographed on a 1.5 × 83-cm dextran column[8], prepared and operated in 20 m*M* sodium phosphate buffer at pH 7.0. [The column had been precalibrated by separate successive applications of 3 μmoles each of I hydrochloride, II oxalate, and 8-hydroxybunolol (V) hydrochloride and by monitoring the effluent at 255 nm for I and V and at 272 nm for II.] The urine extract of unconjugated bases yielded two radioactive fractions. One was the column effluent from 65 to 80 ml, and the other was the effluent from 80 to 105 ml.

Isolation of Unconjugated IV—The fraction eluted from the dextran column from 65 to 80 ml was lyophilized, and the residue was extracted with methanol. The extracts were reduced to 0.2 ml, and the concentrate was streaked onto two 20 × 20-cm TLC plates. After development in Solvent E, the plates were air dried and the radioactivity was located by two-dimensional scanning. A single radioactive band at R_f 0.37 was scraped from the plates and eluted with methanol. The volume of the methanol extract was reduced to 0.2 ml, and the concentrate was streaked onto a 20 × 20-cm plate. After development in Solvent B, two-dimensional scanning revealed a single radioactive band at R_f 0.65.

The plate was cut 46 mm above the origin, and the lower section of the plate was discarded. The upper section now contained the radioactive band 25 mm above the lower edge. This section was developed a second time in Solvent B, with the solvent front moving in the same direction as during the first development. The second development improved the separation of the radioactivity from a nonradioactive band visible under UV light. A band containing radioactive material (22 mm wide) was lo-

[3] Packard model 7201.
[4] Keuffel and Esser.
[5] Varian-Berthold.
[6] AEI Scientific MS902C.
[7] Beckman DU spectrophotometer.
[8] Sephadex G-10.

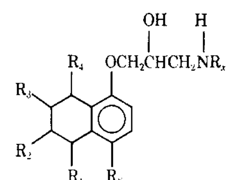


Table I—Structures[a] and R_f Values of Bunolol and Its Urinary Metabolites and Analogs[b]

Compound	R_1	R_2	R_3	R_4	R_8	R_x	R_f Values in Solvent[c] A	B	C	D	E	F
I	=O	H	H	H	H	$C(CH_3)_3$	0.78	0.71	0.31	0.38	0.53	0.43
^{14}C-III[d]	=O	H	H	OH[e]	H	$C(CH_3)_3$	0.75	0.67	0.27	0.24	0.40	0.34
^{14}C-IV[d]	OH	H	H	OH[e]	H	$C(CH_3)_3$	0.75	0.64	0.30	0.24	0.33	0.21
^{14}C-II[d]	OH	H	H	H	H	$C(CH_3)_3$	0.80	0.73	0.40	0.33	0.46	0.41
II[f]	OH	H	H	H	H	$C(CH_3)_3$	0.80	0.74	0.39	0.32	0.45	0.40
V[f]	=O	H	H	H	OH	$C(CH_3)_3$	0.79	0.70	0.31	0.37	0.53	0.43
VI[f]	=O	H	H	H	H	H	0.76	0.46	0.28	0.24	0.20	0.16
Nadolol	H	OH	OH	H	H	$C(CH_3)_3$	0.70	0.57	0.27	0.20	0.25	0.16

[a] Subscripts for substituents are numbered according to the system for the carbon atoms in the tetralone ring system. [b] Approximately 10 μg of each compound was applied to each TLC plate. [c] Unwashed plates were used. Some R_f values were changed by prewashing with methanol. [d] Pure isolate from the unconjugated basic fraction. [e] The hydroxyl group probably is in this position, but conclusive structural proof is lacking. [f] Synthetic.

cated 65 mm above the cut lower edge of the plate. Following scraping, the radioactivity was eluted with methanol.

The eluate was concentrated to 0.2 ml, and the concentrate was purified by preparative TLC in Solvent F. The metabolite was obtained from a 12-mm wide band at R_f 0.27 by scraping and eluting with methanol. The methanol solution was concentrated to 0.2 ml, and 2 ml of chloroform was added. The metabolite solution was purified by passage over a 4 × 6-mm column of diatomaceous earth[9]. After removal of the solvent, the sample weighed 3.8 mg; the calculated weight based on the radioactivity of bunolol was 2.1 mg.

Isolation of Unconjugated III—The fraction eluted from the dextran column from 80 to 105 ml was lyophilized, and the residue was extracted with methanol. The extracts were concentrated to approximately 0.3 ml. The concentrate containing 11-mg equivalents of bunolol was chromatographed in Solvent E using seven prewashed 20 × 20-cm TLC plates. The major radioactive peak at R_f 0.48 was scraped and eluted with methanol. The volume of the methanol extract was reduced to approximately 0.2 ml, and the concentrate was chromatographed in Solvent D on four 20 × 20-cm TLC plates.

A radioactive band at R_f 0.29 represented 74% of the radioactivity on the plate; the balance of the isotope was at R_f 0.41. For further purification, the radioactive band at R_f 0.29 was scraped from the plates and eluted with methanol. The methanol was concentrated to 0.5 ml and then passed over a 1 × 54-cm dextran column[10] equilibrated with methanol. A radioactive peak emerged from 29 to 31.6 ml. After evaporation of the methanol, the residue weighed 3.4 mg; the calculated weight based on the radioactivity of bunolol was 2.4 mg.

Isolation of Unconjugated II—The radioactive band at R_f 0.41 was eluted with methanol. Solvent evaporation left a residue weighing 30 mg, whereas the carbon-14 content corresponded to only 1.3 mg of bunolol. The material was purified by rechromatography in Solvent B and elution of the band at R_f 0.71 with methanol. After solvent removal, the residue weighed 1.7 mg; its radioactivity corresponded to 0.83 mg of bunolol.

Isolation of II and IV after Enzymic Hydrolysis—A 368-ml aliquot of pooled urine from Dogs 1–3, containing 55-mg equivalents of bunolol, was extracted repeatedly at pH 1.8 and 10.5 with ether to remove unconjugated bunolol metabolites. The pH was brought to 6.5, and air was blown over the solution for 4 hr to remove residual ether. For each 100 ml of urine, 0.2 ml of a preparation of aryl sulfatase–β-glucuronidase[11] was added. The amounts corresponded to 0.6 international unit of aryl sulfatase and 1.2 international units of β-glucuronidase.

After incubation at 37° for 15 hr, another 0.2 ml of enzyme solution was added and incubation was resumed for 7 hr. Then deconjugated acids were removed by three extractions at pH 1.8 with ether. Bases were collected by four ether extractions at pH 10.5; radioactivity counting showed the presence of 8.6-mg equivalents of bunolol. The solution of bases was taken almost to dryness and chromatographed in Solvent E on 10 TLC plates. The two radioactive bands were eluted repeatedly with 10-ml portions of methanol.

The eluate of the slower moving material was purified further by preparative TLC in Solvent B on 12 TLC plates. The radioactive bands were eluted and contained carbon-14 equivalent to 1.3 mg of bunolol. This fraction represented IV. The fraction obtained by elution of the faster moving band was rechromatographed on nine plates in Solvent B, and elution yielded 1.3-mg equivalents of II.

Determination of Quantities of Metabolites in Urine—All quantification experiments were conducted with urine pooled from Dogs 1–3. The amounts of metabolites in pooled urine were estimated by a sequence of fractionation steps. Conjugated bases were enzymatically hydrolyzed after ether extraction of the unconjugated bases. Following separation in Solvent E, the radioactive bands were collected by scraping the silica gel containing the radioactive metabolites off the plate, extracting the scrapings four or five times with 1- or 2-ml portions of methanol, and concentrating the pooled methanol fractions to 0.1 or 0.2 ml. After detection of the major peak by radioscanning, its relative quantities were determined by planimetry. The amount of each urinary metabolite was calculated by multiplying the percentage of the dose in the urine by the

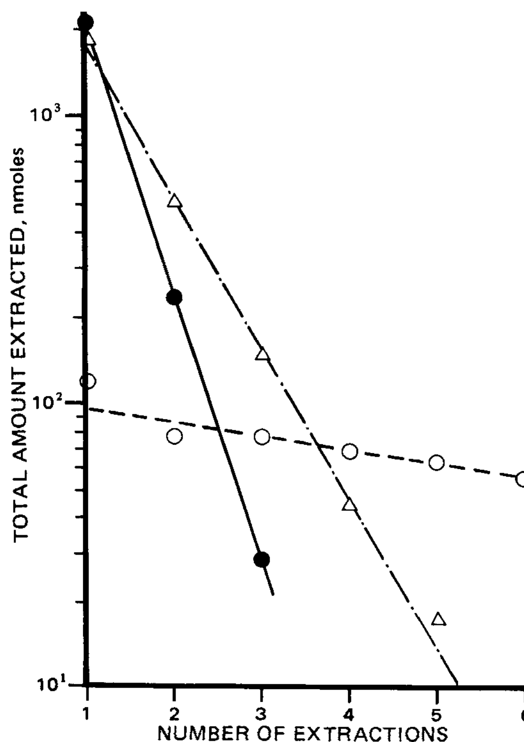


Figure 3—*Ether extraction of I (●), 2.7 μmoles; II (△), 2.0 μmoles; and IV (○), 2.3 μmoles, from aqueous solution. Each aqueous solution (16 ml) was extracted at pH 10 each time with 8 ml of ether.*

[9] Hyflo Supercel Celite.
[10] Sephadex LH-20.
[11] Calbiochem.

Table II—UV Absorption Maxima and Molar Absorptivities of Synthetic Bunolol, Nadolol, and Unconjugated and Deconjugated Basic Bunolol Metabolites

Compound	UV Absorption Peak, nm	Molar Absorptivity[a]
I	253	9000
	310	2400
V	260	8210
Nadolol	270	1080
	277	1060
III	253	8800
	308	2740
II	271	2450
	279	2380
II (deconjugated)	271	2210
	279	2080
IV	272	1810
	278	1760
IV[b]	272	2550
	278	2490

[a] The absorption coefficients of I, V, and nadolol were determined from weighed samples. Coefficients for the other compounds were estimated from the carbon-14 content of solutions. [b] From deconjugated fraction.

percentage of the dose in the ether fraction and by the percentages of the metabolite found on each successive TLC plate.

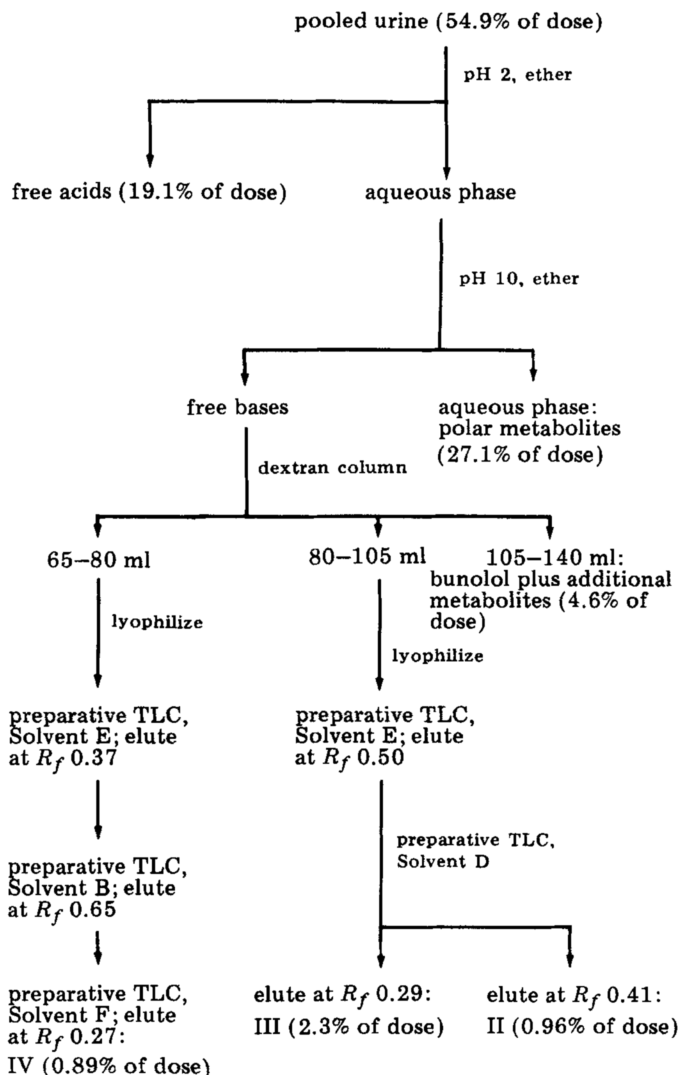
Unconjugated III and IV were assayed in the same urine aliquot (32 ml of the total pool of 805 ml). The specimens were extracted five times at pH 1.8 with two volumes of ether. The aqueous phase pH was brought to 10.5, and the solution was extracted with ether to collect the unconjugated bases. The latter ether extract was reduced to ~0.2 ml and chromatographed on 20 × 20-cm plates in Solvent E. The radioactivity at R_f 0.43 represented III, and the radioactivity at R_f 0.33 represented IV. Compound III was collected, eluted, and chromatographed successively in Solvents C and B. Compound IV was assayed independently by successive TLC in Solvents B and C.

Unconjugated II was assayed similarly using a 22-ml aliquot of the pooled urine. Since synthetic II was available, it was chromatographed in parallel with the urinary metabolite and located in TLC plates by exposure to iodine vapor. The sequence of developing solvents employed was E, C, and B.

Table III—Recovery of Bunolol Metabolites at Major Stages of Purification from Pooled 0–24-hr Urine of Dogs Dosed with ^{14}C-Bunolol (10 mg/kg po)

Metabolite	Purification Step	Percent of Preceding Step
II	Unfractionated urine	61.2[a]
	Ether extract, pH 10	24.6
	Eluate at R_f 0.57 from TLC, Solvent E	52.4
	Eluate at R_f 0.54 from TLC, Solvent C	4.7
	Eluate at R_f 0.73 from TLC, Solvent B	81.6
III	Unfractionated urine	61.2[a]
	Ether extract, pH 10	24.6
	Eluate at R_f 0.43 from TLC, Solvent E	60.7
	Eluate at R_f 0.29 from TLC, Solvent C	83.5
	Eluate at R_f 0.65 from TLC, Solvent B	81.0
IV	Unfractionated urine	61.2[a]
	Ether extract, pH 10	24.6
	Eluate at R_f 0.22 from TLC, Solvent E	29.6
	Eluate at R_f 0.50 from TLC, Solvent B	88.2
	Eluate at R_f 0.32 from TLC, Solvent C	98.0
II (deconjugated)	Unfractionated urine	61.2[a]
	Ether extract, pH 10[b]	7.8
	Eluate at R_f 0.43 from TLC, Solvent E	45.6
	Eluate at R_f 0.53 from TLC, Solvent C	77.6
	Eluate at R_f 0.83 from TLC, Solvent A	100
IV[c]	Unfractionated urine	61.2[a]
	Ether extract, pH 10[b]	7.8
	Eluate at R_f 0.23 from TLC, Solvent E	50.8
	Eluate at R_f 0.35 from TLC, Solvent C	94.1
	Eluate at R_f 0.78 from TLC, Solvent A	92.0
VI	Unfractionated urine	61.2[a]
	Ether extract, pH 10	26.6
	Eluate at R_f 0.16 from TLC, Solvent E	0.46
	Eluate at R_f 0.28 from TLC, Solvent C	24.1
	Eluate at R_f 0.40 from TLC, Solvent B	11.0

[a] Percent of total dose excreted. [b] Preceded by aryl sulfatase–glucuronidase treatment. [c] From deconjugated fraction.



Scheme I

The quantities of basic metabolites extracted after enzyme hydrolysis were determined from a 100-ml aliquot of urine. After extracting unconjugated acids and bases at pH 1.8 and 10.5 with ether and evaporating residual solvent, the pH was adjusted to 6.6 for incubation with aryl sulfatase–β-glucuronidase as described. An ether extract containing both deconjugated bases was chromatographed in Solvent E. The slower moving radioactive band contained IV, which was eluted and rechromatographed sequentially in Solvents C and A. The faster moving band contained II, which was eluted and also rechromatographed in Solvents C and A.

Attempt to Identify Des-*tert*-butylbunolol (VI) in Urine—A 68-ml aliquot of pooled urine was extracted thoroughly with ether to remove acids at pH 1.8 and bases at pH 10.5. The extract of bases was concentrated to 0.3 ml and then streaked onto several 20 × 20-cm TLC plates. Synthetic VI was spotted on both sides of the urine extract, and the plates were developed in Solvent E.

Each plate had three major bands of radioactivity; none corresponded to the synthetic amine. The small quantity of radioactivity at the R_f value of the reference compound was scraped from the plates, eluted, and rechromatographed in Solvent C in parallel with the authentic compound. The process was repeated with Solvent B.

RESULTS

Figure 1 shows that I, II, IV, and V are separable by adsorption chromatography using a dextran[10] column. Scheme I shows how this resolution capability was employed to isolate II–IV from the urine of Dog 4 (which received three doses of ^{14}C-bunolol). Scheme II shows the mass

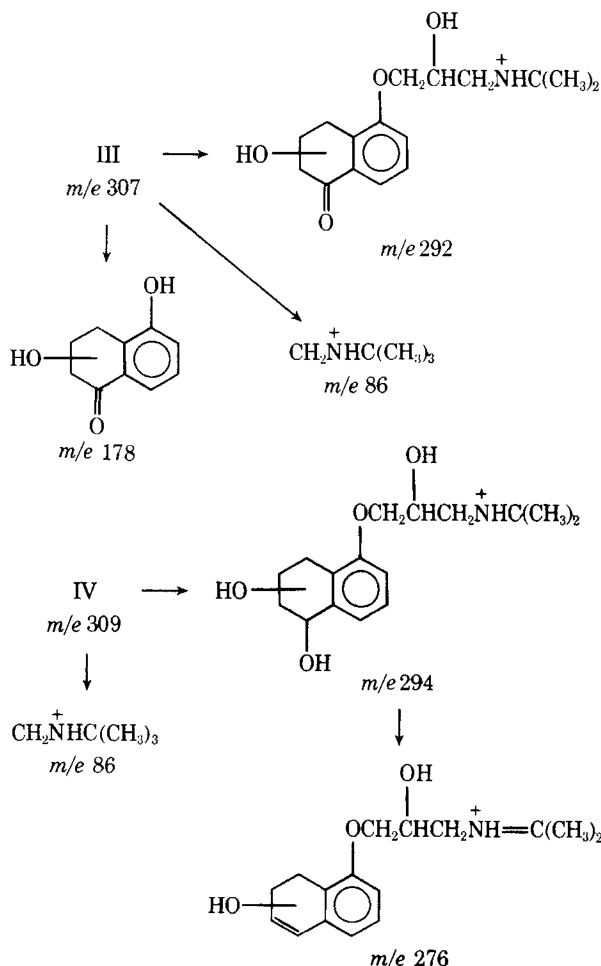
spectral fragmentation patterns of III and IV, metabolites previously discussed (4) and now identified for the first time. The UV spectra of these metabolites are compared with the nadolol spectrum in Fig. 2. The positional isomers, nadolol and IV (Table I), have similar spectra (Table II), a finding that supports the identification of the metabolite by interpreting mass spectral data. The spectrum of III is very similar to that of bunolol (Table II).

Table I presents the structures and R_f values in six solvents of the reference compounds used in this study and of purified bunolol metabolites from dog urine. Table II lists the UV absorption maxima and the molar absorption coefficients of bunolol, nadolol, and unconjugated and deconjugated basic bunolol metabolites isolated from dog urine.

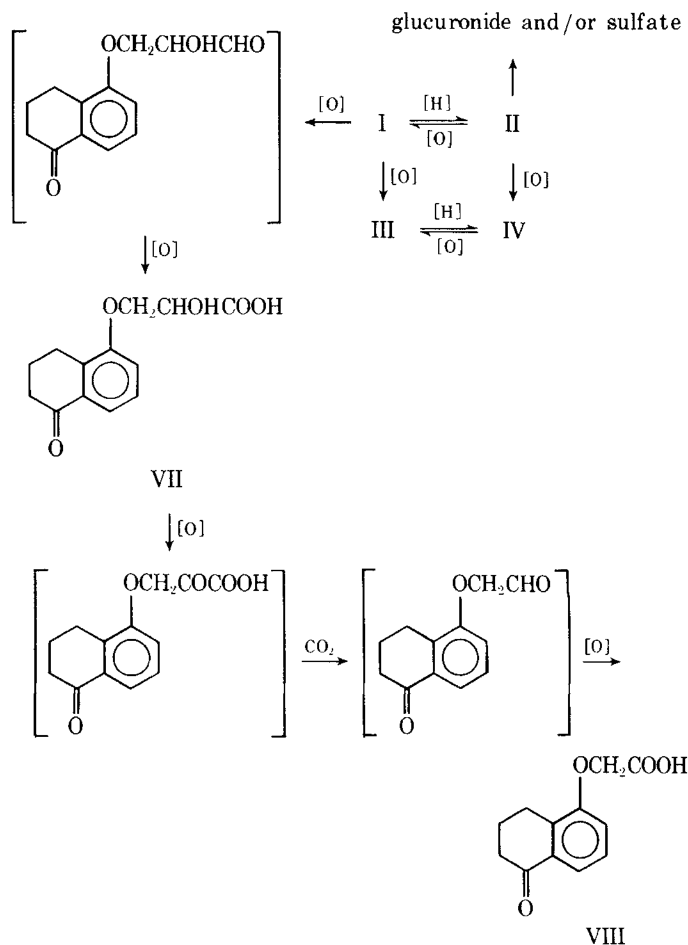
Table III is a condensation of quantitative estimates at the major steps involved in the purification of unconjugated II–IV and of II and IV after enzyme hydrolysis. Table III also shows that the total urinary excretion of the label in 24 hr was 61.2% of the administered dose and that VI could not be isolated from dog urine.

The fractionation and chromatography studies showed that the pooled urine of Dogs 1–3 contained at least 18 isotopically labeled compounds. The major fractions were four unconjugated acids that accounted for 22.5% of the dose, three unconjugated bases (14.7% of dose), and an unknown number of unclassified polar metabolites (14.2% of dose). The minor fractions consisted of six conjugated acids (2.8% of dose) and four conjugated bases (7.0% of dose).

Table IV further illustrates the extensive biotransformation of bunolol in the dog. Seven compounds represented 47% of the urinary radioactivity, and two acidic metabolites, β-(5-oxytetralonyl)lactic acid (VII) and (5-oxytetralonyl)acetic acid (VIII), accounted for almost half of the material identified. The balance consisted of unconjugated and conjugated bases. Compounds I–IV were found in unconjugated form, and conjugated II was also present. Although IV was extracted after enzyme hydrolysis (Table III), this material was assigned to the unconjugated base. This assignment was based upon an extraction study that indicated that IV, collected after enzyme treatment, represented carryover from the unconjugated fraction. Briefly, the extraction experiments with pure compounds in aqueous solutions at pH 10 showed that six ether extractions removed only 20% of IV whereas only three extractions removed at least 96% of I and II (Fig. 3).



Scheme II



Scheme III

DISCUSSION

The pathways proposed for bunolol biotransformation in the dog are illustrated in Scheme III. Reductive as well as oxidative reactions are involved. The acidic metabolites were described earlier (5). The present study supports the depicted oxidation of the side chain to yield *tert*-butylamine rather than desalkylbunolol because the latter compound could not be detected in dog urine. This experimental observation and interpretation are consistent with the literature showing that the *tert*-butyl group is refractory to *N*-dealkylation (10, 11).

The presence of II in dog urine was mentioned previously (6), but its extensive conjugation was unknown. Compound III is a newly identified metabolite. Although synthetic work is needed for assignment of the position of the hydroxyl group, it is clear that the radical is in the saturated ring because the metabolite did not respond to the phenol spray test (9) whereas reference compound 8-hydroxybunolol (V) gave a strong color reaction. The hydroxylated metabolite was also readily distin-

Table IV—Quantities of Identified Labeled Compounds in Pooled Urine in Dogs 1–3

Compound	Percent of Dose	Percent of Urinary Carbon-14
I[a]	0.4	0.7
III[a]	6.2	10.1
II	0.3	0.5
II (conjugated)	1.7	2.8
IV	6.0	9.8
VII[a]	10.0	16.3
VIII[a]	4.3	7.1
Total	28.9	47.3

[a] No conjugate detected.

guishable from the synthetic hydroxyl compound by dextran adsorption chromatography (Fig. 1) and by migration in Solvents D–F (Table I). Compound V could not be detected as a metabolite.

Another newly identified metabolite is unconjugated IV. It is assumed tentatively that the second ring hydroxyl group is in the same position as in III and that the diol metabolite may be formed from this intermediate and from II.

One cannot be certain about the oxidation of II or IV *in vivo*. Nevertheless, these conversions are postulated in Scheme III because II was oxidized readily *in vitro* by human and rat erythrocytes (7). In view of the β-adrenoceptor blocking activity of both I and II (6), it seems reasonable to speculate that III and IV are active metabolites and, therefore, to consider that I is not only an active compound but a prodrug which is converted to three active metabolites.

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Effects of Adenine Nucleotides on Oxidation of Phenothiazine Tranquilizers

IRA BLEI

Abstract ☐ The effects of adenosine diphosphate and triphosphate on the periodic acid oxidation of the phenothiazine tranquilizing drugs were studied. The principal effect was a marked reduction in the rate of formation and decay of the drug free radical. The oxidation rates of the nucleotide free drugs seemed to be most strongly influenced by the inductive effects of substituents at the 2-position of the phenothiazine nucleus. However, the oxidation rates of the drugs in the presence of nucleotide were most strongly influenced by the substituents at the 10-position. Variations of the structure of substituents at the 10-position have only a modest effect on the electronic state of the phenothiazine nucleus. Therefore, the marked effect of structural variation in the 10-substituents in the presence of nucleotide on the periodate oxidation rate most likely is an expression of steric effects related to an interaction between drug and nucleotide.

Keyphrases ☐ Adenosine diphosphate and triphosphate—effect on periodic acid oxidation of various phenothiazines ☐ Phenothiazines, various—periodic acid oxidation, effect of adenosine diphosphate and triphosphate ☐ Oxidation—various phenothiazines by periodic acid, effect of adenosine diphosphate and triphosphate ☐ Nucleotides—adenosine diphosphate and triphosphate, effect on periodic acid oxidation of various phenothiazines ☐ Tranquilizers—various phenothiazines, periodic acid oxidation, effect of adenosine diphosphate and triphosphate

This study was based on an effect observed during a fluorescence quenching study of chlorpromazine (I)–adenosine triphosphate (II) complex formation, previously studied by surface chemical methods (1) and later verified by spectroscopic techniques (2). In the presence of nucleotides, the phenothiazine drug oxidation rates caused by UV irradiation were markedly reduced compared with nucleotide free drug systems. It was felt that a study of this effect might reveal useful details regarding the nucleotide–drug interaction. The general objective of this work was not to obtain absolute rate constants or other kinetic parameters but rather to obtain relative reaction rates from oxidation systems identical with respect to all variables except for structural variations of a group of phenothiazine tranquilizing drugs.

EXPERIMENTAL

Qualitative and quantitative analyses for phenothiazine free radicals were performed principally by spectrophotometry. The identity of the free radical was verified by parallel electron spin resonance measurements (3). The products of oxidation were characterized by TLC, spot tests (4), and UV–visible spectrophotometry (5).

To use spectrophotometry for quantitative analysis, the initial drug concentration had to be about 1×10^{-3} *M*. The system then had to be poised with respect to both nucleotide and oxidant concentrations. The nucleotide concentration was made high enough to assure maximal free radical formation. The periodic acid concentration was not too low so that too small a fraction of drug would be converted to semiquinone nor too high so that the reaction would proceed too rapidly for adequate quantitation. A typical final reaction mixture was: drug, 1×10^{-3} *M*; II, 1×10^{-2} *M*, tromethamine (III)–hydrochloride buffer, pH 7.0, 2×10^{-2} *M*; and periodic acid, 1×10^{-2} *M*.

A typical experiment was performed as follows. Drug, nucleotide, and buffer were added to a volumetric flask and diluted to a precalibrated mark with distilled water. An aliquot of periodic acid stock solution was then added to begin the reaction, followed by final adjustment of volume. Under these conditions, the final pH ranged from 3.0 to 4.0.

Molar absorptivities were determined by periodic acid oxidation of