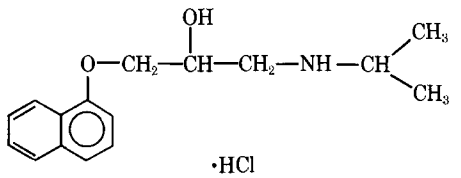
# Absorption, Tissue Distribution, and Excretion of Bunolol-$^{14}$C by Dogs

FRANZ-JOSEF LEINWEBER, LLOYD J. HAYNES, MALCOLM C. CREW, and FREDERICK J. DI CARLO

**Abstract** □ Encapsulated bunolol-$^{14}$C was absorbed quickly by dogs. The peak blood level 1 hr. after treatment corresponded to 4.6% of the dose or 5.1 mcg./ml. Within 72 hr., 54–75% of the radioactivity was excreted in the urine and 15–17% in the feces. At 72 hr., the highest tissue levels were only 0.6–1.4% of the $^{14}$C dose in the GI tract and 0.6–1.1% in the liver. The radioactivity in the 0–24-hr. pooled urine included four unconjugated (37%) and six conjugated acids (5%), three unconjugated (24%) and five conjugated bases (11%), and 24% ether-insoluble metabolites. A radioactive unconjugated basic compound was purified from the 0–24-hr. urine. The compound, comprising 0.7% of the urinary $^{14}$C, migrated as bunolol on TLC. Further identification was made by GLC; retention times for the acetates and trifluoroacetates prepared from authentic and urinary bunolol were identical. Bunolol glucuronide was not found in the 0–24-hr. urine.

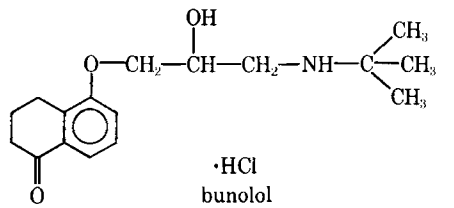
**Keyphrases** □ Bunolol-$^{14}$C, metabolites—absorption, excretion, tissue distribution, dogs □ Absorption kinetics—bunolol-$^{14}$C, dogs □ Excretion kinetics—bunolol-$^{14}$C, dogs □ Urinary drug metabolism—bunolol-$^{14}$C, dogs

Bunolol {*dl*-5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride} was shown to be the most active analog among 30 compounds displaying β-adrenergic blocking activity (1). Following intravenous administration to barbiturate-anesthetized dogs, bunolol was estimated to be 2–3 times more potent than propranolol in antagonizing the isoproterenol-induced changes in heart rate, blood pressure, contractile force, and tachycardia (2, 3). Furthermore, bunolol displayed a greater separation between the β-adrenergic blocking and myocardial depressant doses than did propranolol (2). A study in anesthetized vagotomized dogs challenged with isoproterenol showed bunolol to be 20 times more potent than propranolol after oral administration (4). On the other hand, bunolol was found to be less active than propranolol against ouabain-induced arrhythmia in dogs (5). Bunolol and propranolol were equipotent in blocking isoproterenol protection of histamine-induced bronchoconstriction (6).



·HCl

propranolol



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bunolol

The purpose of the present study was to investigate the absorption, excretion, and tissue distribution of bunolol in the dog after oral administration. In addition, efforts were made to estimate quantitatively the unaltered drug and to determine the number and types of drug metabolites.

Table I—Blood Levels of Total $^{14}$C after Oral Administration of Bunolol-$^{14}$C to Dogs

| Dog Number | Radioactive Dose in Blood, mcg./ml. 30 min. | 1 hr. | 2 hr. | 4 hr. | 6 hr. | 24 hr. | 48 hr. |
|---|---|---|---|---|---|---|---|
| 1 | 5.0 | 5.2 | 3.2 | 2.0 | 1.0 | 0.05 | 0.08 |
| 2 | 1.0 | 6.1 | 4.1 | 1.7 | 1.5 | 0.2 | 0.08 |
| 3 | 5.3 | 4.4 | 3.0 | 1.6 | 0.7 | 0.2 | 0.1 |
| Mean | 3.8 | 5.2 | 3.4 | 1.8 | 1.1 | 0.15 | 0.09 |

## MATERIALS AND METHODS

**Radioactive Bunolol**—Radioactive bunolol was synthesized with $^{14}$C in position 1 (carbonyl-C) of the saturated ring (7). The preparation was 99.0% pure, both chemically and radiochemically, as judged by TLC; it had a specific radioactivity of 4.90 mc./g.

**Protocol**—Twenty-milligram portions of bunolol-$^{14}$C were weighed into three gelatin capsules, and enough bunolol was added to provide 10-mg./kg. doses. The capsules were administered to female beagles weighing: Dog 1, 11.5 kg.; Dog 2, 11.5 kg.; and Dog 3, 13.0 kg. The dogs had free access to food and water.

**Blood Levels and Radioactivity**—Blood samples (about 2 ml.) were withdrawn from each dog at the following time intervals after treatment: 30 min. and 1, 2, 4, 6, 24, 48, and 72 hr. Duplicate aliquots of each blood specimen (1 ml. each) were diluted with distilled water (100-fold for the first five samples, 50-fold for the 24- and 48-hr. specimens, and 10-fold for the 72-hr. specimens) and stored overnight under refrigeration. These lysed blood mixtures were counted for radioactivity with a liquid scintillation spectrometer[1]. One milliliter of each urine collection was diluted with 18 ml. of scintillation solution and counted directly. The counting efficiency was determined by the external standardization method. The scintillation solution consisted of 7.0 g. 2,5-diphenyloxazole, 0.3 g. dimethyl 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene, and 100 g. naphthalene in 1.0 l. reagent grade dioxane.

**TLC**—Chromatograms for analytical purposes were run on 5 × 20-cm. glass plates coated with 250 μ of silica gel G bound with calcium sulfate[2]. For preparative efforts, 20 × 20-cm. plates were used. Before sample application, the plates were developed in methanol, dried in air, and heated in an oven at 140° for at least 30 min. One-dimensional chromatograms were developed using the following solvents: 1, chloroform–glacial acetic acid–methanol (15:1:4); 2, butanol–29.6% ammonia–water (4:1:3, upper phase); and 3, butanol–glacial acetic acid–ether–water (9:6:3:1).

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

Table II—Excretion of $^{14}C$ after Oral Administration of Bunolol-$^{14}C$ to Dogs

| Dog | Radioactive Dose in Urine, % | | | | Radioactive Dose in Feces[a], % | | | |
|---|---|---|---|---|---|---|---|---|
| | 0–24 hr. | 24–48 hr. | 48–72 hr. | 0–72 hr. | 0–24 hr. | 24–48 hr. | 48–72 hr. | 0–72 hr. |
| 1 | 49.5 | 2.9 | 1.4 | 53.8 | No feces | 14.7 | 2.5 | 17.2 |
| 2 | 65.0 | 2.2 | 1.5 | 68.7 | 14.4 | 1.0 | 1.2 | 16.6 |
| 3 | 69.1 | 4.8 | 1.4 | 75.3 | 7.8 | 4.9 | 2.1 | 14.8 |
| Mean | | | | 65.9 | | | | 16.2 |

[a] Almost half of the labeled end-products were not extracted and were measured by digesting the stool with hydroxide of hyamine 10-X.

The chromatograms were scanned for radioactivity with a radiochromatogram scanner[3]. The area under each peak was determined with a compensating polar planimeter[4]. Estimates of relative peak area were used to calculate the amounts of bunolol metabolized. To measure the $^{14}C$ on 20 × 20-cm. thin-layer plates, the plates were cut into 5-cm. strips and scanned.

**GLC**—The samples were injected into a gas chromatograph[5] fitted with dual columns and a differential flame-ionization detector. The columns were 1.8 m. × 0.31 cm. (6 ft. × 0.125 in.) [0.26 cm. (0.105 in.) i.d.] stainless steel, each packed with 3.45 g. of 3% OV-1 on 80–100 mesh Chromosorb W (HP). The columns were operated at 198° for assay of the acetate and at 173° for assay of the trifluoroacetate. The injector temperature was 278°; the helium carrier gas flow rate was 19 ml./min. for the sample column and 38 ml./min. for the reference column.

## RESULTS

**Blood Levels**—The data presented in Table I indicate that bunolol was absorbed rapidly. The maximum blood concentration was observed after 1 hr. and corresponded to a mean of 4.6% of the dose in the systemic circulation. Subsequently, the blood $^{14}C$ level decreased gradually, and radioactivity was measurable after 48 hr. but not after 72 hr.

**Urinary Excretion**—All excreted urine was collected from each dog over the periods from 0 to 24 hr., from 24 to 48 hr., and from 48 to 72 hr. Radioactivity measurements (Table II) showed that most of the drug and its metabolites (49.5–69.1%) were excreted during the first 24 hr.; only small amounts (1.4–4.8%) were excreted in successive 24-hr. intervals. The cumulative urinary excretion reached 53.8–75.4% of the dose by 72 hr.

**Fecal Excretion**—The complete fecal output was collected at 24-hr. intervals for 3 days. Each collection was extracted repeatedly with 75% dioxane, and aliquots of the residues were digested by shaking overnight at 37° with hydroxide of hyamine 10-X[6]. The extracts and the digestion mixtures were counted for radioactivity, and the total value for each collection is listed in Table II. Despite the failure of Dog 1 to defecate during the 1st day, there was close agreement in the total fecal elimination of $^{14}C$ by the three dogs (14.8–17.2% of the dose in 72 hr.).

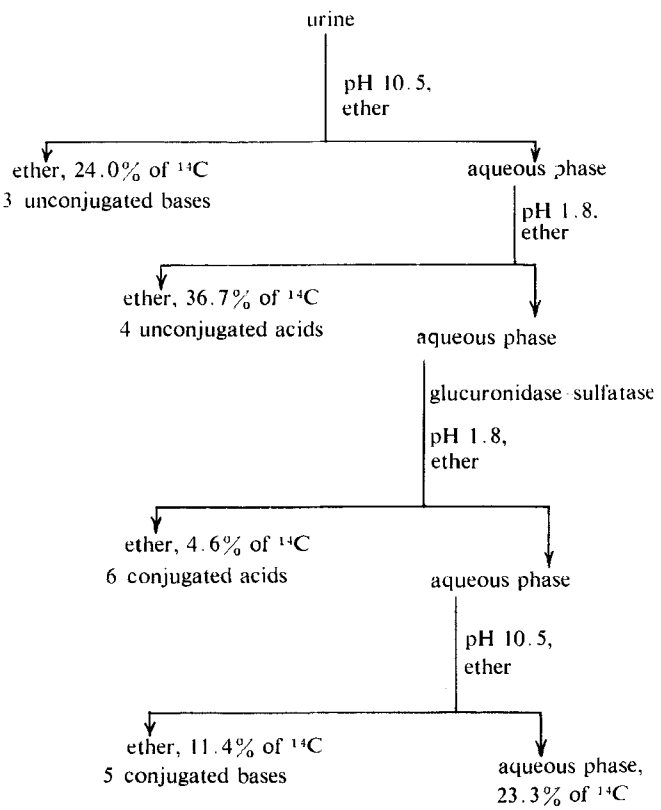
**Tissue Distribution**—Each dog was sacrificed 72 hr. after drug administration. The brain, heart, entire GI tract and its contents, kidneys, spleen, and liver were excised from each animal. Also removed were portions of fat (87–157 g.) and of muscle (102–123 g.). All of these tissues were homogenized and extracted thoroughly with 75% dioxane before portions of the residues were digested with hyamine. The quantities of radioactivity found were greatest in the GI tract and liver (Table III), possibly indicating prolonged enterohepatic circulation of drug metabolites.

**Ether Fractionation of Urinary Metabolites**—A 1-ml. aliquot of pooled urine collected from the three dogs from 0–24 hr. was adjusted to pH 10.5 and extracted three times with 4 ml. of ether. The aqueous layer was adjusted to pH 1.8 and again extracted three times with ether. The pH of the aqueous phase was adjusted to 6.5, and traces of ether were removed by aeration. A preparation containing 7500 Fishman units of β-glucuronidase and 8000 Whitehead units of aryl sulfatase[7] in 50 μl. was added and incubated at 37°. Two hours later, an additional 50 μl. of this enzyme preparation was added. After a total of 45 hr. of incubation, ether extractions were performed at pH 1.8 and 10.5. The corresponding ether extracts were pooled and assayed for $^{14}C$. Practically all of the urinary $^{14}C$ was recovered (Scheme I).

The consideration of neutral structures was set aside, and the urine fractions were designated as unconjugated acids (37%), unconjugated bases (24%), conjugated acids (5%), and conjugated bases (11%). Approximately 23% of the urinary $^{14}C$ fell into none of these categories (Scheme I).

**Number of Urinary Bunolol Metabolites**—Ether extracts, obtained as described, were subjected to TLC in Solvent 1. As noted in Scheme I, radioscanning revealed the presence of three unconjugated and five conjugated bases and of four unconjugated and six conjugated acids. The elucidation of the structure of two of the unconjugated acids is the subject of another report (8), and an investigation of the structures of basic metabolites is in progress.

**Purification of Bunolol from Dog Urine**—The plan followed is shown in Scheme II. The starting material was 56 ml. of the urine collected from 0 to 24 hr. after treating three dogs with $^{14}C$-bunolol. To remove unconjugated acids, the urine was extracted five times with two volumes of ether after pH adjustment to 1.8. To collect the bases for study, the urine was brought to pH 10.5 and extracted thoroughly with ether. The ether phase obtained by extraction of the alkalinized urine contained 24.6% of the $^{14}C$. Following evapo-




*Scheme I—Fractionation and TLC of drug metabolites in dog urine after bunolol-$^{14}C$ administration*

[3] Packard model 7201.
[4] Keuffel and Esser.
[5] Model 1670, HCL Scientific Inc., Rockford, Ill.
[6] Packard Instrument Co.
[7] Calbiochem.

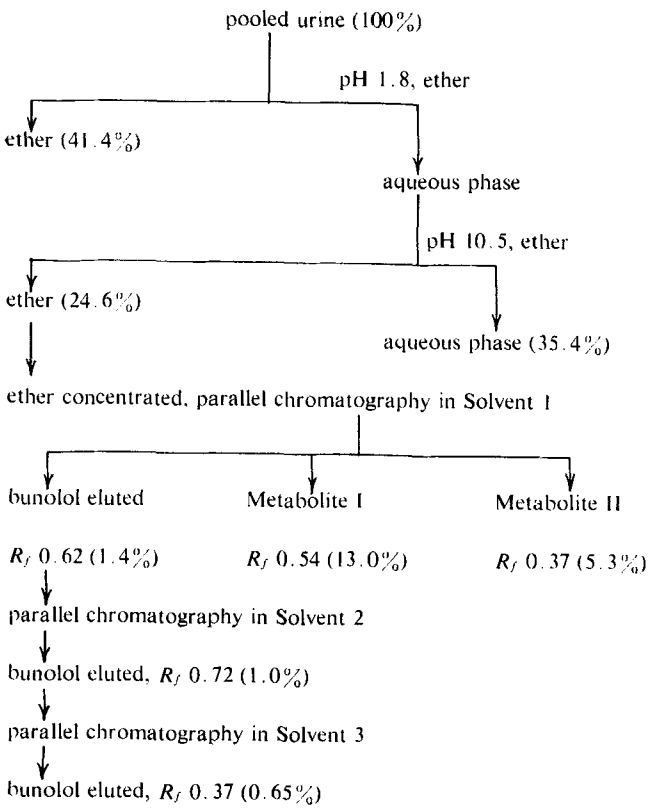
**Table III**—$^{14}C$ Tissue Levels 72 Hr. after Oral Administration of Bunolol-$^{14}C$ to Dogs

| Tissue | Percent of Radioactive Dose | | |
|---|---|---|---|
| | Dog 1 | Dog 2 | Dog 3 |
| Brain | 0.02 | 0.03 | 0.02 |
| Fat[a] | 0.02 | 0.03 | 0.01 |
| GI tract, including contents | 1.31 | 0.57 | 1.35 |
| Heart | 0.81 | 0.12 | 0.19 |
| Kidney | 0.03 | 0.46 | 0.03 |
| Liver | 0.55 | 1.13 | 0.76 |
| Muscle[b] | 0.18 | 0.11 | 0.02 |
| Spleen | 0.01 | 0.17 | 0.02 |
| Total | 2.95 | 2.62 | 2.40 |

[a] Samples assayed weighed 122 g. from Dog 1, 157 g. from Dog 2, and 87 g. from Dog 3. [b] Samples assayed weighed 123 g. from Dogs 1 and 2 and 102 g. from Dog 3.

ration to dryness, a few drops of methanol were added to obtain a clear solution which was streaked onto the central 12 cm. of five 20 × 20-cm. TLC plates.

Aliquots of a solution of authentic bunolol were spotted in the free areas flanking the urine extract. The plates were developed in Solvent 1, dried in air, and inspected under UV light to locate the reference bunolol. The band between the two bunolol spots was scraped from each plate. The scrapings from the five plates were pooled, and the radioactivity was extracted six times with 4-ml. portions of methanol. The pooled eluate represented 1.4% of the urinary $^{14}C$. The concentrate was streaked as before onto a 20 × 20-cm. TLC plate. Synthetic bunolol was spotted in the free areas on both sides of the band, and the plate was developed in Solvent 2. Again the band that migrated parallel with bunolol was collected by scraping the plate and eluting with methanol. The eluate, now representing 1.0% of the urinary $^{14}C$, was submitted to parallel chromatography in Solvent 3 as described previously. Elution yielded 0.65% of the urinary $^{14}C$. When an aliquot of the final eluate was chromatographed in Solvent 1, the major radioactive peak migrated as bunolol and represented 73% of the $^{14}C$ in the




*Scheme II—Purification of bunolol by solvent extraction and TLC*

**Table IV**—Retention Times of Reference Bunolol and Bunolol Isolated from Dog Urine

| Preparation | Retention Time, min. | |
|---|---|---|
| | Acetate (198°)[a] | Trifluoroacetate (173°)[a] |
| Reference bunolol | 9.5[b] | 11.2 (1.2)[c] |
| Urinary isolate | 9.4 (1.7)[c] | 11.1 (0.8)[c] |

[a] Column temperature. [b] Average of 10 measurements; amounts injected ranged from 0.75 to 3.0 mcg. [c] Value in parentheses denotes amount (mcg.) injected.

sample. A slower migrating peak, possibly Metabolite I, was also present. Metabolite I, the major radioactive component in the ether extract (*cf.*, Scheme II) prepared from alkalinized urine, is as yet unidentified.

**Identification of Bunolol in Urine**—An entirely different technique was used to confirm the indication from TLC in three solvents that the urinary compound was bunolol. GLC was applied to two derivatives of bunolol. These derivatives were bunolol acetate and trifluoroacetate.

Bunolol acetate was synthesized and measured by injecting a solution of bunolol in acetic anhydride. Assay of the urinary bunolol was performed by injecting 3 μl. of a mixture containing the residue from the third chromatographic purification step, 20 μl. of acetic anhydride, and 10 mcg. of *p*-terphenyl, which served as internal standard, into the sample column. Into the reference column was injected 3 μl. of acetic anhydride 5 sec. earlier; this step stabilized the baseline.

The values of the retention time obtained with reference and urinary bunolol are in close agreement (Table IV). The amount of bunolol, represented by the area under the peak, was determined by chromatographing standard amounts of bunolol, ranging from 0.75 to 3.0 mcg./injection. A calibration curve was prepared by plotting the ratio of areas of bunolol to *p*-terphenyl. The retention time of *p*-terphenyl was 4 min. Duplicate measurements of the urinary sample indicated that 1.88 and 1.52 mcg. of urinary bunolol each were injected. The average value corresponded to 68 mcg. of bunolol eluted after the third chromatographic purification. Based on measurements of $^{14}C$ eluted and a purity of 73%, the amount of urinary bunolol at that purification state was 64 mcg. This correction is believed to be valid because it was found that Metabolite I would not produce peaks (0.8 mcg./injection) when injected in acetic anhydride solution.

Bunolol trifluoroacetate was evaluated by a different procedure. From a standard solution of bunolol, aliquots were transferred to 0.5-ml. conical reaction flasks equipped with Teflon-lined screw caps[8]. The solvent was evaporated to leave residues ranging from 20 to 200 mcg. of bunolol. A 50-μl. aliquot of trifluoroacetic anhydride (25% in methylene chloride)[9] was added to each residue. The flasks were capped quickly, and the reaction was allowed to proceed at room temperature for at least 40 min. Three-microliter aliquots of the various standards were injected onto the column. The calibration curve obtained indicated that the formation of the trifluoroacetate may serve as a sensitive and reproducible assay of bunolol. A suitable internal standard has not been found yet for this assay. The peak area relationship to bunolol concentration was selected over the peak height relationship because the experimental points fell closer to a straight line. The peak area of the urinary sample indicated that 0.78 mcg. of bunolol was injected. Assay of the sample for $^{14}C$ suggested 1.0 mcg. of bunolol, a value that was in reasonable agreement considering the volatility of trifluoroacetic anhydride.

A disadvantage of this method is the variability in the retention time. The values for the retention time increased markedly when smaller amounts of bunolol were employed. This difficulty was reduced by using an amount of reference bunolol of similar magnitude as that of the unknown. The retention times thus observed were 11 min. (Table IV). It is suggested that the observed variability of retention time in this assay is related to the adsorption of bunolol trifluoroacetate by the column.

[8] Kontes.
[9] Regis Chemical Co.

**Recovery of Bunolol after TLC**—To calculate the amounts of bunolol excreted in the urine, it was necessary to measure the recovery of bunolol after TLC. Therefore, varying amounts of bunolol-$^{14}$C, ranging from 2.5 to 50 mcg., were chromatographed. After scraping and elution, the amounts of $^{14}$C were measured. Recoveries ranged from 81.6 to 95.5%. The extent of recovery appears to be unrelated to the amounts of bunolol employed. Based on an average recovery of 87.2%, and assuming identical recovery irrespective of the solvent, the corrected value for the amount of urinary bunolol is 0.71% of the urinary $^{14}$C.

**Presence of Conjugated Bunolol in Dog Urine**—A 10-ml. sample of pooled urine was brought to pH 1.8 and extracted exhaustively with ether to remove unconjugated acids. The pH was raised to 10.5, and ether extractions were performed to remove unconjugated bases. The aqueous phase was then neutralized, freed of ether by aeration, and mixed with 10 μl. of an enzyme solution containing 1100 Fishman units of glucuronidase and 580 Fishman units of aryl sulfatase. After this mixture was incubated at 37° for 3.5 hr., an additional 10 μl. of the same enzyme solution was added, and the incubation was continued overnight. The incubation mixture was adjusted to pH 1.8 and extracted with ether to remove acidic aglycones. Then the pH was raised to 10.5 for ether extraction to collect the basic aglycones which might include bunolol. This latter extract was processed by TLC as described for the purification of unconjugated bunolol, but the radioactivity eluted after the third chromatographic purification corresponded to less than 0.2% of the urinary $^{14}$C and did not permit identification.

## DISCUSSION

Dogs quickly absorbed bunolol and established high blood levels of the drug and its metabolites. The present findings are consistent with an earlier observation of good oral absorption in a study of the cardiovascular pharmacology of bunolol (3). The blood concentration of bunolol and its metabolites reached a maximum within 1 hr. and slowly decreased to trace levels at 24 hr. Corresponding with this observation was the urinary excretion of most of the radioactivity within the first 24 hr. after treatment. These observations are comparable to the results obtained after $^{14}$C-propranolol administration to dogs (9), although the bunolol dose was 10 times higher.

In terms of both intact drug and total radioactivity, the propranolol blood levels were maximal at 1 hr., and the drug was excreted completely in 48 hr. Following $^{14}$C-bunolol administration, 16.2% of the radioactivity was passed into the feces in 72 hr. while 65.9% was excreted into the urine. Although no effort was made to recover radioactivity from the dog carcass, an additional 2.6% of the dose was found in target tissues, bringing the total mean recovery to 84.7%.

The sustained defecation of radioactivity from this readily absorbed drug and the relatively high levels of $^{14}$C found in the GI tract and liver at sacrifice suggest extensive enterohepatic circulation of bunolol and its metabolites. Organs other than the liver and GI tract contained little radioactivity 72 hr. after bunolol administration. While 0.1–0.8% of the $^{14}$C was detected in the heart, small amounts were also found in the brain (0.02–0.03%). Generally, the present tissue values for dogs are of an order of magnitude comparable to data reported in mice 24 and 96 hr. after administration of pronethalol (10).

Twenty-four hours after dosing dogs with bunolol-$^{14}$C (10 mg./kg. p.o.), 61% of the radioactivity was found in the urine. Approximately three-fourths of this radioactivity was distributed among 18 compounds, classified as unconjugated acids and bases and free acids and bases. The present study shows that only 0.7% of the urinary $^{14}$C was excreted unchanged. The extensive and varied biotransformation of bunolol is reminiscent of the situation with pronethalol (10). Values of pronethalol content in urine of mouse, rat, and rabbit were 1.5, 0.3, and 0.7% of free pronethalol, whereas the values of pronethalol glucuronide were 1.7, 0.6, and 1.5%, respectively.

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