Bumetanide: Radioimmunoassay and Pharmacokinetic Profile in Humans

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Abstract □ A simple, specific, and sensitive radioimmunoassay was developed for the determination of the diuretic bumetanide in plasma and urine. Antiserum to bumetanide was obtained from rabbits immunized with an immunogen prepared by covalently coupling the glycine conjugate of bumetanide to bovine serum albumin. Following extraction of the sample at pH 5.5 with ether, radioimmunoassay of the residue from the ether extract allows for the determination of bumetanide with a limit of sensitivity of about 1 ng/ml using 0.1 ml of plasma or urine. The specificity of the radioimmunoassay was established by comparison with specific radiometric and spectrofluorometric techniques. The pharmacokinetic profile of bumetanide in eight human subjects receiving single 2-mg oral doses of the drug was elucidated using the radioimmunoassay. The peak plasma levels ranged from 39 to 50 ng/ml at 1–4 hr after administration and declined with a mean apparent half-life of 1.17 hr. The mean plasma clearance rate was calculated to be 255 ml/min. During the first 24 hr, a mean of 43% of the bumetanide dose was excreted in the urine as intact drug.

Keyphrases □ Bumetanide—analysis, radioimmunoassay, plasma and urine, compared to radiometric and spectrofluorometric methods, pharmacokinetic profiles □ Radioimmunoassay—analysis, bumetanide, plasma and urine, compared to radiometric and spectrofluorometric methods □ Pharmacokinetics—bumetanide, plasma and urine, radioimmunoassay □ Diuretics—bumetanide, analysis, radioimmunoassay, plasma and urine, pharmacokinetic profiles

Bumetanide[1] (I) is a new sulfonamide diuretic and produces a rapid diuretic response with a short duration of action in humans (1, 2). Its pharmacological action is similar to that of furosemide, but it is 40–60 times more potent on a weight basis (1).

Two methods have been employed for the determination of bumetanide in biological samples. A spectrofluorometric technique (3), with modification (2), is capable of determining the drug in urine with a limit of sensitivity of about 100 ng/ml, and a GC method has similar sensitivity (4). In this laboratory, attempts to quantitate bumetanide in plasma and urine at levels below 100 ng/ml were unsuccessful using either spectrofluorometry or GC.

The development of a sensitive and specific radioimmunoassay is now reported for bumetanide. This method has permitted the elucidation of the blood level and urinary excretion profiles of this drug in humans.

EXPERIMENTAL

Preparation of Immunogen—*N*-(3-*n*-Butylamino-4-phenoxy-5-sulfamoylbenzoyl)glycine (II) was covalently coupled to bovine serum albumin by the mixed anhydride procedure of Erlanger *et al.* (5). Following exhaustive dialysis against 0.05 *M* tromethamine buffer (pH 9) and then distilled water, the immunogen was isolated by lyophilization. On the basis of its optical density at 326 nm in 0.1 *N* NaOH against a standard solution of bumetanide, it was estimated that this immunogen consisted of approximately 40 moles of the drug coupled to 1 mole of albumin.

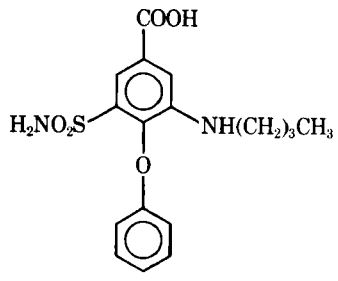
Immunization and Antiserum Preparation—The lyophilized immunogen was dissolved in isotonic saline to give a 2-mg/ml solution. An equal volume of Freund's complete adjuvant[2] was added, and the mixture was emulsified. Two female New Zealand White rabbits were injected intradermally with 0.25 ml of the emulsion at four sites on the back. One month later, each rabbit received 1 mg of the immunogen subcutaneously as a solution in saline. Two weeks later, the rabbits were bled from the ear artery and the serum was harvested as previously described (6).

Radioimmunoassay Procedure—The following stock solutions were prepared:

1. Tritium-labeled bumetanide[3] (specific activity 16.63 mCi/mg); 150,000 cpm/ml in 0.01 *M* sodium phosphate buffer (pH 7.4).
2. Antiserum diluted 1:200 with buffer containing 0.1% bovine γ-globulin[4] and 0.1% sodium azide.
3. Sodium acetate buffer, 1 *M* (pH 5.5).
4. Standard solutions of 1, 5, 10, 20, 50, 100, and 200 ng/ml of unlabeled bumetanide in distilled water.

To generate a calibration curve relating displacement of bound ^{3}H-bumetanide by 0.1–20 ng of unlabeled drug, 0.1 ml of control plasma or urine was added to assay tubes (10 × 75 mm) containing 0.1 ml of each bumetanide standard. Two blanks also were included by adding 0.1 ml of the control plasma or urine to 0.1 ml of water. The unknown samples (0.1 ml) were then added to tubes containing 0.1 ml of water.

To each tube, 0.05 ml of sodium acetate buffer was added followed by 2 ml of ether. The bumetanide was extracted into the ether by vortexing for 5 sec. The tubes were then immersed in a shallow bath of 2-propanol–dry ice at −30°, and the aqueous phases froze solid within about 10 sec. The ether phases were de-



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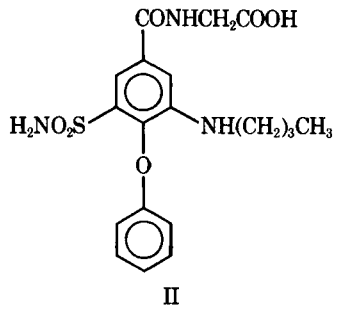

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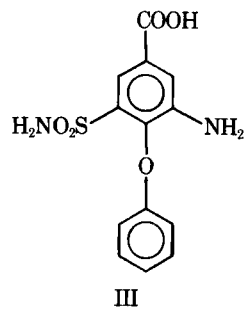

III

[1] 3-*n*-Butylamino-4-phenoxy-5-sulfamoxybenzoic acid.

[2] Grand Island Biological Co., Grand Island, N.Y.

[3] ^{3}H-Bumetanide was prepared by catalytic exchange in tritiated water and was purified by column chromatography.

[4] Miles Laboratories Inc., Kankakee, IL 60901

Table I—Levels of Bumetanide (Nanograms per Milliliter) in the Urine of Four Subjects[a] Who Received 2 mg of the Drug Orally as Measured by the Radioimmunoassay and Fluorometric Method

Collection Period, hr	Subject A		Subject B		Subject C		Subject D	
	Radioimmunoassay	Fluorometric Method	Radioimmunoassay	Fluorometric Method	Radioimmunoassay	Fluorometric Method	Radioimmunoassay	Fluorometric Method
0—2	151	143	160	172	173	219	200	218
2—4	182	193	184	214	202	242	—	—
4—6	120	120	148	147	273	278	240	236
6—8	68	n.m.[b]	23	n.m.	100	123	50	n.m.
8—10	26	n.m.	23	n.m.	18	n.m.	21	n.m.
10—12	15	n.m.	7	n.m.	5	n.m.	7	n.m.
12—24	9	n.m.	8	n.m.	n.m.	n.m.	7	n.m.

[a] These data were obtained during the developmental stages of the radioimmunoassay and are not included in the reported pharmacokinetic results. [b] n.m. = nonmeasurable.

canted into 12 × 75-mm tubes, and the solvent was evaporated by immersing the tubes in a water bath at 40° in a fume hood.

Each dry residue was dissolved in 0.6 ml of phosphate buffer, and 0.2 ml (30,000 cpm) of the ^{3}H-bumetanide solution was added. To each tube, except one blank, 0.2 ml of the diluted antiserum was added and the contents were mixed. After allowing the tubes to stand at 4° overnight, an equal volume (1 ml) of saturated ammonium sulfate was added to precipitate globulin-bound ^{3}H-bumetanide. The tubes were centrifuged at 3000 rpm at 2° for 30 min, and the supernates containing the unbound tracer were decanted into counting vials. After 1 drop of concentrated sulfuric acid was added, 10 ml of toluene scintillator[5] was added and the ^{3}H-bumetanide was extracted into the toluene by placing the covered vials in the vertical position on a reciprocating shaker and shaking for 10 min. Radioactivity in the samples was then determined.

All samples, including the standards and controls, were assayed in duplicate. The average of the tritium counts was used for calculation.

Spectrofluorometric and Radiometric Determinations of Bumetanide—A modification of the spectrofluorometric method of Østergaard *et al.* (3) was developed for the determination of bumetanide in urine. Briefly, the assay involved extraction of bumetanide from urine at pH 2 with ether, followed by TLC on silica gel using chloroform–methanol–acetic acid (85:10:5). The bumetanide (R_f 0.5) was eluted and dissolved in 1 *M* glycine buffer (pH 11.2). The fluorescence was then read in a fluorometer at an emission wavelength of 410 nm with excitation at 325 nm. The limit of sensitivity of this method was about 100 ng/ml of urine.

A radiometric method was used to determine the plasma levels of intact bumetanide in dogs given both oral and intravenous doses of ^{14}C-bumetanide. This procedure entailed an ethyl acetate extraction of acidified plasma and two-dimensional TLC of the concentrated extract with chloroform–methanol–acetic acid (85:10:5) followed by benzene–methanol–acetic acid (9:1:1). The radioactivity, migrating as cochromatographed authentic bumetanide, was determined and converted to nanograms per milliliter of bumetanide from the specific activity of the administered dose.

Clinical Protocol—Eight adult male volunteers, ages 21–60, were enrolled at the research ward[6] for 48 hr. Subjects with a history of renal disease or who were taking medication were excluded. Good health was established by a routine history and physical examination including vital signs, an ECG, and a laboratory battery consisting of complete blood count, urinalysis, fasting blood sugar, blood urea nitrogen, total protein and albumin, serum glutamic oxalic transaminase or serum glutamic pyruvic transaminase, serum bilirubin, alkaline phosphatase, sodium ions, potassium ions, chloride ions, and uric acid. Meals were standardized as to content and amount of fluid. Salt was withheld.

Subjects were admitted to the research ward 24 hr before the start of the study. Each subject was given 1000 ml of tap water to drink over 20–30 min, and then a control (predrug) 24-hr urine collection was initiated. The volume voided was measured. A 20-ml aliquot was taken, labeled with the subject's name, "predrug," and total volume voided, and frozen for shipment and subsequent assay.

On the second morning, the subjects were given 1000 ml of tap water to drink over 20–30 min before drug administration. Subjects were then given a single oral dose of 2 mg (two tablets) of bumetanide. Twelve-milliliter (oxalated) blood specimens were obtained at 0 (control), 0.5, 1.0, 1.5, 2, 4, 6, 10, 12, and 24 hr postadministration. The plasma was separated, labeled, and frozen for subsequent analysis.

Urine specimens (total volume voided) were collected at 0–2, 2–4, 4–6, 6–8, 8–10, 10–12, and 12–24 hr posttreatment. The volume of each specimen was measured, and the subjects were asked to drink an equivalent amount of water. A 20-ml aliquot from each time interval was labeled and frozen for shipment.

Determination of Pharmacokinetic Parameters—Areas under the plasma level–time curves ($0 \rightarrow \infty$) were estimated using the trapezoidal rule; the area from the last data point, $Cp(t)$, to infinity was calculated from the expression $Cp(t)/\beta$. The apparent half-life of elimination of bumetanide from plasma was determined by least-squares analysis of the terminal linear exponential segment of the plasma level–time curve. The apparent half-life of the drug was also calculated by least-squares analysis of the urinary excretion data using "sigma-minus" plots (7).

The total body clearance rate was determined by dividing the dose by the area under the plasma concentration–time curve ($0 \rightarrow \infty$). The latter calculation is made with the assumption that there is 100% bioavailability. The renal clearance rate was obtained by dividing the total amount of drug excreted intact in the urine by the area under the plasma level–time curve.

RESULTS

Antibody Production—Each rabbit produced antiserum capable of binding ^{3}H-bumetanide, as determined by comparison with control serum from nonimmunized rabbits. Almost identical and usable titers of antibodies were obtained from each rabbit at the first bleeding. All reported data were obtained using this first batch of pooled antiserum; 1 ml of a 1:1000 dilution was capable of binding about 70% of the 30,000 cpm of ^{3}H-bumetanide.

Radioimmunoassay Curve—Linearization of the calibration curve was achieved by plotting the percentage inhibition of binding of the ^{3}H-bumetanide, calculated as described previously (6), *versus* the concentration of unlabeled bumetanide on logit–log transformation graph paper[7]. An almost perfect straight line was obtained between 0.1 and 20 ng, resulting in a limit of sensitivity of about 1 ng/ml on analysis of 0.1 ml of plasma or urine. When employing the pooled variance estimate[8] from the replicate data from the whole calibration curve, the percentage inhibition of binding due to 0.1 ng of unlabeled bumetanide was significant at $p = 0.01$. The standard curves obtained in urine and plasma were virtually superimposable. Where necessary, urine samples were diluted with control urine to bring the bumetanide concentration within the limits of the calibration curve.

Recovery of ^{3}H-bumetanide added to plasma and extracted was 80 ± 2% (*SD*, $n = 6$), but no correction was made since standards and unknowns were processed in the same fashion.

Antiserum Specificity—The *N*-desbutyl derivative of bumeta-

[5] Omnifluor, New England Nuclear, Boston, MA 02118
[6] Deer Lodge Research Unit, Deer Lodge, Mont.
[7] TEAM, Box 25, Tamworth, NH 03886
[8] Statistical analyses were carried out by Mr. T. Lewinson, Hoffmann-La Roche Inc.

Table II—Plasma Levels and Urinary Excretion Data of Intact Bumetanide following 2-mg Single Oral Doses of Bumetanide

Hours	Plasma Levels in Eight Subjects, ng/ml							
	1	2	3	4	5	6	7	8
0.5	2.2	1.5	1.3	n.m.	27.5	5.3	n.m.	n.m.
1	13.0	29.5	11.9	4.0	41.0	24.5	12.0	2.2
1.5	44.0	43.0	25.0	6.6	25.5	53.0	38.5	3.8
2	38.0	42.0	38.5	26.2	17.0	63.0	30.5	11.8
4	19.0	16.8	12.6	41.5	7.8	12.8	29.0	49.5
6	1.4	3.7	1.3	11.8	1.3	8.6	9.4	7.3
8	n.m.[a]	3.3	n.m.	5.0	n.m.	4.6	2.3	2.6
10	n.m.	2.6	n.m.	3.0	1.2	2.4	3.3	n.m.
12	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
24	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Interval Time, hr	Dose Excreted in Urine in Eight Subjects, %							
0—2	14.9	14.2	12.1	0.6	10.9	13.7	7.4	0.9
2—4	26.5	20.3	23.3	22.8	13.3	21.4	18.7	18.9
4—6	8.1	6.6	6.5	11.3	3.4	5.6	9.2	17.2
6—8	0.6	0.7	2.0	3.8	1.0	2.1	3.1	4.0
8—10	0.7	0.8	0.6	1.6	0.6	1.4	1.4	1.1
10—12	0.4	0.5	0.2	0.6	0.2	0.6	0.6	0.6
12—24	n.m.	0.8	0.4	0.6	0.2	0.4	0.3	0.1
Total 0—24	51.3	43.9	45.1	41.3	29.7	45.2	40.7	42.8

[a] n.m. = nonmeasurable.

nide (III), which has been reported as a metabolite in several animal species (8) but not specifically in the dog (3) or human, showed less than 0.5% cross-reactivity with the antiserum at a concentration necessary to cause 50% inhibition of the binding of the ^{3}H-bumetanide.

Comparison of Radioimmunoassay with Spectrofluorometric and Radiometric Determinations of Bumetanide—Samples of urine from four subjects who had received a 2-mg oral dose of bumetanide were assayed for intact drug by both the radioimmunoassay and the spectrofluorometric method, which had a limit of sensitivity of about 100 ng/ml. From Table I it can be seen that there was satisfactory agreement for the values (above 100 ng/ml) obtained using both methods. In the four samples for which the difference in values exceeded 10%, the fluorometric assay consistently gave the higher level. This result indicates that the radioimmunoassay is at least as specific as the spectrofluorometric assay, which did include a TLC separation step.

The specificity and accuracy of the radioimmunoassay for the determination of bumetanide in plasma were evaluated by analysis of plasma samples from three dogs who had received ^{14}C-bumetanide intravenously or orally. The levels of intact bumetanide were first determined by the radiometric technique. Samples (50 μl) were then assayed by the radioimmunoassay. The carbon-14 counts present in the aliquots taken for the radioimmunoassay were negligible relative to the amount of ^{3}H-bumetanide added.

The joint determinations were subjected to straight-line analysis by the method of Wald (9). The fitted intercept and slope (3.7; 1.04) were not significantly different ($p \leq 0.05$) from 0 and 1 with a correlation coefficient of 0.98 over a range of 32–540 ng/ml of bumetanide[8]. This finding showed that the radioimmunoassay was giving the same plasma levels in the dog as the radiometric assay that included a separation by TLC.

Pharmacokinetic Profile of Bumetanide in Humans—The plasma levels and urinary excretion data are presented in Table II. The plasma level data were evaluated for each subject in terms of the area under the plasma level curves, the maximum bumetanide level seen and the time of its occurrence, the apparent half-life of elimination of the drug from the plasma, and the plasma clearance rate. These pharmacokinetic parameters are presented in Table III.

Following oral administration of 2 mg of bumetanide, peak plasma levels were seen at 2.2 ± 0.4 (*SE*) hr and averaged 45 ± 3 ng/ml. The mean apparent half-life of bumetanide elimination was calculated from the plasma level data to be 1.2 ± 0.1 hr and was calculated from the urinary excretion of drug (sigma-minus plot) to be 1.6 ± 0.1 hr. The mean plasma clearance was 255 ± 26 ml/min, whereas the renal clearance was 107 ± 11 ml/min. By definition, the renal clearance is the fraction of plasma clearance that equals the fraction of the dose excreted unchanged in the urine (43 ± 2%).

DISCUSSION

The aim of the present investigation was to develop a specific radioimmunoassay for bumetanide, which, by virtue of its sensitivity, would allow the pharmacokinetic profile of this drug to be evaluated in humans following oral administration of a 2-mg dose. Previous attempts to determine the low levels of intact bumetanide that exist in plasma by either GC or spectrofluorometry were unsuccessful in this laboratory. This goal was achieved by producing an antiserum to bumetanide in the rabbit for the subsequent development of a radioimmunoassay. This radioimmunoassay proved to be about 100 times more sensitive than the GC and spectrofluorometric methods.

The specificity of the radioimmunoassay for measuring intact bumetanide was established in a number of ways. First, the antiserum was shown not to cross-react with the *N*-desbutyl derivative, the one reported metabolite of bumetanide (8). Second, when the plasma levels of bumetanide in dogs, who had received ^{14}C-bumetanide, were determined by the radioimmunoassay and a specific TLC–radiometric technique, no significant difference in the observed levels were found. Third, the concentrations of bumetanide in human urine, as determined by the radioimmunoassay, were in close agreement with those obtained using a specific spectrofluorometric method which involved the isolation of intact drug by TLC.

Further evidence for the specificity of the radioimmunoassay becomes apparent when one considers the observation of Davies *et al.* (2) that radioactivity in ether extracts of plasma and urine from subjects who had received ^{14}C-bumetanide accurately reflected the

Table III—Pharmacokinetic Profile of Bumetanide in Humans

Parameter	Subject								Mean	±*SE*
	1	2	3	4	5	6	7	8		
Area under plasma level curve (0→∞), ng/ml/hr	118.2	143.9	95.3	165.5	91.7	216.4	156.3	138.0	140.7	14.4
Time of peak plasma level, hr	1.5	1.5	2	4	1	2	1.5	4	2.2	0.4
Peak level, ng/ml	44.0	43.0	38.5	41.5	41.0	63.0	38.5	49.5	44.9	2.9
Apparent half-life of elimination, hr	0.8	1.1	0.8	1.6	1.1	1.8	1.1	0.9	1.2	0.1
Plasma clearance rate, ml/min	282.0	231.6	350.0	201.4	363.5	154.0	213.3	241.5	254.7	25.7
Dose of intact drug in 0—24-hr urine, %	51.3	43.9	45.1	41.3	29.7	45.2	40.7	42.8	42.5	2.2
Calculated apparent half-life of elimination, hr	1.1	2.0	1.6	1.8	1.6	1.8	1.5	1.1	1.6	0.1
Renal clearance rate, ml/min	144.7	101.7	157.9	83.2	108.0	69.6	86.8	103.4	106.9	10.7

amount of intact bumetanide present. This finding suggests that either no appreciable levels of metabolites were present or that bumetanide was selectively extracted by ether. In any event, the ether extraction step in the radioimmunoassay procedure should ensure that adequate specificity is obtained.

Although the major reason for the development of a radioimmunoassay for bumetanide was to obtain high sensitivity, the radioimmunoassay, due to its simplicity, allows a large number of samples to be assayed with relative ease compared to GC and spectrofluorometric methods.

In humans, the major portion of bumetanide excreted in the urine is excreted at the times when the plasma levels of bumetanide are at their peaks. These results suggest that the amount of excretion is proportional to the plasma level of bumetanide. This conclusion is further supported by the finding that the apparent half-lives calculated from either the urinary excretion data or the plasma level data are essentially the same.

Davies *et al.* (2) found that 47% of a 1-mg intravenous dose of ^{14}C-bumetanide was excreted in the urine within 3 days and that 16% was excreted in the feces within 8 days. Following the 2-mg oral dose of bumetanide to the eight subjects in this study, a mean of 43% of the dose was excreted in the urine during the 1st day, suggesting that these oral doses were completely absorbed. In addition, the mean renal clearance of bumetanide of 102 ml/min following intravenous administration to normal subjects (2) is in excellent agreement with the renal clearance of 107 ml/min found in the present study following oral administration. These results further suggest complete absorption of orally administered drug.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 30, 1975, from the *Department of Biochemistry and Drug Metabolism and the Chemical Research Division, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication July 28, 1975.

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Muscle Relaxant Properties of Chloramphenicol

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Abstract □ Experiments with the guinea pig ileum, guinea pig trachea, rat fundal strip, rat colon, rat vas deferens, and toad heart indicated that chloramphenicol inhibited smooth muscles, decreasing both the height and frequency of spontaneous contraction. Chloramphenicol-induced relaxation was not mediated through adrenergic, cholinergic, or histaminergic mechanisms. The degree of muscle relaxation was related to the concentration of chloramphenicol, and the relaxant effect could be reversed by removing chloramphenicol from the site of action by washing. Its action appears to be direct on the muscle, possibly by interfering with the energy-generating mechanism.

Keyphrases □ Chloramphenicol—effect on smooth muscle relaxation, guinea pig □ Muscle relaxant activity—chloramphenicol, effect on smooth muscles, guinea pig □ Antibacterials—chloramphenicol, effect on smooth muscle relaxation, guinea pig

Chloramphenicol depresses the spontaneous movements of the rabbit ileum (1). It antagonizes the histamine- or acetylcholine-induced response of the rabbit ileum (1) and the dog bronchial chains (2). Chloramphenicol is 15–25 times more active than ephedrine in this respect (2).

This study was concerned with examining the muscle relaxant properties of chloramphenicol to understand its mode of action. Therefore, the effect of chloramphenicol was determined on different tissues containing smooth muscle such as the guinea pig ileum, rat fundal strip, rat colon, and rat uterus and in isolated organ systems representing the adrenergic, cholinergic, and histaminergic systems. The perfused toad heart, rabbit aortic strip, and rabbit ileum preparations were selected to examine the effect of chloramphenicol on the adrenergic system.

The perfused toad heart preparation exemplified a muscle system containing β-adrenergic receptors with strong autogenous contractile drive, whereas the aortic strip and rabbit ileum preparations represented muscle systems innervated with α-adrenergic receptors. The rabbit jejunum, having a muscle contractile system with a moderate autogenous drive, served as the model of the cholinergic system. The guinea pig trachea, having a muscle system with a weak autogenous contractile drive, served as the representative histaminergic system (3).

EXPERIMENTAL

Toad Heart Perfusion (Adrenergic System)—Toads (*Bufo melanostictus*) of either sex, 75–80 g, were pithed. The abdomen was opened by a midline incision, the pericardium was removed, and a venous cannula was inserted into one hepatic vein opening into the sinus venosus for perfusion with a Symes cannula. The perfusion fluid was frog–Ringer solution. A fine hook was fixed at the tip of the ventricle and was connected through a silk thread with an isotonic lever.

The drugs were injected into the rubber tube attached to the cannula to determine their effect on the spontaneously beating heart. The heart perfusion was continued for 30 min to attain equilibrium before any recordings were taken. For each experiment,