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* Present address: 1483 Shulgin Road, Lafayette, CA 94549
ˣ To whom inquiries should be directed.

Rapid Procedure for Assessment of Compounds that Modify Uptake and Release of Tritiated Norepinephrine

MORTON LEVITT ˣ, WAYNE R. CUMISKEY, MICHAEL B. McGRATH, and LEON SHARGEL *

Abstract □ A rapid procedure to estimate tritiated norepinephrine (levarterenol) in a single mouse heart is described. The method is based upon oxidation of the tritium in the tissue to tritiated water, which is then determined by liquid scintillation spectroscopy. Large numbers of samples can be assayed with great facility. The effects of standard compounds that modify the uptake and release of ^{3}H-norepinephrine in heart tissue were determined with this system, and a procedure for studying their interactions is described.

Keyphrases □ Norepinephrine, tritiated—liquid scintillation spectroscopic analysis, effects of various compounds on uptake and release, mouse heart □ Spectroscopy, liquid scintillation—analysis, tritiated norepinephrine, mouse heart □ Radiolabeled compounds—tritiated norepinephrine, liquid scintillation spectroscopic analysis, mouse heart □ Adrenergic agents—norepinephrine, liquid scintillation spectroscopic analysis, mouse heart

Compounds that modify norepinephrine (levarterenol) uptake, release, storage, or turnover are frequently used in clinical medicine and in many procedures for the investigation, development, and characterization of new therapeutic agents (1–4). Norepinephrine metabolism has been studied by measuring changes in tritiated norepinephrine concentrations in the hearts of several species (5–7). Since some of these procedures are tedious and time consuming, only small numbers of individual or pooled samples can be conveniently assayed in a single experiment.

Introduction of instrumentation for the oxidation of small tissue samples allowed the development of a rapid and sensitive assay of ^{3}H-norepinephrine. This report describes a rapid screening procedure for studying compounds that affect the uptake and release of ^{3}H-norepinephrine in individual mouse hearts. The interactions of several test compounds with tyramine, reserpine, metaraminol, amphetamine, and tetrabenazine determined with this procedure are presented.

EXPERIMENTAL

Materials—The radiochemical purity (>95%) of 7-^{3}H-*l*-norepinephrine[1], specific activity of 4.1 Ci/mmole, was verified by ascending paper chromatography in 1-butanol–acetic acid–water (4:1:1). The following compounds were used: *l*-norepinephrine bitartrate[2], *l*-metaraminol bitartrate[2], tyramine hydrochloride[3], reserpine hydrochloride[4], dextroamphetamine sulfate[5], tetrabenazine methanesulfonate[6], chlorpromazine hydrochloride[7], guanethidine sulfate[8], bretylium *p*-toluenesulfonate[9], desipramine hydrochloride[10], and cocaine hydrochloride[10].

Animals and Dosage—Swiss Webster mice, 20 ± 2 g, were used. All compounds were administered as a solution in 0.2 ml of distilled water. ^{3}H-Norepinephrine (1μCi) was administered intravenously in the tail vein. Test compounds were administered subcutaneously.

^{3}H-Norepinephrine Assay—Mice were killed by cervical dislocation. Hearts were removed, blotted, and weighed on tared low ash filter paper disks[11] and dried overnight in a refrigerator (4°). The tissue sample was compressed in a tablet press and oxidized in a sample oxidizer[12]. The resulting tritiated water was collected in counting vials, and 15 ml of scintillation fluid[13] was added. Radioactivity was measured in a liquid scintillation spectrometer[12]. The counting efficiency was determined by the channels ratio method.

Heart tritium concentrations were proportional to dose over the range studied, 0.25–5.0 μCi of tritiated norepinephrine. The dose used in these studies, 1 μCi, yielded about 10,000 cpm/mouse heart (220 nCi/g) after 2 hr.

Uptake and Release Study—Effects on the uptake of ^{3}H-norepinephrine by the heart were determined by administering the test compounds subcutaneously 15 min before the intravenous administration of 1 μCi of ^{3}H-norepinephrine. The animals were sacrificed 2 hr after the administration of the radiotracer. Release of ^{3}H-norepinephrine was determined by administering the test compound subcutaneously 15 min after the administration of ^{3}H-norepinephrine. After 2 hr, the animals were sacrificed, hearts were removed, and radioactivity was determined.

Interaction Study—The interactions of desipramine, chlorpromazine, and cocaine with tyramine, reserpine, metaraminol, amphetamine, and tetrabenazine were estimated by their effects in combination on the release of ^{3}H-norepinephrine from the heart. ^{3}H-Norepinephrine (1 μCi) was administered intravenously to 11 groups of mice, and the test compound was administered to five groups after 15 min. Then 15 min later, the releasing agents were administered to the pretreated animals and to additional control groups. One group served as unmedicated controls. The animals were sacrificed 2 hr after administration of ^{3}H-norepinephrine.

[1] Amersham/Searle.
[2] Sterling-Winthrop Research Institute.
[3] Eastman Organic Chemicals.
[4] Knoll Pharmaceutical.
[5] Mann Research Labs.
[6] Hoffmann-La Roche.
[7] Smith Kline and French Labs.
[8] Ciba-Geigy Pharmaceuticals.
[9] Burroughs Wellcome Co.
[10] Merck & Co.
[11] Carl Schleicher and Scheull.
[12] Packard Tri-Carb.
[13] Consisting of 5 g of 2,5-diphenyloxazole, 0.75 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 100 g of naphthalene, 150 ml of toluene, 40 ml of ethyl alcohol, and dioxane to make 1 liter.

Table I—Effect of Several Compounds on the Uptake and Release of ^{3}H-Norepinephrine[a]

Compound and Dose, mg/kg	Norepinephrine Released, nCi/g[b]	Compound and Dose, mg/kg	Norepinephrine Released, nCi/g[c]
Tyramine, 15	62.8[d]	Desipramine, 1.0	21.6[d]
Reserpine, 1.0	12.6[d]	Cocaine, 10	31.3[d]
Guanethidine, 1.0	64.6[d]	Chlorpromazine, 10	48.5[d]
Metaraminol, 5	19.1[d]	Bretylium, 20	76.9[d]
Control	131	Control	185
Pooled *SE*	±7.0%		±8.5%

[a] Each value (nanocuries per gram) is the geometric mean for six hearts. [b] Compounds were injected 15 min after injection of ^{3}H-norepinephrine. [c] Compounds were injected 15 min before injection of ^{3}H-norepinephrine. [d] Significantly different from corresponding control, $p \leq 0.01$.

Table II—Interaction of Desipramine, Chlorpromazine, and Cocaine with Compounds that Release ^{3}H-Norepinephrine[a]

Compound and Dose, mg/kg	Desipramine		Chlorpromazine		Cocaine	
	None	5 mg/kg	None	5 mg/kg	None	10 mg/kg
Tyramine, 15	94	146	78	114	68	92
Reserpine, 1	42	56	32	133[b]	39	36
Metaraminol, 5	37	58	38	82[b]	34	33
Amphetamine, 10	163	174	111	170	112	122
Tetrabenazine, 10	83	156[c]	80	147	89	112
Control	190	217	195	248	214	215
Pooled *SE*[d]	±11%		±11%		±10%	

[a] Each value (nanocuries per gram) is the geometric mean for six mouse hearts. [b] Significant nonadditivity (interaction), $p \leq 0.01$. [c] $p = 0.05$. [d] Based on residual error estimate from corresponding analysis of variance.

Experimental Design and Statistical Analysis—In all experiments, animals were randomly assigned to treatment groups. The values from the radioactivity determinations were routinely transformed to logarithms prior to analysis to minimize the correlation between means and standard deviations (8) and to facilitate the study of multiplicative interactions.

The primary method for testing hypotheses and determining statistical significance of associated linear contrasts was through analysis of variance techniques applied to the classic fixed-effects model for the resulting data (9).

RESULTS

Uptake and Release of ^{3}H-Norepinephrine—The effects of compounds known to release or block the uptake of ^{3}H-norepinephrine in mouse heart were determined (Table I). Tyramine, reserpine, guanethidine, and metaraminol released ^{3}H-norepinephrine from heart, as demonstrated by the significantly ($p \leq 0.01$) decreased tritium concentrations. Desipramine, cocaine, chlorpromazine, and bretylium significantly ($p \leq 0.01$) blocked uptake of ^{3}H-norepinephrine into cardiac tissue.

Interaction Studies—The interaction of desipramine, chlorpromazine, and cocaine with several compounds that release norepinephrine is illustrated in Table II. Although the interactions were variable, the three compounds slightly decreased the effect of tyramine and tetrabenazine. Chlorpromazine significantly ($p \leq 0.01$) decreased the effect of reserpine and metaraminol. Since these compounds have similar inhibitory effects on norepinephrine uptake but different pharmacological and clinical effects, interaction studies may be of value in elucidating the mechanisms of action (1, 2).

DISCUSSION

Norepinephrine stores in sympathetically innervated tissues such as heart can be labeled by administering ^{3}H-norepinephrine (10). Tritium concentrations in the heart after administration of ^{3}H-norepinephrine are almost entirely due to the parent compound (6, 10, 11). Thus, changes in the concentration of tritium in the heart have been used to determine norepinephrine uptake, release, and turnover (6, 7, 12).

The reported effects of standard compounds that block the uptake or release norepinephrine are consistent with the results obtained with the oxidation procedure (1, 6, 7) where the assay was simplified by isolation of the tritium as tritiated water. This method is rapid, simple, sensitive, and reproducible. The quantity of tissue sample that can be assayed is limited by the tritium concentration of the tissue. Ten to 100 mg are convenient sample sizes, while larger samples (0.5–2 g) may be combusted in several parts.

The test compounds and doses used here were selected after preliminary experiments primarily as examples of the method. Other compounds may be substituted, and different time periods, species, tissues, and numbers of animals can be used within the framework of this procedure.

Studies on the interactions of two or more compounds in animals and humans have provided basic information on the mechanisms of drug action (13, 14). The oxidation procedure provides a convenient means of determining both the actions and interactions of compounds affecting adrenergic neurons.

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* Present address: Department of Pharmacy and Pharmacology, College of Pharmacy and Allied Health Professions, Northeastern University, Boston, MA 02115
ˣ To whom inquiries should be directed. Present address: Division of Neuroscience, New York State Psychiatric Institute, New York, NY 10032

COMMUNICATIONS

Antineoplastic Agents XLV: Sea Cucumber Cytotoxic Saponins

Keyphrases □ Antineoplastic agents, potential—three saponins isolated from sea cucumbers, structures partially determined, activity evaluated □ Cytotoxic activity—evaluated in three saponins isolated from sea cucumbers □ Sea cucumbers—three saponins isolated and identified, screened for antineoplastic and cytotoxic activity □ Saponins—isolated from sea cucumbers, structures partially determined, antineoplastic and cytotoxic activity evaluated

To the Editor:

In Asia, various sea cucumbers (Echinodermata phylum, Holothurioidea class) are readily available under the name Trepang. One of these, *Stichopus japonicus* Selenka, is used for various medicinal purposes[1] (1) and human food. In 1929, Yamanouchi began to explore sea cucumber toxins; in 1942, he reported the isolation of a crystalline saponin mixture from *Holothuria vagabunda* Selenka (2). Subsequently, such saponin mixtures from sea cucumbers have been shown to possess various physiological activities, *e.g.*, neurotoxic (3), hemolytic (4), and the ability to inhibit growth of Crocker mouse sarcoma 180 and Krebs-2 ascites tumors in Swiss mice (5).

The actual composition of these saponin mixtures continues to be a challenging area for chemical study. So far, evidence has been presented only for the structure of the antifungal agent holotoxin A (I) from *S. japonicus* Selenka (6). However, the lanostane derivatives obtained by acid hydrolysis of the saponin mixtures received detailed investigation (7), particularly by Tan *et al.* (8) who recently provided definitive structural evidence for holotoxinogenin (II*a*) (6) and its 25-methyl ether derivative (II*b*).

About 10 years ago, in collaboration with the National Cancer Institute, we began the first systematic and worldwide survey of marine animals as potential sources of new antineoplastic agents (9). Many marine animal species have been located which yield extracts with a confirmed level of activity in the National Cancer Institute's P-388 murine lymphocytic leukemia, 9KB cell culture, and/or the P-388 and L-1210 cell culture evaluation systems (10). Of these potentially important marine animal species, six were Holothurioidea. Three were selected for detailed study: two from the family Stichopodidae, namely, *Stichopus chloronotus* Brandt (from Australia) and *Thelenota ananas* Jaeger (from Taiwan and the Marshall Islands), and one from the family Holothuriidae, *Actinopyga mauritiana* (from Hawaii).

We now wish to report that separation of the cytotoxic and antineoplastic constituents, guided by bioassay, led in each case to a complex mixture of lanostane-type saponins characteristic of sea cucumber Cuvierian organ toxins. The major cytotoxic component of each saponin mixture was isolated and designated stichostatin 1, thelenostatin 1, and actinostatin 1, respectively. A description of the isolation, purification, and partial characterization of stichostatin 1 (P-388, ED_{50} = 2.9) provides a summary of the techniques found most useful in this first detailed study of sea cucumber cytotoxic constituents.

A 2-propanol extract (59 g) obtained from *S. chloronotus* Brandt was partitioned between benzene and water. The water phase was separated and extracted with 1-butanol, and the extracted material was partitioned between ether and water. Freeze drying of the water phase afforded 1 g of the saponins, which were carefully separated on a specially prepared (11) prepacked silica gel column [elution with chloroform–methanol–water (20:5:0.5)].

Fractions 71–106 (6-ml volumes) contained stichostatin 1 (0.19 g). Recrystallization from methanol–chloroform gave needles (0.13 g) melting at 279–280°. Elemental analyses and molecular weight determinations by osmometry methods suggested a molecular weight of 1200 and an empirical formula of $C_{54-56}H_{93-98}O_{27-28}$. A series of mass (molecular ions were not observed), PMR, IR, and UV spectral studies, combined with preliminary chemical evidence, suggests that stichostatin 1 contains a lanostane nucleus related to that of Structure II*c* (8) and a glycoside system related to that of holotoxin A (I).

The structurally related cytotoxic saponins thelenostatin 1 (needles, mp 213–217°, from chloroform–methanol–water) and actinostatin 1 (needles, mp 218–220°, from chloroform–methanol–water) were obtained and partially characterized by similar methods from *T. ananas* Jaeger and *A. mauritiana*, respectively. In addition, both thelenostatin 1 (P-388, ED_{50} = 1.5) and actinostatin 1 (KB, ED_{50} = 2.6 and L-1210, ED_{50} = 2.1) were found to be half-esters of sulfuric acid similar to holothurin A, which is believed to contain the lanostane system illustrated by Structure III (12).

A definitive structural elucidation of these three new cytotoxic saponins, combined with further cytotoxic and antineoplastic evaluations, will be undertaken. Pres-

[1] Trepang is prepared by boiling the sea cucumber to cause evisceration and partial deactivation of the toxins.