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BIOCHEMICAL CORRELATES FOR THE PHARMACOLOGICAL EFFECTS OF L(+)-ISOMERS AND β-DESOXY-SYMPATHOMIMETIC AMINES*

ROBERT R. RUFFOLO, JR., DUANE D. MILLER and POPAT N. PATIL

Division of Pharmacology, School of Pharmacy, The Ohio State University, Columbus, Ohio 43210, U.S.A.

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Abstract The pharmacological effects of metabolically stable α-methylated indirectly acting β-hydroxylated L(+)-isomers and the corresponding β-desoxy-derivatives of phenolic and nonphenolic sympathomimetic amines were investigated. On the isolated, superfused rat vas deferens, preloaded with [^{3}H]norepinephrine (^{3}H-NE), the contraction and the efflux of tritium from the tissue by the drugs were studied. It was observed that a given phenolic amine can block the effects of other phenolic amines. For example, L(+)-cobefrin can block the effect of (+)-desoxycobefrin or tyramine. A nonphenolic amine, however, blocks other nonphenolic amines but not the effects of phenolic amines. For example, L(+)-ψ-ephedrine can block the effect of (+)-desoxyephedrine but not that of tyramine. The efflux of tritium parallels the pharmacologic effect. It is implied that phenolic and nonphenolic amines utilize different transport systems at the adrenergic nerve terminal. Other consistent findings have been that the desoxy-derivative of either phenolic or nonphenolic amines always produces a greater pharmacologic effect than the corresponding L(+)-isomer. Since their ability to block uptake of ^{3}H-NE is the same, as compared to the L(+)-form, the desoxy-derivative must enter the adrenergic neurone faster and/or displace more transmitter to produce its greater pharmacologic effect. Additional studies with isolated bovine splenic nerve granules indicate that the less effective L(+)-isomers do not release NE from storage vesicles, whereas desoxy-analogs release considerable quantities of NE from these sites. The implications are that the desoxy-form may release NE from both cytoplasmic and vesicular stores, while the L(+)-isomer may release NE only from an extravesicular site.

The mechanism by which indirectly acting phenolic and nonphenolic sympathomimetic amines act may be different [1, 2]. It was suggested that tyramine, a phenolic amine, releases intraneuronally bound norepinephrine (NE) from different storage pools than does mephentermine, which is of the nonphenolic type. Subsequently, it was possible to block the response of one phenolic amine by preincubation with a supramaximal concentration of another weaker phenolic amine but not with a nonphenolic compound. Similarly, it was possible to inhibit the response of a nonphenolic sympathomimetic amine by previously exposing the tissue to another nonphenolic indirectly acting amine, but not a phenolic amine [3]. These results suggest that at the adrenergic neurone two different transport processes exist, one being specific for phenolic amines and the other for liposoluble, nonphenolic sympathomimetic amines [4, 5].

In addition to differences between phenolic and nonphenolic indirectly acting amines, sympathomimetics also show a difference in response which is related to the stereochemistry about the β-carbon atom. It was observed [3] that the indirectly acting desoxy-form (without β-hydroxyl group) of a sympathomimetic amine was always more active than the corresponding β-hydroxylated L(+)-analog. The reason for this is unknown, but the working hypothesis is that the desoxy-analog enters the nerve terminal at a faster rate than the corresponding L(+)-analog and therefore liberates more transmitter in a given period of time. Alternatively, it may be that both drugs liberate equal amounts of the neurotransmitter; however, the desoxy-analog is a better inhibitor of re-uptake, resulting in a greater pharmacological response. Thus, it is our aim to examine these biochemical and the pharmacological correlates in the densely adrenergically innervated rat vas deferens.

METHODS

General considerations. Male albino Wistar rats (Laboratory Animal Supply Co., Indianapolis, Ind.) weighing 150–400 g were used throughout the study. The animals were killed by a sharp blow on the head; the vasa deferentia were removed and placed in an oxygenated physiological salt solution (PSS) at room temperature for debridement of extraneous tissue. The composition of the PSS was (mM): NaCl, 118; KCl, 4·7; $MgCl_2 . 6H_2O$, 0·54; $CaCl_2 . 2H_2O$, 2·5; NaH_2PO_4, 1; $NaHCO_3$, 25; and glucose, 11. Ethylenediaminetetraacetic acid (EDTA) was added (10 μg/ml) to retard the spontaneous oxidation of catecholamines. PSS always contained tropolone (3×10^{-5} M) to inhibit catechol-*O*-methyl transferase (COMT).

All drug solutions were made daily (double-distilled demineralized water) except for a stock solution of (−)-[^{3}H]norepinephrine, which was kept at 4° in 1% sodium metabisulfite.

Response of superfused vasa deferentia to desoxy-derivatives and L(+)-isomers. Vasa deferentia from one rat were allowed to equilibrate at 38° for 15 min in

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PSS before incubation with iproniazid (10^{-4} M) to inhibit monoamine oxidase (MAO). After two washings with PSS, the tissues were incubated with ^{3}H-NE (200 ng/0·63 μCi/ml) for 60 min at 38°. Each tissue was washed five times with 10 ml PSS to remove the amine from extracellular spaces.

The vasa deferentia were then suspended in separate superfusion chambers (warmed to 38°) and connected via a thin thread to a Grass force-displacement transducer (model FT.03C) under 250 mg tension. Superfusion with PSS (38°) at a rate of 4 ml/min was allowed to proceed for 30 min before infusions of drugs into the system were begun. Drug-induced contractions were recorded on a Grass ink-writing polygraph. This superfusion procedure is a modification of that described by Su and Bevan [6].

After a superfusion equilibrium period of 30 min, a solution of the indirectly acting sympathomimetic amine was infused into the system by a Harvard infusion pump (model 931) at a predetermined rate and concentration so that the PSS reaching the tissue contained 3×10^{-4} M of each amine. At this concentration the contractile effect of each drug is maximal and is completely blocked by reserpine pretreatment [3, 7]. On one of the two tissues, only the desoxy-analog was infused and the tissue was allowed to reach its maximum response (100 per cent). On the contralateral tissue, first the L(+)-isomer was infused and allowed to cause its maximum response, after which the desoxy-analog was added and also permitted to respond maximally. At this point, tyramine was infused and allowed to elicit its maximum effect. No drug was superfused until the preceding compound had elicited its maximum effect on the tissue. Figure 1 illustrates a typical tracing from an experiment. During the entire drug infusion period, and for 2 min prior, the superfusate was collected at 1-min intervals and assayed for tritium by dissolving a 2-ml aliquot in 13 ml Aquasol (New England Nuclear) and counting by liquid scintillation spectroscopy. No correction was made for NE metabolites, which were assumed to be minimal, since both major metabolic enzymes were inhibited.

Inhibition of ^{3}H-NE uptake in the normal and reserpine-pretreated rat vasa deferentia. Vasa deferentia from a normal or reserpine-pretreated rat (5 mg/kg, i.p. 18–21 hr prior to sacrifice) were placed in separate beakers containing 5 ml of oxygenated PSS and allowed to equilibrate for 15 min at 38°. After equilibration, the tissues were incubated in PSS containing 10^{-4} M iproniazid for 30 min. Both tissues were then washed twice (at 1-min intervals) with 5 ml PSS and allowed to re-equilibrate for an additional 15 min. One tissue was exposed to the drug [either the desoxy- or L(+)-isomer] while the contralateral tissue served as a control. The drug, always in the concentration of 3×10^{-4} M, was allowed to incubate for 3 min before the ^{3}H-NE was added (20 ng 0·11 μCi/ml) to both the experimental and control tissues. The vasa deferentia were exposed to ^{3}H-NE for 5 min, then washed five times at 1-min intervals with 5 ml PSS at 38° to remove extracellularly bound NE. The tissues were then transferred to separate beakers containing 2 ml 0·4 N $HClO_4$ in which they were homogenized with a Brinkman Polytron for 30 sec. The polytron was washed with 2 ml fresh 0·4 N $HClO_4$ which was combined with the original homogenate. The homogenate was centrifuged in a clinical centrifuge for 20 min and then 2 ml of the resulting supernatant was assayed for its tritium content by dissolving it in 13 ml Aquasol and counting by liquid scintillation. The counts in the control tissue represent 100 per cent.

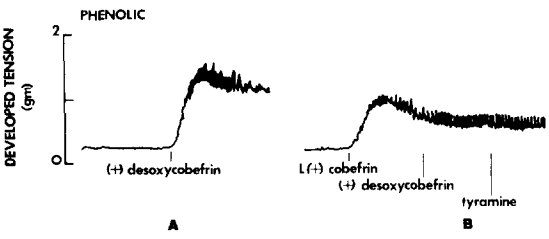

Fig. 1. Experimental protocol for investigating the effects of desoxy-amines and their corresponding L(+)-isomers on superfused rat vas deferens. A and B are two tissues from the same animal. The superfusion concentrations were 3×10^{-4} M for each drug. On tissue B, tyramine was infused in the presence of on-going infusions of L(+)-cobefrin and (+)-desoxycobefrin. Tritium efflux was collected at regular intervals.

Effect on ^{3}H-NE efflux from bovine splenic nerve storage granules. NE storage granules were obtained by a modification of the technique described by von Euler and Lishajko [8]. Briefly, clean, desheathed bovine splenic nerves (3·0 to 7·7 g) were placed in 30 ml of 0·13 M potassium phosphate buffer (pH 7·4) and minced with scissors at 0°. The mince was homogenized with a Brinkman Polytron for 30 sec and the resulting homogenate was filtered by suction through muslin to remove large particles and cell debris. Five ml of the filtrate was added to each of five ultracentrifuge tubes and centrifuged at 10,000 *g* in a Beckman ultracentrifuge (model L) for 10 min at 4°. The resulting supernatant, which contains most of the NE storage granules, was incubated at 38° for 10 min to partially deplete endogenous NE [9]. At this time, exogenous ^{3}H-NE (20 ng 0·11 μCi/ml) was added and incubated for 5 min at 38°. Incubation was terminated by immersing each tube in an ice bath. The suspension was centrifuged at 70,000 *g* for 30 min and the resulting supernatant discarded. The pellet (containing NE storage granules) was resuspended in 5 ml of ice-cold buffer to wash and centrifuged as described above for 30 min. The washing process was repeated and the resulting 70,000 *g* supernatant replaced with 4·8 ml fresh buffer (38°) and the pellet resuspended. The desoxy- or L(+)-analogs studied were added to each tube in a 0·2 ml vol. of buffer (3×10^{-4} M concentration in the tube) and incubated at 38° for 5 min. Incubation was terminated by placing the tubes in ice. The suspension was centrifuged as described above and the supernatant assayed for tritium by dissolving 2 ml in 13 ml Aquasol and counting by liquid scintillation spectrometry. The tritium content of the pellet was determined by extracting twice with a 1-ml vol. of 0·4 N $HClO_4$ and adding the combined extracts to 13 ml Aquasol and counting by liquid scintillation. Efflux of ^{3}H-NE is expressed as the supernatant:pellet ratio (S:P). Higher ratios represent greater ^{3}H-NE efflux. The S:P ratio for the control was considered to be 100 per cent.

Drugs. Only metabolically stable α-methylated sympathomimetic amines were used. Since D(−)-isomers of sympathomimetic amines produce considerable direct sympathomimetic effects, only the desoxy- and L(+)-isomers were used. The following drugs were used in this study: (−)-^{3}H-norepinephrine bitartrate, sp. act. 6·6 Ci/m-mole (Amersham/Searle Corp.); (+)-desoxycobefrin HCl (methyldopamine), (±)-desoxymetaraminol (α-methyl-*m*-tyramine), L(+)-metaraminol bitartrate (Merck, Sharp & Dohme); L(+)-cobefrin (Sterling Winthrop); (+)-amphetamine SO_4, (+)-methamphetamine HCl (Smith, Kline & French); L(+)-*pseudo*-norephedrine HCl (Light & Co.); L(+)-*pseudo*-ephedrine HCl (Burroughs Wellcome); tyramine HCl (General Biochemicals); iproniazid (Hoffman-LaRoche, Inc.); reserpine (Ciba); and tropolone (Aldrich).

RESULTS

The normal efflux of ^{3}H-NE during the first 30 min of superfusion is quite high and obeys first-order kinetics with a rate constant of 0·033 min^{-1} and a half-time of 21 min. However, the tissue retained approximately 8–10 × 10^5 cpm/g at the time drug-induced release was studied. The basal efflux of ^{3}H-NE in an identical preparation has been reported previously [10].

Generally, during the superfusion of the desoxy-amine, the drug-induced release varied between 200 and 300 cpm/ml. The maximum release value of desoxy-derivative in every experiment is considered to be 100 per cent. Although tissue-to-tissue variability in the drug-induced release of ^{3}H-NE occurred, the release pattern in experiments paralleled the pharmacologic response.

A phenolic amine, (+)-desoxycobefrin, produces a pharmacological response and ^{3}H-NE release greater than that of L(+)-cobefrin, which is also phenolic. However, when (+)-desoxycobefrin is superfused after a supra-maximal concentration of L(+)-cobefrin (3 × 10^{-4} M), no additional response or ^{3}H-NE release is obtained (Fig. 2). Furthermore, superfusion of tyramine, which is also phenolic, after the two previous amines, produces no increase in response or ^{3}H-NE release even though the activity of tyramine in control tissue is greater than that of either the desoxy- or L(+)-analogs [3]. When (+)-desoxycobefrin was administered during the infusion of L(+)-cobefrin, the response was maintained but the release declined. The relatively small release of ^{3}H-NE, with the relatively fast on-going decline in the basal release, could explain the discrepancies between the patterns of release and contraction. Qualitatively similar results were obtained with the phenolic amines, (±)-desoxymetaraminol and L(+)-metaraminol. Among the nonphenolic amines, (+)-methamphetamine has greater pharmacological activity than L(+)-ψ-ephedrine; however, when (+)-methamphetamine is administered after L(+)-ψ-ephedrine, the former is unable to elicit any additional response or ^{3}H-NE release. Tyramine (phenolic), when superfused in the presence of L(+)-ψ-ephedrine and (+)-methamphetamine, was able to cause an additional pharmacological response and ^{3}H-NE release (Fig. 3). The same pattern was obtained for (+)-amphetamine and L(+)-ψ-norephedrine, except that no clear-cut increase in ^{3}H-NE release was observed when tyramine was superfused after the former amines, even though the pharmacological response to tyramine was increased. It is possible that the on-going spontaneous decrease in basal efflux [10] has masked the increase in tritium outflow induced by tyramine.

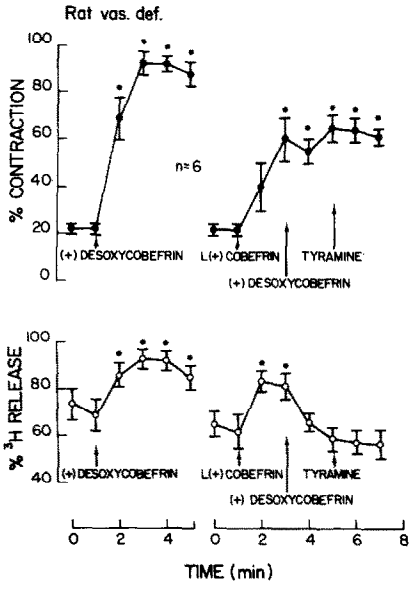

Fig. 2. Pharmacological effects and tritium efflux (presumably NE) from the superfused rat vas deferens. Note that tyramine, a phenolic amine, did not produce contraction or efflux in the presence of L(+)-cobefrin and (+)-desoxycobefrin, both of which are also phenolic. Vertical lines are S. E. M. The asterisks represent a significant difference ($P < 0·05$) from resting tension or basal tritium efflux.

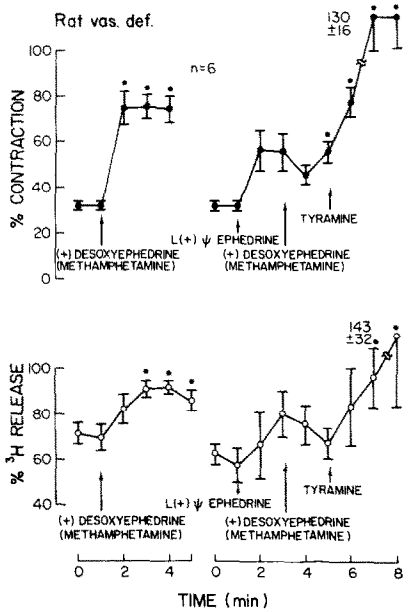

Fig. 3. Pharmacological effects and tritium efflux (presumably NE) from the superfused rat vas deferens. Note that tyramine produced additional effects in the presence of other nonphenolic amines. Vertical lines are S. E. M. The asterisks represent a significant difference ($P < 0·05$) from resting tension or basal tritium efflux.

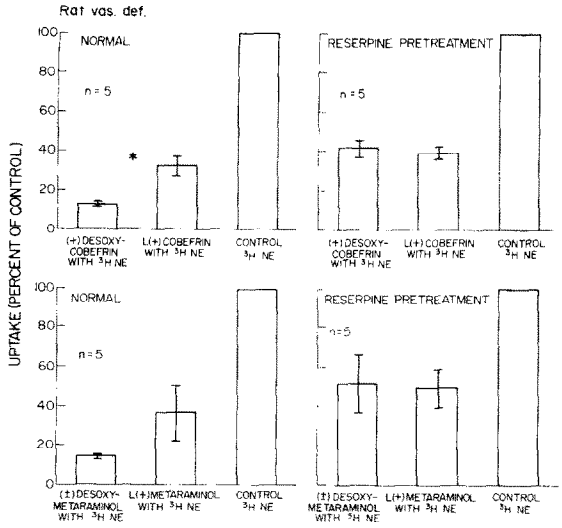

Fig. 4. Uptake of ^{3}H-NE in the presence of the desoxy- and L(+)-isomers of phenolic amines. The amines (3×10^{-4} M) were incubated for 3 min prior to the addition of ^{3}H-NE. The vertical lines are S. E. M. As compared to that in the normal tissue, the ability of these amines to block the uptake in the reserpine-treated tissues is identical; the asterisk indicates $P < 0.05$.

In all cases but one, the desoxy-analog was more effective than the L(+)-form in inhibiting ^{3}H-NE uptake into the normal vas deferens (Figs. 4 and 5). This conceivably could explain the greater action of the desoxy-analogs observed in Figs. 2 and 3. One problem arises, however, from this type of experiment in that sympathomimetic amines, while inhibiting the uptake of exogenously administered ^{3}H-NE, are at the same time releasing endogenous NE. Since the endogenous NE will also be taken up by the nerve terminal, exogenously administered ^{3}H-NE will then compete with both the drug [desoxy- or L(+)-form] and the liberated endogenous NE. The compound releasing more NE may be credited with being a greater inhibitor of uptake when actually it may not be. For this reason, the uptake study was conducted in vasa deferentia from rats pretreated with reserpine, which depletes endogenous catecholamines. Reserpine will also block the vesicular uptake of ^{3}H-NE, which permits study of the various sympathomimetic amines on the membrane of the nerve terminal without complication due to the effects at the level of the vesicular membrane. The absolute accumulation of ^{3}H-NE was decreased to one-third that of normal vasa deferentia (4.9 ± 2.2 ng of ^{3}H-NE/g of tissue in reserpinized vasa deferentia as compared to 14.2 ± 3.3 ng/g in normal tissues). It was determined that both the desoxy- and L(+)-analog possessed equal abilities to inhibit the uptake of exogenously administered ^{3}H-NE at the level of the neuronal membrane (Figs. 4 and 5). These results are significant, since they refute the hypothesis that desoxy- and L(+)-analogs release equal amounts of noradrenaline while the desoxy-form is a greater inhibitor of re-uptake, and support the view that the desoxy-analog enters the nerve terminal faster and releases more NE than the L(+)-form.

In view of these findings, studies on the isolated storage granules of the bovine splenic nerve were performed using one pair of phenolic and one pair of nonphenolic desoxy- and L(+)-analogs. The results are presented in Figs. 6 and 7. As may be seen, both desoxy-analogs studied [(+)-desoxycobefrin and (+)-methamphetamine] produce a considerable increase in S/P ratio and hence ^{3}H-NE efflux from the isolated granules. The L(+)-isomers [L(+)-cobefrin and L(+)-ψ-ephedrine] were observed to cause little or no enhancement of ^{3}H-NE efflux. To eliminate the effects of day-to-day variation, the mean difference (Δ) in

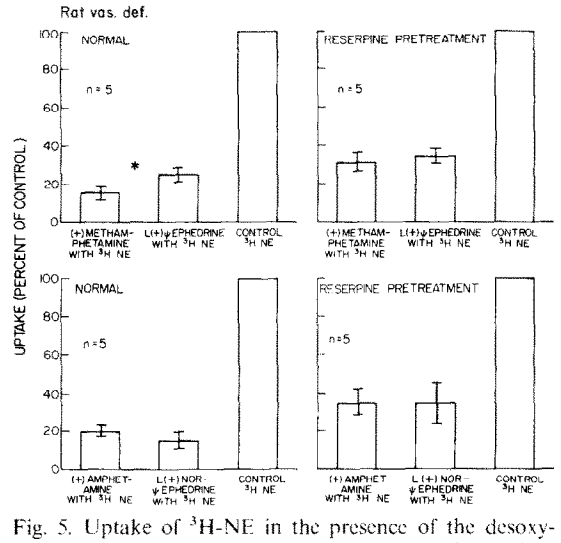

Fig. 5. Uptake of ^{3}H-NE in the presence of the desoxy- and L(+)-isomers of nonphenolic amines. The amines (3×10^{-4} M) were incubated for 3 min prior to the addition of ^{3}H-NE. The vertical lines are S. E. M. As compared to that in the normal tissue, the ability of the pair of amines to block the uptake in the reserpine-treated tissue is identical; the asterisk indicates $P < 0.05$.

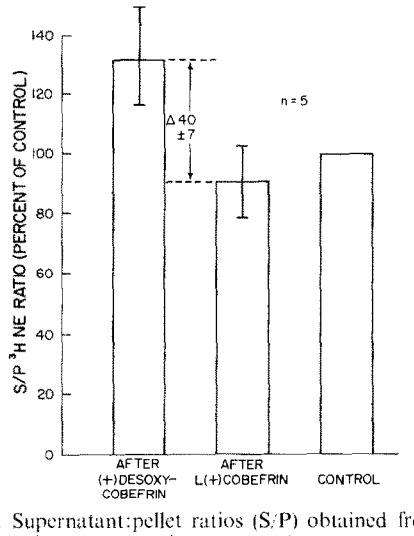

Fig. 6. Supernatant:pellet ratios (S/P) obtained from bovine splenic nerve granules, expressed as per cent of control, for (+)-desoxycobefrin and L(+)-cobefrin. The concentration of each amine was 3×10^{-4} M. The vertical lines are S. E. M. The mean per cent difference between the desoxy- and L(+)-isomer calculated from each experiment is Δ. The higher S/P ratio indicates greater release of ^{3}H-NE into the medium by the drug.

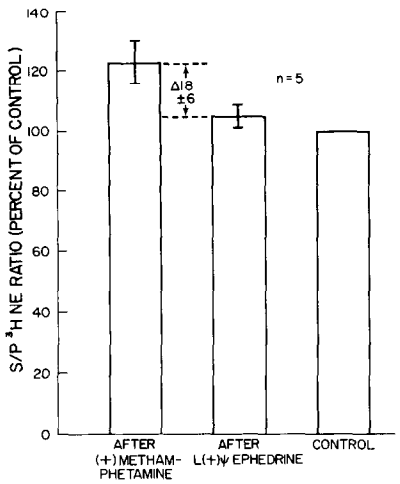

Fig. 7. Supernatant:pellet ratios (S/P) obtained from bovine splenic nerve granules, expressed as per cent of control, for (+)-methamphetamine and L(+)-ψ-ephedrine. The concentration of each amine was 3×10^{-4} M. The vertical lines are S. E. M. The mean per cent difference between the desoxy- and L(+)-isomer calculated from each experiment is Δ.

S/P ratios between the desoxy- and its corresponding L(+)-analog was calculated and presented in Figs. 6 and 7. In both pairs of drugs studied, this Δ represents a statistically significant difference ($P < 0.05$) from zero. The absolute S/P ratios for (+)-desoxycobefrin, L(+)-cobefrin, (+)-methamphetamine and L(+)-ψ-ephedrine were 2·41, 1·65, 2·34 and 2·02 respectively. The control ratio was 1·90.

DISCUSSION

The results of the present study are in agreement with the postulation that the indirectly acting phenolic and nonphenolic amines may utilize different transport systems for neuronal uptake. Figures 2 and 3 show that when a tissue is exposed first to a supramaximal concentration of a lesser active phenolic amine and then to a more active phenolic compound, the latter is unable to elicit any further pharmacological response or ^{3}H-NE release. Similarly, the activity of a nonphenolic amine is inhibited by previously saturating the tissue with another less active nonphenolic compound. Thus, in all pairs studied, the desoxy-analogs, when administered after the corresponding L(+)-form, could release no additional ^{3}H-NE and therefore cause no further response. This also explains the difference in the response of tyramine when superfused on tissues previously exposed to phenolic or nonphenolic amines. In the former case, presumably all carriers for the phenolic uptake process are occupied by L(+)-phenolic amines (and to a lesser extent by desoxy-phenolic amines), which prevent tyramine (phenolic) from being transported into the nerve terminal and releasing stores of NE. However, when tyramine is superfused after supramaximal concentrations of L(+)- and desoxy-nonphenolic amines, tyramine does cause additional ^{3}H-NE release and therefore additional tissue response. In this case, presumably the L(+)- and desoxy-analogs are saturating the nonphenolic uptake process and leaving the phenolic transport mechanism relatively free to take up tyramine, which may then release NE. Thus, based on these biochemical findings, combined with the fact that the physical-chemical properties of the phenolic and the nonphenolic amines differ, the different mechanisms of indirect effect appear highly probable.

α-Methylated amines with 2S configuration appear to be substrate for the enzyme dopamine-β-oxidase [11]. Hence, it can be argued that greater effects of the desoxy-derivative over the corresponding L(+)-form is due to the conversion to the corresponding β-hydroxylated D(−)-isomer, which could be an effective releaser of the NE. Although this possibility is likely, the pharmacological activity of (−)- and (+)-isomers of α-methylated amines are very similar. The (−)-isomers of desoxy-derivatives are not the substrate for the enzyme. In other words, desoxy-derivatives which are not substrate for the dopamine-β-oxidase are more potent than the corresponding L(+)-isomers. Thus, the greater pharmacologic effects of desoxy-derivatives could not be solely explained on the basis of their conversion to beta-hydroxylated D(−)-isomers.

The consistent findings of the present and past investigations have been the greater indirect pharmacologic effects of the desoxy-derivatives of sympathomimetic amines over the corresponding β-hydroxylated L(+)-isomers. Muscholl [12] has pointed out that indirectly acting amines not only release endogenous neurotransmitter, but they also inhibit the uptake of the released neurotransmitter. Since, after reserpine pretreatment, the inhibition of uptake of exogenous ^{3}H-NE at the neuronal membrane is the same by the desoxy- and the L(+)-analogs, the greater indirect effects of desoxy-derivatives might be due to faster entry and/or greater effective displacement of the transmitter from the adrenergic nerve terminal by this amine. The converse should occur with the L(+)-forms of the amine. The precise anatomical location from which these drugs liberate the neurotransmitter is not known. Our findings indicate that the desoxy-form of a sympathomimetic amine may release NE from granular stores, whereas the L(+)-analogs do not (Figs. 6 and 7). The implication is that the desoxy-derivatives liberate neurotransmitter from both vesicular and cytoplasmic sites, while the L(+)-analogs release NE from only the latter site. It should be noted, however, that vesicles isolated from bovine splenic nerve and used in the present study are predominantly large dense core vesicles, whereas those in the nerve terminals of the vas deferens are mainly small dense core vesicles. Although there are several differences between the two vesicle types, extrapolation of data from one type to another is possible in many instances [13].

On the normal tissue, desoxy-derivatives are apparently better inhibitors of uptake than the corresponding L(+)-isomers. Since desoxy-derivatives possess more indirect effects than the L(+)-isomers, the relatively more endogenously released noradrenaline will compete with the exogenously administered ^{3}H-NE. Hence, it is not surprising that on the normal tissue desoxy-derivatives are apparently better inhibitors of ^{3}H-NE uptake than the L(+)-isomers. In other words, endogenously liberated noradrenaline obscures the

quantitation of true affinity of the indirect-acting amine for the transport system. Burgen and Iversen [14] described a structure activity profile for the inhibition of the uptake of NE by many sympathomimetic amines. The studies were carried out on normal tissues. Conclusions drawn from the present study indicate that the work should be re-examined in catecholamine-depleted tissues.

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