Steric Aspects of Adrenergic Drugs XXII: Retention of (+)- and (−)-^{14}C-Norepinephrine by Mouse Heart

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Abstract □ After intravenous infusion (total dose 40 mcg./kg.) of the isomers of norepinephrine to normal or reserpine-pretreated mice, the initial accumulation by the heart was the same for both isomers. The injected isomers disappeared from the normal heart in a single exponential phase. The unnatural isomer disappeared faster, $t_{1/2}$ 2.5 hr., than the natural (−)-isomer, $t_{1/2}$ 7.6 hr. In mice pretreated with iproniazid, iproniazid and guanethidine, or iproniazid and α-methyl-*p*-tyrosine, the rate constants and the $t_{1/2}$ values for the isomers were unaltered. The results indicate that retention, probably by storage vesicles, favors the natural isomer; hence, it is stereoselective. Reserpine, which inhibited the accumulation by storage vesicles, drastically reduced the accumulation of isomers, and the accumulated isomers disappeared at a similar rate constant with a $t_{1/2}$ of approximately 1 hr. Racemic norepinephrine disappeared in two phases, each having a separate rate constant which approximate the values obtained with (+)- and (−)-norepinephrine. In the iproniazid-pretreated mice, injection of equal amounts of (−)- or (+)-^{14}C-norepinephrine resulted in higher *O*-methylated products from the (+)-form.

Keyphrases □ Norepinephrine, ^{14}C-labeled—retention of (+)- and (−)-isomers by mouse heart, effects of iproniazid, guanethidine, or α-methyl-*p*-tyrosine pretreatment, steric aspects of adrenergic drugs □ Adrenergic drugs—steric aspects, retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart □ Catecholamines—retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart, steric aspects of adrenergic drugs □ Neurochemical transmitters—retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart, steric aspects of adrenergic drugs □ False neurotransmitter—retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart

Since 1963 there has been considerable interest in the fate of optical isomers of catecholamines (1–5). Lack of optically pure radiolabeled isomers, however, impeded investigations. Many, if not all, physiological processes can discriminate between the stereoisomers of catecholamines, as discussed in previous reviews (6, 7). Since the transport of isomers across the neuronal membrane shows marginal selectivity in favor of the natural isomer, it has been possible to study uptake and release of both isomers from tissues (8–10). Recently, Allen *et al.* (8) suggested that (+)-norepinephrine might be a valuable false neurochemical transmitter. In view of the large differences in adrenergic receptor-activating properties of the isomers (6, 7), the proposal appears quite attractive. However, for an unnatural substance to be an effective false neurotransmitter, the drug must be retained by adrenergic storage vesicles (11, 12). This paper reports studies on the time course of retention of the isomers by the mouse heart. Other pharmacological tools such as α-methyl-*p*-tyrosine, reserpine, 6-hydroxydopamine, and guanethidine pretreatment were used to gain information about the retention of the isomers.

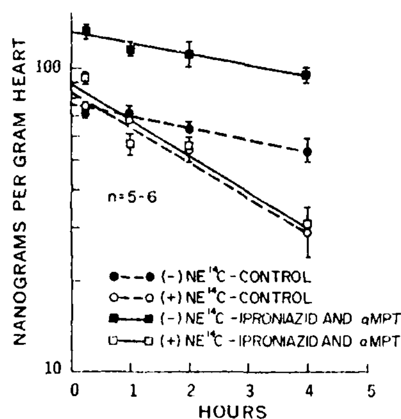

Figure 1—*Uptake and retention of (−)- and (+)-^{14}C-norepinephrines in the normal and drug-treated mouse hearts after intravenous infusion of (−)- and (+)-^{14}C-norepinephrines, 40 mcg./kg. The schedule of treatment with iproniazid and α-methyl-*p*-tyrosine was described in the* Methods *section. Key:* n = *number of observations, vertical bars = standard error of the mean, NE = norepinephrine, and αMPT = α-methyl-*p*-tyrosine.*

METHODS

Infusion of (−)- and (+)-^{14}C-Norepinephrine Isomers—Male albino ICR mice (16–26 g.) were used. Each animal was infused with either (−)- or (+)-^{14}C-norepinephrine *via* one of the lateral tail veins. The total dose infused was 0.8 mcg./20 g., which corresponded to 0.1 μc./0.1 ml./20 g. for each isomer. Infusion[1] was constant at a rate of 0.2 ml./min. and required 25–40 sec. for completion.

Mice were decapitated at varying periods postinfusion (5 min. to 24 hr.), and hearts were quickly removed, cleaned of extraneous tissue, minced with scissors, and rinsed five times with cold 0.9% sodium chloride solution. The minced material was blotted with filter paper, weighed, and transferred to 20-ml. beakers containing 5 ml. of 5% cold trichloroacetic acid containing 0.2 ml. of 10% ethylenediaminetetraacetic acid. Hearts were minced with scissors and homogenized[2] for 20 sec. at moderate speed. The homogenates were centrifuged for 10 min. in a clinical centrifuge at room temperature. The resulting supernate was filtered under suction, and the pellet was resuspended in 0.5 ml. of 0.4 *N* $HClO_4$ and recentrifuged for another 10 min. The second supernate was mixed with the first, and the final volume was made up to 8 ml. The filtrates were transferred to 20-ml. beakers containing 10 mg. ethylenediaminetetraacetic acid. The pH of these extracts was adjusted to between 6.2 and 6.5 with 2 *N* NaOH. The unchanged ^{14}C-norepinephrine was separated from the contaminating metabolites by passing the slightly acidic extracts through strong ion-exchange columns prepared[3] according to the method of Häggendal (13, 14) with a small modification. The modification made was in the use of columns 9.0 mm. in diameter and a resin height of 3.5 cm. The fractions of the eluate containing ^{14}C-norepinephrine were placed in 20-ml. beakers and evaporated to dryness. The recovery of ^{14}C-

[1] Harvard infusion pump model 901, Harvard Apparatus Co., Millis, Mass.
[2] Using an Ultra-Turrax, Brinkmann Instruments.
[3] From Amberlite CG-120 (200–400 mesh), Na^+ form, Mallinckrodt Chemical Co.

Table I—Rate Constants and Half-Lives ($t_{1/2}$) of Disappearance of ^{14}C-Norepinephrine Isomers from the Mouse Heart after Different Treatments

Treatment	Isomer	Rate Constant, hr.$^{-1}$	$t_{1/2}$, hr.
Control	(−)	0.09	7.62
	(+)	0.26	2.57
Iproniazid[a]	(−)	0.07	9.32
Iproniazid[a]	(+)	0.17	3.92
Iproniazid[a]	(±)	0.27	2.19
		0.06	10.10
Iproniazid[a] + guanethidine[b]	(−)	0.09	7.52
	(+)	0.15	4.57
Iproniazid[a] + reserpine[c]	(−)	0.64	1.07
	(+)	0.87	0.78
Iproniazid[a] + α-methyl-*p*-tyrosine[d]	(−)	0.08	8.00
	(+)	0.2	2.56

[a] Injected 100 mg./kg. i.p., 16–20 hr. before ^{14}C-norepinephrine infusion. [b] Injected 20 mg./kg. i.p., 8–10 hr. before iproniazid. [c] Injected 5 mg./kg. i.p., 8–10 hr. before iproniazid. [d] Injected in two divided doses, 200 mg./kg. each, at 3-hr. intervals and 10–12 hr. after iproniazid.

norepinephrine from the columns was 72.14 ± 0.76%. Values reported were corrected for 72% recovery. Residues for the air-dried hydrochloride eluates were taken up in 1 ml. of water and counted in 14 ml. of a liquid scintillation solution[4].

In experiments where animals were treated with iproniazid to inhibit MAO, no attempt was made to separate unchanged ^{14}C-norepinephrine from the *O*-methylated metabolites. In a few experiments, where the mice were sacrificed 10 min. after ^{14}C-norepinephrine injection, the *O*-methylated derivatives did not exceed 5% of the unchanged amine. Since metabolites do not accumulate in the tissues and are easily washed out (15), it was expected that when animals were sacrificed at longer time periods after ^{14}C-norepinephrine infusion, the amount of metabolites present would be lower still. Moreover, the minced heart was washed thoroughly to reduce contamination by *O*-methylated derivatives. The trichloroacetic acid extracts were air dried, taken up in 2 ml. of water, mixed with 13 ml. of a liquid scintillation solution, and counted[5].

Schedule of Drug Treatments in Mice—MAO inhibition was accomplished by administering iproniazid, 100 mg./kg. i.p., 16–20 hr. prior to infusion of ^{14}C-norepinephrine isomers. Effects of all other pharmacological treatments were studied in the enzyme-inhibited animals.

Guanethidine, 20 mg./kg. i.p., was given 8–10 hr. before iproniazid. ^{14}C-Norepinephrine isomers were infused 24–30 hr. after guanethidine.

Reserpine, 5 mg./kg. i.p., was given 8–10 hr. before iproniazid. ^{14}C-Norepinephrine isomers were infused 24–30 hr. after reserpine,

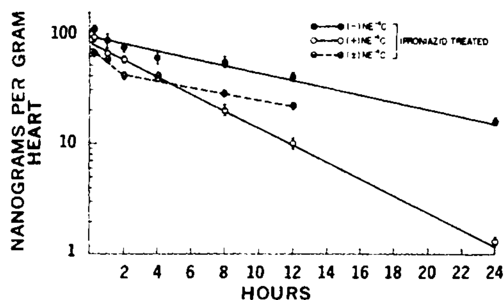

Figure 2—*Uptake and retention of (−)-, (+)-, or racemic ^{14}C-norepinephrines in the hearts of mice that received iproniazid, 100 mg./kg., 16–24 hr. before they received 40 mcg./kg. i.v. of ^{14}C-norepinephrine infusion. Each point is a mean of five to nine observations. Key: vertical bars = standard error of the mean, and NE = norepinephrine.*

[4] Aquasol, New England Nuclear.
[5] Tri-Carb liquid scintillation spectrometer.

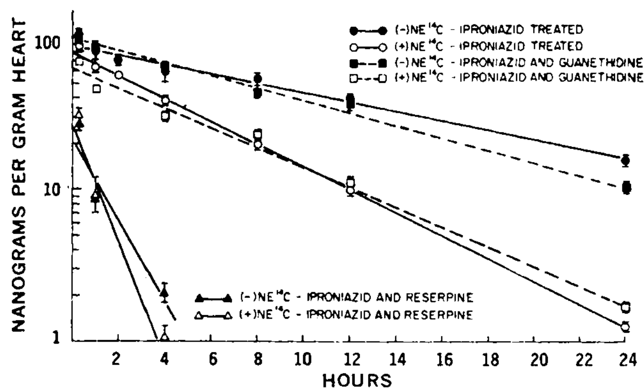

Figure 3—*Uptake and retention of (−)- and (+)-^{14}C-norepinephrines in hearts of mice treated with iproniazid and guanethidine or iproniazid and reserpine after intravenous infusion of ^{14}C-norepinephrine isomers, 40 mcg./kg. The schedule of treatments given is described in the* Methods *section. Each point is a mean of five to nine observations. Key: vertical bars = standard error of the mean, and NE = norepinephrine.*

and animals were sacrificed in the time interval from 15 min. to 4 hr. thereafter.

Tyrosine hydroxylase was inhibited by the administration of two doses of α-methyl-*p*-tyrosine, 200 mg./kg. i.p., 3 hr. apart. Iproniazid was injected 16–20 hr. before the second dose of α-methyl-*p*-tyrosine, which was given 1 hr. before ^{14}C-norepinephrine infusion.

6-Hydroxydopamine was administered in two doses of 75 mg./kg. i.p. at an interval of 1 hr., about 24 hr. before ^{14}C-norepinephrine infusion. Iproniazid was injected as already described. Animals were sacrificed 24–28 hr. after 6-hydroxydopamine treatment.

In other experiments, (−)- and (+)-^{14}C-norepinephrines were mixed in equal amounts (adjusted to equal specific activity), and the resulting (±)-^{14}C-norepinephrine was infused in the iproniazid-treated mice. Animals were sacrificed in the time interval from 15 min. to 12 hr. after (+)-^{14}C-norepinephrine infusion.

Determination of *O*-Methylated Products—Iproniazid-treated mice were given either (−)- or (+)-^{14}C-norepinephrine, 40 mcg./kg. i.v. Five hearts were pooled in one beaker and extracted in trichloroacetic acid as described earlier. *O*-Methylated derivatives were separated from the unchanged or oxidized ^{14}C-norepinephrine by ion-exchange chromatography according to the method of Häggendal (14). The eluates were air dried and counted in the manner described for ^{14}C-norepinephrine estimation.

Drugs and Chemicals—Drugs used in this study were: (−)-norepinephrine (+)-bitartrate monohydrate and (+)-norepinephrine (+)-bitartrate[6]; (−)-^{14}C-norepinephrine-7 (+)-bitartrate, specific activity 57.0 mc./mmole, and (+)-^{14}C-norepinephrine-7 (+)-bitartrate, specific activity 21.2 mc./mmole[7]; iproniazid phosphate[8]; guanethidine hemisulfate and reserpine powder[9]; 6-hydroxydopamine hydrobromide[10], and α-methyl-*p*-tyrosine methyl ester hydrochloride[10].

Reserpine was prepared as a 0.5% solution in 10% ascorbic acid. The solution of 6-hydroxydopamine was prepared in 0.1% sodium metabisulfite at 0° and was deaerated for at least 10 min. using nitrogen. Stock aqueous solutions of iproniazid and guanethidine were prepared and used as needed. Solutions of α-methyl-*p*-tyrosine were prepared fresh daily. Working solutions of labeled (−)- and (+)-norepinephrines were prepared in 1% sodium metabisulfite, protected from light and air, and stored in the refrigerator at 4°. All drugs and chemicals were dissolved in demineralized double-distilled water.

RESULTS

Accumulation and Retention of (−)- and (+)-^{14}C-Norepinephrine Isomers in Mouse Hearts—Mice were given either (−)- or (+)-

[6] Sterling-Winthrop Research Institute, Rennsselaer, N. Y.
[7] Amersham-Searle, Arlington Heights, Ill.
[8] Hoffmann-La Roche, Inc., Nutley, N. J.
[9] Ciba Pharmaceutical Co., Summit, N. Y.
[10] Regis Chemical Co., Chicago, Ill.

Table II—Accumulation and Retention of ^{14}C-Norepinephrine Isomers in Hearts of 6-Hydroxydopamine-Treated[a] Mice[b]

Time after ^{14}C-Norepinephrine Infusion (Total Dose 40 mcg./kg.)	$(-)$-^{14}C-Norepinephrine ng./g. Heart	% of Control	$(+)$-^{14}C-Norepinephrine ng./g. Heart	% of Control[c]
15 min.	4.90 ± 0.23	4.63	6.01 ± 0.76	6.71
4 hr.	0.81 ± 0.21	1.36	0.01	0.02

[a] Treatment with 6-hydroxydopamine was accomplished as described under *Methods*. [b] The results are mean ± standard error of margin of six independent observations. [c] Control refers to the values obtained at corresponding times in the iproniazid-treated animals.

^{14}C-norepinephrine, and the amount of ^{14}C-norepinephrine accumulated and retained in hearts at 15 min. and 1, 2, and 4 hr. was determined. The results obtained were plotted semilogarithmically against time in Fig. 1. It may be seen that at 15 min. both isomers were taken up almost to the same extent but that the $(+)$-^{14}C-norepinephrine loss was faster than the $(-)$-isomer loss. First-order rate constants and half-lives for the disappearance of the isomers were calculated (Table I). Both isomers showed a single exponential loss.

Effect of Iproniazid Treatment—$(-)$-Norepinephrine is a better substrate for MAO than is $(+)$-norepinephrine (16). Hence, it was necessary to compare accumulation after enzyme inhibition. Mice were pretreated with iproniazid before infusion of ^{14}C-norepinephrine as described in the *Methods* section. Five minutes after infusion, it was found that both isomers accumulated to the same extent (data are not illustrated). The accumulation of both isomers at 15 min. was higher than at 5 min. postinfusion. After 15 min., the concentration of both isomers began to decline. Thus, the maximum accumulation occurred between 5 and 15 min. The concentration of $(+)$-^{14}C-norepinephrine declined faster than that of $(-)$-^{14}C-norepinephrine (Fig. 2). Previously, biphasic disappearance of radioactivity was observed after intravenous infusion of $(\pm)$-^{3}H-norepinephrine. It is possible that the disappearance of $(\pm)$-^{3}H-norepinephrine in two phases may be partly due to the decline of $(+)$- and $(-)$-^{3}H-norepinephrine isomers with separate rate constants.

To investigate the disappearance of ^{14}C-norepinephrine, iproniazid-treated animals were infused with racemic ^{14}C-norepinephrine prepared by mixing equal amounts of $(-)$- and $(+)$-^{14}C-norepinephrines in the dose and infusion rate identical to those used for the pure isomers. The animals were sacrificed at different times from 15 min. to 12 hr. (Fig. 2 and Table I). Clearly, the racemic ^{14}C-norepinephrine disappeared biexponentially. After feathering the data, the first rate constant was closer to that of $(+)$-^{14}C-norepinephrine and the second to that of $(-)$-^{14}C-norepinephrine. It appears that exogenously administered norepinephrine is stored in a single kinetic pool from which it disappears as a first-order process; or if it is stored in multiple pools, they are in rapid equilibrium with each other. For subsequent experiments involving the effects of certain drugs on the accumulation and retention of ^{14}C-norepinephrine isomers, iproniazid-pretreated animals were used.

Disappearance of ^{14}C-Norepinephrine Isomers after Guanethidine or Reserpine Treatment—Both reserpine and guanethidine are known to deplete endogenous norepinephrine from tissues. Reserpine is known to produce this action by blocking uptake by vesicular storage mechanisms (17), and guanethidine is utilized to inhibit the same mechanism (18, 19). Hence, the effects of both these drugs should be similar as far as the accumulation and retention of ^{14}C-norepinephrine are concerned. The disappearance curves obtained are illustrated in Fig. 3, and the first kinetic analysis is given in Table I. The results show that guanethidine had very little influence on the disappearance of the isomers. Reserpine, on the other hand, appeared to cause marked inhibition of uptake and storage of both isomers. Both $(-)$- and $(+)$-^{14}C-norepinephrines disappeared at a much faster rate. Reserpine also abolished the stereoselectivity of efflux as observed in the control experiments. Within 4 hr. postinfusion, there was almost the complete disappearance of isomers in reserpine-treated tissues, whereas detectable amounts of ^{14}C-norepinephrine could be found even after 24 hr. in guanethidine-treated tissues.

Table III—*O*-Methylated Metabolites Present in Mouse Hearts[a] 10 min. after Intravenous Injection of $(-)$- and $(+)$-^{14}C-Norepinephrines

Isomer	^{14}C-*O*-Methylated Metabolites (c.p.m./g. heart)	Significance
$(-)$-Norepinephrine	1500[b] ± 221	$p < 0.01$
$(+)$-Norepinephrine	2672 ± 165[c]	

[a] Total dose for each isomer was 40 mcg./kg. Five hearts were pooled together. [b] Mean of nine observations ± standard error to margin. [c] Mean of eight observations ± standard error of margin.

Effect of α-Methyl-*p*-tyrosine—α-Methyl-*p*-tyrosine was given to inhibit tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of norepinephrine. By inhibiting this enzyme, endogenous norepinephrine can be significantly depleted. Accumulation and disappearance of exogenously administered ^{14}C-norepinephrine could thus be studied more clearly without being diluted or exchanged with endogenous norepinephrine. In animals pretreated with iproniazid and α-methyl-*p*-tyrosine, the accumulation and disappearance of ^{14}C-norepinephrine were observed in the interval from 15 min. to 4 hr. (Fig. 1 and Table I). There was a higher accumulation of $(-)$-^{14}C-norepinephrine as compared to $(+)$-^{14}C-norepinephrine. The disappearance rates were not significantly different from those observed in control animals.

Effect of 6-Hydroxydopamine Treatment—6-Hydroxydopamine has been shown to cause severe damage to, and specific degeneration of, the adrenergic nerves (20). In mice treated with this drug, the amount of ^{14}C-norepinephrine was determined at 15 min. and 4 hr. after infusion of each isomer of ^{14}C-norepinephrine (Table II). At 15 min., the concentration of ^{14}C-norepinephrine for either isomer was 4–6% of the control; at 4 hr., it was barely above the background.

Slight accumulation of radioactivity at 15 min. postinfusion probably represents the accumulation of ^{14}C-norepinephrine by extraneuronal uptake of *O*-methylated metabolites and, to some extent, accumulation of norepinephrine by the surviving adrenergic nerves. Thus, the accumulation of ^{14}C-norepinephrine by "uptake$_2$" (3) and *O*-methylated metabolites in the present experiments was rather small and was observed only at shorter time intervals.

Metabolism of $(-)$- and $(+)$-^{14}C-Norepinephrines by Catechol-*O*-methyltransferase—In the iproniazid-treated animals, normetanephrine formed in hearts of mice after the administration of equal amounts of $(-)$- or $(+)$-^{14}C-norepinephrine was isolated by ion-exchange chromatography. As shown in Table III, significantly more *O*-methylated metabolites formed from $(+)$-^{14}C-norepinephrine than from $(-)$-^{14}C-norepinephrine. $(+)$-Norepinephrine may be a better substrate than $(-)$-norepinephrine for the enzyme or, alternatively, more $(+)$-isomer may be available to the enzyme due to low vasoconstrictor activity.

DISCUSSION

The infusion of isomers in mice indicates that the accumulation of ^{14}C-norepinephrine by the heart was higher after 15 min. than after 5 min. No selectivity was observed in the initial accumulation of the isomers. Even after blockade of vesicular accumulation by reserpine treatment, the initial uptake was the same for both isomers. If the initial accumulation is considered mainly neuronal (21–23), the results suggest that the transport of the isomers across the neuronal membrane of the mouse heart lacks stereoselectivity. The interpretation is consistent with *in vitro* findings that mouse heart shows little or no stereoselectivity (24).

Neuronal uptake (3) and efflux (25) are considered active processes. The similar rates of disappearance of the isomers from the reserpine-pretreated animals indicate that neuronal efflux from the mouse heart may also be nonstereoselective. However, it could be that reserpine treatment itself destroys the sodium-dependent and optically specific amine carrier mechanism at the adrenergic neuron (26).

In vivo, the selectivity in the vesicular retention is clearly demonstrated. The manipulation of the accumulation by pharmacological tools suggests that the retention of the isomers by the vesicles is truly stereoselective in favor of the (−)-isomer:

1. Reserpine, which is known to inhibit the uptake by the vesicles, abolishes the stereoselectivity.
2. Pretreatment of mice with α-methyl-*p*-tyrosine, which depletes the vesicular stores by inhibiting the enzyme tyrosine hydroxylase, favors the accumulation of the (−)-isomer only.
3. Chemical sympathectomy with 6-hydroxydopamine drastically reduces the accumulation.
4. Almost all of the (+)-isomer is lost from the heart after 24 hr., while a considerable amount of the (−)-form can be found in the tissue.

The results favor the view that the stereochemically "correct" orientation of the β-hydroxyl group is important for the ATP–magnesium-dependent process in the adrenergic nerve granules (2, 27).

An unexpected finding of the present study is that uptake and retention of isomers were not influenced by a relatively large dose of guanethidine (Fig. 3). These observations are similar to those reported by Krell and Patil (9), who could not show the inhibition of uptake of catecholamines in vas deferens obtained from guanethidine-pretreated animals. In line with these observations, Maitre and Staehelin (28) were unable to find inhibition of uptake of catecholamine by the isolated splenic nerve granules in the presence of guanethidine. It may be that guanethidine depletes endogenous norepinephrine without affecting the vesicular uptake. The uptake of isomers would then appear normal. In the present report, the levels of endogenous norepinephrine after guanethidine were not estimated. It may be that the drug was administered at a time when guanethidine was without a depleting effect. However, as low as 8 mg./kg. of guanethidine is known to deplete 80–90% cardiac norepinephrine for 10–15 hr. after intravenous injection in rats (29). Further work is necessary to investigate the mechanism of action of guanethidine and its influence on the accumulation of exogenous norepinephrine.

In many studies, a multiphasic disappearance of labeled norepinephrine from the heart was observed. Most earlier workers used ^{3}H-labeled racemic norepinephrine as a valid tracer of the natural neurotransmitter. Since (±)-^{3}H-norepinephrine is an equal mixture of two compounds, it could be that these isomers are released from the storage sites at different rates and thus manifest the multiphasic disappearance. In this study, a biphasic decline was observed when (±)-^{14}C-norepinephrine was used. After administering pure (−)- or (+)-isomers, in control or drug-treated mice, the radioactivity disappeared in a single exponential phase (Figs. 1–3). (+)-Norepinephrine disappeared much faster than the (−)-isomer. Iversen *et al.* (23) also employed a pure ^{14}C-isomer of norepinephrine, yet they observed that norepinephrine disappeared in two phases for each isomer. Since the experimental conditions in the present study are different from those reported by Iversen *et al.*, the data cannot be critically compared.

Data on the metabolism of (−)- and (+)-norepinephrines by catechol-*O*-methyltransferase are particularly interesting. From Table III, it may be seen that there are more *O*-methylated metabolites formed from (+)-norepinephrine than from equal doses of (−)-norepinephrine. In earlier studies, this enzyme was reported to possess no stereoselectivity for either isomer of norepinephrine [review by Patil *et al.* (7)]. Iversen *et al.* (23) also reported that catechol-*O*-methyltransferase lacked any stereospecificity. Their data, similar to ours, indicated that after equal doses of (−)- or (+)-^{14}C-norepinephrine there were twice as many *O*-methylated metabolites from (+)-norepinephrine as compared to those from (−)-^{14}C-norepinephrine. Furthermore, detailed kinetic analysis of the influence of the enzyme on optical isomers is necessary for the correct interpretation of the results. As far as the false neurotransmitter status of the (+)-norepinephrine is concerned, rapid *O*-methylation and rapid loss from tissue of the substance may be undesirable properties.

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