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A TRITIUM-RELEASE *IN VIVO* ASSAY OF DOPAMINE-β-HYDROXYLASE ACTIVITY IN SYMPATHETIC NEURONS

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Abstract—Measurement of the rate of release *in vivo* of tritium from [β-^{3}H]dopamine is proposed as an index of dopamine-β-hydroxylase activity in sympathetic neurons. After the systemic administration of this labeled compound to rats, the accumulation of tritium in body water is roughly linear with time and is partially inhibited by pretreatment of the animals with the dopamine-β-hydroxylase inhibitors, disulfiram and fusaric acid. Pretreatment of the animals with the monoamine oxidase inhibitor, tranylcypromine, alters neither the rate of tritiated water accumulation nor the extent to which this rate is slowed by the dopamine-β-hydroxylase inhibitors. The tritium-release assay has been tested in animals subjected to experimental protocols known to alter the levels of dopamine-β-hydroxylase in sympathetic neurons. Chemical sympathectomy with 6-hydroxydopamine, which leads to a decrease in dopamine-β-hydroxylase activity in tissue extracts, decreased the rate of tritiated water release *in vivo*. Chronic exposure of animals to cold, which leads to an increase in dopamine-β-hydroxylase activity in tissue extracts, increased the rate of tritiated water release *in vivo*. Furthermore, this effect of cold stress is prevented by dopamine-β-hydroxylase inhibitors. Our data thus suggest that accumulation of tritiated water in the whole animal after the administration of [β-^{3}H]dopamine can be used as an index of dopamine-β-hydroxylase activity in sympathetic neurons.

Dopamine-β-hydroxylase [EC1. 14.17.1; 3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating) (DβH)] catalyzes the final step in the biosynthesis of norepinephrine according to equation 1.

$$\text{Dopamine} + O_2 + \text{ascorbate} \rightarrow \text{L-norepinephrine} + \text{dehydroascorbate} + H_2O \quad (1)$$

The bovine adrenal enzyme has been purified to homogeneity and found to be a copper-containing glycoprotein [1–3]. The enzyme acts on a wide variety of ring substituted phenylethylamines, both *in vitro* [4] and *in vivo* [5]. After the administration of [^{3}H]tyramine to rats, it has been possible to isolate its β-hydroxylated derivative, [^{3}H]octopamine, from urine [5] and tissues [6]. Similarly, after the administration of [^{14}C]dopamine to rats [^{14}C]norepinephrine has been isolated from extracts of spleen and heart [7]. These administered phenylethylamines are taken up into dense core vesicles in sympathetic nerve terminals where the enzyme-catalyzed β-hydroxylation takes place. A number of drugs are known to block this conversion *in vivo*, including disulfiram [6, 7], which is reduced to the copper-chelating agent diethyldithiocarbamate, and fusaric acid, the mechanism of action of which is unknown [8].

There is little information in the literature on the overall rate at which dopamine is β-hydroxylated immediately after its administration to the whole animal. Since only a small fraction of systemically administered dopamine gains access to those peripheral tissues (sympathetic neurons and adrenal medulla) which contain dopamine-β-hydroxylase, a very low rate of conversion would be anticipated. Studies of [^{14}C]dopamine metabolism in man have shown that, even over 24-hr periods, only 10–15 per cent of the administered isotope can be accounted for as excreted metabolites of the β-hydroxylated product, norepinephrine [9, 10]. Studies *in vivo* of dopamine-β-hydroxylation are complicated since, in addition to the free amines, a variety of free and conjugated dopamine and norepinephrine metabolites are formed and eventually excreted [11]. All previous investigations of dopamine-β-hydroxylation in the whole animal have accordingly necessitated the separation of the small pool of norepinephrine metabolites from the large pool of dopamine metabolites. To avoid this difficulty, we have devised a tritium-release assay by which dopamine-β-hydroxylase activity can be directly measured *in vivo*. The assay is based upon the principle that, when [^{3}H]-dopamine which is specifically labeled in the β-position is used as the substrate for the hydroxylase, a molecule of tritiated water (THO) is released for each molecule of [^{3}H]norepinephrine formed [12]. The former product of the enzymatic reaction, unlike norepinephrine, does not undergo subsequent metabolism. Thus, when β-labeled [^{3}H]dopamine is administered to the whole animal, the rate of accumulation of THO in blood is an index of the rate of [^{3}H]norepinephrine formation. This principle has been used previously to measure dopamine-β hydroxylase activity *in vitro* [13–15]. An *in vivo* assay for another hydroxylase, phenylalanine hydroxylase, based on release of deuterium or tritium from phenylalanine has previously been developed [16].

MATERIALS AND METHODS

All rats used weighed between 100 and 175 g and were of the Sprague–Dawley strain. They were allowed free access to food and water up until the time of experimentation. Dopamine with a tritium label in the β-position on the side chain (sp. act. 6.7 Ci/m-mole) was obtained from New England Nuclear (NET-131), Boston, MA. L-Norepinephrine [7-^{3}H] (sp. act. 6.4 Ci/m-mole, NET-377) and 3-methoxy-4-hydroxyphenylethylamine [5-^{3}H] (250 Ci/m-mole, NET-321) were also obtained from New England Nuclear. Disulfiram (tetraethylthiuram disulfide, antabuse), diethyldithiocarbamic acid (sodium salt), fusaric acid (5-butyl picolinic acid), tranylcypromine (*trans*-2-phenylcyclopropylamine), and iproniazid phosphate (isonicotinic acid 2-isopropylhydrazide phosphate) were obtained from Sigma Chemical Co., St. Louis, MO. 6-Hydroxydopamine hydrobromide was purchased from Regis. Calcium cyanamid was obtained from the American Cyanamid Co., Wayne, NJ.

Dopamine-β-hydroxylase assay in vivo. Prior to the administration of the [β-^{3}H]dopamine, any THO contaminating the sample was first removed by flash evaporation; the resulting solid was then taken up in physiological saline containing 0.1 % sodium ascorbate. The [β-^{3}H]dopamine was kept on ice until it was used. To perform the assay of dopamine-β-hydroxylase *in vivo*, rats were injected intraperitoneally* with 100 μCi/kg of the isotope. After 10 min (or other stated time intervals), the animals were sacrificed by decapitation and the blood was collected from the cervial fracture into heparinized beakers. The whole blood was then lyophilized as described by Stansell and Mojica [17]. Tritiated water was determined by counting an aliquot of the collected water in a liquid scintillation counter (Beckman LS-250).

In order to calculate the THO formation in the whole animal, we assumed that the concentration of THO in blood was equal to its concentration in total body water, which was assumed to be equal to 70 per cent of the animal's weight. We corrected the measured total body THO by substrating the small amount of THO which we injected. This THO presumably resulted from the spontaneous loss of THO from the dopamine which took place in the time interval between the evaporation of the sample and its actual injection. Generally, only 0.15 to 0.20 per cent of the injected radioactivity was preformed THO. The percentage of the [β-^{3}H]dopamine which was converted to THO can thus be calculated with the following equation:

$$\text{Rate of THO release}\left(\frac{\%\text{ conversion}}{10\text{ min}}\right) = \frac{(\text{Wt of animal})(0.7)(\text{THO/ml blood}) - \text{injected THO}}{\text{total administered cpm}} \quad (2)$$

Two additional factors must be considered in attempting to relate the amount of THO released to the amount of norepinephrine formed. The first is the fact that the administered [β-^{3}H]dopamine is a mixture of two enantiomeric substrates, only one of which releases tritium during its conversion to norepinephrine [18, 19]. This stereochemical effect is due to the fact that the enzymatic reaction takes place with the net retention of the configuration at the hydroxylated center [18, 19]. Hydroxylation of (R)-[β-^{3}H]dopamine leads to a loss of tritium, whereas hydroxylation of (S)-[β-^{3}H]dopamine does not. This means that when dopamine-β-hydroxylase is assayed *in vivo* by the tritium-release method, the amount of tritium released is only half of the amount of norepinephrine formed. We corrected for this factor by multiplying the rate of THO release in untreated animals by a factor of two.

The second correction that must be made in relating THO release to norepinephrine formation is that due to the release of THO from reactions unrelated to dopamine-β-hydroxylation. In order to make this correction, we have determined the amount of THO released during a 10-min period in animals pretreated with an inhibitor of dopamine-β-hydroxylase, such as disulfiram. We have assumed that the rate of [^{3}H]norepinephrine formation in disulfiram-treated animals is negligible. We have also assumed that those sources of THO in the whole animal other than that due to dopamine-β-hydroxylation are not affected by the isotopic asymmetry of the tritium label. Using these assumptions, we have calculated the rate of norepinephrine formation from the relationship shown in equation 3.

$$\text{Rate of norepinephrine formation} = 2 \times (\text{rate of THO released in untreated animals} - \text{rate of THO released in disulfiram-treated animals}) \quad (3)$$

Determination of labeled catecholamines and catecholamine metabolites. Tissues were homogenized with a Duall glass homogenizer in ice-cold 0.4 N perchloric acid, and the homogenates were centrifuged for 15 min at 24,000 *g*. [^{3}H]norepinephrine was separated from [^{3}H]dopamine on Dowex 50W-X4 (hydrogen form, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA) columns eluted with increasing concentrations of HCl according to the method of Laverty and Taylor [20]. The [^{3}H]catecholamines were then concentrated and further purified by passage of the Dowex eluates over alumina at pH 8.6 [21]. The [β-^{3}H]dopamine and [^{3}H]norepinephrine were determined by counting aliquots of the final alumina eluates by liquid scintillation spectrophotometry (Beckman LS-250). Data were corrected for the recovery of added standards (50 per cent) through the two column chromatographic procedures. Some of the alumina effluents were adjusted to pH 10 with sodium borate buffer, 0.25 M, and the [^{3}H]normetanephrine was extracted by mechanical shaking for 5 min into an equal volume of a toluene–isoamyl alcohol mixture (3 : 2) [22]. After centrifugation, aliquots of the organic phase were eva-

* We used intraperitoneal injection of [β-^{3}H]dopamine because it was difficult to perform properly timed intravenous injections on the large number of animals involved in these experiments. We have given a small number of animals [β-^{3}H]dopamine intravenously and found that the rate of accumulation of THO in blood does not differ significantly from that observed after intraperitoneal injections.

ported in a sand bath at 110° and radioactivity was determined. The measured [^{3}H]norepinephrine and [^{3}H]normetanephrine were multiplied by a factor of 2 to correct for stereospecificty (see dopamine-β-hydroxylase assay *in vivo*). Authentic norepinephrine was measured in the alumina eluates by the technique of von Euler and Lishajko [23].

Dopamine metabolites were extracted from rat liver and separated according to Spano and Neff [24]. (The Dowex columns were not employed for the liver extracts since the levels of [^{3}H]norepinephrine were assumed to be negligible.) The [^{3}H]dopamine in the 0.2 N acetic acid eluates from the alumina columns was counted directly. The [^{3}H]dihydroxyphenylacetic acid in the 1 N sulfuric acid eluate was further purified by extraction into anhydrous diethyl ether by mechanical shaking for 5 min. Aliquots of the organic phase were evaporated in a scintillation vial and radioactivity was determined. The deaminated, *O*-methylated metabolites in the alumina effluents were determined by extraction of the neutral compounds into ethyl acetate at pH 6.0 [25] and of the neutral plus acidic compounds into ethyl acetate at pH 1.0. The major neutral metabolite, [^{3}H]3-methoxy-4-hydroxyphenylethanol, could then be estimated directly by counting evaporated aliquots of the organic phase obtained with the pH 6.0 extraction. The major acidic metabolite, [^{3}H]3-methoxy-4-hydroxyphenylacetic acid ([^{3}H]homovanillic acid) was estimated by counting evaporated aliquots of the organic phase obtained with the pH 1.0 extraction, and then correcting for the small percentage of radioactivity representing neutral compounds.

The remaining portion of the alumina effluents was hydrolyzed at pH 1.0 under nitrogen [26]. The resulting hydrolysate was recycled through alumina, and conjugated [β-^{3}H]dopamine and conjugated [^{3}H]dihydroxyphenylacetic acid (now present as free compounds) were determined [27]. All data were corrected for the recovery of added standards.

Table 1. Comparison of THO release and [^{3}H]norepinephrine formation from [β-^{3}H]dopamine *in vitro* with purified dopamine-β-hydroxylase*

	μmoles
[^{3}H]dopamine reacted	0.34
[^{3}H]norepinephrine formed	0.20
THO formed	0.17

* Values have been corrected for any reaction that took place in the presence of boiled enzyme. For the dopamine and norepinephrine, this blank was equal to about 20 per cent of the value in the presence of native enzyme; for THO, it was equal to 2 per cent of the value with the native enzyme. The enzymatic reaction was performed for 90 min with bovine adrenal dopamine-β-hydroxylase purified through the calcium phosphate gel step [28]. The enzyme was added to a reaction mixture containing 1.0 mM dopamine (3,929,000 cpm); 100 mM potassium phosphate, pH 6.4; potassium ascorbate, 7.5 mM; potassium fumarate, 62.5 mM; tranylcypromine, 0.7 mM; and 1000 units of catalase (beef liver) in a volume of 1 cc.

RESULTS

Before using the radioactive dopamine for studies *in vivo*, we attempted to determine the position of the tritium in the molecule. To do this, we tested the compound as a substrate in an *in vitro* assay with purified dopamine-β-hydroxylase [28]. The formation of [^{3}H]norepinephrine was only slightly greater than the amount of THO released (Table 1), an indication that only a small fraction of the tritium could be present in the α-position of the side chain or in any other position in the molecule. The small difference may be due to an isotope effect.

After the administration of [β-^{3}H]dopamine to the whole animal, the appearance of THO in blood was roughly linear with time and partially inhibited by pretreatment of the rats with disulfiram (Fig. 1). Fusaric acid treatment also significantly decreased THO release in the whole animal (Table 2). Disulfiram pretreatment, however, had a less dramatic effect on THO release than on the accumulation of [^{3}H]norepinephrine in heart and spleen. As can be

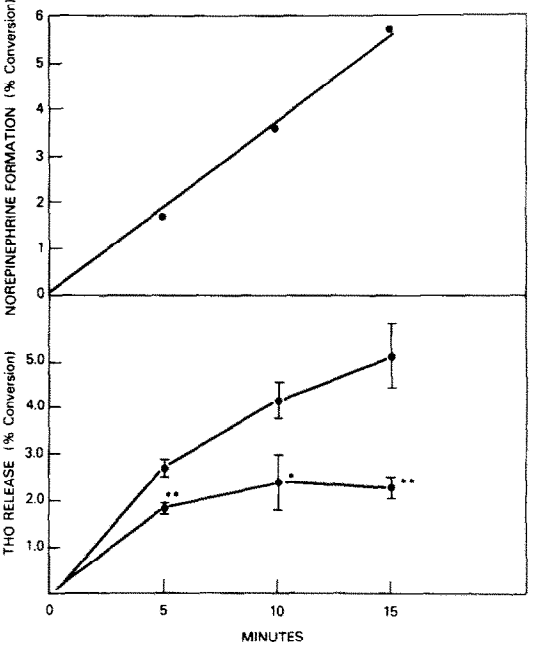

Fig. 1. Effect of disulfiram on the time course of the accumulation of ^3H_2O (THO) in blood after the administration of [β-^{3}H]dopamine. THO release is expressed as per cent of administered [β-^{3}H]dopamine released as THO ± S.E.M. The disulfiram-treated groups of animals differed significantly (a single asterisk, $P < 0.1$; a double asterisk, $P < 0.01$) from the control group when compared by the non-paired *t*-test. The amount of [^{3}H]norepinephrine formed as calculated according to the equation given in Materials and Methods and includes the correction for stereospecificity. Disulfiram was injected intraperitoneally (250 mg/kg) 2.5 hr prior to administration of [β-^{3}H]dopamine. The number of animals (N) used in the experiment was as follows: for the control group, at 5 min, N = 5; at 10 min, N = 3; and 15 min, N = 5. For the disulfiram group, at 5 min, N = 5; at 10 min, N = 4; and at 15 min, N = 6. The calculated values for norepinephrine formation with their standard errors are as follows: at 5 min, 1.76 ± 0.49; at 10 min, 3.44 ± 1.53; and at 15 min, 5.78 ± 1.49.

Table 2.—Effect of dopamine-β-hydroxylase inhibitors on THO release *in vivo**

	Rate of THO release (% of [β-^{3}H]-dopamine converted to THO/10 min)	Estimated rate of dopamine-β-hydroxylation (% dopamine converted to norepinephrine/10 min)
Control animals	3.68 ± 0.26	3.38 ± 0.72
Animals pretreated with fusaric acid	2.86 ± 0.29†	1.74 ± 0.76
Animals pretreated with disulfiram	1.99 ± 0.25‡	0

* Disulfiram was suspended in warm water containing 1% carboxymethyl-cellulose, sodium salt, and administered intraperitoneally (150 mg/kg) 2.5 hr prior to [β-^{3}H]dopamine. Fusaric acid, 80 mg/kg, was given intraperitoneally 1.25 hr prior to the radioactive dopamine. The control animals received an intraperitoneal injection of 1% carboxymethylcellulose sodium salt. The number of animals (N) in each group was as follows: controls, N = 12; fusaric acid-treated, N = 7; and disulfiram-treated, N = 7. Data represent mean ± S. E. M.

† The experimental groups were significantly different ($P < 0.005$) when compared to the control group by non-paired *t*-test.

‡ $P < 0.001$

Table 3. Effect of disulfiram pretreatment on THO release from [β-^{3}H]dopamine in the whole animal and [^{3}H]catecholamine accumulation in heart and spleen*

		Control animals	Disulfiram-pretreated animals	Significance level
THO release (% conversion)	Measured rate	4.35 ± 0.36	3.02 ± 0.19	$P < 0.01$
Total radioactivity (cpm/organ)	Spleen	195,100 ± 8,400	218,314 ± 25,100	
	Heart	261,000 ± 22,700	201,886 ± 7,483	$P < 0.05$
[^{3}H]dopamine (cpm/organ)	Spleen	19,145 ± 2,454	36,834 ± 7,260	$P < 0.1$
	Heart	12,917 ± 1,839	10,505 ± 1,580	
[^{3}H]norpinephrine (cpm/organ)	Spleen	21,996 ± 3,276	3,914 ± 1,480	$P < 0.001$
	Heart	14,460 ± 1,480	3,000 ± 556	$P < 0.001$
[^{3}H]normetanephrine (cpm/organ)	Spleen	697 ± 181	331 ± 96	$P < 0.1$
Authentic norepinephrine (μg/organ)	Spleen	0.65 ± 0.03	0.53 ± 0.04	$P < 0.05$
	Heart	0.66 ± 0.09	0.54 ± 0.03	

* All data represent mean ± S. E. M. Significance level was determined by comparing the disulfiram-pretreated animals with the control group by the non-paired *t*-test. Disulfiram was suspended in carboxymethylcellulose and injected intraperitoneally (300 mg/kg) 2 hr before the [β-^{3}H]dopamine. Control animals received an injection of an equal volume of carboxymethylcellulose. There were seven animals in the control group and six animals in the disulfiram-treated group.

Table 4. Effect of tranylcypromine on THO accumulation in blood, and [β-^{3}H]dopamine metabolites in rat liver extracts*

	Control	Tranylcypromine (2 mg/kg)	Tranylcypromine (8 mg/kg)
THO release (% conversion)	3.63 ± 0.38	3.96 ± 0.22	4.03 ± 0.05
[^{3}H]dopamine	4.34 ± 0.79	3.52 ± 0.27	2.27 ± 0.37†
[^{3}H]dihydroxyphenylacetic acid	3.13 ± 0.28	0.31 ± 0.04‡	0.19 ± 0.05‡
Conjugated [^{3}H]dopamine	20.0 ± 2.2	17.4 ± 2.7	21.4 ± 4.9
Conjugated [^{3}H]dihydroxyphenylacetic acid	1.73 ± 0.18	0.34 ± 0.07‡	0.28 ± 0.02‡
Neutral deaminated *O*-methylated metabolites (mainly [^{3}H]3-methoxy-4-hydroxyphenylethanol)	0.53 ± 0.07	0.18 ± 0.02†	0.12 ± 0.01§
Acidic deaminated *O*-methylated metabolites (mainly) [^{3}H]homovanillic acid)	3.66 ± 0.73	1.40 ± 0.23†	1.25 ± 0.33†

* Data for the catecholamine metabolites represent mean percentage of total radioactivity present in the liver ± S. E. M. The individual doses of tranylcypromaine were administered intraperitoneally 16 hr and 2 hr prior to the [β-^{3}H]dopamine. The catecholamine metabolites were separated as described in Materials and Methods. We assumed in this experiment that norepinephrine metabolites in liver were negligible. The number of animals (N) in each group was as follows: control, N = 6; tranylcypromine (2 mg/kg) N = 7; and tranylcypromine (8 mg/kg) N = 5.

† The experimental group was significantly different from the control group ($P < 0.05$) when compared by the non-paired *t*-test.

‡ $P < 0.001$.

§ $P < 0.005$.

Table 5. Tranylcypromine pretreatment and the effect of dopamine-β-hydroxylase inhibitors on THO release *in vivo**

	Rate of THO release (% of [β-^{3}H]dopamine converted to THO/10 min)
Control animals	4.36 ± 0.25
Animals pretreated with tranylcypromine	4.05 ± 0.26
Animals pretreated with tranylcypromine and fusaric acid	2.41 ± 0.17†
Animals pretreated with tranylcypromine and disulfiram	2.17 ± 0.14†

* Data represent mean per cent ± S. E. M. Tranylcypromine (2 mg/kg) was dissolved in H_2O and administered 16 hr and 2 hr prior to the dopamine-β-hydroxylase assay *in vivo*. Disulfiram (300 mg/kg) was suspended in warm 1% carboxymethylcellulose and injected intraperitoneally 2.5 hr before the [β-^{3}H]dopamine. Fusaric acid (80 mg/kg) was administered intraperitoneally 1.25 hr before the isotope. The number of animals (N) in each group was as follows: controls, N = 6; tranylcypromine, N = 7; tranylcypromine + fusaric acid, N = 8; and tranylcypromine + disulfiram, N = 6.

† Experimental groups were statistically different ($P < 0.001$) when compared to the control group by the nonpaired *t*-test.

seen in Table 3, THO release, without correction for stereospecificity of tritium release during the hydroxylation reaction, was inhibited 30 per cent, whereas [^{3}H]norepinephrine formation in spleen and heart was inhibited about 80 per cent. These results suggest that some other pathway of [β-^{3}H]dopamine metabolism, such as monoamine oxidation, contributed to the formation of THO. Treatment of animals with a monoamine oxidase inhibitor, tranylcypromine [29], though effectively decreasing the conversion of [β-^{3}H]dopamine to its deaminated metabolites (Table 4), failed to alter either net THO release in the whole animal or the degree of inhibition caused by inhibitors of dopamine-β-hydroxylase (Table 5). In independent experiments, we obtained further evidence for the *in vivo* effectiveness of tranylcypromine by demonstrating that treatment with this drug (2 mg/kg 16 hr and 2 hr prior to sacrifice) caused a greater than 90 per cent inhibition of monoamine oxidase activity measured in extracts of kidney and liver with either [β-^{3}H]dopamine or [^{3}H]3-methoxy-4-hydroxyphenylethylamine as substrates [30]. Treatment with another monoamine oxidase inhibitor, iproniazid [31] (100 mg/kg 18 hr prior to sacrifice), also failed to alter THO release *in vivo*.

Although our studies with various inhibitors have not identified the reaction that leads to the disulfiram-insensitive release of tritium, it should be noted that the extent of inhibition of THO release by disulfiram treatment appears to increase with time, i.e. after 5 min there is little, if any, additional release of THO in the disulfiram-treated rats (see Fig. 1).

We tested the tritium-release *in vivo* assay of dopamine-β-hydroxylase with physiological and pharmacological manipulations known to alter the activity of the sympathetic nervous system. When administered intravenously [32] or intraperitoneally [33], 6-hydroxydopamine is known to destroy large populations of sympathetic neurons and to lead to an irreversible chemical sympathectomy. Treatment with 6-hydroxydopamine was found to lead to a slight (though highly significant) decrease in THO release *in vivo* which is equal to a 40 per cent inhibition of norepinephrine formation (Table 6). We also tested the effect of cold exposure with our assay, since this form of stress is known to stimulate catecholamine synthesis [34], and to lead to increased levels of dopamine-β-hydroxylase in sympathetic neurons [35]. Cold stress led to an increase in THO release in the whole animal and the effect could be inhibited by both disulfiram and fusaric acid (Table 7).

DISCUSSION

A tritium-release *in vivo* assay for dopamine-β-hydroxylase is possible only if the isotope employed is labeled specifically in the β-position. The [^{3}H]-dopamine used in these experiments was prepared by catalytic reduction of arterenone hydrochloride, a

Table 6. Effects of 6-hydroxydopamine pretreatment on THO release from [β-^{3}H]dopamine*

	Rate of THO release	Rate of norepinephine formation
Control animals	4.38 ± 0.12	3.96 ± 0.65
6-Hydroxydopamine-treated animals	3.56 ± 0.13†	2.32 ± 0.65
Disulfiram-treated animals	2.40 ± 0.30†	0

* All data represent mean per cent conversion ± S. E. M. Rates are expressed as per cent conversion of [β-^{3}H]-dopamine to product in 10 min. Disulfiram was administered intraperitoneally (200 mg/kg) as a warm aqueous suspension of carboxymethylcellulose. 6-Hydroxydopamine hydrobromide was dissolved in 1% sodium ascorbate, pH 6.0, and 100 mg/kg (free base) was administered intraperitoneally 96 hr and 72 hr prior to the dopamine-β-hydroxylase assay *in vivo*. The amount of isotope administered to the 6-hydroxydopamine-treated animals was adjusted for their 15 per cent decrease in body weight. The number of animals (N) in each group was as follows: control, N = 7; 6-hydroxydopamine, N = 7; and disulfiram, N = 4.

† Experimental groups were statistically different from the control group ($P < 0.001$) when compared by the nonpaired *t*-test.

Table 7. Effect of cold stress on rate of THO release from [β-^{3}H]dopamine*

	Rate of THO release	Rate of norephrine formation
Experiment 1		
Control animals (6)	3.73 ± 0.27	3.36 ± 0.24
Animals subjected to cold stress (7)	4.61 ± 0.38†	6.08 ± 1.09
Animals, subjected to cold stress, pretreated with disulfiram (4)	1.57 ± 0.39‡	
Experiment 2		
Control animals (9)	4.25 ± 0.08	3.82 ± 0.07
Animals subjected to cold stress (8)	5.30 ± 0.10§	5.60 ± 0.73
Animals, subjected to cold stress, pretreated with fusaric acid (6)	2.50 ± 0.35§	

* All data represent mean per cent conversion ± S. E. M. Rates are expressed as per cent conversion of [β-^{3}H]-dopamine to product in 10 min. The cold stressed animals were placed in the cold room at 0–4° for 72 hr, and their fur wetted gently with H_2O twice daily. The amount of isotope given these animals (100 μCi/kg) was corrected for the 10 per cent decrease in body weight associated with this stress. Disulfiram was administered intraperitoneally (300 mg/kg to some of the cold stressed animals 3 hr prior to the dopamine-β-hydroxylase assay *in vivo*. The number of animals is given in parentheses. Fusaric acid was administered intraperitoneally (80 mg/kg) to some of the cold stressed animals 1.25 hr prior to the dopamine-β-hydroxylase assay *in vivo*. The rate of norepinephrine formation was calculated from equation 3 on the assumption that in this experiment, as in most of the others (see Fig. 1 and Table 2), disulfiram inhibits the rate of THO release in control animals by about 44 per cent. In experiment 2, we have also assumed that the rate of [^{3}H]norephinephrine formation in the fusaric acid-treated animals is negligible.

† The experimental group was statistically different from the control group ($P < 0.05$) when compared by the non-paired t-test (one-tailed).

‡ The experimental group was statistically different from each of the other groups ($P < 0.01$).

§ Each group was significantly different from each other group ($P < 0.001$).

procedure that is known to concentrate a large fraction (greater than 90 per cent) of the tritium on the β-positions of the side chain, although some labeling of the α-position is possible.* The results of our experiment with purified bovine adrenal dopamine-β-hydroxylase (Table 1) suggest that most, if not all, of the tritium is in the β-position. The small discrepancy between [^{3}H]norepinephrine formation (20 per cent of the starting isotope was converted to [^{3}H]-norepinephrine) and ^3H_2O formation (17 per cent of the isotope was converted to THO) may reflect labeling of the α-position. Even if this discrepancy does represent α-labeling, the data suggest that no more than 15 per cent of the [^{3}H]dopamine molecules have their tritium in this position. The failure of monoamine oxidase inhibitors to affect THO release in the whole animal also suggests that α-labeling of the [^{3}H]dopamine is slight. Moreover, the overall pattern of the data obtained in our experiments *in vivo* provides further evidence that most of the tritium is present in the β-position, and that an appreciable fraction of the THO which accumulates in blood after [^{3}H]dopamine administration reflects dopamine-β-hydroxylation *in vivo*. THO release in the whole animal is decreased by known inhibitors of dopamine-β-hydroxylase, decreased by chemical sympathectomy, and increased by chronic exposure of the animals to cold temperature. Since monoamine oxidation is quantitatively a much more important pathway of dopamine metabolism than is β-hydroxylation [9, 10], a significant amount of label in the α-position (which would be lost during the monoamine oxidation reaction) would, in all probability, make it impossible to detect these physiological alterations *in vivo* in the rate of dopamine-β-hydroxylation.

Although our data indicate that a large fraction (approximately 30–50 per cent) of the THO which accumulates in blood after [β-^{3}H]dopamine administration reflects release associated with β-hydroxylation, a high rate of THO release persists in animals after their treatment with dopamine-β-hydroxylase inhibitors. The reaction(s) responsible for this high blank rate has not been identified. It is unlikely that more than a small fraction of this THO release reflects persistent, uninhibited dopamine-β-hydroxylation since, as previously observed [6, 7], we find that disulfiram causes an 80–85 per cent inhibition of the conversion of [β-^{3}H]dopamine to [^{3}H]norepinephrine in heart and spleen (Table 3). It is possible that disulfiram is a less effective inhibitor of dopamine-β-hydroxylation in other organs, and that this is the source of the persistent THO release observed in animals treated with this drug. We are unaware, however, of evidence in the literature for a disulfiram-resistant population of dopamine-β-hydroxylase molecules. It is also possible that the disulfiram-resistant THO release results from a dopamine monoamine oxidase pathway which is distinct from the one we studied in rat liver (Table 4) and is unaffected by tranylcypromine. It has been reported that purified plasma amine oxidase releases THO from the [β-^{3}H]dopamine that we have employed in these studies [36]. We have found, however, that administration of iproniazid, an inhibitor of pyridoxal-containing amine oxidases [31], did not alter the rate of THO release in the whole animal. We have also explored the possibility that tritium release was associated with some further step along the monoamine oxidation pathway, such as the dehydrogenation of the aldehyde intermediate. Treatment

* Dr. Richard Young, New England Nuclear Corp., personal communication.

with the aldehyde dehydrogenase inhibitor, calcium cyanamid [37], however, led to an increase rather than a decrease in THO release (data not shown). Since disulfiram is an aldehyde dehydrogenase inhibitor [38], in addition to a dopamine-β-hydroxylase inhibitor, it is possible that this drug traps the unstable aldehyde intermediate in the monoamine oxidase pathway and thereby promotes the release of tritium. Such an effect of disulfiram would clearly obscure its inhibition of dopamine-β-hydroxylase in our tritium release assay. It is unlikely that this effect is important, however, since pretreatment with tranylcypromine, which prevents the formation of the aldehyde, did not increase the percentage inhibition of THO release achieved with disculfiram (Table 5). Whatever the source of the THO in the disulfiram-treated animals, the reaction that is responsible for it appears to be specific for [β-^{3}H]-dopamine. After the administration of β-labeled [^{3}H]norepinephrine of comparable specific activity (6.4 Ci/m-mole), the rate of accumulation of THO in blood was only 10 per cent of that seen after the administration of [β-^{3}H]dopamine.

The effects of 6-hydroxydopamine treatment on the release *in vivo* of THO are in reasonable agreement with the results expected from studies of the effect of this drug on enzyme levels in sympathetic neurons. Chemical sympathectomy causes a 50 per cent decrease in dopamine-β-hydroxylase levels in the stellate ganglia [39], a decrease that is consistent with our finding that chemical sympathectomy causes about a 41 per cent decrease in the calculated rate of norepinephrine formation *in vivo* (Table 6). It is possible that the somewhat smaller effect on the hydroxylation rate was observed because certain populations of sympathetic neurons, such as those in the mesenteric vasculature [40], though immune to the effects of 6-hydroxydopamine, are quantitatively important sites of dopamine-β-hydroxylation and THO release. On the other hand, the effect of cold exposure on rate of THO release *in vivo* may even exceed that expected from studies of dopamine-β-hydroxylase levels in animals subjected to this form of stress. Chronic cold exposure causes only a 20–25 per cent increase in dopamine-β-hydroxylase levels in the superior cervical ganglia [35], an increase that must be compared to the 50–80 per cent increase in the rate of norepinephrine formation that can be calculated from the results of the *in vivo* assay (Table 7).

The sensitivity of the *in vivo* assay, as described, is limited by the relatively high blank reaction, i.e. the disulfiram-insensitive liberation of THO. It is possible that the contribution of this blank rate can be minimized by selection of a later time period for the measurement of the amount of THO release. Thus, as has been noted earlier, the results shown in Fig. 1 indicate that a larger fraction of the THO release *in vivo* is sensitive to the dopamine-β-hydroxylase inhibitor, disulfiram, at later rather than earlier times. Indeed, in the 5- to 15-min period, most of the THO released appears to be inhibited by disulfiram. This consideration is especially pertinent to the clinical application of the method. If our *in vivo* assay were to be used in humans to measure the activity of dopamine-β-hydroxylase in various disease states, it seems likely that the contribution of the blank reaction would not be excessive if the THO in blood (or any other body fluid) were measured at several time periods after administration of the tritiated dopamine. Rates of conversion would then be calculated only from the later time periods.

REFERENCES

1. E. Y. Levin, B. Levenberg and S. Kaufman, *J. biol. Chem.* **235,** 2080 (1960).
2. S. Friedman and S. Kaufman, *J. biol. Chem.* **240,** 4763 (1965).
3. E. F. Wallace, M. J. Krantz and W. Lovenberg, *Proc. natn. Acad. Sci. U.S.A.* **70,** 2253 (1973).
4. S. Kaufman and S. Friedman, *Pharmac. Rev.* **17,** 71 (1965).
5. C. R. Creveling, M. Levitt and S. Udenfriend, *Life Sci.* **10,** 523 (1962).
6. J. Musacchio, I. J. Kopin and S. Snyder, *Life Sci.* **3,** 769 (1964).
7. M. Goldstein, E. Lauber and M. R. McKereghan, *Biochem. Pharmac.* **13,** 1103 (1964).
8. T. Nagatsu, H. Hidaka, H. Kuzuya and K. Takeya, *Biochem. Pharmac.* **19,** 35 (1970).
9. McC. Goodall, S. E. Gitlow and H. Alton, *J. clin. Invest.* **50,** 2734 (1971).
10. McC. Goodall and H. Alton, *Biochem. Pharmac.* **17,** 905 (1968).
11. J. Axelrod, *Pharmac. Rev.* **18,** 95 (1966).
12. S. Senoh, B. Witkop, C. R. Creveling and S. Udenfriend, *J. Am chem. Soc.* **81,** 1768 (1959).
13. M. Goldstein, N. Prochoroff and J. Sirlin, *Experientia* **21,** 592 (1965).
14. S. Kaufman, W. F. Bridgers and J. Baron, in *Advances in Chemistry Series* (Eds W. F. Bridgers and J. Baron), Number 77, p. 172. American Chemical Society, Washington, DC (1968).
15. G. Wilcox and M. A. Beaven, *Analyt. Biochem.* **75,** 484 (1976).
16. S. Milstein and S. Kaufman, *J. biol. Chem.* **250,** 4782 (1975).
17. M. J. Stansell and L. Mojica, *Clin. Chem.* **14,** 1112 (1968).
18. L. Bachan, C. B. Storm, J. W. Wheeler and S. Kaufman, *J. Am. chem. Soc.* **96,** 21 (1974).
19. K. B. Taylor, *J. biol. Chem.* **249,** 454 (1974).
20. R. Laverty and K. M. Taylor, *Analyt. Biochem.* **22,** 269 (1968).
21. A. H. Anton and D. F. Sayre, *J. Pharmac. exp. Ther.* **145,** 326 (1964).
22. J. Axelrod, *Meth. Enzym.* **5,** 748 (1962).
23. U. S. von Euler and F. Lishajko, *Acta physiol. scand.* **45,** 122 (1959).
24. P. F. Spano and N. H. Neff, *Analyt. Biochem.* **42,** 113 (1971).
25. C. Braestrap, M. Nielsen and J. Scheel-Kruger, *J. Neurochem.* **23,** 569 (1974).
26. R. Hoeldtke and J. W. Sloan, *J. Lab. clin. Med.* **75,** 159 (1970).
27. U. S. von Euler, I. Floding and F. Lishajko, *Acta Soc. Med. upsal.* **64,** 217 (1959).
28. S. Kaufman, *Meth. Enzym.* **17,** 754 (1971).
29. C. G. S. Collins, M. B. H. Youdim and M. Sandler *Biochem. Pharmac.* **21,** 1995 (1972).
30. D. S. Robinson, W. Lovenberg, H. Keiser and A. Sjoerdsma, *Biochem. Pharmac.* **17,** 109 (1968).
31. P. A. Shore and V. H. Cohn, *Biochem. Pharmac.* **5,** 91 (1960).

32. H. Thoenen and J. P. Tranzer, *Naunyn-Schmiedberg's Arch. Pharmak.* **261**, 271 (1968).
33. L. D. Lytle, O. Hurko, J. A. Romero, K. Cohman, D. Leehey and R. J. Wurtman, *J. Neural Transm.* **33**, 63 (1972).
34. J. Leduc, *Acta physiol. scand.* **53** (suppl. 183), 1 (1961).
35. H. Thoenen, R. Ketter, W. Burkard and A. Saner, *Naunyn-Schmiedeberg Arch. Pharmak.* **270**, 146 (1971).
36. W. Lovenberg and M. A. Beaven, *Biochim. biophys. Acta* **251**, 452 (1971).
37. M. J. Walsh, E. B. Truitt and V. E. Davis, *Molec. Pharmac.* **6**, 416 (1970).
38. R. A. Deitrich and V. G. Erwin, *Molec. Pharmac.* **7**, 301 (1971).
39. S. Brimijoin and P. B. Molinoff, *J. Pharmac. exp. Ther.* **178**, 417 (1971).
40. B. A. Berkowitz, S. Spector and J. H. Tarver, *Br. J. Pharmac.* **44**, 10 (1972).