Brit. J. Pharmacol. (1965), **24**, 477–484.

β-HYDROXYLATED SYMPATHOMIMETIC AMINES AS FALSE NEUROTRANSMITTERS

BY

J. E. FISCHER, W. D. HORST AND I. J. KOPIN

From the Laboratory of Clinical Science, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland, U.S.A.

(*Received August* 5, 1964)

While noradrenaline is generally thought to be the major transmitter in adrenergic neurones (Euler, 1956), recently a number of other substances have been shown to be released by nerve stimulation under some circumstances (Crout & Shore, 1964; Muscholl & Maître, 1963; Rosell, Kopin & Axelrod, 1964). We have tested a number of sympathomimetic amines for their ability to be released by nerve stimulation in the isolated perfused spleen of the cat. The results obtained with tyramine and α-methyltyramine are presented in detail. The formulae of these compounds, and of their β-hydroxylated derivatives, octopamine and α-methyloctopamine, are shown in Fig. 1.

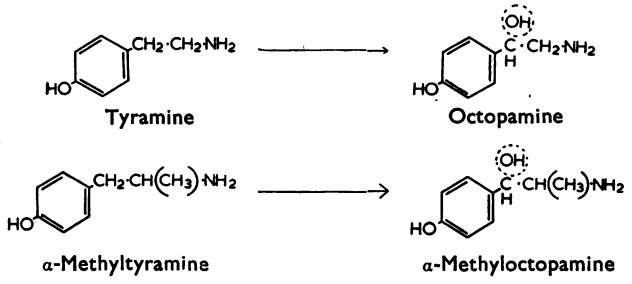

Fig. 1. Tyramine and α-methyltyramine, and their β-hydroxylated derivatives.

METHODS

Spleens of cats were perfused by a modification of the technique of Brown & Gillespie (1957). Cats of either sex, weighing between 2 and 4 kg, were anaesthetized with pentobarbitone sodium (35 mg/kg, intraperitoneally) and a tracheostomy performed. The abdomen was opened through a midline incision and the intestines were divided at the second portion of the duodenum and at the rectum. The superior mesenteric artery was ligated and the superior mesenteric vein cannulated with a polyethylene cannula (PE No. 240) to the level of the junction with the splenic vein. The omentum and vascular connexions between stomach and spleen were then divided and the left gastric artery was ligated. The splenic nerves were then isolated and crushed. A catheter (PE No. 60) was introduced into the splenic artery and the spleen perfused with warmed (37° C) Krebs-Ringer bicarbonate solution saturated with 95% oxygen and 5% carbon dioxide and containing heparin and 100 mg/l. of glucose from a Harvard infusion pump. The portal vein was then ligated and perfusion pressure was monitored on a Grass model 5d Polygraph with Statham P 23 AC transducers. [^{3}H]-Tyramine (50 μc) or α-methyltyramine (50 μc) (or other tritiated amines) was infused for a 2-min interval. The effluent solution was collected from the splenic vein and reinfused

for an additional 2 min. Perfusion was continued with the same solution containing no tritiated amine. About 15 min after the infusion of the labelled amine the splenic nerves distal to the crush were placed on shielded platinum electrodes. After the perfusion had continued for 20 min at 7.6 ml./min contraction was induced with two trains of stimuli 4 min apart, lasting 10 sec each (30 shocks/sec, 1 msec duration, at 10 V), to empty the spleen. After a further 10 min, collections of the effluent solution over 2-min intervals were begun. After two control collections, each of 2 min duration, the splenic nerve was stimulated with rectangular monophasic pulses of 1 msec duration and of 10 V. A total of 100 or 300 stimuli were given at frequencies varying from 10 to 100 shocks/sec. The splenic vein effluent solutions during the 2-min period which included nerve stimulation and an additional 2-min period following were also collected. Splenic contractions were also induced by noradrenaline in doses of 10-100 ng administered into the splenic artery. In some experiments, phenoxybenzamine (2 mg) was given arterially; 15 min later the stimulations were repeated. After the conclusion of the experiment the spleens were homogenized in 0.4 N-perchloric acid and assayed for tritiated amines.

Identification and assay of products released by nerve stimulation. Effluent solutions were centrifuged to sediment the few red cells and the clear supernatant fluid was assayed for tritiated amines as previously described (Fischer, Musacchio, Kopin & Axelrod, 1964). The amine contents of these supernatant fluids will henceforth be referred to as output. In some experiments the effluent solutions from several control periods and several periods of nerve stimulation were pooled separately. After absorption on Dowex 50 ammonium ion the amines were eluted with 3 N-ammonium hydroxide solution in 80% methanol. The eluates were concentrated in a stream of nitrogen and spotted on Whatman No. 3 mm filter paper. After paper chromatography (*n*-butanol saturated with N-hydrochloric acid, descending) with authentic tyramine and octopamine or α-methyltyramine and α-methyloctopamine, the radioactivity in the effluent solution was shown to correspond to the amine and its β-hydroxylated derivative. The strips were then cut, eluted with water, and the tritium in the eluate was assayed in a liquid scintillation spectrometer. The amounts of the amine and its β-hydroxylated derivative found were identical with the amounts estimated by chemical methods (Fischer *et al.*, 1964).

RESULTS

Release of [^{3}H]-tyramine and [^{3}H]-octopamine from the spleen

After infusion of [^{3}H]-tyramine, the labelled amine and its β-hydroxylated derivative, [^{3}H]-octopamine, were both present in the effluent solution from the isolated perfused spleen. These were identified by paper chromatography and estimated chemically as described in the methods. At 30 min after the administration of [^{3}H]-tyramine, this amine accounted for about 70 to 80% of the total tritiated amine in the effluent solution.

Effect of nerve stimulation on the release of [^{3}H]-octopamine

When the splenic nerve was stimulated there was a considerable increase in [^{3}H]-octopamine appearing in the effluent solution, as well as some increase in [^{3}H]-tyramine (Fig. 2). [^{3}H]-Octopamine was not released when the spleen was made to contract by infusion of 100 ng of (−)-noradrenaline (Fig. 2). Some release, however, could be obtained with doses of the order of 1 μg of the catechol amine.

Increased amounts of [^{3}H]-tyramine appeared in the effluent solution not only during nerve stimulation, but also when splenic contraction was induced by doses of noradrenaline as low as 1 ng. After phenoxybenzamine had been administered, the levels of [^{3}H]-octopamine, but not of [^{3}H]-tyramine, in the effluent solution were increased. Nerve stimulation again resulted in considerable increases in concentration of [^{3}H]-octopamine in the effluent solution, but the spleen did not contract, the perfusion pressure did not increase (Fig. 2) and there was no increase in [^{3}H]-tyramine in the effluent solution. At the end of the experiment, analysis of the spleens showed the presence of large quantities of

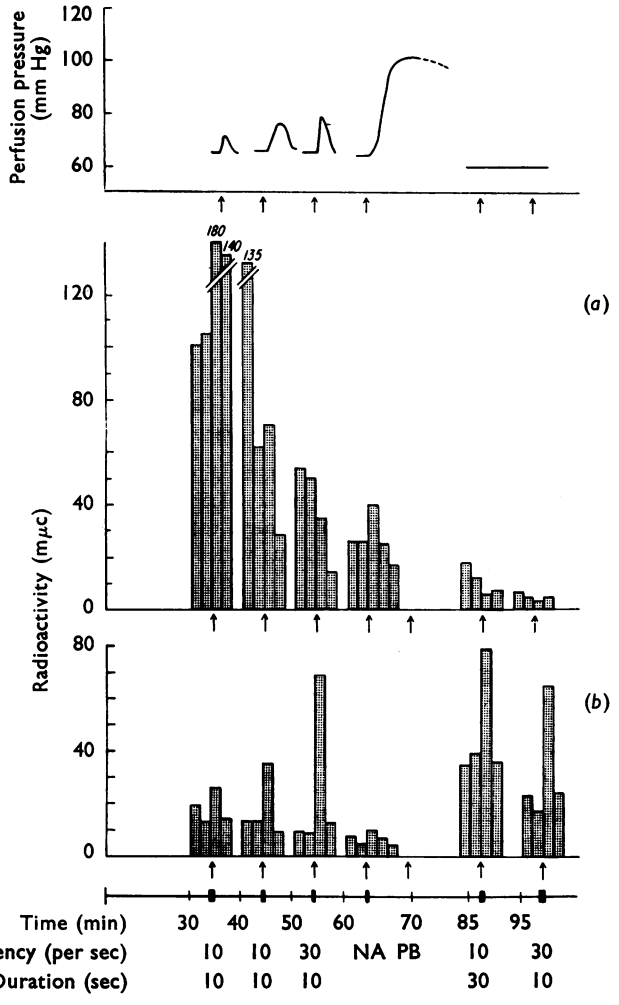

Fig. 2. Release of [^{3}H]-tyramine (upper histogram, *a*) and [^{3}H]-octopamine (lower histogram, *b*) from the cat isolated perfused spleen. The perfusion pressures and labelled amines appearing in the perfusate were determined as described in the text. Frequency and duration of each train of stimuli (at arrows) is indicated. At NA, noradrenaline (10 ng) was injected intra-arterially. Phenoxybenzamine (PB, 2 mg) was administered intra-arterially at the indicated time. These results were typical of six such experiments summarized in Table 1. Each bar represents a 2-min collection period.

[^{3}H]-tyramine as well as [^{3}H]-octopamine. The total amount of [^{3}H]-octopamine in the effluent solution represented less than 5% of the [^{3}H]-octopamine found in the spleens.

Relationship of frequency of nerve stimulation to release of [^{3}H]-octopamine

When 300 shocks were delivered to the splenic nerve at various frequencies the total output for the period of stimulation varied. The relationship between [^{3}H]-octopamine per stimulus to rate of stimulation is shown in Fig. 3. At 10 V, 30 shocks/sec appeared to be optimal for releasing the labelled β-hydroxylated amine. At higher or lower frequencies

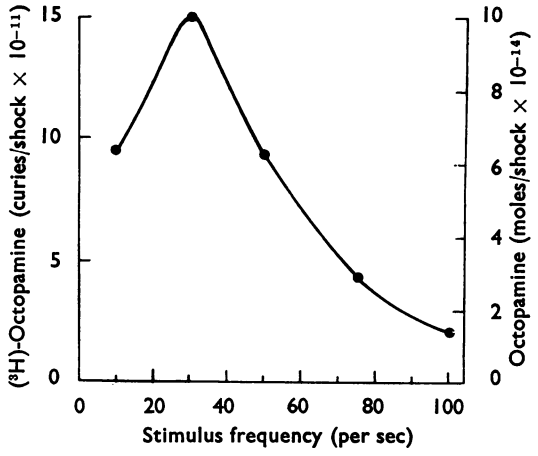

Fig. 3. The relationship between [^{3}H]-octopamine output and frequency of nerve stimulation. Each point represents the mean of values derived from six spleens, into which [^{3}H]-tyramine was infused. The splenic nerve was stimulated every 10 min with a total of 300 shocks at various frequencies. Sequence of stimulation at different frequencies was varied with different experiments.

less [^{3}H]-octopamine appeared in the effluent solution. After phenoxybenzamine, the amount of amine released at the optimal frequency of 30 shocks/sec was unchanged. At the lower rates of stimulation, however, the output of [^{3}H]-octopamine was greatly enhanced (Fig. 2).

Effects of alterations in rate of perfusion

At 30 min after infusion of [^{3}H]-tyramine, the rate of appearance of [^{3}H]-octopamine in the effluent solution varied with the rate of perfusion (Table 1). At flow rates of 7.6 ml./min there was about twice as much [^{3}H]-octopamine as at flow rates of 3.8 ml./min; this was true during rest as well as after different frequencies of nerve stimulation.

TABLE 1

EFFECT OF FLOW RATE ON [^{3}H]-OCTOPAMINE IN EFFLUENT SOLUTION FROM PERFUSED SPLEEN

Six spleens from cats weighing 2 to 4 kg received [^{3}H]-tryptamine and were perfused with Krebs-Ringer-bicarbonate solution at flow rates of 3·8 or 7·6 ml./min. [^{3}H]-Octopamine (expressed as mμc of tritium per 2-min interval, means and standard errors) was determined in the effluent solution at rest and after stimulation of the splenic nerve with a total of 300 shocks delivered at 10 or 30 shocks/sec

	[^{3}H]-Octopamine release for perfusion rate	
No. of shocks	3·8 ml./min	7·6 ml./min
None	3·7±0·58	13·0±0·75
300 (at 10/sec)	20·0±6·2	40·3±2·6
300 (at 30/sec)	25·0±4·2	57·2±5·7

Release of α-methyl-[^{3}H]-octopamine from the spleen

When α-methyl-[^{3}H]-tyramine was infused into an isolated spleen, results similar to those with [^{3}H]-tyramine were obtained (Fig. 4). Because the α-methyl compound is not destroyed by monoamine oxidase the levels of radioactivity recovered in the effluent solution were greater. The relationship between frequency of nerve stimulation and

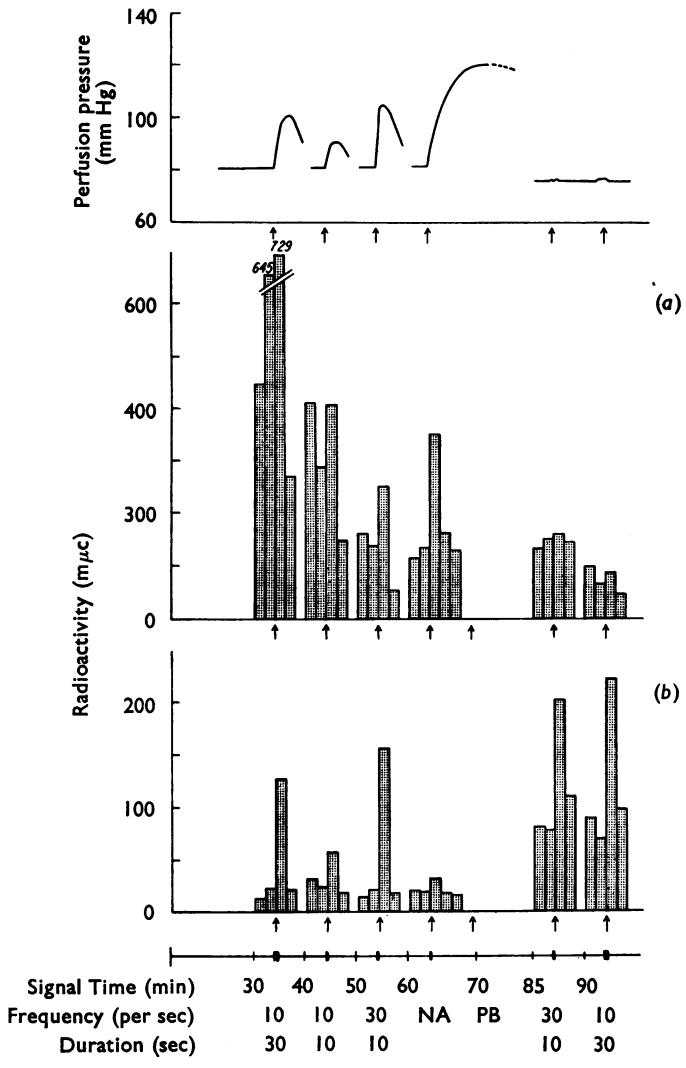

Fig. 4. Release of α-methyl-[^{3}H]-tyramine (upper histogram, *a*) and α-methyl-[^{3}H]-octopamine (lower histogram, *b*) from the cat isolated perfused spleen after infusion of α-methyl-[^{3}H]-tyramine. This Figure is to be compared to Fig. 2, in which release of [^{3}H]-octopamine after infusion of [^{3}H]-tyramine is presented. This result was typical of five such experiments.

amounts of α-methyl-[^{3}H]-octopamine in the effluent solution was also similar to what was found for octopamine after tyramine infusion (Fig. 5). In the experiments with the α-methyl compound, however, the rate of release of the β-hydroxylated derivative could not be increased by increasing the rate of perfusion. Analysis of the spleens at the ends of experiments showed large amounts of α-methyl-[^{3}H]-tyramine as well as α-methyl-[^{3}H]-octopamine.

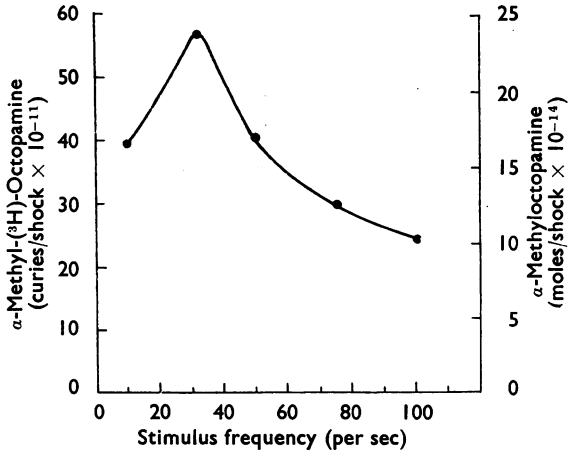

Fig. 5. Relationship between release of α-methyl-[³H]-octopamine (*p*-hydroxynorephedrine) and frequency of nerve stimulation. This Figure should be compared with Fig. 3, in which release of [³H]-octopamine is represented. Each point represents the mean of values derived from four spleens.

DISCUSSION

The results presented provide further evidence that the β-hydroxylated derivatives of tyramine and α-methyltyramine behave very much like noradrenaline. It has previously been shown that, following administration of these amines, there is greater uptake in innervated than in chronically denervated rat salivary glands, with rapid conversion to their β-hydroxylated derivatives, octopamine and α-methyloctopamine, only in the innervated glands. These compounds are formed apparently in the granules found in sympathetic nerve endings (Carlsson & Waldeck, 1964; Fischer *et al.*, 1964; Musacchio, Snyder & Kopin, 1964; Musacchio, Fischer & Kopin, 1965). The β-hydroxylated compounds are released by reserpine, guanethidine and tyramine, all of which deplete noradrenaline. We have shown that the β-hydroxylated derivatives are released by nerve stimulation. While the outputs of both [³H]-tyramine and α-methyl-[³H]-tyramine were also increased during nerve stimulation, this appears to be related to splenic contraction. Minute doses (10 ng) of noradrenaline, which cause splenic contraction, result in more than an equimolar increase in [³H]-tyramine and α-methyl-[³H]-tyramine output. Small doses of noradrenaline, which induce splenic contraction, do not increase [³H]-octopamine output; large doses (10 μg) result in some release but may do so by replacement at the binding sites. The amines not hydroxylated in position β are not released by nerve stimulation when splenic contraction has been blocked by phenoxybenzamine, although large amounts of these amines remain in the spleen. Furthermore, these nonhydroxylated amines do not appear to be retained by the granules (Musacchio *et al.*, 1965). Splenic contraction might result in extrusion of platelets which have bound the amine, but failed to convert them to the β-hydroxylated derivative.

The relationship of stimulus frequency and treatment with phenoxybenzamine to the amount of β-hydroxylated amine released is similar to that observed by Brown & Gillespie (1957) for noradrenaline. Both noradrenaline and the β-hydroxylated derivatives of the sympathomimetic amines which we have investigated are optimally released at frequencies of 30 shocks/sec. Hertting (personal communication) has found that noradrenaline released

from spleens perfused with Krebs-Ringer solution varies with the flow rate. We have observed this phenomenon with [^{3}H]-octopamine but not with the α-methyl derivative.

While noradrenaline is generally considered to be the adrenergic transmitter in mammals (Euler, 1956), a number of related compounds have been shown to act like this catechol amine. [^{3}H]-Adrenaline is taken up by the sympathetic nerve endings and released in response to nerve stimulation (Rosell *et al.*, 1964). After administration of α-methyldopa, α-methylnoradrenaline can be demonstrated in tissues (Carlsson & Lindqvist, 1962) and the α-methyl amino acid has been shown to restore the action of nerve stimulation in reserpinized animals (Day & Rand, 1964). It was suggested that α-methylnoradrenaline acts as a false neurotransmitter. Muscholl & Maître (1963) demonstrated that upon nerve stimulation the α-methyl catechol amine was released from the hearts of animals previously treated with α-methyldopa. Crout & Shore (1964) demonstrated that metaraminol, the β-hydroxylated derivative of α-methylmetatyramine, is taken up, stored, and released by nerve stimulation in the isolated heart. It is not surprising, therefore, that other amines can function as false neurochemical transmitters. Preliminary observations with [^{3}H]-phenylethylamine, (+)-[^{3}H]-amphetamine, [^{3}H]-metatyramine and α-methyl-[^{3}H]-metatyramine indicate that all these amines are converted to some extent to their β-hydroxylated derivatives *in vivo* and that these products can also be released by nerve stimulation from the spleen.

The accumulation of α-methyl ring hydroxylated derivatives of phenylethylamine is enhanced by the fact that these foreign substances are not substrates for monoamine oxidase.

After administration of monoamine oxidase inhibitors, however, *p*-hydroxy- and *m*-hydroxyphenylethylamine (metatyramine) are excreted in increased amounts in man (Sjoerdsma, Lovenberg, Oates, Crout & Udenfriend, 1959). Octopamine has also been found in human urine and accumulates in the tissues of animals treated with monoamine oxidase inhibitors (Kakimoto & Armstrong, 1962). The demonstration that octopamine accumulates in the sympathetic nerves (Kopin, Fischer, Musacchio & Horst, 1964) and can be released by nerve stimulation has provided the basis for a hypothesis regarding the apparent sympathetic blockade induced by chronic treatment with monoamine oxidase inhibitors. Octopamine and similar β-hydroxylated amines which accumulate in the sympathetic nerve after inhibition of monoamine oxidase can function as false neurochemical transmitters. It was suggested that these biologically relatively inactive counterfeit transmitters replace a portion of the noradrenaline released by nerve stimulation and result in partial adrenergic blockade (Kopin *et al.*, 1964).

This may also be the mechanism of the partial adrenergic blockade noted after the development of tachyphylaxis to sympathomimetic amines. Day & Rand (1963) suggested that " amphetamine (and related drugs) as well as guanethidine attach to the storage sites for noradrenaline and replace the drug." Our results suggest that the β-hydroxylated derivatives of amphetamine rather than amphetamine itself are the compounds which replace noradrenaline. The norephedrine formed (or other β-hydroxylated derivatives of other sympathomimetic amines and perhaps guanethidine as well) could be released by nerve stimulation, functioning as a counterfeit transmitter. These amines replace noradrenaline, resulting in diminished noradrenaline release by nerve stimulation and thus causing apparent sympathetic blockade.

SUMMARY

1. The ability of sympathomimetic amines to serve as false neurotransmitters was investigated. [^{3}H]-Tyramine or α-methyl-[^{3}H]-tyramine was infused into cat isolated perfused spleens. The components of the splenic vein effluent solution were determined both chemically and chromatographically.

2. Splenic nerve stimulation resulted in release of [^{3}H]-octopamine or α-methyl-[^{3}H]-octopamine, the β-hydroxylated derivatives, after the two amines administered. [^{3}H]-Tyramine or α-methyl-[^{3}H]-tyramine levels in the splenic effluent solution were increased by nerve stimulation, but this appeared to be related to splenic contraction. When splenic contraction was blocked by phenoxybenzamine and the splenic nerve stimulated, output of amines not hydroxylated in position β was not increased.

3. Octopamine and α-methyloctopamine behaved like noradrenaline in the relation between output and stimulus at various frequencies of stimulation.

4. It is suggested that the β-hydroxylated derivatives of some sympathomimetic amines are capable of serving as false neurotransmitters.

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