EFFECT OF DRUGS ON THE UPTAKE, RELEASE, AND METABOLISM OF H^3-NOREPINEPHRINE IN THE RAT BRAIN

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Accepted for publication March 9, 1965

Norepinephrine metabolism in the brain has been studied by a variety of experimental approaches. In some studies, changes in the concentration of endogenous catecholamines have been observed after the administration of inhibitors of amine metabolism and many other drugs. Reserpine (Carlsson *et al.*, 1957; Holzbauer and Vogt, 1956) and amphetamine (Vogt, 1954) have been shown to lower the norepinephrine content of the brain. Monoamine oxidase (MAO) inhibition elevates brain norepinephrine in the rat (Shore *et al.*, 1957) but not in the cat (Vogt, 1959). Catechol O-methyl transferase (COMT) inhibition had no effect on the concentration of norepinephrine in the rat brain (Crout *et al.*, 1961; Weil-Malherbe *et al.*, 1961). Treatment with MAO inhibitors resulted in elevated C^{14}-norepinephrine levels after C^{14}-dopa administration, while COMT inhibition alone had no significant effect in the rat brain (Goldstein, 1964). In the cat brain, MAO inhibition did not elevate C^{14}-norepinephrine levels after intraventricular injection of C^{14}-norepinephrine (Mannarino *et al.*, 1963).

Recently, it has been shown that H^3-norepinephrine injected in the lateral ventricle of the rat can be taken up and retained in the brain (Glowinski *et al.*, 1965). Subcellular distribution and metabolism studies have shown that exogenous norepinephrine introduced in the brain in this way mixes with the endogenous store (Glowinski *et al.*, 1965). Our work, as well as that of others (Burack and Draskoczy, 1964), also indicated that norepinephrine is stored in more than one compartment in the brain. The ability of H^3-norepinephrine to be taken up and stored in the brain allowed us to examine the effect of drugs and metabolic inhibitors on the uptake, release and metabolism of H^3-norepinephrine in the rat brain.

METHODS. H^3-norepinephrine (7.2 c/mmol, New England Nuclear, Boston, Mass.) was injected into the lateral ventricle of the brain by a procedure previously described (Milhaud and Glowinski, 1962). Sprague-Dawley male rats weighing 280 to 300 grams received 0.07 μg of H^3-norepinephrine in 20 μl of Merles solution.[1] Animals were killed by a blow on the head and their brains were homogenized in 15 ml of 0.4 N perchloric acid. After centrifugation the total radioactivity was measured in a 0.2-ml aliquot of the supernatant fluid. H^3-norepinephrine (Whitby *et al.*, 1961), normetanephrine, and catechol metabolites were assayed by a procedure previously described (Kopin *et al.*, 1961). The O-methylated deaminated metabolites (3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylglycol) were estimated by the difference between total radioactivity and the sum of H^3-norepinephrine and H^3-normetanephrine. Endogenous norepinephrine was determined by the procedure of Anton and Sayre (1962).

Catron (β-phenylisopropyl hydrazine), tropolone-4-acetamide, reserpine (Serpasil, Ciba), *d*-amphetamine sulfate, cocaine hydrochloride, D-lysergic acid diethylamide (LSD) and mescaline hydrochloride were injected intraperitoneally. Bretylium tosylate and guanethidine sulfate were injected intraventricularly in the same manner as H^3-norepinephrine. All dosages were expressed on the basis of the salts. Tropolone-4-acetamide and bretylium tosylate were generously supplied by Dr. H. Corrodi and Dr. J. J. Burns.

RESULTS. *Effects of monoamine oxidase and catechol O-methyl transferase inhibitors on metabolism of H^3-norepinephrine in the rat brain.* Rats received Catron (a MAO inhibitor) or tropolone-4-acetamide (a COMT inhibitor) before the intraventricular administration of H^3-norepinephrine. The animals were killed 2 hours after the catecholamine injection and their brains were assayed for H^3-norepinephrine and its metabolites (table 1). In the Catron-treated animals the levels of H^3-norepinephrine and H^3-normetanephrine were increased as

Received for publication December 31, 1964.

[1] Merles solution can be made by dissolving NaCl 8.98 g, KCl 0.25 g, $CaCl_2$ 0.14 g, $MgCl_2$ 0.11 g, NaH_2PO_4 0.07 g, urea 0.13 g, glucose 0.61 g, in one liter of water.

TABLE 1

Effect of enzyme inhibitors on the metabolism of H^3-norepinephrine in the intact rat brain

Expt.	Drug	Dose	Time Drug Given	H^3-Norepinephrine	H^3-Normetanephrine	H^3 O-Methylated Deaminated Metabolites	H^3-Catechol Deaminated Metabolites
1	Control			100 ± 6.2	100 ± 4.7	100 ± 4.9	100 ± 5.6
	Catron	5 mg/kg	2 hr before	174.0 ± 15*	640.0 ± 8.5*	0	0
	Tropolone-4 acetamide	25 mg/kg	1 hr before	114.1 ± 10	23.2 ± 1.0*	87.3 ± 7.2	102.9 ± 3.5
2	Control			100 ± 9.4	100 ± 12.3	100 ± 16.4	—
	Catron	2 × 5 mg/kg	1 hr and 12 hr after	334.2 ± 4.0*	453 ± 7.9*	141 ± 22.0	—

* $P < .001$ when compared with control values.

Results are expressed as per cent of the control values ± standard error of the mean.

Expt. 1: Inhibitors were given before H^3-norepinephrine, groups of six rats then received 0.07 μg of H^3-norepinephrine (2260 mμc) in the lateral ventricle and were killed 2 hours later. At this time H^3-norepinephrine, H^3-normetanephrine, H^3 O-methylated deaminated metabolites and H^3-catechol deaminated metabolites represented 55 to 60%, 5 to 8%, 30 to 35% and 2 to 3%, respectively, of the total radioactivity in brain of control rats (400.3 mμc/g).

Expt. 2: Groups of six rats received 0.2 μg of H^3-norepinephrine (6460 mμc) in the lateral ventricle and were killed 24 hours later. Catron was given after H^3-norepinephrine. At this time H^3-norepinephrine, H^3-normetanephrine and H^3 O-methylated deaminated metabolites represented 60 to 65%, 4 to 6% and 25 to 30%, respectively, of the total radioactivity in brain of control rats (48.7 mμc/g).

compared to the controls; deaminated metabolites could not be detected. These results indicate that the inhibition of MAO in the rat brain blocks the deamination of both norepinephrine and normetanephrine. Tropolone-4-acetamide had no significant effect on the levels of norepinephrine and the O-methylated deaminated metabolites. However, the drug markedly reduced the formation of the O-methylated amine, normetanephrine.

In another experiment, Catron was given 1 hour and 12 hours after H^3-norepinephrine and the brains were assayed 24 hours later for the catecholamine and its metabolites. In this experiment, the H^3-norepinephrine was elevated to a greater extent than when the drug was given before the H^3-catecholamine and the rats killed 2 hours later. In addition, there was a large amount of deaminated O-methylated metabolites. This distribution of metabolites after 24 hours could be explained by the ability of Catron to interfere with the spontaneous release of norepinephrine (Axelrod *et al.*, 1961a), as well as to block MAO activity. It also could represent deamination of norepinephrine accumulated after prolonged MAO inhibition.

Effects of drugs on the uptake of H^3-norepinephrine in the rat brain. Rats were treated with a number of drugs and then they received H^3-norepinephrine intraventricularly. The animals were killed 2 hours later and their brains assayed for H^3-norepinephrine and its metabolites. Pretreatment with reserpine, amphetamine and guanethidine markedly reduced the concentration of H^3-norepinephrine in the brain (table 2). Bretylium, cocaine, LSD and mescaline had no effect. Although reserpine, amphetamine and guanethidine caused a diminution in the level of H^3-norepinephrine, they had different effects on the metabolism of the catecholamine (table 3). Reserpine reduced the concentration of normetanephrine, while amphetamine and guanethidine elevated this metabolite. Reserpine and guanethidine increased the formation of O-methylated deaminated compounds, while amphetamine did not change the percentage of these metabolites.

Effect of reserpine and amphetamine on the release and metabolism of H^3-norepinephrine from the rat brain. Previous work had shown that norepinephrine injected intraventricularly into the rat disappears from this tissue in a multiphasic fashion (Glowinski *et al.*, 1965). These results suggested the existence of more than one store of the catecholamine in the brain, a store having a rapid turnover and a store having a much slower turnover. In the

TABLE 2

Effect of drugs on the uptake of H^3-norepinephrine in the rat brain

Drug	Dose	Time between Drug Administration and H^3-Norepinephrine Injection (min)	No. of Animals in Group	Mean Uptake of H^3-Norepinephrine as % of Control ± S.E.M.
Control			15	100 ± 2.9
Reserpine	5 mg/kg	120	8	22.4 ± 4.0*
Amphetamine	20 mg/kg	60	8	35.8 ± 3.4*
Guanethidine†	200 μg/brain	10	8	52.3 ± 4.0*
Bretylium†	100 μg/brain	10	6	99.6 ± 5.9
Cocaine	15 mg/kg	30	12	99.2 ± 3.0
LSD	2 mg/kg	60	4	99.3 ± 5.2
Mescaline	40 mg/kg	45	4	96.8 ± 2.0

* $P < .001$ when compared with control values.

† Drugs administered intraventricularly.

Groups of animals received 0.07 μg of H^3-norepinephrine in the lateral ventricle at various times after the administration of the drug. Except where indicated all drugs were administered intraperitoneally. Rats were killed 2 hours later and their brains assayed for H^3-norepinephrine. The amount of H^3-norepinephrine in the control brains was 259.8 mμc/g.

TABLE 3

Effect of drugs on the metabolism of H^3-norepinephrine in the rat brain

Drug	H^3 O-Methylated Deaminated Metabolites	H^3-Normetanephrine
Control	100 ± 4.9	100.0 ± 6.9
Reserpine	177 ± 4.2*	61.5 ± 3.6*
Amphetamine	106 ± 2.7	292.0 ± 4.5*
Guanethidine	155 ± 3.5*	146.0 ± 3.3*
Bretylium	96.5 ± 6.5	105.6 ± 6.2
Cocaine	75.8 ± 7.7**	92.5 ± 3.7

* $P < .001$; ** $P < .05$ when compared with the control values.

Animals were treated as described in table 2 and their brains assayed for metabolites of H^3-norepinephrine 2 hours after the intraventricular injection of the H^3-catecholamine. Results are expressed as per cent of control value ± standard error of the mean. The amounts of H^3 O-methylated deaminated metabolites and H^3-normetanephrine in control brains were 114.0 mμc/g and 26.8 mμc/g, respectively.

experiment described above, amphetamine and reserpine reduced the concentration of H^3-norepinephrine in the brain when the drugs were given before the radioactive catecholamine. This reduction in the level of catecholamine might be due to an inhibition of the uptake or to a release of the H^3 amine, or to a combination of these actions. To study the releasing action of these drugs, amphetamine or reserpine was given after the injection of H^3-norepinephrine, when the radioactive catecholamine was present in the brain in a bound form. In the first experiment the drugs were given 1 hour after the radioactive catecholamine and the animals were killed 3 hours later. It has previously been established that the disappearance of the bound H^3-norepinephrine at this time is rapid (half-life 3 hours). In the second experiment, amphetamine and reserpine were given 21 hours after the intraventricular administration of H^3-norepinephrine and the animals were killed 3 hours later. At this time, the half-life for H^3-norepinephrine disappearance in the brain is much slower (17 hours). Both drugs released the endogenous and radioactive norepinephrine (table 4). However, they released a greater percentage of the H^3-norepinephrine in the period of slow disappearance. At this time the percentage of H^3-norepinephrine released by amphetamine was the same as that of the endogenous catecholamine. Reserpine released a significantly greater percentage of endogenous norepinephrine than H^3-norepinephrine at both earlier and later times. For this reason the specific activity of the norepinephrine remaining in the brain was calculated after amphetamine and reserpine (table 5). The specific activity of norepinephrine remaining in the brain after reserpine was higher than in

TABLE 4

Release of H^3-norepinephrine from the rat brain by amphetamine and reserpine

Drug	Dose mg/kg	(1) 4 Hr % H^3-Norepinephrine Release	(2) 24 Hr % H^3-Norepinephrine Release	(3) % Endogenous Norepinephrine Release
Amphetamine	20	26.7 ± 4.1	39.4 ± 2.3[a]*	36.3 ± 2.5
Reserpine	5	60.6 ± 5.7	79.2 ± 3.0[a]**	89.5 ± 1.8[b]

[a] Comparison between columns (2) and (1). * $P < .05$. ** $P < .02$.

[b] Comparison between columns (3) and (2). $P < .02$.

Rats were given 0.2 μg of H^3-norepinephrine intraventricularly. Groups of six rats were given intraperitoneal injections of amphetamine or reserpine 1 hour and 21 hours after, and were killed 4 hours and 24 hours after the H^3-norepinephrine administration, respectively. Results are expressed as per cent of endogenous and radioactive norepinephrine when compared to the control value ± standard error of the mean.

TABLE 5

Specific activity of H^3-norepinephrine in the brain after amphetamine and reserpine

Drug	4 Hr after Administration of H^3-Norepinephrine			24 Hr after Administration of H^3-Norepinephrine		
	H^3-NE (mμc/g)	Endogenous NE (mμg/g)	Specific activity (mμc/mμg)	H^3-NE (mμc/g)	Endogenous NE (mμg/g)	Specific activity (mμc/mμg)
Control	346 ± 12.2	587 ± 17	0.59 ± 0.03	27.2 ± 2.1	591 ± 25	0.046 ± 0.002
Amphetamine	271 ± 21.6	381 ± 7	0.71 ± 0.05	10.5 ± 0.6	376 ± 4	0.044 ± 0.003
Reserpine	136 ± 20.2	59 ± 4	2.30 ± 0.04*	6.1 ± 0.5	61.6 ± 3	0.098 ± 0.004*

* $P < .001$ when compared with control.

Further analysis of the experiments described in table 4. Results are expressed as mean value ± standard error of the mean of endogenous and radioactive norepinephrine (NE) after various treatments.

the control animals. At the early time, the ratio of the specific activity of norepinephrine in the reserpinized animals to the specific activity in control animals was about 4, but at the later time this ratio was reduced to 2. This indicates that the more recently bound norepinephrine is more resistant to the depleting action of reserpine, or that there is more free H^3-norepinephrine in the brain at this time which cannot be released by reserpine. This latter possibility is less probable because unbound H^3-norepinephrine would be rapidly metabolized.

The metabolites of H^3-norepinephrine present in the brain were also examined after the administration of amphetamine and reserpine in these experiments (table 6). As in the experiment in which the drugs were given before H^3-norepinephrine, considerably more normetanephrine was found in the brain after amphetamine. However, this metabolite represents a small fraction of the total radioactivity in the brain. There was a slight increase in the concentration of O-methylated deaminated metabolites after both drugs, and a reduction of the deaminated catechols after amphetamine. In the later period, the metabolites represented a smaller percentage of the total radioactivity in both treated and untreated animals but the relative increase in the level of normetanephrine after amphetamine was the same when compared with the early time.

DISCUSSION. Previous work has shown that H^3-norepinephrine is stored in a heterogeneous fashion in the rat brain (Glowinski *et al.*, 1965). Norepinephrine was found to disappear from the brain in a multiphasic manner; at first the disappearance was rapid and then it became increasingly slower. The experiments described in this report further support the concept of more than one storage form of norepinephrine in the brain. Heterogeneous storage forms also have been demonstrated in peripheral sympathetic nerves (Trendelenburg, 1961; Axelrod *et al.*, 1961a; Potter and Axelrod, 1963; Iversen, 1963).

TABLE 6

Metabolites of H^3-norepinephrine in the brain after reserpine and amphetamine

Drug	H^3-Norepinephrine	H^3-Normetanephrine	H^3 O-Methylated Deaminated Metabolites	H^3-Catechol Deaminated Metabolites
Control	100 ± 4.2	100 ± 17.2	100 ± 2.2	100 ± 2.7
Amphetamine	73 ± 3.9*	228 ± 22.1*	118 ± 4.0**	47 ± 5.4**
Reserpine	37 ± 5.5*	98 ± 18.4	133 ± 10.0**	107 ± 12.0

* $P < .001$; ** $P < .01$ when compared with control values.

Rats were treated as described in column (1) of table 4. In control animals killed 4 hours after the administration of H^3-catecholamine, H^3-norepinephrine and its metabolites represented the following percentages of the total radioactivity: H^3-norepinephrine 60%, H^3-normetanephrine 4.3%, H^3 O-methylated deaminated metabolites 33.1% and H^3-catechol deaminated metabolites 2.6%. Results are expressed as per cent of control value ± standard error of the mean.

Both amphetamine and reserpine release H^3-norepinephrine as well as the endogenous catecholamine from the brain. Although these drugs reduce the level of H^3-norepinephrine in the brain, they appear to release the amine in a different manner. After both drugs, the major metabolites present in the brain are O-methylated deaminated products, but amphetamine administration results in the formation of more normetanephrine while reserpine causes the formation of more deaminated metabolites. The same observations have been found for the metabolites present in the brain when the drugs were given before the radioactive catecholamine. Reserpine also has been shown to decrease the concentration of endogenous normetanephrine in the brain (Haggendal, 1963), and to elevate the concentration of deaminated products (Anden *et al.*, 1963). The difference in the metabolic fate of the norepinephrine released by these two drugs indicates that amphetamine and reserpine release norepinephrine from different stores in the brain. It is also possible but unlikely that these drugs may be acting directly on the enzyme involved in the metabolism of norepinephrine by different mechanisms. The increase in the concentration of normetanephrine after amphetamine suggests that this drug releases norepinephrine from the neurone as the unchanged amine and it is then O-methylated by COMT. The increased O-methylated deaminated metabolites after reserpine suggest that norepinephrine released from the storage sites is probably deaminated before it leaves the neurone. It has been suggested that the norepinephrine released by reserpine from peripheral sympathetic nerves is inactivated by intraneuronal MAO before it leaves the nerve (Kopin *et al.*, 1962; Kopin and Gordon, 1963). These authors also suggested that sympathomimetic amines such as tyramine, on the other hand, act by releasing norepinephrine from sympathetic nerves in a physiologically active form which is then metabolized by COMT. From the metabolic products present in the brain after amphetamine and reserpine it would appear that similar processes of release occur in this tissue. The differences in the pharmacological actions of these two drugs on both peripheral and central nervous tissues may be related to the fact that in the case of amphetamine, norepinephrine is released in a physiologically active form, whereas with reserpine only physiologically inactive metabolites are released.

The experiments with reserpine and amphetamine indicate that the H^3-norepinephrine taken up by the brain does not immediately mix completely with the endogenous store. These drugs release a greater percentage of labeled catecholamine the longer it is stored. The proportion of H^3-norepinephrine retained in the brain after reserpine is greater 4 hours after the administration of the catecholamine than after 24 hours. This would suggest that the norepinephrine is distributed in a "reserpine resistant" pool and in a "reserpine releasable" pool. The changes in the percentage of norepinephrine released by reserpine at various times suggest either that the H^3-norepinephrine is transferred from the "reserpine resistant" pool to the "reserpine releasable" pool, or that there is a different rate of turnover in these compartments, the turnover in the "reserpine resistant" pool being faster. Additional evidence for changes in the distribution of H^3-norepinephrine in the different pools with time has been obtained in subcellular studies (J. Glowinski, S. Snyder and J. Axelrod, unpublished observation, 1965). The relative amounts of H^3-norepinephrine in the soluble and particulate fractions change with time.

This report, as well as others (Mannarino *et al.*, 1963; Milhaud and Glowinski, 1963), has demonstrated that O-methylation and deamination of norepinephrine occur in the brain. The relative importance of MAO and COMT in the inactivation of norepinephrine in the brain has often been discussed. After inhibition of MAO, we found an increase in the concentration of H^3-norepinephrine, but an even greater relative increase for H^3-normetanephrine. On the other hand, the administration of the COMT inhibitor tropolone-4-acetamide lowered H^3-normetanephrine but did not elevate H^3-norepinephrine. It is difficult on the basis of these data to assess the comparative roles of MAO and COMT, since Catron is a more complete inhibitor of MAO than tropolone-4-acetamide is of COMT. The absence of H^3-norepinephrine elevation after tropolone-4-acetamide might be due to its weaker action on COMT. It is also possible that there are two sites of action of COMT and that one of these sites is inaccessible to tropolone-4-acetamide. On the basis of the elevation of

norepinephrine in the brain after MAO inhibition, it has been assumed that MAO plays a more important role than COMT in the inactivation of norepinephrine in the rat brain (Crout *et al.*, 1961; Weil-Malherbe *et al.*, 1961). However, changes in the total catecholamine content may be misleading. Since norepinephrine appears to be stored in more than one compartment, the relative action of MAO and COMT may differ from one compartment to another. Furthermore, the size of the storage compartment is not necessarily related to the physiological activity of norepinephrine in that compartment. It has been shown that in peripheral sympathetic nerves the physiologically active pool of norepinephrine is only a small fraction of the total store (Crout *et al.*, 1962). Studies with reserpinized animals have shown that the functional pool of norepinephrine in the brain is only a small percentage of the total store (Haggendal and Lindquist, 1964). Consequently, the inactivation of norepinephrine in the brain is a complex process depending on the compartment from which it is released, the physiological activity of the released norepinephrine, the accessibility of degradative enzymes and the processes by which it can be taken up again and bound.

As in the peripheral nervous system, many drugs affect the uptake of H^3-norepinephrine in the brain. Amphetamine, reserpine and guanethidine blocked the uptake of H^3-norepinephrine into the brain, and these drugs also have been found to inhibit the uptake of H^3-norepinephrine into peripheral sympathetic nerves (Axelrod *et al.*, 1961b; Hertting *et al.*, 1961). However, cocaine and bretylium (Whitby *et al.*, 1960; Hertting *et al.*, 1962), drugs which block the uptake of H^3-norepinephrine in the periphery, did not affect this process in the brain. It was found also that chlorpromazine (Axelrod *et al.*, 1961b), which like cocaine can block the uptake of H^3-norepinephrine in peripheral tissues and brain slices (Dengler *et al.*, 1961), had no effect on the uptake of H^3-norepinephrine in the intact brain (Glowinski and Axelrod, 1964). These results would indicate that there are both similarities and differences between peripheral sympathetic nerves, brain slices and the intact brain.

Drugs can affect the level of H^3-norepinephrine by inhibiting the uptake and binding, or by releasing the bound amine. Amphetamine has a greater effect on the uptake of H^3-norepinephrine in the brain than on the release of bound H^3-norepinephrine since it caused a 30% release of bound H^3-norepinephrine but a 70% blockage of uptake. Thus the actions on the uptake and on the release are well differentiated for amphetamine, but in the case of reserpine the same reduction of the H^3-norepinephrine level in the brain was obtained when the drug was given before or after the H^3-catecholamine. From these results it is difficult to separate the effect of reserpine on uptake and release.

A number of hypotheses have been proposed to explain the central excitation produced by amphetamine. The drug may act indirectly by releasing norepinephrine in the brain (Stein, 1964). It may act directly either on catecholamine receptors (Van Rossum *et al.*, 1962; Smith, 1963) or on serotonin receptors (Vane, 1960; Gelder and Vane, 1962). Our results suggest that amphetamine not only releases norepinephrine in the brain but also prevents its re-uptake. Thus, it may have a dual action on norepinephrine, the net effect of both actions being to elevate the concentration of norepinephrine which is available to the central adrenergic receptors.

SUMMARY

The effects of drugs and metabolic inhibitors on the uptake, release and metabolism of intraventricularly administered H^3-norepinephrine in the rat brain are described. Amphetamine, reserpine and guanethidine, but not cocaine, D-lysergic acid diethylamide, mescaline or bretylium, block the uptake or accumulation of H^3-norepinephrine in the brain. Monoamine oxidase inhibition produces an elevation of H^3-norepinephrine and H^3-normetanephrine in the brain. A catechol O-methyl transferase inhibitor, tropolone-4-acetamide, reduces the level of H^3-normetanephrine but has no effect on the level of H^3-norepinephrine. Amphetamine and reserpine release H^3-norepinephrine in a different manner. Amphetamine administration results in an elevation of normetanephrine levels, while reserpine administration causes an elevation of O-methylated deaminated metabolites. Both drugs release a greater proportion of the brain content of H^3-norepinephrine when they are administered a long time after the injection of

the radioactive catecholamine than after a short time. The different actions of amphetamine and reserpine on the metabolism of H^3-norepinephrine indicate the presence of more than one storage form of the brain catecholamine.

ACKNOWLEDGMENT. We thank Miss Helen Hunt for her excellent technical assistance.

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