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ACTION OF DRUGS UPON THE FORMATION OF TRITIATED WATER FROM *dl*-7-H³-NORADRENALINE IN THE RAT*

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Abstract—The formation of tritiated water (THO) following the injection of *dl*-7-H³-noradrenaline (H³-NA) was investigated in rats. Already 15 min after the H³-NA) injection THO is detectable in the plasma and the concentration rises up to the 16th hr. 32 Hr after the H³-NA injection practically all radioactivity found in the plasma can be identified as THO. Groups of rats received in addition to 100 μc/kg H³-NA increasing amounts of unlabelled *d*-NA. The decrease in the tissue concentration of H³-NA was parallel to the decrease in THO-formation.

Drugs which block the tissue uptake of NA like cocaine or chlorpromazine, or which interfere with the NA-binding properties of tissue such as reserpine decreased also the formation of THO. Drugs that increased the H³-NA accumulation in the tissues like the COMT blockers pyrogallol and catechol, and drugs that decreased the rate of disappearance of H³-NA like the MAO-inhibitor pargyline, increased the concentration of THO found in the plasma 4 hr after the H³-NA injection. Diethyldithiocarbamate decreased THO formation. The THO is probably produced by an enzyme which is localized in the sympathetic nerve. The enzyme involved may be identical with the catechol oxidase described by Axelrod in tissue homogenates.

THE FATE of catecholamines (CA) in the body has been thoroughly investigated during the last decades. The major part of the administered CA leaves the body metabolized and only a small proportion is excreted unchanged. Two enzymes are responsible for the degradation of these neurohormones, the monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT). COMT seems to be primarily responsible for the inactivation of circulating CA, whereas MAO metabolizes the intraneuronally bound CA.[1, 2] Most of the investigations concerning the isolation and identification of the metabolites of the CA involved radioactive labelled compounds. The availability of tritium-labelled CA with high sp. act's made it possible to administer these compounds in nearly physiological amounts. Usually, the tritium atom of H³-noradrenaline or H³-adrenaline is attached to the β-C-atom of the side chain. In this position the tritium seems to be fairly stable and does not exchange under normal experimental conditions. It has been observed however, by Gitlow *et al.*,[3] that after the administration of *dl*-7-H³-noradrenaline to man increasing amounts of tritium water were detectable in plasma and urine. We made a similar observation in cats.[4]

It seemed of interest to study whether this formation of tritiated water is caused either by an unspecific exchange of the tritium atom or by an enzymatic transformation of the noradrenaline molecule.

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METHODS

Male Wistar rats (200–250 g) were used for all experiments. 100 μc/kg of 7-*dl*-H^3-noradrenaline-HCl (NENCO, Boston, Mass., spec. act. 6·2 c/m-mole) was injected into the tail vein. The animals were kept in a constant temperature room at 24°. 10 min before the operation, the animals were anaesthetized with 1·2 g/kg urethane i.p., and exsanguinated from the carotid artery. The blood (5–8 ml) was collected in plastic tubes containing 0·15 ml heparine solution (Heparin Novo, 5000 i.u./ml). The blood was centrifuged and 2–3 ml plasma were deproteinized with 5 ml 0·4 N perchloric acid. After centrifugation, the clear supernatant was used for the determination of H^3-noradrenaline (H^3-NA), total radioactivity (TA) and the concentration of tritium water (THO).

The hearts were cut out immediately after the collection of the blood, homogenized in icecold 0·4 N perchloric acid and the clear supernatant processed in the same way as the deproteinized plasma.

The radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer. In order to determine the TA, 2 ml of the deproteinized plasma was added to 20 ml of a liquid scintillator[5].

The H^3-NA was isolated by adsorption on aluminium oxide at pH 8·5 and elution with 0·25 N HCl. The individual quenching of the samples was corrected by adding an internal standard.

The deproteinized plasma was distilled at room temperature *in vacuo* in a microevaporator. The radioactivity of the distillates was measured as described above. The specific activities of the distillates remained constant after re-distillation. The distribution of the distilled radioactivity at different pH values between the aqueous phase and several organic phases (butanol, isoamyl alcohol-toluene, ethyl acetate, benzene) corresponded to the values obtained with THO. No radioactive peaks were found after paper chromatography of the distillates in several solvent systems (phenol-HCl butanol-acetic acid-H_2O 4-1-1, isopropyl alcohol-ammonia-H_2O 8-1-1). Therefore it can be concluded that the distillable radioactivity was tritium water.

0·94 Per cent of the radioactivity of the H^3-NA solution injected was THO. All values were corrected by this factor.

Total body water and the biological half life of the body water was determined as follows: groups of 6 animals received 9·16 μc/kg THO i.v. and were killed 4, 32, 64 and 128 hr after the injection. The radioactivity in the plasma was determined as above.

In order to exclude the possibility of tritium water formation as a result of an unspecific exchange of the tritium atom, groups of 6 rats received i.v. 100 μc/kg H^3-NA of decreasing sp. act's. It was not possible to add *dl*-noradrenaline to the labelled H^3-NA in amounts, that decreased considerably the H^3-NA taken up and bound in the tissues, because of the toxicity. Therefore, *d*-noradrenaline was added to these solutions. The controls received intravenously 2·0 μg/100·0 μc/kg of 7-H^3-*dl*-NA, groups 2–4 received *d*-noradrenaline in addition, group 2: 200 μg, group 3: 600 μg and group 4: 1800 μg/kg. The animals were killed 4 hr after the injection, blood and hearts processed as described above. In group 4 only one animal survived.

It seemed of interest to determine whether drugs known to interfere either with the uptake of noradrenaline (NA), such as cocaine or chlorpromazine, or with binding of NA, such as reserpine, have any influence on the formation of tritium water from the administered H^3-NA. The following experiment was performed: groups of 6 animals

were pretreated respectively with (1) 2 mg/kg reserpine (RES) (Serpasil CIBA) i.m. 30 min, (2) chlorpromazine-HCl (CPZ) 20 mg/kg i.m. 30 min, or (3) cocaine-HCl (COC) 10 mg/kg i.v. 10 min prior to the H³-NA injection. The rats were sacrificed 4 hr after the H³-NA injection.

In a further experiment 4 groups of 6 rats were pretreated as follows: (1) pargyline (PARG) 25 mg/kg i.m. 16 hr before the H³-NA injection; (2) pyrogallol (PG) (2 × 200 mg/kg) i.p. 10 min before, and 2 hr after H³-NA; (3) catechol (CA) (2 × 50 mg/kg) i.p. 10 min before, and 2 hr after H³-NA; and (4) diethyldithiocarbamate-NA (DDTC) (800 mg/kg) i.p. 30 min before the H³-NA injection.

The animals were killed 4 hr after the administration of the labelled noradrenaline.

RESULTS

In the first experiment groups of 6 rats received H³-NA and were exsanguinated 15 min, 4 hr, 8 hr, 16 hr, 32 hr, 64 hr and 128 hr respectively after the injection. Fig. 1 shows the exponential decrease of the H³-NA in the hearts, the TA of the plasma and

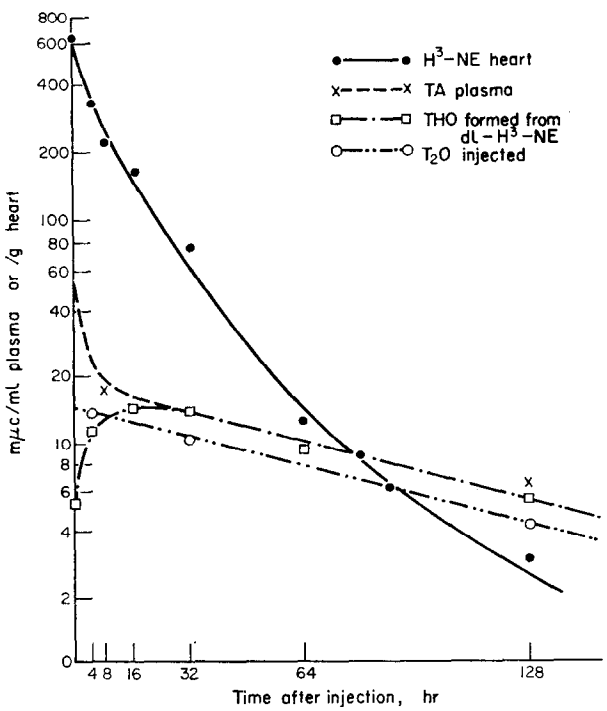

FIG. 1. Groups of 6 rats received i.v. 100 μc/kg *dl*-7-H³-noradrenaline (H³-NA). The animals were killed at different time intervals as indicated on the abscissa. The cardiac H³-NA content and plasma total radioactivity (TA) and tritium water activity (THO) were determined in each group. In a parallel experiment rats were injected with 9·16 μc/kg THO i.v. and the plasma THO concentration determined after different time intervals.

the THO activity in the plasma. The THO concentration of the plasma increased rapidly over the first 8 hr, and continued to increase at a slower rate up to 16 hr following noradrenaline administration. At the same time, the TA of the plasma decreased. 32 Hr after the administration of H³-NA practically all the radioactivity found in the plasma is represented by THO. Only minute, insignificant amounts of H³-NA and

its metabolites could be detected after this time in the plasma. The biological half life ($t_{1/2}$) of the body water, determined by the disappearance of THO from the plasma, was found to be 71 hr in our experiments. The plasma TA and the radioactivity of the plasma distillates after H^3-NA administration decreased from the 32nd hr onwards at the same rate of disappearance as found for THO. This suggests that from this time on there is no detectable formation of THO from the remaining small quantities of H^3-NA.

The total body water, determined 4 hr after the administration of THO by the concentration of THO in the plasma, was found to be 72·3 ± 1·5 per cent of the body wt. in our animals. It can be calculated from the total tritium water formed after the administration of H^3-NA that about 10 per cent of the H^3-NA administered undergoes a transformation in the body, that leads to a loss of the tritium atom attached to the side chain of the NA molecule. Fig. 2 shows the concentration of H^3-NA in the

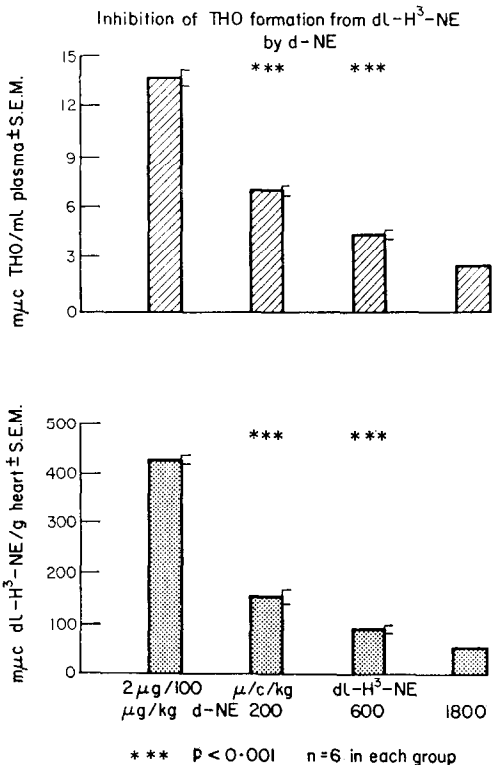

FIG. 2. Groups of 6 rats were i.v. injected with 100 μc/2 μg/kg *dl*-7-H^3-noradrenaline. In addition, group 2 received 200 μg, group 3 600 μg, and group 4 1800 μg unlabelled *d*-noradrenaline simultaneously. In group 4 only one animal survived. The animals were sacrificed 4 hr after the injection. H^3-NA was determined in the heart and THO in the plasma. The columns represent the mean values ± S.E.M. of H^3-NA/g heart and THO/ml plasma.

hearts and the concentration of THO in the plasma of animals that received, in addition to 2 μg/100 μc/kg H^3-NA, increasing amounts of unlabelled *d*-noradrenaline. The addition of the unlabelled CA decreased the proportion of the labelled amine that is taken up and bound in the heart tissue. Concomitantly, the amount of the THO formed from the H^3-NA decreased. An unspecific exchange of the tritium atom would

be independent of the amount of total NA administered, depending only on the amount of H³-NA given. The correlation between the tritium water formed and the amount of H³-NA bound in the tissue implies that the removal of the tritium occurs in the intraneuronal bound fraction of NA. It has been shown that the specific uptake and binding takes place in the sympathetic nerve ending.[6–8] It seemed therefore appropriate to study the influence of drugs known to interfere with the uptake or binding mechanisms of NA, on the formation of THO from H³-NA.

Cocaine and CPZ inhibit the active transport of NA into the sympathetic nerve.[9–11] Reserpine diminishes the binding ability of the intraneuronally-located granules.[12–14] In animals pretreated with COC or CPZ, smaller amounts of NA are taken up into the sympathetic neuron. In reserpine-treated animals the uptake of NA into the nerves is practically not altered, but once taken up the amine cannot be transported into the granules; it leaves the sympathetic nerve, or is metabolized by intraneuronal MAO.[2, 15]

It can be seen in Fig. 3, that the interference of uptake or binding by the drugs also led to a diminished formation of THO from the injected H³-NA.

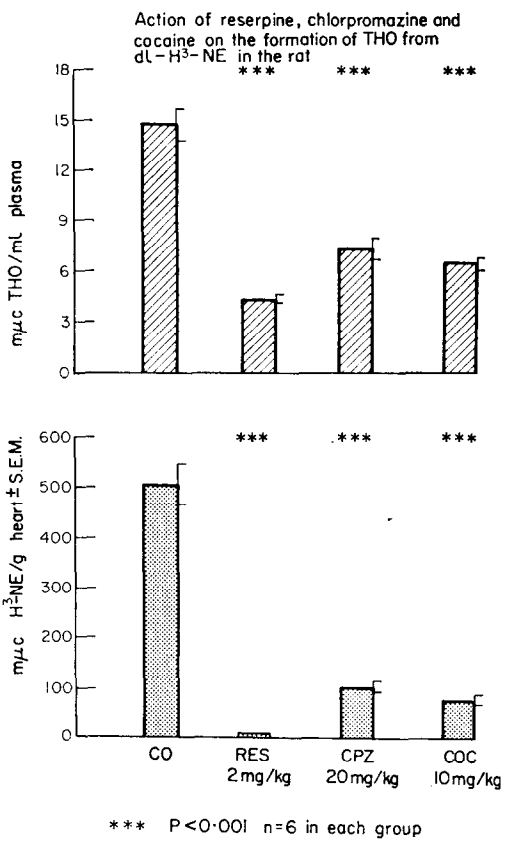

FIG. 3. Rats received 100 μg/kg *dl*-7-H³-noradrenaline i.v. and were killed 4 hr after the injection. Group 2 received 2·0 mg/kg reserpine (RES) and group 3 20 mg/kg chlorpromazine (CPZ) i.m. 30 min prior to the H³-NA injection. Group 4 was treated i.v. with 10 mg/kg cocaine (COC) 10 min prior to the H³-NA injection. The columns represent the mean values ± S.E.M. of H³-NA/g heart and THO/ml plasma.

Whereas the initial uptake of NA after MAO inhibition is unchanged, the rate of disappearance of the bound NA is decreased.[16] Raised H^3-NA tissue levels can be found with MAO inhibition after H^3-NA administration only in later time periods. In our experiments, the cardiac H^3-NA level of animals treated with pargyline, a long-acting MAO inhibitor, was significantly higher than that of the controls. Concomitantly, the THO level was found to be increased in the plasma of the pargyline-treated animals.

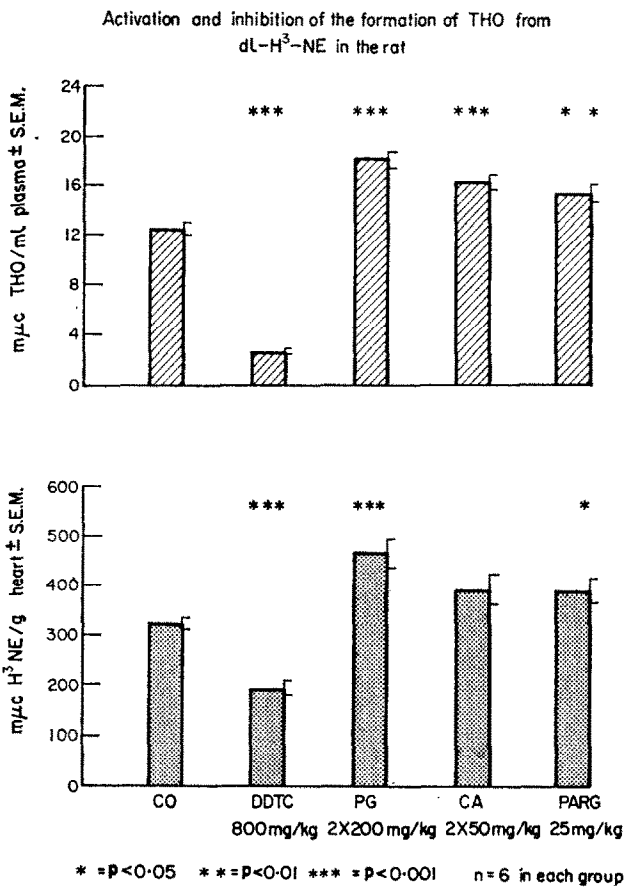

FIG. 4. Groups of 6 rats were injected with 100 μc/kg *dl*-7-H^3-noradrenaline i.v. Group 2 was treated with 800 mg/kg diethyldithiocarbamate (DDTC) i.p. 30 min before the H^3-Na injection. Group 3 was injected twice with 200 mg/kg pyrogallol (PG) i.p. 10 min before, and 2 hr after the H^3-NA injection. Group 4 was injected twice with 50 mg/kg catechol (CA) i.p. 10 min before, and 2 hr after the H^3-NA injection and group 5 was pretreated 16 hr prior to the H^3-NA injection with 25 mg/kg pargyline (PARG) i.m. The animals were killed 4 hr after the H^3-NA injection. The columns express the mean values ± S.E.M. of H^3-NA/g heart and the THO/ml plasma.

COMT is the enzyme responsible for the catabolism of circulating catecholamines. Inhibition of this enzyme increases the concentration, and prolonges the presence of the injected H^3-NA in the blood, and, hence more can be taken up and bound.[1, 16] We used pyrogallol and catechol to block COMT. PG administration resulted in an

increased tissue content of H³-NA, and at the same time the formation of THO was also increased. Although the H³-NA content of the hearts after the pretreatment with catechol appears to be increased, the values are on the borderline of significance. Yet, the formation of THO in this group was significantly increased. It can be assumed that with a larger number of animals the rise in the H³-NA tissue concentration would also become significant.

Diethyldithiocarbamate has been shown by Axelrod[17] to inhibit catechol oxidase, an enzyme that forms adrenochrome. DDTC is known to inhibit a number of enzyme containing copper. In our experiments DDTC strongly reduced the formation of THO in the plasma following the injection of H³-NA. The tissue concentration of H³-NA were also decreased by DDTC. Some of the results have been presented at the spring meeting 1967 of the German Pharmacological Society and are published in the proceedings of this meeting.[18]

DISCUSSION

There are several possibilities regarding the formation of THO from H³-NA labelled on the β-atom of the side chain. It could be an unspecific exchange of a labile H³-atom, that exchanges with free H^+ of the tissue water. This can be excluded from the findings of experiment 2, in which increasing amounts of unlabelled NA decreased

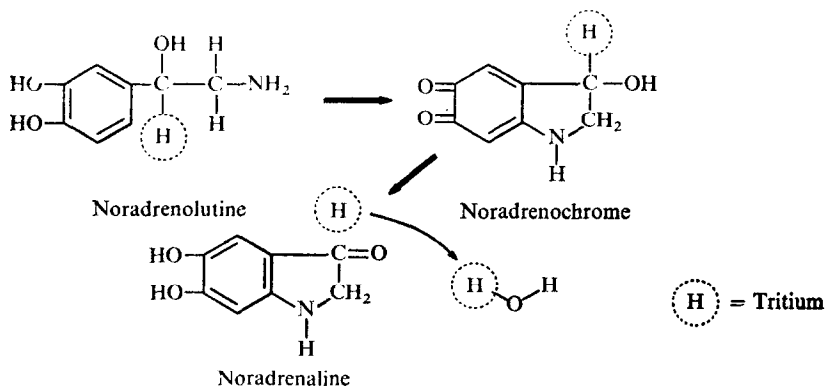

FIG. 5. A schematic representation of the formation of THO from 7-H³-noradrenaline over the proposed oxidative pathway: noradrenaline-noradrenochrome-noradrenalutine.

the THO concentration of the plasma, although all animals received equal amounts of tritium-labelled NA. Correspondingly, the tissue content of H³-NA was also decreased, indicating that the locus of THO formation is inside the sympathetic nerves. In the case of an unspecific exchange no differences between the groups would be expected.

Another possibility would be the conversion of NA to vanillic acid.[19] Here, tritium on the β-C-atom would be split off. Since the inhibition of MAO oxidase did not prevent THO formation, this minor metabolic pathway of NA seems unlikely to be responsible for the THO yielded in our experiments.

The oxidation of the OH-group of the side chain would result in the compound noradrenolone. Here also, the tritium would be split off. If this process were reversible,

a certain loss of the radioactive label would occur without detectable new metabolites. We cannot exclude this possibility, but this reaction is not known to occur with catecholamines *in vivo* or *in vitro* in biological media

Throughout the last decades, the oxidation of the catecholamines to adrenochrome or noradrenochrome has been repeatedly put to discussion.[20, 21] These compounds have never been identified with certainty *in vivo*. *In vitro*, however, Axelrod recently described an enzyme present in the supernatant of many tissue homogenates, called catechol oxidase, that was capable of oxidizing adrenaline, noradrenaline and dopamine to the respective oxidized products The further oxidation of these compounds was prevented by adding β-phenylisopropyl hydrazine to the incubation mixture to form a stable hydrazone. The hydrazones were then extracted into toluene-isoamyl alcohol and their activities determined. Axelrod failed to find any adrenochrome formed *in vivo*.[17]

If NA were to undergo a transformation into noradrenolutine via noradrenochrome the tritium atom would be also removed. At the moment this catabolic process seems to us the most probable pathway for the formation of THO.

The highest activity of catechol oxidase was found in the salivary glands in Axelrod-S *in vitro* experiments.[17] A small amount of activity was also found in the skin and in the lungs, but practically no activity was found in the heart. In some of our preliminary experiments parallel estimations of THO were made in the heart and in the plasma at different time intervals after the administration of H^3-NA. The THO content of the heart was always the same as the THO concentration of the plasma, if both values were corrected for the water content of the heart tissue and plasma. We consider it likely that the THO of the heart tissue came into the heart by exchange of the tissue water with the plasma water. It is not possible to conclude from our experiments in what organs the conversion of NA to noradrenolutine via adrenochrome takes place *in vivo*. Only minute amounts of i.v. administered NA pass the blood–brain barrier. It can therefore be concluded that the THO found in our experiments originated mainly from peripherial tissues. It cannot be said if such a metabolic pathway exists in the central nervous system.

In our experiments the heart served the purpose of showing the correlation between the ability of a tissue to take up and retain H^3-NA and the formation of THO in the peripheral tissues. Drugs that interfere with the uptake of NA like chlorpromazine or cocaine also decrease the amount of THO formed. Reserpine interferes with the ability of the tissue to retain NA, probably by an inhibition of the transport of NA into the granules.[12–14]

In reserpine-pretreated animals, the time over which NA remains inside the nerve endings is very much shortened. In our experiments the reserpinized animals also showed a markedly decreased formation of THO. In animals treated with MAO blockers the turnover of NA is slowed down. The H^3-NA that has been taken up remains for a longer period of time inside of the nerve endings. In these groups also the formation of THO was significantly higher than in control animals. When COMT was inhibited in rats more H^3-NA was taken up and bound at the nerve endings. In these groups also a higher THO formation could be observed.

The formation of THO is dependent on the presence of H^3-NA inside the sympathetic nerves. It can therefore be concluded that this metabolism takes place inside the sympathetic nerve. This is also supported by the correlation between the decrease in the

H^3-NA tissue concentration and the rate of THO formation following the H^3-NA injection.

The inhibition of this pathway by DDTC indicates, that the enzyme involved may contain copper. It is, however, not clear why at the same time the amount of H^3-NA found in the tissue was decreased. This finding remains to be clarified and is at present under investigation, as well as the isolation and identification of the presumable metabolites involved in the process of the THO formation.

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