Extraction of brain radioactivity (^{3}H) by glutaraldehyde fixation 3 h after intravenous injection of tritiated norepinephrine (NA-^{3}H) in newborn rats pretreated or not with enzymatic inhibitors

	Total ^{3}H (dpm/mg)	NA-^{3}H (%)	^{3}H extracted by fixative (%)
Controls	5,717 ± 457 (12)	4.5 ± 0.5 (3)	70.2 ± 2.6 (9)
IMAO + ICOMT	13,265 ± 1,144 (11)	72.6 ± 1.3 (4)	44 ± 3.5 (7)

Mean values ± S.E.M. Number of rats in each group given in brackets. All differences between control and pretreated rats significant with $p < 0.001$ (Student's test).

Estimations of NA-^{3}H in unfixed brains indicate that the amine itself accounts for 72.6% of the radioactivity in pretreated rats, whereas, in control rats, less than 5% of total brain ^{3}H persists as NA-^{3}H. The extraction of radioactivity during immersion of brain slices in glutaraldehyde, taking place almost entirely within the first two baths of fixative, is significantly different in the two groups: it represents 44% versus 70% of total brain ^{3}H, in pretreated and control rats respectively.

In control rats, however, despite the fact that 95% of the radioactivity is in the form of labeled metabolites of NA-^{3}H, the extraction by the glutaraldehyde fixative amounts only to 70% of total brain ^{3}H. This confirms that glutaraldehyde has a capacity to bind some metabolites of NA-^{3}H to nervous tissue, as previously revealed by radioautographs of the rat brain, which exhibited a diffuse reaction 3 h after intraventricular administration of normetanephrine-^{3}H[3]. Conversely, in pretreated rats, slightly more radioactivity is removed from brain slices than can be accounted for by the labeled metabolites alone. Such a small loss of NA-^{3}H could have been due to slow penetration of glutaraldehyde, during a fixation procedure carried out by immersion rather than vascular perfusion[13].

From these results, it may be inferred that at least 75% of NA-^{3}H is retained in brain tissue after glutaraldehyde fixation. This figure is in agreement with previously reported estimates derived from experiments where the exact nature of the radioactivity present in nervous tissue could not be precisely determined at the time of fixation[14, 15]. The data also emphasize the value of glutaraldehyde as a primary fixative for the preservation of NA-^{3}H, as opposed to paraformaldehyde or ice cold $KMnO_4$ solutions, which extract much greater proportions of this tritiated amine from nervous tissue[15–17,3].

The preferential extraction of NA-^{3}H metabolites by glutaraldehyde fixation, demonstrated in the present investigation, had already been suspected in earlier radio-isotopic[9] and radioautographic[18,14] studies. It is likely that deaminated metabolites constitute the main component of the extractable radioactivity, since they lack the amino group presumably responsible for the binding of catecholamines to glutaraldehyde in vitro[19].

Since the greater fraction of NA-^{3}H removed from nervous tissue during the elaborate preparative sequences required for electron microscopy or high-resolution radioautography appears to be lost in the primary fixative[9, 15], two properties of glutaraldehyde fixation will concur to the in situ preservation of this exogenous amine within brain: retention of a major proportion of NA-^{3}H itself, and preferential extraction of its labeled metabolites[20].

Résumé. Trois heures après une injection i.v. de noradrénaline tritée (NA-^{3}H) chez le rat nouveau-né, moins de 5% de la radioactivité mesurée dans le cerveau correspond à cette amine marquée. Par contre, lorsque la monoamine oxydase et la catéchol-*O*-méthyl transférase sont inhibées, la NA-^{3}H constitue 73% de la radioactivité cérébrale. Dans ces conditions, l'extraction de la radioactivité par la fixation au glutaraldéhyde 3.5% dans le tampon phosphate diffère également de façon significative et indique qu'une fraction majeure de la NA-^{3}H est liée au tissu par le glutaraldéhyde, tandis que les métabolites marqués sont préférentiellement extraits.

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Stereospecificity of Oxotremorine Antagonists

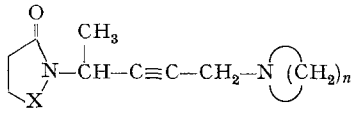
Previous work in our laboratories has shown that a number of N-(*tert*-aminoalkynyl)-substituted succinimides and pyrrolidones are rather potent in blocking the motor effects of the muscarinic agent oxotremorine, 1-(2-oxopyrrolidino)-4-pyrrolidino-2-butyne, while the effects on peripheral cholinergic symptoms, such as mydriasis and acetylcholine-induced spasms of guinea-pig ileal strips, are of lower magnitude[1–6]. Consequently, these compounds can be regarded as anticholinergic agents with selectivity for the central nervous system.

Physical and pharmacological data for the enantiomers of compounds I–III

Compound	Derivative	Melting point (°C)	$[\alpha]_D^{20}$ [a]	In vivo dose (μmoles/kg) in mice required to produce Oxotremorine blockade [b]	Mydriasis [c]	Acetylcholine antagonism on isolated guinea-pig ileum (pA_2)
(R)-(+)-I	Sesquioxalate	125–127	+ 80.7	0.26	1	6.9
(S)-(−)-I		124–126	− 80.4	[d]	95	[d]
(±)-I				0.51	2	6.5
(R)-(+)-II	Base	73–75	+ 58.3	0.48	2	7.0
(S)-(−)-II		73–75	− 61.3	28	75	[d]
(±)-II				0.76	3	6.0
(R)-(+)-III	Perchlorate	191–192	+ 37.5	0.65	2.5	6.8
(S)-(−)-III		191–192	− 38.0	[d]	[d]	4.7
(±)-III				1.3	3.6	6.6
Atropine				2.8	0.29	8.8

[a] All rotations were measured in ethanol (c 0.6–1.0). [b] Dose of test compound required to double the dose of oxotremorine inducing a grade 2 tremor in 50% of the mice. [c] Dose of test compound required to double the pupil size relative to the control. [d] Very weak effect, not possible to evaluate.

The most active compounds all have the same intermediate chain connecting the 2 nitrogens in the molecule. As this chain contains an asymmetrically substituted carbon atom, we decided to prepare the enantiomers of compounds I–III in order to study their stereospecificity.



I X = CH_2 $n = 4$
II X = C=O $n = 4$
III X = C=O $n = 6$

Since we used a common intermediate, 1-methyl-2-propynylamine, in the preparation of compounds I–III, we preferred to perform the resolution step at this stage of the synthetic procedure. 1-Methyl-2-propynylamine has been resolved by MARSZAK-FLEURY[7] using (+)-bromocamphorsulphonic acid and (+)-tartaric acid. We resolved the amine into its (+)- and (−)-enantiomers by fractional crystallization of its (+)- and (−)-tartrate, respectively. In addition to the usual measurement of the optical rotation, we found it convenient to follow the resolution process by NMR-spectroscopy, using the anisochronous signals from the diastereotopic groups of the two diastereomeric amides, formed when optically impure amine is acylated with (−)-*O*-methylmandelyl chloride.

The two enantiomers were characterized as their benzoyl derivatives, which melted at 89°–90 °C. The (+)-amine gave a benzamide with $[\alpha]_D^{20}$ +42.6° (*c* 1, ethanol); for the benzamide of the (−)-amine the corresponding value was −42.9°. The enantiomers of the resolved amine were transformed to the enantiomers of compounds I–III according to methods described for the racemates[2,5,6]. The sign of rotation remained unchanged throughout the reaction sequences.

The 6 optically active compounds and the corresponding racemates were tested in mice for antagonism towards tremor induced by oxotremorine, and for mydriatic activity, according to methods described previously[3,5] and the dose required to produce oxotremorine blockade and mydriasis was determined. They were also investigated for antagonistic activity towards acetylcholine on isolated guinea-pig ileum preparations[5]. The results of these in vitro tests are expressed as pA_2 values[8]. The physical data for the enantiomers and the results of the pharmacological tests are summarized in the Table which also contains atropine as a reference compound.

It is evident from the Table that the (+)-isomers are considerably more active than their enantiomers. In several tests the (+)-isomers were about twice as active as their corresponding racemates, whereas the (−)-isomers were practically inactive. It is of interest to note that the compound (+)-II was 10 times more active than its racemate in antagonizing the effects of acetylcholine-induced contractions of guinea-pig ileum, but only about 1.5 times as active as its racemate in producing oxotremorine blockade and mydriasis in mice. This phenomenon will be the subject of further investigations.

As the 3 (+)-enantiomers were all prepared from (+)-1-methyl-2-propynylamine, we were interested in establishing the absolute configuration of this amine. The corresponding saturated amine, 1-methylpropylamine was chosen as the reference compound. Upon catalytic hydrogenation (+)-1-methyl-2-propynylamine

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afforded (R)-(−)-1-methylpropylamine, the absolute configuration of which has been established by several workers[9,10]. Thus, (+)-methyl-2-propynylamine can be ascribed the R configuration.

Zusammenfassung. Die optischen Antipoden der 3 sehr wirksamen Oxotremorinantagonisten, N-(1-Methyl-4-pyrrolidino-2-butinyl)pyrrolidon, N-(1-Methyl-4-pyrrolidino-2-butinyl)succinimid und N-(1-Methyl-4-perhydroazepino-2-butinyl)succinimid, sind hergestellt und auf ihre pharmakologische Aktivität geprüft worden: Die (+)-Antipoden, die R-Konfiguration haben, wurden als Träger der Aktivität erkannt, während die (−)-Antipoden praktisch unwirksam sind.

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Increase in Monoamine Concentration in Rat Brain Following Melatonin Administration

Melatonin is a hormone which is secreted from the pineal gland of mammals in relation to illumination[1]. Previously, Anton-Tay, et al.[2] have shown that melatonin administered i.p. significantly increases brain serotonin concentration, but this change was not associated with changes in norepinephrine content. We decided to investigate the possibility that melatonin may alter brain norepinephrine and dopamine concentrations when administered by methods where sufficient brain levels of melatonin could be achieved prior to its metabolism to 6-hydroxymelatonin by the liver[3]. To accomplish this, melatonin was injected into the common carotid artery or the cisterna magna of rats.

Materials and methods. Male Sprague-Dawley rats (150–200 g) maintained on a 12-h light-dark cycle, 3 to a cage, with free access to Purina Rat Chow and water, were used in these experiments.

Rats to be injected intraarterially were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). A ventral midline incision of approximately 1 cm was made, and the left common carotid artery was exposed. A cannula was inserted into the artery toward the brain, secured with sutures and the incision closed. Following a 24-h recovery period, rats were injected with either melatonin in saline or saline only. All injections were made between the hours of 10.00 and 12.00.

Rats to be injected intracisternally by the method of Schanberg et al.[4], were lightly anesthetized with ether and given specified amounts of melatonin (Sigma Chemical Company, St. Louis, Mo.) dissolved in 10 µl of distilled water. Control rats were injected with 10 µl of water only.

Rats were decapitated at the time specified after injection. Brains were removed and immediately frozen, halved through the mid-saggital plane, homogenized in 0.4 *N* perchloric acid containing disodium ethylenediaminetetraacetate and norepinephrine and dopamine content determined by the method of Shellenberger and Gordon[5].

Results and discussion. Cotzias et al.[6] found the i.p. injection of melatonin (400 mg/kg) in mice did not result in significant alterations in brain dopamine content. Anton-Tay et al.[2] reported that the i.p. administration of melatonin (2.8 mg/kg) did not alter rat brain norepinephrine concentrations. However, both of the above investigators did report a melatonin induced increase in brain serotonin levels[2,6].

In our experiments, melatonin was given by intraarterial injection and intracisternal injection; both routes of administration led to significant increases in rat brain dopamine and norepinephrine content. The intraarterial

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Effect of melatonin on rat brain catecholamines when administered by intraaterial or intracisternal injection[a]

		Dopamine (µg/g)	Norepinephrine (µg/g)
Intraarterial (controls)	T = 0[b]	0.71 ± 0.03	0.49 ± 0.01
	T = 1 h[c]	0.65 ± 0.03	0.52 ± 0.03
Intraarterial melatonin (250 µg/kg)	T = 0	0.75 ± 0.02	0.45 ± 0.03
	T = 1 h	1.40 ± 0.09[d]	0.68 ± 0.03[d]
Intracisternal (controls)	T = 0	0.64 ± 0.03	0.39 ± 0.01
	T = 1 h	0.76 ± 0.04	0.46 ± 0.03
Intracisternal melatonin (40 µg/kg)	T = 0	0.67 ± 0.08	0.45 ± 0.02
	T = 1 h	1.02 ± 0.06[d]	0.67 ± 0.03[d]

[a] $n = 4$ rats/group. [b] T = 0 are rats sacrificed immediately following injection. [c] T = 1 h are rats sacrificed 1 h following injection. [d] significantly different from appropriate controls as well as T = 0 melatonin treated rats ($p < 0.05$).