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N-Nitrosospermidine: the principal nitrosation product of spermidine[1]

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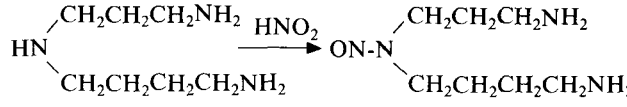
Summary. N-(4-Aminobutyl)-N-(3-aminopropyl)nitrosamine (N-nitrosospermidine) was identified as a major product in the reaction between spermidine and nitrous acid. N-Nitrosospermidine was not significantly mutagenic in the Ames assay.

The carcinogenic N-nitroso compounds[2] have not only been detected in foods, but demonstrated to be formed from nitrite and N-alkyl compounds in vitro with human gastric juice, as well as in vivo in animals[3]. A large number of N-alkyl compounds, which yield N-nitroso compounds on reaction with nitrous acid, are known to be present in foodstuffs[4]. Among them, polyamines, spermidine and spermine, have secondary amino groups, and are normal constituents of various kinds of foods[5]. The investigation of nitrosation products of polyamines has been limited to the identification of nitrosamines produced by the reaction of polyamines with a large excess of nitrous acid[6]; however, the studies on the reaction of polyamines with a limited amount of nitrous acid seems important for the evaluation of the possible biohazards caused by the N-nitroso compounds derived from polyamines. In this paper, the results of identification and mutagenicity studies on a major N-nitroso compound formed by the reaction of spermidine with a limited amount of nitrite are presented.

At pH 3.5, 50 mM [tetramethylene-1,4-^{14}C]-spermidine (New England Nuclear, Boston, Mass.) was reacted with 65 mM sodium nitrite for 16 h at room temperature. The major N-nitroso compound formed by the reaction was isolated from the reaction mixture by ion-exchange chromatography (SP-Sephadex, linear gradient elution with pyridinium acetate buffer, pH 5.0, from 0.1 M to 1.5 M), followed by cellulose column chromatography (isopropylalcohol - acetic acid - water, 4:1:1). The product, a diamino compound as judged by the elution position in the ion-exchange chromatography, was homogeneous in thin-layer chromatography (table 1). The overall yield of the N-nitroso compound was 22.5%, which was calculated from the radioactivity recovered in the purified product.

The absorption spectrum of the product [$\lambda_{max}^{H_2O}$ nm (ε): 232 (7100), 343 (87)] agreed closely with those of dialkylnitrosamines[7]. Removal of the nitroso group from the nitroso compound by treatment with hydrogen bromide in glacial acetic acid[8] yielded spermidine as the sole product (table 1). From these results, the structure of the nitroso compound was identified as N-(4-aminobutyl)-N-(3-aminopropyl)nitrosamine (N-nitrosospermidine).



Spermidine N-Nitrosospermidine

The content of N-nitrosospermidine in the reaction mixture described above was found to be 27% of the starting material by use of the reversed isotope dilution method. Since 40% of the spermidine used for the reaction was recovered unchanged from the reaction mixture, the yield of N-nitrosospermidine corrected for unchanged spermidine was 45%. Therefore, N-nitrosospermidine is the major product of the initial reaction between spermidine and nitrous acid.

At pH 3-4, 100 mM spermidine and 120 mM sodium nitrite were allowed to react for 16 h at room temperature. A 200-µl aliquot of the reaction mixture, which was free from nitrite, was assayed for bacterial mutagenicity as described by T. Yahagi et al.[9] using *Salmonella typhimurium* TA1535, TA1537, TA98 and TA100 as the tester strains. In the absence of 9000×g supernatant of rat liver homogenate

Table 1. Thin-layer chromatography of spermidine and its derivatives

	R_f-values i-PrOH–conc. HCl–H_2O (8:3:2)	i-PrOH–acetic acid–H_2O (4:1:1)	n-BuOH–conc. HCl–H_2O (7:2:1)
N-Nitrosospermidine	0.43	0.25	0.19
N-Nitrosospermidine treated with HBr	0.23	0.08	0.08
Spermidine	0.23	0.07	0.09

Samples were chromatographed on thin-layer plates of microcrystalline cellulose (Avicell SF, Funakoshi Pharm. Co. Ltd., Tokyo). Radioactive spots were located by autoradiography and amine spots by ninhydrin spray.

Table 2. Mutagenic effect of the product of spermidine-nitrite interaction

Sample	His+ revertants/plate							
	TA 1535		TA 1537		TA 100		TA 98	
	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
None	14	11	10	11	88	88	21	29
100 mM $NaNO_2$	155	171	15	9	130	111	30	39
100 mM spermidine	16	17	-	-	111	87	-	-
100 mM spermidine + 120 mM $NaNO_2$	112	91	19	20	396	108	65	33

A 200-µl aliquot of the sample was preincubated with bacterial cells in 0.1 M phosphate buffer, pH 7.4 (−S-9) or in S-9 mix (+S-9) for 20 min at 37 °C and then deposited in a soft agar overlay on minimal medium according to the procedure of Yahagi et al.[9]. Each value represents the mean of the numbers of revertant colonies on 2 plates after 48 h of incubation at 37 °C. −, Not determined.

prepared from PCB-pretreated rats (S-9), positive results were obtained with strains TA1535, TA100 and TA98 (table 2). In the presence of S-9, positive results were obtained with strain TA1535. Similar results were recently reported by Kokatnur et al.[10]. Using TA 1535 and TA100, N-nitrosospermidine was assayed in a range from 7.5 to 30 µmoles per plate. No significant mutagenicity was observed. The weak direct-acting mutagenicity found in the reaction mixture might be attributed to the reaction product(s) other than N-nitrosospermidine.

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Increase in in vivo (^{3}H) spiperone binding in the rat hippocampal formation and striatum after repeated treatment with haloperidol

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Summary. An increase in in vivo (^{3}H) spiperone binding was observed in rat hippocampal formation and striatum after repeated treatment with haloperidol. This suggests that in hippocampus as well as in striatum prolonged blockade of dopaminergic transmission by a neuroleptic agent results in the development of a supersensitivity of the dopamine receptors.

Recent biochemical[2-5] and electrophysiological[6] studies have provided evidence for a dopaminergic innervation of the mammalian hippocampal formation originating from the substantia nigra and ventral tegmental area[7,8]. Moreover, the recent demonstration of specific (^{3}H) spiperone hippocampal binding sites selectively inhibited by dopamine (DA) agonists and antagonists[3,4] suggests the existence of a discrete population of DA receptors in the rat hippocampal formation. This view was supported by the increase in hippocampal dihydroxyphenylacetic acid (DOPAC) levels induced by acute administration of neuroleptics[2,3,9]. Repeated treatment with neuroleptic agents is known to result in an increase in the number of striatal DA receptors in rat[10] and mouse[11], probably as a compensatory response to the sustained blockade of dopaminergic transmission. It was therefore of interest to study whether a similar phenomenon may also occur in the hippocampal formation. For this purpose, we have investigated the effects of repeated treatment with haloperidol on in vivo (^{3}H) spiperone binding in rat striatum and hippocampal formation.

Methods. Male rats [Tif:RAI f (SPF) weighing 120–130 g at the beginning of the experiment] were injected s.c. daily for 10 or 21 days with haloperidol (1 mg/kg). Control animals were similarly treated with saline. In vivo radioreceptor assay was performed 4–12 days following the last injection of the drug. 12 µCi (^{3}H) spiperone (sp.act. 39 Ci/mmole, NEN) was administered i.v. and the animals were killed 2 h later. Specific (^{3}H) spiperone binding was measured as previously described[4].

Results. As shown in the table repeated treatment with haloperidol for 10 days followed by a 4-day washout period produced an increase of (^{3}H) spiperone binding in both hippocampus and striatum; however, this effect was statistically significant only in the former region. In vivo (^{3}H) spiperone binding was increased significantly by more than