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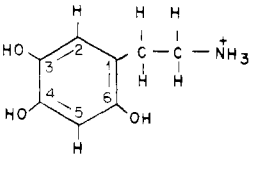
Nuclear Magnetic Resonance Studies of 6-Hydroxydopamine and Its Interactions with SH-Containing Model Compounds. Evaluation of Possible Mechanism for Neurocytotoxicity†

Joseph Granot and Avner Rotman*,‡

ABSTRACT: 6-Hydroxydopamine (I) is a well-known neurocytotoxic agent which has become an important tool in many neurochemical studies in recent years. Biochemical investigations of the mechanism of action of 6-hydroxydopamine indicated that this amine binds covalently and irreversibly to proteins. In the present work, molecular properties of 6-hydroxydopamine in aqueous solution such as self-association, ionization, intramolecular conformations, and possible cyclization were investigated using ^{1}H nuclear magnetic resonance spectroscopy. A model study for the interaction of 6-hydroxydopamine with proteins was undertaken by using SH-containing molecules: cysteine, glutathione, and bovine serum albumin. The binding of these compounds to 6-hydroxydopamine was found to cause labilization of the hydrogen attached to C_2 of the amine aromatic ring. This effect was interpreted in terms of nucleophilic attack of RS^- on C_1 of 6-hydroxydopamine. A proposed model for neurocytotoxicity is discussed.

The compound 6-hydroxydopamine (6-OHDA;[1] 2,4,5-trihydroxyphenylethylamine; I) is well-known for its selective neurotoxic action on catecholamine-containing neurons in both the peripheral and central nervous systems (Kostrzewa & Jacobowitz, 1974). This selectivity of the cytotoxic action is associated with effective uptake transport and accumulation of this compound within catecholamine neurons by the axonal amine "membrane pump", whereas the neuronal degeneration effects seem to relate to the ease of the autoxidation of 6-OHDA (Lundström et al., 1973; Sachs et al., 1975; Creveling et al., 1975). These properties have made 6-OHDA a valuable denervation tool in experimental studies in the central and the peripheral nervous system (Malmfors & Thoenen, 1971; Jonsson et al., 1975).



I

Numerous data have been published concerning the mechanism of action of 6-OHDA (Rotman, 1978). It is known that, for nerve degeneration to occur, accumulation of 6-OHDA in the nerve terminals up to a critical concentration is required (Blank et al., 1972) and that autoxidation is also a prerequisite (Jonsson & Sachs, 1975). However, the specific steps resulting in the cytotoxicity are still obscure. Two possible

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[1] Abbreviations used: ^{1}H NMR, proton magnetic resonance; 6-OHDA, 6-hydroxydopamine; BSA, bovine serum albumin.

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mechanisms have been proposed: one relates cytotoxicity to the rapid generation and injurious effects of hydrogen peroxide and/or the superoxide radical anion formed during intraneuronal oxidation of the amine (Cohen & Heikkila, 1974; Heikkila & Cohen, 1972); the other suggests that the covalent binding of one or more of the oxidation products of the amine with neuronal proteins results in a destruction of essential macromolecular constituents (Saner & Thoenen, 1971; Rotman et al., 1975, 1976; Rotman, 1978; Jonsson, 1976). Most of the binding studies were done by measurements of the covalent binding of radioactive 6-OHDA to macromolecules in different systems such as proteins (mainly bovine serum albumin), neuroblastoma cells or rat and mouse hearts.

It is the purpose of the present work to provide a basis, on the molecular level, for elucidation of the cytotoxic action of 6-OHDA. Proton NMR spectroscopy was applied to study several relevant chemical properties of 6-OHDA in aqueous solution, as well as its interactions with sulfhydryl-containing model compounds such as cysteine, glutathione, and bovine serum albumin. The results were interpreted in terms of a possible mechanism for cytotoxicity.

Experimental Section

Materials. 6-OHDA[1] hydrobromide salt and BSA were obtained from Sigma Chemical Co. L-Glutathione (reduced) and cysteine were purchased from Fluka. The *p*-quinone of 6-OHDA was prepared according to the method of Wehrli et al. (1972). Solutions were made by dissolving the materials either in doubly distilled water or in deuterium oxide (99.7%). The concentrations used were 0.06–0.15 M for 6-OHDA and 0.1–0.3 M for the amino acids. pH values were adjusted by the addition of concentrated acid or base solutions and are given as the pH meter readings without correction for isotope effects in the deuterated solutions.

NMR Spectra. ^{1}H NMR spectra were recorded on a Bruker HFX-10 spectrometer operating at 90 MHz with an internal deuterium lock. Ten percent D_2O was added to water solutions for locking. A trace of dioxane or *tert*-butyl alcohol in the experimental solutions served as internal reference for shift measurements. Upfield shifts (expressed in Hz) are denoted by positive signs. The experimental error in the chemical shifts is estimated as ±1 Hz. Proton spin coupling constants were measured on a Bruker WH-270 spectrometer, equipped with a Nicolet Model 1180 32K computer, operating at 270 MHz in the Fourier transform mode. All measurements were performed at an ambient probe temperature of 27 °C.

Assignment of the ^{1}H NMR Resonances. The spectrum of 6-OHDA (I) consists of two multiplets in the aliphatic region and two singlets in the aromatic region. As previously described (Granot, 1976), the low-field multiplet in the aliphatic region was assigned to the $H_{\alpha\alpha'}$ resonances, and the high-field multiplet to $H_{\beta\beta'}$. As for the aromatic resonances, chemical shift calculations using the shielding parameters of Lambert et al. (1975) yielded a lower resonance field for H_2 relative to H_5. This assignment was further confirmed by homonuclear irradiation of the H_α resonances. The decoupling of the H_α–H_5 spin–spin coupling resulted in an increase in the intensity of the high-field signal in the aromatic region, in agreement with its assignment as H_5.

Results and Discussion

Characterization of 6-OHDA Properties in Aqueous Solutions

Self-Association. Chemical shifts of the resonances of 6-OHDA were measured as a function of its concentration, in the range 0.014–0.34 M, and in the pH region 5.0–7.5. No shifts were observed, within experimental error, throughout the whole concentration range. As self-association between aromatic molecules results in upfield shifts of the proton resonances, the above finding indicates that 6-OHDA does not self-associate in aqueous solution. Similar observations were also obtained with dopamine and other catecholamines (Granot & Fiat, 1977).

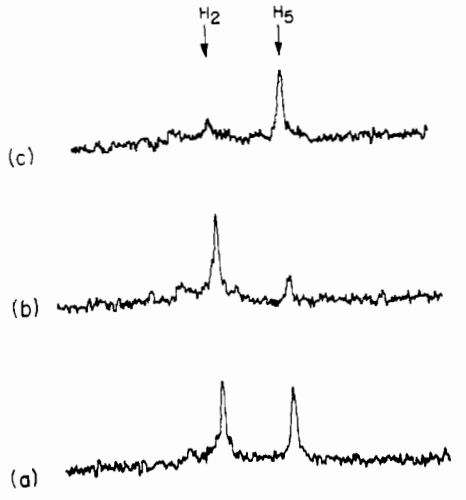

FIGURE 1: ^{1}H NMR spectra of the aromatic region of 6-OHDA (0.1 M) in D_2O solution. (a) Free in solution, immediately after dissolution at pH 7.5. (b) Solution a, after 30 min. (c) 6-OHDA with added cysteine (0.15 M) at pH 8.5, immediately after dissolution.

Lability of Aromatic Hydrogens. Dissolution of 6-OHDA in D_2O was followed by significant decrease, not involving broadening, in the intensity of the H_5 signal (Figure 1b), which was time and pH dependent. The chemical shifts in the 6-OHDA resonances were found to be insignificantly altered during this process. The rate of change in the H_5 amplitude was found to be quite slow, with a half-time of about 1.5 h at pH 8.5, and decreasing rate with increasing acidity of the solution. At pH 3 the half-time was found to be about 11 h. Acceleration of the process was achieved by bubbling oxygen gas through the solution. The H_2 resonance was found to remain relatively unmodified and actually no significant effect on its integrated intensity could be observed, even in the presence of O_2, for long periods of time (12–24 h). Repeating the experiments, under the same conditions but in H_2O solutions, yielded no effect at all on either of the aromatic resonances. Evidently, the observed effect in D_2O arises from exchange between the H_5 proton of the 6-OHDA molecules and the solvent deuterons. A possible origin for the lability of H_5 is the formation of β-diketone (IV), through the intermediate oxidation forms, the *p*-quinone and the less abundant *o*-quinone (II and III, respectively, in Figure 2). These quinone products of 6-OHDA are readily formed in neutral or basic aqueous solutions (autoxidation of 6-OHDA may start quantitatively at pH ~7). However, even the presence of small amount of oxidized 6-OHDA at acidic pH can account for the slow exchange of H_5 in D_2O solution observed at this pH region. The rate of this process increases with pH and in the presence of molecular oxygen since these factors control the amounts of forms II and III. A phenomenon as described above was not observed with dopamine, evidently due to the lack of possible quinone forms such as II and III.

It is noteworthy that no significant difference in the chemical shifts was observed by comparing the ^{1}H NMR spectra of

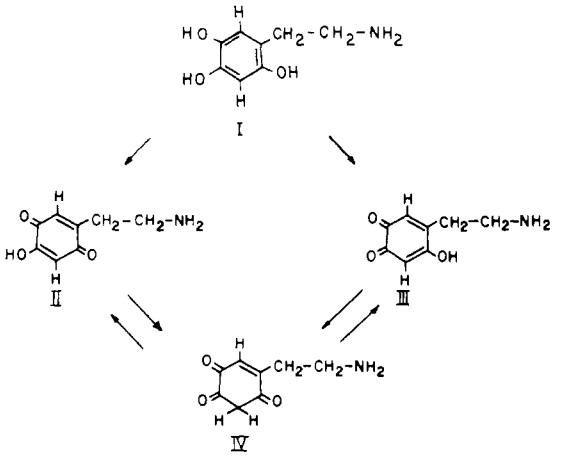

FIGURE 2: Ketoenol tautomerization of 6-OHDA oxidation products. Proposed mechanism for H_5 lability.

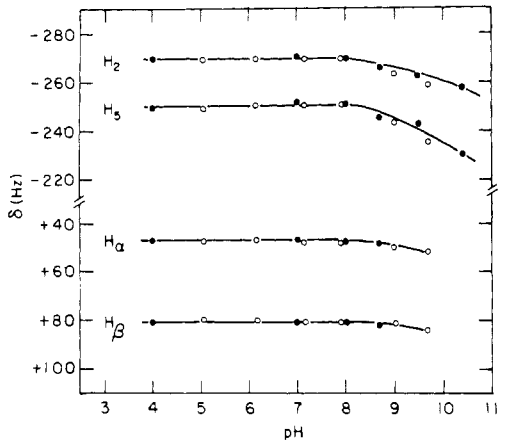

FIGURE 3: pH dependence of the proton resonances of 6-OHDA in H_2O (○) and D_2O (●) solutions. The shifts are measured relative to dioxane. Negative shifts are to low field.

6-OHDA and its *p*-quinone. A small shift difference (of ca. 6 Hz) was found between the resonance of hydroquinone and benzoquinone. It appears that the formation of quinone forms does not alter profoundly the ^1H NMR spectra of the parent compounds. These observations imply that the absence of significant effect on the chemical shifts of 6-OHda upon the H–D substitution of H_5 does not necessarily indicate that only a minor fraction of IV is formed.

Cyclization. Intramolecular cyclization of oxidized 6-OHDA to form 5,6-dihydroxyindole may occur in aqueous solutions (Harley-Mason, 1953; Powell & Heacock, 1972). Such a process, of transferring aliphatic hydrogens to cyclic forms, should cause a marked effect on the ^1H NMR spectrum of 6-OHDA. However, inspection of spectra of oxidized 6-OHDA (at pH 7–8) for several hours revealed no significant change, implying, in agreement with previous observations (Powell & Heacock, 1972), that the cyclization process is very slow.

Acid Ionization. 6-OHDA has two major ionization sites: the three-ring hydroxyls and the protonated ammonium group. Chemical shifts of the proton resonances of 6-OHDA in D_2O and H_2O solutions were measured as a function of pH. Sodium sulfite was added to the solutions to prevent rapid oxidation. The results are shown in Figure 3. Line broadening and decrease in the signal intensities, due to precipitation, precluded the extension of the pH profile to the high pH region. A complete analysis of the titration curves thus could not be carried out. However, a constant trend in the changes of the chemical shifts with pH is observed, i.e., $\Delta\delta(H_5) > \Delta\delta(H_2) > \Delta\delta(H_\alpha) > \Delta\delta(H_\beta)$. Clearly, similarly to other phenolic amines (Granot, 1976), the ammonium group of 6-OHDA is less acidic than the first aromatic hydroxyl.

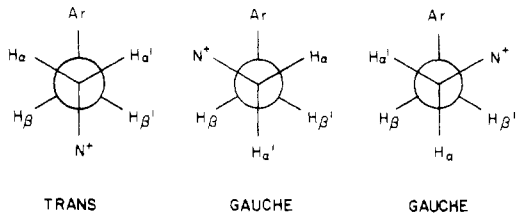

FIGURE 4: Newman projections of 6-OHDA.

TABLE I: Vicinal Coupling Constants and Rotamer Populations of 6-OHDA and Dopamine.

	6-OHDA (Hz)	Dopamine[a] (Hz)
$J_{\alpha\beta} + J_{\alpha\beta'}$	13.8	14.2
P_t[b]	0.39	0.47
P_g[b]	0.61	0.53

[a] Granot, 1978. [b] Fractional populations of the trans (P_t) and the gauche (P_g) conformers.

Inspection of Figure 3 actually reveals no isotope effect on the pH profiles of the 6-OHDA resonances in D_2O or H_2O solutions. This can be rationalized as follows: the pD values in D_2O solutions are 0.4 log unit higher than the pH meter readings (Glasoe & Long, 1960). On the other hand, the acidic ionization constants in D_2O solutions were found to be 0.5–0.6 log unit higher than the respective values of H_2O solutions (Schwarzenbach, 1938; Rule & La Mer, 1938). Hence, using uncorrected pH meter readings to present pH dependencies would approximately compensate for the isotope effects on the pK_a values.

Intramolecular Conformations. 6-OHDA has three possible rotamers staggered about the C_α–C_β bond: a trans and two mirror-image gauche rotamers (Figure 4). The vicinal coupling constants ($J_{\alpha\beta}$, $J_{\alpha\beta'}$) are the average of the contributions of the three rotamers, weighted according to their relative populations, and thus can be used to derive these populations. The method of calculation has been described elsewhere (Granot, 1978). At 270 MHz, the pattern of the side-chain of 6-OHDA is very nearly first-order AA′XX′, and the vicinal coupling constants can be directly measured. The results for coupling constants and rotamer populations, compared with the respective values for dopamine, are given in Table I. Evidently, 6-OHDA shows higher preference for the gauche conformations than dopamine. This result is in agreement with recent CNDO calculations of the conformations of 6-OHDA (Katz et al., 1974). The preference of the gauche conformer may be attributed to intramolecular interaction between the ammonium group and the orthohydroxyl of 6-OHDA. By using atomic models it is indeed observed that due to such an interaction the molecule would tend to assume a gauche rather than trans conformation.

Interactions of 6-OHDA with SH-Containing Compounds

Cysteine. Addition of cysteine to acidic D_2O solution of 6-OHDA was found to produce no effect on the proton resonances of the latter. However, at pH >7, a decrease in the intensity of the H_2 resonance was observed (Figure 1c). This

process was fast as compared with the exchange rate of H_5 and was enhanced as the pH was raised. At pH ~9, H_2 completely disappeared, whereas the intensity of H_5 has only slightly changed (5–10 min after preparation). This pH-dependent effect was not observed in H_2O solution even after a long period of time (5–10 h), implying that the interaction between the 6-OHDA and cysteine causes H–D substitution at the C_2 site of 6-OHDA in D_2O solution. The above effect was accompanied by slight changes (3–4 Hz, upfield) in the chemical shift of the 6-OHDA ring protons. In addition to the changes in the aromatic region, the resonances of the side chain of 6-OHDA and those of cysteine were broadened, both in the D_2O and the H_2O solutions.

In order to examine the dependence of the interaction between 6-OHDA and cysteine on the oxidation state of 6-OHDA, the experiments described above were repeated, once in the presence of sodium sulfite and a second time after bubbling oxygen gas through the solutions. It was found that sodium sulfite inhibits the interaction, and the exchange of H_2 began only at pH >10. On the other hand, oxygen enhanced this process and complete H–D substitution could be achieved at pH ~7. It can thus be concluded that only the oxidized form of 6-OHDA is readily available for interaction with cysteine.

Glutathione and BSA. Glutathione and BSA were selected as additional model compounds for investigation of the 6-OHDA-RS^- interaction. The amino acid compositions of these compounds consist of ca. 33% and 6% of potential cysteine, respectively. The effect of glutathione and BSA on 6-OHDA in either water or deuterium oxide solutions was studied as a function of pH, in the absence and presence of sodium sulfite or molecular oxygen. In general the effects were found to be similar to those obtained with cysteine. However, the rate of H–D substitution in D_2O solution was considerably reduced upon increasing the molecular weight of the interacting compounds. At pH 8.4 the $T_{1/2}$ values for the decrease in the amplitude of the H_2 resonance upon addition of glutathione or BSA were ca. 1.0 and 2.5 h, respectively.

Proposed Mechanism for the Interaction. The present study supports previous observations that RS^--containing molecules interact with oxidized 6-OHDA in aqueous solution and provides new evidence regarding the site of the nucleophilic substitution. It has been previously suggested that RS^- attacks at carbon 2 or 5 of the aromatic ring of 6-OHDA (Rotman et al., 1976). Recently, the product of oxidized 6-OHDA and glutathione has been synthesized and analyzed (Liang et al., 1977). Yet, on the basis of the observation of a single line (assigned as H_5) in the aromatic region of the ^1H NMR spectrum of the product, in D_2O solution, the site of glutathione substitution was assigned as occurring at C_2. However, as shown in the present study, this observation does not imply replacement of H_2 but rather its exchange with a solvent deuteron.

The present results indicate that the interaction of 6-OHDA with RS^- reagent is responsible for the disappearance of the H_2 resonance of the former in D_2O but not in H_2O solutions. The absence of effect in H_2O solution cannot be attributed to the presence of unreacted 6-OHDA since we observed, by thin-layer chromatography done under the same conditions as the NMR studies, that 6-OHDA interacted completely with the nucleophiles. Clearly C_2 cannot be the site of the RS^-–6-OHDA interaction. A proposed mechanism for this interaction is described in Figure 5. The approaching RS^- is attacking the *p*-quinone (II) at position 1, initiating electron movement toward the oxygen on C_3 and formation of double bond between C_2 and C_3 (V). The insertion of RS^- in position

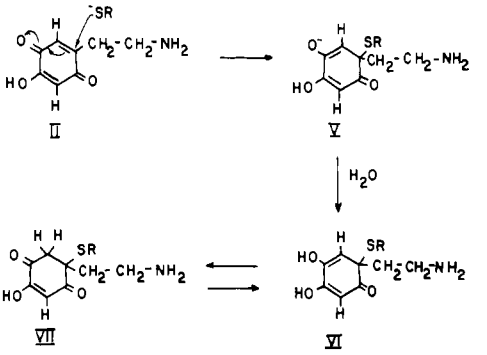

FIGURE 5: Proposed mechanism of interaction between RS^- and oxidized 6-OHDA.

1 prevents the regeneration of the *p*-quinone, leaving only the two tautomers VI and VII. This tautomerism can explain the rapid exchange of H_2 in D_2O.

The observed slower process with glutathione and BSA can be attributed to increasing inaccessibility of the sulfhydryl groups for 6-OHDA binding in the more bulky compounds. The observation that the ^1H NMR resonances of cysteine as well as those of the 6-OHDA side chain were broadened upon their binding supports the conclusion that the binding site is at C_1. Using a space-filling model it is found that the presence of an RS substituent at this site would cause restriction of the internal rotation of both the amine side chain and the substituent itself, which consequently would be reflected in line broadening of the NMR resonances.

Relevancy to Neurocytotoxicity. It has been previously suggested (Rotman, 1978, and references therein) that irreversible covalent binding of oxidation products of 6-OHDA to proteins in the neuron might be the cause of nerve degeneration. The present results clearly indicate that oxidized 6-OHDA (most probably uncyclized) does bind to SH-containing molecules through a rapid pH-dependent process. Possible cross-linking of protein molecules (Creveling et al., 1975) was suggested to occur by binding of a second protein molecule to 6-OHDA via interaction between the sulfhydryl anion and the C_5 carbon. It should be noted that, while the last suggestion seems reasonable in view of the arguments brought above, it could not be substantiated from the experimental data since no significant effect on H_5 could be observed upon interaction with the model compounds. However, due to marked broadening, beyond detection limit, of the aromatic resonances of 6-OHDA with increased oxidation followed by precipitation, the role of C_5 in binding and consequent cross-linking could not be established. It has been previously shown that polymerization occurs in a later step of the interaction between RS^- and 6-OHDA and involves precipitation of polymerized material (Rotman et al., 1976). This precipitate was shown to contain cross-linked material but it could not be analyzed with the NMR technique.

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Preparation of Coenzyme M Analogues and Their Activity in the Methyl Coenzyme M Reductase System of *Methanobacterium thermoautotrophicum*†

Robert P. Gunsalus, James A. Romesser, and Ralph S. Wolfe*

ABSTRACT: A number of 2-(methylthio)ethanesulfonate (methyl-coenzyme M) analogues were synthesized and investigated as substrates for methyl-coenzyme M reductase, an enzyme system found in extracts of *Methanobacterium thermoautotrophicum.* Replacement of the methyl moiety by an ethyl group yielded an analogue which served as a precursor for ethane formation. Propyl-coenzyme M, however, was not converted to propane. Analogues which contained additional methylene carbons such as 3-(methylthio)propanesulfonate or 4-(methylthio)butanesulfonate or analogues modified at the sulfide or sulfonate position, *N*-methyltaurine and 2-(methylthio)ethanol, were inactive. These analogues, in addition to a number of commercially available compounds, also were tested for their ability to inhibit the reduction of methyl-coenzyme M to methane. Bromoethanesulfonate and chloroethanesulfonate proved to be potent inhibitors of the reductase, resulting in 50% inhibition at 7.9×10^{-6} M and 7.5×10^{-5} M. Analogues to coenzyme M which contained modifications to other regions were evaluated also and found to be weak inhibitors of methane biosynthesis.

The cofactor, coenzyme M, was demonstrated by McBride & Wolfe (1971) to be involved in the terminal steps of methane biosynthesis by the methanogenic bacterium, *Methanobacterium* strain M.o.H. The structure of the novel coenzyme was determined by Taylor & Wolfe (1974a) to be 2-mercaptoethanesulfonic acid (HS-CoM, **1**).[1] The methylated form of the cofactor, 2-(methylthio)ethanesulfonic acid (CH_3-S-CoM, **4**), was found to be reductively demethylated by cell extracts to methane with molecular hydrogen as the reductant (Taylor & Wolfe, 1974a). Little is known about the chemical specificity of the methyl-coenzyme M reductase. The dimethylsulfonium analogue of coenzyme M, 2-(dimethylsulfonium)ethanesulfonate (**10**), however, was unable to serve as a substrate for methane formation (Taylor & Wolfe, 1974b). Here we describe the synthesis of a number of coenzyme M analogues which contain modifications to the alkyl sulfide, ethylene carbon, and sulfonate regions. The ability of these analogues to serve as potential substrates or inhibitors for methane formation was tested using the cell-free assay system developed for the thermophilic methane organism *M. thermoautotrophicum* (Gunsalus & Wolfe, 1977).

Experimental Section

Preparation of Analogues. Ammonium 2-mercaptoeth-

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[1] Abbreviations used: CH_3-S-CoM, methyl-coenzyme M or 2-(methylthio)ethanesulfonic acid; HS-CoM, 2-mercaptoethanesulfonic acid; $(S\text{-CoM})_2$, 2,2′-dithiodiethanesulfonic acid; $(CH_3)_2$-S^+-CoM, 2-(dimethylsulfonium)ethanesulfonate; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

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