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New Water-soluble Hydrogen Donors for the Enzymatic Photometric Determination of Hydrogen Peroxide. II.[1] *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline Derivatives

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Five *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline derivatives have been synthesized and assessed as water-soluble hydrogen donors for the photometric determination of hydrogen peroxide in the presence of peroxidase. These compounds, sodium salts of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-anisidine, 3,5-dimethyl-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline and 3,5-dimethoxy-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-aniline, gave high absorbances at visible wavelengths in media in the weakly alkaline to fairly acidic pH range. Further, the urea adduct of hydrogen peroxide could be conveniently used as a standard material.

Keywords——photometric determination; hydrogen peroxide; hydrogen donors; sodium salt of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline; sodium salt of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine; sodium salt of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-anisidine; sodium salt of 3,5-dimethyl-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-aniline; sodium salt of 3,5-dimethoxy-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline; urea-hydrogen peroxide adduct

In a previous paper, we reported a series of *N*-alkyl-*N*-sulfopropylaniline derivatives as water-soluble hydrogen donors for the photometric determination of hydrogen peroxide in the presence of peroxidase.[1]

We now report syntheses of a series of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline derivatives (Chart 1) and their application to the photometric determination of hydrogen peroxide. The new reagents [sodium salt of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline (ALOS), sodium salt of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS), sodium salt of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-anisidine (ADOS), sodium salt of 3,5-dimethyl-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline (MAOS) and sodium salt of 3,5-dimethoxy-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline (DAOS)] can be prepared from readily available starting materials and can be purified easily by recrystallization from water. The spectral characteristics of the chromogens resulting from the oxidative condensation of the reagents with 4-amino antipyrine (4-AAP) were found to be similar or superior to those of the reagents reported in the previous paper.[1]

Experimental

Syntheses of Hydrogen Donors——Syntheses of hydrogen donors were carried out by the condensation of the *N*-ethylaniline, *N*-ethyl-*m*-toluidine, *N*-ethyl-*m*-anisidine, 3,5-dimethyl-*N*-ethylaniline and 3,5-dimethoxy-*N*-ethylaniline with sodium 3-chloro-2-hydroxypropanesulfonate.

The preparation of ALOS will be described as an example; the other *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline derivatives can be prepared under similar conditions.

An aqueous solution of 4.0 g (0.1 mol) of NaOH was added to a mixture of an aqueous solution of 19.6 g (0.1 mol) of sodium 3-chloro-2-hydroxypropanesulfonate in 200 ml of water and a solution of 12.1 g (0.1 mol) of *N*-ethylaniline in 100 ml of isopropanol. During the addition, the mixture was gradually heated with stirring, then refluxed for 3 h. The reaction mixture was evaporated to dryness *in vacuo* and the residue

was dissolved in 100 ml of water. The aqueous solution was extracted with ether to remove unreacted *N*-ethylaniline, then the aqueous layer was concentrated *in vacuo* and cooled, and the separated crystals were filtered off. The crystals were recrystallized from water to give colorless plates (yield 22.2 g, 79.0%). mp 260—262°C (dec.). Infrared (IR) cm^{-1}: ν_{CN} 1340; ν_{SO_3} 1040; ν_{OH} 1460, 3350; ν_{arom} 1600. *Anal.* Calcd for $C_{11}H_{16}NNaO_4S$: C, 46.97; H, 5.73; N, 4.98. Found: C, 46.57; H, 5.80; N, 4.97.

The yield, mp, IR and analytical data for the other hydrogen donors are as follows:

TOOS, colorless plates, yield 83.4%, mp 75—76°C (dec., $1.5H_2O$), IR cm^{-1}: ν_{CN} 1330; ν_{SO_3} 1050; ν_{OH} 1450, 3350; ν_{arom} 1620. *Anal.* Calcd for $C_{12}H_{18}NNaO_4S$ (anhydrous): C, 48.80; H, 6.14; N, 4.74. Found: C, 48.42; H, 6.20; N, 4.73.

ADOS, white amorphous powder, yield 65.0%, mp 143—144°C (dec.), IR cm^{-1}: ν_{CN} 1320; ν_{SO_3} 1040; ν_{HO} 1460, 3400; ν_{arom} 1610. *Anal.* Calcd for $C_{12}H_{18}NNaO_5S$ C, 46.29; H, 5.83; N, 4.50. Found C, 46.10; H, 5.81; N, 4.49.

MAOS, colorless minute crystals, yield 68.0%, mp 273—274°C (dec.), IR cm^{-1}: ν_{CN} 1330; ν_{SO_3} 1040; ν_{OH} 1450, 3340; ν_{arom} 1600. *Anal.* Calcd for $C_{13}H_{20}NNaO_4S$: C, 59.47; H, 6.52; N, 4.53. Found: C, 50.15; H, 6.57; N, 4.55.

DAOS, white amorphous powder, yield 61.0%, mp 270—271°C (dec.), IR cm^{-1}: ν_{CN} 1340; ν_{SO_3} 1050; ν_{OH} 1450, 3350; ν_{arom} 1600. *Anal.* Calcd for $C_{13}H_{20}NNaO_6S$: C, 45.74; H, 5.91; N, 4.10. Found: C, 45.52; H, 5.93; N, 4.10.

Synthesis of Urea Hydrogen Peroxide Adduct——To a solution of 6 g (0.1 mol) of urea in 6 ml of water, 12 ml of 30% hydrogen peroxide solution was added. The mixture was left to stand for 3 h at room temperature, then the urea hydrogen peroxide adduct was filtered off to obtain 3.6 g of colorless plates. Another 3 g of the adduct was obtained from mother liquor by concentrating it to half the initial volume.

The urea adduct contained 35% hydrogen peroxide as determined by acid permanganate titration. The urea adduct was stored by suspending it in a saturated solution of urea below 25°C. Under these conditions, the adduct was stable for over 3 months. Before usage of the adduct, the suspension was filtered and the adduct was washed with a small portion of water and dried in a desiccator over phosphorus pentoxide.

Reagents——Aqueous 4-AAP solution (10 mM).

Aqueous hydrogen donor solution (10 mM).

Aqueous hydrogen peroxide solution (0.45 mM).

Aqueous urea hydrogen peroxide solution (0.45 mM). The concentrations of the hydrogen peroxide and urea-hydrogen peroxide solutions were determined by acid permanganate titration.

Aqueous urea solution (01 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, and 10 mM).

Aqueous peroxidase solution (100 U/ml). The solution was prepared from peroxidase (280 U/mg) obtained from BDH Chemicals Ltd. The solution was stored under refrigeration.

Moni-TROL-II (DADE Division, American Hospital Supply Corp.) was used as a control serum.

Buffer solutions were prepared from Good's buffer reagents and NaOH solution. The pH ranges covered by the buffer reagents are: pH 5.5—6.5 with NES, pH 7.0 with MOPS, pH 7.5—8.5 with HEPES and pH 9.0—9.5 with CAPS.

All reagents were of analytical grade. Good's buffer reagents were obtained from Dojindo Labs. (Kumamoto-shi).

Apparatus——Photometric measurements were carried out with a Shimadzu UV-210A double-beam recording spectrophotometer using 1 cm-cells. A pH meter (Toa Denpa HM-20B) was used for pH measurements.

Standard Procedure for Color Development——The color reactions were carried out according to the procedure described in the previous paper.[1] The optimum pH range for the color reaction, the minimum requirement of the hydrogen donors, the time dependences of the color intensity and the preparation of calibration curves were investigated in the same way as reported previously.[1] In these experiments, the standard urea adduct solution was used instead of the standard hydrogen peroxide solution.

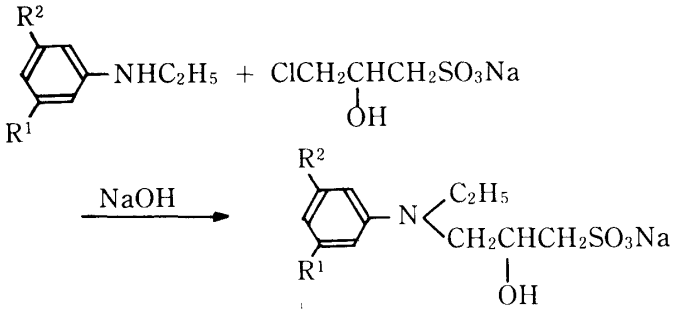
Influence of Urea on the Color Development——A solution consisting of 2.5 ml of 0.1 M Good's buffer solution (MOPS, pH 7.0), 0.1 ml of 10 mM 4-AAP solution, 0.1 ml of 10 mM TOOS solution, 0.1 ml of 0.45 mM hydrogen peroxide solution, 0.1 ml of peroxidase solution and 0.1 ml of urea solution of various concentrations (0.1—10 mM) was incubated at 37°C for 10 min. The absorption spectrum of the resulting colored solution was observed against the reagent blank.

Results and Discussion

Synthesis and Physical Properties of the Hydrogen Donors

N-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline derivatives were prepared according to Chart 1.

The hydrogen donors reported in the previous paper were prepared by the sulfopropylation of the appropriate *N*-alkylaniline with propanesultone, which is now suspected to be a carci-



ALOS: $R^1=R^2=H$, TOOS: $R^1=CH_3$, $R^2=H$,
ADOS: $R^1=OCH_3$, $R^2=H$, MAOS: $R^1=R^2=CH_3$,
DAOS: $R^1=R^2=OCH_3$.

Chart 1. Syntheses of Hydrogen Donors

nogen. On the other hand, the hydrogen donors reported in this paper are prepared from *N*-alkylanilines and sodium 3-chloro-2-hydroxypropanesulfonate, which is readily available.[2)]

The crude products obtained from the reaction mixture can easily be purified by recrystallization from water, because the products are moderately water-soluble. The hydrogen donors (*N*-sulfopropyl derivatives) which were reported in the previous paper are more soluble in water and have to be recrystallized from alcohol–acetone, which can present difficulties.

Color Reaction with the Hydrogen Donors

The oxidative condensation of the hydrogen donors with 4-AAP in the presence of hydrogen peroxide and peroxidase (Chart 2) was carried out at various pH values, and the dependences of the color intensity of the chromogens upon pH values are shown in Fig. 1. The data for phenol, a known hydrogen donor, are also included for the sake of comparison. The extent of influence of pH is dependent on the kind and number of the substituents on the phenyl ring of the hydrogen donors.

The color intensity of the chromogens decreases with the introduction of substituents on the phenyl ring, and this effect is more pronounced with methoxy substitution (ADOS and DAOS) than with methyl substitution (TOOS and MAOS). The color intensity also decreases with increasing number of substituents, but the pH dependences of the color intensity are greater for the chromogens with higher color intensity.

The absorption spectra of the chromogens resulting from the oxidative condensation of these hydrogen donors with 4-AAP are shown in Fig. 2. The absorption maxima of the

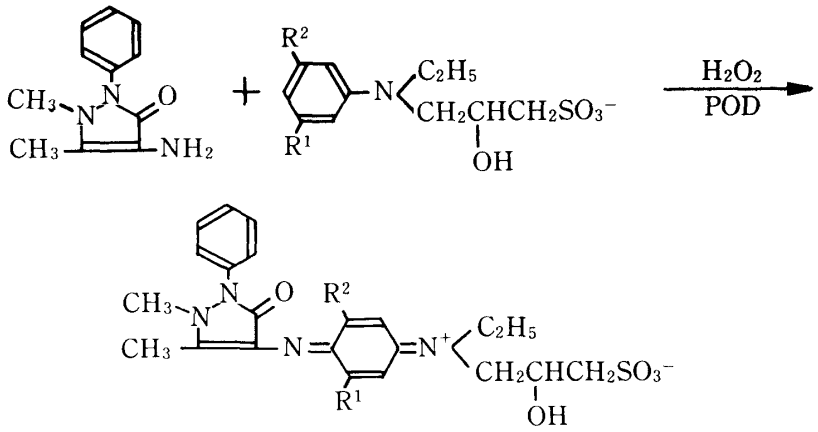


Chart 2. Oxidative Condensation of 4-AAP with Hydrogen Donors

R^1 and R^2 are the same as in Chart 1.

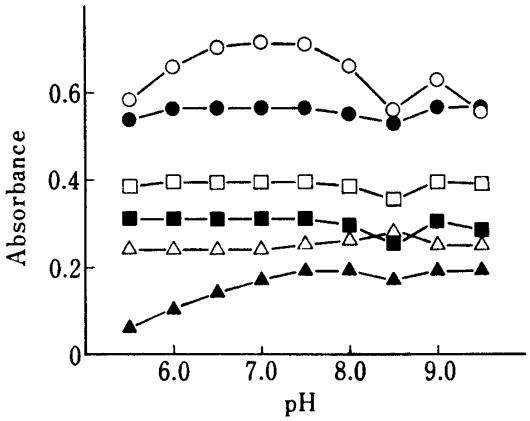


Fig. 1. pH Dependences of the Color Reactions of Hydrogen Donors

Absorbance was measured at λ_{max} of each chromogen shown in Table I.
○, ALOS; ●, TOOS; □, ADOS; ■, MAOS; △, DAOS; ▲, phenol. Concentration of reactants: 4-AAP 3.3×10^{-4} M; $(NH_2)_2CO\cdot H_2O_2$ 1.5×10^{-5}M; POD 3.3 units; hydrogen donor 3.3×10^{-4} M.

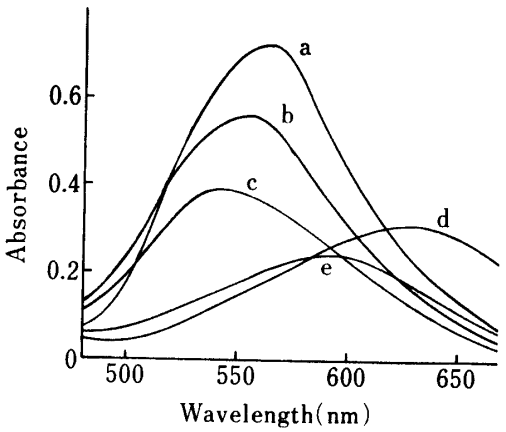


Fig. 2. Absorption Spectra of Chromogens resulting from the Oxidative Condensation of Hydrogen Donors with 4-AAP at pH 7.0

a, ALOS; b, TOOS; c, ADOS; d, MAOS; e, DAOS.
The concentrations of the reactants were the same as those described in Fig. 1.

chromogens are all in the range of 540—630 nm, where the absorption by bilirubin in serum does not interfere.[3,4]

A similar approach has been reported using sodium 3,5-dichloro-2-hydroxybenzene sulfonate as a water-soluble hydrogen donor, but the absorption maximum of the chromogen resulting from the oxidative condensation of this hydrogen donor with 4-AAP appears at 520 nm.[5]

The absorption maximum of the chromogen shifts slightly to shorter wavelength upon mono-methyl or mono-methoxy substitution. However, dimethyl or dimethoxy substitution results in a remarkable bathochromic shift as compared with that of the mother compounds (ALOS). The spectral characteristics of the chromogens are summarized in Table I.

Table I. Characteristics of Hydrogen Donors

Compound	mp(°C) (dec.)	Water solubility (5°C, %)	Optimum pH range	Chromogen (with 4-AAP) λ_{max} (nm),	$\varepsilon\times10^4$	Relative sensitivity
ALOS	260—262	38	6.5—7.5	565	5.02	3.9
TOOS	75—76 ($1.5\ H_2O$)	11	5.5—9.5	555	3.92	3.1
ADOS	143—144	29	5.5—9.5	542	2.72	2.1
MAOS	273—274	8	5.5—9.5	630	2.25	1.8
DAOS	271—273	19	5.5—9.5	593	1.75	1.4
Phenol	38	6	7.0—9.5	505	1.27	1.0

Optimum Conditions for the Color Reaction

The minimum requirement of the hydrogen donor was determined by carrying out the color reaction with equimolar hydrogen donor and 4-AAP at various concentrations in the presence of hydrogen peroxide and peroxidase at pH 7.0. As shown in Fig. 3, the maximum color intensity was obtained with the use of a 15—20 fold excess of the hydrogen donor to hydrogen peroxide (used as the urea adduct). As illustrated in Fig. 4, the absorbances of the chromogens reach maximum within 5 min and remain constant for 30 min (with the exceptions of ALOS and MAOS), then decrease slightly. The reason for the color fading is not clear, but

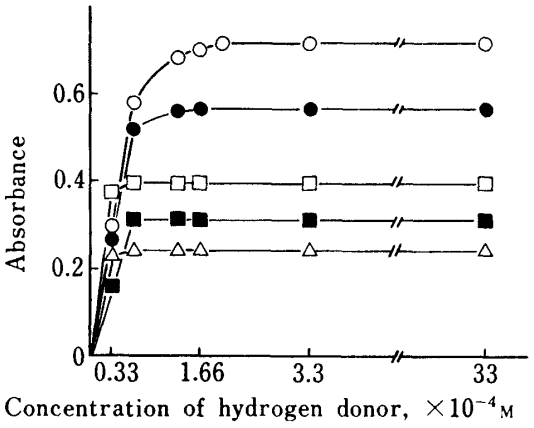


Fig. 3. Minimum Requirement of Hydrogen Donor Concentration for the Color Reaction

○, ALOS; ●, TOOS; □, ADOS; ■, MAOS; △, DAOS.
The concentrations of $(NH_2)_2CO \cdot H_2O_2$ and POD were the same as those shown in Fig. 1 and that of 4-AAP was equal to the hydrogen donor concentration.

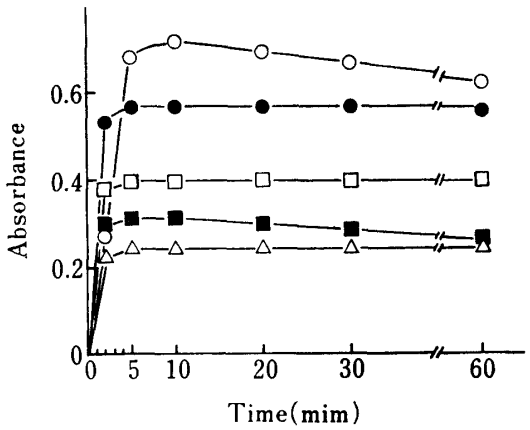


Fig. 4. Time Dependence of Color Development at pH 7.0

○, ALOS; ●, TOOS; □, ADOS; ■, MAOS; △, DAOS.
The concentrations of the reactants were the same as those described in Fig. 1.

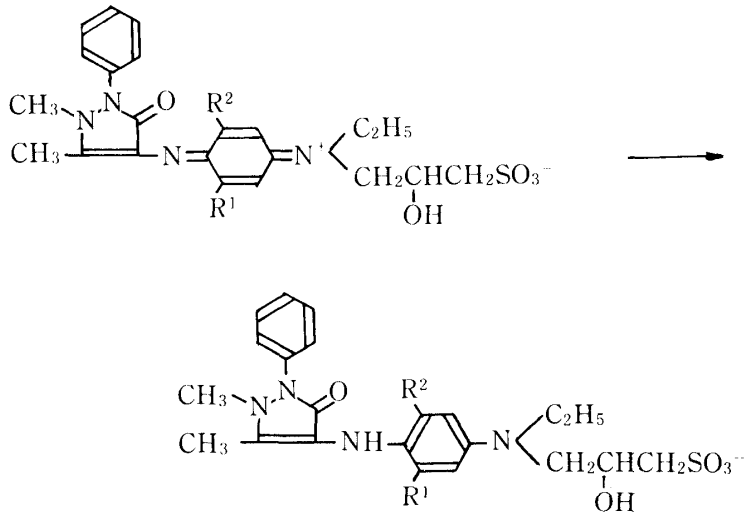


Chart 3. Reduction of Chromogens to the Corresponding Quinone Diamides

R^1 and R^2 are the same as in Chart 1.

it may be due to reduction of the chromogens, in other words, reduction of the quinone diimides to the quinone diamides, as shown in Chart 3.[6-8] The results of a detailed investigation of the reduction will be reported elsewhere.

As shown in Table I, the chromogen from ALOS shows the highest color intensity, but it suffers from rather rapid color fading. The optimum pH range is also narrower than those for the other hydrogen donors.

The chromogen from TOOS shows the second highest color intensity, and the chromogen from TOOS is fairly stable. Thus, TOOS was found to be the most useful hydrogen donor among those investigated.

The chromogen from MAOS has the advantage that the absorption maximum appears at the longest wavelength, but the color fades fairly quickly. That from DAOS shows the lowest color intensity, but the absorption maximum appears at longer wavelength, so that DAOS may be useful in the determination of analytes which are present at higher concentrations.

Stabilization of the Standard Solution of Hydrogen Peroxide

Aqueous hydrogen peroxide solution is known to be unstable due to the catalytic decomposition of hydrogen peroxide in the presence of trace amounts of various inorganic or organic materials.[9] This property becomes critical when a very dilute aqueous solution of hydrogen peroxide is used as a standard solution in microdetermination. The urea adduct of hydrogen peroxide is thus employed as a standard material for hydrogen peroxide.[10-12] The adduct is obtained as a stable solid which can easily weighed and dissolved in water to make a standard solution.

The effect of the use of urea on the determination of hydrogen peroxide was examined using TOOS under the conditions described in "Experimental," in the presence of various concentrations of urea. The urea had no effect at concentrations up to at least 10 mM.

As a preliminary investigation, the use of these hydrogen donors for the determination of hydrogen peroxide was carried out in the presence of a control serum and peroxidase. The calibration curves of hydrogen peroxide with these hydrogen donors are linear over the concentration range of 0.007—0.04 mM, and the sensitivity of determination is 1.4—3.8 times higher than that with phenol.

These results are very promising, and indicate that these hydrogen donors may be useful for the determination of clinically important constituents in serum through the enzymatic evolution of hydrogen peroxide. The results of practical applications of this procedure will be the subject of the succeeding paper.

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