# Inhibitory Effects of Catechol Derivatives on Hydrophilic Free Radical Initiator-Induced Hemolysis and Their Interaction with Hemoglobin

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**The effects of pyrocatechol and its monosubstituents on the hemolysis of bovine erythrocytes induced by the hydrophilic free radical initiator, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), were investigated. Relatively hydrophilic derivatives such as the 4-$COO^-$ substituent (protocatechuic acid), which are almost completely ionized at physiological pH, have a more inhibitory effect than the more hydrophobic derivatives such as the 4-$C(CH_3)_3$ substituent. In the presence of relatively low concentrations of the latter derivatives, the onset of hemolysis was retarded, but the hemolysis then proceeded more rapidly and the time, at which almost complete hemolysis occurred, was almost the same as that in their absence. Regression analysis on the relationships between the inhibitory effects of the derivatives and their redox potentials and hydrophobic parameters revealed that the inhibitory activity of the catechol derivatives on AAPH-induced hemolysis was controlled by low hydrophobicity as well as electron donor activity. In the presence of relatively hydrophobic catechol derivatives, oxidation of hemoglobin was observed. These findings suggest that interaction of these derivatives with hemoglobin after their penetration into erythrocytes reduced the scavenging activity against free radicals. Their interaction with membrane or cytoplasmic components may cause the increased hemolysis rate after the onset of hemolysis at relatively low concentrations.**

**Key words** hemolysis; catechol derivative; radical scavenger; hemoglobin

Nonenzymatic, free radical-mediated oxidation of biological membranes has been suggested as being associated with the pathogenesis of various tissue injuries.[1,2] It has been shown that various phenolic antioxidants like flavonoids, tannins and α-tocopherol scavenge free radicals such as superoxide anion radicals and lipid peroxyl radicals, thus preventing cell damage.[3–6] In a previous paper we demonstrated that the scavenging activity of catechol derivatives on the hydrophilic free radical initiator-induced lipid peroxidation of soybean phosphatidylcholine liposomes was controlled by their electron donor activities, whereas the effects on hydrophobic free radical initiator-induced lipid peroxidation were controlled by their hydrophobicity, in addition to their electron-donor activity.[7] However, interaction of antioxidants with free radicals in biological membranes seem to be very complicated due to the interaction with various membrane and cytoplasmic components, if they are membrane-permeable.

Erythrocytes are good models for investigating cell injuries induced by free radicals and the protective effect of antioxidants. It has been reported that hemolysis is induced by free-radical initiators such as AAPH and suppressed by antioxidants such as α-tocopherol.[8,9] In this work we examined the effects of pyrocatechol and its monosubstituents on hemolysis induced by the hydrophilic free radical initiator, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH). Erythrocytes are also good subjects considering that heme proteins such as hemoglobin have been suggested to be involved in the free radical-induced injury to various tissues.[10,11] Therefore, we also investigated the interaction of the catechol derivatives with hemoglobin.

## Experimental

**Materials** Azo compound, AAPH was purchased from Wako Pure Chemical Industries (Osaka). All catechol compounds and ascorbic acid were from Wako Pure Chemicals and Nakarai Tesque, Inc. (Kyoto, Japan).

**Measurement of Hemolysis** Erythrocyte suspension (hematocrit 10%) in phosphate buffered saline (pH 7.4) was incubated in air in a shaking water bath at 37 °C in the presence of 100 mM AAPH, either with or without catechol derivatives. An aliquot (0.4 ml) of this mixture was taken out periodically to measure the extent of hemolysis spectrophotometrically by the cyanmethemoglobin method.[12]

**Measurement of Hemoglobin Oxidation** Oxidation of hemoglobin inside erythrocytes in the presence of catechol compounds was observed by measuring the visible spectra of hemoglobin. Erythrocyte suspension (hematocrit 10%) in phosphate buffered saline (pH 7.4) was incubated in air in the absence of AAPH as described above in the presence of various concentrations of catechol derivatives. An aliquot (100 μl) of the erythrocyte suspension was taken out periodically, washed with phosphate buffered saline (PBS) and lysed in 4 ml hypotonic phosphate buffer (pH 7.4) and the hemoglobin spectrum was measured in a Shimadzu UV-1600 spectrophotometer.

## Results and Discussion

**Effects of Catechol Derivatives on AAPH-Induced Hemolysis** We examined the effects of pyrocatechol and six monosubstituents on hemolysis induced by the hydrophilic free radical initiator, AAPH. As has been reported,[8,9] AAPH induced hemolysis due to oxidative damage to the erythrocyte membrane. As shown in Figs. 1 through 3 for the effects of the 4-Cl, 4-$CH_3$ and 4-$COO^-$ (protocatechuic acid) substituents, respectively, in the presence of the catechol derivatives, hemolysis was retarded in a concentration-dependent manner. The time after addition of AAPH at which 50% of erythrocytes caused hemolysis was determined in the presence of various concentrations of catechol derivatives from the hemolysis curves as shown in Figs. 1—3. The mean value of this time in the absence of catechol derivatives was $2.6 \pm 0.4$ h ($n=27$); the concentration of the derivatives causing a two-fold increase in that time, the $IC_2$, was determined. The results was shown in Table 1, in which the value for levodopa, which is also a monosubstituent of pyrocatechol, and that of ascorbic acid

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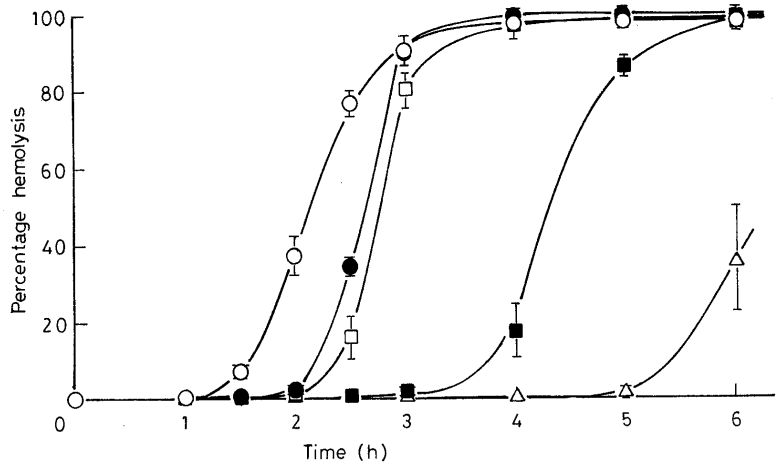


Fig. 1. Effect of 4-Cl Substituent on 100 mM AAPH-Induced Hemolysis

○, control; ●, 25 μM; □, 50 μM; ■, 100 μM; △, 200 μM. Data are means±S.D. of three experiments with same preparation.

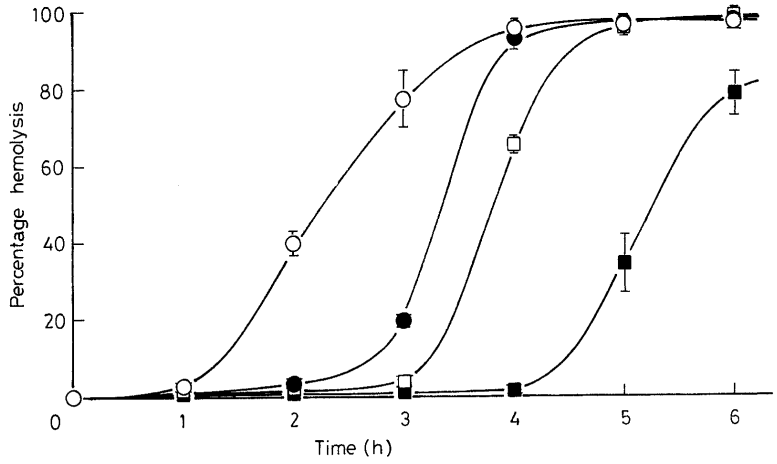


Fig. 2. Effect of 4-$CH_3$ Substituent on 100 mM AAPH-Induced Hemolysis

○, control; ●, 100 μM; □, 200 μM; ■, 300 μM. Data are means±S.D. of three experiments with same preparation.

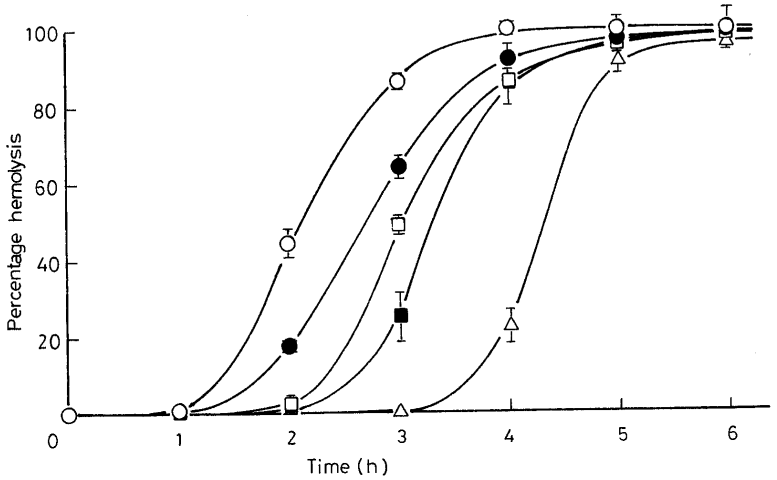


Fig. 3. Effect of 4-$COO^-$ Substituent (Protocatechuic Acid) on 100 mM AAPH-Induced Hemolysis

○, control; ●, 25 μM; □, 50 μM; ■, 100 μM; △, 200 μM. Data are means±S.D. of three experiments with same preparation.

were also listed for comparison. The inhibitory effect of all the catechol derivatives tested was stronger than that of ascorbic acid. The effect of the derivatives including levodopa, which are present in their ionic forms at physiological pH, was stronger than the other derivatives regardless of their electron-donor activity. These results are quite different from those on the inhibitory effects of the same derivatives on the AAPH-induced lipid peroxidation of soybean phosphatidylcholine liposomes which we reported previously.[7]

We next sought to determine the relationship between the physico-chemical properties of the derivatives and their inhibitory effect on hemolysis. The correlation of their inhibitory effect with two derivative parameters,

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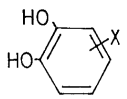
Table 1. Concentrations of Catechol Derivatives Producing a Two-Fold Increase in the Time to Cause 50% Hemolysis of Bovine Erythrocytes Induced by AAPH, $IC_2$,[a] Their Redox Potentials, $E_0$, and Their Hydrophobic Parameter, $\pi$



| X | $IC_2$ ($\mu$M)[b] | $E_0$[c] | $\pi$[d] |
|---|---|---|---|
| H | 229±46 | 0.795 | 0.00 |
| 4-$CH_3$ | 233±47 | 0.753 | 0.56 |
| 4-$C(CH_3)_3$ | 208±20 | 0.732 | 1.98 |
| 4-Cl | 214±30 | 0.801 | 0.71 |
| 3-OH | 126±13 | 0.680 | −0.67 |
| 4-$COO^-$ | 120±31 | 0.833 | −4.36 |
| 4-CH=CH-$COO^-$ | 107±21 | 0.794 | −3.89[e] |
| Levodopa | 63.7±3.2 | | |
| Ascorbic acid | 603±130 | | |

*a*) Inhibitory effects of levodopa and ascorbic acid are also listed for comparison. *b*) Values represent means±S.D. of data from duplicate or triplicate experiments on three different preparations. *c*) Cited from reference 13. *d*) Cited from reference 14. *e*) Calculated according to reference 17.

redox potential and hydrophobic parameter, was examined by regression analysis. The inhibitory effect of the catechol derivatives was expressed as the logarithm of the $1/IC_2$. The values of the redox potential, $E_0$, of the catechol derivatives were taken from Horner and Geyer[13] and those of the hydrophobic parameter, $\pi$, of the derivatives were taken from Leo *et al.*[14] The relation between the inhibitory effect and these parameters is shown in Eqs. 1 through 3:

$$\log(1/IC_2)(\mathrm{M}^{-1}) = -0.054\pi + 3.73 \quad (n=7,\ r=0.86,\ s=0.08,\ F=14.4) \tag{1}$$

$$\log(1/IC_2)(\mathrm{M}^{-1}) = -0.016E_0 + 3.66 \quad (n=7,\ r=0.05,\ s=0.15,\ F=0.02) \tag{2}$$

$$\log(1/IC_2)(\mathrm{M}^{-1}) = -1.46E_0 - 0.069\pi + 4.85 \quad (n=7,\ r=0.96,\ s=0.04,\ F=26.9) \tag{3}$$

Here, $n$ is the number of compounds tested, $r$ is the correlation coefficient, $s$ is the standard deviation, and $F$ is the ratio between regression and residual variances. Equations 1—3 and Fig. 4 reveal that catechol derivatives with lower hydrophobicity have stronger inhibitory effects on AAPH-induced hemolysis, although the difference is small as shown by the slope. Comparison of Eq. 3 with Eq. 1 revealed that the addition of the redox potential term, which has also a negative slope, significantly improved the quality of the correlation. Thus, according to this equation, the inhibitory effect of the catechol derivatives on AAPH-induced hemolysis is controlled by their low hydrophobicity as well as their electron donor activity.

Although all the catechol derivatives tested exhibited inhibitory effects on AAPH-induced hemolysis, the derivatives, which have a greater hydrophobicity and are present almost completely in undissociated form at physiological pH, differ in the characteristics of their effects on hemolysis from those of ionic derivatives such as the 4-$COO^-$ substituent. As shown in Fig. 3 for the 4-$COO^-$

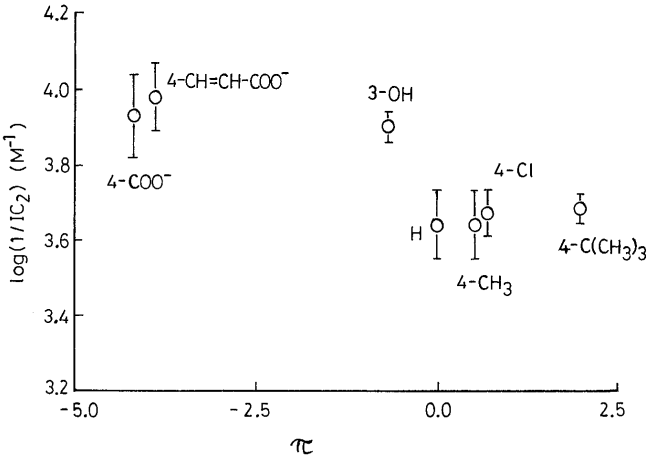



Fig. 4. Relationship between the Hydrophobic Parameter, $\pi$, of the Substituents of Pyrocatechol and Their Inhibitory Effects on AAPH-Induced Hemolysis, Expressed as $1/IC_2$ Values

substituent, in the presence of these derivatives a parallel shift in the hemolysis curve was observed with an increase in their concentration. However, in the presence of the other derivatives, as shown in Figs. 1 and 2 for the 4-Cl and 4-$CH_3$ substituents, there was a clear tendency for the hemolysis rate to be accelerated after onset of hemolysis at relatively low concentrations. In these cases, the time at which almost complete hemolysis occurred, was similar to that in the absence of these derivatives, although the onset of hemolysis was delayed by the same derivatives. According to their physico-chemical properties, these derivatives seem to penetrate easily into the erythrocyte cytoplasm. Therefore, it is very probable that they interact with cytoplasmic components due to their reducing nature. This interaction seems to reduce the inhibitory effect of these relatively hydrophobic derivatives on hemolysis. It may also cause the increased hemolysis at relatively low concentrations of these derivatives after its onset.

**Effects of Catechol Derivatives on Oxidation of Hemoglobin Inside Erythrocytes** In order to clarify the interaction of the catechol derivatives with cytoplasmic components as speculated above, we examined the effects of these derivatives on hemoglobin inside the erythrocytes by observing its spectrum after incubation of the erythrocytes with the derivatives. As shown in Fig. 5, for the effect of incubation of erythrocytes with 200 $\mu$M—1.0 mM 4-Cl substituent at 37 °C for 3 h, oxidation of hemoglobin to methemoglobin was observed, accompanied by incubation of the catechol derivatives which are present almost completely in their undissociated form at pH 7.4. Methemoglobin formation depended on the concentration of 4-Cl substituent. These results suggest that oxidation of hemoglobin was induced following penetration of the derivatives into cytoplasm. On the other hand, as shown in Fig. 6 for the effect of 1.0 mM 4-$COO^-$ substituent (protocatechuic acid), oxidation of hemoglobin was not observed following incubation of the erythrocytes even at relatively high concentrations of the ionic derivatives. These derivatives are present almost completely in their ionic form at pH 7.4 and, therefore, have relatively poor membrane permeability. The present findings indicate that penetration of the catechol derivatives into cytoplasm and interaction with hemoglobin

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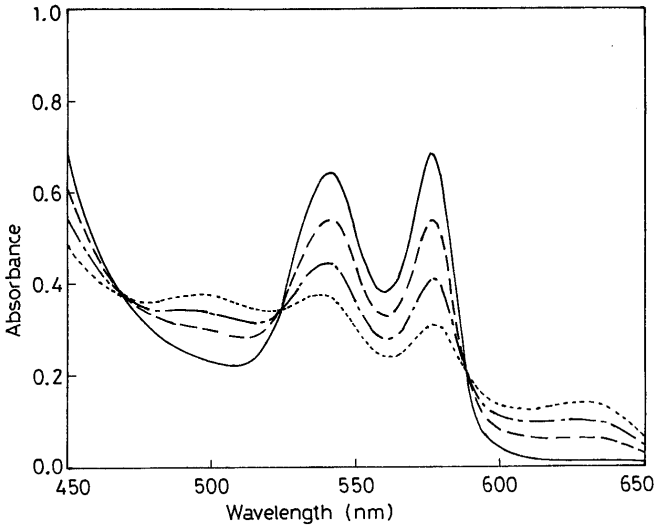


Fig. 5. Effect of 4-Cl Substituent on Absorption Spectrum of Oxyhemoglobin after Incubation of Erythrocytes at 37 °C for 3 h

——, control; – – –, 200 μM; —·—, 500 μM; ------, 1.0 mM.

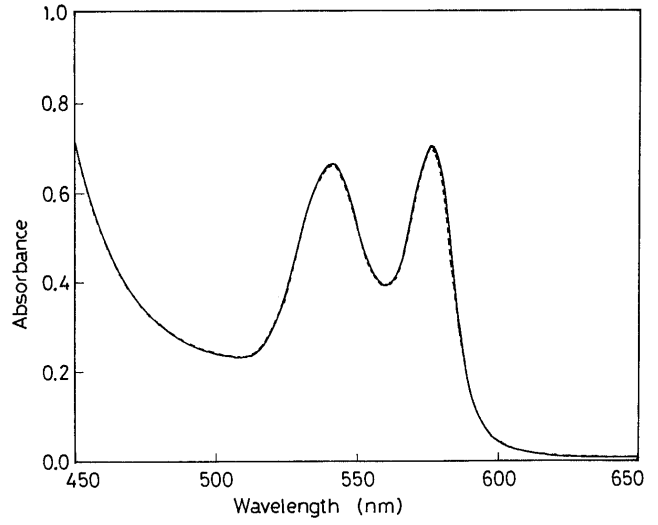


Fig. 6. Effect of 4-$COO^-$ Substituent (Protocatechuic Acid) on Absorption Spectrum of Oxyhemoglobin after Incubation of Erythrocytes at 37 °C for 3 h

——, control; ------, 1.0 mM.

reduces their inhibitory effect on hemolysis.

Formation of phenoxyl radicals, ArO·, by the interaction between *p*-hydroxyanisole and oxyhemoglobin has been reported by electron spin resonance spectroscopy study and it involves the following reactions[15]:

$$[Hb^{2+}O_2]+ArOH \rightarrow [MetHb^{3+}-O_2^{2-}]+ArO\cdot+H^+ \quad (4)$$

$$[MetHb^{3+}-O_2^{2-}]+2ArOH \rightarrow 2ArO\cdot+MetHb^{3+}+2OH^- \quad (5)$$

Here, $[MetHb^{3+}-O_2^{2-}]$ represents the perferryl species of hemoglobin, which is assumed to be formed as a reaction intermediate.[15,16] Products of these reactions inside the erythrocytes themselves did not induce hemolysis, because hemolysis was not observed in the absence of AAPH. Therefore, oxidation of the catechol derivatives due to the reaction with oxyhemoglobin mentioned above seems to reduce their free radical scavenging activity. It is suggested that the negative dependence of the antihemolytic activity on hydrophobicity is probably due to the rapid penetration into cytoplasm and fast oxidation of the derivatives with greater hydrophobicity.

Catechol derivatives such as the 4-Cl and 4-$CH_3$ substituents of pyrocatechol, which can easily penetrate into cytoplasm, delayed the onset of free radical-induced hemolysis, but at relatively low concentrations the time at which almost complete hemolysis is produced is similar to that in the absence of these derivatives due to the increased hemolysis rate after its onset. However, the mechanism is still not clear. Catechol derivatives interact with AAPH-derived peroxyl radicals, ROO·, and subsequent lipid peroxyl radicals, LOO·, and prevent hemolysis as shown by the following reactions.[7]

$$ROO\cdot(LOO\cdot)+ArOH \rightarrow ROOH(LOOH)+ArO\cdot \quad (6)$$

One explanation is the stimulation of lipid peroxidation and membrane damage by catechol radicals produced by this reaction. Hydrogen abstraction from biological membrane components by phenoxyl radicals has been suggested.[15,16] The effect may be significant when the concentration of the catechol derivatives is relatively low and the derivatives are oxidized rapidly according to Eq. 6.

Interaction of the catechol derivatives with hemoglobin, as mentioned above, may also be involved. The radicals may stimulate hemolysis after its onset by cooperation between AAPH-derived peroxyl radicals and lipid peroxyl radicals, when the concentration of the catechol derivatives is relatively low and, therefore, the free radical scavenging activity of the derivatives is insufficient. Oxidized hemoglobin may also affect hemolysis, because it has been suggested to be involved in the production of the side effects of a number of therapeutic drugs.[10,11]

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