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# Platelet Aggregation Inhibitors. I. Hydroxylamine: A Potent Inhibitor of Platelet Aggregation

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Platelets have been known to function in the formation of thrombi and plug,[2] and the key role of adenosine diphosphate (ADP) as an important initiator of platelet aggregation has been well documented.[3] A wide variety of compounds that inhibit platelet aggregation have been extensively investigated.[2] Among them inhibitors of several enzymes such as monoiodoacetate, *p*-chloromercuribenzoate, N-ethyl maleimide and methyl mercuric nitrate, have been shown to inhibit ADP-induced platelet reaction.[4,5] This paper describes strong alteration in the ability of platelets to agglutinate with ADP, collagen and thrombin, when rabbit platelets were treated with hydroxylamine, a kind of enzyme inhibitors.

## Experimental

**Materials**——Hydroxylamine·HCl was purchased from Wako Pure Chemical Industries, Ltd., ADP ($Na_2$) and hydroxylurea were from Sigma Chemical Co. Collagen (bovine achilles tendon) and O-methyl-hydroxylamine·HCl were from Tokyo Chemical Industry Co., Ltd., and thrombin was from Sankyo Co., Ltd. Phenylhydroxylamine was the product of Nakarai Chemicals Ltd. 6-Hydroxylaminopurine was synthesized according to the method of Giner-Sorolla and Bendich.[6] N-Trimethyl-hydroxylammonium iodide was synthesized as follows. Methyl iodide (2.8 g, 20 mmoles) was added to anhydrous ethanol containing 16 mmoles of hydroxylamine,[6] and the mixture was heated at 50—60° for 6 hr and then was evaporated to dryness. The residue was crystallized and recrystallized from ethanol affording white plates (500 mg, yield 16%) of N-Trimethylhydroxylammonium iodide; mp 128—134° (decomp., uncorr.). *Anal.* Calcd. for $C_3H_{10}ONI$: C, 17.75; H, 4.97; N, 6.90. Found: C, 17.78; H, 4.87; N, 6.97.

**Platelet-rich Citrated Plasma (PRCP)**——All glassware coming into contact with the whole blood or PRCP was siliconized using 10% solution of Shin Etsu Silicone-Kc 88 in petroleum ether.

Male rabbits (weight, 2—3 kg) were anesthetized with ethylether. The carotid artery was carefully cut and cannulated with a 20 cm piece of siliconized polyethylene tubing. About 60—90 ml portion of blood was transfered into siliconized polyethylene centrifuge tubes which contained 1/10 volume of 3.8% of sodium citrate. The blood was centrifuged at 1000 rpm for 10 min. The supernatant PRCP (20—40 ml) was removed, stored at room temperature and used within 10 hr. The PRCP contained $6—8 \times 10^8$ platelets per ml.

**Platelet Aggregation**——The rate and extent of platelet aggregation were measured by the optical density method of Born and Cross[7] by using Evans EEL 169 platelet aggregation meter.

A cuvette contained 1.0 ml of PRCP and 10 $\mu$l of saline (for controls) or 10 $\mu$l of a solution of the compound in saline (for test compounds) was placed in the aggregation meter and allowed to incubate at 37° for 3 or 60 minutes. At this point, the PRCP was challenged with 10 $\mu$l of a solution of ADP, thrombin or 100 $\mu$l of a solution of collagen in saline. The amount of ADP required for the remarkable aggregation was a final concentration of $10^{-5}$M, that of collagen was 0.53 mg/ml and that of thrombin was 0.3 unit/ml. The percent inhibition of aggregation by a test compound was calculated by dividing the maximum deflection in the optical density curve in the presence of the compound by that observed in the control, then multiplying by 100.

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3) A. Gaarder, J. Jonsen, S. Laland, A. Hellem and P.A. Owren, *Nature* (London), **192**, 531 (1961).
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6) A. Giner-Sorolla and A. Bendich, *J. Am. Chem. Soc.*, **80**, 3932 (1958).
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## Result and Discussion

Rabbit platelet-rich citrated plasma (PRCP) was incubated with hydroxylamine at 37° for 3 minutes, and then challenged with ADP. Profiles of the aggregation are shown in Fig. 1. Platelet aggregation and its inhibition were estimated by the extent of the decrease in the optical density after the addition of ADP. It showed that hydroxylamine completely inhibited ADP-induced platelet aggregation at $10^{-4}$M, whereas adenosine, a known powerful inhibitor of platelet aggregation,[8] showed 60% inhibition at the same concentration. The efficiency of hydroxylamine at $10^{-5}$M was the same as that of adenosine at $10^{-4}$M.

The agent also inhibited collagen- and thrombin-induced platelet aggregation and had a stronger activity than adenosine (Table I).

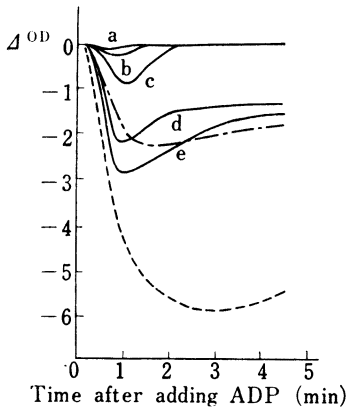


Fig. 1. Inhibitory Effect of Hydroxylamine against ADP-induced Platelet Aggregation

Rabbit PRCP treated with $NH_2OH \cdot HCl$; ——, (a) $10^{-4}$M, (b) $5\times10^{-5}$M, (c) $2.5\times10^{-5}$M, (d) $10^{-5}$M, (e) $5\times10^{-6}$M, adenosine; – – –, $10^{-4}$M and none (control); ····· at 37° for 3min was challenged with ADP ($10^{-5}$M).

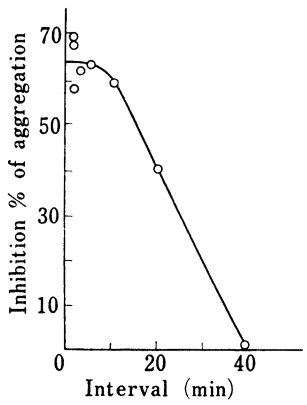


Fig. 2. Inhibition of ADP-induced Rabbit Platelet Aggregation brought about by Adding $NH_2OH \cdot HCl$ ($10^{-5}$M) at Increasing Intervals of Time before the Addition of ADP ($10^{-5}$M).

Table I. Activity of Hydroxylamine and Its Derivatives as Inhibitors of Rabbit Platelet Aggregation

| Compound | Final concentration (M) | Inhibition %[a] of platelet aggregation induced by | | |
|---|---|---|---|---|
| | | ADP | collagen | thrombin |
| $NH_2OH \cdot HCl$ | $10^{-4}$ | 98 | 100 | 98 |
| | $10^{-5}$ | 61 | 13 | 70 |
| $NH_2OMe \cdot HCl$ | $10^{-4}$ | 7 | | |
| $(CH_3)_3N^+OH \cdot I^-$ | $10^{-4}$ | 16 | | |
| $C_6H_5$-NHOH | $10^{-4}$ | 66 | | |
| $NH_2CONHOH$ | $10^{-4}$ | 8 | | |
| 6-Hydroxylaminopurine | $10^{-4}$ | 0 | | |
| Adenosine | $10^{-4}$ | 60 | 46 | 96 |

a) The values were obtained from the maximal aggregation after the addition of ADP, collagen or thrombin.

8) G.V.R. Born and M.J. Cross, *Proc. Physiol. Soc.*, **1962**, 29P.

Several N- and O-substituted derivatives of hydroxylamine, O-methylhydroxylamine, N-trimethyl-hydroxylammonium iodide, phenylhydroxylamine, hydroxylurea and 6-hydroxylaminopurine, were tested for inhibitory activity against ADP-induced platelet aggregation. The results are summarized in Table I. None of the derivatives showed significant inhibitory activities except phenylhydroxylamine which showed the same potency as adenosine.

Inhibitory action of hydroxylamine against ADP-induced platelet aggregation was characteristic. Thus, the maximal aggregations were observed at about 1 minute after the addition of ADP and the deaggregations were observed thereafter (Fig. 1). The reasons for this deaggregation were not known. Similar observation has been reported with the inhibition by adenosine.[8]

Figure 2 showed that the inhibitory activity of hydroxylamine decreased the longer the interval of time between its addition to PRCP and the subsequent addition of ADP. The probable explanation for the loss of activity is that hydroxylamine was increasingly inactivated by some factors from platelets or plasma.

It has been proposed by Salzman, *et al.*[9] that ATPase plays an important role in the platelet function. Recently, hydroxylamine has been shown to affect ATPases involved in active transport at cell membrane.[10] Hence, the inhibitory activity of hydroxylamine against platelet aggregation might be related to the alterations of the enzyme function of platelets.

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# Stereochemical Studies. XII.[1] Effects of Neighboring Functional Groups on 1,2-Asymmetric Induction in the Reduction of Propiophenone Derivatives by Catalytic Hydrogenation

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In preceding papers[1,3] of this series, it has been reported that the stereochemical course of 1,2-asymmetric induction in the reduction of propiophenone derivatives with sodium borohydride depends on neighboring functional groups such as $-NH_2HCl$, -OH, and $-OCH_3$, attached at $\alpha$- and/or $\beta$-positions to the carbonyl group. Catalytic hydrogenation is a convenient general procedure for the reduction of carbonyl compounds to the corresponding alcohols. The present paper describes the effects of neighboring functional groups on the direction and the degree of 1,2-asymmetric induction in the reduction of propiophenone derivatives (I—IV) having a functional group at $\alpha$- or $\beta$-position to the carbonyl group by

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2) Location: *Hongo, Bunkyo-ku, Tokyo.*
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