

bath immediately. The mixture was decomposed with ice water and extracted with  $\text{CHCl}_3$ . After washing with 5%  $\text{NaHCO}_3$  and water, the extract solution was evaporated and the residue was chromatographed over silica gel. Elution with hexane gave *ca.* 350 mg of the unsaturated nitrile (IX) and further elution with hexane-benzene gave an oily residue which was crystallized from  $\text{CHCl}_3$ -MeOH to afford 93 mg of pure 4-methylcholestenone (I), mp 101.5—102°, mixed mp 101—101.5°,  $[\alpha]_D +104.7^\circ$  ( $c=0.43$ ,  $\text{CHCl}_3$ ). IR  $\nu_{\text{max}}^{\text{Nujol}}$   $\text{cm}^{-1}$ : 1670, 1607. UV  $\lambda_{\text{max}}^{\text{EtOH}}$  nm (log  $\epsilon$ ): 250 (4.22).

[Chem. Pharm. Bull.]  
20(7)1569—1571(1972)

UDC 612.1.014.4

## Effect of Plasma pH on Stability and Capacity of Aggregation of Platelets

KIYOMI KIKUGAWA, KAZUHIRO IZUKA, and MOTONOBU ICHINO

Research Laboratory, Division of Fermentation and  
Chemical Products, Kohjin Co., Ltd.<sup>1)</sup>

(Received November 15, 1971)

It has been known that blood platelets form aggregates in hemostasis and in thrombogenesis.<sup>2)</sup> The key role of adenosine diphosphate (ADP) as an important initiator of platelet aggregation has been well documented.<sup>3)</sup> Born<sup>4)</sup> and O'Brien<sup>5)</sup> have established an *in vitro* turbidimetric method for the estimation of ADP-induced platelet aggregation and its inhibition. Thus, platelet aggregation and its inhibition are estimated by measuring the change in the optical density of platelet-rich plasma.

It has been found in the course of our investigations of the platelet aggregation inhibitors<sup>6,7)</sup> that the extent of ADP-induced platelet aggregation of platelet-rich citrated plasma (PRCP) fluctuated over the storage period of several hours. These fluctuations were then found due to the alterations in pH of the plasma. Although it has been described that aggregation capacity of platelet is low at pH 6.5,<sup>8)</sup> effect of plasma pH on platelet aggregation has not yet been demonstrated. This paper describes that alterations in plasma pH greatly influenced the stability of platelet aggregating capacity and the extent of ADP-induced platelet aggregation.

### Experimental<sup>9)</sup>

**Platelet-rich Citrated Plasma (PRCP)**—Blood (pH 7.5) was collected from male rabbits anesthetized with ethyl ether and was mixed with 1/10 volume of 3.8% sodium citrate (pH 7.65). The mixture was then centrifuged at 1000 rpm at room temperature for 10 min and the supernatant (PRCP) was removed with a siliconized pipett. The PRCP thus prepared showed pH 7.7—7.9 and was stored at room temperature.

**Bufferized PRCP**—Tris(hydroxymethyl)aminomethane and sodium veronal were purchased from Sigma Chemical Company and Wako Pure Chemical Industries, Ltd. respectively. PRCP was bufferized with an equal volume of either of the following buffers; 0.1M Tris-HCl (0.1M Tris-HCl+0.08M NaCl), 0.1M

1) Location: Higashihama, Saiki, Oita.

2) J.F. Mustard and M.A. Packham, *Pharmacol. Rev.*, **22**, 97 (1970).

3) A. Gaarder, J. Jonsen, S. Laland, A. Hellem, and P.A. Owren, *Nature* (London), **192**, 531 (1961).

4) a) G.V.R. Born, *Nature* (London), **194**, 927 (1962); b) G.V.R. Born and M.J. Cross, *J. Physiol.*, **168**, 178 (1963).

5) J.R. O'Brien, *J. clin. Path.*, **15**, 452 (1963).

6) K. Iizuka and K. Kikugawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 614 (1972).

7) K. Kikugawa, K. Iizuka, Y. Higuchi, H. Hirayama, and M. Ichino, *J. Med. Chem.*, **15**, 387 (1972).

8) K. Yasunaga, *Ketsueki to Miyakukan*, **1**, 627 (1970) and the references cited therein.

9) Unless otherwise mentioned, materials and methods were same as those described in ref. 6 and 7.

Tris-acetate (0.1M Tris-acetate+0.08M NaCl), 0.05M Tris-HCl (0.05M Tris-HCl+0.15M NaCl) and physiologically isotonic Veronal buffer.<sup>10)</sup> Recording of pH of PRCP (bufferized and unbufferized) was carried out using Hitachi-Horiba pH meter M-5. Before and after the aggregation pH of the plasma was maintained almost constant. In bufferization of PRCP, pH of the bufferized plasma was not always identical with that of the buffer added.

**Platelet Aggregation**—The extent of platelet aggregation was measured according to the method of Born and Cross<sup>4b)</sup> using Evans EEL 169 platelet aggregation meter. A cuvet containing 1.0 ml of PRCP (or bufferized PRCP) and 10  $\mu$ l of physiological saline (or 10  $\mu$ l solution of adenosine ( $10^{-2}$ M) in saline) was placed in the aggregation meter and allowed to stir at 37° for 3 min. At this point, the PRCP (or the bufferized PRCP) was challenged with 10  $\mu$ l of  $10^{-3}$ M solution of ADP in saline. Decrease in optical density of the stirred plasma was recorded using Rikadenki 1-pen recorder Model, N-14 for 5 min, and a maximal deflection of the optical density curve could be read on the recorder during the period.

As the sensitivity of platelet to the aggregating agent varies from preparation to preparation of PRCP, comparisons of the extent of aggregation under variable conditions were made using the same preparation of PRCP, and the direct effect of plasma pH on platelet aggregation was investigated within a minimal interval.

## Result and Discussion

When rabbit PRCP was stored at room temperature during the period of 5—9 hr, extent of ADP-induced platelet aggregation fluctuated between 45—71 in maximal deflections. During this period the pH (pH 7.7) of the plasma changed gradually to a higher value of 8.3 (Fig. 1-A). Thus, these fluctuations were considered to be partly due to the gradual increase in plasma pH, which may result in loss of platelet aggregating capacity.

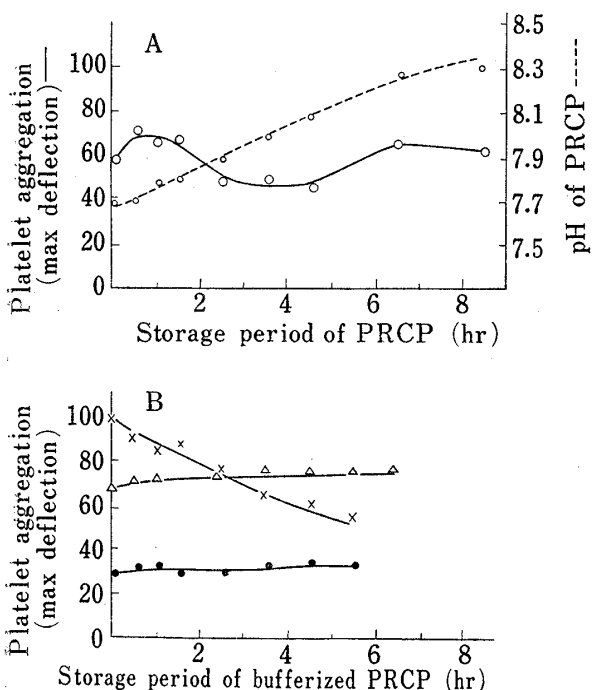


Fig. 1. Stability of Platelet Aggregating Capacity During the Storage Period of PRCP

- A: unbuffered PRCP  
 B: bufferized PRCP —×— :PRCP bufferized with 0.1 M Tris-HCl (pH 8.1) (pH of the plasma was maintained at 8.1), —●— :PRCP bufferized with 0.1M Tris-HCl (pH 7.45) (pH of the plasma was maintained at 7.6), —△— :PRCP bufferized with Veronal buffer (pH 7.30) (pH of the plasma was maintained at 7.6).

Platelet aggregation of the PRCP with added 0.1M Tris-HCl (pH 8.10), 0.1M Tris-HCl (pH 7.45) or Veronal buffer (pH 7.30) was examined during the period of 5.5—6.5 hr, and the results are shown in Fig. 1-B. The pH of the plasma was maintained constant at 8.1 or 7.6 during the period. With the plasma bufferized at pH 8.1, extent of

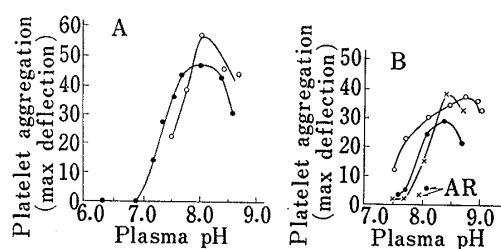


Fig. 2. Effect of Plasma pH on Platelet Aggregation

- A: The extent of aggregation was compared after 2 hours storage of the following bufferized PRCP's; —○— :PRCP bufferized with 0.1M Tris-HCl, —●— :PRCP bufferized with Veronal.  
 B: PRCP stored for about 2 hours was bufferized just before the examination of aggregation. —●— :PRCP bufferized with 0.1M Tris-HCl, —×— :PRCP bufferized with 0.1M Tris-Acetate, —○— :PRCP bufferized with 0.05M Tris-HCl. AR indicated the aggregation observed in the presence of  $10^{-4}$ M of adenosine.

10) K. Chitani and Y. Kondoh, "Zikken Kagaku Koza", Vol. 24, ed. by Nippon Kagakukai, Maruzen Inc., Tokyo, 1963, p. 211.

platelet aggregation was reduced gradually to about 60% of the initial aggregation during the period of 5.5 hr. This indicated that platelet gradually lost its aggregating capacity at pH 8.1. With the plasmas bufferized with Tris or Veronal buffer at pH 7.6, extents of platelet aggregation were kept almost constant during the period of 5.5—6.5 hr. Thus, the aggregating capacity of platelet was maintained at around pH 7.6. The result mentioned above indicated that the stability of platelet aggregating capacity was pH dependent and the aggregating capacity of platelet was maintained at pH 7.6 (pH of whole blood) over several hours but was lost at pH values higher than 8.1.

Direct effect of plasma pH on ADP-induced platelet aggregation was further investigated. ADP-induced platelet aggregation was examined with PRCP with added 0.1M Tris-HCl buffers or Veronal buffers which had been stored for about 2 hr. The results shown in Fig. 2-A indicated that platelet aggregated most strongly at pH 8.0 over the pH range of 6.3—8.7. The results, however, reflected both the effect of pH on platelet stability during the period of 2 hr and the direct effect of pH on platelet aggregation.

In order to study the direct effect of pH on platelet aggregation separately, PRCP which had been stored for about 2 hr was bufferized with Tris buffers just before the examination of ADP-induced platelet aggregation (Fig. 2-B). With PRCP bufferized with 0.1M Tris-HCl or 0.1M Tris-acetate, the extent of aggregation was found largest at around pH 8.4, and the extent of aggregation observed at pH 7.7 was much smaller than that observed at above pH 8.0. It would be noticeable that the extent of aggregation at pH 7.7 was approximately equal to that at around pH 8.0 with inhibition by a known powerful inhibitor, adenosine ( $10^{-4}$ M).<sup>11)</sup> With PRCP bufferized with 0.05M Tris-HCl, the extent of aggregation at above pH 8.5 was larger than that at pH values lower than pH 8.0. In this case, the effect of pH on the extent of aggregation was not so great as that observed with 0.1M Tris buffered PRCP's probably because of the different concentration of Tris ion. The results mentioned above indicated that the aggregation of blood platelet was largely influenced by plasma pH, and the maximal extent of aggregation was observed at above pH 8.

In summary, platelet aggregating capacity was maintained at around pH 7.6 over several hours with a considerable extent of ADP-induced platelet aggregation, and was lost at pH values higher than 8 with the larger extent of platelet aggregation. Thus, the fluctuations in the extent of aggregation with unbufferized PRCP were concluded to be due to the increase in plasma pH which caused the loss of platelet aggregating capacity and increased the extent of platelet aggregation.

In most cases,<sup>4,8)</sup> PRCP has been directly used without bufferization in the *in vitro* turbidimetric studies of platelet aggregation, but in some cases,<sup>12-14)</sup> PRCP has been bufferized with Tris, imidazole or Veronal buffers. From the results obtained above, bufferization of PRCP at around physiological pH would be necessary in order to maintain platelet aggregating capacity with a considerable extent of ADP-induced platelet aggregation. In bufferization of PRCP some care must be taken as pH of the bufferized plasma was not always identical with that of the buffer added.

**Acknowledgement** The authors wish to express their sincere thanks to Dr. H. Hirayama of the laboratory for his supply of experimental animals and to Miss R. Sugata for her technical assistances.

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

12) M. Nakanishi, H. Imamura, and K. Goto, *Yakugaku Zasshi*, **90**, 564 (1970).

13) W.B. Laceyfield, R.G. Herrmann, J. Mills, W.M. Mills, and J.D. Frank, *J. Med. Chem.*, **14**, 133 (1971).

14) E.F. Elslager, J.R. Mclean, S.C. Perricone, D. Potoczak, H. Veloso, D.F. Worth, and R.H. Wheelock, *J. Med. Chem.*, **14**, 397 (1971).