Influence of Histamine or Histamine-Liberator (48/80) on the ADP-Induced Platelet Aggregation

In a previous work[1] it was found that the addition of histamine to the rat platelet-rich citrated plasma (PRP) does not interfere with the clumping effect of ADP added to the plasma simultaneously with histamine. This result is similar to that obtained by O'BRIEN[2], MITCHELL and SHARP[3] on human or rabbit platelets, but the contrary to that of CONSTANTINE[4] who observed that histamine inhibits the ADP-induced platelet clumping in rabbit plasma. It is very difficult to reconcile the opposite results obtained by O'BRIEN[2], MITCHELL and SHARP[3] and ourselves[1] with those of CONSTANTINE[4], especially in view of the fact that histamine provokes platelet aggregation in the decalcified whole blood[1,5,6].

The purpose of our experiments was to study whether the clumping effect of histamine in the whole blood is effectively abolished in PRP, and, subordinately, to compare the influence of the added histamine with that of the endogenous one as released by histamine-liberator.

Methods. Blood from the right ventricle of adult rats, lightly anaesthetized with ether, was drawn into a plastic syringe and immediately added to the sodium citrate solution in a siliconized centrifuge tube (1 part of 3.8% sodium citrate solution to 9 parts of blood). The samples were centrifuged for 13 min at 1000 rpm. The supernatant platelet-rich plasma was collected by siliconized Pasteur pipets and transferred into a plastic container; the platelet count was estimated according to REES and ECKER[7]. The PRP was then divided into polystyrene tubes, stored at room-temperature (18–20 °C) for the subsequent examination of the optical density by '169 Platelet Aggregation Meter' (Evans electroselenium Ltd). The stirring rate was 2800 rpm.

Since the change of optical density varies with the platelet count each result obtained was corrected by referring it to a standard number of cells (250,000/ml)[8]. The drugs used were: Na_3ADP (C.F. Boehringer & Söhne GmbH, Mannheim); histamine dichloride (Merck AG, Darmstadt); 48/80 (Wellcome Research Laboratories, Beckenham); $NaH_2PO_4 \cdot H_2O$ (Merck AG, Darmstadt).

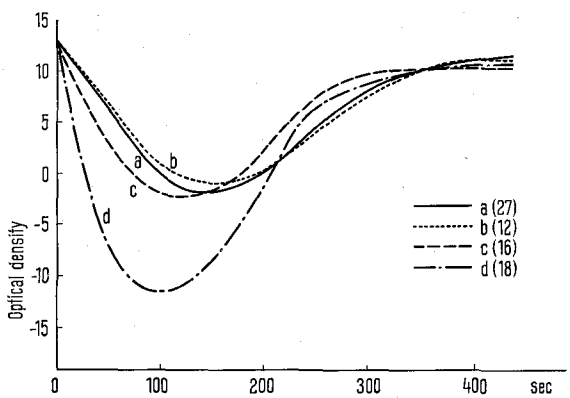

Fig. 1. Changes in O.D. observed: a) in PRP + ADP; b) in PRP + histamine + ADP (both added at 0 time); c) in PRP + histamine (added 60 sec before ADP) + ADP (added at 0 time); d) in PRP + histamine (added 300 sec before ADP) + ADP (added at 0 time). The final concentration of the drugs was: ADP $9.12 \times 10^{-5} M$; histamine $2.5 \times 10^{-4} M$. Each curve was obtained by averaging the single values recorded in the experiments; the n values in parenthesis are the number of experiments in each group. We computed the two sample t-test for the results obtained in the a) and d) groups of experiments at 50–100–150 sec. The values of the observed t were greater than the limiting value for 0.05 probability.

Results. 1. Figure 1 shows that histamine does not interfere with ADP-induced platelet clumping if added at the same time as ADP; but if histamine is previously (1–5 min) added to the plasma, the subsequent addition of ADP provokes a greater clumping effect. This phenomenon is well marked if the lapse of time between the addition of the two substances is 5 min. It is, however, possible to inhibit the potentiating effect of the pretreatment of platelets with histamine if Na H_2PO_4 (final concentration 6.5×10^{-6} M) is contemporary added to PRP with histamine. In this case, however, the clumping effect of ADP is also greatly reduced. The influence of histamine on the effect of ADP, as shown by Figure 2, involves only the aggregation phase because the platelet disaggregation has the same time course either with or without histamine pretreatment.

2. In 71 experiments we used a histamine-liberator (48/80) which was added to PRP at the same lapses of time before ADP as histamine. The final concentration of 48/80 was 9 μg/ml. By using higher concentrations of 48/80 to 45 μg/ml we have not observed an increased effect. After the subsequent addition of ADP (final concentration $9.12 \times 10^{-5} M$) the behaviour of platelets thus treated was roughly similar to that of platelets treated with histamine; nevertheless the clumping effect of ADP appeared increased to a lesser extent with 48/80 rather than with histamine.

Discussion. The present results show that the clumping effect of ADP is increased if histamine is incubated with plasma for a brief lapse of time (1–5 min) before ADP ad-

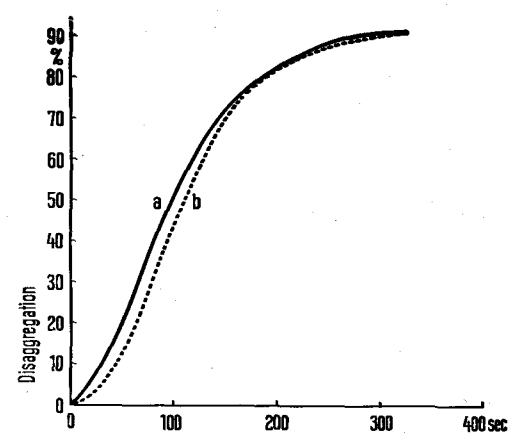

Fig. 2. The average raising in the O.D. observed in 27 (a) and 18 (b) experiments following its maximal reduction. Note that 0 time indicates the peak of the maximal platelet aggregation a) in PRP + ADP; b) in PRP + histamine (added 300 sec before ADP) + ADP, respectively.

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dition; such an increase is abolished by phosphate. Consequently we can suppose that the inhibitory effect of histamine on ADP-induced aggregation, previously described by CONSTANTINE[4] could be due to the use of histamine-phosphate. We can put forward a similar supposition to explain the absence of any effect of histamine observed by O'BRIEN[2]. In actual fact, O'BRIEN[2] and CONSTANTINE[4] unfortunately do not specify the histamine salt they used. It seems interesting to quote also that we have obtained similar results with histamine and 48/80. As platelets are known to contain histamine, we can emphasize that, so far as we had observed, the effect of 48/80 above 9 µg/ml is independent of the concentration. Therefore we can suppose that histamine-liberator, even at low concentration, releases the maximum of histamine disposal. According to HUMPHREY[9], the content of histamine in the rat platelets is less than 1 µg/10^9 cells; on the contrary, by histamine addition every platelet could have at its disposal roughly 27×20^{-6} µg of histamine.

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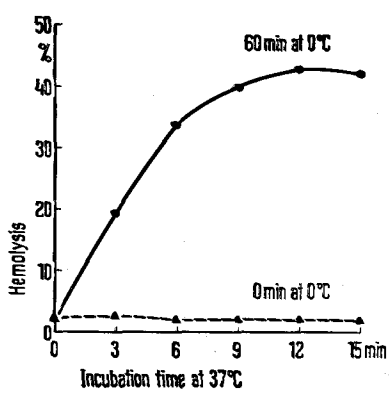
Hot-Cold Hemolysis: The Role of Positively Charged Membrane Phospholipids

The erythrocytes of several species exhibit a startling response to many bacterial exotoxins (e.g. *Cl. welchii* α-toxin or staphylococcal β-toxin): the classic 'hot-cold' hemolytic phenomenon; hemolysis caused by the lowest dilutions of some exotoxins might be incomplete or absent during incubation of susceptible erythrocytes for a fixed time at 37°C. However, additional incubation at 0°C, rather than inhibiting the hemolytic process, permits it to proceed to completion; hence the term 'hot-cold'.

In an attempt to explain this effect we advanced the working hypothesis that the 'hot' phase of the phenomenon may result from chemical alterations of the choline residues of membrane sphingomyelin. Our observation of lytic effects of the triiodide ion on the sheep, human, and rat erythrocytes fully supports this concept. The I_3^- ion was formed either non-enzymatically, by reaction of hydrogen peroxide with iodide, or enzymatically, using glucose oxidase and glucose as a source of H_2O_2 and horse radish peroxidase to oxidize iodide. Hemolysis was determined by measuring the amount of hemoglobin as hemin[1]. The lytic effects of I_3^- depend, *Caeteris paribus*, on the concentration of the triiodide ion identified by its absorption spectrum (max_1 at 3530 Å with molar extinction coeff. 26,400; max_2 at 2875 Å with *m/e* coeff. 40,000[2]) and determined using its max_1. The I_3^- induced hemolysis occurs between pH 6.0 and 7.9 with the optimum at pH 7.0. Hemolysis is usually complete within 60 min of incubation at 37°C. However, when the incubation time is shorter, or when less than optimal amounts of I_3^- are employed, the extent of the 'hot' hemolysis is reduced. Now, the erythrocytes left at 0°C, exhibit additional leakage of hemoglobin: the 'cold' effect which is proportional to the dose of I_3^- used in the 'hot' phase of the experiment or, with suboptimal time of incubation, to the duration of the 'hot' phase (Figure).



'Hot-cold' hemolysis of sheep RBC induced by I_3^-. Two series of duplicate tubes each containing 2.5 ml of 0.6% sheep RBC suspended in 0.1*M* potassium phosphate buffer, pH 6.9, and 0.8 ml of a solution of I_3^- (350 nanomoles; with molar ratio I_3^-/RBC phospholipid = 4.3) were incubated at 37°C ('hot' phase) and the percentage hemolysis determined in one series of tubes (▲---▲) at the indicated time intervals. At each time period duplicate tubes of the second series were removed to an ice bath and held for an additional 60 min ('cold' phase) and the percentage hemolysis again determined (●—●).

I_3^- hemolysis could be prevented by the addition of dipalmitoyl lecithin (Nutr. Biochem. Corp., Cleveland, Ohio; in form of sonicates) to the incubation medium before the initiation of the 'hot' phase. We have also demonstrated that this saturated lecithin binds I_3^- in a stable complex, apparently a triiodide, that can be identified by thin layer chromatography (Eastman Chromagram K301R2; continuous run in diethyl ether, methanol: 1:1). Similar interaction of I_3^- with $-N^+(CH_3)_3$ groups of membrane phospholipids, at pH 7.0 fully protonated (pK about 9.0) and accessible to influences of the medium[3], apparently leads to hot-cold hemolysis. Of the membrane phospholipids, the sphingomyelins which are net positive over wide pH[4], may well be the most active substrate for interaction with I_3^-. The erythrocytes of sheep, man, and rat show (in this order) both decreasing sensitivity to the lytic effects of I_3^- (Table), and decreasing contents of their sphingomyelins (14.66; 10.0; 4.91 nmoles/10^8 cells with the molar ratios total membrane phospholipid/membrane sphingomyelins of 1.58, 3.97, and 7.6, respectively[5–7]).

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