SPECTROPHOTOMETRIC PLATELET AGGREGATION ASSAY TO MEASURE SINGLE PLATELET DISAPPEARANCE: AN EVIDENCE

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A recent report from this laboratory described a spectrophotometric method of monitoring agonist-induced aggregation of thin suspensions of gel-filtered calf blood platelets by Brownian motion[1]. It has been observed subsequently that aggregation induced by ADP occurred after virtually all optical effects of shape-change reactions ceased and that the aggregates formed at the initial stages (< 30 sec) were predominantly dimeric (unpublished results of Jamaluddin, Krishnan and Sreedevi). This indicated that the initial rate measured by the method could represent single platelet recruitment into aggregates. Here we present microscopic evidence in support of this possibility in ADP-induced aggregation.

Treatment of gel-filtered calf platelets with ADP results in their transformation to apparently spheroidal forms, judging by the optical effects[1]. Assuming that the spheroidal forms are monodisperse and that they are the ones taking part in the aggregatory reactions, the kinetics may be represented, in analogy with the aggregation of monodisperse spherical colloidal particles[2], as:

$$dN_1/dt = -k_{11}N_1^2 - k_{12}N_1N_2, \quad (1)$$

$$dN_2/dt = (k_{11}/2)N_1^2 - k_{12}N_1N_2, \quad (2)$$

where N_1 and N_2 are, respectively, the numbers ot monomeric and dimeric platelets ml^{-1}, t is the time and k_{11} and k_{12} are the rate constants of dimer and trimer formation, respectively.

The turbidity, τ_0 of the platelet suspension at the beginning of the aggregatory reaction ($t = 0$) is given by the equation:

$$\tau_0 = N_1R_1 + N_2R_2 + \text{---} \quad (3)$$

where R_1 and R_2 are the scattering cross-sections of the monomeric and dimeric platelets, respectively[3].

Differentiation of (3) with respect to time and substitution of (1) and (2) into the differentiated forms assuming that as $t \to 0$, $N_2 \to 0$, and rearrangement gives:

$$-\left(\frac{dN_1}{dt}\right)_{t\to 0} = \left(\frac{d\tau_0}{dt}\right)_{t\to 0} \Big/ \left(\frac{R_2}{2} - R_1\right). \quad (4)$$

The initial rate of single platelet disappearance is thus seen to be equal to the initial rate of turbidity change divided by an optical factor, $(R_2/2) - R_1$). The value of $(d\tau_0/dt)_{t\to 0}$ can be obtained as the initial slope of the downward pen deflection in the spectrophotometric platelet aggregation assay described before[1]. The initial rate of decrease of single platelets, $-(dN_1/dt)$, may be obtained microscopically as shown in figure 1A.

Following treatment with ADP, aggregation of gel-filtered calf platelets occurred, reversibly, without any requirement for exogenous fibrinogen, after a lag period (figure 1A). The lag period which was the same as that observed in the spectrophotometric assay[1], was inversely related to the ADP concentration. Platelet preparations that had undergone activation prior to agonist treatment aggregated without showing significant shape-change and lag periods and were not used for the purposes of the present study.

The rates of aggregation determined spectrophotometrically as well as by microscopy were hyperbolic functions of ADP concentration:

$$r = \frac{R(ADP)}{S_{0.5} + (ADP)}$$

or

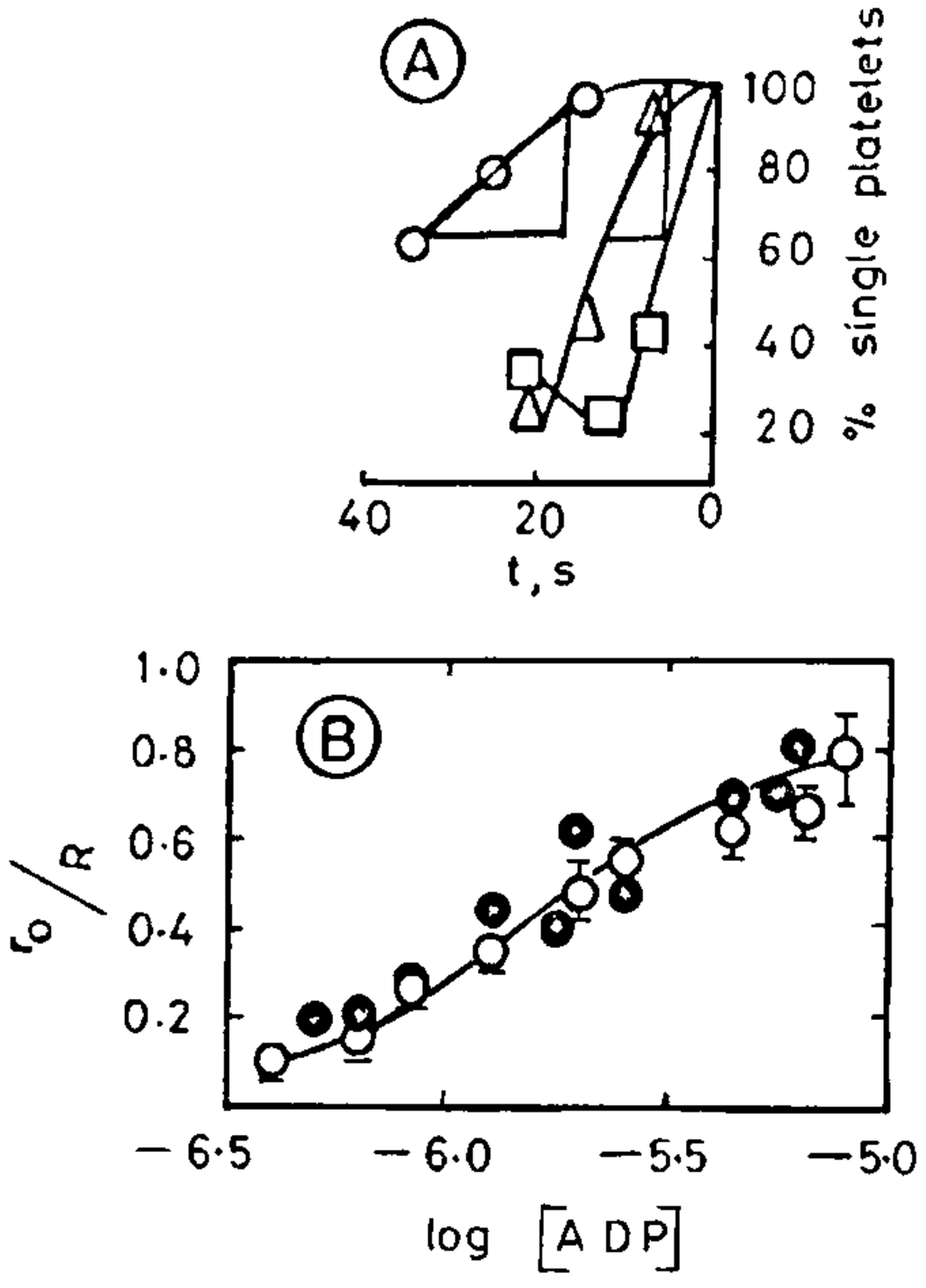
$$\frac{r}{R} = \frac{(ADP)}{S_{0.5} + (ADP)} \quad (5)$$

where r is the rate, R the maximum rate attained at (ADP) $\gg S_{0.5}$, and $S_{0.5}$ the concentration of ADP at which $r = 0.5R$.

R was obtained as the reciprocal of the ordinate intercept of linear plots of r^{-1} vs $(ADP)^{-1}$. $S_{0.5}$ was obtained as the reciprocal of the extrapolated abscissa intercept of such plots. The values of R and $S_{0.5}$ determined by the two methods could differ owing to the effect of the optical factor (equation (4)) but the ratio, $[(d\tau_0/dt)/(d\tau_0/dt)_{max}]_{t\to 0}$ that is, the ratio of the rate at any concentration of ADP to the maximum rate, determined spectrophotometrically, is independent of the optical factor.

Differentiation of (5) with respect to the logarithm of ADP concentration gives:

$$\frac{d(r/R)}{d\ln(ADP)} = \frac{S_{0.5}(ADP)}{[S_{0.5} + (ADP)]^2}. \quad (6)$$



Figures 1A and B. **A.** Time course of single platelet disappearance following ADP-treatment of gel-filtered calf platelets. Gel-filtered platelets obtained as described before[1] were diluted with the gel-filtration column buffer to a platelet concentration of $3–4 \times 10^7$ ml^{-1} and 1 ml of the diluted sample was treated with various concentrations of ADP (in 2μl volume) at 30 ± 1°C (ambient temperature). Aliquots (0.1 ml) were withdrawn into 0.4 ml of 1% formaldehyde in gel-filtration column buffer, at the indicated intervals of time. It was assumed that formaldehyde cross-linking was faster than the disaggregation by dilution. The single platelets in the samples were counted without delay in 5/25 squares in a haemo-cytometer, under the microscope. The percentage of single platelets remaining at each time interval was calculated relative to the single platelets present in 0.1 ml aliquots of the untreated platelet sample fixed in formaldehyde and the values were plotted against time. ADP concentrations used were 0.64μM (○), 3.8μM (△), and 6.6μM (□). Results of one experiment representative of three other similar experimental results, reported. The manner of obtaining initial rate, in percent single platelet disappeared per se, is also illustrated. **B.** Plot of fractional rate of aggregation (r/R) obtained by the spectropnotometric (○) and microscopic (●) methods. The spectrophotometric method of initial rate measurement was described before[1]. The values are mean ± SD (n = 5). Data points of the microscopic experiments were obtained in several experiments. Values of R were obtained as described in the text.

A plot of (r/R) against log (ADP) will have slopes approaching zero as (ADP) $\ll S_{0\,5}$ and (ADP) $\gg S_{0\,5}$. At (ADP) = $S_{0\,5}$ the slope has its maximum value of 0.57 (2.303/4). Therefore in case the spectrophotometric and microscopic methods measure one and the same phenomenon, plots of (r/R) determined by either method as a function of log (ADP) should coincide. The data presented in figure 1B show that this indeed might be the case.

Because of the need to minimize artifactual crosslinking by formaldehyde as well as to make aggregation rates amenable to manual sampling platelet concentrations in the microscopic experiments had to be kept low. Therefore the number of particles counted, had, of necessity, remained low and so statistical treatment of data from different experiments was not possible. But the microscopic method was a true indicator of decrease in platelet counts by aggregation. This was verified by serially diluting platelet suspensions used in the experiments and determining the platelet counts in the diluted samples. The expected decrease in platelet counts was obtained in each case (within 5%). This coupled with the consistency of the data in repeated experiments lent strong support to the view that the spectrophotometric and the microscopic methods are both measuring the same process. This view was further strengthened by the observation that the predominant form of aggregates found at early times, under the microscope, was dimeric.

Furthermore, since 90% or more of platelets used in the present study were present initially as monomers and since the scattering cross-section of a dimer is significantly lower than that of two monomers[3] it is easily seen from (3) that the dominant contribution to the initial turbidity decrease comes from the decrease in the N_1R_1 term or from the decrease in single platelets.

Thus experimental observation and theoretical expectation both support the view that the spec-

trophotometric method measures the kinetics of single platelet recruitment into aggregates. There can be little doubt that the method is suitable for kinetic investigations into the mechanisms and modulations of interplatelet interactions. In this respect the spectrophotometric method is vastly superior to the conventional aggregometric method. The aggregometer apparently measures the clumping of primary aggregates and is not suitable for kinetic purposes.

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A SIMPLE AND ECONOMIC METHOD FOR LONG-TERM PRESERVATION OF MUSHROOM CULTURE

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In an attempt to establish culture collection of mushrooms (Agaricales) from South India at this Institute, a simple and low cost method was developed for a long-term preservation of Oyster mushroom (*Pleurotus pulmonarius* (Fr.) Quél.) and the culture could be kept viable by this method at least for eight years, maintaining the same cultural characters.

This method is simple, economic, requiring minimum space, needs no cryostatic or low temperature arrangements and therefore, most suitable in tropical developing countries. The method is described below:

One ml liquid paraffin was dispensed in small-sized (9 × 75 mm) glass tubes (Corning make); were plugged with non-absorbant cotton; the mouths were covered by tin foil and the tubes were sterilized at 15 lb steam pressure for 15 min and then cooled to room temperature.

The petri plates showing actively growing cultures of mushroom were selected and circular agar punches (3 mm diam) were cut aseptically using sterilized cork borer. Three such agar punches with growing cultures were inoculated in the tubes prepared as in above, and the tin foil cover was replaced. The tubes were stored in a cupboard at room temperature. The cultures thus prepared were tested for their ability to survive after each year and were found to remain viable even after the lapse of eight years. The productivity of the preserved cultures was tested and compared with that of the freshly maintained cultures and both were found to be at par.

Of the other methods to preserve fungal cultures (including those of mushrooms), the liquid nitrogen storage method is very effective and cultures can be preserved in polypropylene straw ampoules[1], but it is considerably expensive and subjected to availability of liquid nitrogen.

The storage of fungal cultures in water[2] poses the problem of evaporation under tropical conditions and is, therefore, not suitable.

The proposed method, like the conventional method[3], has the same survival rate i.e. minimum of eight years, and additionally has certain advantages. Firstly, the cost accrued is half that of the conventional method. Secondly, the space required for storage is much less than the former one. Thirdly, the productivity of the preserved culture is at par with that of the freshly maintained culture. It is, therefore, claimed that the method described here is the most suitable, simple and economic, but equally effective as the conventional one, for long-term preservation of mushroom cultures.

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