Uptake of a New Hydrophobic Fluorescent Dye by Human Platelets.* (29701)

R. M. LYCETTE,† W. F. DANFORTH, J. L. KOPPEL AND J. H. OLWIN
Coagulation Research Laboratory, Department of Surgery, Presbyterian-St. Luke's Hospital, Chicago, and Biology Department, Illinois Institute of Technology, Chicago

It was reported previously that a new hydrophobic, fluorescent dye (STC) could be adsorbed by microorganisms, with a correlation between the amount of this dye adsorbed by brewing yeast and the ability of yeast to flocculate(1,2). STC adsorption data fit the Langmuir equation for monomolecular adsorption(2); and yeast flocs were dispersed either by solvents which attack bound lipids or by the uranyl ion which is chelated by phospholipids(3). This ion is not transported into yeast cells(4). These data suggested that STC uptake by yeast cells may be related to concentration of surface phospholipids.

The aggregation of platelets, which is analogous to the flocculation of yeast, may also involve surface phospholipids. Bangham *et al* have shown that migration of sheep platelets in an electrical field resembles that of egg lecithin micelles(5). This migration can be reversed by the uranyl ion which also binds to synthetic lecithin monolayers(6).

This paper describes STC uptake by human platelets under a given set of experimental conditions and shows how uptake differs from usual findings in adsorption and dye-binding studies.

Materials and methods. STC, the dye used here, was of the 2-(stilbyl 4′-)-4,5-arylo-1,2,3-triazole class sold by the Geigy Chemical Corp., Ardsley, N. Y., under the trade name "Tinopal PCR." It was applied to platelets in an acetone-water mixture.

Platelet concentrates were prepared in plastic bags by the Red Cross Method for Platelet Packs, with EDTA as anticoagulant. The method simulated the procedure of Gardner *et al*(7) except as stated below.

To platelets derived from one unit of blood and free of excess plasma was added 100 ml of cold physiological saline. Cells were held overnight at 4°C, and 15 ml aliquots were placed in 27 mm plastic tubes and centrifuged 5 minutes at 125 × *g* in a PR-1 International Centrifuge. The supernatant was decanted to other tubes and centrifuged for 20 minutes at 900 × *g*. This was followed by 2 additional washings of platelets in saline containing 0.01 M Na_2EDTA buffered at pH 3.5. The pellet was transferred with a siliconized spatula to 10 × 75 mm siliconized glass tubes and suspended in buffer to give an optical density of 0.200 at 610 mμ in a Coleman spectrophotometer. Appropriate dilutions were made with buffer to serially reduce platelet concentration.

While the condition of pH 3.5 is unphysiological, earlier work showed that maximum uptake of STC occurred near the isoelectric point of the cells, where their net charge is zero and where hydrophobic interactions may predominate. No measurable dye was taken up at pH 7.0 to 7.4. Earlier work indicated that dye uptake increases with decreasing pH and that varying the platelet suspension back and forth between pH 3.5 and 7.4 does not significantly alter dye uptake at the final pH. EDTA has the added advantage of chelating divalent cations, which affect platelet adhesive or cohesive properties. Dye uptake was identical whether cells were checked immediately or examined after several days at 4°C.

A stock solution was prepared with 1 mg STC/ml of acetone. Further stepwise dilutions were made with acetone to yield concentrations ranging from 10 to 1000 μg/ml. The use of acetone was necessary to insure reproducible solutions of extremely hydrophobic dye. Although the final acetone con-

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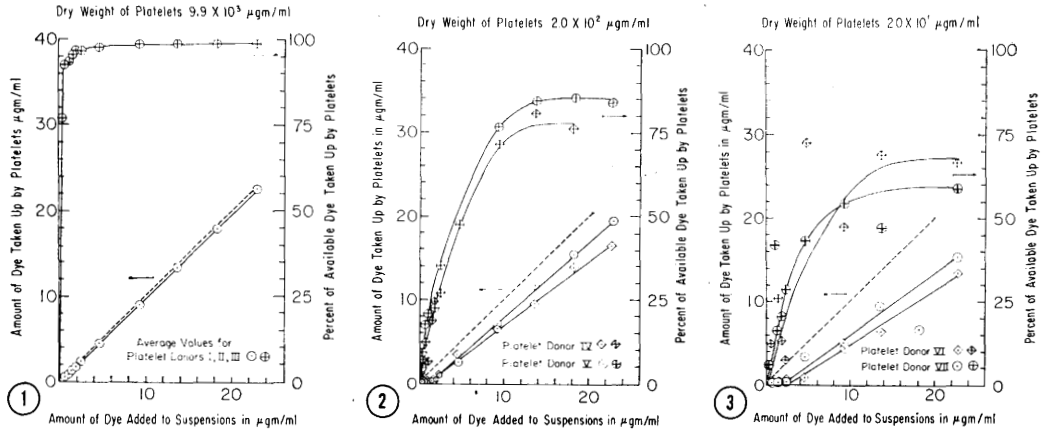

FIG. 1. Effect of dye concentration on dye uptake expressed in terms of actual amount as well as percent of available dye taken up. Values plotted are average results obtained with platelets from 3 different donors. Platelet concentration in the incubation system was 9.9×10^3 µg dry weight per ml. Dotted line represents theoretical state where all of dye is taken up, as µg/ml.

FIG. 2. Effect of dye concentration on dye uptake expressed in terms of actual amount as well as percent of available dye taken up. Values plotted are separate results obtained with platelets from 2 individual donors. Platelet concentration in the incubation system was 2.0×10^2 µg dry weight per ml. Dotted line represents theoretical state where all of dye is taken up, as µg/ml.

FIG. 3. Effect of dye concentration on dye uptake expressed in terms of actual amount as well as percent of available dye taken up. Values plotted are separate results obtained with platelets from 2 individual donors. Platelet concentration in the incubation system was 2.0×10^1 µg dry weight per ml. Dotted line represents theoretical state where all of dye is taken up, as µg/ml.

centration (c.a. 2% by volume) did not visibly affect platelet morphology or cause measurable leakage of ultraviolet adsorbing material from platelets, it may have altered cell properties.

One ml of dye solution was diluted with 3 ml of distilled water, and 0.2 ml of the mixture added to 2.0 ml of cell suspension. The concentration of dye in the final systems ranged from 0.227 to 22.7 µg/ml. The tubes were plugged with "Parafilm"‡-covered corks and rotated end-over-end in a controlled temperature box§ at a rate of 18 times/minute for 30 minutes at 37°C.

Following the rotation period, the tubes were centrifuged at $650 \times g$ for 10 minutes at 26°C. The fluorescence of supernatants was determined using 10×75 mm tubes in a Turner fluorometer. Platelet dye uptake was measured as the difference between fluorescence of a platelet-free control and the supernatant. The filters used were a 7-60 primary, and a 47B secondary backed up by a 2ND. One µg/ml of quinine sulfate at pH 1.0 was the fluorescence standard. Glassware was rinsed with acetone before usual washing methods.

Results. The data in Fig. 1-3 were obtained by incubating various strengths of dye with 3 different amounts of platelets, the highest containing about 500 × as many cells as the lowest.

The cells were never completely saturated with dye even when the ratio of dye to dry weight of platelets was about 1.0 (Fig. 3). When the ratio was much greater than 1.0, the points were more scattered than in Fig. 3 and could not be interpreted. On the other hand, when relatively large numbers of platelets were in suspension resulting in a dye-platelet ratio of much less than 1.0 (Fig. 1), a small amount of free dye remained in solution over the entire 100-fold range of dye strengths. This residual dye could be readily taken up by fresh platelets.

At all 3 concentrations of platelets the per cent of dye taken up rose with increasing dye concentration to plateaus whose heights were influenced by the total number of platelets present. Differences in the maximum per cent

‡ Sold by American Can Co., Menasha, Wisc.

§ Built by Elmac Engineering Co., Chicago, Ill.

of dye taken up, shown by platelets from 4 donors under 2 sets of experimental conditions, suggest this as a suitable criterion in comparing cells from many donors.

Platelets were also able to take up large quantities of uranyl nitrate under similar experimental conditions.

Discussion. Since human platelets took up large quantities of both STC dye and the uranyl ion, perhaps both agents entered the cells and were bound to internal lipoid structures, in addition to being adsorbed at the surface. Further work may establish exact binding sites for both agents.

Although no substances were released from platelets which change STC fluorescence in solution, an increased efficiency (3-4 ×) occurred whether the dye was affixed to cells (9.8 mg/ml dry weight) or whether associated with micelles in an aqueous emulsion of soybean phosphatides (0.0125 mg % Asolectin). It is not known if other lipids produce a fluorescence increase, but this enhancement suggests some type of physical or chemical bonding.

Since differences in per cent of dye taken up by platelets from normal donors are not large, it is hoped that large variations from the norm may be seen when platelets from persons with coagulation abnormalities are studied.

At all platelet weights, the weight of added dye bound increases with increasing concentrations of dye, contrary to the usual findings in adsorption and dye-binding studies. The most plausible reason for this is that small aggregates of dye molecules are bound more readily than single molecules. At all platelet concentrations, the weight of dye bound is nearly proportional to the square (1.6-2.4 power) of the strength of free dye, suggesting that a dimer may be the form most readily bound.

These results are in contrast with those obtained with yeast cells(2). Whereas yeast cells were saturated with 0.77 μg STC/mg of cells, platelets were not saturated when suspended in concentrations as high as 750 μg STC/mg of cells. Thus the nature of uptake by platelets differs markedly from that by yeast.

STC could not be desorbed from platelets unless acetone concentration was increased 4-fold; this indicates that dye is held by a process other than simple adsorption and that residual supernatant dye is not the result of a simple equilibrium between free and bound dye.

Summary. A method for uptake of a new hydrophobic fluorescent dye by human blood platelets is described in which cell concentrations are constant and those of dye varied widely. Uptake is measured in isotonic saline buffered with EDTA at a pH of 3.5, where cells are near their isoelectric point and dye maximally affixed to them. Dye uptake did not fit usual patterns of adsorption. At all platelet weights, the amount of added dye bound increased with increasing strengths of dye, being nearly proportional to the square of the concentration of remaining dye. Data from several normal platelet donors show maxima in per cent of dye taken up and slight differences in this per cent of dye over a wide range of dye added. This suggests varying amount of hydrophobic substances in or on platelets and a means for their assay.

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