

Fluorescent Labeling of Human Platelets

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Noncovalently bound fluorescent probes have been used to study changes in the platelet which may occur during platelet aggregation. Platelets were exposed to either N-phenyl-naphthylamine (NPN) or 8-anilino-1-naphthalene-sulfonic acid (ANS). Both dyes were bound by the platelet, and platelet aggregation by collagen or thrombin was unaffected by the presence of the label. No change in fluorescence intensity or wavelength of maximum intensity was observed during platelet aggregation.

P RIMARY HEMOSTASIS, the collagen-triggered aggregation of platelets, reportedly occurs when platelets come in contact with the newly exposed collagen in the subendothelium of a severed or injured blood vessel.¹

Much is known about the properties of the collagen which are required for this interaction,² but little is known about the properties of the platelet surface which are involved in the interaction with collagen. We have begun to investigate this question using noncovalent fluorescent probes, since these are capable of giving information not only on binding but on environmental changes near the bound probe. The use of fluorescent probes as a technique for investigation of biologic membranes has been discussed recently by Radda and Vanderkooi³ and by Waggoner and Stryer.⁴ Since fluorescence emission properties of some chromophores are responsive to the polarity of their environment,⁵ specific probes have been designed which bind at the surface or at various distances into the lipid bilayer and reflect changes in the membranes at these locations.⁴ Noncovalent fluorescent probes are particularly useful in studying dynamic systems, such as the aggregating platelet, where the microenvironment of the probe may be changing. The advantage of noncovalent probes over covalently bound ones is that they are less likely to interfere with functional groups in the membrane, possibly those which are involved in the interaction we are studying. To our knowledge, previous attempts to bind covalent or noncovalent probes of this type to platelets have destroyed platelet aggregability, while our technique allowed the platelets to maintain their function.

Our aim was to find a fluorescent probe which would bind to the platelet membrane and would reflect changes occurring during the platelet-collagen interaction. For these studies we have used N-phenyl-naphthylamine (NPN), an uncharged molecule which binds in the hydrocarbon chain region of the membrane⁶ and 8-aniline-1-naphthalenesulfonic acid (ANS), a negatively charged molecule which binds at the polar-nonpolar interface of the membrane. These two probes have been used in other systems to detect and study changes in con-

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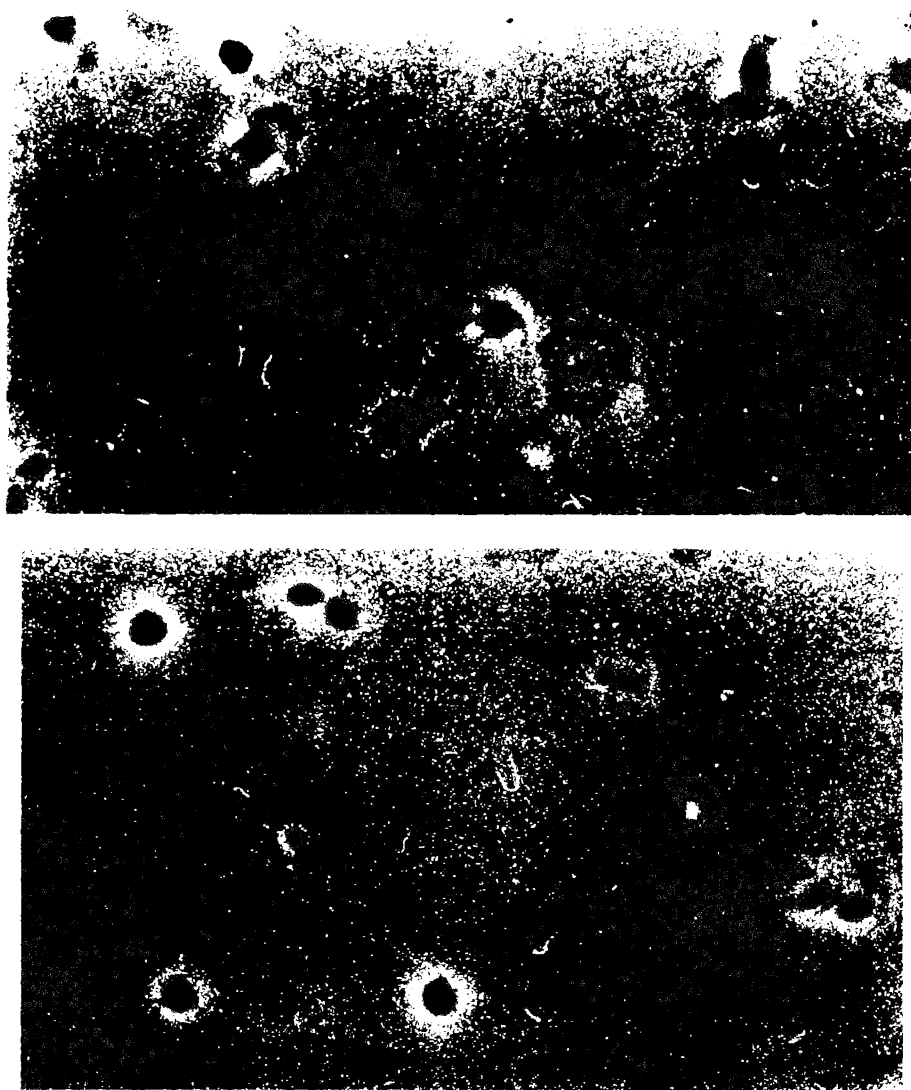
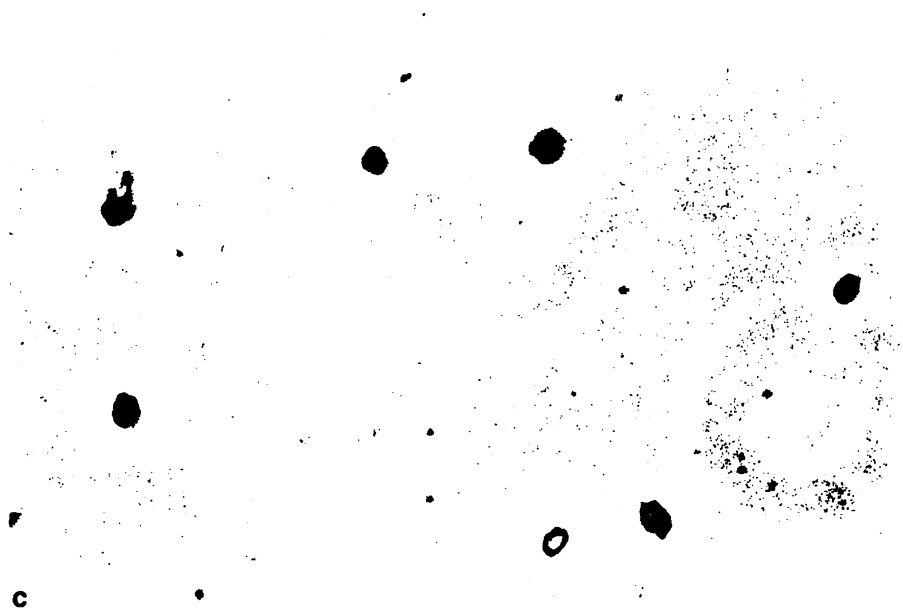


Fig. 1. Phase photomicrographs of platelets washed by density gradient centrifugation and gel filtration and labeled with fluorescent probes. Magnification, $\times 2750$. (A) PRP. (B) Washed, unlabeled control. (C) Labeled with N-phenyl-naphthylamine (NPN). (D) Labeled with 8-anilino-1-naphthalene sulfonic acid (ANS).

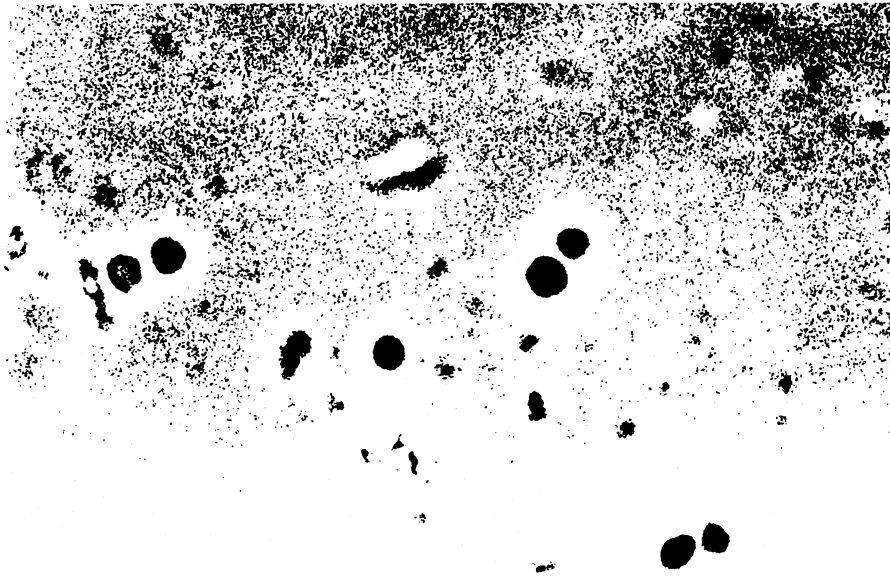
formation of biologic membranes, including erythrocytes^{7,8} and bacterial cell walls.⁹ We have succeeded in binding both ANS and NPN to the platelets without affecting their aggregability and have examined this labeling as well as its applicability in studies of platelet-collagen interactions.

MATERIALS AND METHODS

All procedures involving whole blood or platelets were performed using plastic or siliconized glass vessels.



C



D

See legend on facing page.

Platelet-rich Plasma (PRP)

Blood was drawn by peripheral venipuncture from normal human volunteers and mixed with 3.8% sodium citrate (9 volumes blood: 1 volume citrate). The citrated blood was centrifuged for 10 min at 126 *g* using an International HN-S centrifuge. The PRP was pipetted off.

Platelet-poor Plasma (PPP)

PRP was centrifuged for 10 min at 1200 *g* to remove the platelets, and the supernatant was used for PPP.

Washed Platelets

Published methods for "washing" platelets, i.e., freeing them of plasma,¹⁰ were found inadequate for our purposes since they failed to remove lipid components such as chylomicrons which have a great affinity for NPN. Therefore, platelets were washed by underlaying 7 ml of PRP (350,000 platelets/ μ l) with 3 ml of 0.5 *M* sucrose dissolved in 0.126 *M* NaCl, 0.015 *M* Tris-HCl, pH 7.6, 0.001 *M* MgCl₂, and 0.0054 *M* KCl and 2 ml 1.7 *M* sucrose dissolved in 0.126 NaCl, 0.015 *M* Tris-HCl, pH 7.6, 0.001 *M* MgCl₂, and 0.0054 *M* KCl and centrifuging in a swinging bucket rotor for 15 min at 1200 *g*. The plasma was aspirated from the layer above the sucrose, and the platelets, which banded at the interface between the 0.5 *M* and 1.7 *M* sucroses, were removed in a volume of about 2 ml and resuspended in the sucrose by gentle stirring. Sucrose was removed using a modification of the technique of Tangen and Berman.¹⁰ The platelets (2 ml) were passed over an 8 × 1-cm Sepharose 2B column (Pharmacia) equilibrated with 0.1% glucose, 0.001 *M* MgCl₂, and 0.0054 *M* KCl dissolved in 1 part of 0.145 *M* Tris-HCl, pH 7.6, to 9 parts of 0.140 *M* NaCl, and 2-ml fractions were collected. Platelet concentrations were determined by means of a Coulter Counter and/or turbidity measurements at 436 and 625 nm. The above method of preparation yielded platelets which appeared intact by phase microscopy (Fig. 1A-D) and which were still able to aggregate fully, in the absence as well as in the presence of NPN and ANS, upon addition of thrombin (Upjohn) and Ca²⁺ (Fig. 2A-C).

Since the use of fluorescent probes in investigations of cell-protein interactions has great potential utility, it seemed important to eliminate or at least identify all sources of irrelevant binding. Some components capable of such binding were found in the supernatant after centrifugation or aggregation of washed platelets, even when these were obtained from blood from fasting volunteers and thus were devoid of chylomicrons. Composed both of protein, as shown by a Lowry determination,¹¹ and of lipid, as determined by extraction and chromatography according to Owens,¹² the components contained small amounts of lecithin and lysolecithin, plus six as yet unidentified neutral lipids.

Collagen

Rat-tail collagen was prepared by the method of Dimitru and Garrett.¹³ The resulting solution was lyophilized and stored at -20°C for future use. The lyophilized collagen was redissolved according to a modification of the procedure of Gross.¹⁴ Twenty milligrams of collagen was dissolved in 5 ml 0.5 *N* acetic acid by gently stirring in the cold overnight. The resulting solution was dialyzed against a large volume (1 liter) of cold 0.145 *M* phosphate buffer, pH 7.6, for 24 hr and then against a large volume of 0.4 *M* NaCl for 24 hr, with at least one buffer change. The collagen solution was centrifuged for 60 min at 190,000 *g*, using a Beckman

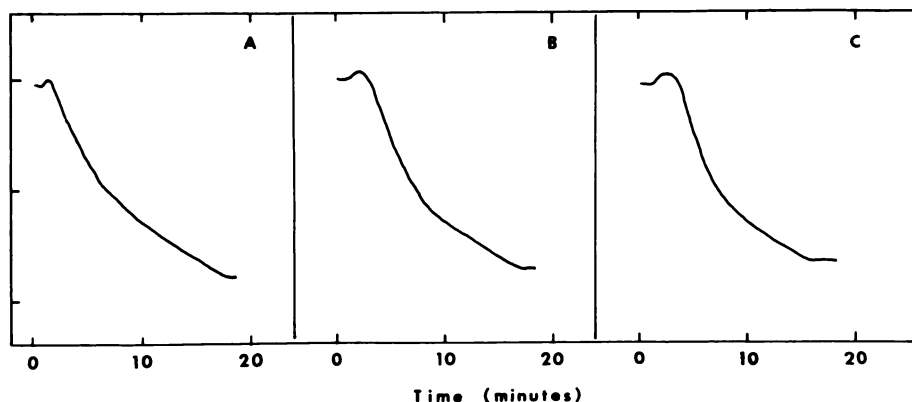


Fig. 2. Typical aggregation curves of washed platelets by thrombin (final conc., 1 U/ml). (A) Unlabeled control. (B) Labeled with N-phenyl-naphthylamine (NPN). (C) Labeled with 8-anilino-1-naphthalene sulfonic acid (ANS).

50Ti rotor. The supernatants were divided into aliquots in disposable, sterile plastic tubes and stored at 4°C.

Chemicals

All chemicals were reagent grade.

Aggregometry

Aggregation of platelets was monitored by measuring the change in turbidity.¹⁵ The intensity of light scattered at right angles, I_{90} , was monitored on a Zeiss PMQ II spectrophotometer set up in the fluorescence mode for observation perpendicular to the incident beam. The cuvette chamber was heated to 37°C using a circulating waterbath (Neslab). Constant stirring of the sample was accomplished by use of a micromagnetic stirring bar and a small motorized magnet at one side of the quartz cuvette (Hellma, Inc.). PRP or washed platelets served to set an arbitrary intensity $I_{90} = 10$ mv CaCl_2 (final concentration, 1 mM) and, for collagen-initiated aggregation, fibrinogen (Käbi; final concentration, 250 mg/100 ml) were added to washed platelets before aggregating. One milliliter of washed platelets, with a platelet concentration of 100,000–150,000/ μl , was brought to 37°C in the chamber, 1 U of thrombin or 25 μg of solubilized collagen was added, and the change in I_{90} with time recorded. The small amount of NaCl (25 μl of 0.4 N) introduced as collagen solvent did not interfere.

Fluorescence

Fluorescence measurements were performed in the Perkin-Elmer MPF-2A fluorescence spectrophotometer. Measurements were made using an excitation slit width of 3 nm and an emission slit width of 6 nm.

Probe Binding

ANS or NPN (Eastman) were weighed and dissolved in methanol (Fisher Spectranalysed) to a final concentration of 0.005 mg/ml. Aliquots of the stock solution were added to the washed platelets with a Unimetric microliter syringe.

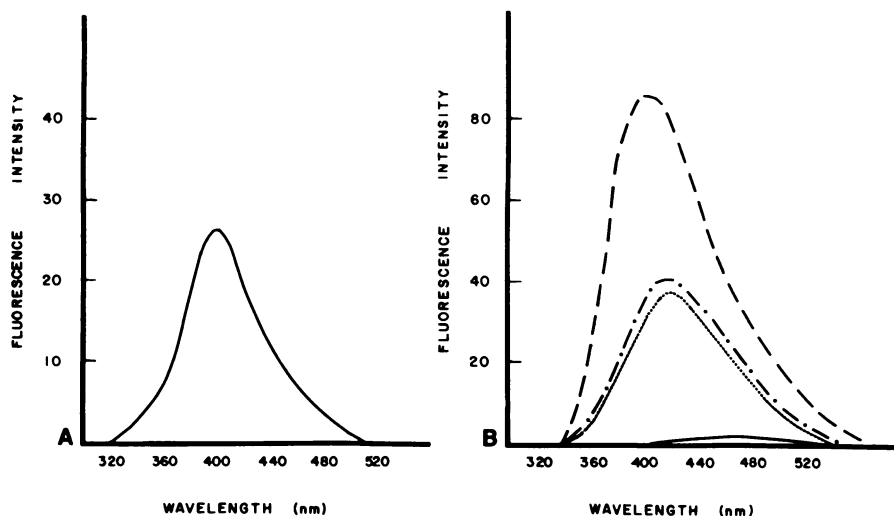


Fig. 3. (A) Emission spectrum of washed platelets labeled with NPN (1.25×10^{-4} mg/ml). Excitation wavelength, 340 nm; excitation slit width, 3 nm; emission slit width, 6 nm. (B) Emission spectra of NPN (1.25×10^{-4} mg/ml) in solvents of different polarity. Excitation wavelength, 340 nm; excitation slit width, 3 nm; emission slit width, 6 nm. — platelet buffer; ●●● methanol; -●-●- chloroform; ---- benzene.

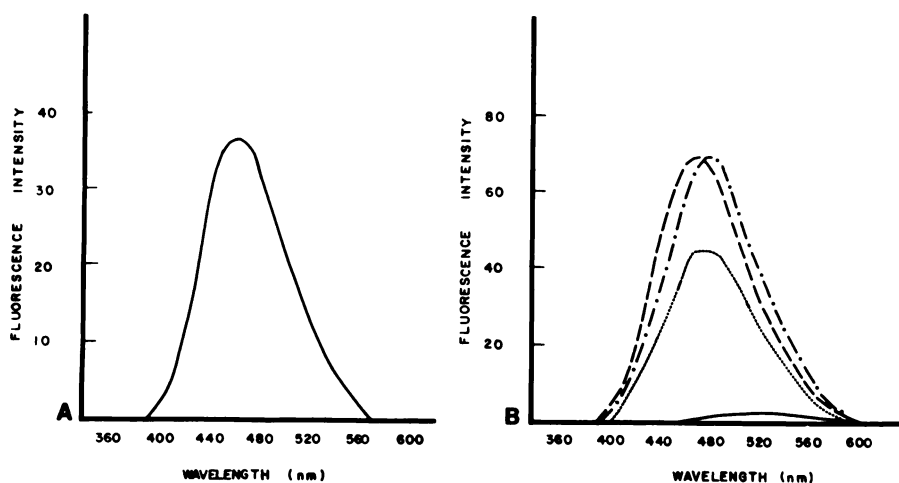


Fig. 4. (A) Emission spectrum of washed platelets labeled with ANS (1.25×10^{-3} mg/ml). Excitation wavelength, 375 nm; excitation slit width, 3 nm; emission slit width, 6 nm. (B) Emission spectra of ANS (1.25×10^{-3} mg/ml) in solvents of different polarity. Excitation wavelength, 375 nm; excitation slit width, 3 nm; emission slit width, 6 nm. — platelet buffer; ●●● methanol; -●- chloroform; ---- benzene.

RESULTS

Behavior of Probes in Solvents of Different Polarity

The fluorescence of NPN and ANS at dye concentrations comparable to those used in the platelet experiments was measured in platelet buffer, methanol, chloroform, and benzene. The resulting emission spectra are plotted in Figs. 3B and 4B.

Binding of Probes to Platelets

Since NPN and ANS readily bind to many components of serum, attempts to detect labeling of platelets in PRP were totally unsuccessful with both probes. Hence, all results reported here were obtained with washed platelets. Labels were bound to the washed platelets by adding aliquots of a stock solution to the platelets and stirring at room temperature. NPN platelet complexes fluoresced with a maximum at 400 nm when excited at 340 nm; ANS platelet complexes fluoresced with a maximum at 460 nm when excited at 375 nm (Figs. 3A and 4A). The wavelength and intensity of both NPN and ANS indicated that in each case the probe molecule was in a nonpolar environment in or on the platelet.

The amount of fluorescence from dye bound to the platelets was determined by difference. After measuring the fluorescence of the labeled platelets, the suspensions of platelets were centrifuged for 20 min at 1200 g and the fluorescence of the supernatant measured. There was invariably some fluorescence present in the supernatant (35%–40% of the amount seen with the platelet suspension), with emission maxima at wavelengths identical to those seen when the platelets were present, indicating that the probe in the supernatant was still in a nonpolar environment, and therefore that the probe was bound to a supernatant compo-

Table 1. Relative Supernatant Fluorescence Intensity

I		II		III	
Observed	Normalized	Observed	Normalized	Observed	Normalized
38	100	56	147	—	—
30	100	39	133	29	97
31	100	36	116	31	100
39	100	50	128	42	108
55	100	41	76	60	110
39 av.		44 av.	120 av.	40 av.	104 av.

Fluorescence (in relative fluorescence units) was measured in the supernatants of aliquots of washed platelets. Washed platelets were pooled, and 2-ml aliquots were taken and treated as follows: I. Centrifuged to remove the platelets, and the supernatant was labeled with 10 μ l of 0.5 mg/ml NPN; set = 100. II. Labeled with 10 μ l of a 0.5 mg/ml solution of NPN in CH₃OH, and the platelets centrifuged out. III. Treated with 10 μ l CH₃OH, the platelets centrifuged out, and the supernatant labeled with 10 μ l of 0.5 mg/ml NPN.

ment since the unbound probe in aqueous medium would exhibit very different fluorescence characteristics. No change in the amount of fluorescence measured in the supernatant relative to the amount of fluorescence of the platelet suspension was observed with time or with changing the probe concentration over a range of 2.5×10^{-4} – 5×10^{-3} mg/ml. Further centrifugation for 20 min at 12,000 g, to remove any platelet fragments which might still be present, reduced the fluorescence in the supernatant by only 25%. The fluorescence emission of the supernatant was then still characteristic of probe bound in a nonpolar environment ($\lambda_{\max} = 400$ nm for NPN, $\lambda_{\max} = 460$ nm for ANS). Thus the major contribution to the fluorescence of the supernatant came from moieties which were not sedimented at 12,000 g and which provided the probe with a hydrophobic environment.

Experiments were designed to examine the effect of exposure of the platelet to the NPN molecule on the amount of lipid and protein detected in the supernatant. These experiments involved comparing the levels of fluorescence in the supernatant of three different samples: (1) the platelets were spun out and the NPN added to the supernatant, (2) the NPN was added in the presence of the platelets and the platelets then spun out, and (3) a volume of methanol equal to the volume of the probe solution was added to the platelets, the platelets were spun out, and NPN then added to the supernatant. Table 1 shows the average results of these experiments. The presence of one or more components in the supernatants from aggregated or centrifuged washed platelets has been described in Materials and Methods. Column I presents the results of a number of such experiments, with platelets from different donors, in terms of NPN-labelable components.* In contrast, if the same platelets are first NPN labeled and then centrifuged (column II), there is clearly an additional release of NPN-binding component into the supernatant, as shown by an average fluorescence increase of 20% over the levels detected in column I. This increase is not due to artifactual solubilization by the methanol label solvent, as shown by column III.

*To date we have not determined whether one or more than one component is found solubilized in the supernatant.

Aggregation

The fluorescence of the platelets during aggregation was followed. As noted in the Materials and Methods section, fibrinogen was added to the washed platelets to facilitate aggregation by collagen. The order in which the probe and fibrinogen were added was found to have no effect on the quantity of NPN bound. It was found, however, that 10% more ANS bound to the platelets when the probe was added after the fibrinogen than when the order was reversed.

The platelets were aggregated by adding thrombin or collagen, and the fluorescence was measured as the aggregation proceeded. No change in either the maximum wavelength or the intensity of fluorescence was observed during aggregation of washed platelets labeled with either NPN or ANS. The overall aggregation of the platelets themselves was unaffected (Fig. 2). The aggregated platelets were spun out and the fluorescence of the supernatants measured. There was no difference between the fluorescence remaining in the supernatant after platelet aggregation and that in the supernatant of controls in which unaggregated platelets were spun down. Thus the lipoprotein labeled by NPN and ANS and described above was not involved in the platelet aggregation reaction.

DISCUSSION

The aim of this study was to examine the feasibility of using noncovalent fluorescent probes to study changes in the platelet membrane during the interaction with collagen. We have shown that it is possible to bind noncovalent fluorescent probes to the platelet membrane *without* affecting the platelet aggregation properties. One factor which strongly affects the amount of dye bound by the platelet is the presence of serum lipids which remained in sepharose-washed platelets¹⁶ even from fasting donors. Our modification (cf. Materials and Methods) reduced residual supernatant NPN binding markedly.

We have carefully examined the question of possible damage to the platelet during the labeling process. The collagen or thrombin aggregation of labeled platelets was compared with that of unlabeled platelets, and it was shown that there were no differences in the lag time between the addition of thrombin or collagen and the beginning of aggregation, the rate of aggregation, or the amount of aggregation. No increase in soluble protein was observed after labeling. These facts support our contention that noncovalent probes can be used to label platelets without damaging them or interfering with the aggregation process.

The particular probes evaluated in this communication indicated that no changes in their environment occurred during the platelet aggregation process. Therefore, these two probes were bound in regions of the platelet surface which do *not* change their physical properties during thrombin- or collagen-induced aggregation. However, the technique developed and described here, marking the first successful labeling of residually intact platelets, is now being applied to other probes which, it is hoped, will reflect changes in the properties of the platelet membrane which occur during aggregation.

During revision of this paper, Hawiger and Timmons¹⁶ have presented the results of their work using NPN and ANS to label human platelets. They, too,

have been successful in binding the probes to the platelets, but no attempt was made to quantitate the relative amounts of the probes binding to the platelets or to verify that the platelets would aggregate after washing and addition of the probes.

REFERENCES

1. Packham MA, Mustard JF: Platelet reactions. *Semin Hematol* 8:30-64, 1971
2. Brass LF, Bensusan HB: The role of collagen quaternary structure in the platelet: collagen interaction. *J Clin Invest* 54:1480-1487, 1974
3. Radda GK, Vanderkooi J: Can fluorescent probes tell us anything about membranes? *Biochim Biophys Acta* 265:509-549, 1972
4. Waggoner AS, Stryer L: Fluorescent probes of biological membranes. *Proc Natl Acad Sci USA* 67:579-589, 1970
5. Stryer L: Fluorescence spectroscopy of proteins. *Science* 162:526-533, 1968
6. Radda GK: Enzyme and membrane conformation in biochemical control. *Biochem J* 122:385-396, 1972
7. Gomperts B, Stock R: ANS fluorescence as an indicator of ionic interaction with membranes, in Chance B, Lee CP, Blasie JK (eds): *Probes of Structure and Function of Macromolecules and Membranes*, vol 1. New York, Academic Press, 1971, p 303
8. Gitler C, Rubalcava B: Interaction of fluorescent probes with hemoglobin free erythrocyte membranes, in Chance B, Lee CP, Blasie JK (eds): *Probes of Structure and Function of Macromolecules and Membranes*, vol 1. New York, Academic, 1971, p 311
9. Cramer WA, Phillips SK, Keenan TW: On the role of membrane phase in the transmission mechanism of colicin E1. *Biochemistry* 12:1177-1181, 1973
10. Tangen O, Berman HJ: Gel filtration of blood platelets: A methodological report, in Mannucci PM, Gorini S (eds): *Platelet Function and Thrombosis. A Review of Methods*. *Adv Exp Med Biol* 34:235-243, 1972
11. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275, 1951
12. Owens K: A two-dimensional thin layer chromatographic procedure for the estimation of plasmalogens. *Biochem J* 100:354-361, 1966
13. Dimitru ER, Garrett RR: Solubilization of rat tail tendon collagen. *Arch Biochem Biophys* 66:245-47, 1957
14. Gross J: Studies on the formation of collagen. I. Properties and fractionation of neutral salt extracts of normal guinea pig connective tissue. *J Exp Med* 107:247-263, 1958
15. Michal F, Born GVR: Effect of the rapid shape change of platelets on the transmission and scattering of light through plasma. *Nature (New Biol)* 231:220-222, 1971
16. Hawiger J, Timmons S: Human platelet membrane changes detected with fluorescent probe. The role of fibrinogen. *Fed Proc* 34:281, 1975