Intracellular Viscosity Changes during Activation of Blood Platelets: Studies by Fluorescence Polarization†

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ABSTRACT: The intracellular viscosity changes that occur in washed human platelets as a result of activation by thrombin or ADP were studied by the use of a fluorescent probe. Results obtained showed a sharp and quick decrease of the intracellular viscosity when platelets were activated by thrombin. This decrease preceded both the release and the aggregation. When platelets were activated by ADP, the decrease in the polarization of fluorescence (and the viscosity) was more moderate. The fluorescent probe is bound to small proteins and peptides in the cytoplasm and not in the granules. Therefore, these changes in the fluorescence polarization reflect changes in the cytoplasmic viscosity which might be due to reorganization of the contractile proteins' system.

It is well demonstrated that platelets undergo dramatic intracellular changes during activation by various reagents. These include a reduction in diameter of the ring of the microtubules and contraction toward the center of the cell followed by disassembly and the reassembly in the fully activated platelets (Crawford et al., 1980). In addition, the granules move toward the center of the cell, pseudopods appear, a change in the state of actin is observed, and other events occur (Gordon & Milner, 1976). These changes which take place as a result of transfer of signals through the platelet membrane are very rapid and are sometimes terminated within a few seconds. A change in the state of actin during platelet activation was reported recently by Carlssson et al. (1979) and by us (Pribluda et al., 1981). It is therefore only logical to assume that these processes will affect the intracellular viscosity, which can be monitored by the use of the fluorescence polarization technique.

Fluorescence polarization has been used to study membrane microviscosity changes (Shinitzky & Barenholz, 1974; Shinitzky & Inbar, 1974; Shattil & Cooper, 1976; Nathan et al., 1979) as well as the rotational relaxation time of lectins bound to the cell-surface membrane (Inbar et al., 1973). Vertical displacement of membrane proteins has also been examined (Borochov & Shinitzky, 1976). Fluorescence polarization was used by Udenfriend et al. (1966) to study the state of intracellular amino acids taken up by living cells. Recently, this technique was used in the study of intracellular microviscosity on a sample of cell suspension (Cercek & Cercek, 1977; Cercek et al., 1978), or on a single-cell basis (Cercek & Cercek, 1976), or on a single-cell basis using a flow cytometer (Lindmo & Steen, 1977).

The method of introduction of fluorescein into cells was developed previously (Ganesan & Rotman, 1964; Rotman & Papermaster, 1966). This technique is based on the principle that fluorescein diacetate, being a nonpolar compound, penetrates readily into the cell and is hydrolyzed by esterases to produce fluorescein. The product, fluorescein, which is water soluble and a polar compound, cannot diffuse out of the cell as fast as its esters can enter. Consequently, there is a progressive accumulation of fluorescein inside the cell.

The use of free fluorescein as an intracellular probe suffers from the disadvantage of its fairly rapid leakage from the cell, especially at 37 °C (Rotman & Heldman, 1981). Therefore, we developed a new version of this probe, azidofluorescein, which can photochemically undergo covalent binding to cytoplasmic proteins, its leakage thus being prevented. We describe here the synthesis of this probe, its physical characterization and spectral properties, and its use as a tool to monitor the intracellular cytoplasmic viscosity changes during the activation of washed human platelets by thrombin, and ADP.

Experimental Procedures

Materials. ADP, thrombin, fluorescein amine (isomer I), and fluorescein diacetate were purchased from Sigma (St. Louis, MO). [^{3}H]Serotonin (15 Ci/mmol) was purchased from The Radiochemical Center (Amersham, England). *N*-2-(Hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffered modified Tyrode solution was prepared according to Peerschke et al. (1980).

Azidofluorescein. Fluorescein amine (isomer I, 100 mg) was dissolved in 5 mL of dimethylformamide (DMF). To this solution was added 5 mL of water, and if a precipitate appeared, the DMF concentration was increased until a clear solution was obtained. The solution was acidified by the addition of 1 mL of concentrated sulfuric acid followed by 200 μL of amyl nitrite. After the solution was stirred for 30 min at 4 °C, sodium azide (200 mg in 4 mL of water) was added. The mixture was stirred and cooled for a further 30 min, and then a saturated solution of sodium chloride was added until a volume of 20 mL was reached. The dark red precipitate was filtered and washed with a cold solution of sodium chloride. All steps of this preparation were performed under a red light. The yield was 70 mg.

Azidofluorescein Diacetate. Azidofluorescein (50 mg) was dissolved in 2 mL of dry pyridine, and to this solution was added 1 mL of freshly distilled acetic anhydride. The mixture was allowed to stand overnight at room temperature and then poured into crushed ice. The orange-red precipitate was filtered, washed with cold water, and dried in vacuo. All steps of this preparation were carried out under a red light. The yield was 40 mg.

Incorporation of Azidofluorescein in Platelets. Platelet-rich plasma (PRP) was prepared according to the method of Peerschke et al. (1980), and to this suspension was added (in the dark) azidofluorescein diacetate in ethanol to a final

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concentration of 4×10^{-5} M. The platelet suspension was incubated at 37 °C for 15 min and then centrifuged at 1000*g* for 20 min. The platelet concentrate (collected in the dark) was irradiated for 5 min at room temperature. The platelets were gel filtered on a column (Sepharose 2B CL or Bio-Gel A50 M) and brought to a concentration of 2×10^7 platelets/mL, in Hepes-buffered modified Tyrode solution. The suspension was incubated at 37 °C for 30 min. The incubation medium was removed by centrifugation at 550*g* for 15 min, and a second incubation was performed. The platelets were again centrifuged at 550*g* for 15 min and brought to a concentration of 2×10^8 platelets/mL. In control experiments, platelets were prepared by identical procedures except for the addition of the azidofluorescein diacetate. Carrier ethanol was added in these cases.

Aggregation. Aggregation was studied with a Chronolog aggregometer. Platelets were activated by the addition of 0.08–1 unit/mL thrombin (as specified under Results) or 10 μM (final concentration) ADP. Aggregation was stopped by the addition of formaldehyde to a final concentration of 0.6% (Costa & Murphy, 1975). EDTA when present was at a final concentration of 3 mM. The concentration of aspirin (when present) was 50 μM.

Release. Platelets were preloaded with [^{3}H]serotonin (usually about 20 pmol/10^8 platelets) and activated in the aggregometer, and at the desired time, formaldehyde was added to a final concentration of 0.6% (Costa & Murphy, 1975). After measurement of the fluorescence or fluorescence polarization, the platelet suspension was centrifuged in a Beckman microfuge, and the radioactivity in the supernatant was counted, using a Packard Tri-Carb 3255 liquid scintillation counter.

Fluorescence Polarization. Fluorescence and fluorescence polarization were determined on a Perkin-Elmer 1000 M fluorometer. An excitation wavelength of 474 nm and an emission wavelength of 541 nm were used: filter bandwidths were ±20 nm. All values were corrected for light scattering by using the same preparation of nonfluorescent platelets.

Nuclear Magnetic Resonance (NMR). NMR spectra were recorded on a Varian A-60 instrument (for fluorescein diacetate) and on a Brooker 90-MHz instrument (azidofluorescein diacetate). In both cases, the solvent was $CDCl_3$, and Me_4Si (tetramethylsilane) was used as internal standard.

Infrared (IR). Infrared spectra were recorded on a Perkin-Elmer 467 infrared spectrophotometer. Azidofluorescein diacetate, fluorescein diacetate, and diazofluorescein diacetate were dissolved in chloroform.

Ultraviolet (UV). Fluorescein and azidofluorescein were dissolved in ethanol, and the UV spectra were measured at concentrations of 3.2×10^{-6} M with a Cary 14 spectrometer.

Perrin Plot. Platelets were labeled with azidofluorescein as described above, and the fluorescent lysate was dissolved in a sucrose solution. The polarization of fluorescence of these solutions as well as a solution of the fluorescent lysate in glycerol was measured as described above.

The light source for activation of azidofluorescein was a Wild microscope UV source containing a 200-W mercury arc lamp (Osram, HBO); a filter omitting light below 300 nm was used.

Results

Synthesis. Several synthetic routes for azidofluorescein diacetate were considered, with the one described here proving to be the most successful (Figure 1). The starting material was the commercial fluroescein amine (I), which was converted to the diazonium derivative (II). Treatment of the diazonium

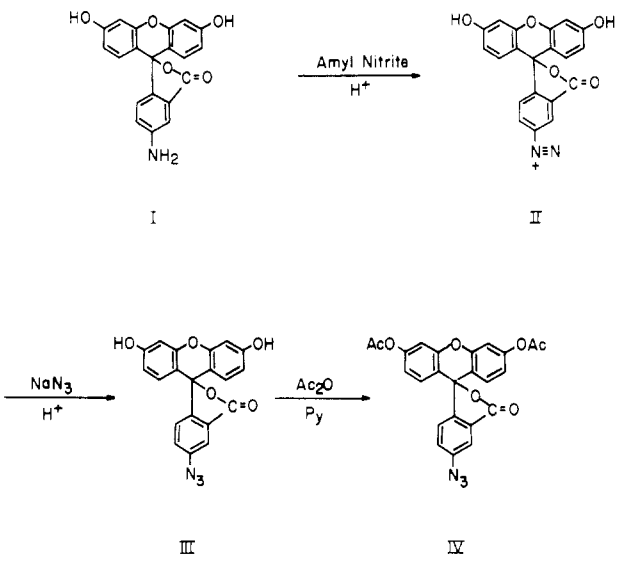

FIGURE 1: Synthesis of azidofluorescein diacetate. Fluorescein amine (I) is diazotized with amyl nitrite at low pH to form diazofluorescein (II), which is converted with sodium azide to azidofluorescein (III). Azidofluorescein is acetylated with acetic anhydride (Ac_2O) and pyridine (Py) to form azidofluorescein diacetate (IV).

salt (II) with sodium azide resulted in almost quantitative conversion to the azidofluorescein (III). The azide is stable at basic pH, and this enabled the esterification of the aromatic alcohols, using the standard method of acetic anhydride and pyridine to give the final product IV. The products III and IV were characterized by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. The IR spectra of fluorescein diacetate, diazofluorescein diacetate, and azidofluorescein show that the only difference between these three fluorescein derivatives is the appearance of an absorption at 4.70 μm, which is due to the presence of the azido group, and an absorption at 4.4 μm resulting from the presence of a diazo bond. Fluorescein and azidofluorescein gave almost identical UV spectra. The proton NMR spectra of fluorescein diacetate and azidofluorescein diacetate were also compared, and it is not surprising that the two spectra are very similar. The acetate absorption is clear at $\delta = 2.2$. The excitation and emission fluorescence spectra of fluorescein and azidofluorescein are very similar.

Aggregation and Release. When the incorporation of fluorescein was carried out at an azidofluorescein diacetate concentration of 4×10^{-5} M, no effect on the ADP- or thrombin-induced aggregation or on serotonin release was observed. When the concentration of azidofluorescein diacetate was increased to 1.2×10^{-4} M, there was some reduction in the ADP-induced aggregation but not in that caused by thrombin. The rate of intracellular hydrolysis of the diacetates of fluorescein diacetate and azidofluorescein diacetate was identical (data not shown).

Effect of Thrombin Activation on the Polarization of Fluorescence (P). The effect of 1 unit/mL thrombin on the polarization of fluorescence of platelets incorporated with fluorescein (via azidofluorescein) is shown in Figure 2. There is a very sharp decrease of the *P* value which is almost maximal after only 5 s. At this time, the aggregation has not yet started, and the release is at only one-third of its maximal value. When platelets were activated with different concentrations of thrombin (0.04–1 unit/mL) for 10 s, the change in the *P* value was almost identical while the release increased with thrombin concentration (Figure 3). When platelets were activated with thrombin in the presence of EDTA to block aggregation but not release, the change in the *P* value was practically identical

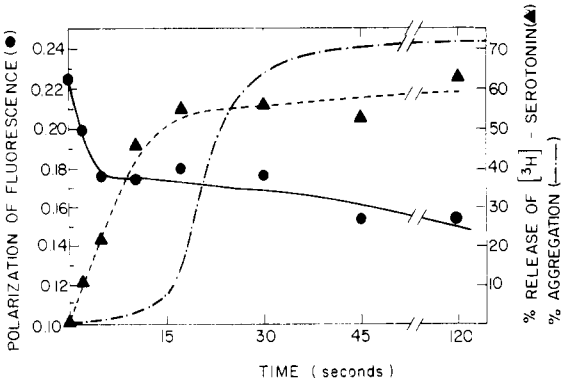

FIGURE 2: Thrombin activation of platelets incorporated with azidofluorescein (1 unit/mL thrombin). (●) Polarization of fluorescence; (▲) release of [^{3}H]serotonin; (----) aggregation. All measurements were carried out at 22 °C.

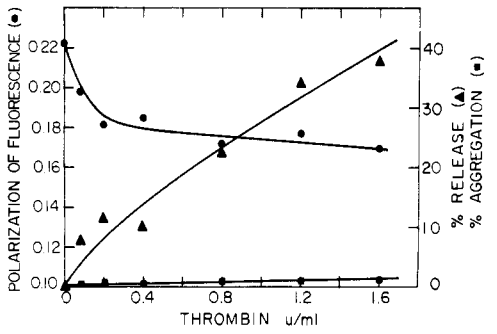

FIGURE 3: Platelets (with azidofluorescein) activated with different concentrations of thrombin for 10 s. (●) Polarization of fluorescence; (▲) release of [^{3}H]serotonin; (■) aggregation. All measurements were carried out at 22 °C.

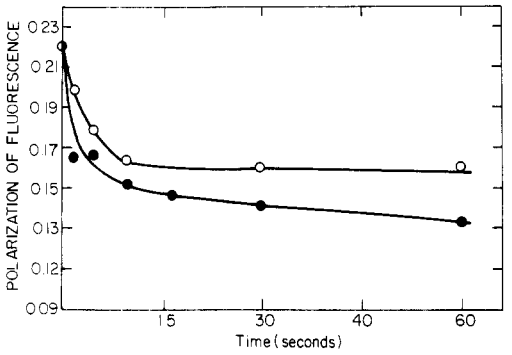

FIGURE 4: Change in polarization of fluorescence of incorporated azidofluorescein in platelets activated with 1 unit/mL thrombin in the presence (○) and in the absence (●) of 3 mM EDTA. All measurements were carried out at 22 °C.

with that of platelets activated with thrombin in the absence of EDTA (Figure 4).

Effect of ADP Activation on the Polarization of Fluorescence (P). The effect of ADP activation on the polarization of fluorescence of fluorescein incorporated in platelets is shown in Figure 5. It is clear that after 10 s, when neither aggregation nor release has started, there is already a very significant decrease in the P value. At 30 s, when aggregation is about 75% completed and release has not yet started, the decrease in the P value is already at its maximum. When platelets were pretreated with aspirin to block the ADP-induced release, the change in the P value was similar to that shown in Figure 5. These experiments were performed with 150 μM (final concentration) fibrinogen. No effect on polarization was observed when platelets fixed with formaldehyde were used.

Estimation of Intracellular Viscosity (Perrin Plot). The value of $1/r$ as a function of $1/\eta$ is shown in Figure 6. Extrapolation of the straight line to infinite viscosity give a value of 305 for $1/r_0$ or 0.325 for r_0. A similar value (0.318) was obtained when the labeled protein mixture was dissolved in glycerol and the fluorescence polarization was measured.

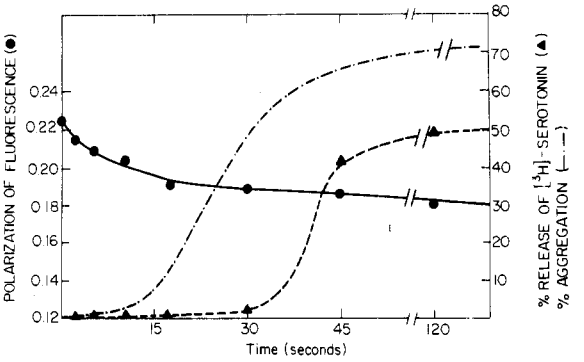

FIGURE 5: ADP activation of platelets incorporated with azidofluorescein (10 μM ADP final concentration). (●) Polarization of fluorescence; (▲) release of [^{3}H]serotonin; (----) aggregation. All measurements were carried out at 22 °C.

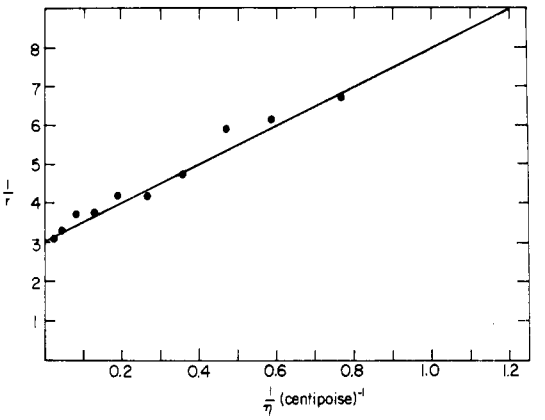

FIGURE 6: Reciprocal of fluorescence anisotropy ($1/r$) as a function of the reciprocal of sucrose solution viscosity ($1/\eta$) for fluorescein azide bound to platelet lysate at a temperature of 25 °C.

Discussion

Azidofluorescein is a very promising intracellular labeling reagent. Its synthesis is fairly simple and results in very good yields. The compound has very similar UV and NMR spectra to those of fluorescein. The partition coefficients of the two diols, as compared to those of the respective diacetates, are almost identical (Rotman & Heldman, 1981). Azidofluorescein had the advantage that it can be covalently attached to intracellular proteins and peptides, and thus, its diffusion from the cell is prevented. In a previous report (Rotman & Heldman, 1981), we have shown that azidofluorescein is mainly attached to peptides or low molecuar weight proteins, and only very little, if any, is bound to actin or other contractile proteins. The fluorescent probe is not located in the dense bodies or in the α granules but rather in the cytoplasm. This was concluded from the fact that activation of platelets with thrombin (1 unit/mL) which results in the secretion of both types of granules (Day & Holmsen, 1971) was not accompanied by release of fluorescent material to the medium. When platelets containing the probe were lysed, almost all the fluorescence was recovered in the soluble part of the cytoplasm.

The addition of the azido group to the fluorescein skeleton had little if any effect on the hydrophobicity of the molecule (Rotman & Heldman, 1981) or on its susceptibility to the hydrolytic enzymes that cleave the acetate groups. This was deduced from the similar rate of fluorescence upon addition

of azidofluorescein diacetate or fluorescein diacetate to platelet suspensions (the diacetates are not fluorescent, and the fluorescence of the diol is observed only after the hydrolysis). Our experiments so far with this probe indicate that the photochemical covalent binding of the azidofluorescein has no effect on platelet functions. Thus, aggregation with ADP or thrombin and the release reactions were unaffected by the incorporation of the fluorescent probe.

The fact that the fluorescein probe was not bound to the contractile proteins but remained in the soluble part of the cytoplasm opens the exciting possibility that changes in the contractile system due to polymerization or depolymerization (which will affect the intracellular viscosity) could be measured by the polarization of fluorescence of this probe. The relationship between the viscosity and the polarization of fluorescence is given in the Perrin equation (Perrin, 1926; Shinitzky & Barenholz, 1978)

$$\frac{r_0}{r} = 1 + C_{(r)}\frac{T\tau}{\eta}$$

where r is the fluorescence anisotropy which is obtained from P (polarization of fluorescence) by the relationship

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} = \frac{(I_\parallel/I_\perp) - 1}{(I_\parallel/I_\perp) + 2} = \frac{2P}{3 - P}$$

r_0 is the upper theoretical limit of $I_\parallel/I_\perp$, τ is the lifetime of the excited state, T is the absolute temperature, and $C_{(r)}$ is the parameter which relates to the molecular shape and the location of the transition dipoles of the rotating fluorophore as expressed in the determined r value. P, the polarization of fluorescence value, is defined (and was calculated) as

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$$

where $I_\parallel$ and $I_\perp$ are the intensities of emission parallel and perpendicular to the vertically plane polarized exciting light, respectively.

Results shown in Figures 2–5 clearly indicate that the P value decreases when platelets are activated by thrombin, or ADP. In the case of thrombin, this decrease is very rapid and is almost completed with 2–5 s, well before the beginning of the release and aggregation. When platelets were activated with ADP, the decrease in the P value was much slower and more moderate and did not reach the value obtained in the case of thrombin activation (compare Figures 2 and 5).

This decrease in the P value is not due to clumping of the platelets during aggregation for the following reasons: (a) A decrease in the P value was observed even when platelets were activated with thrombin in the presence of EDTA, conditions under which no aggregation occurs. (b) When platelets were activated with ADP or thrombin, the decrease in the polarization of fluorescence was very significant and almost maximal even before aggregation began.

From Figure 6, the absolute intracellular viscosity of the platelets before and after activation could be calculated. Thus, a P value of 0.22 for resting platelets corresponds to a $1/r$ value of 6.38 and to a viscosity of $\eta = 1.48$ cP. The P value of thrombin-activated platelets of 0.18 corresponds to a $1/r$ value of 7.83 and a viscosity of 1.03 cP. The straight line obtained when $1/r$ was plotted as a function of $1/\eta$ indicates that there is no free rotation of the fluorescein molecule but only of the whole protein together with the fluorophore.

These results can be explained by the mobilization of actin and the formation of microfilaments during platelet activation. It was previously reported that when platelets are activated by thrombin (Carlsson et al., 1979; Pribluda et al., 1981) or ADP (Pribluda et al., 1981) there is a reorganization of actin which is manifested as a decrease in the DNase-available actin. It was also reported that microfilaments appear in the activated platelets (White, 1971). This polymerization of G-actin to F-actin which would at first sight be expected to lead to an increase in viscosity is actually, according to our view, causing a decrease in the intracellular viscosity. Since the fluorescent probe is not bound to actin and in view of the high actin concentration in the cells and the reduction in the DNase-available actin observed after activation (when microfilaments appear), it is logical to assume that the newly polymerized actin is less soluble. Alternatively, it may be deposited in various compartments in the cell. The phenomenon will result in a depletion of proteins from the cytoplasm and consequently to a microviscosity decrease in the soluble part of the cytosol (where the label is located).

Thus, we conclude that one of the first events in platelet activation by thrombin is a rapid and quite dramatic decrease in the cytoplasmic viscosity, possibly due to transformation of actin to either insoluble or compartmentable F-actin. In the case of ADP activation, this change is less dramatic but still significant and, again, precedes both the aggregation and release.

A similar phenomenon to that described above, i.e., decrease in the polarization of fluorescence upon activation, was observed by Cercek & Cercek (1976) when lymphocytes were activated with phytohemagglutinin, but unfortunately no mention was made of the possibility of involvement of contractile proteins. However, it would not be surprising if this decrease in polarization of fluorescence during lymphocyte stimulation was due to actin polymerization and removal of the fibrous actin from the cytosol.

The results presented in this paper are compatible with those of our previous report (Pribluda et al., 1981) on the mobilization of actin during platelet activation. Thus, there is a correlation between the kinetics of actin mobilization as measured by the DNase fluorescent method (Pribluda et al., 1981) and that of change in the polarization of fluorescence (and viscosity). In both cases, there is a rapid and pronounced effect by thrombin and a moderate one by ADP. The possibility that this decrease in viscosity is caused by the disassembly of the microtubule ring after platelet activation cannot be ruled out. However, although the disassembly of the microtubule ring is of the same time order as the observed decrease in viscosity, we could not observe any further change in viscosity due to the reassembly of the microtubule ring.

It is noteworthy to mention that the microfilaments are believed to serve as mechanochemical tools in the movement of the platelet granules toward the center of the cell. If at the same time this formation of the microfilaments causes a reduction in the intracellular viscosity, then obviously the movement of the granules would be facilitated.

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Changes in the Circular Dichroic Spectrum of Colchicine Associated with Its Binding to Tubulin†

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ABSTRACT: Circular dichroism has been used to study the interaction of colchicine with the tubulin $\alpha\beta$ dimer at 26 °C. Tubulin purified from bovine brain microtubule protein exhibits negligible circular dichroism at wavelengths above 310 nm. Free colchicine exhibits a negative circular dichroic band at 340 nm characterized by an extremum in molar ellipticity $[\theta]$ of $-3.35\ (\pm 0.27) \times 10^4$ deg·cm^2/dmol. This negative band either vanished or was greatly reduced, i.e., $[\theta] = 0\ (\pm 0.57) \times 10^4$ deg·cm^2/dmol, when colchicine was bound to tubulin. This was demonstrated by circular dichroic studies on stable [^{3}H]colchicine–tubulin complexes which were separated from all unbound colchicine by means of gel filtration. Also, tubulin was titrated with colchicine, and at low colchicine concentrations, the observed ellipticity at 340 nm could be extrapolated to zero at 0 M colchicine; saturation occurred at a molar ratio of colchicine to tubulin of about 1:1. The association constant characterizing the drug–protein interaction was estimated to be about 0.9 μM^{-1}. As controls, three other acidic proteins were studied at a molar ratio of colchicine to protein of 2:1, and their presence had no effect on the circular dichroic properties of colchicine. These results are consistent with the idea that a conformational change in colchicine accompanies its binding to tubulin. The spectrum of the complex between 250 and 300 nm was quite similar to that expected from simple additivity of the spectra of drug and protein except between 255 and 265 nm. The technique described herein should be applicable to other protein–drug systems.

Colchicine, the active antimitotic agent of the meadow saffron *Colchicum autumnale*, binds specifically and with high affinity to the tubulin dimer, the major protein subunit of microtubules (Shelanski & Taylor, 1968; Weisenberg et al., 1968; Wilson, 1970; Wilson & Meza, 1973). Approximately 1 mol of colchicine binds per mol of tubulin (Owellen et al., 1972; Wilson et al., 1974; Bhattacharyya & Wolff, 1976) with an affinity constant (for vertebrate tubulins) that ranges from 1 to 40 μM^{-1}, depending upon the source of tubulin and the conditions of measurement (Owellen et al., 1972, 1974; Wilson et al., 1974; Bhattacharyya & Wolff, 1974, 1976; Sherline et al., 1975; McClure & Paulson, 1977). Colchicine binding requires native tubulin. Binding activity is abolished by denaturants (Wilson, 1970) or by extensive treatment of tubulin with sulfhydryl reagents (Kuriyama & Sakai, 1974; Ikeda & Steiner, 1978). The colchicine binding activity of tubulin is also unstable in the buffers routinely used, decaying according to first-order kinetics (Weisenberg et al., 1968; Wilson, 1970; Wilson & Meza, 1973; Sherline et al., 1975; Cortese et al., 1977). The rate of decay is a sensitive function of pH and temperature, with optimal stability of binding activity observed at pH 6.75 and 0 °C (Wilson, 1970). Furthermore, the colchicine binding activity of tubulin is stabilized by vin-

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