Reflectance Spectroscopy of Clotting Blood

A Description of the Time-Dependent Behavior

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• ***Context.*—Research into whether cyclooxygenase-2 (COX-2) inhibitors affect thrombosis has been hampered by the lack of a specific assay. Erythrocytes modulate the effect of aspirin on platelets, which suggests that tests of whole blood clotting may be more sensitive.**

***Objectives.*—To determine whether reflectance spectroscopy of clotting blood generates useful information about coagulation and whether it shows an effect of COX-2 inhibitors.**

***Design.*—A survey of 14 adults examined the range of phenomena demonstrated by reflectance spectroscopy. These phenomena were compared before and after treatment with a COX-2 inhibitor in 4 subjects.**

***Setting.*—Out-patient clinic.**

***Main Outcome Measure.*—Reflected light intensity was measured from blood as it clotted in a cuvette thermostated at 37°C.**

***Results.*—The survey of healthy adults showed that the time course of reflected light intensity is similar at all wavelengths and may be divided into 4 stages: a monotonic decrease, a sigmoidal increase, a linear region, and a terminal phase. Clot formation as determined by tube inversion occurs at the transition between the first and second phases; the sigmoidal increase cannot be due to fibrin polymerization. The terminal phase coincides with clot retraction. Similar results are obtained in native whole blood and in recalcified citrated blood. Cyclooxygenase-2 inhibitors have an intrinsic effect on the sigmoidal increase ex vivo ($P < .001$).**

***Conclusions.*—Reflectance spectroscopy generates unique information about clotting blood. It is feasible to use anticoagulated blood to elucidate the events underlying the time course and to investigate the effects of COX-2 inhibitors.**

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The VIGOR study comparing naproxen to rofecoxib, a cyclooxygenase-2 (COX-2) inhibitor, recorded a higher incidence of thrombotic events in the rofecoxib-treated arm.[1] No consensus has emerged concerning the explanation of this finding. To account for the difference, one school has proposed that naproxen protects against thrombosis,[2,3] while another has suggested that COX-2 inhibitors promote thrombosis, perhaps by inhibiting prostaglandin I_2 synthesis in the vascular endothelium.[4,5] One problem with the latter hypothesis is that it requires factors extrinsic to blood and, therefore, is difficult to test. The absence of a standard method, like the prothrombin time for monitoring warfarin, to assess the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on clotting has hampered research in this area.

Santos and colleagues[6,7] showed that the red cell membrane modulates the effect of aspirin on platelets, which suggests that the study of whole blood clotting would better probe the factors contributing to NSAID action. Routine techniques, such as the Lee-White clotting time, simply lack the sensitivity to distinguish subtle effects. More sophisticated techniques record the time course of clot formation and lysis. One of these techniques, thrombelastography, measures the mechanical displacement of a probe immersed in blood held in an oscillating cup; however, thrombelastography is generally recognized as not detecting the effect of aspirin on coagulation.[8] Another technique follows changes in the mechanical properties of clotting blood, as computed from vibrations in the 200-Hz range (Sonoclot Coagulation & Platelet Function Analyzer). Although parameters generated by this method correlate with platelet count and function,[9] one prospective study showed that this method also could not detect the effect of aspirin.[10]

Optical methods allow nondestructive probing of blood and have been used extensively to study plasma coagulation, platelet function,[7] blood rheology,[11,12] and oximetry.[13] This article describes the application of reflectance spectrometry to the clotting of whole blood, particularly the time-dependent behavior in healthy young adults. To show potential utility, a pilot study demonstrating a COX-2 effect intrinsic to blood is included.

SUBJECTS AND METHODS

Reflectance Measurements

All measurements recorded light reflected from blood placed in a quartz cuvette thermostatted at 37°C. Cleaning the cuvettes between measurements followed the recommendations of Biggs and Macfarlane.[14] For monochromatic illumination, light from a 75-W xenon arc lamp passed through a grating monochromator (Alphascan, Photon Technology International, Inc, Lawrenceville,

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Table 1. Effect of Treatment on Logistic Parameters* Fit to Sigmoidal Increase at 470 nm in Recalcified Blood					
Subject No.	Treatment	Dose, mg/d	Duration	*B* Posttreatment/ *B* Pretreatment†	ρ Posttreatment/ ρ Pretreatment
1	Celecoxib	400	30 d	0.094	1.00
2	Celecoxib	400	7 d	0.011	1.01
3	Rofecoxib	50	7 d	0.002	1.02
4	Rofecoxib	50	90 d	0.012	1.01
5	Indomethacin	150	7 d	33.3	0.96
2	Aspirin	325	2 h	2.9	1.00

* Parameters *B* and ρ are calculated from equation 1.
† $P < .001$ for COX-2 inhibitors.

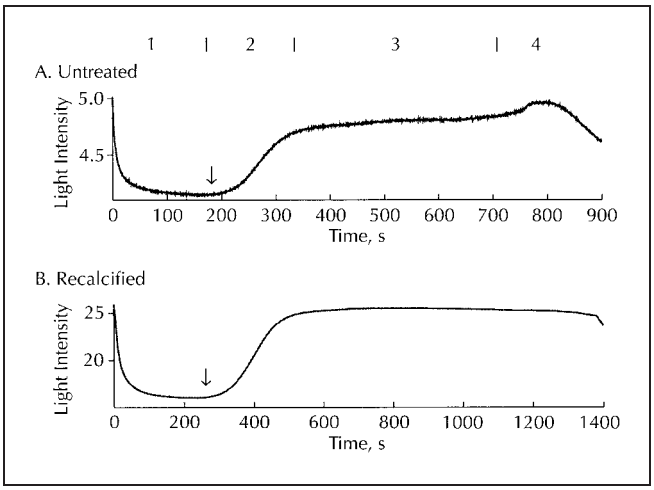

Figure 1. *Time course of total light intensity. A, Variation of total light intensity with time for freshly drawn blood. B, Comparable tracing from blood drawn into citrate anticoagulant and recalcified. Both curves demonstrate 4 regions, indicated across the top of the figure: a monotonic decrease, a sigmoidal increase, a linear region, and a terminal phase. The arrows mark the times of clot formation, as determined by tube inversion; solidification always occurred during the transition between first and second phases. The terminal phase corresponds to the onset of clot retraction and was not seen in all subjects.*

NJ). When monochromatic illumination was used, the incident beam was normal to the cuvette surface; a mirror collected light reflected between 20° and 30° and passed it through a second monochromator, set to the illuminating wavelength, to a photomultiplier tube (Alphascan, Photon Technology International). For broadband illumination, a 100-W tungsten halogen lamp (Applied Photophysics, Leatherhead, United Kingdom) was used. All spectra were acquired by a charge-coupled device array spectrometer (USB2000, Ocean Optics, Dundee, Fla), which measured light intensity simultaneously at 1980 wavelengths between 200 and 875 nm. For all spectral measurements, a probe was used in which a central read fiber carried the signal to the spectrometer and 6 circumferential fibers illuminated the specimen. The 400-μm read fiber effectively determines the slit width of the spectrometer. Except where noted, the probe was fixed at 45°.

Reflectance spectra are reported relative to a reference whose spectral characteristics are independent of time:

$$R(\lambda, t) = \frac{I(\lambda, t)}{I^0(\lambda)} = \frac{C(\lambda, t) - D(\lambda)}{C^0(\lambda) - D(\lambda)}$$

where R is the relative reflectance, λ is wavelength, t is time, I is the intensity of reflected light, superscript 0 indicates the reference state, C is the number of photons per second reported by the spectrometer, and D is the dark reading from the spectrometer. For determining spectral reflectance, an opaque aqueous solution of barium sulfate (50% wt/wt) was used as a diffuse white standard. For time-course measurements, the initial state of blood in the cuvette was estimated by linear extrapolation from the first 5 time points at each wavelength and used as reference.

Both spectrometers were interfaced to computers for data analysis. The spectra were smoothed by the Savitzky-Golay algorithm.[15] Curve fitting used the nonlinear least-squares Marquardt-Levenberg algorithm,[16] as implemented in the program gnuplot (Free Software Foundation, Cambridge, Mass). A Student t test was used to compare means.

Survey of Healthy Subjects

Subjects were selected from individuals presenting to an ambulatory clinic. All gave informed consent according to a protocol approved by the institutional review board. Procedures conformed to the principles of the Declaration of Helsinki. Fourteen individuals who requested premarital blood testing were enrolled for the study of healthy individuals (8 men, 6 women; average age, 27 years; range, 18–40 years). None required medical treatment and all denied use of nonprescription products in the preceding 2 weeks. Blood was drawn into siliconized tubes without anticoagulant (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). One milliliter of blood was transferred into a 10 × 10 × 30-mm quartz cuvette; reflectance measurements began within 45 seconds. To assess the effect of anticoagulant, some samples were drawn into sodium citrate and later recalcified with 1.8% calcium chloride (1:10 vol/vol). Reflectance spectra were acquired by broadband illumination through the fiber optic probe; the probe was fixed at 45° for all measurements.

Effect of COX-2 Inhibitors

The effect of COX-2 inhibitors was measured on patients presenting with a complaint of pain determined to be of musculoskeletal origin. Four patients were studied (2 men, 2 women; average age, 67 years; range, 49–77 years). One subject (subject 2; Table 1) took no other drugs regularly; the other 3 were being treated for hypertension in addition to musculoskeletal pain. No attempt was made to control for antihypertensive regimen or for duration of COX-2 treatment. In each case, the patient reported taking the COX-2 inhibitor daily as prescribed and denied taking any medicines other than his or her usual regimen. The opportunity arose to study the effect of indomethacin in a 34-year-old man who had no other medical conditions, and these results are presented for comparison with those of the COX-2 study (subject 5; Table 1). Blood drawn into sodium citrate anticoagulant was refrigerated and studied within 24 hours; recalcification was brought about by 0.4% calcium chloride (1:3 vol/vol). For these measurements, 0.5 mL of blood was placed into a mini 10 × 10 × 30-mm cuvette. After recalcification, the time course of reflectance at 470 nm was measured.

RESULTS

Four Regions of the Time Course of Total Light Intensity May Be Distinguished for Both Freshly Drawn and Recalcified Blood

Figure 1, A, shows the time course of total light intensity detected using broadband illumination as freshly drawn blood clots. Four regions can be grossly distin-

guished: a rapid monotonic decrease, a sigmoidal increase, a linear region, and a terminal phase. The arrow marks clotting as determined by tube inversion; clotting regularly occurred at the transition between the first and second regions. The terminal phase coincides with the appearance of clot retraction in the specimen, grossly visible at the edges of the cuvette but not in that area illuminated by the probe. Figure 1, B, shows the time course of total light intensity detected from blood drawn into citrate and recalcified within 1 hour of phlebotomy. The same 4 regions may be distinguished for these samples as with freshly drawn blood. Blood from all healthy subjects exhibited the first 3 regions, but some did not develop clot retraction during the period of observation. Therefore, I will focus on the first 3 regions of the time course.

Detected Light Is Diffuse Reflection of the Irradiating Beam

The detector range of the array spectrometer is 200 to 875 nm, from ultraviolet into the near infrared. Because of this bandwidth, several physical processes could contribute to the detected light signal in principle: thermoluminescence, photoluminescence, chemiluminescence, and reflection of the irradiating beam. Thermostatting the specimen obviates any contribution of thermoluminescence to the time-dependent behavior. For the remaining factors, a series of measurements was made on clotted and citrate-anticoagulated specimens to determine whether these factors added to the detected spectra or, more particularly, to the time-dependent signal. To assess the role of photoluminescence, 300-nm light irradiated the specimen and an emission spectrum between 200 and 875 nm was obtained; the wavelength of the irradiating beam was increased in 50-nm steps, and corresponding emission spectra were recorded. The system detected no emission peaks other than reflection of the irradiating beam (data not shown). Shuttering the light source during these measurements led to a step-change in the emitted light signal, the amplitude of which was less than 1% of baseline for wavelengths other than those near the irradiating beam. In one series of experiments, citrate-anticoagulated blood was recalcified; irradiating the specimen with 350 nm light caused no time-dependent emission at any wavelength during coagulation (data not shown). To assess the role of chemiluminescence, citrate-anticoagulated blood was recalcified without irradiation. No time-dependent emission was detected (data not shown).

Simple specular reflection, being dominated by light from the 2 glass surfaces of the cuvette, should be essentially constant and was minimized by the configuration of source and detector during the acquisition of spectra. Nonspecular reflection was assessed by fixing a broadband irradiating beam normal to the cuvette and varying the position of the detector; total intensity of nonspecular reflection varied as the cosine of the angle of the detector (Figure 2, A). The nonspecular signal, therefore, follows the Lambert law.[17] This conclusion must be viewed as a first-order approximation. These measurements were made on clots after the sigmoidal increase and on anticoagulated blood, neither of which is in a constant state. Anticoagulated blood sediments and clots mature (Figure 1). To this level of approximation, however, we may state that the light detected constitutes diffuse reflection of the irradiating beam.

Reflectance at some wavelengths depends on the orientation of the probe. However, this angular dependence is the same in liquid and clotted blood (Figure 2, B) and should introduce no error into the spectral analysis of the time course as long as the angle of the probe is kept fixed, as it was in the experiments described here.

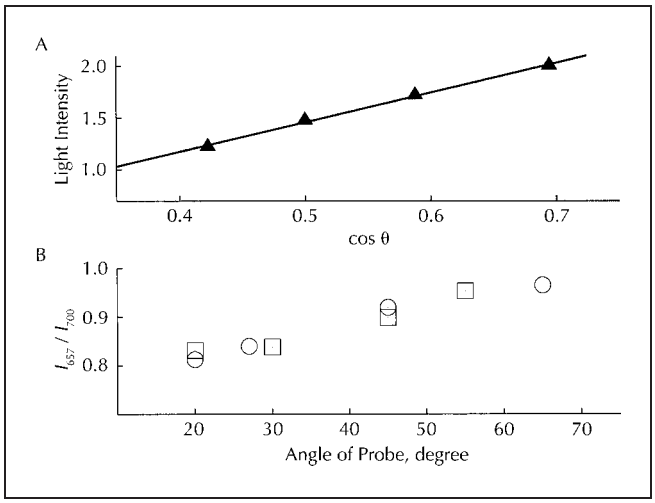

Figure 2. *Effect of system configuration on light signal. A, Total light intensity varies as the cosine of the angle (θ) between the detector and the direction normal to the surface; in these measurements, the incident beam was also normal to the cuvette. Within experimental error, the line fit to the data points passes through the origin. Total light intensity follows the Lambert law. B, Light intensity at 657 nm relative to that at 700 nm varies as the angle of the probe. In the probe, the beam and detector lie in the same direction with respect to the surface. This variation is comparable in both liquid (□) and clotted (○) blood and does not affect the measurement of spectra during coagulation. Error bars fall within the symbols.*

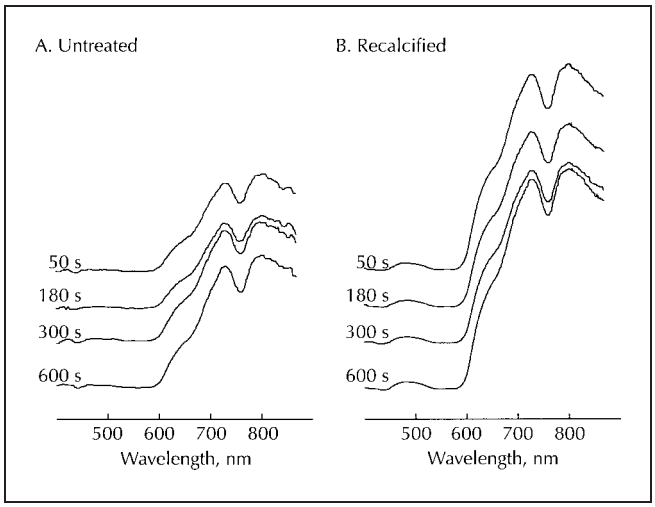

Figure 3. *Time dependence of spectral reflectance. These spectra, taken from the same specimens shown in Figure 1, have been corrected for lamp characteristics by using a diffuse white surface as reference. To prevent overlap, the spectra have been translated. The vertical scale is the same for all spectra; the region of each spectrum under the label corresponds to 0. There is a broad peak around 485 nm in the recalcified specimen that is not apparent in freshly drawn blood. The distribution of intensities varies with time for both recalcified and untreated blood.*

Spectra of the Detected Light Vary With Time

Figure 3, A, compares spectra of the detected signal from freshly drawn blood at times selected to correspond

to representative points of the first 3 regions of Figure 1, A. Detectable shifts occur in the distribution of intensities as blood clots. Figure 3, B, shows the same time series for recalcified blood. Comparison of the spectra between fresh and recalcified specimens reveals a similar distribution of intensities, the most obvious difference being a broad peak centered around 485 nm in the recalcified specimen that is not apparent in fresh blood. Therefore, clotting blood, whether freshly drawn or recalcified, responds to illumination with a detected signal that varies with time both in total intensity and spectral characteristics.

Gas Diffusion Has No Detectable Effect on the Spectra During the Period of Observation

Because the top of the cuvette is open to the atmosphere, diffusion of gases across this surface could, in principle, affect the spectrum of reflected light and confound the changes due to clotting. To assess the effect of gas diffusion, the cuvette was filled with blood, which was allowed to clot for 10 minutes. The probe initially illuminated the blood immediately below the atmospheric interface; it was then lowered in 5-mm increments to the bottom of the cuvette. No regular variation of the spectrum was evident (data not shown). Although carbon dioxide content does not have a significant effect on the absorption spectrum of hemoglobin, oxygen content clearly does. The ratio of light reflected at 805 nm to that at 660 nm has been used to monitor changes in the concentration of oxyhemoglobin.[13] The time course of this ratio showed no regular pattern, increasing in some subjects but decreasing in others as clotting progressed (data not shown), behavior which is not consistent with a steady influx of oxygen across the interface. Therefore, the diffusion of gases across the interface has no detectable effect on the spectra acquired during coagulation.

Time Course of Relative Light Intensity at Each Wave Length Has the Same 4 Regions as That of Total Light Intensity

To compare time dependence of spectra such as those in Figure 3, it is more convenient to choose a standard state of the specimen as reference than a diffuse white surface. Therefore, an initial spectrum was calculated by linear extrapolation from the first 5 time points and was used as the reference for calculating reflectance from the specimen. The initial relative intensity at every wavelength becomes 1 by definition, thereby allowing comparisons between the blue (450–500 nm) and the red (>610 nm) ends of the spectrum, which otherwise would be obscured, as in Figure 3. Figure 4 shows a subset of the spectra displayed in Figure 3 with the extrapolated initial state of the specimen as reference. In the freshly drawn sample (Figure 4, A), relative changes in the red region of the spectrum exceed those in the yellow-green; furthermore, the greatest relative changes in the red end of the spectrum occur in the 600- to 650-nm range. The recalcified specimen (Figure 4, B) shows more complex behavior; although relative changes across the spectrum are more uniform, intensities at 610 and 700 nm demonstrate distinct regions.

Time courses of relative reflectance at selected wavelengths (Figure 5) roughly track the time course of total light intensity (Figure 1) and can be divided into the same 4 regions. As mentioned, I will focus on the first 3 of these regions. Although only a few representative wavelengths

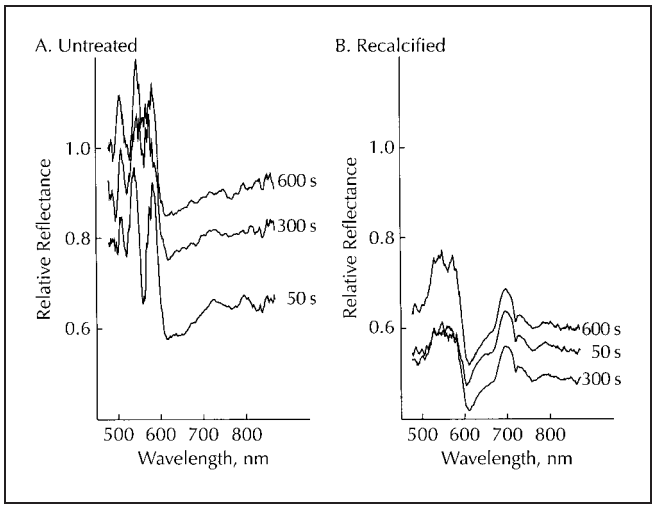

Figure 4. *Time dependence of normalized spectra. Using the initial spectrum as a reference state allows the comparison of relative changes across the entire range of wavelengths. The spectra from both freshly drawn blood (A) and recalcified blood (B) show regions around 550 and 610 nm that change at similar rates. The recalcified specimen shows another such region at 700 nm.*

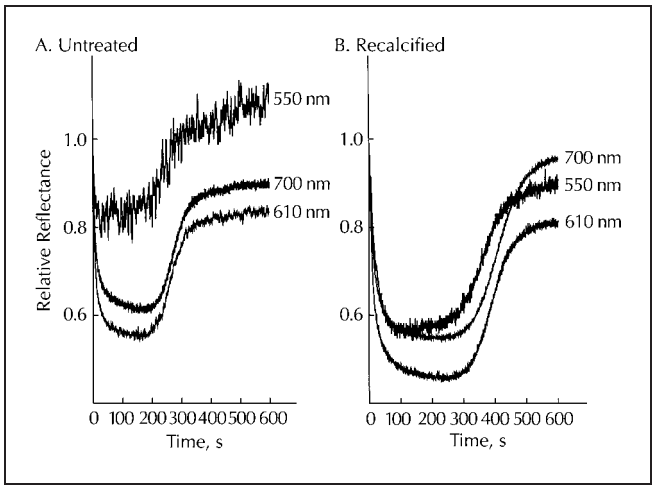

Figure 5. *Time dependence at selected wavelengths. Freshly drawn blood (A) and recalcified blood (B) illustrate that the relative reflectance at each wavelength follows a time course similar to that of total light intensity (Figure 1). All wavelengths studied demonstrated similar time dependence in all healthy subjects.*

are shown, it should be noted that no change corresponding to solidification occurred at any wavelength.

Evidence That the Time Courses at Different Wavelengths Track Different Biochemical Entities

All individuals showed similar monotonic decreases at all wavelengths. The data plotted in Figure 5 show the sigmoidal increase in light intensity occurring approximately at the same time at the 3 wavelengths; 64% of the specimens behaved this way. In the remaining 36%, the sigmoidal increase at 550 nm occurred before the corresponding changes at 610 and 700 nm (Figure 6). Figures 5 and 6 demonstrate that considerable variation occurs in the linear region after the sigmoidal increase. Several generalities appear concerning this region from the survey of healthy subjects. At 550 nm, the light intensity either

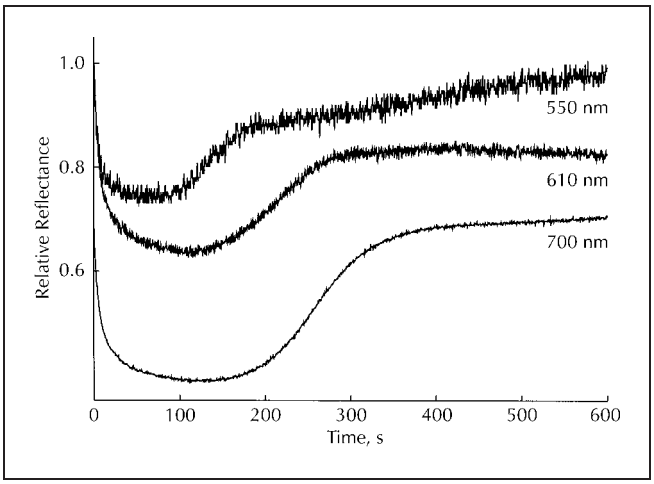

Figure 6. *Intersubject variation of time dependence. The 3 tracings are from the same individual and illustrate that the sigmoidal increase at 550 nm may precede those at other wavelengths. The 700 nm tracing has been translated downward to prevent overlap. Note also the variable slopes of the linear region.*

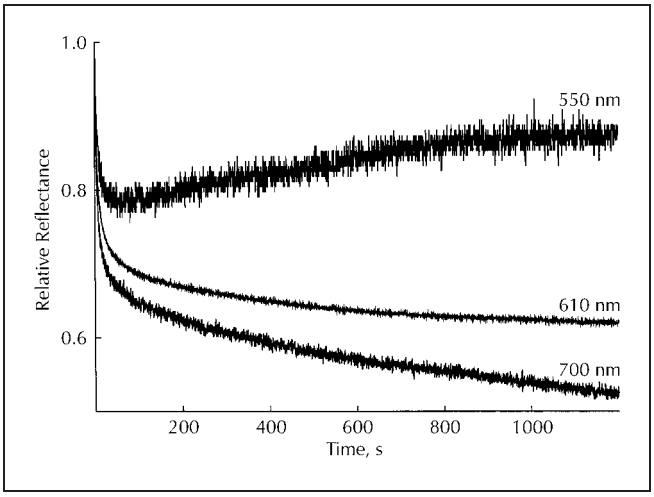

Figure 7. *Effect of Alzheimer disease. Blood was drawn at presentation of a 64-year-old man with dementia, who was ultimately diagnosed with probable Alzheimer disease. Measurements were made immediately after phlebotomy. Although there is a slow sigmoidal increase at 550 nm, none appears at either 610 or 700 nm, even with a prolonged period of observation (compare Figure 1). This result demonstrates that the biochemical events determining the time course at 550 nm are independent of those determining the courses at 610 and 700 nm.*

trends upward or plateaus. At 610 nm, the light intensity either trends downward or plateaus. The intensity at 700 nm may increase or decrease. No correlation exists among the behaviors at these 3 wavelengths in the linear region.

The conclusions drawn from the survey of healthy subjects suggest the hypothesis that time courses at different wavelengths track different biochemical entities, at least during the sigmoidal increase and in the linear region. Further evidence for this hypothesis comes from studies done on a 64-year-old patient with probable Alzheimer disease. The patient had no remarkable medical history and took no drugs. The dementia investigation[18] resulted in no other medical diagnoses. A blood sample drawn at presentation was examined (Figure 7); unlike all other

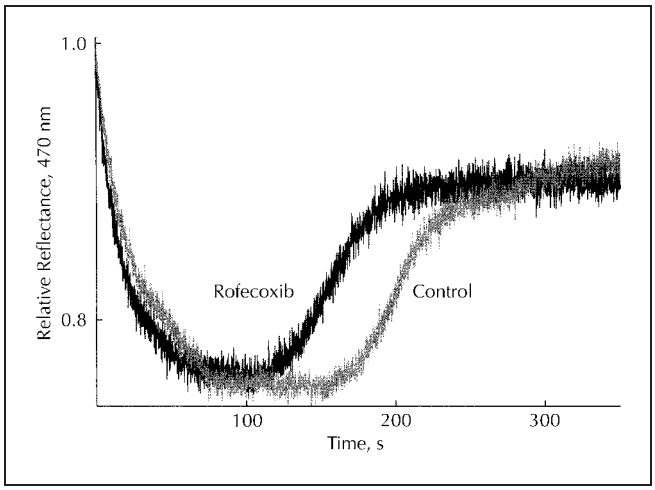

Figure 8. *Effect of cyclooxygenase-2 inhibitors. The rofecoxib tracing represents the time course of light intensity at 470 nm after taking 50 mg of rofecoxib daily for 90 days (subject 4). The control tracing shows the time course before therapy. Blood was drawn into citrate and recalcified. All individuals studied showed a similar change for the region of the sigmoidal increase, but no regular effects in the other regions.*

specimens studied, there was a sigmoidal increase at 550 nm, but not at 610 or 700 nm.

COX-2 Inhibitors Affect the Region of the Sigmoidal Increase

To determine whether the time course of reflected light could be pharmacologically modified, several pilot studies were performed to investigate the effect of NSAIDs. In one study, we examined the effect of COX-2 inhibitors. An initial sample was drawn from a patient about to be treated for musculoskeletal pain. At a follow-up visit, after confirming that the patient had been taking the prescribed treatment, a second blood sample was drawn and studied. Comparison of pretreatment and posttreatment time courses showed that the region of the sigmoidal increase was altered in every subject after taking COX-2 inhibitors (Figure 8). No pattern of a COX-2 effect could be discerned during the monotonic decrease or in the linear region (Figure 8). To quantify the effect on the sigmoidal region, the time course was fit by the following logistic function:

$$R(470, t) = \frac{A}{1 + B\rho^t} + C \qquad (1)$$

All 4 parameters were allowed to vary in fitting the curve. The results of the pilot study, comparing the ratios of the parameters B and ρ after treatment to the initial values, are given in Table 1. Although COX-2 inhibitors did not affect the logistic parameter ρ, they reduced the parameter B ($P < .001$). Two other measurements are included in Table 1 for comparison because they were made on citrate-anticoagulated blood under the same conditions as the COX-2 measurements. In one, blood drawn before the ingestion of 325 mg of aspirin was compared with a sample drawn 2 hours after ingestion. In another, blood drawn from a patient about to be started on indomethacin was compared with a sample taken after 5 days of therapy. Like the COX-2 inhibitors, these NSAIDs had no effect on the parameter ρ. However, unlike the COX-2 inhibitors, these NSAIDs increased the parameter B.

Two pharmacologic studies were performed on freshly

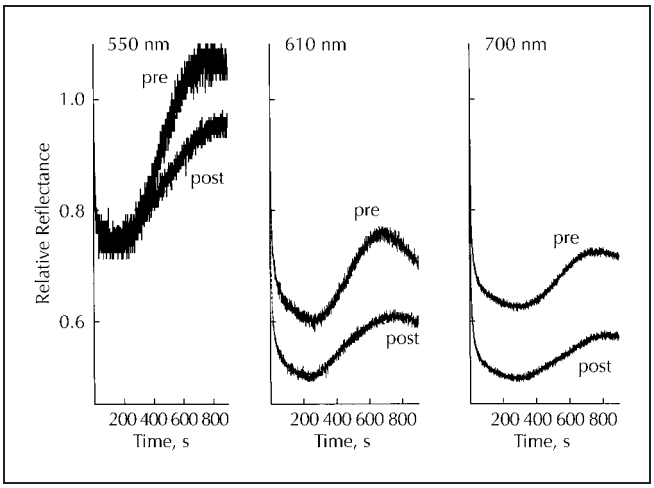

Figure 9. *COX-2 Inhibitor 2 hours after dose. The 3 panels show the effect of a single 400-mg dose of celecoxib after 2 hours (subject 2). Parameters from curve fitting are given in Table 2. Both specimens were studied immediately after phlebotomy.*

Table 2. Effect of Nonsteroidal Anti-inflammatory Drugs on Logistic Parameters* Fit to Sigmoidal Increase in Freshly Drawn Blood

Wavelength, nm	*B* Posttreatment/ *B* Pretreatment	ρ Posttreatment/ ρ Pretreatment
	Celecoxib	
470	0.087	1.01
550	0.154	1.00
610	0.055	1.01
700	0.017	1.01
	Aspirin	
470	4.48	0.99
550	1.45	0.99
610	2.18	0.99
700	1.85	0.99

* Parameters *B* and ρ are calculated from equation 1.

drawn blood using the array spectrometer. One studied the effect of 400 mg of celecoxib 2 hours after ingestion. Three representative wavelengths are shown in Figure 9, and the logistic parameters are compiled in Table 2. To allow comparison with the studies done in recalcified blood, the parameters were also determined at 470 nm; there was a decrease in the parameter *B*, whereas the parameter ρ was unaffected. A similar study was performed with a single 325-mg dose of aspirin; the results are compiled in Table 2. Unlike celecoxib, aspirin increased the parameter *B*, whereas ρ was again unaffected.

COMMENT

In contrast to plasma, the appearance of whole blood during coagulation does not change significantly to the eye. Nonetheless, photometry detects differences in reflected light intensity and distinguishes 4 regions in the time course (Figure 1). This article examines whether useful information can be extracted from the first 3 phases of that time course. The difference between plasma and whole blood suggests that any explanation of optical properties during coagulation will require fundamentally different models. Furthermore, the optical effects of fibrin polymerization in vitro precede the development of rigidity.[19,20] The sigmoidal increase in the intensity of light reflected from blood, however, occurs after clot formation (Figure 1). The experiments done on blood to determine the angular dependence of reflected light intensity and to ascertain which physical processes contribute to the signal lead to the conclusion that, as a first-order approximation, the time course manifests changes in the chemical nature of the reflecting surface, as opposed to, for example, volume changes of light-scattering centers.

Of the physical processes that could return light into the hemisphere of the irradiating beam, 2 have any time dependence minimized by experimental design (namely, thermoluminescence and specular reflection), and 2 have been eliminated by control experiments (namely, photoluminescence and chemiluminescence). The detected light, therefore, constitutes nonspecular reflection of the irradiating beam. Nonspecular reflection may be further characterized by the angular dependence of reflected light intensity. Figure 2, A, demonstrates that the intensity of reflected light is proportional to cos θ, the angle between detector and normal to blood surface, which is also the direction of the incident beam. This relationship is known as the Lambert law, and surfaces that obey this law produce diffuse reflection. Physically, diffuse reflection may be viewed as absorption of incident light followed by emission at the same wavelength[17,21]; it thus depends essentially on the resonant frequencies of the electrons and hence on the chemical nature of the surface. It has been aptly called resonant scattering[22] to distinguish it from Rayleigh scattering, which has an angular dependence determined by $1 + \cos^2 \theta$ for nonpolarized incident radiation. These considerations underscore the difference between clotting plasma and blood. Rayleigh scattering results in some light emitted at 90° to the incident beam,[22] as occurs with plasma, whereas a purely lambertian surface reflects no light in that direction. The line fit to the data in Figure 2, A, passes through the origin within experimental error, which further confirms the lambertian behavior of blood. Undoubtedly, some light will emerge from blood at right angles to the beam. This is another reason why these interpretations must be viewed as a first-order approximation. When light enters a lambertian surface, its conversion into heat precludes its reflection; therefore, diffuse reflection spectra carry the same information as absorption spectra for a plane surface and follow chemical changes of the constituents.

The reflectance spectrum depends on the orientation of the probe (Figure 2, B). The glass interface, therefore, must impose some ordering on the constituents of liquid blood. The same angular dependence occurs in both liquid and clotted blood (Figure 2, B), which has 2 important consequences. First, structural changes due to polymerization of fibrin do not contribute to this phenomenon. Second, as a practical matter, the dependence of spectral intensities on the angle of the probe will not affect the time course of light intensity. The probe orientation remained the same for all data reported in this article, deferring the investigation of this observation to future work.

Dispersing the reflected light into its spectrum showed similar time courses at each wavelength in all healthy subjects (Figures 5 and 6). Several lines of evidence show that different biochemical processes during coagulation can be followed by examining different regions of the time course at each wavelength. That the sigmoidal increase at 550 nm may precede the correspondent phases at 610 and 700 nm

(Figure 6) implies that the time course follows a different biochemical process; the blood of the patient with Alzheimer disease (Figure 7) demonstrates that the process reported at 550 nm can occur without those at 610 or 700 nm. These facts suggest the hypothesis that the biochemical processes underlying the time course at 550 nm are coupled to those manifested at 610 and 700 nm and may drive them. Concerning the linear region, the lack of correlation between the time courses at any 2 wavelengths implies that, here as well, different regions of the spectrum report different biochemical events. Concerning the initial monotonic decrease, no inferences can be drawn at this stage, because all specimens at all wavelengths exhibited similar behavior. Moreover, the fact that COX-2 inhibitors exert their primary effect during the sigmoidal increase with no regular effect during the monotonic decrease or in the linear region raises the possibility that the intensity of light at a given wavelength may track different processes at different times. Taken together, the data reported support the notion that these 4 regions correspond to distinct events during whole blood coagulation. Only 2 statements can be made concerning the identities of these events at this point. The terminal phase indicates clot retraction, and the sigmoidal increase does not mark the onset of fibrin polymerization.

To identify which biochemical processes correspond to the changes in reflected light will require studies on anticoagulated blood. Although the behavior of recalcified citrated blood differs from that of freshly drawn blood (Figure 3), the persistence of the 4 regions (Figures 1 and 5) suggests that the factors determining the shape of the time course can be elucidated by such studies. Aspirin and celecoxib effects are similar in both freshly drawn (Table 2) and anticoagulated blood (Table 1). Furthermore, the appearance of these effects (Table 2; Figure 9) 2 hours after ingestion implies that in vitro studies may reproduce the ex vivo phenomena.

Several authors have expressed concern that prolonged use of COX-2 inhibitors may increase the risk of thromboembolic events.[5] It has been proposed that the increased risk may result from interactions between elements in the blood and the vascular endothelium.[4] At this time, however, there is no consensus that COX-2 inhibitors promote thrombosis clinically,[2,23] and the differential effects of COX-active agents in various experimental models make their extrapolation to clinical practice difficult.[23] The data presented here show that COX-2 inhibitors affect whole blood coagulation intrinsically (Tables 1 and 2). No inferences can be drawn at this stage concerning the relationship of this effect to thrombosis, although the data suggest that the effects of rofecoxib and celecoxib are opposite to those of indomethacin and aspirin.

Although Alzheimer disease is a neurodegenerative disorder, several studies have discovered abnormalities in blood, primarily in platelets and erythrocytes.[24–26] Alzheimer disease shows no regular effect on the proteins of the coagulation cascade. It is likely, therefore, that the results shown in Figure 7 will be traced to the cellular elements of the blood or to some interaction between them. It is intriguing that the sigmoidal increase is affected by both Alzheimer disease and NSAIDs, because several epidemiological studies have demonstrated that NSAID use reduces the risk of Alzheimer disease.[27] Furthermore, some studies suggest that the protective effect in Alzheimer disease may not involve the COX pathway.[28]

The time course of light reflected from clotting blood contains unique information. It demonstrates an effect of COX-2 inhibitors on coagulation without the presence of endothelium, and the data suggest that it may discern effects of aspirin and other NSAIDs. The attempt to unravel the threads of the biochemical processes that contribute to this time course is worthwhile because of the potential utility in evaluating effects of NSAIDs on coagulation and gaining insight into the pathology of Alzheimer disease. Conversely, the ability to modify the time course introduces practical methods for beginning the attempt, using either pharmacologic probes or factor-deficient blood. That the general features of the time course survive in the clotting of blood collected into anticoagulant brings into play a wide array of techniques to elucidate the phenomena, all of which makes the goal of using reflectance spectroscopy to study the clotting of whole blood not only desirable, but also feasible.

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