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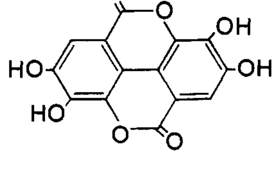
Activation of Intrinsic Blood Coagulation by Ellagic Acid: Insoluble Ellagic Acid–Metal Ion Complexes Are the Activating Species[†]

Paul E. Bock, K. R. Srinivasan, and Joseph D. Shore*

ABSTRACT: The species of ellagic acid responsible for initiating intrinsic blood coagulation has been characterized. Ellagic acid is soluble at a level of 30 ± 10 μM in pH 7.4 Tris–NaCl buffer at 22 °C. Dilution of soluble ellagic acid resulted in enhanced procoagulant and kallikrein-generating activity in normal human plasma and the appearance of a new absorbance spectrum. These effects were prevented by 1 mM EDTA, and the new species could be removed by centrifugation. Addition of stoichiometric Cu^{2+} to Millipore-filtered soluble ellagic acid generated an absorbance spectrum similar to that caused by dilution in the absence of EDTA, as well as procoagulant and kallikrein-generating activities. Although no turbidity was visible and the spectral properties did not indicate extensive light scattering, centrifugation resulted in loss of the absorbance spectrum and kallikrein-generating activity. Titration of ellagic acid with Cu^{2+} showed that the absorbance spectral change was correlated with the formation of insoluble complexes and increased procoagulant activity. Zn^{2+}, Co^{2+}, and Fe^{3+} caused similar spectral changes and the formation of insoluble species capable of inducing prekallikrein activation. We conclude that the procoagulant and kallikrein-generating activity of ellagic acid solutions can be ascribed to slowly settling insoluble aggregates of ellagic acid–metal ion complexes, which are formed with adventitious metal ions present in the diluting buffer. Formation of these aggregates, and the development of biological activity, could be prevented but not reversed by 1 mM EDTA. The activity of these complexes and Cu^{2+}–ellagic acid complexes was dependent on the presence of each of the four proteins of the contact activation system. Although soluble ellagic acid may bind to factor XII, this interaction does not initiate blood coagulation or prekallikrein activation, since these activities were only associated with the insoluble species.

Initiation of the intrinsic blood coagulation system occurs when normal plasma contacts a negatively charged surface. The surface-dependent conversion of factor XII to an active serine protease is the first event in the cascade of reactions leading to the formation of an insoluble fibrin clot. The participation of prekallikrein and high molecular weight kininogen in contact activation results in the generation of fibrinolytic activity and the formation of kinins (Griffin & Cochrane, 1979; Ulevitch & Cochrane, 1977).

Activation of the contact system can be achieved with a variety of negatively charged insoluble substances. The action of these substances involves the specific adsorption of factor XII and the other proteins of the contact activation system, leading to an acceleration of the proteolytic activation of factor XII, prekallikrein, and factor XI (Wiggins et al., 1977; Griffin, 1978; Griffin & Cochrane, 1976; Meier et al., 1977; Revak et al., 1977). Although most known activators are insoluble, activation has been reported to occur in solution by the polyanions heparin (Moskowitz et al., 1970), dextran sulfate (Kluft, 1978), and carrageenans (Schwartz & Kellermeyer, 1969) as well as by soluble ellagic acid (Ratnoff & Crum, 1964). Unique among these substances, ellagic acid was of particular interest since this compound has a low molecular weight and the following chemical structure:



Studies of the mechanism of human factor XII activation

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involving ellagic acid (Saito, 1977; Ratnoff & Saito, 1979a,b) have been interpreted to provide evidence for a nonproteolytic activation mechanism, while studies involving insoluble substances have not (Griffin & Beretta, 1978; Meier et al., 1977). As part of our interest in the kinetics and mechanism of the reactions involved in contact activation, we have investigated the activation of this system by low concentrations of ellagic acid with the initial goal of characterizing the physical and chemical properties of the activating species.

Experimental Procedures

Ellagic Acid. Ellagic acid (97%) (2,3,7,8-tetrahydroxy-[1]benzopyrano[5,4,3-*cde*][1]benzopyran-5,10-dione) was purchased from Aldrich Chemical Co. and used without further purification. Thin-layer chromatography on silica gel 60 F_{254} aluminum-backed plates (E. Merck, Darmstadt) in toluene–ethyl formate–formic acid (5:4:1) showed a single UV-absorbing spot with an $R_f = 0.15$. An extinction coefficient for ellagic acid at 360 nm was determined from the absorbance of solutions prepared by heating known weights of this compound in 0.02 M Tris-HCl–0.15 M NaCl, pH 7.4. The value of $\epsilon_{360\text{nm}} = (1.62 \pm 0.04) \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ obtained by this procedure was used to calculate ellagic acid concentrations. An indistinguishable value for the extinction coefficient was obtained by measuring the difference in absorbance between ellagic acid dissolved in ethanol and diluted in buffer, using $\epsilon_{366\text{nm}} = 1.0 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ (Jurd, 1956) to determine the concentration in ethanol.

As will be shown, the method of preparation of ellagic acid solutions is of critical importance to the measurement of biological activity for this compound. The following procedure was developed for preparation of stable solutions of ellagic acid. Suspensions of ellagic acid at approximately 0.5 mg/mL in 0.02 M Tris-HCl–0.15 M NaCl, pH 7.4, were prepared in the presence or absence of 1 mM EDTA at room temperature. The suspension was Millipore filtered (0.22 or 0.45 μm) and rapidly diluted to nearly the desired final concentration (<30 μM) with the appropriate buffer. After incubation at room temperature for at least 1 h, the solution was carefully Millipore filtered a second time and not further diluted. As will be shown, undiluted solutions prepared either in the presence or in the absence of EDTA are stable for more than 1 h, do not contain appreciable amounts of insoluble material, and have minimal clotting activity. During the course of these studies, we found that additional precautions were often required in order to obtain reproducible results, particularly for solutions prepared in the absence of EDTA. Spectrophotometer cuvettes, glassware, and centrifuge tubes were soaked in 0.1 M EDTA, pH ~7, and thoroughly rinsed with double-deionized water before contact with ellagic acid solutions. Millipore filters were soaked overnight in deionized water and rinsed with buffer to remove metal ions and UV-absorbing substances (Cooney, 1980).

Clotting Assay. The clotting activity of ellagic acid solutions was measured by the acceleration of the partial thromboplastin time (PTT) of normal human plasma (George King Biomedical). A mixture of 50 μL of normal plasma, 50 μL of rabbit brain cephalin (a 10-fold dilution of the stock solution prepared according to the procedure recommended by Sigma), and 100 μL of ellagic acid solution or kaolin in 0.02 M Tris-HCl–0.15 M NaCl, pH 7.4, was incubated in a plastic tube at 37 °C. After 8 min, 50 μL of 0.05 M $CaCl_2$ was added and the clotting time determined. In order to compare the clotting activity of ellagic acid solutions containing various amounts of added Cu^{2+}, solutions of ellagic acid which had been incubated at room temperature for 1 h with an equal concentration of $CuCl_2$ were diluted and immediately assayed at final concentrations ranging from 1 nM to 10 μM. A plot of log PTT vs. log Cu^{2+}–ellagic acid concentration was linear over the range of clotting times between 200 and 60 s and had a similar slope to the line obtained from experiments in which dilutions of a kaolin suspension were assayed. The relative activity of ellagic acid solutions containing less than saturating Cu^{2+} concentration (Figure 7B) was determined by using this plot from the ratio of concentrations of Cu^{2+}–ellagic acid which would give equivalent clotting times. Hageman, Fletcher, and factor XI deficient plasmas were purchased from George King Biomedical. Fitzgerald plasma was obtained from Dr. R. Waldmann.

Prekallikrein Activation Assay. The generation of kallikrein in normal human plasma induced by ellagic acid was measured in masked plastic semimicro cuvettes by the increase in the rate of hydrolysis of H-D-Pro-Phe-Arg-*p*-nitroanilide (S2302, Kabi, U.S.) in a Gilford 2400S spectrophotometer at 22 °C. Normal plasma (20 μL) was added to a solution of 500 μM S2302 in 0.02 M Tris-HCl–0.15 M NaCl, pH 7.4, ±1 mM EDTA, followed by 5–100 μL of ellagic acid to give a final volume of the assay mixture of 1.0 mL. The increase in absorbance at 405 nm due to amide hydrolysis by kallikrein, corresponding to less than 5% substrate hydrolysis, was recorded as a function of time after addition of ellagic acid. Substrate concentrations were determined by absorbance, using a molar extinction coefficient of $1.3 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ at 316 nm.

Spectrophotometry. Absorbance spectra were recorded by using a Cary 17 spectrophotometer with matched quartz cuvettes. The spectra of dilute ellagic acid (0.2 μM) solutions were obtained by using 10-cm path-length cylindrical quartz cuvettes. Spectrophotometric titrations of ellagic acid were performed by adding small volumes of a concentrated solution of metal ion (chloride or sulfate salts) to 3.0-mL aliquots of ellagic acid in 0.02 M Tris-HCl–0.15 M NaCl, pH 7.4, and identical buffer solutions. After incubation for 1 h at room temperature, the absorbance spectra were recorded for the appropriate sample–reference pairs.

Four 1-cm path-length matched quartz cuvettes and tandem cuvette holders were used for the ellagic acid–metal ion difference spectra. A base line was recorded between pairs of cuvettes containing 3.0 mL of ellagic acid and 3.0 mL of buffer in both sample and reference compartments. The difference spectrum was obtained 1 h after addition of the metal ion in a small volume to both the ellagic acid solution in the sample compartment and buffer in the reference compartment.

Concentration difference spectra were obtained as the difference in absorbance between concentrated ellagic acid in a 1-mm path-length cylindrical quartz cell in the reference compartment and a 100-fold dilution of this solution in a 10-cm path-length cylindrical cell in the sample compartment. The difference spectra were corrected for the base line obtained with buffer in the same cuvettes. The high absorbance difference due to the presence of EDTA prevented obtaining good spectral data below 260 nm in this experiment.

Centrifugation. Ellagic acid solutions were centrifuged in the SS34 rotor of a Sorvall RC2B centrifuge in plastic 50-mL centrifuge tubes at 12100*g* for 45 min with the temperature maintained at 21–24 °C. These conditions were found to sediment all of the ellagic acid–metal ion complexes studied.

Results

Our initial studies concerned determination of whether ellagic acid is soluble, with a quantitative estimate of its solubility at room temperature. This was measured in two

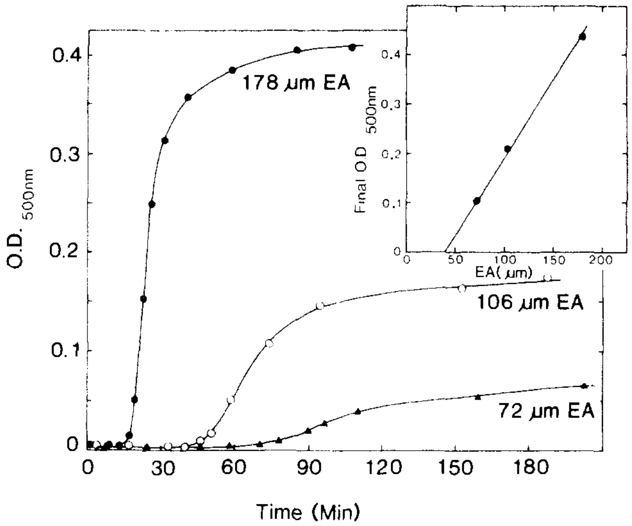

FIGURE 1: Kinetics of precipitation of ellagic acid from supersaturated solutions in 0.02 M Tris-HCl–0.15 M NaCl, pH 7.4, at the indicated concentrations of ellagic acid. Inset: The extrapolated final levels of turbidity reached in these experiments plotted as a function of the ellagic acid concentration.

ways: from the kinetics of precipitation from supersaturated solutions, and determination of the equilibrium attained between soluble and insoluble ellagic acid starting from higher and lower temperatures. Heated suspensions of ellagic acid were Millipore filtered and rapidly brought to 22 °C in an ice–water bath. Figure 1 shows the kinetics of precipitation in the stirred supersaturated solutions monitored by the appearance of turbidity at 500 nm. The appearance of turbidity is time dependent with a variable latent period, followed by a rapid increase. In unstirred solutions, the length of the latent period at a given ellagic acid concentration is much longer. This kinetic behavior is consistent with the slow formation of a critical nucleus composed of a few molecules of ellagic acid in solution, followed by rapid growth of the nucleus to form large insoluble aggregates. The final levels of turbidity reached in these experiments plotted against the starting ellagic acid concentration are presented in the inset of Figure 1. This extrapolated relationship indicates a solubility of approximately 40 µM at 22 °C. Values of 20–40 µM were obtained from experiments in which ellagic acid was allowed to precipitate from supersaturated solutions overnight, and the concentration remaining in solution was determined after centrifugation.

For corroboration of these experiments, stirred suspensions of ellagic acid starting at 0–5 °C and at 37 °C were allowed to equilibrate at room temperature for 5 h. These suspensions were centrifuged, and the concentration of ellagic acid in the supernatant was determined by the absorbance. The effect of contaminant metal ions in the buffer was evaluated by doing the same experiment in the presence of 1 mM EDTA. In all cases, the final soluble concentration of ellagic acid was 30 ± 10 µM at 22 °C. Millipore filtration of these solutions did not decrease the absorbance by more than 10%. The concentration of ellagic acid in Millipore-filtered solutions of 20–30 µM was not changed by repeated Millipore filtration after incubation for more than 15 h at room temperature. These studies demonstrate that ellagic acid is soluble at room temperature and that the presence of small amounts of metal ions does not measurably affect its solubility. On the basis of these results, stock solutions of ellagic acid were prepared at concentrations less than 30 µM.

Our next experiments involved evaluation of the possible effects of metal ion contaminants and aggregation on the procoagulant activity of ellagic acid. It will become clear that the method of preparation of ellagic acid solutions is closely related to the presence of procoagulant activity. Specifically, clotting activity is generated on dilution of solutions prepared essentially free of metal ion contaminants and insoluble material by repeated Millipore filtration. The following experiments indicate that dilution of these solutions is equivalent to readdition of such trace contaminants, resulting in the generation of activity. Stock ellagic acid solutions at concentrations of approximately 10 µM were prepared in the presence and absence of 1 mM EDTA. These solutions were diluted in the presence and absence of EDTA to a concentration of 0.5 µM and incubated for 1 h at 22 °C. Clotting activity was measured before and after centrifugation by the shortening of the partial thromboplastin time of normal human plasma at a final concentration of 0.2 µM ellagic acid. Experiments 1 and 2 of Table I show that only ellagic acid prepared and diluted in the absence of EDTA acquired procoagulant activity. Experiment 3 was performed with a solution prepared, diluted, and incubated 1 h in the absence of EDTA, with 1 mM EDTA added subsequently, followed by another hour of incubation prior to addition to the coagulation assay. This experiment demonstrated that the time-dependent increase in procoagulant activity which occurs in the absence of EDTA is not rapidly reversible by subsequent incubation with 1 mM EDTA. It also showed that the presence of 1 mM EDTA has no effect on the coagulation assay. The development of clotting activity in dilute ellagic acid solutions in the absence of EDTA was a slow process. Estimates of the rate of this process indicated that 1 h was sufficient time to ensure that the reaction had gone to completion under the conditions of our experiments.[1]

Table I: Effect of EDTA and Centrifugation on Clotting Activity of Ellagic Acid Solutions[a]

		clotting time (PTT, s)	
expt	soln	before centrifugation	after centrifugation
1	ellagic acid	98	359
	buffer	400	
2	ellagic acid prepared in buffer containing 1 mM EDTA	341	352
3	ellagic acid	94	
	ellagic acid + 1 mM EDTA after 1 h of incubation	87	306

[a] Clotting activity was measured as described under Experimental Procedures 1 h after dilution to a concentration of 0.5 µM from stock solutions of 9.9 µM ellagic acid in 0.02 M Tris–Cl buffer, 0.15 M NaCl, and 1 mM EDTA, pH 7.4, and 10.3 µM ellagic acid in the same buffer without EDTA, prepared as described under Experimental Procedures. Diluted ellagic acid solutions were centrifuged at 12100g for 45 min.

The second column of Table I shows that the substance responsible for the procoagulant activity of those ellagic acid solutions which were active could be completely removed by centrifugation. Experiments similar to those of Table I were performed with ellagic acid concentrations ranging from 10^{-9}

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[1] At higher ellagic acid concentrations and in the absence of EDTA, the rate of this process is faster and can occur on a time scale comparable to that of the clotting assay. This presumably accounts for lower clotting times obtained with rapidly diluted solutions in the absence of EDTA at high final concentrations in the assay. In order to avoid this complicating effect, clotting assays shown in Table I and in similar experiments were performed at concentrations (0.1–0.2 µM) for which the activity determined immediately after dilution was nearly zero, while the activity after 1 h of incubation was easily measured.

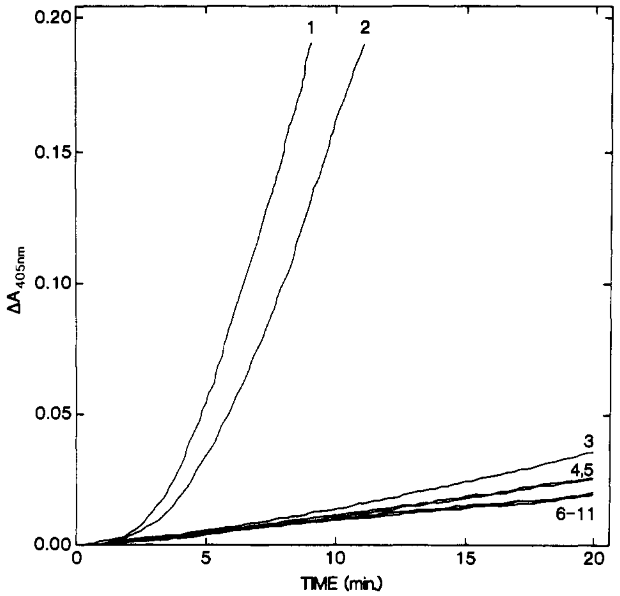

FIGURE 2: Effect of dilution, 1 mM EDTA, and centrifugation of ellagic acid on prekallikrein activation in normal plasma. Reactions were initiated by the addition of ellagic acid at a final concentration of 0.1 μM in the assay mixture described under Experimental Procedures. Stock ellagic acid solutions were 20 μM; dilutions at a concentration of 1 μM were incubated for 1 h before addition to the assay mixture. Curve 1, ellagic acid prepared, diluted, and assayed in the absence of EDTA; curve 2, ellagic acid prepared and diluted without EDTA and assayed in the presence of EDTA; curve 3, ellagic acid prepared, diluted, and assayed in the presence of EDTA; curves 4 and 5, undiluted ellagic acid prepared and assayed in the presence or absence of EDTA; curves 6–9, supernatants from centrifugation of the ellagic acid solutions used in assays 1–5; curves 10 and 11, assays without ellagic acid in the presence and absence of EDTA.

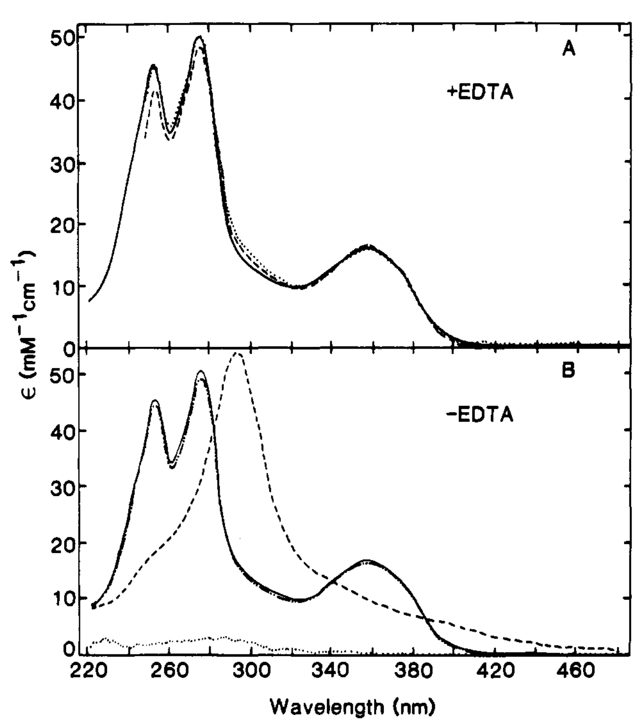

FIGURE 3: Effect of dilution and centrifugation on the absorbance spectrum of ellagic acid prepared in the presence or absence of EDTA. (A) Absorbance spectrum of 10 μM ellagic acid prepared in 0.02 M Tris-HCl, 0.15 M NaCl, and 1 mM EDTA, pH 7.4, at 22 °C: after centrifugation (—, two identical spectra); the spectrum of ellagic acid 100 min after dilution to a concentration of 0.2 μM in buffer containing 1 mM EDTA (- - -); after centrifugation of this solution (···). (B) Absorbance spectrum of 9.3 μM ellagic acid in buffer without EDTA (—) and after centrifugation (-···-); the spectrum of ellagic acid 100 min after dilution to a concentration of 0.18 μM in the absence of EDTA (- - -) and after centrifugation of this solution (···).

to 10^{-5} M in the assay mixture. Ellagic acid solutions prepared in the presence of 1 mM EDTA all yielded clotting times indistinguishable from those of the controls without ellagic acid over this concentration range, indicating no detectable clotting activity, but they consistently showed activity when prepared and diluted in the absence of EDTA between approximately 10^{-8} and 10^{-5} M.

To provide a more specific measure of ellagic acid activation of the contact system, we investigated the ability of ellagic acid solutions to stimulate kallikrein generation in normal human plasma as measured by hydrolysis of a chromogenic substrate (H-D-Pro-Phe-Arg-*p*-nitroanilide). Considering the relative plasma concentrations of contact activation enzymes and the approximately 10 times higher specific activity of kallikrein with this substrate as compared with factor XII$_a$ (Silverberg et al., 1980) or factor XI$_a$ (P. E. Bock and J. D. Shore, unpublished observations), we have assigned increased substrate hydrolysis in these experiments to kallikrein generation. However, some component of the observed hydrolytic activity, probably less than 10%, may be due to factor XII$_a$. Curve 1 of Figure 2 shows that 0.1 μM ellagic acid, prepared and diluted in the absence of EDTA, can cause substantial activation of plasma prekallikrein as evidenced by the rapidly accelerating rate of substrate hydrolysis. Curve 2 demonstrates that ellagic acid diluted in the absence of EDTA, added to an assay mixture containing 1 mM EDTA, is also active. Although the activity was slightly lower than that of curve 1, it is qualitatively similar and indicates again that the activity of ellagic acid is not significantly changed by subsequent addition of 1 mM EDTA. Curve 3 shows that if ellagic acid is prepared and diluted in the presence of 1 mM EDTA, negligible kallikrein is generated. Curves 4 and 5 show that with the same final ellagic acid concentration in the assay mixture, if the ellagic acid is added directly from a concentrated stock solution, there is no difference with or without EDTA, and in both cases, negligible kallikrein generation occurs. The absence of activity obtained for rapidly diluted stock solutions of ellagic acid without EDTA is a consequence of the slow time dependence of the process forming the active species at this low ellagic acid concentration. This experiment also demonstrated that the stock ellagic acid solutions did not contain appreciable amounts of the activating species prior to dilution. Finally, curves 6–9 show that if the ellagic acid solutions used in curves 1–5 were centrifuged, the ability to generate kallikrein was lost, and they became equal to the controls, curves 10 and 11, obtained without ellagic acid.

In order to explain the dependence of the biological effects of ellagic acid on EDTA, dilution, and centrifugation, we evaluated these effects with regard to the absorbance spectral properties of ellagic acid. Figure 3A shows that the absorbance spectrum of ellagic acid in buffer containing EDTA is essentially unchanged by dilution from 10 to 0.2 μM or centrifugation, with absorbance maxima at 253, 276, and 357 nm. Figure 3B shows that a 9.3 μM solution of ellagic acid in the absence of EDTA has the same spectrum as that in the presence of EDTA and is unaffected by centrifugation. This result and similar experiments indicate that undiluted ellagic acid solutions can be prepared which contain negligible insoluble material (less than 5%). However, when such an ellagic acid solution is diluted to 0.18 μM in the absence of EDTA, all absorbance peaks characteristic of soluble ellagic acid are lost, and a new spectrum with a maximum at 294 nm emerges. Centrifugation of this dilute solution results in loss of the absorbance spectrum. Therefore, the new spectrum can be assigned to an insoluble aggregated species whose formation

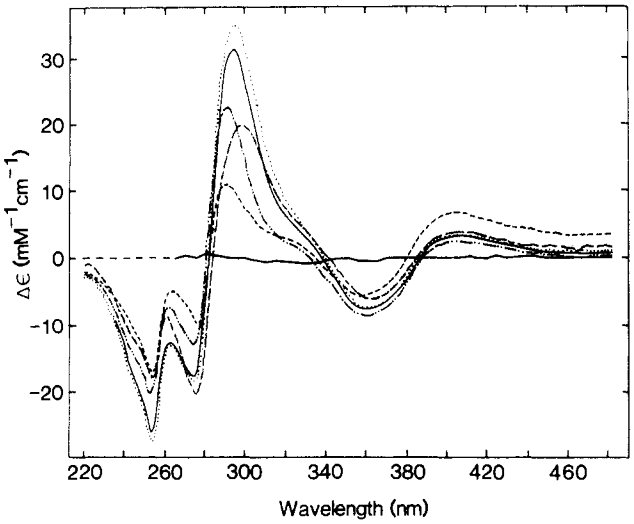

FIGURE 4: Comparison of ellagic acid concentration difference spectrum with ellagic acid – metal ion difference spectra. Concentration difference spectra (solid lines): The difference spectrum between 19.1 μM ellagic acid in 0.02 M Tris-HCl–0.15 M NaCl, pH 7.4, and 0.19 μM ellagic acid 1 h after dilution in the same buffer. The second solid spectrum shows no significant difference in absorbance between 20.4 μM ellagic acid in buffer containing 1 mM EDTA and a 100-fold dilution in this buffer after 100 min. Ellagic acid – metal ion difference spectra: Difference spectra recorded 1 h after addition of a 10-fold excess of $CuCl_2$ (···) $ZnSO_4$ (-···-) $CoSO_4$ (– – –), or $FeCl_3$ (- - -) to ellagic acid at a concentration of 2.2 μM.

in dilute solution is prevented by EDTA. On the basis of the residual absorbance in this experiment, the solubility of this new species must be less than 10^{-8} M. No turbidity was observed in this suspension, the spectral properties do not indicate extensive light scattering, and the spectrum was stable for at least 1 h. It thus seems likely that the aggregate forms a very fine particulate suspension, and the formation of this species is clearly correlated with the presence of procoagulant activity.

The ability of EDTA to prevent the dilution effect on clotting activity, kallikrein generation, and spectral properties strongly indicates that these effects result from metal ion contaminants in the buffer. The impurities likely to be present in 0.15 M NaCl are substantial enough to account for the observed phenomena at low ellagic acid concentrations. For further investigation of this possibility, the effects of various metal ions on the spectral properties of ellagic acid were studied and compared with the spectral change caused by dilution. Figure 4 shows the ellagic acid concentration difference spectrum in the absence of EDTA which is a consequence of the spectral changes at low concentration shown in Figure 3B. In the presence of EDTA, no concentration difference spectrum was obtained, reaffirming the role of chelation by adventitious metal ions. To simulate the effect of these contaminants, we added a 10-fold excess of various metal cations to ellagic acid solutions, and the difference spectra were taken after incubation for 1 h. As shown in Figure 4, addition of copper, zinc, cobalt, or iron resulted in difference spectra qualitatively similar to the concentration difference spectrum. Although similar, the position of the major positive difference peak and the magnitude of the extinction changes were functions of the specific metal ion used. Of the metal ions studied, Cu^{2+} showed the largest extinction change and greatest similarity to the concentration difference spectrum. Calcium and magnesium produced smaller changes in absorbance and required much higher concentrations, indicating weaker affinity for ellagic acid (not shown). Difference spectra obtained at this concentration of ellagic acid (2.2 μM) were stable for more than 1 h, typically showing changes of 10% or less in the difference absorbance. At higher ellagic acid concentrations, a slow decrease in absorbance throughout the spectrum was more pronounced, which may be due to agglomeration of the insoluble metal chelates. The absolute changes in extinction obtained in experiments with Cu^{2+} were somewhat variable, $\Delta\epsilon_{295\text{nm}}$ ranging from 31 to 39 mM^{-1} cm^{-1} at ellagic acid concentrations in the range 1.7–2.4 μM. This may reflect slow changes in the spectra due to secondary aggregation of the insoluble complexes coupled with the probable concentration dependence of the extinction coefficients. At these concentrations of ellagic acid, no turbidity was visible when saturating metal ions were added, and there is no evidence for a significant contribution from light scattering in the difference spectra. Relatively clear solutions were also obtained at approximately 20 μM ellagic acid after addition of metal ions; however, during overnight incubation, a flocculent yellow-green precipitate formed in solutions containing Cu^{2+}, Zn^{2+}, and Co^{2+}. A charcoal-colored precipitate was observed in solutions containing Fe^{3+}.

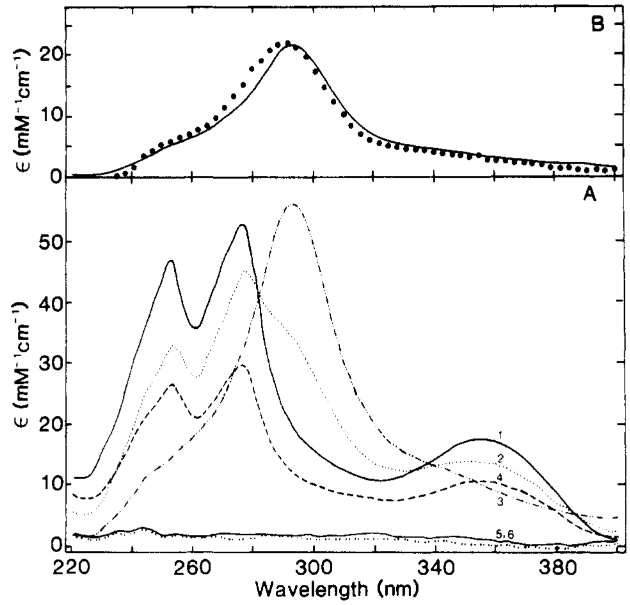

FIGURE 5: Solubility and spectral properties of complexes formed in Cu^{2+}–ellagic acid mixtures. (A) Absorbance spectrum of 2.0 μM ellagic acid and the buffer base line (—, curves 1 and 5); the spectrum of ellagic acid at 40% saturation with Cu^{2+} (0.33 Cu^{2+}/ellagic acid, ···, curve 2); ellagic acid saturated with Cu^{2+} (1.5 Cu^{2+}/ellagic acid, -···-, curve 3); the residual spectrum after centrifugation of the 40% saturated mixture (– – –, curve 4); and the residual spectrum after centrifugation of the Cu^{2+}-saturated ellagic acid solution (···, curve 6). (B) Comparison of the spectrum of ellagic acid saturated with Cu^{2+} multiplied by 0.4 (solid line) with the calculated spectrum of the insoluble component removed by centrifugation of the 40% Cu^{2+}-saturated ellagic acid solution (curve 2 minus curve 4) (●) (mean of two identical experiments).

Considering the large extinction coefficient of the Cu^{2+}–ellagic acid complex and similarity to the complexes formed by dilution we chose this metal ion for further detailed study. Figure 5A shows the spectra of 2.0 μM ellagic acid alone, with saturating Cu^{2+}, and with 40% saturating Cu^{2+} and the residual spectra after centrifugation. Centrifugation of the ellagic acid solution produced an insignificant change in the absorbance spectrum as previously shown (Figure 3). Saturating Cu^{2+} creates a spectrum virtually identical with that of dilute ellagic acid (Figure 3B) which can be abolished by centrifugation. This result clearly shows that the spectrum obtained with saturating Cu^{2+} can be assigned to insoluble species, with no detectable contribution from soluble ellagic

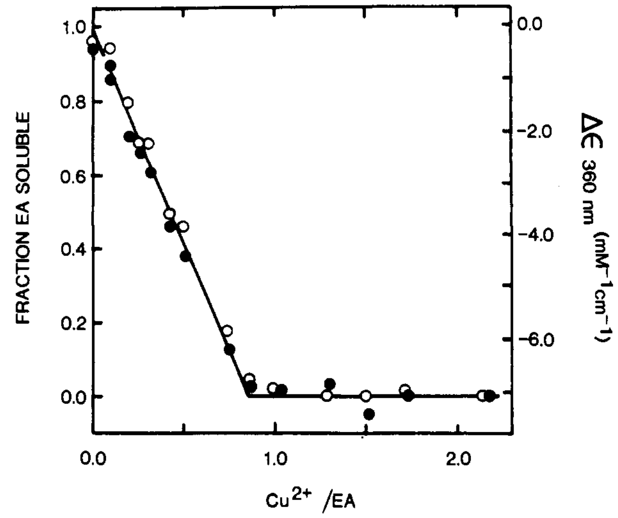

FIGURE 6: Correlation of Cu^{2+}-induced ellagic acid spectral changes with the formation of insoluble complexes. Decrease in extinction at 360 nm 1 h after addition of increasing concentrations of Cu^{2+} to ellagic acid at 1.9 and 2.3 μM (●). Fraction of ellagic acid soluble (○) determined from the residual absorbance at 360 nm after centrifugation.

acid or soluble Cu^{2+}–ellagic acid complexes. Addition of copper to 40% saturation results in an intermediate spectrum, and centrifugation produces a residual spectrum indistinguishable from that of soluble ellagic acid alone between 240 and 380 nm, indicating that this spectrum does not contain a detectable contribution from soluble Cu^{2+}–ellagic acid chelates. Figure 5B shows the calculated spectrum of the insoluble component removed by centrifugation of the 40% copper-saturated ellagic acid (curve 2 minus curve 4) compared with the spectrum of copper-saturated ellagic acid multiplied by 0.4. The peak absorbance is identical, but the saturated Cu^{2+}–ellagic acid spectrum is slightly red shifted. This indicates heterogeneity of the insoluble species, since that formed at less than saturation has a slightly different spectral property.

On the basis of these results, a titration of ellagic acid with Cu^{2+} was performed by using two different methods of measurement. The absorbance change at 360 nm after Cu^{2+} addition and incubation for 1 h was measured, using the difference in extinction at that wavelength as a measure of complex formation (curves 1 and 3 of Figure 5A). The suspensions were centrifuged, and residual soluble ellagic acid was measured by the absorbance at 360 nm remaining in the supernatants, which is a quantitative measure of that species. As shown in Figure 6, both measurements could be plotted on the same curve and indicated very tight binding with saturation at an approximate ratio of 0.85 Cu^{2+} per ellagic acid molecule. At any level of saturation, the 360-nm spectral change, monitoring the formation of Cu^{2+}–ellagic acid complexes, and fraction of insoluble material formed were proportional.

Having demonstrated a positive correlation between absorbance spectral properties and the formation of insoluble aggregates, we correlated these with procoagulant activity. Figure 7A shows representative spectra from a titration of ellagic acid with copper. An apparently good isosbestic point was observed at 340 nm, while in the 280-nm region the spectra deviate from isosbestic behavior. Similar small deviations from isosbestic behavior were observed in spectrophotometric titrations of ellagic acid with Co^{2+} and Zn^{2+} as well as Cu^{2+}. This further supports the results of Figure 5B indicating multiple forms of aggregated Cu^{2+}–ellagic acid complexes with slightly different spectral properties. The

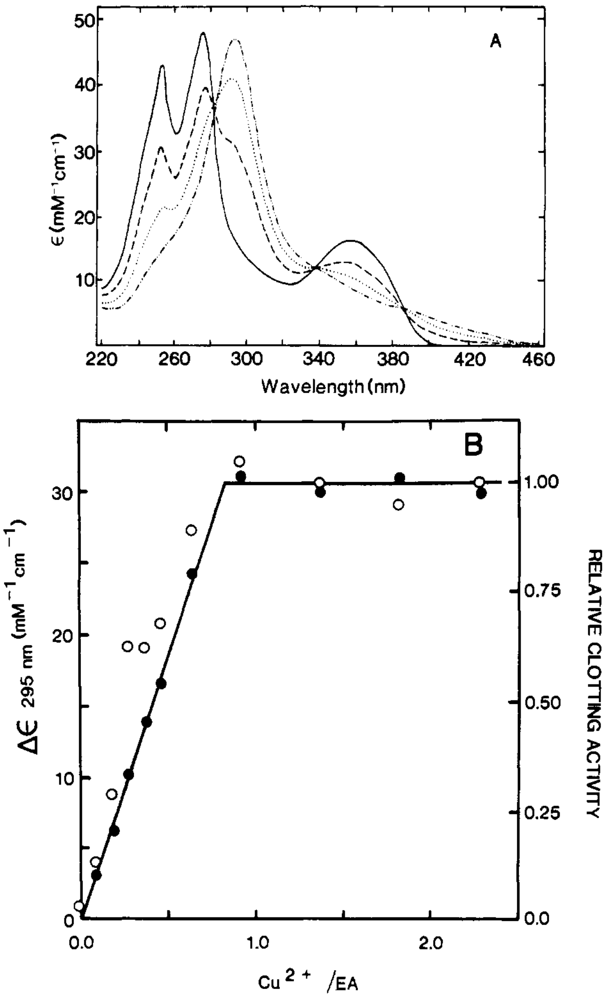

FIGURE 7: Correlation of Cu^{2+}-induced ellagic acid spectral changes with increased clotting activity. (A) representative spectra from a titration of 2.4 μM ellagic acid with $CuCl_2$. Absorbance spectra are shown for ellagic acid (—), 0.37 Cu^{2+}/ellagic acid (---), 0.64 Cu^{2+}/ellagic acid (···), and 1.8 Cu^{2+}/ellagic acid (-··-) recorded 1 h after addition of Cu^{2+}. (B) Extinction change at 295 nm (●) plotted as a function of the Cu^{2+}/ellagic acid ratio along with the relative clotting activity of the same solutions (○) determined as described under Experimental Procedures at a concentration of 0.1 μM in the assay.

suspensions used for the spectra in Figure 7A, and many more obtained at intermediate Cu^{2+} to ellagic acid ratios, were assayed for procoagulant activity. Figure 7B shows the plot of increased extinction at 295 nm and the increased relative clotting activity as a function of the Cu^{2+} to ellagic acid ratio. The results demonstrate a good correlation between spectral changes measuring the formation of insoluble aggregates and the 40-fold increase in ability to initiate clotting. Saturation was observed at a ratio of 0.85 Cu^{2+} per ellagic acid in both clotting activity and spectral change. As a consequence of the nature of this interaction, the apparent stoichiometry obtained in these experiments may be dependent on the ellagic acid concentration. An average value of 0.79 Cu^{2+} per ellagic acid was obtained from three experiments at ellagic acid concentrations between 2.0 and 2.4 μM.

The concentration dependence of clotting activity for Cu^{2+}–ellagic acid suspensions in normal plasma was similar to that obtained with diluted suspensions of kaolin. However, on a weight basis, Cu^{2+}–ellagic acid suspensions were approximately 300 times as effective as kaolin. Equivalent concentrations of Cu^{2+} alone had no measurable effect on the clotting assays. The ability of ellagic acid diluted in the absence of EDTA, Cu^{2+}–ellagic acid, and kaolin to initiate

Table II: Clotting Activity of Ellagic Acid Suspensions in Normal and Contact Factor Deficient Human Plasmas[a]

	clotting time (PTT, s)			
plasma	kaolin (0.02 mg/mL)	ellagic acid (0.2 μM)	Cu^{2+}-ellagic acid (0.2 μM)	buffer
normal	80	98	81	320
Hageman	>600	>600	>600	>600
Fitzgerald	550	>600	472	>600
factor XI deficient	507	597	552	>600
Fletcher[b]	415	489	534	591
normal[b]	155	175	173	346

[a] Clotting activity in normal, Hageman (factor XII deficient), Fitzgerald (high molecular weight kininogen deficient), and factor XI deficient plasma was determined as described under Experimental Procedures with an 8-min incubation period. Ellagic acid, 12.0 μM, was diluted to 0.5 μM in 0.02 M Tris-HCl-0.15 M NaCl, pH 7.4, and incubated at 22 °C for 1 h before addition to the assay. Cu^{2+}-ellagic acid was prepared by incubation of 12.0 μM ellagic acid with an equal concentration of $CuCl_2$ for 1 h followed by dilution to 0.5 μM. [b] Assays with Fletcher (prekallikrein deficient) plasma were incubated for 1 min before addition of calcium and are compared with assays in which normal plasma was incubated for the same length of time.

clotting in normal plasma was compared with their effect on plasmas deficient in each of the known contact factors. The results of these clotting assays, presented in Table II, show that each of the four proteins of the contact activation system is required for a normal rate of clotting when induced by either ellagic acid–metal ion complexes or kaolin.

Using the generation of plasma kallikrein activity as a sensitive measure of biological activity, we studied various ellagic acid–metal ion complexes in this system. Figure 8 shows that in all cases these complexes could initiate kallikrein production and that centrifugation removes the activating species. Saturating levels of Cu^{2+}, Zn^{2+}, and Co^{2+} were used in this experiment. The results shown for Fe^{3+} were obtained with a concentration equal to the ellagic acid concentration since higher levels of this metal ion resulted in less activity. Addition of equivalent concentrations of the metal ions alone did not result in generation of activity (not shown). Similar rates of prekallikrein activation to those shown in Figures 2 and 8 were obtained in normal and factor XI deficient plasmas with 0.1 μM ellagic acid diluted in the absence of EDTA or Cu^{2+}–ellagic acid. Negligible kallikrein generation occurred over the same time period in plasmas deficient in factor XII, high molecular weight kininogen, or prekallikrein (not shown).

Discussion

Blood coagulation can be activated by a variety of insoluble substances with negatively charged surfaces. However, the requirement for an insoluble surface is apparently not absolute, since certain soluble anionic polymers are active (Moskowitz et al., 1970; Kluft, 1978; Schwartz & Kellermeyer, 1969). When the nature of these substances is considered, the proposal of Ratnoff & Crum (1964) that the same system of plasma proteins could also be activated in solution by low concentrations of soluble ellagic acid was of potential importance. Studies of the biological effects of this compound showed that factor XII activation was required for the generation of procoagulant activity, and several biological activities dependent on the contact activation system were elaborated (Ratnoff & Crum, 1964; Saito et al., 1974; Colman et al., 1969). We estimated the solubility of ellagic acid in buffer solutions since it appeared possible that small amounts of undissolved ellagic acid could be responsible for these effects. Our solubility estimate of 30 ± 10 μM suggests that experiments involving unstable solutions of more concentrated ellagic acid may not be clearly ascribed to solution properties of this compound. However, the factor XII dependent procoagulant effects of ellagic acid at concentrations well below its solubility, as originally reported (Ratnoff & Crum, 1964) and studied in a system of purified contact activation proteins (Saito, 1977), were confirmed by this laboratory (Bock et al., 1979).

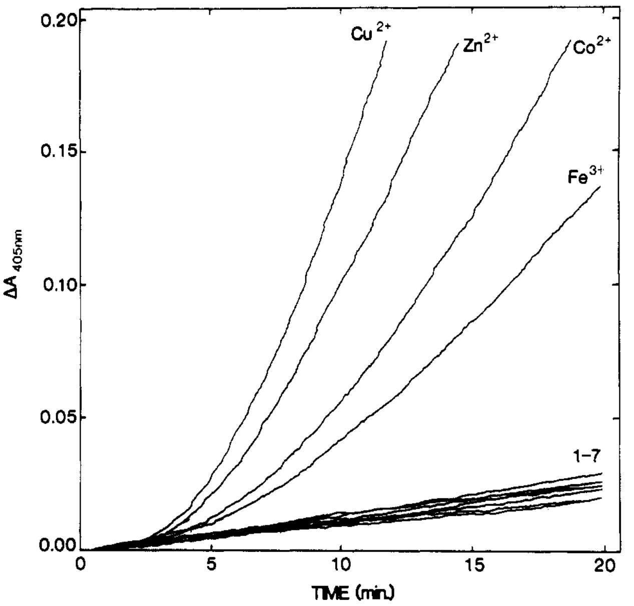

FIGURE 8: Activation of prekallikrein in normal plasma induced by ellagic acid–metal ion complexes. Ellagic acid (20 μM) was incubated for 1 h with an equal concentration of $FeCl_3$ or a 5-fold excess of $CuCl_2$, $ZnSO_4$, or $CoSO_4$. Reactions were initiated by addition of aliquots of these suspensions at a final concentration of 0.1 μM to the assay mixture described under Experimental Procedures. Curves 1–7 show results obtained with the supernatants from centrifugation of these solutions, the stock ellagic acid solution without added metal ions, the supernatant from centrifugation of the stock solution, and the control without ellagic acid.

In more detailed studies of ellagic acid induced blood coagulation, we found that the procoagulant and prekallikrein activating effects of this compound increased on dilution, and this process could be prevented but not reversed by EDTA. Surprisingly, the substance responsible for the activity of these dilute solutions could be completely removed by centrifugation. Correlation of the biological activity of diluted ellagic acid solutions with changes in its absorbance spectrum and solubility, and the effect of EDTA on these properties, indicates that the procoagulant species are insoluble complexes formed between ellagic acid and trace metal ions. The dependence of activity on dilution can therefore be explained as the readdition of trace metal ions present in the buffers used to dilute ellagic acid stock solutions which have been depleted of insoluble complexes by Millipore filtration. The most likely source of these trace metals is the 0.15 M NaCl used to approximate physiological ionic strength. This conclusion contradicts a previous suggestion that contact activation could be initiated by soluble ellagic acid (Ratnoff & Crum, 1964). Furthermore, solutions of ellagic acid prepared in the presence of EDTA which do not contain insoluble material and obey Beer's law (Figure 4) have no procoagulant activity in normal human plasma at concentrations between 1 nM and 10 μM. These results suggest that the activity of other low molecular weight compounds reported to act in solution (Ratnoff & Crum, 1964; Becker & Dubin, 1977) should be reevaluated.

In view of these observations, it is important to reevaluate the physical studies of the interaction of factor XII with ellagic acid. Studies of the circular dichroism spectrum of factor XII (McMillin et al., 1974), sedimentation of factor XII in a sucrose gradient (Donaldson & Ratnoff, 1965), and protein fluorescence quenching in the presence of ellagic acid (Fair et al., 1977) have been cited as evidence for an ellagic acid induced conformational change in factor XII essential to its activation. Ellagic acid solutions were reported by Donaldson & Ratnoff (1965) to greatly increase the sedimentation rate of factor XII in a sucrose gradient, an observation which may involve the low solubility of ellagic acid at the temperature of their experiment or interaction with the insoluble complexes characterized in this study. The absorbance spectrum of soluble ellagic acid (Figure 3) indicates that a substantial portion of the quenching of factor XII protein fluorescence reported by Fair et al. (1977) may be explained by the action of ellagic acid as a potent fluorescence excitation and emission inner filter. After correcting for inner filter effects, we observed a small true quenching of the protein fluorescence which was apparently correlated with the procoagulant activity of the ellagic acid solutions (Bock et al., 1979). However, in light of our present results and further studies of this effect, it is not clear which form of ellagic acid is responsible for the fluorescence change. Although soluble ellagic acid may bind to factor XII and quench the protein fluorescence, our results indicate that this interaction is not responsible for contact activation. Additionally, this interaction is apparently not specific since bovine serum albumin and other proteins (unpublished results) exhibit similar behavior. Finally, the spectral overlap between the fluorescence emission of factor XII and ellagic acid absorbance demonstrates the possibility of protein tryptophan fluorescence quenching by a resonance energy transfer mechanism which precludes the unambiguous assignment of this effect to a protein conformational change. Therefore, studies of the interaction of factor XII with ellagic acid performed thus far cannot be unequivocally interpreted in terms of an ellagic acid induced conformational change associated with procoagulant activity.

The reaction of Cu^{2+} with soluble ellagic acid has been studied as a model system. We have shown that Cu^{2+} binds tightly to ellagic acid, forming highly insoluble complexes concomitant with characteristic changes in the ellagic acid absorbance spectrum and the development of potent procoagulant activity. These complexes have spectral properties similar to those of the active species of ellagic acid formed in dilute buffer solution. Although the complexes are insoluble, only slowly settling suspensions are formed under our conditions, and the large extinction coefficients permit study of their absorbance spectra without substantial contributions from light scattering. The formation of insoluble aggregates suggests that the stoichiometries and extinction changes accompanying complex formation may be dependent on ellagic acid concentration, and we have therefore confined our spectral studies to a narrow concentration range. Analysis of the spectral changes produced by Cu^{2+} indicates that a heterogeneous mixture of insoluble complexes with slightly different absorbance characteristics is formed. The absorbance spectra are dependent on the specific metal ion incorporated, although the origin of these spectral changes is not fully understood. With our present results, we cannot rule out the possible participation of ionization, lactone hydrolysis, or oxidation of ellagic acid in the formation of the active complexes or their spectral properties.

It is likely that the aggregated ellagic acid–metal ion complexes interact with factor XII and other contact activation proteins to exert their biological effect. Figure 8 demonstrates that prekallikrein activation is not dependent on a specific metal ion, since qualitatively similar effects were produced by different metal cations. The quantitative differences in prekallikrein activation between various metal complexes are probably due to differences in the size and shape of the aggregates formed and are beyond the scope of our current investigation. Spectral properties of the insoluble ellagic acid–Cu^{2+} chelates, and their high procoagulant and kallikrein generating activities, indicate that they may enable a more quantitative approach to understanding the activation phenomenon.

It is interesting that studies involving ellagic acid are primarily responsible for the proposal that initiation of contact activation may occur through nonproteolytic activation of the human factor XII zymogen (Saito, 1977; Ratnoff & Saito, 1979a,b). In contrast, studies involving kaolin, particulate ellagic acid, or similar substances have not provided support for this mechanism (Griffin & Beretta, 1978; Meier et al., 1977). On the other hand, Kurachi et al. (1980) observed the same rate of factor XI activation by bovine factor XII or factor XII_a in the presence of dextran sulfate or sulfatide and concluded that the zymogen and proteolytically activated enzyme had equivalent catalytic activity toward factor XI. Greengard & Griffin (1980) found a much slower rate of factor XI activation by the factor XII zymogen compared with factor XII_a in the presence of ellagic acid and high molecular weight kininogen. Our experiments do not address this question directly, but they indicate that ellagic acid cannot be distinguished from other activating substances based on its solubility. It appears doubtful that activation of the contact system can occur in solution by low molecular weight compounds, although we cannot exclude such a possibility for compounds other than ellagic acid. Another possibility which cannot be excluded is that activation of factor XII by insoluble ellagic acid–metal ion complexes and by other substances occurs by different mechanisms or reflects different aspects of the same process.

In conclusion, although ellagic acid is soluble in buffer solutions at concentrations less than 30 μM, homogeneous solutions of this compound in the presence of EDTA have no procoagulant or kallikrein generating activity. Insoluble ellagic acid–metal ion complexes formed in buffers containing adventitious metal ions or by the deliberate addition of a variety of metal cations are biologically active and have similar absorbance spectral properties. These insoluble complexes account for the contact activity of ellagic acid solutions.

Acknowledgments

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Cytochrome P-450 Inactivation: Structure of the Prosthetic Heme Adduct with Propyne[†]

Paul R. Ortiz de Montellano*,[‡] and Kent L. Kunze

ABSTRACT: Hepatic microsomal cytochrome P-450 from phenobarbital-pretreated rats is destroyed by propyne in a reduced nicotinamide adenine dinucleotide dependent process which also results in vivo in the accumulation of an abnormal green porphyrin. The green porphyrin has been identified by its electronic absorption, mass spectrometric, and nuclear magnetic resonance properties as the isomer of *N*-(2-oxopropyl)protoporphyrin IX in which the alkylated nitrogen is that of pyrrole ring A. Alkylation of the other nitrogens in the parent heme is quantitatively unimportant, although evidence for traces of the resulting adducts has been obtained. The green porphyrin exhibits a circular dichroism spectrum and is therefore the result of a chirally selective or specific interaction. The structure of the green porphyrin implicates a cytochrome P-450 destructive mechanism in which a species formed by catalytic oxidation of the acetylenic moiety reacts with the nitrogens of prosthetic heme. The possible nature of the reactive intermediate is discussed.

Hepatic microsomal cytochrome P-450 is destroyed during catalytic processing of olefins (De Matteis, 1971, 1978; Levin et al., 1972; Ortiz de Montellano & Mico, 1980), acetylenes (White & Müller-Eberhard, 1977; Ortiz de Montellano & Kunze, 1980a), and allenes (Ortiz de Montellano & Kunze, 1980b). Suicidal π-bond metabolism by the cytochrome P-450 enzyme appears to underlie the destructive process, although its suicidal nature has only been explicitly documented in the case of 2-isopropyl-4-pentenamide (Ortiz de Montellano & Mico, 1981). The destruction of the enzyme by monosubstituted olefins and acetylenes is paralleled by an approximately equimolar decrease in microsomal heme content (De Matteis, 1971; Bradshaw et al., 1978; White, 1978) and by the concurrent appearance of abnormal hepatic green porphyrins (De Matteis, 1978; White & Müller-Eberhard, 1977; Ortiz de Montellano & Kunze, 1980a; Ortiz de Montellano & Mico, 1980). Analogous green porphyrins, however, have not been observed to accompany destruction of the enzyme by disubstituted olefins and acetylenes or by allenes (Ortiz de Montellano & Kunze, 1980a,b; Ortiz de Montellano & Mico, 1980). We have isolated the green porphyrins obtained with a number of unsaturated agents and have shown that their mass spectrometric molecular ions correspond to the stoichiometric sum of the molecular weights of protoporphyrin IX (as the dimethyl ester due to the isolation procedure) plus the administered agent plus an oxygen atom (Ortiz de Montellano et al., 1979, 1980; Ortiz de Montellano & Kunze, 1980a). These results have led us to postulate that terminal olefins (Ortiz de Montellano et al., 1979, 1981a,b) and acetylenes (Ortiz de Montellano & Kunze, 1980a) are oxidized by cytochrome P-450 to transient intermediates that irreversibly alkylate the prosthetic heme of the enzyme. The validity of this proposal has remained uncertain, however, and the details of the interaction have remained experimentally inaccessible, due to the absence of precise structural information on the isolated green porphyrins. We have recently, as the result of a major effort to obtain such structural information, unambiguously identified the ethylene-derived green porphyrin as one of the four possible regioisomers of *N*-(2-hydroxyethyl)protoporphyrin IX (Ortiz de Montellano et al., 1980, 1981a,b). Similar efforts to determine the structure of the porphyrin isolated from acetylene-treated rats have been

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