0.5 percent aqueous Na_2CO_3) showed that more than 90 percent of the radioactivity extracted chromatographed as methotrexate.
8. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
9. For examples of the use of L1210 in drug screening programs, together with a discussion of the method of calculation of drug-promoted survival of host animals, see A. Goldin, J. Vendetti, S. Humphreys, N. Mantel, *J. Nat. Cancer Inst.* **17**, 203 (1956); **21**, 496 (1958).
10. D. J. Hutchison, *Advanc. Cancer Res.* **7**, 130 (1963).
11. G. Fischer, *Biochem. Pharmacol.* **7**, 75 (1961); M. T. Hakala, S. F. Zakrzewski, and C. A. Nichol, *J. Biol. Chem.* **236**, 952 (1961); M. Friedkin, E. Crawford, S. R. Humphreys and A. Goldin, *Cancer Res.* **22**, 600 (1962); D. K. Misra, S. R. Humphreys, M. Friedkin, A. Goldin, E. J. Crawford, *Nature* **189**, 39 (1961).
12. G. A. Fischer, *Cancer Res.* **19**, 372 (1959).
13. ———, *Biochem. Pharmacol.* **11**, 1233 (1962).
14. W. C. Werkheiser, L. W. Law, R. A. Roosa, C. A. Nichol, *Proc. Amer. Ass. Cancer Res.* **4**, 71 (1963).
15. D. Kessel and T. C. Hall, *Proc. Amer. Fed. Clin. Res.* **13**, 338 (1965).
16. We thank Miss Leanne Nigrosh for technical assistance. Supported by Cancer Chemotherapy National Service Center contracts PH 43-62-169 and PH 43-6561 and by grant C6516 of NIH.

20 August 1965

Hageman Factor: Alterations in Physical Properties during Activation

Abstract. *Highly purified preparations of Hageman factor, a potent clot-promoting agent in normal mammalian plasma, had a sedimentation coefficient of approximately 5S before activation. After activation, the Hageman factor behaved as a much less soluble or larger molecule during ultracentrifugation and gel filtration. No significant change in sedimentation behavior was noted when the Hageman factor in plasma deficient in plasma thromboplastin antecedent was activated. The altered sedimentation behavior of purified activated Hageman factor probably reflects its decreased solubility in aqueous media.*

Blood readily clots in vitro upon contact with certain adsorbent surfaces (*1*). Generation of clot-promoting activity by surfaces depends partly upon the activation of Hageman factor, a plasma protein deficient in persons with Hageman trait (*2*). Once activated, Hageman factor behaves as an enzyme and initiates a series of enzymatic reactions culminating in the generation of thrombin and the formation of fibrin (*3*).

The experiments we report here demonstrate that highly purified Hageman factor undergoes a change in its physical properties during activation. Hageman factor had a sedimentation coefficient of approximately 5*S* before activation. In contrast, activated Hageman factor was associated either with a macromolecule or with an insoluble substance.

Hageman factor was purified 3000- to 5000-fold with respect to human serum (*4*). Although this preparation was originally thought to be activated Hageman factor (*4*), it has been shown to contain only 5 to 10 percent of activated material (*5*). The term "Hageman factor" refers to the precursor, or unactivated form, whereas "activated Hageman factor" refers to the form which has enzymatic activity and accelerates clotting.

The preparation of purified Hageman factor was dissolved and applied to linear sucrose density-gradient mixtures (5 to 20 percent) and ultracentrifuged (*6*). The Hageman factor and sucrose were each dissolved in 0.05*M* tris buffer, *p*H 7.4. After centrifugation in a Spinco model L-2 ultracentrifuge at 100,000 or 105,000*g* for 24 hours at 0°C in an SW-39 head, the gradient mixtures were fractionated. Usually, successive 0.2-ml portions were removed from the upper surfaces of the gradient mixtures, and added to 0.5 ml of buffered 1 percent bovine serum albumin. These diluted fractions were then tested for their Hageman factor content (*4, 5*). Hageman factor isolated from gradient fractions shortened the abnormally long recalcified clotting time of plasma from a person known to be deficient in Hageman factor. This assay was performed in the presence of kaolin (a substance which activates Hageman factor) and a crude phospholipid ("cephalin") (*7*). Activated Hageman factor in fractions shortened the recalcification time of the same plasma in the presence of "cephalin" alone in silicone-coated test tubes. Plasma was prepared in silicone-coated apparatus.

Table 1. Clot-promoting action of purified Hageman factor upon Hageman plasma deficient in Hageman factor. A mixture of 0.1 ml of test material, 0.1 ml of "cephalin" (in barbital-saline buffer, 0.06 mg/ml, *p*H 7.4). and 0.1 ml of plasma deficient in Hageman factor was incubated in silicone-coated Pyrex tubes (10 by 75 mm) for 8 minutes. Then 0.1 ml of 0.025*M* calcium chloride was added, mixed, and the time required for visible fibrin formation at 37°C was measured.

Hageman factor	Clotting time (sec)
Untreated	>400
Ellagic acid-activated	79
Barium carbonate-activated	70

After purified Hageman factor was subjected to sucrose gradient ultracentrifugation, this activity was located in fractions containing proteins with sedimentation coefficients of 4.5 to 5.5*S* (Fig. 1).

The same preparation of Hageman factor was activated by solutions of ellagic acid or by barium carbonate (Table 1), and subjected to ultracentrifugation in the same way as Hageman factor. Ellagic acid (4,4′,5,5′6,6′-hexahydroxydiphenic acid 2,6:2′,6′-dilactone), dissolved in concentrations as low as $10^{-8}M$, activated Hageman factor by an unknown mechanism (*5*).

The ellagic acid was synthesized (*8*) by the method of Perkin and Nierenstein (*9*) by oxidative coupling of gallic acid (*10*). It was dissolved at a concentration of $2 \times 10^{-4}M$ in tris buffer and carefully centrifuged to remove any insoluble material before mixing it with Hageman factor. After exposure to Hageman-factor solutions, barium carbonate was removed by dialysis after its conversion to soluble acetate by the addition of equivalent amounts of acetic acid.

Once activated by either technique, coagulant properties attributable to activated Hageman factor resided in density-gradient fractions containing the heaviest or least soluble materials (Fig. 1). In other experiments, Hageman factor activated by ellagic acid was centrifuged at 100,000*g* for only 100 minutes, or 31,000*g* for 120 minutes. Here too, the activated Hageman factor was located principally in fractions at the bottom of the gradient mixture. Activated Hageman factor was also separated from Hageman factor during column chromatography by Sephadex G-200 gel filtration. During elution with buffer composed of 0.025*M* barbital in 0.0125*M* saline at *p*H 7.4, activated Hageman factor was found in fractions known to contain the heaviest materials. In contrast, Hageman factor was in effluent fractions known to contain 4.5*S* materials.

The preparations of purified Hageman factor tested invariably contained a small proportion of activated en-

zyme (*4*, *5*). Without further treatment before ultracentrifugation, the activated Hageman factor in these preparations was also located in the heaviest gradient fractions (Fig. 1). Conversely, activation with ellagic acid was usually incomplete, and residual Hageman factor was located in gradient fractions containing material with sedimentation coefficients of 4.5 to 5.5*S*. Similar results were obtained when sucrose gradients were fractionated from the bottom, as suggested by Martin and Ames (*6*).

These experiments appeared to demonstrate that the activation of purified Hageman factor altered it from a substance with a sedimentation coefficient of about 5*S* to one with the properties of a heavy macromolecule or of a substance poorly soluble in water. Although this question was unresolved, the following observations seem more consistent with the possibility that the activated Hageman factor is relatively insoluble in aqueous media.

Human plasma, deficient in plasma thromboplastin antecedent (PTA) and prepared in silicone-coated apparatus and subjected to ultracentrifugation by the technique described. This plasma contained native Hageman factor. It was activated with $10^{-5}M$ ellagic acid and then ultracentrifuged. Both unactivated and activated Hageman factors were found principally in fractions containing material with sedimentation constants between 5.0 and 7.0*S*. Presumably, then, the rapid sedimentation of activated Hageman factor did not occur in plasma, as a macromolecule would.

A purified Hageman factor preparation was mixed with plasma deficient in this activity, and then activated with ellagic acid. After ultracentrifugation, most of this activated material was in fractions containing 5.5*S* substances. When the same preparation was activated before being mixed with plasma deficient in Hageman factor, most of the activated Hageman factor sedimented to the bottom of the gradient mixture during ultracentrifugation. When ellagic acid-activated Hageman factor was ultracentrifuged in 5 to 20 percent sucrose density gradients containing either 5 percent bovine serum albumin (Crystalline, Pentex, Kankakee, Ill.) or 0.5*M* NaCl, it again sedimented to the bottom of these gradients. Once sedimented, activated Hageman factor could not be dissolved in 5 percent bovine serum albumin, 0.5*M* NaCl, or 5*M* urea. The Hageman factor remaining unactivated by ellagic acid sedimented with 4.5*S* materials in gradients with added albumin or 0.5*M* NaCl. In addition, during ultracentrifugation at 100,000*g* in 10 to 40 percent sucrose density gradients for only 100 minutes, activated Hageman factor again sedimented to the bottom of the gradient.

Since, during clotting, Hageman factor reacts enzymatically with PTA to form activated PTA (*11*, *12*), it was of interest to test the sedimentability of both forms of PTA. Crude PTA was prepared from plasma deficient in Hageman factor (*12*) and

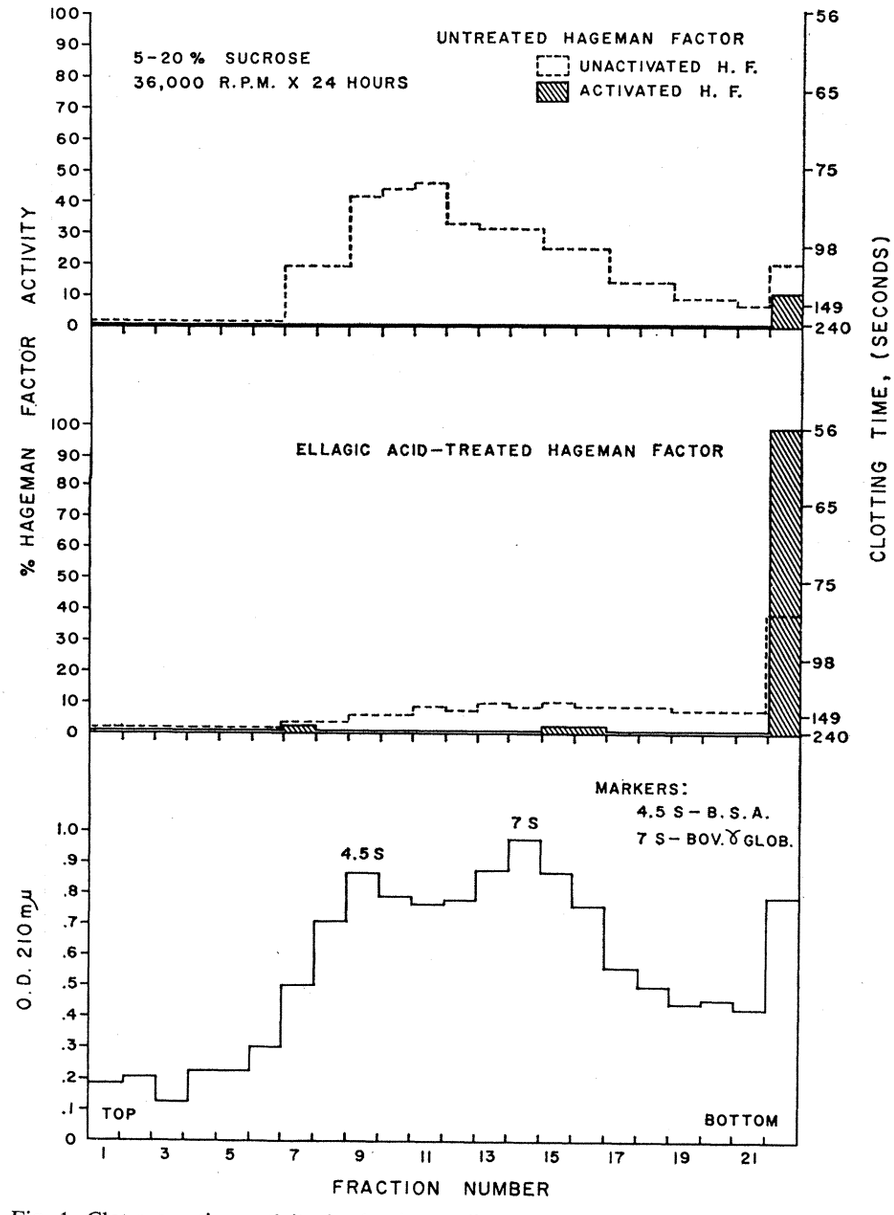

Fig. 1. Clot-promoting activity in density-gradient fractions after ultracentrifugation at 105,000*g* (36,000 rev/min) for 24 hours: (Top) Hageman factor dissolved in tris buffer before ultracentrifugation. (Center) Hageman factor dissolved in $10^{-4}M$ ellagic acid in tris buffer before ultracentrifugation. The quantity of activated Hageman factor or that which was not activated before assay is expressed as a percentage of the maximum activity in fraction number 22 of the material treated with ellagic acid (center panel). Corresponding clotting times (logarithmic) are indicated on the right ordinates. The activity attributable to unactivated Hageman factor in the bottom fraction of the center panel is less than that due to already activated material. This loss of activity occurred during the delay in performing the assays for activated material. Relative concentrations of proteins measured at 210 $m\mu$ in a Beckman DU spectrophotometer are shown in the bottom panel. A mixture of equal volumes of 1 percent bovine serum albumin (4.5*S*) and 1 percent bovine γ-globulin (7*S*) in tris buffer was applied to the same sucrose gradient used to fractionate test solutions.

then activated by ellagic acid–activated Hageman factor. After ultracentrifugation, the bulk of both PTA and activated PTA (*13*) was located in gradient fractions containing substances with sedimentation coefficients between 5 and 6*S*. Thus, the physical properties of the crude PTA had not changed appreciably during its activation.

The formation of a dimer as a requirement for enzymatic activity has been reported in the case of phosphorylase B (*14*). If the change in the physical properties of Hageman factor after activation also represented polymerization of the inactive protein, an active polymer must contain many monomeric units. Activated Hageman factor behaved as if it were larger than a 19*S* porcine thyroglobulin during gel filtration. Appreciable amounts of activated Hageman factor were readily sedimented during centrifugation at only 31,000*g*. The additional fact that 2*M* urea and 0.5*M* NaCl failed to impair activation of Hageman factor with ellagic acid militates against the hypothesis that polymerization is important in activation.

Vroman (*15*) has postulated that during the generation of clot-promoting activity coagulant proteins may be altered so that hydrophobic sites are exposed. He suggested that Hageman factor becomes hydrophobic during activation and that the exposed hydrophobic areas are attracted to similar portions of the PTA molecule. The complex formed might then enhance the development of clot-promoting activity in blood. Our experiments support this possibility but do not define the mechanism of altered physical behavior of activated Hageman factor. Even though Hageman factor in plasma did not demonstrate this change after activation, the data regarding purified material are relevant to the phenomenon of activation once Hageman factor has been extracted from its normal colloidal environment.

Botti (*10*) has defined an experimental "hypercoagulable" state effected by intravenous injection of ellagic acid. Intravascular fibrin formation occurred only where stasis was induced by ligation of a vessel. Presumably, Hageman factor activation is crucial to the induction of this experimental thrombus. These experiments support the possibility that clearance of clot-promoting activity by the reticuloendothelial system may be important in protection against thrombosis, as suggested by Spaet and his associates (*16*). Perhaps activated Hageman factor in vivo can adhere to endothelial surfaces and then be cleared from the vascular compartment by the reticuloendothelial system. Such a clearance mechanism might protect an organism against the clot-promoting action of activated Hageman factor.

VIRGINIA H. DONALDSON*
OSCAR D. RATNOFF†
St. Vincent Charity Hospital, University Hospitals of Cleveland, and *Department of Medicine, Western Reserve University, Cleveland, Ohio*

References and Notes

1. J. Lister, *Proc. Roy. Soc. Med.* **12**, 580 (1862–63).
2. O. D. Ratnoff and J. Colopy, *J. Clin. Invest.* **34**, 602 (1955); O. D. Ratnoff and J. Rosenblum, *Am. J. Med.* **25**, 160 (1958); E. Shafrir and A. de Vries, *J. Clin. Invest.* **35**, 1183 (1956); J. Margolis, *Nature* **178**, 805 (1956).
3. R. G. Macfarlane, *Nature* **202**, 498 (1964); E. W. Davie and O. D. Ratnoff, *Science* **145**, 1310 (1964).
4. O. D. Ratnoff and E. W. Davie, *Biochemistry* **1**, 967 (1962).
5. O. D. Ratnoff and J. Crum, *J. Lab. Clin. Med.* **63**, 359 (1964).
6. R. G. Martin and B. N. Ames, *J. Biol. Chem.* **236**, 1372 (1961).
7. W. N. Bell and H. G. Alton, *Nature* **174**, 880 (1954).
8. Dr. J. Crum synthesized the ellagic acid.
9. G. Perkin and M. Nierenstein, *J. Chem. Soc.* **87**, 1412 (1905).
10. R. E. Botti and O. D. Ratnoff, *J. Lab. Clin. Med.* **64**, 385 (1964).
11. B. A. Waaler, *Scand. J. Clin. Lab. Invest.* **11**, *Suppl.* 37, 1 (1959); J. Margolis, *J. Physiol.* **144**, 1 (1958); R. M. Hardisty and J. Margolis, *Brit. J. Haematol.* **5**, 203 (1959); J. P. Soulier, O. Wartelle, D. Menache, *Rev. Franc. Etudes Clin. Biol.* **3**, 263 (1958); O. D. Ratnoff, *Thromb. Diath. Haemorrhag.* **4**, *Suppl.* 166 (1960).
12. O. D. Ratnoff, E. W. Davie, D. L. Mallett, *J. Clin. Invest.* **40**, 803 (1961).
13. J. Margolis, *J. Clin. Path.* **11**, 406 (1958); S. I. Rapaport, *et al., J. Lab. Clin. Med.* **57**, 771 (1961).
14. A. B. Kent, E. G. Krebs, E. H. Fischer, *J. Biol. Chem.* **232**, 549 (1958).
15. L. Vroman, *Thromb. Diath. Haemorrhag.* **10**, 455 (1964).
16. T. Spaet, H. Horowitz, D. Zucker-Franklin, J. Cintron, J. Biezenski, *Blood* **17**, 196 (1961).
17. Supported by grants from U.S. Public Health Service (HE. 08865-01 and HE-01661C), American Heart Association, Cleveland Area Heart Society, and Henry County and Fulton County Branches of the Ohio State Heart Association.
* Established investigator of the American Heart Association.
† Career investigator of the American Heart Association.

1 September 1965

Selective Mitochondrial Damage by a Ruby Laser Microbeam: An Electron Microscopic Study

Abstract. *A pulsed ruby laser microbeam produces changes in single living cells stained with a low concentration of Janus green B. Electron micrographs show that such alterations are the result of morphological damage sustained by and restricted to the mitochondria in the irradiated area.*

Mitochondria have been selectively damaged in single KB cells by a focused ruby laser microbeam. The cells had been vitally stained prior to irradiation with Janus green B, well known for its mitochondrial specificity. Presumably the stain enhanced the organelle's ability to absorb laser light since unstained cells show no detectable reaction to such radiation. Morphological evidence for the fact that the induced damage is restricted to the mitochondria is based on electron micrographs of irradiated cells. Other cellular constituents in the exposed area show no significant ultrastructural alteration.

A ruby laser integrally mounted on a phase-contrast microscope was used for irradiations (*1*). Its air-cooled, ruby-rod source had an optical pumping system input of 2.6 kv and a rated maximum output of 0.5 joule delivered in 500 μsec. The energy of the beam was controlled by a neutral density filter of graded transmittance interposed between the laser head and the microscope ocular. An observation eyepiece and both television and photographic cameras were incorporated into the system so that viewing and photographing were possible at any time during an experiment. The target area of the cell was accurately located before each irradiation by focusing a beam of ordinary visible light, which passed through the same optical path as the laser light, onto the selected region. Under these conditions, when the laser was energized, a focused beam of coherent light (wavelength λ = 6943 Å) approximately 6 μ in diameter was produced at the predetermined spot.

We used KB tissue-culture cells (*2*) that were growing in a monolayer on formvar-coated glass slips. In order to facilitate the subsequent location and identification of irradiated cells, we cut, with a diamond scribe, a number of circles, 200 μ in diameter, on the up-