THE EFFECT OF PHOSPHORYLATED HESPERIDIN ON THE POLYMERIZATION OF FIBRINOGEN AS STUDIED BY LIGHT SCATTERING*

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In previous publications from this laboratory (1, 2), it was demonstrated that phosphorylated hesperidin acted as a potent anticoagulant when administered intravenously to rabbits. The mode of action of this material appeared to be similar to that of heparin, since only the plasma recalcification times and antithrombin titers showed significant changes, whereas the prothrombin times, antithromboplastin titers, and serum calcium levels were within the normal range. Also the injection of protamine nullified the hypocoagulability of blood produced by phosphorylated hesperidin.

Beiler and Martin (3) have shown that phosphorylated and sulfonated hesperidins and to a lesser degree the acetylated derivative are hyaluronidase inhibitors. Heparin likewise possesses antihyaluronidase activity (4), but this property is less utilized than its anticoagulant action. However, when heparin is desulfated both these properties are destroyed (4). In the presence of blood, the anticoagulant action of heparin is due to its combination with a heparin cofactor to form an antithrombin (5) which blocks the fibrinogen activation step in the blood coagulation mechanism. On the other hand, the anticoagulant property of heparin acting on a fibrinogen-thrombin system in vitro has been attributed to its high negative surface charge (6) which provides sufficient repulsive forces in the polymerizing environment to hinder both the activation and the association of fibrinogen molecules.

It was reasoned that if the anticoagulant and antihyaluronidase properties of heparin were due to electrostatic interaction with neighboring charged particles, then perhaps the same mechanism could account for the anticoagulant properties of the highly charged phosphorylated hesperidin.

In this paper are reported the results of an investigation of the action of phosphorylated hesperidin on the thrombin-induced conversion of fibrinogen studied by the light scattering technique as a function of ionic strength,

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concentration, and time of addition. The absence of a cofactor comparable to that for heparin is demonstrated. The effect of rabbit sera obtained at various times after the injection of phosphorylated hesperidin on the conversion of fibrinogen is also described.

**EXPERIMENTAL**

**Materials**—The ammonium sulfate fractionation technique of Laki (7) was used to purify the Armour Bovine Fraction I. Two preparations were employed in these investigations and have been designated Bovine Fractions I-L9 and I-L10. These fibrinogen solutions were dialyzed against 0.3 M KCl and contained 1.49 and 1.53 mg. of N per ml. respectively. With a nitrogen to protein conversion factor of 5.9 (7), the protein concentrations were 8.8 and 9.0 mg. per ml. respectively. The clottability of the solutions was found to equal approximately 97 per cent. These, therefore, are pure fibrinogen solutions, since Lorand (8) has shown that 3 per cent of the total N appears as non-protein nitrogen during the activation of fibrinogen by thrombin. The thrombin was the Parke, Davis and Company commercial preparation for topical use and contained 22 National Institutes of Health units per mg. A 10 per cent aqueous solution of phosphorylated hesperidin (hereinafter designated PH) for intravenous administration preserved with 0.5 per cent phenol was supplied by The National Drug Company, Philadelphia, Pennsylvania. A sample of an anhydrous sodium salt of heparin, lot No. 3390, containing 117 U. S. P. units per mg., was generously supplied by Dr. K. W. Thompson of Organon, Inc., Orange, New Jersey.

Rabbit blood, obtained either by venipuncture or by cleanly cutting the denuded ear vein, was the source for the sera used in these studies. Blood samples were taken before injection of PH and periodically thereafter at the time intervals noted in the text. For the cofactor studies, specimens of human sera were obtained from the blood of normal subjects.

Phosphate buffers (KH$_2$PO$_4$-K$_2$HPO$_4$), pH 7.4, of varying ionic strength, $\mu$, were prepared according to the data of Cohn (9) and Green (10). Analytical reagent grade materials were used throughout these investigations.

**Methods**—Dissymmetry and turbidity measurements were taken concomitantly on the thrombin-catalyzed polymerizing fibrinogen solutions. The intensities of the scattered light were measured either at $-45^\circ$ and $-135^\circ$ or at $+90^\circ$ and $0^\circ$. The wave-length of the incident light was 436 m.$\mu$. The procedures and methods of calculation of the dissymmetry coefficient, $q$, and turbidity, $I_{90}/I_0$, were those described by Brice, Halwer, and Speiser (11). Depolarization and dissymmetry corrections were not applied to the turbidity values because the systems under investigation were dynamic. Therefore absolute turbidities were not evaluated, but instead
the ratio of light intensities at 90° and 0° is recorded as \( I_{90}/I_0 \). This ratio is proportional to the absolute turbidity (11). The working standard method was employed for the turbidity measurements by means of a working standard comprised of calibrated neutral filters and an opal glass diffuser.

The standard reacting mixture was prepared by adding 1.0 ml. of Bovine Fraction I-L solution to 50 ml. of phosphate buffer (pH 7.4, varying ionic strength) in a semioctagonal scattering cell, followed by the addition of 0.5 ml. of 0.01 per cent thrombin solution. For the series of experiments in which 0.5 ml. of 1 per cent PH was introduced into the reacting mixture after the reaction had started, the control experiment, containing only fibrinogen and thrombin, was stirred at a comparable reaction time. The addition was generally made at an early reaction time when the solution was slightly turbid (\( q \) between 1 and 2.5). The stirring procedure, with a rubber-tipped glass rod, was also kept uniform for all experiments. In the second type of experiment, called the zero time addition, 0.5 ml. of 1 per cent PH or serum was introduced before the addition of thrombin. A stop-watch was started at the moment the thrombin was added to the mixture.

The protein concentrations of the stock fibrinogen solutions were obtained by comparing the optical densities of diluted fibrinogen samples at 280 \( \mu \) with the values obtained for similar preparations whose nitrogen content had been determined by the Dumas procedure. The purity of the fibrinogen solutions was assayed according to the method of Laki (7). The fibrinogen and thrombin solutions were centrifuged for 20 minutes at high speeds in a model SS-1 Servall centrifuge, the former at 5000 r.p.m. and the latter at 10,000 r.p.m. The buffer solutions were clarified by suction filtration through ultrafine sintered glass funnels.

**Apparatus**—The light scattering photometer used was model LS1 manufactured by Process and Instruments, Brooklyn, New York, and essentially follows the design and circuit of Brice, Halwer, and Speiser (11). Detents placed on the rotating calibrated disk at \(-135°\), \(-45°\), 0°, and \(+90°\) provided fixed positions which facilitated the rapid selection of the angles of observation for the dissymmetry and turbidity measurements.

The optical densities were taken with a model DU Beckman quartz spectrophotometer. The pH values for the solutions were determined with a model G Beckman pH meter.

**Results**

**Zero Time Addition of PH to Fibrinogen-Thrombin System As Function of Ionic Strength**—The purpose of this series of experiments was to observe the effect of phosphorylated hesperidin on the conversion of fibrinogen when
it is added before thrombin to buffered fibrinogen solutions at constant pH and varying ionic strength. Phosphate buffers, pH 7.4, at \( \mu = 0.05, 0.15, 0.3, \) and 0.4 were selected as solvents for the purified fibrinogen. The concentrations of reactants were 0.2 mg. of clottable fibrinogen per ml., 96 \( \gamma \) of PH per ml., and 0.02 unit of thrombin per ml. except in the control experiments, which did not contain PH. Dissymmetry and turbidity measurements were taken concomitantly on these solutions as a function of time.

It was found that the zero time addition of PH blocked indefinitely the polymerization of fibrinogen in media of \( \mu = 0.05 \) and 0.15, whereas at \( \mu = 0.3 \) and 0.4 the initiation of the clotting process was markedly slower than in the control experiments. Fig. 1 is a plot of the dissymmetry coefficient, \( q \), and \( I_{90}/I_0 \times 10^4 \) as a function of reaction time, \( t \), for the control and phosphorylated hesperidin mixtures in phosphate buffer, \( \mu = 0.3 \) and pH 7.4. It was observed that approximately 5 minutes elapsed before the growth of the fibrinogen particles in the control experiment could be detected optically. This is considered the induction period, during which time the activation of fibrinogen by thrombin is undoubtedly predominating. The induction period is increased to 48 minutes in the presence of 96 \( \gamma \) of PH per ml. (\( q \) versus \( t \) curve).

Similar results were found at \( \mu = 0.4 \), with the difference that in solvent of this ionic strength the control and PH mixtures polymerized more slowly because of the higher salt concentration.

**Addition of PH at Early Stage of Fibrinogen Conversion (\( \mu = 0.1, \text{pH 7.4} \))**—In the next experiment 0.5 ml. of 1 per cent PH was added to 51.5 ml. of the fibrinogen solution 3 minutes after the addition of thrombin. This particular reaction time, after the induction period, when the polymerization was beginning to progress rapidly, was selected for the addition of PH in order to observe its effect on the association of fibrinogen intermediates. For comparison, the effect on the fibrinogen conversion of the addition, at \( t = 3 \) minutes, of 0.5 ml. of 1 per cent heparin to an identical fibrinogen-thrombin mixture was also investigated. The solutions prepared in phosphate buffer (\( \mu = 0.1, \text{pH 7.4} \)) contained 0.2 mg. of clottable fibrinogen per ml., 0.02 unit of thrombin per ml., and either 96 \( \gamma \) of PH or heparin per ml. The control containing only fibrinogen and thrombin was stirred at \( t = 2.5 \) minutes. The scattering data for these experiments are reported in Fig. 2 in which \( q \) and \( I_{90}/I_0 \times 10^4 \) are plotted against reaction time.

It was found that the polymerization of fibrinogen was arrested after the addition of PH as indicated by the low, constant \( I_{90}/I_0 \) values and also by the dissymmetry coefficients which increased slightly and then leveled off to an average \( q = 3.0 \). A gel network did not appear after 24 hours. On the other hand, the addition of an equal weight of heparin under similar conditions merely retarded the association of fibrinogen intermediates, as
evidenced by the $q$ and $I_{90}/I_0$ values which were significantly lower at the end of the observation period than those of the control experiment. A coarse gel network, which readily condensed upon agitation to form a clot smaller than the control, was also observed.

**Effect of PH-Serum Mixtures on Fibrinogen Conversion**—It has been found\(^1\) that when 0.5 ml. of 1 per cent heparin in human serum is added at zero time to the standard reacting mixture (buffer, $\mu = 0.1$) the reaction was permanently blocked. However, 0.5 ml. of 1 per cent heparin (aqueous) added to a similar fibrinogen-thrombin system at zero time prolonged the induction period, after which the polymerization took place. It was concluded that the heparin-cofactor complex, acting as an antithrombin, blocked the activation and consequently the conversion of fibrinogen, whereas heparin *per se* in this ionic strength merely retarded the initiation of the polymerization. The aim of the next group of experiments was to detect the presence or absence of a similar serum cofactor for phosphorylated hesperidin.

Initially, a concentration of PH was sought which would not block completely the polymerization of fibrinogen. It was found that a concentration of 9.6 $\gamma$ of PH per ml. of reacting solution added at $t = 3$ minutes retarded the conversion in the manner shown in Fig. 3. When added at

\(^1\) Sheppard, E., Imperante, J., and Wright, I. S., in preparation.
zero time, the induction period was prolonged to 14 minutes as compared to the control value of 2 minutes. It was reasoned that if a phosphorylated hesperidin cofactor existed the addition of a mixture of 0.5 ml. of 0.1 per cent PH and 0.5 ml. of serum at zero time to the standard reacting mixture would prevent the polymerization. As is illustrated in Fig. 3, this was not the case, since the reaction proceeded at a rapid rate after a 12 minute induction period.

These experiments were repeated in phosphate buffer, $\mu = 0.3$, with 10 times the concentration of PH in the serum mixture. The reactions followed essentially the pathways of the experiments depicted in Fig. 1, except for a shorter induction period for the PH-serum mixture than for
the comparable PH experiment. This observation, owing presumably to the removal of active material by complex formation of PH with the serum proteins, as well as the fact that the reaction was not blocked, corresponds with the results in Fig. 3.

**Effect of Hesperidinized Rabbit Sera on Conversion of Fibrinogen**—Sera obtained from rabbit blood before and at various times after the injection of 25, 100, and 150 mg. of PH per kilo of body weight were added to the fibrinogen-thrombin mixtures at zero time, and the polymerization of fibrinogen was followed by the light scattering technique. The solutions contained 0.2 mg. of clottable fibrinogen per ml., 0.5 ml. of rabbit serum, and 0.08 unit of thrombin per ml. in phosphate buffer, pH 7.4 and μ = 0.1.

The light scattering findings for a dose of 100 mg. of PH per kilo of body weight are presented in Fig. 4, A and B, which are plots of q and $I_{90}/I_0 \times 10^4$ versus the reaction time, respectively, for the serum-fibrinogen-thrombin mixtures. It will be noted that the sera obtained 20, 60, and 180 minutes after injection prolonged the induction period from a control value.
of 30 seconds to 3 minutes. However, at 4 hours after the injection, the induction period for the hesperidinized serum mixture was 1.5 minutes. Another significant finding was the presence of persistently higher $q$ values for all the hesperidinized serum mixtures than those obtained for the control. However, the $I_{90}/I_0$ versus $t$ curves showed that the lowest degree of conversion occurred for the mixture containing the serum taken 20 minutes after injection, whereas the serum 4 hours after injection did not appreciably affect the degree of conversion.

Similar results were obtained for 150 mg. of PH per kilo. Sera obtained 20 and 60 minutes after the injection of PH produced identical $q$ and $I_{90}/I_0$ versus $t$ curves, having significantly higher $q$ values and lower turbidities than the preinjection control. The induction period was also prolonged from a control value of 1.5 minutes to 8 minutes for the hesperidinized serum mixtures. The sera, however, obtained after the injection of 25 mg. of PH per kilo did not alter the induction period or the degree of the fibrinogen conversion. None the less, curves for $q$ versus $t$ were higher than for the control. These curves were highest for the sera obtained 1 and 2 hours after injection and had almost returned to the control curve for the 6 hour serum mixture.

DISCUSSION

The results of the light scattering study of the action of phosphorylated hesperidin on the conversion of fibrinogen to fibrin demonstrated that this highly charged compound interfered with the polymerization at two different stages. For example, at $\mu = 0.1$ it either prevented the initial activation of fibrinogen by thrombin (addition at $t = 0$) or blocked the association of activated fibrinogen molecules or intermediates (addition at $t = 3$ minutes). It will be recalled that the zero time addition of PH (96 $\gamma$ per ml. of reacting mixture) to the fibrinogen-thrombin system in phosphate buffers at $\mu = 0.05$ and 0.15 inhibited the initiation of the fibrinogen conversion, whereas at higher values (i.e. $\mu = 0.3$ and 0.4) the polymerizations occurred after a prolongation of the induction period. Thus, the ability of PH to interfere with the polymerization by electrostatic interaction is reduced, owing to a decrease in the energy of interaction of the charged particles in the solvents of higher ionic strength. It was also found that the kinetics of the fibrinogen polymerization were affected by the concentration of PH in the reacting mixture. Thus the presence of 96 $\gamma$ of PH per ml. in phosphate buffer of $\mu = 0.1$, $t = 3$ minutes, permanently blocked the further polymerization of growing $x$-mers, whereas 9.6 $\gamma$ of PH per ml. temporarily arrested the reaction.

Phosphorylated hesperidin was found to be more potent on an equal weight basis than heparin in effecting the fibrinogen conversion in vitro. It is apparent from Fig. 2 that the addition of heparin at $t = 3$ minutes
lowered the degree of polymerization, but did not markedly affect the orientation of fibrinogen intermediates. On the other hand, the presence of an equal weight of PH introduced at the same reaction time to the polymerizing fibrinogen solution interrupted the growth of fibrinogen x-mers shortly after the addition.

Evidence that a PH cofactor does not exist was gleaned from the studies in vitro. Studies in vivo also demonstrated its absence, since 150 mg. of PH per kilo given intravenously to a rabbit produced a hypocoagulability of the blood for several hours after the injection. Had a cofactor existed, then blood specimens, drawn as long as the PH remained in the blood, would not clot. This was the case when 50 mg. of heparin per kilo of body weight were injected in a rabbit. Samples of blood taken up to 2 hours after the injection of heparin did not clot within 24 hours, whereas a sample drawn 3 hours after the injection clotted in 18 hours.

The addition of hesperidinized rabbit serum to the fibrinogen-thrombin system at zero time demonstrated the mode of action of PH in vivo. The series of experiments with sera from a rabbit before and after the injection of 100 mg. of PH per kilo (Fig. 4, A and B) showed that q values higher than those of the control were attained and persisted even for the mixture containing serum obtained 6 hours after injection. By this time the induction period, which had been prolonged from 30 seconds for the control to 3 minutes for the hesperidinized serum mixtures, was returning to the control value. These findings illustrate the fact that the orientation of fibrinogen intermediates was more sensitive to the presence of residual PH in the serum than was the inhibition of the fibrinogen activation step. The increase in the dissymmetry coefficient for the mixtures containing the hesperidinized serum indicates that end to end association of polymers predominates and produces, in effect, narrow fibrin strands. The same type of orientation may be achieved by increasing the pH or decreasing the ionic strength (12). An examination of the turbidity curves revealed that the degree of polymerization was smallest for the serum obtained 20 minutes after injection and increased progressively as a function of time after injection. Finally, turbidity values for the 6 hour curve were comparable to those of the control. Actually the gel was more turbid than the control, but this was due to the presence of large asymmetric particles in the network which contributed additional scattering to the system.

The sera obtained from rabbits that had received 25 and 150 mg. of PH per kilo produced similar effects on identical fibrinogen-thrombin mixtures. Sera obtained from the former dose did not prolong the induction period or alter the degree of conversion, but yielded higher q values than did the control, whereas sera obtained 20 and 60 minutes after the latter dose prolonged the induction, reduced the degree of conversion, and affected the orientation of the fibrinogen particles.
The effect of phosphorylated hesperidin on the kinetics and pathway of fibrinogen polymerization was studied by the light scattering technique as a function of concentration, time of addition, and ionic strength. It was found that PH (96 γ per ml. of reacting mixture), added before thrombin, permanently blocked the fibrinogen conversion in phosphate buffers of 0.05 and 0.15 ionic strength, pH 7.4, whereas in buffers of μ = 0.3 and 0.4 the reaction proceeded to the gel stage after a prolongation of the induction period.

At μ = 0.1, the addition of PH to the polymerizing fibrinogen solution at a 3 minute reaction time interrupted the further growth of particles. The presence of a lower concentration of PH (9.6 γ per ml.) in the mixture retarded the initiation of conversion when added at t = 0 and temporarily inhibited the association of fibrinogen intermediates when it was added at t = 3 minutes. These results indicate that the action of PH is due to an electrostatic mechanism and that it interferes with the fibrinogen conversion at two stages (i.e. activation and association of fibrinogen).

In vitro, PH is a more active inhibitor of fibrinogen conversion than is an equal weight of heparin. However, in the presence of serum, heparin combines with its cofactor to form an antithrombin, thereby increasing its inhibitory action. A comparable cofactor for phosphorylated hesperidin was not found in human or rabbit serum.

The addition of sera, obtained from rabbit blood before and at various times after the intravenous administration of varying dosages of PH, to the fibrinogen-thrombin system at zero reaction time affected the induction period and the degree of conversion in direct proportion to the concentration of residual PH in the blood. However, the orientation of the polymerizing particles remained altered, even when these conditions returned to normal.

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