# Studies on the Agglutination of Erythrocytes Treated with Human Plasmin and Some Other Enzymes* (33781)

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Human plasmin, a proteolytic enzyme possessing a specificity similar to trypsin (1), is capable of hydrolyzing a number of plasma proteins (2). Erythrocytes of the various blood groups were treated with highly purified human plasmin (3). In addition, bovine plasmin, thrombin (both human and bovine), α-chymotrypsin, as well as a number of enzymes commonly employed in serological studies, such as, papain, trypsin, ficin, and bromelin were used in concurrent experiments to obtain comparative data. The present report evaluates the immunological significance of the results obtained.

*Materials and Methods.* Human plasminogen, prepared from Cohn Fraction III-2,3, was activated to plasmin with low molar ratios of urokinase in 25% glycerol (3, 4). This highly purified plasmin was isolated by ammonium sulfate precipitation and dissolved in 10% glycerol–0.05 *M* Tris–0.02 *M* lysine buffer, pH 8.0, at 0°. The protein concentration of this preparation was 12.8 mg/ml. The specific enzyme activity was 30 casein units/mg of protein (5). The preparation was sterilized with a Millipore filter at 0° and stored in small, sterile vials at −20°. The human plasmin stock solution was diluted with the sterile phosphate buffered saline, pH 7.2, in different proportions so that the final protein concentrations of the enzyme varied from 0.7 to 5.1 mg/ml.

Bovine plasminogen (Parke, Davis and Company, Detroit) was activated at pH 7.4 with a human plasmin–streptokinase complex (bovine plasminogen activator) for 30 min at 25°. The specific activity of the bovine plasmin was found to be 0.7 casein units/mg of protein (5). The bovine plasmin was sterile filtered and was stored as a 10% glycerol solution at −20°. The stock solution was diluted with the phosphate buffered saline to obtain final enzyme concentrations of 0.013–2.6 mg/ml.

Human thrombin (Fibrindex, Ortho Diagnostics, Raritan, New Jersey) and bovine thrombin (Parke, Davis and Company) were dissolved in the phosphate buffered saline immediately before use. The final concentrations of human and bovine thrombin were 50–200 units and 200–400 units, respectively.

A 1% solution of crystalline trypsin (Wilson Company, Chicago) was prepared in the phosphate buffered saline and stored at −20°. The specific activity of this preparation was 165 casein units/mg of protein (5). The final concentration of the enzyme in the erythrocyte suspensions was 1 mg/ml.

One percent solutions of ficin (Sigma Chemical Company, St. Louis) and papain (Nutritional Biochemicals Corporation, Cleveland) and a 0.5% solution of bromelin (General Biochemicals Incorporated, Chagrin Falls, Ohio) were prepared and stored at −20° (6).

Crystalline α-chymotrypsin, grade A, (6 units/mg) was obtained from Calbiochem, Los Angeles, and dissolved in phosphate buffered saline at 0.0004 *M* concentration. The solution was treated with 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) for 90 min at 25° in order to inhibit any trypsin contaminant (7). The enzyme solution was sterile filtered and stored at −20°. The final concentration of the enzyme in the erythrocyte suspensions was 1 mg/ml.

The following antisera (Michael Reese Research Foundation, Chicago) were used: three different samples of "incomplete" anti-D

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($Rh_o$), anti-E (rh″) anti-c (hr′), and anti-e (hr″) human sera; anti-A, anti-B, anti-S, anti-s, anti-U, anti-K, anti-$Lu^b$, anti-$Fy^b$, anti-$Jk^b$, anti-$Js^a$, anti-$Js^b$, and anti-I human sera; anti-M, anti-N, and antihuman rabbit sera. Samples of anti-P, anti-k (Cellano), anti-$Fy^a$ and anti-$Jk^a$ were obtained from Pfizer Laboratories, New York. "Incomplete" anti-C (rh′) human sera were obtained from Michael Reese Research Foundation and from Ortho Pharmaceutical Corporation, Raritan, New Jersey.

The erythrocytes were obtained from six known local donors using sterile precautions and from a Knickerbocker-Pfizer panel.

Sterile phosphate buffered saline, pH 7.2, was obtained from Hollister-Stier Laboratories, Oakland.

Erythrocytes were used within 24 hr after collection. The erythrocytes were washed three times in the buffered saline prior to treatment with the enzymes. The erythrocytes were treated with human plasmin at room temperature and at 37° for time intervals varying from 5 min to 2 hr. Similar experiments were performed with bovine plasmin, chymotrypsin, and human and bovine thrombin preparations. The standard methods were followed for treatment of the erythrocytes with trypsin, papain, ficin, and bromelin (6). The erythrocytes were then washed three times in buffered saline to remove the excess enzyme and 2% suspensions were prepared. Special attention to avoid bacterial contamination was given to every step of the procedure.

Twofold serial dilutions of antisera and erythrocyte suspensions were mixed in equal volumes in 10 × 75-mm tubes. Following incubation according to the instructions of the serum supplier, the tubes were centrifuged for 15 sec at 3400 rpm, and the visible agglutination was noted. If no visible agglutination occurred, the erythrocyte suspensions were subjected to the indirect antiglobulin test. An equal volume of antihuman rabbit serum was added to each tube after washing the erythrocytes three times with saline. The tubes were centrifuged and the visible agglutination was examined as above. Controls consisted of the same erythrocytes treated with an equal volume of the buffered saline instead of the enzyme solution as well as erythrocytes treated with the enzyme and suspended in their own serum.

*Results.* The agglutination of erythrocytes with the various Rh antisera tested was markedly increased following incubation with human plasmin at 37°. A slight rise in titer without hemolysis was observed with the anti-$LE^a$ serum used. Anti-K and anti-k antisera did not directly agglutinate the erythrocytes treated with human plasmin. However, slightly increased agglutination was observed in the subsequent antiglobulin tests. No change in the agglutinations of groups A, B, MNSsU, Duffy, $Lu^b$, P, I, $Js^a$, and $Js^b$ was noticeable. Incubation of erythrocytes with human plasmin at room temperature was ineffective and did not produce any alteration in agglutination. The agglutination was enhanced by increasing the incubation time from 5 to 10 min at 37°. Longer incubation periods did not produce further increase in titers in the serologic tests. The enhancement of erythrocyte agglutination was found to be dependent upon the concentration of plasmin. Thus, the highest titers with the different antisera were obtained when the erythrocytes were treated with the maximum concentration of plasmin (5.1 mg/ml).

Trypsin, bromelin, papain, and ficin increased the agglutination of erythrocytes in the Rh system in these studies and as reported by others (8). However, a loss or diminution in agglutinability of the MNS and Duffy antigens was seen as expected (Table I). No alteration in the agglutination of erythrocytes was observed following treatment with α-chymotrypsin, bovine plasmin, human and bovine thrombin.

*Discussion.* The effect of human plasmin on the agglutination of erythrocytes appears to be similar in certain aspects to that of other proteolytic enzymes. A significant difference, however, was observed with the MNS and $Fy^a$ antigens. In contrast to the effects of other enzymes, human plasmin did not abolish agglutination of erythrocytes with anti-M, anti-N, anti-S and anti-$Fy^a$ antibodies.

TABLE I. Agglutination of Erythrocytes Following Treatment with Various Enzymes.[a]

| | Agglutinin titers following | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Saline test | | | | | | | | | | Antiglobulin test | | | | |
| Enzymes | C | D | E | c | e | M | N | $Le^a$ | S | s | $Fy^a$ | $Fy^b$ | $Jk^a$ | K | k |
| Control | 4 | 1 | 4 | 2 | 8 | 16 | 8 | 1 | 2 | 4 | 4 | 1 | 2 | 4 | 4 |
| Human plasmin | 32 | 8 | 32 | 16 | 32 | 16 | 8 | 4 | 2 | 4 | 4 | 1 | 2 | 16 | 16 |
| Trypsin | 64 | 512 | 64 | 256 | 128 | 4 | 8 | 1 | 2 | 4 | 8 | 0 | 2 | 4 | 4 |
| Bromelin | 64 | 256 | 64 | 128 | 256 | 4 | 4 | 4 | 1 | 1 | 0 | 0 | 0 | 4 | 4 |
| Papain | 128 | 256 | 32 | 32 | 64 | 2 | 1 | 1 | 0 | 0 | 4 | 0 | 2 | 16 | 16 |
| Ficin | 128 | 512 | 256 | 512 | 512 | 4 | 8 | 4 | 0 | 0 | 0 | 0 | 0 | 8 | 4 |

[a] α-chymotrypsin, bovine plasmin, human and bovine thrombin did not alter the agglutinability of erythrocytes.

Furthermore, no increased agglutination could be observed with anti-, and anti-I. Different specificities of the enzymes might account for some of the serological differences observed. Previously, Prager *et al.* reported treatment of erythrocytes with commercially available plasmin and found no change in the agglutination reaction (9). The origin (whether bovine or human) and the specific activity of the enzyme were not mentioned. The difference in results is probably due to the varying experimental conditions, and the source and purity of the plasmin preparations. The highest concentration of bovine plasmin, not showing nonspecific agglutinating activity, contained merely 1.82 casein units/ml. The absence of serological reactivity of the preparation might be due to the small quantity of plasmin used.

Human plasmin, like trypsin, is a serine protease (10). Both enzymes catalyze the hydrolysis of arginyl and lysyl bonds. In view of their common serological effects toward the Rh antigens, it appears that the hydrolysis of arginyl and lysyl bonds of these antigens may be the reason why enzyme treated erythrocytes are agglutinated by incomplete antibodies. Winzler *et al.* (11) have recently isolated the glycopeptides released from the erythrocyte cell surface after incubation with trypsin. Their analyses indicate that these glycopeptides are the free terminal portions of the cell surface glycoproteins split at the trypsin accessible lysyl or arginyl residues. TLCK-treated α-chymotrypsin did not enhance the agglutination of the Rh–Hr system, probably due to the difference in substrate specificity. Since human plasmin enhanced agglutination by the Rh–Hr antibodies, but did not abolish agglutination of erythrocytes with anti-M, anti-N, anti-S, and anti-$Fy^a$ antibodies, it indicates that human plasmin may either possess a greater degree of specificity than trypsin, or that it hydrolyzes arginyl and lysyl bonds at a different rate.

Thrombin, which is also able to hydrolyze arginyl and lysyl bonds, was found serologically inactive as previously reported by Prager *et al.* (9). Although capable of cleaving synthetic substrates, such as tosylarginine methyl esters (12), thrombin has a narrow specificity in its proteolytic action on fibrinogen. Only a few arginyl–glycine bonds linking the fibrinopeptides to the rest of the fibrinogen molecule are hydrolyzed by the enzyme (13). Additional arginyl and possibly lysyl bonds in fibrinogen may be split only at a much slower rate (14). Because of the narrow specificity of thrombin this enzyme may be different from the other proteolytic enzymes regarding its activity toward erythrocyte antigens.

*Summary.* The serological activity of human plasmin was compared with those of trypsin, bromelin, papain, and ficin. Human plasmin increased agglutination of erythrocytes with Rh–Hr antibodies, and to a lesser extent, with anti-$Le^a$, anti-K, and anti-k (Cellano) antibodies. It did not decrease the agglutination of erythrocytes with anti-M, anti-N, anti-S, and anti-$Fy^a$ antibodies. No in-

creased agglutination could be observed with anti-P and anti-I antibodies. Bovine plasmin, human and bovine thrombin, and α-chymotrypsin did not alter the agglutinability of erythrocytes.

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## Mass Isolation of Malaria Sporozoites from Mosquitoes by Density Gradient Centrifugation* (33782)

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Biochemical and immunological studies of malaria sporozoites are hampered by the lack of efficient mass isolation methods. Dissection of the paired salivary glands from individual mosquitoes is tedious and time consuming. Culturing mature oocysts *in vitro* and collecting the sporozoites liberated shortly thereafter does provide greater numbers and cleaner preparations (1, 2). However, with either method the removal must be accomplished manually and this precludes obtaining extremely large numbers.

As an alternative we investigated the technique of density gradient centrifugation. Although frequently employed to separate cellular and subcellular particles, the technique has recently been extended to the isolation of whole cells and organs (3, 4). The success of Rawley *et al.* (5) in using density gradients to separate mammalian erythrocytes infected with different developmental stages of *Plasmodium berghei* indicated that the technique might be applicable to the isolation of the invertebrate stages of plasmodia.

This report describes the mass isolation of *Plasmodium gallinaceum* sporozoites from *Aedes aegypti* by using a sucrose density gradient.

*Materials and Methods.* Donor mosquitoes had a 70–90% infection rate with a mean oocyst count of 75/midgut. Since infectivity varied with different lots of mosquitoes progress in purification was based on the reduction of mosquito mass with simultaneous increase in sporozoite concentration for each fractionation step. The results of successive steps were viewed throughout the course of each run under a phase contrast microscope as well as with Giemsa stained preparations.

One thousand (2.6–3.3 g) 9–11-day postinfected mosquitoes were subdivided into 10 lots

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