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# Inhibition of Hageman Factor, Plasma Thromboplastin Antecedent, Thrombin and Other Clotting Factors by Phenylglyoxal Hydrate[1] (38500)

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The first recognized reaction in the intrinsic pathway of thrombin formation, the activation of Hageman factor (HF, Factor XII), may be brought about by exposure of plasma or purified preparations of HF to negatively charged solids (1–3). Neutralization of this negative charge by exposure to positively charged agents inhibits the activation of HF (4, 5). HF can also be activated by several soluble agents, notably ellagic acid, an effect probably related to a net negative charge, although special spatial configurations may also be required (6).

A reasonable assumption is that the effect of appropriate negatively charged substances is mediated through reactions involving the participation of positively charged amino acid residues in the HF molecule. Purified preparations of HF contain three such amino acids, arginine, lysine and histidine (7, 8). Under mild conditions, phenylglyoxal hydrate (PGH) combines almost exclusively with the guanido group of arginyl residues of proteins (9). Incubation of purified HF with PGH sharply impeded the subsequent generation of clot-promoting activity upon addition of kaolin, a negatively charged solid, or ellagic acid. Exposure of HF to kaolin or ellagic acid before addition of PGH significantly lessened this inhibitory effect. Additional experiments are described demonstrating that PGH also interfered with the function of other clotting factors.

*Materials and Methods.* Pooled normal human citrated plasma and plasma from patients with hereditary deficiencies of clotting factors, none of which had been allowed to come into contact with glass, were prepared as described earlier (10) and stored at −70°. Partially purified Hageman factor (HF, 53 units/mg protein) was separated from human oxalated plasma as reported recently (11). $^{125}$I-labeled Hageman factor ($^{125}$I-HF) was prepared by enzymatic iodination of HF with Na $^{125}$I using lactoperoxidase (12), as reported earlier (13). Partially purified human plasma thromboplastin antecedent (PTA, Factor XI, 84 units/mg protein) and Enzite trypsin-activated PTA were prepared by an earlier method (14). One unit of HF or PTA is the amount found in 1 ml of a pool of 25 normal human plasmas. Purified human thrombin, the gift of Dr. Kent Miller, University of Miami School of Medicine, Miami, FL, was dissolved at a concentration of 116 NIH units/ml of buffer and stored at −70° until used.

Phenylglyoxal hydrate (PGH) was dissolved in buffer at concentrations ranging from 5 to 15 mg per ml by heating in a boiling water bath, and then diluted in buffer as needed. Preparations obtained from K. and K. Laboratories, Jamaica, NY and Aldrich Chemical Co., Milwaukee, WI were equally effective. Ellagic acid (K. and K.) was dissolved at a concentration of $10^{-4}$ *M* in buffer. Kaolin (acid-washed, N.F., Fisher Scientific Co., Pittsburgh, PA) was suspended in buffer or in 0.1% crude soybean phosphatides (Centrolex "O", the gift of Central Soya Co., Chicago, IL) in 0.15 *M* sodium chloride. Cytochrome C (horse heart) was obtained from Mann Research Lab., N.Y., NY.

Unless otherwise specified, all dilutions were made in barbital-saline *buffer* (pH 7.5) containing 2.76 g of barbital, 2.06 g of sodium barbital and 7.3 g of sodium chloride per liter.

The effect of PGH on the thrombin time, one-stage prothrombin time, partial thromboplastin time (PTT) and Russell's viper

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venom time was studied by incubating a mixture of 0.1 ml normal pooled plasma and 0.1 ml PGH (at several concentrations) at 25° for 2 hr in 10 × 75 mm disposable tubes. Incubation of proteins under these conditions induces binding of PGH to arginine, with essentially no effect on other amino acid residues (9). Thereafter, the mixture was incubated at 37° for 1 min. The *thrombin time* was measured by adding 0.05 ml crude bovine thrombin (Topical Thrombin, Parke, Davis & Co., Detroit, MI, 10 NIH units/ml) and recording the clotting time at 37°. The *prothrombin time* was measured by adding 0.2 ml of an equal mixture of rabbit brain thromboplastin (Permaplastin, Alban & Co., St. Louis, MO) and 0.025 *M* calcium chloride, prewarmed to 37°, and recording the clotting time. The PTT was measured upon addition of 0.1 ml of a suspension of 10 mg kaolin/ml of 0.1% Centrolex "O" and 0.1 ml 0.025 *M* calcium chloride, both prewarmed to 37°. The *Russell's viper venom time* was measured upon addition of 0.1 ml of 0.0067 mg Russell's viper venom (Sigma Chemical Co., St. Louis, MO) in 0.001% Centrolex "O" in buffer, and 0.1 ml 0.025 *M* calcium chloride.

*The effect of PGH on individual clotting factors in plasma* was tested by incubating 0.5 ml pooled normal plasma and 0.5 ml PGH (at several concentrations) for 2 hr at 25° in 12 × 75 mm polystyrene tubes. Thereafter, the concentration of fibrinogen was assayed by a modification (15) of a previously described technique (16). Concentrations of HF, PTA, Christmas factor (Factor IX) and antihemophilic factor (AHF, Factor VIII) were measured after diluting the mixture 20-fold or more with buffer, using specific assays based upon the PTT and substrate plasmas obtained from patients with hereditary functional deficiencies of each factor (17); the clotting times were recorded in comparison with those of plasma incubated for 2 hr at 25° with buffer instead of PGH, and equalizing the concentration of PGH just before assay. Assays for Factor VII and proaccelerin (Factor V) were performed after similar dilution, using a one-stage prothrombin time technique (18) and substrates of human Factor VII-deficient and artificial proaccelerin-deficient (19) plasmas respectively. Assays for prothrombin (Factor II) were performed by the method of Owren and Aas (20), and for Stuart factor (Factor X), by the technique of Bachman *et al.* (21).

*The effect of PGH on partially purified HF* was studied by incubating mixtures of 0.05 ml each of PGH, HF and kaolin (10 mg/ml buffer), in various combinations, for 2 hr at 25° in 10 × 75 mm polystyrene tubes, maintaining the volume at 0.15 ml with buffer. Thereafter, 0.05 ml PGH or kaolin was added to appropriate tubes and the volumes brought to 1.0 ml with buffer. A sample of 0.1 ml of each tube was then incubated in 10 × 75 mm polystyrene tubes at 37° for 8 min with 0.1 ml 0.1% Centrolex "O" in 0.15 *M* sodium chloride and 0.1 ml Hageman factor-deficient plasma. The clotting time was then measured after addition of 0.1 ml 0.025 *M* calcium chloride, prewarmed to 37°. The effect of ellagic acid was tested in similar fashion, substituting ellagic acid for kaolin.

*The effect of PGH on PTA* was measured in similar fashion, incubating 0.05 ml PTA or activated PTA and 0.05 ml PGH or buffer at 25° for 2 hr. After dilution to 1.0 ml with buffer, with or without 0.05 ml PGH, PTA was assayed on a substrate of bovine PTA-deficient plasma (22). Kaolin-Centrolex "O" was used to assay PTA, and Centrolex "O" alone, to assay activated PTA.

*The effect of PGH on the binding of $^{125}$I-HF to kaolin* was studied by incubating 0.07 ml of $^{125}$I-HF (1.6 units/ml, approximately 15,000 cpm) with 0.05 ml of either kaolin (10 mg/ml), PGH (5 mg per ml) or Cytochrome C (5 mg/ml) for 2 hr at 25° in 10 × 75 mm polystyrene tubes, maintaining the volume at 0.17 ml with buffer. Thereafter, 0.05 ml of kaolin, PGH or Cytochrome C were added to appropriate tubes and the tubes were further incubated at 37° for 5 min. The tubes were then centrifuged at 1800 *g* for 5 min at 4°. The precipitated kaolin was washed with 1 ml buffer four times and was counted for radioactivity using a Nuclear-Chicago γ-counter (Model 1085).

*The effect of PGH on thrombin* was tested by incubating human thrombin (116 NIH units/ml) with an equal volume of PGH or buffer for 2 hr at 25° in 12 × 75 mm polystyrene tubes. The contents of the tubes

TABLE I. THE EFFECT OF PHENYLGLYOXAL HYDRATE (PGH) ON THE THROMBIN TIME, PROTHROMBIN TIME, PARTIAL THROMBOPLASTIN TIME AND RUSSELL'S VIPER VENOM TIME OF NORMAL PLASMA.

| Assay | Concentration of PGH: Preliminary incubation (mg/ml) | Concentration of PGH: During assay (mg/ml) | Clotting time (sec) |
|---|---|---|---|
| Thrombin time | 1.87 | 1.50 | >60.0 |
| | 0.94 | 0.75 | 34.6 |
| | 0.47 | 0.38 | 15.3 |
| | 0.00 | 0.00 | 15.5 |
| Prothrombin time | 1.87 | 0.94 | >300.0 |
| | 0.94 | 0.47 | 66.9 |
| | 0.47 | 0.23 | 26.5 |
| | 0.23 | 0.12 | 16.1 |
| | 0.12 | 0.06 | 15.3 |
| | 0.06 | 0.03 | 14.7 |
| | 0.00 | 0.00 | 14.7 |
| Partial thromboplastin time | 7.50 | 3.75 | >300.0 |
| | 3.75 | 1.87 | 138.8 |
| | 1.87 | 0.94 | 113.2 |
| | 0.94 | 0.47 | 97.2 |
| | 0.47 | 0.23 | 89.6 |
| | 0.23 | 0.12 | 85.5 |
| | 0.00 | 0.00 | 85.5 |
| Russell's viper venom time | 0.94 | 0.47 | >90.0 |
| | 0.47 | 0.23 | 47.3 |
| | 0.23 | 0.12 | 39.4 |
| | 0.12 | 0.06 | 36.7 |
| | 0.00 | 0.00 | 35.5 |

were then diluted so that the concentration of thrombin was 5 units/ml, and 0.2 ml samples were incubated at 37° for 1 min in 10 × 75 mm disposable glass tubes. An equal volume of normal pooled plasma was added and the clotting time determined. The effect of PGH was compared to that of thrombin, incubated for 2 hr with buffer, and then diluted serially with buffer before assay.

Results. *1. The effect of phenylglyoxal hydrate on plasma.* Incubation of normal pooled human plasma with phenylglyoxal hydrate for 2 hr at 25° lengthened the thrombin time, prothrombin time, partial thromboplastin time and Russell's viper venom time (Table I). At concentrations of PGH of 1.25 mg/ml or more, a visible precipitate formed during the 2 hr period. The concentration of coagulable fibrinogen in such plasma was diminished (Table II). The titer of other clotting factors was also strikingly reduced, as measured after sufficient dilution of plasma to minimize interference by PGH in the assay procedure. Exceptionally, HF, and to a lesser extent, Stuart factor, seemed more resistant than other factors to incubation with PGH at a concentration of 5 mg/ml.

*2. The effect of phenylglyoxal hydrate on partially purified Hageman factor.* Incubation of partially purified HF with PGH (1.6 mg/ml of the initial mixture) for 2 hr at 25° sharply diminished its coagulant properties, as tested by addition of kaolin (Table III). As little as 0.62 mg PGH/ml of the initial mixture reduced potential clot-promoting activity by 75%. When kaolin was mixed with HF before its incubation with PGH, the diminution in clot-promoting activity was much less pronounced.

One possible explanation for the protective effect of kaolin is that HF was adsorbed to its surface and in this way protected against the action of PGH. Essentially similar results were obtained, however, when HF was incubated with ellagic acid, a soluble activator of HF (Table IV).

Incubation of HF with PGH at 25° for 2 hr did not diminish the ability of HF to bind to kaolin, whereas cytochrome C inhibited HF binding to kaolin more than 50% (Table V).

*3. The effect of phenylglyoxal hydrate on partially purified plasma thromboplastin antecedent and thrombin.* Incubation of partially purified PTA or Enzite trypsin-activated PTA with PGH (final concentration 2.5 mg/ml) for 2 hr at 25° abolished their clot-promoting properties (Table VI). Purified human thrombin was even more susceptible to inhibition by PGH, which was effective in concentrations as low as 0.625 mg/ml under these conditions; after dilution of the mixture at a concentration of 5 NIH units of thrombin/ml, the thrombin time of a sample was 350 sec, whereas that of thrombin, similarly incubated with buffer and then diluted and tested in the same way, was 13.2 sec. This prolongation of the thrombin time represented a reduction of approximately 93% in

TABLE II. THE EFFECT OF PHENYLGLYOXAL HYDRATE (PGH) ON SOME CLOTTING FACTORS IN PLASMA.

| Factor | Concentration of PGH | | Clotting time | | Residual coagulant activity (%) |
|---|---|---|---|---|---|
| | Preliminary incubation[a] (mg/ml) | During assay (mg/ml) | Buffer[b] (sec) | PGH (sec) | |
| Fibrinogen | 5.0 | — | 300[c] | 65[c] | |
| | 2.5 | — | | 148[c] | |
| | 1.25 | — | | 130[c] | |
| | 0.62 | — | | 262[c] | |
| Prothrombin (II) | 5.0 | 0.125 | 78.1 | 166.1 | <1 |
| Proaccelerin (V) | 5.0 | 0.125 | 33.8 | 95.9 | <1 |
| Factor VII | 5.0 | 0.125 | 50.8 | 143.3 | <1 |
| Stuart factor (X) | 5.0 | 0.125 | 39.7 | 92.0 | 2 |
| AHF (VIII) | 2.5 | 0.062 | 76.2 | 104.2 | <1 |
| Christmas factor (IX) | 5.0 | 0.125 | 86.1 | 162.3 | <1 |
| PTA (XI) | 2.5 | 0.062 | 76.1 | 213.0 | <1 |
| Hageman factor | 5.0 | 0.125 | 52.8 | 73.2 | 25 |

[a] Concentration of PGH during preliminary incubation with plasma for 2 hr at 25°.

[b] Sufficient PGH added after preliminary incubation to equalize its concentration in the coagulant assay.

[c] Milligram/100 ml plasma.

TABLE III. THE EFFECT OF PHENYLGLYOXAL HYDRATE UPON KAOLIN-ACTIVATED HAGEMAN FACTOR (FACTOR XII).[a]

| Initial mixture | Addition after 2 hr | Clotting time (sec) | Clot-promoting activity (%) |
|---|---|---|---|
| HF + Buffer | Kaolin | 64 | 100 |
| HF + Kaolin | Buffer | 66 | 90 |
| HF + Kaolin | PGH | 66 | 90 |
| HF + PGH | Kaolin | 168 | 5 |
| HF + Kaolin + PGH | Buffer | 95 | 31 |

[a] Mixtures of HF (1.6 units/ml), and either kaolin, PGH (5 mg/ml) or both, and sufficient buffer to bring the total volume to 0.15 ml were incubated at 25° for 2 hr (see Materials and Methods section). Thereafter, kaolin, PGH or buffer were added and the total volume brought to 1.0 ml with buffer. The clot-promoting activity of a 0.1 ml sample was then tested on a substrate of HF-deficient plasma, and the percent of clot-promoting activity calculated related to the tube with the greatest activity.

TABLE IV. THE EFFECT OF PHENYLGLYOXAL HYDRATE UPON ELLAGIC ACID-ACTIVATED HAGEMAN FACTOR.[a]

| Initial mixture | Addition after 2 hr | Clotting time (sec) | Clot-promoting activity (%) |
|---|---|---|---|
| HF + EA | Buffer | 57 | 96 |
| HF + EA | PGH | 56 | 100 |
| HF + PGH | EA | 156 | 3 |
| HF + EA + PGH | Buffer | 92 | 20 |
| HF | EA + PGH | 75 | 40 |

[a] Mixtures of HF (0.8 units/ml) and either ellagic acid (EA, $10^{-4}$ *M*), PGH (5 mg/ml) or both, and sufficient buffer to bring the total volume to 0.15 ml were incubated at 25° for 2 hr (see Materials and Methods section). Thereafter, PGH, EA or both were added and the total volume brought to 1.0 ml with buffer. The clot-promoting activity of a 0.1 ml sample was then tested upon a substrate of HF-deficient plasma, and the percent of clot-promoting activity calculated relative to the tube with the greatest activity.

clot-promoting activity. Comparable results were obtained with crude bovine thrombin preparations. This effect of PGH on thrombin did not interfere with other experiments described because the mixtures were diluted before assay, and tested immediately. The effect of PGH upon thrombin is time-dependent, and was therefore not a significant factor in the results obtained.

TABLE V. THE EFFECT OF PHENYLGLYOXAL HYDRATE UPON THE BINDING OF $^{125}$I-HF TO KAOLIN.[a]

| Initial mixture | Addition after 2 hr | % Kaolin-bound radio-activity |
|---|---|---|
| $^{125}$I-HF + Buffer | Kaolin | 61 |
| $^{125}$I-HF + Kaolin | Buffer | 59 |
| $^{125}$I-HF + Kaolin | PGH | 58 |
| $^{125}$I-HF + PGH | Kaolin | 62 |
| $^{125}$I-HF + Kaolin + PGH | Buffer | 60 |
| $^{125}$I-HF + Kaolin | Cytochrome C | 48 |
| $^{125}$I-HF + Cyto-chrome C | Kaolin | 28 |

[a] Mixtures of $^{125}$I-HF (1.6 units/ml, approximately 15,000 cpm), and either kaolin, PGH, Cytochrome C or buffer were incubated at 25° for 2 hr (see Materials and Methods section). Thereafter, kaolin, PGH, Cytochrome C or buffer were added and the mixtures were further incubated at 37° for 5 min. Kaolin-bound radioactivity in each tube was counted after washing kaolin four times with buffer. The results were expressed as the percent of kaolin-bound radioactivity related to that added to each tube.

TABLE VI. THE EFFECT OF PHENYLGLYOXAL HYDRATE UPON PLASMA THROMBOPLASTIN ANTECEDENT (FACTOR XI).[a]

| Initial mixture | Addition after 2 hr | Clotting time (sec) | Clot-promoting activity (%) |
|---|---|---|---|
| PTA | Buffer | 70 | 100 |
| PTA + PGH | Buffer | 212 | <1 |
| PTA | PGH | 74 | 80 |
| Activated PTA | Buffer | 81 | 60 |
| PTA + PGH | Buffer | >325 | <1 |
| PTA | PGH | 86 | 44 |

[a] Mixtures of PTA or activated PTA were incubated at 25° for 2 hr with equal volumes of buffer or phenylglyoxal hydrate (5 mg/ml) in 10 × 75 mm polystyrene tubes (see Materials and Methods section). Phenylglyoxal hydrate (5 mg/ml) was added to appropriate tubes, all of which were diluted to 1.0 ml with buffer. PTA was assayed in the presence of activated PTA in the absence of kaolin.

*Discussion.* How negatively charged surfaces activate Hageman factor (HF) is only partially understood. Activation appears to be accompanied by alteration of HF to a more hydrophobic state (23) and by conformational changes (8).

The capacity of PGH to combine with guanido groups of arginine suggested that it might be useful in identifying the amino acid residues with which negatively charged surfaces react. In the experiments described, preliminary incubation of partially purified HF with PGH effectively inhibited its subsequent activation by kaolin or ellagic acid. This was not due to the inhibition of HF binding to kaolin by PGH, since PGH-treated HF was bound to kaolin as much as untreated HF was. Thus, the adsorption of HF to negatively charged surfaces is not necessarily equated with the activation of HF under these conditions. Even momentary exposure of HF to either kaolin or ellagic acid before addition of PGH diminished but did not abolish inhibition by PGH. One possible conclusion, then, is that activation of HF involves the participation of arginine residues. Once activation has taken place, the molecule is much less susceptible to inactivation by exposure to a reagent reacting almost specifically with arginine residues.

Only a partial investigation of other activated clotting factors was undertaken. Both activated PTA and thrombin were inhibited by exposure to PGH. Incomplete experiments with activated Christmas factor and activated Stuart factor gave inconclusive results. Other studies demonstrated that exposure of plasma to PGH reduced the titer of all clotting factors tested.

*Summary.* Exposure of purified Hageman factor (HF, Factor XII) to phenylglyoxal hydrate (PGH), an agent reacting with arginine residues in protein, inhibited its coagulant properties upon subsequent exposure of negatively charged agents. Once HF had been exposed to kaolin or ellagic acid, however, subsequent addition of PGH was much less inhibitory. PGH had no effect upon the ability of HF to bind to negatively charged surfaces. PGH also inhibited preparations of activated PTA (Factor XI) and thrombin, and, when incubated with plasma, reduced the titer of coagulable fibrinogen, PTA,

Christmas factor (Factor IX), antihemophilic factor (Factor VIII), Factor VII, Stuart factor (Factor X), proaccelerin (Factor V) and prothrombin (Factor II), and to a lesser degree, HF.

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1. Hubbard, D. and Lucas, G. L., J. Appl. Physiol. **15,** 265 (1960).
2. Ratnoff, O. D. and Rosenblum, J. M., Amer. J. Med. **25,** 160 (1958).
3. Margolis, J., Ann. N. Y. Acad. Sci. **104,** 133 (1963).
4. Ratnoff, O. D. and Miles, A. A., Brit. J. Exp. Pathol. **45,** 328 (1964).
5. Nossel, H. L., Rubin, H., Drillings, M. and Hsieh, R., J. Clin. Invest. **47,** 1172 (1968).
6. Ratnoff, O. D. and Crum, J. D., J. Lab. Clin. Med. **63,** 359 (1964).
7. Spear, R. J., Ridgway, H. and Hill, J. M., Thromb. Diath. Haemorrh. **14,** 1 (1965).
8. McMillin, C. R., Saito, H., Ratnoff, O. D. and Walton, A. G., J. Clin. Invest. **54,** 1312 (1974).
9. Takahashi, K., J. Biol. Chem. **243,** 6171 (1968).
10. Forbes, C. D. and Ratnoff, O. D., J. Lab. Clin. Med. **79,** 113 (1972).
11. Saito, H., Ratnoff, O. D. and Donaldson, V. H., Cir. Res. **34,** 641 (1974).
12. Thorell, J. I. and Johansson, B. G., Biochim. Biophys. Acta **251,** 363 (1971).
13. Saito, H., Ratnoff, O. D., Donaldson, V. H., Haney, G., and Pensky, J., J. Lab. Clin. Med. **84,** 62 (1974).
14. Saito, H., Ratnoff, O. D., Marshall, J. S. and Pensky, J., J. Clin. Invest. **52,** 850 (1973).
15. Ogston, D., Ogston, C. M. and Bennett, N. B., Thromb. Diath. Haemorrh. **16,** 32 (1966).
16. Ratnoff, O. D. and Menzie, C., J. Lab. Clin. Med. **37,** 316 (1951).
17. Ratnoff, O. D. and Davie, E. W., Biochemistry **1,** 967 (1962).
18. Seibert, R. H., Margolius, A., Jr. and Ratnoff, O. D., J. Lab. Clin. Med. **52,** 449 (1958).
19. Wolf, P. J., Clin. Pathol. **6,** 34 (1953).
20. Alexander, B., *in* "The Coagulation of Blood, Methods of Study" (L.M. Tocantins, ed.), p. 89. Grune and Stratton, New York (1965).
21. Bachmann, F., Duckert, F., and Keller, F., Thromb. Diath. Haemorrh. **2,** 24 (1958).
22. Kociba, G. J., Ratnoff, O. D., Loeb, W. F., Wall, R. L., and Heider, L. E., J. Lab. Clin. Med. **74,** 37 (1969).
23. Donaldson, V. H. and Ratnoff, O. D., Science **150,** 754 (1965).

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