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Studies on the Anticoagulant Properties of Chlorazol Fast Pink (Benzo Fast Pink 2BL)[1] (41000)

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Abstract. Chlorazol fast pink is representative of a group of azo dyes that are anticoagulant both *in vitro* and *in vivo*. At a concentration of 2.5 mg/ml, it completely inhibited the thrombin time, prothrombin time, and partial thromboplastin time of human plasma, and the thrombin time and Reptilase time of purified human fibrinogen. Among modes of action considered, it was found to inhibit the cleavage of fibrinopeptides A and B from fibrinogen, and the augmentation of the coagulant action of antihemophilic factor (factor VIII) by thrombin. It did not retard the aggregation of fibrin monomers, but the clots that formed were fragmentary. Clot formation returned to normal after removal of all but tightly bound dye from fibrinogen and thrombin. This and similar experiments suggested that the dye interfered reversibly with the interaction of thrombin and fibrinogen to a degree that accounted for much of its anticoagulant activity. The studies suggest that chlorazol fast pink may be an unusual tool for clarifying the steps involved in the formation of a fibrin clot.

Introduction. Chlorazol fast pink (benzo fast pink 2BL) is representative of a number of azo dyes that found usage as *in vivo* anticoagulants about 50 years ago (1, 2). Huggett and his coworkers (3–6) believed that such dyes inhibited the conversion of prothrombin to thrombin and the subsequent action of thrombin upon fibrinogen. Reinvestigation of the mode of action of chlorazol fast pink suggests that its principal action is inhibition of the interaction between thrombin and its substrates and that this inhibition is reversible.

Materials and methods. Chlorazol fast pink (benzo fast pink 2BL, Color Index No. 25380, MW 1034.9, MCB Reagents, Cincinnati, Ohio) was dissolved at concentrations up to 8% (w/v) (8×10^{-3} *M*) in barbital–saline buffer ("buffer"), stored at 4°, and further diluted in the same buffer before use (Fig. 1).

Normal and abnormal plasmas were separated from venous blood to which 1/50 volume of sodium citrate buffer (0.5 *M*, pH 5.0) had been added, as described earlier (7). Citrated Fletcher trait (prekallikrein-deficient), Christmas disease (factor IX-deficient) and Stuart factor (factor X-deficient) plasmas were purchased from Geo. King Bio Medical Inc., Overland, Kansas. High-molecular-weight kininogen-deficient (Fitzgerald trait) plasma was the gift of Dr. Robert Waldmann, Detroit, Michigan, and bovine plasma thromboplastin antecedent (PTA, factor XI)-deficient plasma was the gift of Dr. Gary Kociba, Columbus, Ohio. Pooled normal plasma was prepared and stored as described earlier (7).

Purified human fibrinogen (97% coagulable, Imco Corp., Stockholm, Sweden) was dissolved in water at concentrations up to 8 mg/ml. Partially purified human fibrinogen (65% coagulable) was the gift of Dr. Walter Forman, Cleveland Veterans Administration Hospital.

Crude bovine thrombin (Topical Thrombin, Parke, Davis Co., Detroit, Mich.) was

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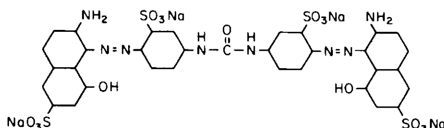

FIG. 1. Structure of chlorazol fast pink (benzo fast pink 2BL).

dissolved in buffer at a concentration of 1000 NIH U/ml and diluted further as required. Purified human thrombin (specific activity 3328 U/mg protein) was the gift of Dr. John W. Fenton, Albany, New York. Partially purified human thrombin ("minimum activity 2000 NIH units/mg protein," Sigma Chemical Co., St. Louis, Mo.) was used at an initial concentration of 50 U/ml.

Partially purified human antihemophilic factor (AHF, factor VIII) was separated by gel filtration through Ultrogel AcA22 (LKB Instruments, Inc., Rockville, Md.) of commercial human AHF (Profilate, Alpha Therapeutic Corp., South Pasadena, Calif.) (8). The collected void volume, in 0.02 *M* imidazole buffer, 0.15 *M* sodium chloride (pH 6.5) had a coagulant titer of 0.30–0.55 U/ml.

Tissue thromboplastin for measurement of the prothrombin time and of serum prothrombic activity was derived from rabbit brain (Permaplastin, Alban Scientific Co., St. Louis, Mo.).

Crude hirudin (Grade III, Sigma Chem. Co., St. Louis, Mo.) was dissolved in Michaelis buffer (pH 9.6) (9) at a concentration of 1000 U/ml.

Unless otherwise noted, the term buffer refers to barbital–saline buffer (0.025 *M* sodium barbital in 0.125 *M* sodium chloride, pH 7.5).

Except as noted, all clotting tests were performed in 10 × 75-mm disposable glass tubes (internal diameter 8 mm).

Human plasma prepared in our laboratory was obtained from blood drawn with the permission of the Committee on Human Experimentation, University Hospitals of Cleveland.

Kaolin-Centrolex "P", used for measurement of the partial thromboplastin time and for specific assays of clotting factors, was kaolin (acid-washed, American standard, Fisher Scientific Co., Fair Lawn, N.J.), 10 mg/ml 0.1% crude soybean phosphatides (Centrolex "P", the gift of Central Soya Co., Fort Wayne, Ind.) in 0.15 *M* sodium chloride.

The prothrombin time (10), partial thromboplastin time (11) (modified by substitution of kaolin-Centrolex "P" for crude "cephalin"), thrombin time (12), and serum prothrombic activity (prothrombin consumption) (13) were measured by previously published methods modified by addition to the reaction mixtures of 0.1 ml of buffer or 0.1 ml chlorazol fast pink (at various concentrations) in buffer. The Reptilase time was measured by incubating 0.3 ml purified human fibrinogen (Imco, 4 mg/ml) with 0.1 ml buffer or chlorazol fast pink (10–40 mg/ml buffer) for 1 min at 37°. Thereafter, 0.1 ml Reptilase "R" (Bothrops Atrox venom, lyophilized, Abbott Laboratories, N. Chicago, Ill.) was added and the clotting time was then measured at 37°.

The effect of calcium chloride on the thrombin time was tested by incubating 0.2 ml normal human plasma for 1 min at 37° with 0.05 ml of chlorazol fast pink (10 mg/ml buffer) and 0.05 ml calcium chloride, serially diluted with 0.15 *M* sodium chloride. Thereafter, 0.1 ml crude bovine thrombin (5 NIH U/ml) was added and the clotting time was measured by continual tilting. The concentration of chlorazol fast pink in the final mixture was 1.25 mg/ml.

The release of fibrinopeptides in a mixture of crude bovine thrombin and purified fibrinogen was measured by a technique described earlier (14, 15), modified by inclusion of chlorazol fast pink at 0.25 and 2.5 mg/ml in the fibrinogen mixture. Concomitant coagulation was determined from the nitrogen content of Kjeldahl digests of clots extruded from the reaction mixture.

Polymerization of fibrin monomers was tested by a previously described method in which plasma was incubated at pH 5.2 with thrombin, and the clotting time was then measured upon addition of hirudin and adjustment of the pH to 7.2 (16); this procedure was modified by addition of chlorazol fast pink or buffer to the initial mixture.

The effect of chlorazol fast pink upon individual clotting factors was assayed by mixing 0.1 ml chlorazol fast pink (20 mg/ml)

with 0.9 ml normal pooled plasma. The mixture was then immediately assayed for individual clotting factors by published methods (17–19). The concentration of dye in the final assay mixture, 0.025 mg/ml, was such that it did not alter the thrombin time, partial thromboplastin time, or prothrombin time.

To assess the reversibility of effect of chlorazol fast pink and the degree of tight binding, 1.5 ml purified fibrinogen (Imco, 4 mg/ml) was mixed with an equal volume of chlorazol fast pink (2–8 mg/ml) in barbital–saline buffer or buffer, and then separated from excess dye by precipitation. The precipitation was carried out in separate trials either with 1/3 volume of ammonium sulfate solution (pH 7.0, saturated at 4°) followed by three washes with a 25% saturated solution, or with 8% ethanol followed by a glycine wash (20) repeated 14 times. The precipitates were dissolved (5 ml) and dialyzed against water (1 hr) and then 1 liter of buffer (18 hr) at 4°. Retained chlorazol fast pink was estimated by measuring absorbance at 520 nm, and comparing the absorbance with that of known concentrations of dye, effects of protein on absorbency being negligible. The concentration of protein in the various fibrinogen solutions was measured by the method of Lowry *et al.* (21). The amount of dye adsorbed to the protein was then estimated, assuming a MW for fibrinogen of 340,000.

To determine the effect of chlorazol fast pink upon the thrombin-induced augmentation of coagulant activity of AHF, 0.015 ml partially purified human thrombin (Sigma, 1.6 U/ml buffer) was added to a mixture of 0.55 ml partially purified AHF (0.50 U/ml imidazole–saline buffer) and 0.05 ml chlorazol fast pink (1.9–60 mg/ml) or buffer, and incubated at 37°. At intervals, the AHF coagulant activity of the incubated mixture was assayed (16). The concentrations of the dye and thrombin in the final assay mixture were 0.025 mg/ml and 0.002 U/ml, respectively. A volume of 0.05 ml dye (60 mg/ml) was also added to a mixture of 0.65 ml partially purified AHF (0.50 U/ml) and 0.012 ml thrombin (2.2 U/ml) that had been incubated at 37° for 8 min. The coagulant activity of the mixture was assayed at intervals before and after the addition of dye.

Other techniques used are noted in the footnotes of tables.

Centrifugations were performed at 2° at low speed in an International PR-2 centrifuge, and at high speed in a Sorvall RC-2 centrifuge.

Results. Anticoagulant properties of chlorazol fast pink. Chlorazol fast pink, at a concentration of 0.5 mg/ml, prolonged the thrombin time, prothrombin time, and partial thromboplastin time of normal human plasma and, at somewhat larger concentrations, completely inhibited clotting (Table I). The effect of the dye was immediate; prolonged incubation of plasma with submaximal amounts of dye did not augment anticoagulant action. Chlorazol fast pink similarly inhibited the coagulation of purified human fibrinogen by human or bovine thrombin.

Calcium ions, which enhance aggregation

TABLE I. THE EFFECT OF CHLORAZOL FAST PINK ON THE THROMBIN TIME, PROTHROMBIN TIME, AND ACTIVATED PARTIAL THROMBOPLASTIN TIME OF NORMAL PLASMA

Concentration of dye[a] (mg/ml)	Thrombin time (sec)	Prothrombin time (sec)	Act. Part. thromboplastin time (sec)
2.5	>600	>600	>600
1.25	27.9[b]	44.0	>600
0.50	17.1	23.2	88.3
0.25	14.6	15.2	50.5
0.125	14.6	14.3	50.0
0	14.3	14.1	46.5

[a] Final concentration.
[b] Fragmentary clot.

of fibrin monomers without enhancing the enzymatic activity of thrombin (22, 23), slightly accelerated the thrombin time of normal plasma in the presence of chlorazol fast pink, but did not overcome the dye's inhibitory properties. Thus, at a concentration of 6.25 m*M*, added calcium chloride shortened the thrombin time of normal plasma from 16.2 to 10.6 sec, and that of normal plasma with dye (final concentration 1.25 mg/ml) from 28.2 to 20.6 sec. Similar results were observed when the thrombin time of purified fibrinogen was measured.

Localization of the site of action of chlorazol fast pink. The action of chlorazol fast pink, which contains four sulfonate groups, did not appear to be comparable to that of heparin. As noted above, the dye inhibited clotting in a mixture of purified thrombin and fibrinogen, in the apparent absence of antithrombin III. Further, it also inhibited the coagulant effect of Reptilase R upon purified fibrinogen. At a concentration of 2.0 mg/ml, it prolonged the Reptilase time from 25.1 to 90.0 sec, and at higher concentrations, clotting was not observed. Nonetheless, protamine sulfate, at a concentration of 1 mg/ml, completely neutralized the anticoagulant properties of chlorazol fast pink (2 mg/ml), as measured by the thrombin time of normal plasma; lesser amounts of protamine sulfate were ineffective. This observation suggests that the anticoagulant action of chlorazol fast pink was related to its negatively charged sulfonate radicals.

No evidence was obtained that addition of chlorazol fast pink to plasma inhibited the precursor form of any recognized plasma protein clotting factor other than fibrinogen. Normal plasma was incubated at 37° with chlorazol fast pink (final concentration 2 mg/ml) or buffer. The titers of Hageman factor (factor XII), plasma prekallikrein, high-molecular-weight kininogen, plasma thromboplastin antecedent (PTA, factor XI), Christmas factor (factor IX), antihemophilic factor (AHF, factor VIII), factor VII, Stuart factor (factor X), proaccelerin (factor V), and prothrombin were then measured after 20-fold dilution of plasma with buffer. Under these conditions, chlorazol fast pink did not decrease the coagulant titer of any of these factors. This experiment did not preclude an effect of the dye that was reversible upon dilution (vide infra). But chlorazol fast pink, at a concentration in whole blood of 5 mg/ml, did not decrease prothrombin consumption in 1 hr.

The rate of release of fibrin monomers in a mixture of thrombin and fibrinogen was sharply reduced, to about one-eighth the control, in the presence of chlorazol fast pink (2.5 mg/ml), but the dye had little effect at 0.25 mg/ml (Fig. 2). This observation did not differentiate between an action upon thrombin or fibrinogen. Attempts to demonstrate inhibition of esterolysis of *p*-toluene-sulfonyl-L-arginine methyl ester or acetyl-L-arginine methyl ester were vitiated by precipitation of the substrate by the dye. Similarly, its apparent interaction with the synthetic substrate made it impossible to assess the effect of the dye upon amidolysis by thrombin of H-D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide. But chlorazol fast pink, at concentrations as low as 0.15 mg/ml, inhibited thrombin-induced augmentation of AHF-like coagulant activity (Fig. 3). This experiment did not differentiate between an action of the dye upon thrombin or upon its protein substrate, AHF, but incubation of AHF with chlorazol fast pink (5 mg/ml) for 4 min sharply decreased its titer in the absence of added thrombin; no loss of activity was observed in the absence of dye during the same time interval. When chlorazol fast pink was added to a mixture of AHF and thrombin that had achieved maximal coagulant activity, the decay of AHF-like activity was hastened.

Chlorazol fast pink did not alter the *rate* of aggregation of fibrin monomers in plasma, but the clot that formed was fragmentary (Table II). This phenomenon may have been related to an interaction of dye with fibrinogen. To demonstrate this, fibrinopeptide release was allowed to proceed in the absence of chlorazol fast pink. When dye was then added simultaneous with the addition of hirudin, which neutralizes the coagulant action of thrombin, only a fragmentary clot formed (Table II C). That chlorazol fast pink interacted strongly with fibrinogen was demonstrated by substantial

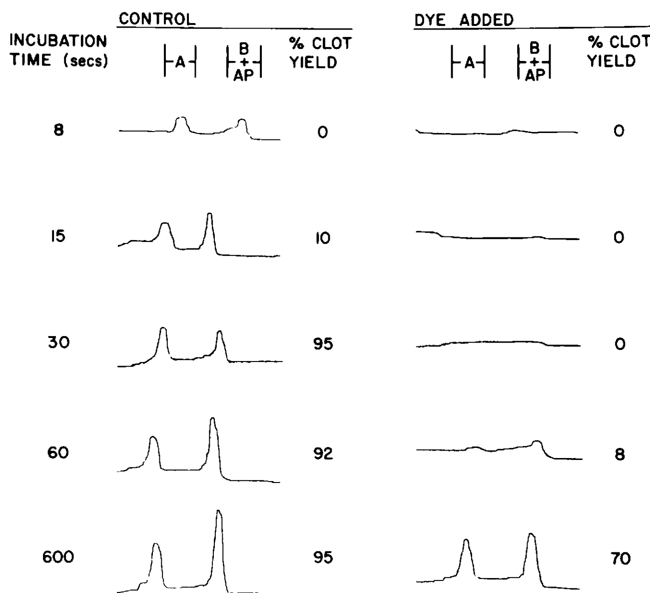

FIG. 2. Fibrinopeptide release and clot yields in reactions between thrombin (Parke–Davis, 2.5 U/ml) and fibrinogen (2 mg/ml) in presence and absence (control) of chlorazol fast pink (2.5 mg/ml). Concentrates of fibrinopeptides from 0.8-ml aliquots were separated by electrophoresis at pH 2, and stained by a modification (14) of Sukaguchi's reaction. The densitometric tracings display the relative amounts of fibrinopeptide A and of coincident migrating fibrinopeptides B and AP after the indicated periods of incubation at 37°. Confirmatory electrophoresis of equal aliquots at pH 8.6 (not shown) indicated that peptide AP was released together with A in amounts on the order of one-third of A.

retention of the dye after repetitive ammonium sulfate precipitation and subsequent dialysis of the fibrinogen. Under the specific conditions used, the amount of dye apparently retained by fibrinogen was a function of the concentration of dye in the original mixture, and ranged proportionately from 1 to 5 moles of dye/demimole of fibrinogen, for dye concentrations ranging from 1 to 4 mg/ml.

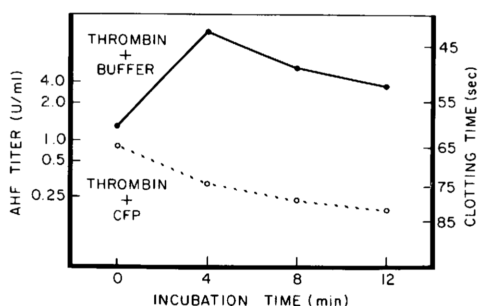

FIG. 3. The effect of chlorazol fast pink on thrombin-induced augmentation of AHF-like coagulant activity. 0.015 ml of partially purified human thrombin (1.6 U/ml) was added to a mixture of 0.055 ml partially purified human AHF (0.5 U/ml) and 0.05 ml of chlorazol fast pink (60 mg/ml) or barbital–saline buffer. The mixtures were incubated at 37° and AHF coagulant activity assayed at the times indicated on the horizontal axis.

In a further attempt to isolate the action of chlorazol fast pink, the dye was incubated, at a concentration of 0.8 mg/ml, with purified human thrombin or fibrinogen. After 1 min, each mixture was diluted with buffer, a sample was mixed with fibrinogen or thrombin, respectively, and the clotting time was measured (Table III). These clotting times were compared with those of experiments in which chlorazol fast pink was added only after thrombin or fibrinogen had been diluted. Premixture of thrombin or fibrinogen with dye did not augment the coagulant defect. The clots that formed in the presence of chlorazol fast pink were small, as though the dye exerted an effect upon the forming fibrin strands, in agreement with the experiment illustrated in Table II.

These experiments suggest that the effects of chlorazol fast pink were reversible and dependent upon high concentrations of

TABLE II. THE EFFECT OF CHLORAZOL FAST PINK UPON POLYMERIZATION OF FIBRIN

Solvent composition during fibrin monomer production	Clotting time upon neutralization (sec)		
	Incubation time		
	(10 sec)	(4 min)	(16 min)
(A) Barbital–saline buffer	∞	14.4	11.1
(B) Chlorazol fast pink	∞	9.7[a]	12.6[a]
(C) Barbital–saline buffer; chlorazol fast pink added at 16 min			10.4[a]

Note. In A and C, 4/10 ml plasma, 1.0 ml Michaelis buffer (pH 2.6), 0.1 ml thrombin [1000 NIH units/ml of 0.15 *M* Na acetate buffer (pH 5.2)], and 0.1 ml barbital–saline buffer were incubated at 37°. At the indicated times, 0.2 ml of the mixture was added to 0.2 ml hirudin [1000 U/ml of Michaelis buffer (pH 9.6)]. The pH of the second mixture was 7.2. In B, 0.1 ml chlorazol fast pink (5 mg/ml) was substituted for barbital–saline buffer in the initial mixture. In C, 10 μl chlorazol fast pink (50 mg/ml) was added after 16 min to 0.2 ml hirudin simultaneously with the addition of 0.2 ml of the initial mixture.

[a] Fragmentary clot.

the dye. The reversibility of its effect upon fibrinogen was confirmed by studies in which this protein was separated from excess dye by precipitation with 8% ethanol. The thrombin time of the reprecipitated fibrinogen was not significantly different from that which had not been exposed to the dye (Table IV).

A different technique was used to separate chlorazol fast pink from thrombin. Purified human thrombin was mixed with barbital–saline buffer or chlorazol fast pink and filtered through small columns of Sephadex G-25 (Table V). Under these conditions, the coagulant properties of the filtered thrombin were uninfluenced by pretreatment with the dye. Identical results were observed when fibrinogen was treated in the same way.

The effect of chlorazol fast pink upon the thrombin time of dysfibrinogenemic plasmas. Chlorazol fast pink, at a concentration of 0.8 mg/ml, signficantly prolonged the thrombin time of plasma of patients with fibrinogen Cleveland I (in which fibrin aggregation is impaired) and fibrogen Cleveland II (in which fibrinopeptide A release is impaired) (Table IV). The prolongation of the thrombin time of the dysfibrinogenemic plasmas was of approximately the same degree as that of normal plasma similarly mixed with chlorazol fast pink, being about twice that in absence of the dye in each of the tests.

Discussion. About 50 years ago, Rous, Gilding and Smith (1), incidental to a study of capillary permeability, discovered that the azo dye, Chicago blue 6B, had an an-

TABLE III. THE EFFECT OF PREINCUBATION WITH CHLORAZOL FAST PINK (CFP) ON THE INTERACTION BETWEEN THROMBIN AND FIBRINOGEN

Initial mixture	First addition	Second addition	Clotting time (sec)
Thrombin + buffer	Buffer	Fibrinogen	12.4
Thrombin + CFP	Buffer	Fibrinogen	16.1
Thrombin + buffer	Buffer + CFP	Fibrinogen	19.4
Fibrinogen + CFP	Buffer	Thrombin	18.3
Fibrinogen + buffer	Buffer + CFP	Thrombin	17.0

Note. In step 1, 0.1 ml purified human thrombin (Fenton, 200 U/ml) or purified human fibrinogen (Imco, 8 mg/ml) was incubated for 1 min at 37° with 0.1 ml chlorazol fast pink (1.6 mg/ml) or buffer. In step 2, 0.6 ml buffer or, in order, 0.5 ml buffer and 0.1 ml chlorazol fast pink (CFP, 1.6 mg/ml), was added to the initial mixture. In step 3, 0.2 ml aliquots of these mixtures was added to 0.1 ml fibrinogen (2 mg/ml) or 0.1 ml thrombin (50 U/ml), respectively, and the clotting time was measured at 37°. The clots formed in the absence of chlorazol fast pink were firm, while those found in the presence of the dye were fragmentary. The results shown are the average of duplicate determinations.

TABLE IV. THE THROMBIN TIME OF FIBRINOGEN TREATED WITH CHLORAZOL FAST PINK (CFP) AND THEN SEPARATED FROM THE DYE

Test mixture	Clotting time (sec)
(A)	
Fibrinogen + buffer	9.5
Fibrinogen + CFP	>60
(B)	
Reprecipitated [fibrinogen + buffer]	9.8
Reprecipitated [fibrinogen + CFP]	7.6

Note. In A, 0.1 ml partially purified human fibrinogen (2.6 mg coagulable protein/ml) was incubated at 37° with 0.1 ml buffer or chlorazol fast pink (2.5 mg/ml buffer) for 1 min. 0.1 ml crude bovine thrombin (15 U/ml) was added and the clotting time was measured at the same temperature. In B, 3 ml of fibrinogen was mixed with 3 ml of buffer or chlorazol fast pink (2.5 mg/ml), and the fibrinogen was reprecipitated at −3° by slow addition of 95% ethanol to a concentration of 8%. After 30 min, the precipitates were separated by centrifugation at 900*g* for 10 min at −3°, resuspended in 30 ml/1 *M* glycine in 4% sodium citrate dihydrate and 6% ethanol, and recentrifuged. The washing procedure was repeated a total of 14 times. The final precipitate was suspended in 2 ml barbital–saline buffer and dialyzed against the same buffer overnight. 0.2-ml samples of the washed precipitates were then incubated at 37° for 1 min after which 0.1 ml thrombin (15 U/ml) was added and the clotting time was measured at the same temperature.

ticoagulant action on whole blood. This observation was exploited by Brambell and Parks (2) to preserve the fluidity of blood during perfusion experiments, and for a few years, azo dyes enjoyed a vogue as anticoagulants in experimental animals. Among various dyes tested, Huggett and Rowe (4) found that Chicago blue 6B and the closely related compound chlorazol fast pink were the most effective.

Huggett (3−6) believed that the anticoagulant dyes interfered with the formation of thrombin and with the subsequent effect of thrombin upon fibrinogen. The action of the dyes might seem to be analogous to that of heparin, with which it had some structural similarities, notably the presence of sulfonate radicals (24). In the present studies, the inhibition of the interaction of thrombin and fibrinogen was readily confirmed, but this did not seem to be due to a heparin-like property, as it could be demonstrated in the apparent absence of antithrombin III, and the dye inhibited the coagulant properties of Reptilase R. The dye also inhibited interaction of thrombin upon AHF, but at least part of this action appeared to be upon AHF itself. Although this conforms with the earlier idea that chlorazol fast pink inhibited both the formation and action of thrombin, no impairment of conversion of prothrombin to thrombin was observed in whole blood. The action of the dye was inhibited by protamine sulfate, as though the negatively charged sulfonate groups of chlorazol fast pink had been neutralized.

The molecular mechanisms responsible for the anticoagulant properties of chlorazol fast pink were only partly defined in the present experiments. The dye interfered with the release of fibrinopeptides from fibrinogen, and with the thrombin-induced augmentation of AHF-like coagulant activ-

TABLE V. THE EFFECT OF FILTRATION OF CHLORAZOL FAST PINK-TREATED THROMBIN THROUGH SEPHADEX G-25

Sample tested	Clotting time	
	CFP-free thrombin (sec)	CFP-treated thrombin (sec)
Unfiltered thrombin	7.6	28.9
Filtrate		
1st ml	>100	>100
2nd ml	9.9	10.8
3rd ml	49.9	40.8
4th ml	>100	>100

Note. 0.9 ml purified human thrombin (Sigma, 45 NIH U/ml) was mixed with 0.1 ml chlorazol fask pink (CFP, 10 mg/ml buffer) or with buffer and filtered through 13 × 16-mm columns of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with buffer, collecting 1-ml samples in 12 × 75-mm polystyrene tubes. 0.1 ml of the samples applied or of each sample of filtrate was mixed with 0.2 ml partially purified human fibrinogen (6 mg/ml) and the clotting time was measured at 37°.

TABLE VI. THE EFFECT OF CHLORAZOL FAST PINK ON THE THROMBIN TIME OF DYSFIBRINOGEMIC PLASMA

Plasma	Thrombin time		
	Buffer (sec)	Chlorazol fast pink (sec)	Ratio CFP/buffer
(A)			
Normal	14.3	27.8	1.94
Fibrinogen Cleveland I	31.0	57.3	1.85
Fibrinogen Cleveland II-a	26.0	57.7	2.22
(B)			
Normal	9.5	15.7	1.65
Fibrinogen Cleveland I	18.4	30.9	1.68
Fibrinogen Cleveland II-b	24.3	49.2	2.02

Note. 0.2 ml plasma was mixed with 0.1 ml chlorazol fast pink (4 mg/ml) or buffer and incubated at 37° for 1 min in 10 × 75-mm glass tubes. 0.1 ml thrombin was then added and the clotting time was measured at 37°. In A, the concentration of thrombin in the final mixture was 1 NIH U/ml, and in B, 2 NIH U/ml. Two different subjects with fibrinogen Cleveland II, designated a and b, were tested.

ity, but whether this reflected an action upon thrombin, upon its protein substrates, or upon their interaction was not clarified. In part this uncertainty was due to the reversibility of the anticoagulant action of chlorazol fast pink.

The apparent decrease in titer of AHF when this factor was incubated with dye alone makes it likely that chlorazol fast pink reacted with this substrate of thrombin. That this was also true of fibrinogen was evident from the observation that chlorazol fast pink appeared to influence the quality, if not the rate, of fibrin aggregation. The clots that formed in the presence of dye were often fragmentary even when the dye had been added after the release of fibrinopeptides. Thus, evidence was obtained for an interaction of dye and two substrates of thrombin. We could not rule out an additional action upon thrombin itself because the dye precipitated synthetic substrates.

The defect in fibrin formation induced by chlorazol fast pink was observed not only with normal plasma but also with plasmas of individuals in whom fibrinopeptide release and fibrin monomer aggregation were defective. Interpretation of these experiments is clouded because the individuals studied were heterozygotes, and had substantial amounts of normal fibrinogen in their plasmas.

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