The Effects of Radio-Opaque Contrast Media on the Structure, and Solubility of the Fibrin Clot

By Karl Verebely, Henn Kutt, Richard M. Torack and Fletcher McDowell

WATER-SOLUBLE radio-opaque contrast media (Fig. 1) are being used with increasing frequency in clinical medicine for diagnostic procedures such as cerebral or cardiac angiography and intravenous pyelography. It has been found that the radio-opaque chemicals interfere with blood coagulation. In the presence of these compounds the clotting mechanism in plasma and

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Supported in part by Research Grants HE-04872 and NB-03346, NB-04161 from USPHS and from the Winthrop Laboratories. Dr. Kutt is a recipient of a Research Career Development Award IKO 3 NB 35003, NINDB, USPH.

First submitted October 2, 1968; accepted for publication December 13, 1968.

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fibrinogen solutions was disturbed, the clotting time was delayed and the clots that formed were less opaque than the normal control. When the concentration reached a certain magnitude which was different for each compound, no clotting at all took place.

Decreases in the opacity of clots have been thought to be related to changes in ionic strength, temperature, pH, thrombin concentration and a variety of inhibitors. These factors were considered as leading to structural changes which were manifested in reduced optical density of the clots.

In this study direct experimental evidence is presented indicating that the presence of radio-opaque contrast media causes structural changes in fibrin and plasma clots, and that the contrast chemicals interfere with normal clot formation at or before the stage of lateral aggregation of the longitudinally polymerized fibrin fibers. The concentrations of contrast media used in this study (0.25 to 40 per cent by volume) were below or within the range of those observed in patients during cerebral arteriography (22 to 65 per cent by volume).

Materials and Methods

The effects of the following compounds on plasma and fibrin clot formation were investigated: Sodium diatrizoate (Hypaque 50 per cent, Winthrop Laboratories); Meglumine iothalamate (Conray 60 per cent, Mallinckrodt Pharmaceuticals); iodopyracet (Diodrast 50 per cent, Winthrop Laboratories); sodium acetrizoate (Urokon Sodium 50 per cent, Mallinckrodt Pharmaceuticals). Glycine methylester and glycine ethylester (Mann Research Laboratories, N.Y.C.) were used as inhibitors of the Fibrin Stabilizing Factor (FSF).

A. Observation of the Effect of Contrast Media on the Plasma Clot Formation

1. Phase contrast microscopy. Five ml. of blood were collected in a glass centrifuge tube containing 9 mg. EDTA (Na₂), then centrifuged at 2000 RPM for 3 minutes and the plasma removed. To 0.1 ml. of the plasma, 0.02 ml. of 0.6 M contrast media was added. A control mixture contained 0.1 ml. of plasma and 0.02 ml. of 0.6 M Na Cl. Clot formation in the above systems was activated by 0.1 ml. of thromboplastin (Simplastin, Warner Chilcott Laboratories). The thromboplastin used was prepared in the following manner. Fifty milligrams of lyophilized simplastin was reconstituted with 4 ml. of distilled water and centrifuged at 1000 rpm, for 5 minutes. The resulting supernatant was free of most suspended large particles which would interfere with the phase contrast microscopic visualization of fibrin formation either under normal conditions or in the presence of contrast media.

The pH values were measured in similarly prepared large-volume samples containing 2.0 ml. of plasma, 2.0 ml. of thromboplastin and 0.4 ml. 0.6 M Na Cl for the control with a Beckman Expandomatic pH meter. Clot formation was viewed through a Carl Zeiss Standard GF research microscope with a phase-contrast attachment (condenser II, and phase objectives 10 X, 25 X and 100X). Still photomicrographs were taken after the fibrin clot formation up to 30 minutes with an Exacta VX IIa, 35 mm., single lens reflex camera using, Kodak plus X or Kodachrome II professional color films (35 X 24 mm.).

2. Electron microscopic observations. Normal and abnormal clots using sodium diatrizoate as the contrast medium were prepared as in part 1. Ten minutes later these clots were fixed in 1 per cent OsO₄ buffered to pH 7.2-7.4 with Dalton’s Chromate. The clots were then dehydrated in a graded series of alcohols, and embedded in Epon. Thin sections were stained with a saturated solution of uranyl acetate or with lead citrate and examined with an RCA EMU 3-G electron microscope.

B. Optical Density Measurement of Fibrin Clots with and without Contrast Media

In two sets of spectrophotometer cuvettes, 2.5 ml. of 700 mg. per cent bovine fibrinogen.
Fig. 2.— (A and B) Normal control clots. 1.0 ml. plasma + 0.2 ml. saline + 0.1 ml. simplastin Magnification: 1280×. (C) Clot formed in the presence of sodium diatrizoate. 1.0 ml. plasma + 0.2 ml. sodium diatrizoate + 0.1 ml. simplastin Magnification: 1280×. (D) Clot formed in the presence of meglumine iothalamate. 1.0 ml. plasma + 0.4 ml. meglumine iothalamate + 0.1 ml. simplastin. Magnification: 1280×, phase contrast microscopy.

(Fraction I, Mann Research Laboratory) was mixed with increasing volumes of 0.6 M contrast media (0.25 ml.; 0.5 ml.; 1 ml.; 2 ml.; 3 ml.; 4 ml.; 5 ml.), and 0.6 M NaCl was used in similar volumes for a control. These mixtures were activated by 1 ml. of 0.2 per cent bovine thrombin. (Mann Research Laboratory). The optical densities of 60 minute clots were measured at 360 μm in a Coleman Junior spectrophotometer.

C. Protein Content of Clots

Protein content was determined by the Folin-Ciocalteau’s method modified by Dr. Nils U. Bang. In this method the clots which formed in 60 minutes were washed three times for 5 minutes in 10 ml. normal saline to free them from most nonfibrin proteins. After the final wash the excess fluid was removed from the clot by pressing it with filter paper. The clot then was placed in a test tube to which 1 ml. of 2.5 N NaOH was added and boiled for 10 minutes in a water bath. After cooling, 4.6 ml. Glycine buffer (0.163 M, pH 12+), 0.8 ml. distilled water and 1.5 ml. 5 per cent trichloracetic acid were added. The test tubes were thoroughly mixed and 1.5 ml. diluted phenol reagent (1:3) was added (Fisher Scientific Co.). After standing 15 minutes the samples were read against a reagent blank at 650 μm in a Coleman Junior spectrophotometer. The values obtained were expressed as a ratio between the clot protein content determined from the test samples and standards (1 mg and 5 mg) of bovine fibrinogen (Fraction I).

D. The 30 per cent Urea Solubility of Plasma Clots

Fresh anticoagulated plasma (preparation described in section A1) was used. The test mixture contained 4 ml. plasma, 0.6 ml. of 0.6 M contrast media; the FSF inhibitor system contained 4 ml. plasma, 0.6 ml. of 0.6 M glycine methyl and ethylesters; and the control system contained 4 ml. plasma and 0.6 ml. of 0.6 M NaCl. All mixtures were activated by 0.1 ml. of thromboplastin. Two identical sets of the above reaction systems were prepared and clot formation was permitted to continue for 60 minutes. One set of clots was imme-
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Fig. 3.—(A and B) Aciform structures formed in the presence of sodium acetrizoate. Magnification: (A) 3200×; (B) 1280×. (C and D) Amorphous protein masses and aciform structures formed in the presence of sodium acetrizoate. 1.0 ml. plasma + 0.2 ml. sodium acetrizoate + 0.1 ml. simplastin Magnification: 1280×. Phase contrast microscopy.

Equimolar Na Cl (0.78 M) was compared with 50 per cent sodium diatrizoate (0.78M) to determine whether the high molar concentration or the specific chemical constitution of the compounds was responsible for the reduced optical density and the abnormal morphologic appearance of the fibrin clot.

RESULTS

I. Clot Formation in the Presence of Contrast Media

The coagulation process was altered to various degrees depending on the type and concentration of the contrast medium present in the reaction mixture. When visualized under the phase contrast microscope the increasing concentrations of the contrast chemicals caused increasing morphologic distortion of the clot. Samples containing 17–37 per cent contrast media by volume showed protein aggregates (Fig. 2), occasional aciform structures, or both, among the reduced amount of fibrin fibers (Fig. 3). At low concentrations, e.g., 2–5 per cent contrast media by volume, the normal fibrin network was visible but the control mixture contained an optically denser fibrin clot.

The addition of contrast media did not greatly alter the hydrogen ion concentration of the clotting mixtures. The pH values of the reaction mixtures ranged between 7.18 and 7.35 (Table 2).

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Table 1.—Gross and Microscopic Changes Observed in the Presence of 0.78 M Na Cl and 0.78 M Na Diatrizoate Compared to the Control

<table>
<thead>
<tr>
<th>Group</th>
<th>Reaction Mixture</th>
<th>Appearance of Clot</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.02 ml Sodium diatrizoate (0.78M)</td>
<td>Microscopic: small amounts of fibers occasional protein masses and aciform structures. Gross: Transparent clear clot.</td>
</tr>
<tr>
<td></td>
<td>0.1 ml Plasma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 ml Simplastin</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.02 ml NaCl (0.78M)</td>
<td>Microscopic: Normal fibrin network. Gross: Dense opaque clot.</td>
</tr>
<tr>
<td></td>
<td>0.1 ml Plasma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 ml Simplastin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02 ml Normal Saline</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 ml Plasma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 ml Simplastin</td>
<td></td>
</tr>
</tbody>
</table>

The reaction mixtures were prepared as presented in Table 1. The Na Cl-plasma clot (Group B) showed neither reduced optical density nor morphologic distortion although the preparation had the same molar concentration as the sodium diatrizoate plasma mixture (Group A).

II. Ultra Structure of Clots

Electron micrographs of normal fibrin clots revealed a periodic, striated fiber structure with a fairly uniform width of 100 mµ (Fig. 4a and 4b). Similar micrographs of clots formed in the presence of sodium diatrizoate revealed virtually no fibers of this type. The fibers were always thinner than 100 mµ with the thinnest fibers being about 50Å in width (Fig. 5a). The thicker fibers had occasional gaps in their continuity and at this point the ends had a frayed appearance (Fig. 5b). The smaller fibers assumed irregular shapes (Fig. 5c) which were quite different from the linear orientation of normal fibers. Some periodic structure however could still be seen in these fibers.
Fig. 5.—Clot containing $6.0 \times 10^{-2}$M sodium diatrizoate. (a) An electron micrograph of a clot in which numerous fibers of varying width are present. $\times 40,000$. (b) An abnormal fiber showing an interruption with a frayed appearance at this point. $\times 58,000$. (c) An electron micrograph of a thin fiber with a greatly irregular shape but having a poorly defined periodic structure (Arrow). $\times 46,000$. (d) An intertwined mass of very thin fibers, some of which measure about 50Å, with virtually no lateral aggregation. $\times 42,000$.

The micrographs always contained numerous extremely thin longitudinally polymerized fibers which showed no tendency for lateral aggregation (Fig. 5d).

III. Optical Density of Clots

The fact that the presence of contrast media reduced the optical density of the clot, led us to study the relationship between optical density and the quantity of fibrinogen incorporated into the fibrin clot. Figures 6 and 7 show the effect of all compounds tested. At a contrast media concentration of 5 per cent by volume ($3.0 \times 10^{-2}$M), most of the fibrinogen was incorporated into the fibrin clot (Fig. 6). Conversely at the same concentration all of the agents except sodium acetrizoate caused considerable reduction in optical density (Fig. 7). At a concentration of 10 per cent by volume ($6.0 \times 10^{-2}$M) of contrast media the optical density of all clots was reduced below 18 per cent of
Fig. 6.—Protein content of clots in the presence of increasing concentrations of various contrast media.

Fig. 7.—Optical density of clots with increasing concentrations of various contrast media. At each dilution the OD of clots formed in the presence of contrast media was calculated as the percentage of the equimolar NaCl control's OD.

the equimolar NaCl control, yet the incorporation of fibrinogen was considerably lower only with iodopyracet. These results showed that a reduction in the fibrin clot’s optical density was not paralleled by a decrease in fibrin monomer incorporation into the clot’s structural protein. An extreme example of this
Protein content of clot

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Fig. 8.—Protein content and optical density of clots formed in the presence of meglumine iothalamate in increasing concentrations.

Table 2.—Urea Solubility of Clots Formed in the Presence of Contrast Media, FSF Inhibitors and Sodium Chloride Controls

<table>
<thead>
<tr>
<th>Chemical agent in the reaction mixture.</th>
<th>Protein Content (mg.) of 60 min. clot</th>
<th>Protein Content (mg.) of 60 min. clot after 24 hrs. in 30% urea</th>
<th>% of fibrin protein dissolved in pH of reaction mixture</th>
<th>pH of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na diatrizoate 0.6M</td>
<td>1.0</td>
<td>1.03</td>
<td>0</td>
<td>7.2</td>
</tr>
<tr>
<td>Meglumine iothalamate 0.6M</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>7.30</td>
</tr>
<tr>
<td>Metaglucamine diatrizoate 0.6M</td>
<td>1.03</td>
<td>1.02</td>
<td>0</td>
<td>7.22</td>
</tr>
<tr>
<td>Glycine Methyl Ester 0.6M</td>
<td>0.83</td>
<td>0.0</td>
<td>100</td>
<td>7.20</td>
</tr>
<tr>
<td>Glycine Ethyl Ester 0.6M</td>
<td>0.82</td>
<td>0.0</td>
<td>100</td>
<td>7.23</td>
</tr>
<tr>
<td>Na Cl 0.6M</td>
<td>0.93</td>
<td>0.94</td>
<td>0</td>
<td>7.25</td>
</tr>
<tr>
<td>Normal Saline</td>
<td>0.90</td>
<td>0.90</td>
<td>0</td>
<td>7.20</td>
</tr>
</tbody>
</table>

* Each milligram value represents the mean of at least four determinations and the S.D. was less than ±0.04 mg in each.

nonparallel relationship is shown in Figure 8. In the presence of meglumine iothalamate the fibrin incorporation into the clot hardly changed with the increase in contrast medium concentration; however the optical density of the clot decreased rapidly.

IV. Urea Solubility

Plasma clots formed in the presence of sodium diatrizoate, methylglucamin diatrizoate and meglumine iothalamate were found insoluble in a solution of 30 per cent urea (Table 2). The stability of these clots in 30 per cent urea demonstrated that the Fibrin Stabilizing Factor (FSF) acted upon the altered fibrin polymers. The enzyme FSF has been known to catalyze covalent bond
formation in the last step of fibrin polymerization. The plasma clots formed in the presence of glycine methyl and ethyl esters, however, totally dissolved. The control clots which contained normal saline or 0.6 M NaCl in their clotting mixture were also stabilized by FSF and did not dissolve in 30 per cent urea.

**Discussion**

The physical and chemical characteristics of fibrin clots appeared to be significantly altered by the presence of various types of contrast media.

Abnormal protein aggregates were observed with the use of light microscopy mainly at contrast medium concentrations of 9 to 37 per cent by volume. It is probable that these protein masses formed from intermediate fibrin polymers which were at different stages of polymerization and aggregated together in an altered manner. The aciform structures were believed to be precipitated fibrinogen molecules which were quite different in appearance from the crystals formed from contrast media.

Electron micrographs of a clot formed in the presence of sodium diatrizoate showed that numerous very thin and intermediate sized fibers were present but that fibers of normal thickness were not found. Since these small fibers still exhibited some periodicity, the chief structural abnormality appeared to be the inability of these fibers to join the main fibrin bundle either before or at the stage of lateral aggregation. This change was very consistent with the finding that a reduced optical density of the clot preceded the diminished fibrin monomer incorporation.

Increased concentration of contrast medium caused a sudden decrease in the optical density of the clots until no clot formed. This suggested that with higher contrast medium concentrations fewer primary fibrils were incorporated into the clot's structure. However, the fibrinogen incorporation curve did not parallel the sudden drop of the optical density curve, in that fibrin monomer incorporation started to decrease only at the highest concentrations of contrast media used. This indicated that at low contrast medium concentrations fibrin polymerization was not blocked but the structural arrangement of the clot was altered. Higher concentrations of contrast media caused further interference with fibrin monomer incorporation into polymerized fibrin as evidenced by the reduction in the clot's protein content.

Several investigators have shown chemical interference with normal fibrin formation. Edsal demonstrated that the structural properties of fibrin are greatly modified by variations of pH, ionic strength of chemical agents and other factors. He made observations on the gross structure of fibrin clot. The presence of iodide, thiocyanate, urea and glucose resulted in the formation of a fine transparent clot grossly similar to those we observed in the presence of contrast media. Edsal described a coarse opaque control clot similar to those that were formed in our control samples.

Our reaction mixtures were prepared with pH values ranging between 7.18 to 7.35. Although Edsal proposed a pH of 6.3 as optimal for fibrin formation in his reaction mixture, we found that our systems at pH 7.25 provided good control clots with thick fibers and high optical density. We believe that the pH values of our test mixtures, which were very close to those of the control,
were not responsible for the altered spatial arrangement of the fibrin polymers.

The experiments conducted with NaCl solution equimolar to the contrast media suggested that high ionic concentration alone is not responsible for the marked morphologic distortion observed in the presence of meglumine iothalamate and other contrast media. Some specific property of the contrast chemicals rather than their high molar concentration seems to interfere with the normal fibrin polymerizing process.

Electrophoresis studies from this laboratory demonstrated that contrast media can reversibly bind plasma proteins and change their electrophoretic mobilities. Since contrast media are capable of binding proteins, it is possible that they also reacted with fibrinogen or enzymes which catalyze fibrin polymerization.

Lorand and Jacobsen introduced glycine methyl and ethyl esters as inhibitors of FSF. In the presence of such inhibitors the covalent bond formation or stabilization of the plasma clot was prevented; thus the clots that formed were soluble in 30 per cent urea or in 1 per cent monochloroacetic acid.

We performed 30 per cent urea solubility tests to see whether the abnormal fibrin structures formed in the presence of contrast media were acted upon by FSF and thus stabilized, or became freely soluble as in the presence of glycine ethyl and methyl esters. The results of such experiments would also shed light upon the ability of FSF to discriminate altered polymer structures present in the abnormal clots. We found that the clots formed in the presence of three different contrast media were insoluble in 30 per cent urea. This indicated that the fibrin stabilizing enzyme could not differentiate between the regular fibrin structure of the normal control clot and the abnormally polymerized clots formed in the presence of contrast media. However, it is possible to postulate that only the arrangement of fibrin monomers was altered and the sites at which fibers joined each other remained unchanged in the abnormal polymer. As a result, FSF catalyzed covalent bond formation as it does in normal plasma clots.

**Summary**

Radio-opaque contrast media interfered with the normal process of fibrin and plasma clot formation. The morphologic changes included a decrease of the fibrin fiber diameter and an increase of protein aggregates. These appeared amorphous in the light microscope and showed a linear relationship with the concentration of contrast agents in the reaction mixture. Above a threshold concentration, which was different for each contrast media, no clot formed. With increasing concentrations of contrast medium between $3.5 \times 10^{-3}$M and $2.0 \times 10^{-2}$M, the optical density of the clots decreased rapidly while the incorporation of fibrin monomers into the polymer structure of the clots stayed within normal limits. This indicated that the spatial arrangements of the clots was altered. Electron micrographs confirmed that the fibrin fibers were reduced by 70 per cent to 90 per cent of the control fiber diameter in the presence of $6.1 \times 10^{-2}$M sodium diatrizoate. The fibrin stabilizing enzyme (FSF, LLF or Factor XIII) catalyzed covalent cross links despite the structural changes in the clot.
Esseva trovate que substantias de contrasto a radio-opacitate interfereva in le processo normal del formation de coagulos fibrinic e plasmatic. Le alterationes morphologic includeva un declino del diametro del fibras de fibrina e un augmento del aggregatos de proteina. Iste ultimes appareva amorphe in le microscopio optic e monstrava un relation lineari con le concentration del agents de contrasto in le mixtura de reaction. Supra un concentration liminal, le qual differeva pro le varie substantias de contrasto, nulle coagulo se formava. Con crescente concentrationes del substantia de contrasto inter 3,5 × 10⁻³ e 2,0 × 10⁻² M, le densitate optic del coagulos declinava rapidemente durante que le incorporation de monomerode fibrina ad in le structura polymeric del coagulos se teneva intra limites normal. Isto indicava que le disposition spatial del coagulos esseva alterate. Micrographias electronic confirmava le facto que le fibras de fibrina esseva reducite in lor diametros per 70 a 90 pro cento in comparation con le valores de controbo in le presentia de 6,1 × 10⁻² M diatrizoate de natrium. Le enzyma de stabilisation de fibrina (FSF, LLF, o Factor XIII) catalysava covalente nexos transversal in respecto del alterationes structural in le coagulo.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. Koloman Laki for his advice and criticism in the preparation of this manuscript.

REFERENCES

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