STUDIES ON THE PATHOGENESIS OF ACUTE INFLAMMATION

III. THE FAILURE OF ANTICOAGULANTS TO PREVENT THE LEUCOCYTIc STICKING REACTION AND THE FORMATION OF SMALL THROMBI IN RABBIT EAR CHAMBERS DAMAGED BY HEAT

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PLATE 50

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The mechanism responsible for the sticking of white blood cells to blood vessel walls in early inflammation continues to pose a challenging problem since this reaction represents the key to an overall understanding of the exudative events set off by injury.

In studies reported earlier, interference with the clotting of blood in vivo failed to prevent the vigorous sticking of leucocytes to vascular endothelium within rabbit ear chambers damaged by heat. As a specific example, maximal activation of the endogenous fibrinolytic system of rabbits by streptokinase (SK) administration did not diminish adherence of circulating white blood cells to endothelium. Since lysis of fibrin in vitro was brisk, failure to modify the sticking reaction could be explained only by assuming that destruction of fibrin on the surfaces of both white cells and endothelium failed to keep pace with newly formed material. In a more direct approach to the problem, the behavior of white cells in damaged chambers was followed after removal of all chemically detectable fibrinogen from the systemic circulation. Even under these conditions, when fibrinogen levels were too low to measure, brisk leucocytic sticking still developed after ear chambers had been damaged by heat (1). From these data it was concluded that the sticking reaction was not causally related to formation of fibrin.

Such consistent failure to halt white cell sticking led to an investigation of specific pharmacological substances known to block the clotting of blood in vivo. From preliminary observations previously reported, it was anticipated that one such agent, heparin, would materially impede the reaction (2). Although many anticoagulants were available, heparin and warfarin sodium were selected because of extensive use by others; both were of low toxicity in the ranges used; and because each could be administered parenterally to insure adequate blood levels. Furthermore, both agents were known to prevent not only the local but also the generalized Shwartzman reac-

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tion; a lesion in which leucocytic sticking figures prominently (3, 4). This as well as other evidence has been regarded as support for the contention that polymerization of fibrinogen to fibrinoid is essential for development of the Shwartzman lesion (5). Unfortunately, the means by which these agents block fibrinoid formation have not been exactly defined, nor has a direct relationship been demonstrated between fibrinoid formation and leucocytic sticking.

In general, the relatively non-specific antiproteolytic properties inherent in heparin and warfarin sodium were thought responsible for their anticoagulant effect. Warfarin sodium interferes with synthesis of prothrombin by hepatic cells; the resultant deficiency of this enzyme in turn impairs conversion of fibrinogen to fibrin (6). On the other hand, heparin is more complex since it acts not only as an antithrombin but also is reputed to modify the stability of fibrinogen in vivo (7). With reference to the last point, when complex sulfonated polysaccharidal polymers structurally similar to heparin were given to animals shortly after endotoxin administration, they not only impaired the clotting of blood but at the same time enhanced polymerization of fibrinogen to either fibrinoid or fibrin in vivo (8). In a similar fashion, when heparin was administered in vivo after endotoxin, it caused a partial polymerization of fibrinogen in vitro when plasma was cooled to 4°C. On warming of such plasma, however, polymerization of fibrinogen not only stopped but was reversed; the material formed in the cold was eventually restored to a stable suspensoid state (9).

Despite lack of a clear appreciation of the mechanisms involved in their action, the influence of heparin and warfarin, both alone and in combination, on the leucocytic sticking reaction in rabbit ear chambers injured by heat was investigated extensively. The following account defines this experience and describes our inability to demonstrate an inhibitory action by anticoagulants on the sticking of white cells to endothelium during acute inflammation. Furthermore, totally unexpected phenomena related to heparin administration and the development of platelet and fibrin-like thrombi were encountered.

**Methods**

White male rabbits weighing 2 to 3 kg of mixed New Zealand-Flemish stock were obtained from Eldridge Rabbitry, St. Louis. Animals were used for only one experiment, and aside from penicillin and streptomycin, received no injections. Plastic ear chambers of the Sandison type but modified as noted previously were employed for microscopic studies (2). Only ear chambers that contain thin, fully mature connective tissue devoid of active inflammation were used for experiments. No ear chambers were employed a second time. Microscopic equipment was as recorded before (2, 10).

Heparin sodium, in 400 mg sterile ampules suitable for intravenous administration was used as indicated in the text. Warfarin sodium (supplied as coumadin sodium) in bulk powdered form was prepared in concentrations of 30 mg/ml in sterile water for intravenous administration. Variadase, data pertaining to enzyme content may be found with experiments.

1 Generously supplied by the Upjohn Company, Kalamazoo, Michigan.
2 Endo Laboratories, Inc., Richmond Hill, New York, kindly provided this agent.
3 Dr. J. M. Ruegsegger, Lederle Medical Research Department, American Cyanamid Company, Pearl River, New York, furnished this material.
mixed in pyrogen-free 5 per cent dextrose in water (Baxter Laboratories) was administered by continuous intravenous infusion at a rate of 25 ml per hour by a technique described earlier (1). Preparation and administration of both thromboplastin and thrombin were as noted before (1).

Clotting times were measured by the 3 tube Lee-White method using blood from ear veins obtained in syringes rinsed with isotonic saline to minimize air leakage and bubble formation. One stage prothrombin times were determined by the method of Quick (11). Blood for prothrombin times was drawn from ear veins into syringes containing 0.1 molar sodium oxalate for anticoagulation; 0.1 ml oxalate was mixed with 0.9 ml blood. Death from blood loss following cardiac puncture occurred so frequently in anticoagulated animals that the approach was not used for bleeding. Ample samples of blood were always obtained by venipuncture when the ear was prewarmed by a jet of hot air.

All substances for intravenous use were screened for content of bacterial pyrogens by methods described previously (1). Whenever possible, exceptions are noted in text, pyrogenic substances were avoided. All glassware and needles were siliconed with desicote (Beckman Instruments, Inc.) and rendered pyrogen-free by sterilizing in dry heat of 170°C for 2 hours.

The method of Sailer and Newhouse was used for chemical measurements of fibrinogen levels except that rabbit fibrin was used as the reference standard (12). It was found that firm clots could be obtained, even after massive heparinization, by adding only thrombin to samples of plasma and it was not necessary to use protamine sulfate. Crystalfine soy bean trypsin inhibitor (Worthington Biochemical Corp.) solution was prepared, 0.01 gm dissolved in 2.5 ml buffered isotonic saline, to counteract proteolytic activity in blood samples from animals receiving varidase; 0.05 ml stock solution was added to 1.0 ml blood.

Electrophoresis of serum and plasma samples was performed on paper with the Spinco model RD-2 electrophoresis apparatus at room temperature using a veronal buffer of pH 8.6 with an ionic strength of 0.075. A few electrophoretic patterns were determined with a veronal buffer of pH 8.6 and an ionic strength of 0.1 at 37°C with runs of 16 hours as described by Smith and Von Korff (13).

Titration to determine the quantity of protamine sulfate required to counteract in vivo heparin were performed by the method of Alien (14). Protamine sulfate in sterile ampules containing 10 mg/ml in sodium chloride solution suitable for intravenous administration was obtained from Eli Lilly and Company.

Antibody against rabbit fibrin was prepared by immunizing mature Rock Island hens intravenously with a partially purified preparation of rabbit fibrinogen (fraction I, Lot No. 6804, Pentex Laboratories). The fibrinogen was clotted, washed first in sterile isotonic saline and then in distilled water to remove as much occluded protein as possible. The resultant fibrin was fragmented in a Ten Broeck tissue grinder and resuspended in saline at a concentration of 15.7 mg/ml; merthiolate (thimerosal, Lilly) was added in a concentration of 1 in 5,000 as preservative. After immunization was completed, the chickens were bled aseptically from wing veins; harvested serum was found to contain precipitating antibody to a number of different rabbit serum proteins as demonstrated in Ouchterlony plates. However, by repeated absorption with small amounts of rabbit serum, it was possible to remove the bulk of cross-reacting precipitins leaving only a small amount of antibody against rabbit gamma globulin and a substantial quantity of precipitin for fibrinogen. The use of this material for detecting small quantities of fibrinogen in experimental plasma will be discussed in the appropriate sections.

Cameras and photographic conditions were as described previously (2, 10). Still photographs were made with Kodak ektachrome color reversal film, type F. Ansco ansochrome color film tungsten (3400kv), was used for motion picture records.
RESULTS

A. Heparin Studies:

1. Effects of Large Doses of Heparin.—In order to obtain evidence for a clear anticoagulant effect of heparin, very large amounts were employed initially. In early experiments, 400 mg heparin sodium given by ear vein over a period of 2 to 3 minutes produced a slight rise in temperature; on the average, less than 0.5°C when tested in 5 rabbits. Likewise, peripheral leucocytic counts were consistently although only modestly increased several hours after administration of heparin but there was no preceding leucopenic phase. Although this was regarded as evidence that the heparin was contaminated by a bacterial pyrogen, the physiological effects were not considered drastic enough to preclude use in ear chamber studies.

This amount of heparin greatly increased clotting times during the first 9 hours after administration. Furthermore, blood usually did not clot when drawn 12 hours later but after 24 hours, clotting times became either near normal or, as in 3 of 5 rabbits, shorter than control values (Table I). Also note that fibrinogen values were not altered by this amount of heparin.

LeQuire et al. reported similar results after administering smaller amounts of heparin to rabbits (15).

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### TABLE I

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Test</th>
<th>Time venous blood obtained after heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control*</td>
</tr>
<tr>
<td>M-447</td>
<td>Clotting time (min.)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen (mg %)</td>
<td>784</td>
</tr>
<tr>
<td>M-410</td>
<td>Clotting time (min.)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen (mg %)</td>
<td>472</td>
</tr>
<tr>
<td>M-478</td>
<td>Clotting time (min.)</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen (mg %)</td>
<td>472</td>
</tr>
<tr>
<td>M-477</td>
<td>Clotting time (min.)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen (mg %)</td>
<td>629</td>
</tr>
<tr>
<td>M-462</td>
<td>Clotting time (min.)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen (mg %)</td>
<td>390</td>
</tr>
</tbody>
</table>

* Control obtained approximately 15 minutes before heparin administered.
‡ N.C., no clot.
Electrophoresis of plasma after administration of heparin revealed a substantial increase in mobility of fibrinogen. Although the nature of this effect of heparin on fibrinogen was not investigated, it indicated a significant chemical as well as electrical change in the molecule as a result of the anticoagulant. Serious hemorrhage from venipuncture sites and minor abrasions was a constant experimental hazard and occasionally caused death 12 hours or more following heparin administration but after acute observations had been concluded.

As soon as 400 mg of heparin was found to be well tolerated, it was given to rabbits with fully mature ear chambers. Although there was no consistent influence on either velocity or volume of ear chamber circulation, occasionally the volume of blood flow decreased immediately after heparin administration. This hemodynamic change sometimes lasted for several hours and seemed to coincide with the peripheral vasoconstriction that preceded the modest fever caused by heparin. Brisk flow of blood returned 2 to 3 hours later after temperature elevations either reached a plateau or started to subside. In no instance was the circulatory disturbance sufficient to prevent microscopic study of ear chambers. Similarly, the number of circulating leucocytes was not detectably altered in the uninjured preparation. In short, heparin failed to modify either the circulatory dynamics or the behavior of intravascular formed elements within uninjured ear chambers.

After injury, the administration of heparin was associated with several events that were not anticipated. In keeping with preliminary evidence cited previously, it was expected leucocytic sticking might be prevented (2). However, following a detailed re-examination of white cell behavior, it was found that heparin did not block adherence of white blood cells to endothelium after local heat injury (Figs. 1 and 2). Rarely, intense sticking was delayed in onset, sometimes for 2 or 3 hours. Such occasional retardation of heavy white blood cell sticking could best be related to the reduced volume of peripheral blood flow attending fever evoked by administration of heparin. It should be stressed that in most experiments early leucocytic sticking developed as in untreated animals and in no instance was the reaction prevented (Table II). In any event, once sticking was well under way, leucocytic diapedesis was brisk so that after 24 hours many white cells were found within damaged tissue.

Another completely unexpected and even more spectacular finding was the increased prominence of thrombotic phenomena within patent blood vessels adjacent to injured tissue (Table II). This was evident sometimes within 30 minutes and always by 1 or 2 hours after trauma and, as must be reemphasized, developed only within the immediate area of trauma. In damaged ear chambers of untreated rabbits, formation of platelet thrombi and the proliferation of fibrin strands is not a prominent finding but can be seen when searched for diligently (1, 2). The first detectable change in the injured chamber after heparin had been given was an increased adhesiveness of platelets. Within a few minutes, platelets began to stick to each other in ever greater numbers and propagated as elastic, veil-like thrombi from isolated points along the endothelium (Fig. 3). Eventually, these clumps of platelets became so bulky that they broke apart to form emboli and were swept away into the venous side of the systemic circulation. New thrombi usually formed promptly from the same
endothelial locations and the process was repeated eventually many times. Concurrently, although blood would not clot spontaneously at the skin surface or in vitro, long thin strands of elastic, colorless, clear material, could be seen within vascular channels at the site of platelet thrombus formation (Fig. 4). Not infrequently these strands, perhaps a form of fibrin, became coated with platelets and red blood cells but such trapping was usually transient. Leuco-

### TABLE II

**Effect of 400 Mg Heparin Sodium Given Intravenously Immediately before Rabbit Ear Chambers Were Injured by Heat**

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Intensity of leucocytic sticking</th>
<th>Incidence of thrombotic phenomena</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-20</td>
<td>Light sticking 6 hrs. after injury</td>
<td>Platelet thrombi 1 hr. after injury</td>
</tr>
<tr>
<td>M-19</td>
<td>Heavy sticking 3 hrs. after injury</td>
<td>Platelet thrombi 1 hr. after injury</td>
</tr>
<tr>
<td>M-24</td>
<td>Heavy sticking 20 min. after injury</td>
<td>None seen</td>
</tr>
<tr>
<td>M-32</td>
<td>Heavy sticking 3 hrs. after injury</td>
<td>None seen</td>
</tr>
<tr>
<td>M-411</td>
<td>Moderately heavy sticking 1 hr., 15 min. after injury</td>
<td>Platelet thrombi 1 hr., 15 min. after injury</td>
</tr>
<tr>
<td>M-494</td>
<td>Moderately heavy sticking 3 hrs. after injury</td>
<td>Platelet thrombi 4 hrs., 30 min. after injury. Strands of &quot;fibrin-like&quot; material 6 hrs. after injury</td>
</tr>
<tr>
<td>M-500</td>
<td>Heavy sticking 2 hrs., 30 min. after injury</td>
<td>Numerous platelet and WBC thrombi 2 hrs., 30 min. after injury</td>
</tr>
<tr>
<td>M-416</td>
<td>Heavy sticking 3 hrs., 30 min. after injury</td>
<td>Platelet thrombi 40 min. after injury. Strands of &quot;fibrin-like&quot; material 2 hrs. after injury</td>
</tr>
<tr>
<td>M-394</td>
<td>Moderate sticking 2 hrs., 30 min. after injury</td>
<td>Platelet thrombi 1 hr., 30 min. after injury. Strands of &quot;fibrin-like&quot; material 6 hrs. after injury</td>
</tr>
<tr>
<td>M-401</td>
<td>Heavy sticking 6 hrs. after injury</td>
<td>Strands of &quot;fibrin-like&quot; material with platelet thrombi 12 hrs. after injury</td>
</tr>
</tbody>
</table>

White cell sticking, often with a unilateral distribution, is most common in damaged and often prestatic blood vessels. Even so, there seemed no common relation between sites of adherence for leucocytes and these heparin-associated thrombi.
halted thrombus formation. As a consequence of this association, occlusion of the circulation by thrombosis and by stasis after 24 hours was no more extensive than in untreated animals despite the exaggerated formation of fibrin-like material associated with the large doses of heparin. Accordingly, extended study of lesions within ear chambers of heparinized rabbits did not reveal a significant difference from untreated animals in size of lesions (Figs. 5 and 6). As the

![Text-Fig. 1. Effect of 3.0 mg/kg heparin sodium given intravenously on clotting times of 6 rabbits.](image)

illustrations also show, there was no intensification of hemorrhage from these static channels despite the vigorous anticoagulation.

2. Effects of Small Doses of Heparin.—

The failure of the 400 mg heparin load to either reduce or prevent leucocytic sticking and at the same time, the exaggerated development of thrombotic phenomena as described in the preceding section suggested an effect related primarily to dose size. Accordingly, heparin was given in amounts comparable to dose ranges employed in human beings for clinical anticoagulation. As may be seen in Text-fig. 1, 2 hours after heparin was given intravenously to 6 rabbits, clotting times were at least 8 times longer than control values. An anticoagulant effect of this order compared favorably with that achieved in human beings and thus seemed satisfactory for ear chamber experiments.
Heparin in the amount of 3.0 mg/kg did not modify the uninjured ear chamber with reference to the volume and velocity of blood flow, vascular fragility, and the intravascular behavior of leucocytes and platelets. Furthermore, ear chambers damaged by heat behaved essentially as after the larger heparin load with but one exception. The formation of platelet thrombi and strands of fibrin-like material was only slightly accentuated even though the small heparin dose was repeated several times at short intervals. Leucocytic sticking progressed without alteration and was eventually associated with brisk diapedesis of white cells. Furthermore, after 24 hours, there was no evidence that the inflammatory reaction was modified by a smaller amount of heparin. As with larger heparin doses, perivascular hemorrhage was not aggravated (Figs. 5 and 6).

Trial of other amounts of heparin was not attempted since there was no reason to suspect a distinct difference from results obtained with the 2 dose ranges investigated.

3. Heparin and Protamine Sulfate Studies.—

In view of the curious platelet and "fibrin-like" thrombi that appeared in heat-damaged ear chambers of rabbits given a single intravenous load of 400 mg heparin, it was of interest to determine the influence of a potent heparin antagonist on the system. Protamine sulfate was selected because of known efficacy in neutralizing heparin anticoagulant activity both in vivo and in vitro and because of the wide safety margin attending its administration. Study of the substance revealed that it did not cause fever, leucopenia, or leucocytosis. Protamine, however, did possess the disadvantage that administration of an excess also produced anticoagulation. Thus, it was necessary to determine the optimal dosage required to accomplish neutralization of heparin in vivo prior to performance of experiments.

Initially, protamine titrations were performed on several rabbits approximately 2 to 3 hours after 400 mg heparin had been administered by vein since thrombotic phenomena after burn injury were most brisk at this time. Under these conditions it was found that approximately 0.1 mg protamine sulfate neutralized heparin activity in 1.0 ml of blood. From this figure, heparin levels not only of the blood sample but of the entire animal could be estimated for performance of ear chamber experiments.

After rabbits were given 400 mg heparin intravenously, ear chambers were damaged by heat; it was then possible to determine when leucocytic sticking and thrombotic phenomena reached peak levels. Just at that time, usually 2 to 3 hours after injury (Table III), a sample of venous blood was obtained for measuring the clotting time and protamine titration. Immediately thereafter, an amount of protamine sulfate was given believed sufficient to restore clotting times to near control values. The adequacy of the estimated protamine dosage for counteracting heparin anticoagulation was checked by two means. First, the heparin content of blood obtained just before administration of protamine was measured by a protamine titration and from this figure the required dose could be calculated retrospectively. As may be seen in Table III, the amount of protamine administered corresponded well with that required to neutralize heparin anticoagulation in vivo. Secondly, approximately 2 hours after protamine had been given, another specimen of venous blood was obtained for a clotting time determination. From Table III, it may be seen that the anticoagulant activity of heparin in this sample was not nullified consistently by the administered protamine since moderate clotting time prolongation often persisted. This was so even though an excess of protamine, according to the
| Animal No. | Time after injury blood sample
taken and protamine given | Animal weight kg. | Blood volume ml. | Quantity protamine mg. given intravenously | Minimal calculated amount of protamine required to counteract heparin mg. | Clotting time 2 hrs. after protamine given | Microscopic events after protamine administered | Incidence of thrombotic phenomena |
<table>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M-494</td>
<td>hrs. 5</td>
<td>2.6</td>
<td>169</td>
<td>30.0</td>
<td>13.5</td>
<td>No clot after 13 hrs.</td>
<td>Moderate sticking persisted</td>
<td>Only few thrombi seen, no change</td>
</tr>
<tr>
<td>M-500</td>
<td>3</td>
<td>2.8</td>
<td>182</td>
<td>36.4</td>
<td>14.6</td>
<td>Clot 1 hr. 15 min. after protamine</td>
<td>Heavy sticking unchanged</td>
<td>Numerous platelet thrombi uninfluenced by protamine</td>
</tr>
<tr>
<td>M-546</td>
<td>2</td>
<td>2.9</td>
<td>188</td>
<td>36.0</td>
<td>37.6</td>
<td>Clot 2 hrs. after first protamine</td>
<td>Heavy sticking unaltered</td>
<td>Platelet thrombi persisted</td>
</tr>
<tr>
<td>(Given protamine twice)</td>
<td>4</td>
<td></td>
<td>38.0</td>
<td>3.7</td>
<td>4½ min. after second protamine</td>
<td></td>
<td>&quot;</td>
<td>“</td>
</tr>
<tr>
<td>M-641</td>
<td>2</td>
<td>3.0</td>
<td>195</td>
<td>39.0</td>
<td>23.4</td>
<td>Clot in 50 min.</td>
<td>Vigorous sticking persisted</td>
<td>Continued heavy thrombus formation</td>
</tr>
<tr>
<td>M-496∥</td>
<td>2</td>
<td>2.4</td>
<td>156</td>
<td>30.0</td>
<td>24.9</td>
<td>Clot after 1 hr. 20 min.</td>
<td>Although sticking continued, may have lessened</td>
<td>Platelet thrombi continued to develop</td>
</tr>
</tbody>
</table>

* Clotting times of all specimens prolonged greater than 24 hrs. except for second sample from No. M-546.
† Blood volume = kg body weight × 65.0.
§ Blood volume × mg protamine required to clot 1.0 ml blood.
∥ This animal also treated with coumadin.
titration data, was given in later studies. Since an excess of either material may cause anti-
coagulation, it was not possible to differentiate their separate effects either in vitro or in vivo.

The inconclusive nature of the studies with heparin and protamine in vitro was also encountered in ear chamber experiments. In this instance, leucocytic sticking induced by heat injury in heparinized animals was not modified after a neutralizing dose of protamine had been given (Table III). Likewise, thrombotic phenomena present at the time of protamine administration persisted without apparent change. This was so regardless of the amount of protamine given. Finally, protracted study of ear chambers of rabbits so treated did not indicate that the inflammatory reaction differed appreciably from that of either untreated or heparinized animals.

4. The Relation of Time after Trauma to Thrombus Formation Associated with Heparin.—

As noted previously, heparin-associated platelet thrombi and streamers of fibrin developed only in vessels bordering damaged tissue. Furthermore, since these aggregations were not detected until 30 minutes or longer after injury and heparinization, it suggested either formation or release of tissue factors active in triggering the reaction. In order to test this possibility, heparin was administered at different times after ear chambers had been damaged by heat. Thus, when 400 mg heparin was given 2 hours after injury, a time of brisk leucocytic sticking but of little thrombotic activity in untreated rabbits, platelet sticking developed promptly, usually within 5 minutes. After 10 minutes, streamers of fibrin and large platelet thrombi were prominent but as noted previously, leucocytic sticking was not altered.

In a small number of experiments, 400 mg heparin was given either 6 or 24 hours after injury. Even though modest leucocytic sticking persisted at these times, thrombotic phenomena were not notably enhanced. It should be noted also that the reaction to injury has already begun to abate to some extent after the 6th hour in this preparation. As a result, even though the results suggested transient release of an active factor or trigger for thrombus formation, the brevity of the inflammatory reaction produced in this model may have artificially terminated clotting activity.

5. Effect of Defibrinogenation upon Heparin Activity within Injured Ear Chambers.—

From data described in preceding sections, it was suspected that a third factor, apparently arising from injured tissue, was needed to catalyze the intra-
vascular reaction between heparin and fibrinogen that lead to accentuated thrombotic phenomena. To test this possibility it seemed worthwhile to attempt a blockage of the heparin effect by removing fibrinogen from the circulation of rabbits before ear chambers were injured. At the same time, such experimental conditions would provide a check on previous observations wherein leucocytic adherence was not substantially altered by either heparinization or defibrinogenation alone.

Defibrinogenation of animals was initially attempted by a technique previously described (1) except that in these experiments, a purified form of streptokinase (SK), 100,000 units per
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A vial, was used. The procedure was designed so as to gradually remove all chemically detectable fibrinogen from the circulation during an 8 hour infusion yet preserve the peripheral circulation in a condition suitable for ear chamber studies. As noted before, administration of thromboplastin (TP) followed by thrombin produced many intravascular platelet emboli and streamers of fibrin that were visible until fibrinogen values became extremely low (1). Although as much as a 90 per cent or more reduction in fibrinogen values was universally accomplished, it was not always possible to remove completely all such material. It was found that the addition of thrombin to plasma obtained from animals after extended defibrinogenation produced a small amount of granular precipitate after the mixtures were allowed to stand at room temperature for 8 to 24 hours. Such prolonged incubation of thrombin with experimental plasma was not done in previous studies (1). Although a fibrin web did not form, this granular material suggested a clottable protein, perhaps an altered form of the fibrinogen-fibrin complex.

With these results in mind, experiments were performed to effect in vivo defibrinogenation in 5 different ear chamber preparations. The results were not conclusive since small amounts of clottable protein were found in plasma samples from 4 of the 5 animals despite 10 to 12 hours of infusion. After injury, heavy leucocytic sticking appeared in all 5 animals. Heparin was not given to one of these animals because thrombotic phenomena persisted after heat injury; subsequently, no fibrinogen was found in the plasma. Heparin was given to the other 4 animals and in 2 of these there seemed an appreciable delay in the appearance of platelet-fibrin clots; whereas, thrombi developed promptly in the remaining 2 animals. These results could not be interpreted.

As a further check on the nature of this clottable protein, experimental plasmas were assayed for precipitatable fibrinogen by using anti-rabbit fibrin chicken (ARFC) serum in Ouchterlony double diffusion plates. A distinct line of precipitate in agar formed between normal rabbit plasma and the antibody (ARFC); whereas, normal rabbit serum did not react in this manner (Text-fig. 2). The specificity of the reaction was verified with purified rabbit fibrinogen. As also shown in Text-fig. 2, a distinct line of precipitate formed between ARFC serum and plasma from “defibrinogenated” rabbits. Since this band of precipitate connected with the band obtained from normal plasma, it indicated that a substance immunologically similar to fibrinogen persisted in the circulation of treated animals. Somewhat confusing, however, was the fact that after normal rabbit fibrin was lysed in vitro by added SK, the same line of precipitate was found (Text-fig. 3). This suggested that depolymerization of rabbit fibrin by fibrinolysin (plasmin) yielded polypeptide fragments that still reacted with the ARFC serum. Thus, it was possible that the precipitate formed between experimental plasma and ARFC serum (Text-fig. 3) represented combination of chicken precipitin with polypeptide fragments of rabbit fibrinogen rather than unclotted traces of unaltered fibrinogen alone. Such a possibility did not seem too unlikely when it was found that disruption of fibrin by another proteolytic enzyme such as trypsin resulted in a polypeptide residue that did not react with the ARFC serum (Text-fig. 3). Unfortunately, trypsin could not
be used *in vivo* in these experiments since it produced death from acute heart failure.

From this information it was determined that 5.0 ml unabsorbed ARFC serum could be given intravenously after 10 hours of defibrinogenation with

**TEXT-FIG. 2**

Ouchterlony double diffusion plate. Absorbed anti-rabbit-fibrin chicken serum (*ACS*) in central well. The contents of the surrounding wells were as follows: (1) Control plasma from rabbit M-623. (2) Plasma from rabbit M-623 after 10 hours of defibrinogenation. (3) Plasma from rabbit M-623 after 11 hours of defibrinogenation and 10.0 ml unabsorbed ARFC serum. No clottable protein present. (4) Same as well (3) but 15 minutes after 400 mg heparin given. (5) Plasma from rabbit M-623 11½ hours after infusion stopped. (6) Serum from rabbit M-623. Note total lack of precipitate.

**TEXT-FIG. 3**

Ouchterlony double diffusion plate. As in Text-fig. 2, absorbed anti-rabbit-fibrin chicken serum (*ACS*) in central well. The surrounding wells contained the following: (1) Normal oxalated rabbit plasma. (2) Plasma from rabbit M-623 after 11 hours of defibrinogenation and 10.0 ml unabsorbed ARFC. No clottable protein was found. (3) Normal oxalated rabbit plasma clotted with thrombin and digested with trypsin. (4) Normal oxalated rabbit plasma clotted with thrombin and lysed with streptokinase. (5) Same as well (2). (6) Normal rabbit serum.

TP-thrombin-SK without disrupting circulatory integrity although the volume of blood flow was often temporarily reduced. Testing of rabbit plasma obtained 20 minutes after administration of chicken antiserum still revealed a precipitate in Ouchterlony plates but no clottable protein was detected even after extended incubation. Accordingly, the procedure for defibrinogenation was modified to include administration of chicken antiserum after 10 hours of infusion.

After ARFC serum administration, prominent platelet–white cell thrombi and strands of fibrin-like material became scarce in 2 of 3 animals; possibly because leucocytes and thrombocytes were decreased in number. Ear chambers
were damaged by heat immediately after the first 5.0 ml of ARFC serum was given. Within 5 to 15 minutes, leucocytic sticking appeared along patent channels next to the damaged tissue. A second 5.0 ml of chicken serum was given about 30 minutes after injury, but it did not alter the sticking reaction. There was less evidence of effect on circulatory dynamics. As these observations were made, no platelet or fibrin thrombi were seen despite moderate numbers of free floating platelets. Blood obtained at this time revealed no fibrinogen by chemical means but a precipitate in agar identical with that seen in Text-fig. 2 was still obtained with specific antibody (ARFC).

At this point, after 10 plus hours of defibrinogenation and 10.0 ml anti-rabbit-fibrin chicken serum, 400 mg. heparin was given sc. the opposite ear. Within a span of 15 to 25 minutes, platelet-fibrin thrombi began to be seen in patent vessels nearest the damaged tissue. Venous blood obtained at this time, even though the infusion of thrombin-SK continued, revealed chemically detectable yet quite small amounts of fibrinogen. Within 1 hour after administration of heparin, the circulatory volume of the ear chamber increased and inflammation evolved much as in untreated animals. Throughout all these extraordinary maneuvers, leucocytic sticking progressed although sometimes not as vigorously as in untreated rabbits; the reduction usually appeared related to either peripheral leucopenia or diminished ear chamber circulation and sometimes a combination of both. Furthermore, lesions in animals so treated revealed considerable infiltration of leucocytes but perivascular hemorrhage was less than expected. A profound fall in hematocrit values from venepunctures and from perhaps unseen sites of hemorrhage attested the rigor of these experiments and undoubtedly lowered peripheral blood pressures to account at least in part for the modest hemorrhage noted about static ear chamber vessels.

To sum up, although there was no evidence of platelet or fibrin thrombi within injured ear chamber blood vessels immediately after 400 mg heparin was given to rabbits cleared of fibrinogen, thrombi began to reappear within 15 or more minutes. Formation of thrombi correlated well with the reappearance of chemically measurable fibrinogen in the circulation. Of especial note, leucocytic sticking persisted throughout despite maximum defibrinogenation and added anticoagulant activity of heparin. From these findings, it seemed that the sticking reaction of leucocytes was clearly separated from the effect of heparin on fibrinogen and platelets. Unfortunately, there was considerable overlap in the times when thrombi appeared. It was thus not possible to state categorically that “defibrinogenated” animals had no heparin-associated thrombi. Nevertheless, there was the distinct impression that this was so.

B. Sodium Warfarin Studies:

The intravenous administration to rabbits of 15 mg/kg sodium warfarin (coumadin) daily for 4 days, according to the schedule of Shapiro and McKay to prevent the generalized Shwartzman reaction (4), did not consistently prolong prothrombin times over control values.
Failure to confirm Shapiro's results may have resulted from our use of larger and older rabbits. At any rate, as seen in Table IV, administration of 50 mg/kg coumadin daily intravenously for 4 or 5 days did increase prothrombin times consistently by at least a factor of 3 over pretreatment levels. Such prolongation was thought adequate for experimental purposes since it not only exceeded by several times the drug effect usually sought in clinical usage but also caused the animals to bleed easily. An appreciable decrease in hematocrit value was found in a few of these animals but there was no obvious blood loss (Table IV). Since coumadin was given at least 3 or 4 hours before ear chamber studies were begun, no estimation of pyrogenicity or of peripheral leucocytes was made although every precaution was taken to prevent bacterial contamination and only sterile solutions were used.

**TABLE IV**

**Effect of Sodium Warfarin, 150 Mg Daily, on the Reaction Produced by Heat Trauma in Rabbit Ear Chambers**

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Weight</th>
<th>Total Na warfarin dosage</th>
<th>Prothrombin time Before therapy</th>
<th>Prothrombin time After therapy</th>
<th>Hematocrit value Before therapy</th>
<th>Hematocrit value After therapy</th>
<th>Microscopic events after injury</th>
<th>Intensity of leucocytic sticking</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-451</td>
<td>2.6</td>
<td>600</td>
<td>13.0 (sec.)</td>
<td>49.2 (sec.)</td>
<td>37.5 (per cent)</td>
<td>39.0 (per cent)</td>
<td>None</td>
<td>Heavy sticking 1 hr., 30 min. after injury</td>
</tr>
<tr>
<td>M-440</td>
<td>3.3</td>
<td>600</td>
<td>15.7 (sec.)</td>
<td>61.8 (sec.)</td>
<td>37.5 (per cent)</td>
<td>39.0 (per cent)</td>
<td>None</td>
<td>Light sticking 1 hr., 30 min. after injury</td>
</tr>
<tr>
<td>M-457</td>
<td>2.6</td>
<td>600</td>
<td>13.7 (sec.)</td>
<td>57.4 (sec.)</td>
<td>37.5 (per cent)</td>
<td>36.0 (per cent)</td>
<td>None</td>
<td>Heavy sticking 4 hrs. after injury</td>
</tr>
<tr>
<td>M-458</td>
<td>2.6</td>
<td>600</td>
<td>11.8 (sec.)</td>
<td>53.0 (sec.)</td>
<td>39.0 (per cent)</td>
<td>39.0 (per cent)</td>
<td>None</td>
<td>Heavy sticking 2 hrs. after injury</td>
</tr>
<tr>
<td>M-472</td>
<td>2.4</td>
<td>600</td>
<td>11.6 (sec.)</td>
<td>52.0 (sec.)</td>
<td>39.0 (per cent)</td>
<td>37.5 (per cent)</td>
<td>None</td>
<td>Light sticking 20 min. after injury</td>
</tr>
</tbody>
</table>

Coumadin treatment did not alter the circulatory dynamics within uninjured ear chambers. Similarly, leucocytes and platelets were neither increased nor decreased in number and there was no suggestion that coumadin changed their stickiness. The 4 days of coumadin anticoagulation did not increase vascular fragility sufficiently to cause spontaneous leakage of red cells from mature ear chamber blood vessels.

The inflammatory response elicited by heat injury in ear chambers of rabbits fully anticoagulated with coumadin was quite similar in most respects to the reaction noted after heparin treatment. Leucocytic sticking was not influenced by coumadin pretreatment since margination developed within 15 to 30 minutes after injury and became quite heavy within 2 to 3 hours (Table IV). After sticking became established, diapedesis of white cells was not delayed or otherwise impaired so that there was considerable infiltration of tissue after 24 hours.
Stasis of blood flow eventuating in perivascular hemorrhage was identical with that of the untreated controls. Likewise, coumadin treatment did not alter the time and intensity of appearance of plasma globules.

By contrast, however, there was no evidence that coumadin augmented formation of either platelet thrombi or strands of fibrin-like material encountered after injury in rabbits given heparin (Table IV). Actually, thrombotic phenomena in rabbits given coumadin were of the same low order of frequency as in untreated controls.

**TABLE V**

**Effect of Combining Sodium Warfarin, 150 Mg Daily with 400 Mg Heparin Sodium Given Intravenously Immediately before Rabbit Ear Chambers Were Damaged by Heat**

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Weight (kg)</th>
<th>Total Na warfarin dosage (mg)</th>
<th>Prothrombin time before therapy (sec.)</th>
<th>Prothrombin time after therapy (sec.)</th>
<th>Hematocrit value before therapy (per cent.)</th>
<th>Hematocrit value after therapy (per cent.)</th>
<th>Intensity of leucocytic sticking</th>
<th>Incidence of thrombotic phenomena</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-466</td>
<td>2.6</td>
<td>600</td>
<td>17.7</td>
<td>64.0</td>
<td>35.0</td>
<td>33.0</td>
<td>Heavy sticking 1 hr., 30 min. after injury</td>
<td>Platelet thrombi and strands of 'fibrin-like' material 30 min. after injury</td>
</tr>
<tr>
<td>M-424</td>
<td>3.0</td>
<td>750</td>
<td>14.4</td>
<td>75.0</td>
<td>42.5</td>
<td>33.5</td>
<td>Heavy sticking 1 hr., 15 min. after injury</td>
<td>Platelet thrombi and strands of 'fibrin-like' material 30 min. after injury</td>
</tr>
<tr>
<td>M-472</td>
<td>3.0</td>
<td>750</td>
<td>12.3</td>
<td>47.6</td>
<td>45.0</td>
<td>35.0</td>
<td>Heavy sticking 1 hr., 15 min. after injury</td>
<td>Platelet thrombi 1 hr., 15 min. after injury</td>
</tr>
<tr>
<td>M-492</td>
<td>3.0</td>
<td>600</td>
<td>14.2</td>
<td>95.0</td>
<td>34.5</td>
<td>31.0</td>
<td>Moderately heavy sticking 3 hrs. after injury</td>
<td>No 'fibrin-like' material noted</td>
</tr>
<tr>
<td>M-496</td>
<td>2.4</td>
<td>750</td>
<td>15.3</td>
<td>44.6</td>
<td>36.5</td>
<td>41.0</td>
<td>Heavy sticking 2 hrs. after injury</td>
<td>Platelet thrombosis 30 min. after injury</td>
</tr>
</tbody>
</table>

**C. Combined Anticoagulant Studies:**

Animals in this series of experiments were given heparin sodium and sodium warfarin at the same time to determine if their combined anticoagulant action would modify leucocytic behavior since neither alone prevented the sticking reaction. Coumadin, 50 mg/kg, was given by vein daily for 4 or 5 days to insure satisfactory prothrombin time prolongation. On the day of study, 400 mg heparin was administered intravenously immediately before ear chambers were damaged by heat. This combination of agents did not modify either circulatory dynamics or behavior of formed elements within uninjured ear chambers. Furthermore, there was no evidence that this regimen caused intralumenal formation of fibrin or that it increased the vascular fragility of uninjured chambers.

Following trauma by heat to ear chambers of rabbits receiving both anticoagulants, leucocytic sticking developed within 15 minutes and reached striking proportions during the next hour or so (Table V). Sticking of other
formed elements, however, was much as noted in animals treated with heparin alone; i.e., platelet–white cell thrombi were prominent. Likewise, the strands of fibrin-like material encountered with heparin alone were also noted within heavily damaged vascular channels prior to their blockage by stasis.

Diapedesis of leucocytes was not reduced by these agents as lesions 24 hours old revealed a moderate infiltration of cells. Interestingly, there was no accentuation of hemorrhage about such lesions and plasma globules appeared despite combined anticoagulation. In short, the simultaneous use of heparin and coumadin with resultant extreme anticoagulation did not modify evolution of microscopic burns during the first 24 hours after their inception.

**DISCUSSION**

The exact means by which heparin sodium and sodium warfarin antagonize the clotting of blood *in vivo* has yet to be fully revealed. It is unlikely that either substance completely blocks the enzymatic polymerization of fibrinogen to fibrin even though high drug levels and gross interference with blood clotting can be achieved. Even so, in the face of the intense pharmacological effect achieved with these substances in our experiments, it seems improbable that fibrin formation figured significantly in the sticking of leucocytes induced by heat injury. The validity of this conclusion was enhanced considerably when it was found that white blood cell sticking developed even after maximum doses of heparin sodium and sodium warfarin were used simultaneously. These results are in agreement with previous data from which it was concluded that fibrin formation on the surface of cells was not responsible for the sticking reaction (1).

At first glance, these heparin-associated thrombi did not seem compatible with the high level of anticoagulation produced by the drug. It is worth recalling that under somewhat different circumstances the undamaged cheek pouch of hamsters previously anticoagulated with heparin showed both leucocytic sticking and formation of white thrombi after several hours of study (16). It must not be forgotten that the preparation of the hamster cheek pouch is an acute technique, always subject to variable but usually slight injury when being prepared for study. This is an important consideration since in our ear chamber experiments trauma, even though ever so slight, was essential for development of thrombi after heparin administration. No thrombi were ever recorded either within undamaged rabbit ear chambers or within vessels located peripherally some distance in the chamber from the zone of injury. Contrary to the experience of Copley who studied other vascular models (17), we did not find increased intravascular platelet adhesiveness or clumping in the mature vasculature of uninjured ear chambers following either large or small amounts of intravenous heparin. It is possible that these contradictions arise from the fact that rabbit platelets may be either more resistant to heparin as suggested by Best (18); or,
as now seems more likely, the studies of others may have always involved either abnormal surfaces or injured blood vessels.

Even though an experimental explanation for the thrombi was not available, it is likely that the fibrin-like substance represented the heparin-precipitatable fibrinogen described by Thomas et al. (9). These experiments are the first documentation of its occurrence in vivo.

If, as seems inescapable, these heparin-associated thrombi were related to fibrinogen, then their presence contradicted the conclusion that anticoagulants eliminated the possibility that fibrinogen is an important factor in the leucocytic sticking reaction. Fortunately, the studies with defibrinogenated animals seemed to clearly separate leucocytic sticking from heparin-associated-thrombi since vigorous sticking of white blood cells continued when no thrombi could be found. As the last series of experiments indicated, the sticking of white cells proceeded when clottable protein could not be detected. Yet, plasma from such defibrinogenated animals formed a precipitate in Ouchterlony plates when set up against antibody to rabbit fibrin (Text-fig. 2). Unfortunately, this reaction was not specific enough to differentiate between intact fibrinogen-fibrin molecules and the polypeptide residue of these substances after depolymerization by plasmin (Text-fig. 3). We are thus still left with the possibility that a monolayer of fibrinogen molecules could account for the sticking reaction.

Finally, it has been difficult to reconcile our finding of enhanced thrombus formation associated with heparin to the established fact that anticoagulants inhibit the Shwartzman reaction, a lesion wherein both platelet aggregation and fibrin formation are prominent. Extended study of heparinized, damaged ear chambers for 24 hours or more, however, indicated that the final extent of thrombus formation and vascular obstruction was neither more nor less than that found in untreated animals. This fact may indicate that the clots were physiologically ineffective and did not permanently occlude the circulation. On the other hand, a more plausible possibility is that heparin-associated-thrombi proliferated only in vessels intensely damaged and therefore most likely to undergo occlusion by stasis. Thus, under such special circumstances the clot did not increase likelihood of obstruction since the vessels were already destined to become static. As described before, neither the extent nor rate of stasis evolution was altered by anticoagulants. If this explanation is then valid, our observations are not actually in discord with those of others concerning anticoagulants and the Shwartzman reaction.

**SUMMARY**

Vigorous anticoagulation with heparin sodium and sodium warfarin singly and in combination did not prevent the margination and endothelial sticking reaction of leucocytes in rabbit ear chambers damaged by heat. The general inflammatory reaction observed in this preparation was similarly uninfluenced.
by the anticoagulants. An unexpected finding after administration of heparin was the enhanced formation of platelet and fibrin-like thrombi within damaged ear chambers. Sodium warfarin did not induce or prevent this heparin effect. Production of these heparin-associated thrombi was minimized in animals subjected to defibrinogenation in vivo whereas leucocytic sticking was not modified. Although defibrinogenation was not absolute, these experiments represent additional proof that the sticking of white blood cells to vascular endothelium is not causally related to the fibrinogen-fibrin system.

BIBLIOGRAPHY

8. Thomas, L., Smith, R. T., and Von Korff, R., Studies on the generalized Shwartzman reaction. VII. The role of fibrinogen in the deposition of fibrinoid after combined injections of endotoxin and synthetic acidic polymer, J. Exp. Med., 1955, 102, 263.
Sanderson, M., Egner, W., and Crosbie, J. M., A protamine titration as an
15. LeQuire, V. A., Augmentation of the thermogenic effects of pyrogens by homolo-
gous plasma in rabbits, Naval Medical Research Institute, Project NM 007 047,
Report No. 6, July 6, 1949.
hamster cheek pouch after trauma, infection and neoplasia, *Circulation*, 1951,
3, 339.
17. Copley, A. L., Embolization of platelet agglutination thrombi in the hamster's
EXPLANATION OF PLATE 50

Pictures were printed as kodachromes from ektachrome positive transparencies by Eastman Kodak Company. Fig. 3 was taken from 16 mm. motion picture film.

Fig. 1. Leucocytic sticking (L) within venule of ear chamber damaged 2 hours previously by heat. Animal untreated. × 54.

Fig. 2. Leucocytic sticking (L) in venule 1 hour 15 minutes after ear chamber damaged by heat. 400 mg heparin given intravenously prior to injury. × 54.

Fig. 3. Platelet (P) thrombus within venule of damaged ear chamber of rabbit given 400 mg heparin intravenously. Note single white blood cell (L) attached to tail of clot. × 108.

Fig. 4. Strand of fibrin-like (F) material in heat-damaged ear chamber despite preceding administration of heparin. India ink (pelikan) given to serve as a marker. Note static venule (S) close by. × 54.

Fig. 5. Appearance of burn lesion in ear chamber of untreated rabbit 24 hours after injury. × 26.

Fig. 6. Heat damaged ear chamber of rabbit given 400 mg heparin after 19 hours of study. Note, despite large size of lesion, lack of accentuated hemorrhage. × 26.
(Allison and Lancaster: Pathogenesis of inflammation. III)