THE ROLE OF VENOUS ENDOTHELIUM IN THE INCEPTION OF THROMBOSIS*

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CLOSE TO A CENTURY AGO, Virchow outlined three variables operating in the pathogenesis of thrombosis: (1) Coagulability of the blood, (2) disturbance of the blood flow, and (3) change in the vessel wall.[42]

As a result of intensive study of the first factor, the principal stages in the clotting process, once it is initiated, have been outlined.[27, 37] The physiology of venous return is well understood and slowing of the venous blood stream after bed-rest and in pathologic states is accepted.[16, 44] The role of the third variable, the vessel wall, and specifically, its lining, the endothelium, has remained unclarified.

The earliest experimental studies of thrombosis were, in a sense, the most complete. Microscopic observation of the circulation in the small blood vessels of the mesentery and other transparent areas in the living animal enabled the pioneer workers to observe the thrombotic process as a whole.

In the period from 1850 to 1890, Wharton Jones,[21] Bizzozero,[5] and Eberth and Schimmelbusch[10] found that injury of any type to the small blood vessels of the living animal was followed by adherence of platelets to the endothelium of the injured vessel. These platelets metamorphosed and coalesced to form platelet thrombi. Fibrin appeared shortly afterward in the area surrounding the platelet thrombi. The question which these early researchers failed to answer was: What is the nature of the changes in the vessel wall, following injury, which leads to the adherence of platelets to the endothelium?

The classic pathologists, using standard histologic methods, in the study of veins procured postmortem, failed to show any structural changes in the endothelium of veins implicated in thrombosis.[1]

Shionoya in 1927[38] and Best in 1938,[3] using transparent, extracorporeal shunts of glass, collodion or cellophane, confirmed the mode of formation of platelet thrombi but added nothing to the knowledge of the vessel wall.

The first successful demonstration of changes in the properties of the endothelium following injury was made by Chambers and Zweifach.[6, 7] They injected a suspension of carbon particles intravenously into a frog. After prodding a mesenteric capillary with a micro-needle, they observed that the carbon particles adhered to the intercellular cement substance in numbers large enough to outline the endothelial cell. This indicated clearly that the intercellular cement substance became more sticky after injury to the endothelium.

In 1947, a great forward stride in the study of the endothelium of larger veins was made by J. F. O'Neill,[32] who devised a method whereby the entire endothelial surface of a vein could be studied. O'Neill used silver nitrate as a staining solution.

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This stain blackens the intercellular cement and outlines the endothelial cell. This technic is limited, in that structures adherent to the endothelial surface appear as structureless silhouettes.

In this contribution, the venous endothelium has been studied by new technics which allow permanent preparations to be made, and enable one to study at once the entire endothelial surface of a vein segment and any structures adherent to it. These technics are also applicable to the arterial endothelium and are at present being used in The Pathological Institute of McGill University in the study of the early changes in the endothelium in arteriosclerosis.

MATERIALS AND METHODS

The veins studied by these methods were obtained from adult dogs. The jugular, femoral, brachial, saphenous, cephalic and inferior vena cava are veins suitable for study. In the dog, veins smaller than the cephalic vein of the forelimb cannot be successfully prepared.

The vein selected is removed surgically from the anesthetized animal. The endothelium of veins is extremely delicate, and seemingly minute trauma to the vein wall will lead to disruption of the lining and subsequent thrombosis. Extreme care is therefore exercised in the removal of the vein and every effort is made to avoid contact with the vessel wall.

The method of mounting and staining the vein preparations has been described in detail in a previous publication.[35] The method of preparing the vein for staining and subsequent steps is shown diagrammatically in Figure 1. The finished preparation appears as in Figure 2. The vein preparation may be stained with silver nitrate, 1:250, for 30 seconds, followed by fixation in neutral formalin and dehydration in graded alcohols. It is cleared in methyl salicylate.

In the new technics, the preparation is immersed in indigo tetrasulfonate, 0.1 per cent, for 20 minutes, followed by one to three minutes in 0.2 per cent methylene blue; 0.1 per cent methylene blue—0.075 per cent azure A, 1:1; toluidin blue, 0.1 per cent, or thionin, 0.1 per cent. It is then fixed in ammonium molybdate and dehydrated successively in ethyl alcohol—butyl alcohol, 1:1; and butyl alcohol. It is then cleared in xylol-methyl-salicylate, 4:1.

The heparin-toluidin blue stain follows the same procedure as the indigo tetrasulfonate method. Heparin, 0.1 per cent, is substituted for indigo tetrasulfonate; toluidin blue, 0.1 per cent, is used as the second stain. Fixation, dehydration and clearing are identical.

EXPERIMENTAL OBSERVATIONS

A. Controls. The veins used as controls were removed atraumatically. Fourteen femorals, 25 jugulars and seven inferior venae cavae were used in establishing the normal appearance of venous endothelium.

The normal histology of endothelium varies little in veins procured from different anatomical sites and from one animal to the other. The endothelium is composed of roughly diamond-shaped cells, whose long axis runs parallel to that of the vessel. The length of the cells averages 50μ, the width 11μ, though wide variations occur in small numbers of cells. The cells in smaller veins, such as the saphenous, tend to be slightly smaller in size. Staining of normal endothelium actually means staining of intercellular cement substance, as the cytoplasm and nuclei of normal endothelium, except in the case of thionin, do not stain with the supravital stains used, unless cell injury or death has occurred (Fig. 18). When thionin is used, the nucleus appears as an egg-shaped body, measuring 11μ at its widest diameter and situated anywhere in the cytoplasm.

After silver nitrate staining, the cement lines are black or brownish-black color, and are smooth and continuous (Fig. 3). Their width may vary from 0.5 to 0.75μ. Indigo tetrasulfonate-methylene blue staining produces light blue lines, 0.75 to 1.5μ wide. These lines show discontinuity and irregularity of staining depth (Fig. 4). Toluidin blue, used following indigo tetrasulfonate or heparin mordants, stains the cement a lilac color and produces a continuous line similar to silver nitrate staining (Fig. 5). Thionin staining differs from toluidin blue only in its reddish-purple color (Fig. 19).

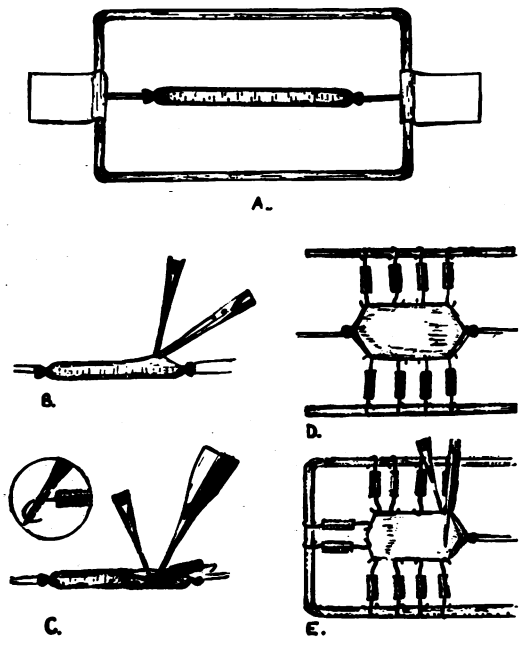


FIG. 1.—(A) The vein segment is suspended from the frame by means of the long ends of the ligatures. (B) The vein wall is tented up by forceps, 1 cm. away from its end, and the initial cut is made in it. (C) The first spring is inserted into the edge of the initial opening in the vein. The insert shows detail of the correct method of holding the spring. (D) The springs have been inserted along both edges. The ends of the vein are still secured to the frame by means of the ligatures. (E) The vein cuff, ligature and 6 mm. of vein adjacent are being removed by means of scissors, while the vein is steadied with a forceps. This has already been done at the other end and the springs have been inserted.

In the course of the cement lines are interspersed circular and oval bodies 3 to 10μ in diameter. They correspond to the "stigmata" described by the classical pathologists.[25, 26] After silver nitrate staining, they are blackened to the same degree as the cement lines. With the supravital stains, they take a deeper stain than the cement substance (Figs. 6 and 7). On the basis of experimental studies below, it is believed that these bodies represent platelets identical in size, shape and staining reaction with those seen on the cement (Fig. 8).

The basement membrane of the endothelium appears in areas of altered endothelial permeability and where the endothelial cells have desquamated following injury (Figs. 13, 16, 17, 18 and 20). It is composed of a homogenous light-staining material, striped by fibers of irregular width, running a roughly parallel course 6 to 9μ apart, in a direction tangential to the long axis of the endothelial cell. The fibers join at intervals. They correspond to descriptions of elastic fibers.

B. Experimental Injury of the Vein Wall. Jugular and femoral veins were used in these experiments.

1. *Mechanical Injury.* The portion of the vein wall to be injured is dissected free from its coverings and is treated by one of the following methods:

a. Application of a bulldog clamp one second to one minute. (The same clamp was used in all experiments.)

b. Application of a ligature for two seconds to one minute.

c. Digital compression 15 to 30 seconds.

Twelve veins were injured by these methods, remaining *in situ* 20 to 60 minutes following injury.

2. *Chemical Injury.* The irritants used were: (a) Croton oil in olive oil, 1, 5, and 10 per cent; and (b) acetic acid in olive oil, 5 per cent.

The selected irritant, 0.025 to 0.2 cc., was injected between the vein wall and its innermost sheath. Twenty-seven veins were subjected to this type of injury, remaining *in situ* 20 minutes to one hour after injury.

3. *Stripping of the Venous Sheath.* The vein selected was stripped completely of its sheath, interrupting the vasa venarum, lymphatics and nerves passing to this vein segment. In some cases the wound was closed without further treatment. The majority of veins were loosely enclosed in rubber dam, tantalum foil, or polyethylene sheeting or tubing to ensure separation from the vein bed. Twenty-seven veins were treated in this manner from three and a half to 72 hours. These were all sterile procedures.

Whatever the type of injury to the vein wall, the endothelium reacts in the same manner. The degree of the reaction varies, however, in proportion to the severity of the injury. The sequence of changes following injury can best be described if it is divided into two stages.

Stage 1—Minimal Reaction Following Injury to the Vein Wall. The structure and configuration of the endothelial cells are unchanged (Figs. 6, 7, 15). On the intercellular cement lines a great number of structures appear. Some of these have the same appearance as the platelet thrombi seen occasionally in normal endothelium. They are circular or oval bodies five to 15μ in diameter, made up of dark-staining granular material. Besides these, there are larger polymorphous bodies, staining lightly. These bodies are composed of clusters of tiny needle-like fibers which radiate from a central dark-staining dot. The material corresponds exactly in description to fibrin, and has been identified as such. On focusing the microscope downwards, these fibrin clumps are seen to overlie platelet thrombi adherent to the cement lines. Thionin has the property of differential staining of fibrin and underlying platelet thrombi. The fibrin stains light brown and the platelet thrombus, deep purple (Fig. 19).

The fibrin tends to spread along the intercellular lines and across the cell surface to join contiguous masses of fibrin (Fig. 15). The sequence of events is shown diagrammatically in Figs. 9, 10, 11, 12, and in Figures 6, 7, 14, 15, the various structures are shown clearly.

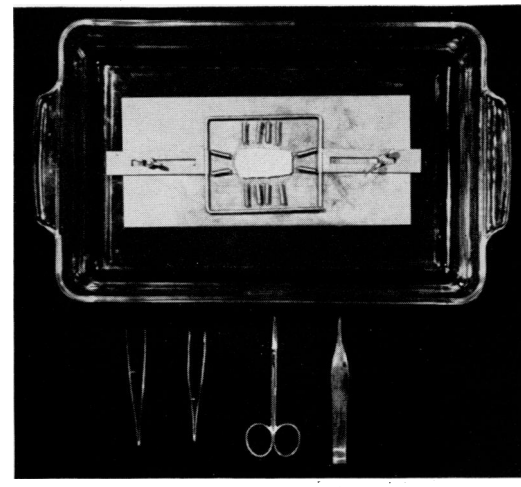


FIG. 2.—Apparatus for holding the vein segment. The vein is attached to the stainless steel frame by means by stainless steel springs; the frame, in turn, being secured by adjustable clamps, held by wing-nuts. The Pyrex dish, in which the whole is placed, serves to hold sufficient Ringer's solution to cover the preparation during the mounting procedure. Instruments used in mounting are ranged in front of the Pyrex dish.

The earliest events in this sequence were worked out in the veins of heparinized animals (Figs. 5, 22). Briefly, the entire sequence is described as follows:

1. The platelets singly and multiply become adherent to the cement substance (Fig. 9).

2. Adherent platelets metamorphose and coalesce to form platelet thrombi (Fig. 10).

3. Fibrin deposits on the surface of the platelet thrombi (Fig. 11).

4. Fibrin masses spread along the intercellular lines and across the cell bodies (Fig. 12).

The platelet thrombi and fibrin masses are estimated to project from ten to 50μ above the endothelial surface.

Injury may stop at this stage, in which the endothelium remains intact, or if the initial injury is severe or long continued the reaction progresses to the next stage.

Stage 2—Maximal Reaction Following Injury to the Vein Wall. The continuity of the endothelial surface is disrupted and

FIG. 3

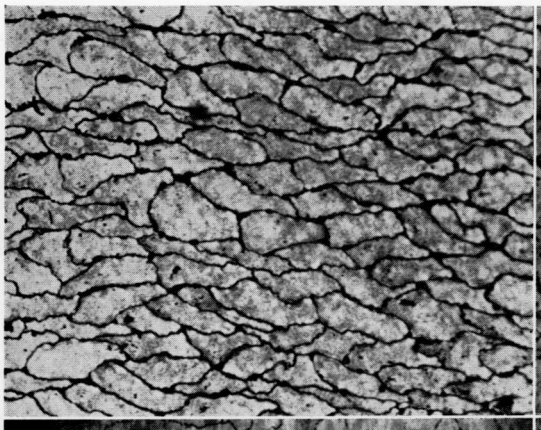


FIG. 4

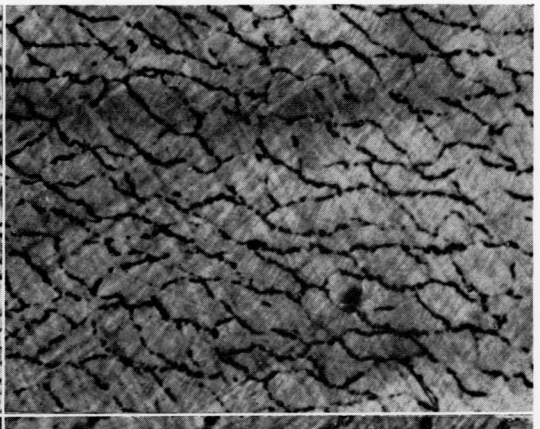
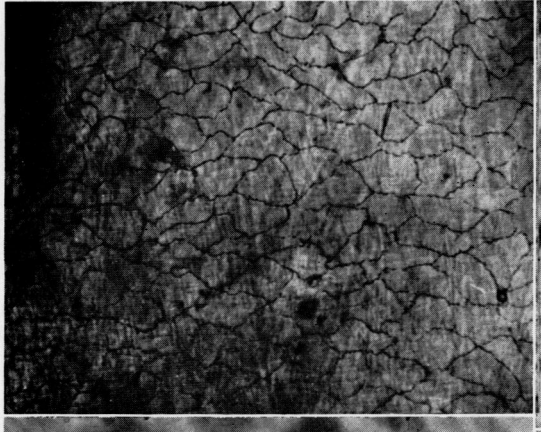


FIG. 5



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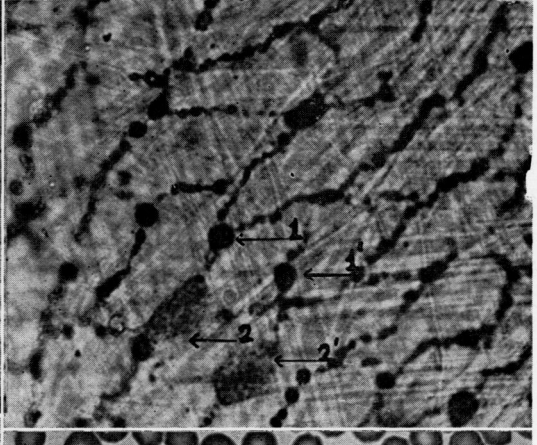


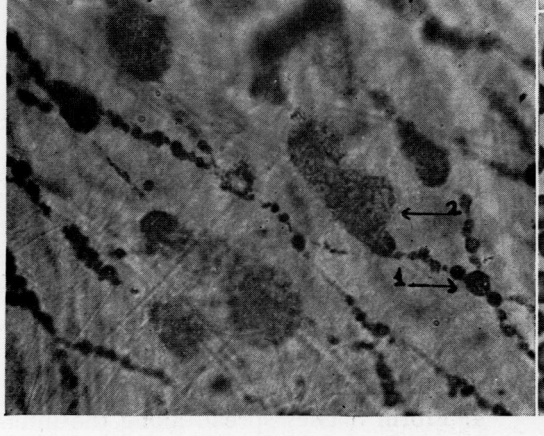


FIG. 7

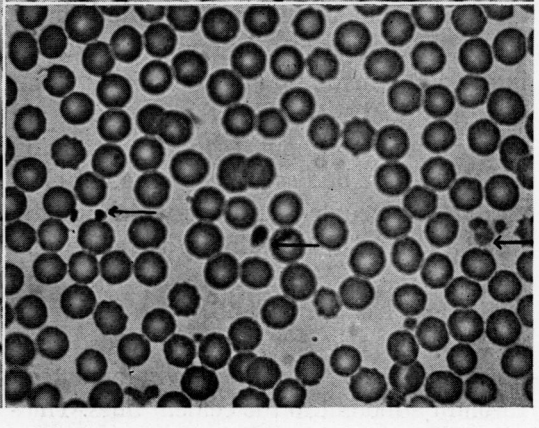


FIG. 8

(See opposite page for legends.)

pathologic anatomic changes appear in the endothelial cells. The appearance of intracellular granules staining the same color as the cement substance marks the first change (Fig. 13, 18). The nucleus may stain lightly at this point. In the area behind the affected cell (or cells) the striations of the basement membrane become stained (Figs. 13, 16). Small circular or oval separations appear in the intercellular lines. These clefts are of increasing size (Figs. 13, 18). In the larger clefts, one or more of the striations in the basement membrane can be seen. The clefts continue to enlarge until the cell body disappears (Figs. 16, 17). Large areas of the vein wall may desquamate their endothelium in this way (Figs. 16, 20). Fibrin is deposited in these desquamated areas. Contrary to expectation, the amount of fibrin on endothelium showing a maximal reaction is usually smaller than the amount produced in minimal injury. This is especially true for areas of total desquamation. Different areas of the vein wall react in different degrees. Evidence of both minimal and maximal endothelial reactions occur in most injured veins (Fig. 14). Occasionally there appears a foamy vacuolization of the endothelial cytoplasm after severe injury. This has been seen most frequently in Croton oil-treated veins and may result from intracellular passage of the toxic agent. Both stages of reaction may be seen with any of the staining methods. Silver nitrate staining, however, destroys the finer structures of fibrin and platelet thrombi.

Results of Mechanical Injury. The degree of the reaction was proportional to the time of application of the crushing injury and to the time the vein remains *in situ* following the injury. Stage 2 injury was usually seen in the area which had been directly under the ligature of clamps (Fig. 14).

Results of Chemical Injury. A much larger series of veins were subjected to chemical injury by Croton oil and acetic acid than to mechanical injury. It was felt that by varying the dosage, maximal or minimal injury might be consistently produced. In general, the smaller doses (0.025 cc. of 5 per cent Croton oil), tended to produce minimal injury, as did shortening the period during which the vein is subjected to injury.

Paradoxical results occur where small doses give a maximal degree of reaction and vice versa. These divergent results have been attributed to inaccurate placement of the injection, with the irritant infiltrating the wall instead of lying between the vein

FIG. 3.—Photomicrograph of the endothelium of the normal jugular vein of a dog, stained with 1:250 silver nitrate solution. The intercellular cement lines are black, smooth and continuous. The oval and circular irregularities on the cement lines correspond to the "stigmata." They are believed to be the outline of platelets and platelet thrombi. (x 160)

FIG. 4.—Photomicrograph of the endothelium of the normal jugular vein of a dog, stained with indigo tetrasulfonate–methylene blue (x 160). Note the discontinuity and unevenness of the staining of the intercellular cement, as compared with the silver nitrate preparations.

FIG. 5.—Photomicrograph of the endothelium of a normal dog, stained with heparin-toluidin blue (x 160). Note the fineness of staining of the intercellular lines. Several platelets are seen on the intercellular cement lines.

FIG. 6.—Photomicrograph of the jugular vein of a dog, stained with indigo tetrasulfonate-methylene blue (x 350). Platelet thrombi, arrows 1 and 1', and fibrin clumps, arrows 2 and 2', are shown. These illustrate the first changes in stage 1, minimal reaction.

FIG. 7.—Photomicrograph of the jugular vein of a dog, stained with indigo tetrasulfonate-methylene blue (x 480). Arrow 1 shows detail of a platelet thrombus. Arrow 2 points to a fibrin clump in which the finder details of the fibrin can be seen.

FIG. 8.—Photomicrograph of a smear of normal dog blood with added heparin, stained with indigo tetrasulfonate–methylene blue: azure A (x 725). The arrows are opposite platelets which are from 3 to 6 micra in size. Note the difference in the depth of staining of the platelets indicated at left and at center, with those indicated on the right. Compare these platelets with those in Figure 7, which appear on the intercellular lines.

wall and its sheath. The degrees of reaction to these irritants are seen in Figs. 15, 16, 17, 18, 19, 23.

Results of Stripping of the Venous Sheath. These experiments are a repetition of those reported by J. F. O'Neill[32] in 1947. O'Neill attempted to show that stripping a vein of its sheath with subsequent interruption of the vasa venarum leads to anoxic injury of the endothelium equivalent in severity to the second stage of reaction to injury, as defined in this paper. O'Neill's observations were correct. However, all his observations were made on veins wrapped in rubber dam. In repeating O'Neill's experiments, polyethylene tubing and sheeting, and tantalum foil were used as wrapping materials in addition to rubber dam. Some veins were stripped but not wrapped. All the veins wrapped in rubber dam for 24 hours showed gross signs of inflammation, such as thickening and increased turgidity and vascularity of the walls. The endothelium of these veins shows total desquamation (Fig. 20). Veins enclosed in polyethylene tubing and sheeting for the same period of time show minimal injury (Fig. 21).

Tantalum foil results in slightly more endothelial injury than polyethylene tubing or sheets.

These veins stripped and left unwrapped, showed patches of minimal injury. These represented the results of a slight inflammatory reaction, subsequent to removal of the sheath. The reactions in wrapped veins are felt to represent an inflammatory foreign-body reaction induced by the wrapping material, and are most severe with rubber dam.

FIG. 9

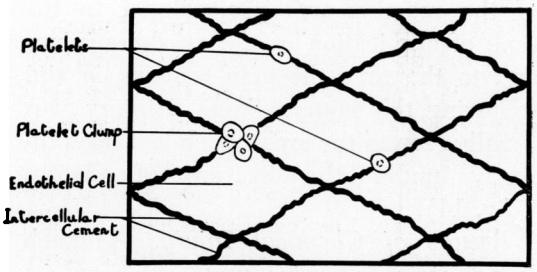
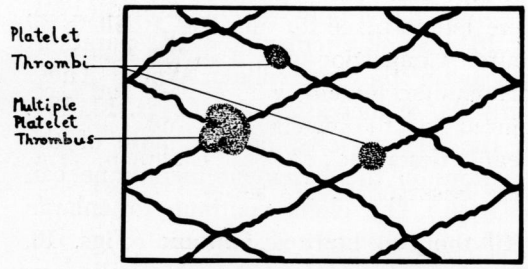


FIG. 10



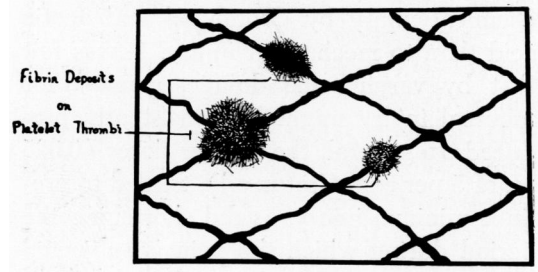


FIG. 11

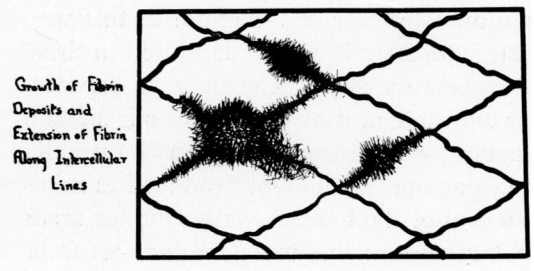


FIG. 12

FIG. 9.—Diagram of platelets depositing on the intercellular cement. This is the first event in the minimal reaction to injury.

FIG. 10.—Diagram showing the second phase of the minimal reaction to injury. Platelets metamorphose and coalesce to form platelet thrombi.

FIG. 11.—Diagram showing the third phase of the minimal reaction to injury. Fibrin deposits appear on the surface of the platelet thrombi.

FIG. 12.—Diagram showing the fourth phase of the minimal reaction to injury. The fibrin deposits are beginning to spread along the intercellular lines and over the cell surface.

C. Congestion. *Acute Congestion.* Acute congestion of a single vein was produced by placing a constricting ligature around it. Constriction was carried to a point just short of interruption of the stream. Tests were made to make sure that blood was flowing past the constriction. Ligatures were left in place for one and a half to 24 hours. Congestion was also produced, in this manner, in veins whose walls had been injured by the mechanical and chemical means described above. Eighteen veins were observed in this series.

Chronic Congestion. Chronic congestion was produced by enclosing the pelvis and one hind limb of a dog in a plaster spica. The unenclosed limb was freely mobile. Immobilization was carried out in this way for five- and 12-day periods. In one animal, ligation of the inferior vena cava was carried out one and one-half months prior to removal of the femoral veins. Five animals had chronic congestion induced by these methods.

Results. Veins acutely congested for periods up to 24 hours showed only slight differences from the control side. Areas of injury appeared at the end of the vein to which the ligature had been applied and could not be separated from the slight injury subsequent to dissection of the vein and to the presence of the constricting ligature.

The uniform appearance of granularity and early desquamation as reported by O'Neill[32] was not seen in this series.

In the eight veins in which constriction was combined with injury of the vein wall for periods of an hour or less, there was no increase in the amount of endothelial injury or early thrombosis over the control side, which had been injured in an identical manner, and remained *in situ* for the same time period (Figs. 16, 17). In one vein, where constriction was combined with an injury induced by 0.1 cc. of 5 per cent acetic acid in olive oil for a 24-hour period, a gross thrombus resulted (Fig. 25).

The opposite side treated in the same manner was free of gross thrombi. As the present study is concerned mainly with the inception of thrombosis, we did only one experiment of this type. Long-term experiments will be described in future studies.

In the five animals subjected to chronic congestion, the veins from the congested side showed no significant difference from the controls.

It is felt that the series of animals, in which congestion was produced, is not large enough to permit any definite statements as to the effects of congestion. In the small number of animals tested, no marked effects of congestion were ascertained.

D. Isolated Vein Segments. Segments of vein, two to four inches long, were enclosed between ligatures and were left *in situ,* or removed to Ringer's solution (37°C.) for 30 minutes to one hour. Six vein segments were prepared in this manner.

In eight other veins, the vessel was injured before or after isolation between ligatures. Injuries included freezing of vein segments at −24°C. for five and ten minutes and the mechanical and chemical injuries already described.

Results. In the uninjured segments, there were no gross thrombi. Microscopically, the endothelium revealed an occasional patch of fibrin, usually near the ends injured by the ligatures.

The segments injured before or after isolation showed minimal or maximal reaction to injury according to the strength of the injury. Fibrin formation, however, tended to localize to the site of injury. In none of the veins was a gross thrombus found, even though the endothelial surface showed numerous fibrin deposits.

E. The Effect of Heparin in Normal and Injured Veins. Nine dogs were treated with heparin in doses ranging from 0.75 to 2 mg.

per Kg. body weight. Two lots of heparin, one containing 103 U.S.P. units per mg. and another 160 U.S.P. units per mg. were used. In five of the dogs in this series, the veins were injured mechanically or chemically prior to heparinization. Clotting times were determined by a modified Lee-White method, in which blood was drawn into a siliconed syringe and placed in test tubes, incubated at 37°C.

FIG. 13

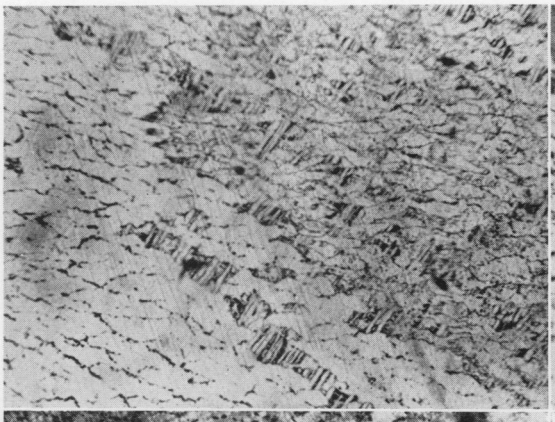


FIG. 14

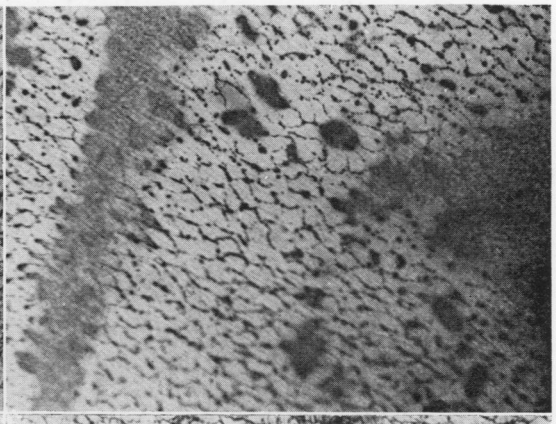


FIG. 15

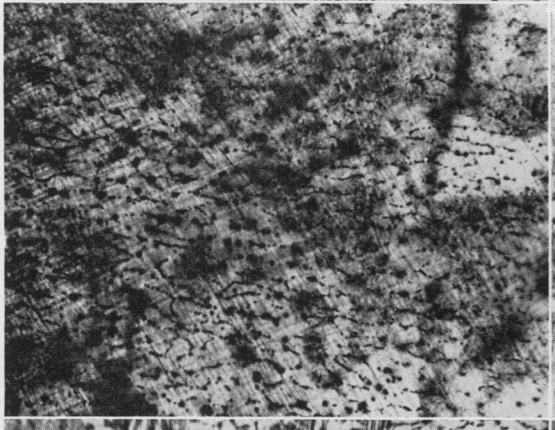


FIG. 16

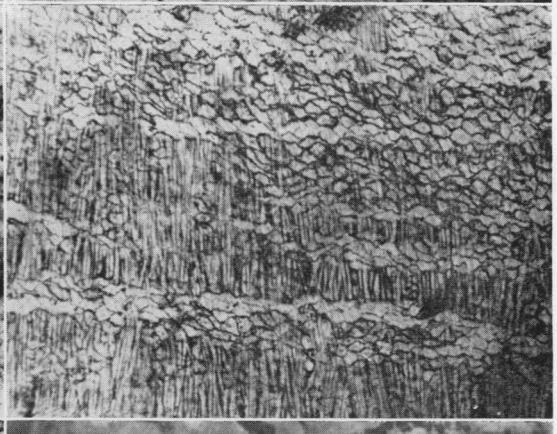
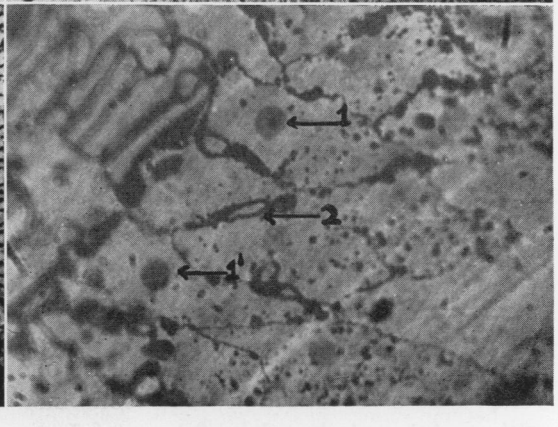


FIG. 17

FIG. 18



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Results. Four normal dogs were treated with heparin, dose 0.75 mg. per Kg. body weight, which produced an average clotting time of 45 minutes over a normal clotting time averaging six and one-half minutes. Veins were removed from these animals at intervals. Veins removed 15 to 20 minutes after injection of heparin were devoid of platelets. As the clotting time approached 15 minutes, platelets appeared in increasing numbers on the intercellular cement. These platelets were unaltered and stained more sharply than platelets that have undergone metamorphosis (Fig. 22). In the saphenous veins, which were the last to be removed, platelet thrombi and fibrin formation was observed. In normal dogs, heparin seems to prevent the adherence of platelets to the intercellular cement substance.

Injured veins in dogs treated with the lower dosages of heparin, *i.e.*, 0.75 mg. per Kg., the reaction to injury proceeded as in the untreated animal and fibrin formation was seen, though somewhat diminished in amount.

Four dogs were given doses of high potency (160 U.S.P. units per mg.) heparin, 2 mg. per Kg. and one dog, 1 mg. per Kg. These doses rendered the blood incoagulable during the experiment and, in the clotting samples, for 48 hours following. The response to clamping along the course of the entire vein, in these animals, was definitely impaired. However, exuberant fibrin formation was seen, on one occasion after this injury, in dogs treated with 2 mg. per Kg. of heparin (Fig. 24). Usually fibrin formation occurred, but in rather small amounts as compared with the results of a similar injury in an unheparinized dog. The clamping produced areas of stage 2 injury, just as in unheparinized dogs.

In all cases where 0.05 cc. and 0.1 cc. of 5 per cent Croton oil in olive oil was injected next to the vein and allowed to remain for at least 30 minutes, thrombus formation appeared in considerable amounts (Fig. 23).

FIG. 13.—Photomicrograph of a femoral vein of a dog, stained with indigo tetrasulfonate-methylene blue: azure A (x 80). The first phase of stage 2 maximal reaction is shown. The cells in the right upper corner show a distinct coarse granularity. Numerous clefts between the cells are present, through which the basement membrane, with its fibers running diagonally, can be seen.

FIG. 14.—Photomicrograph of the jugular vein of a dog, stained with indigo tetrasulfonate-methylene blue : azure A (x 80). This vein had a bulldog arterial clamp applied for one minute. It remained *in situ* ten minutes after injury. Numerous thrombi are seen. There is a fibrin sheet at the right side of the photograph. The linear fibrin streak at the left, overlies an area of early stage 2 injury.

FIG. 15.—Photomicrograph of a femoral vein of a dog, stained with indigo tetrasulfonate-methylene blue : azure A (x 80). This vein was treated with 0.1 cc. of Croton oil in olive oil, 5 per cent. It remained *in situ* 30 minutes before removal. Note the copious fibrin and numerous platelet thrombi on the intact endothelium.

FIG. 16.—Photomicrograph of the endothelium of a femoral vein of a dog, stained with indigo tetrasulfonate-methylene blue : azure A (x 80). This vein was treated with 0.05 cc. of 5 per cent Croton oil in olive oil, plus a constricting ligature for 30 minutes. Note the cellular changes in the transition to desquamation.

FIG. 17.—Photomicrograph of the femoral vein of a dog, stained with indigo tetrasulfonate-methylene blue : azure A (x 80). This vein from the side opposite the vein shown in Figure 16. It was treated with 0.05 cc. of Croton oil in olive oil for 30 minutes. Note the extensive areas of total desquamation in comparison with Figure 16. This illustrates one of the paradoxical effects on the endothelium, after chemical irritants.

FIG. 18.—Photomicrograph of the jugular vein of a dog, stained with indigo tetrasulfonate-toluidin blue (x 350). This vein was treated with 0.05 cc. 10 per cent Croton oil in olive oil and remained 20 minutes *in situ* before removal. Arrows 1 and 1′ point to the nuclei, which become stained in the dying cell. Arrow 2 points to a beginning intercellular cleft. Note the basement membrane in the upper left hand corner appearing in an area where the cells have desquamated.

It appears, from these experiments, that fibrin may appear on the injured vessel walls even in cases where the blood is rendered incoagulable by heparin.

F. The Effect of Venous Distention Produced by a Limb Tourniquet in the Prevention of Injury to the Endothelium. The saphenous veins are extraordinarily susceptible to injury during their dissection and removal, even when extreme care is exercised. This is attributed to their numerous branches, relatively thick adherent sheaths, and their tendency to go into spasm and collapse completely. A small series of veins previously distended by prior application of a tourniquet was dissected out.

Results. In the four veins dissected out while distention was maintained by tourniquet, the endothelium showed less stage 2 reaction to injury and less thrombus formation. In two limbs the veins were relatively free of thrombi. A large number of saphenous veins was dissected out in limbs without tourniquet. There was always a large amount of endothelial injury and thrombosis. Though this series is small, the results clearly indicate that spasm with obliteration of the lumen increases endothelial injury and the production of fibrin.

DISCUSSION

In the introduction to this paper, it was stated that a question regarding the inception of thrombosis remained unanswered. This question was "What is the nature of the changes in the vessel wall following injury which leads to the adherence of platelets to the endothelium?" The experimental observations in this paper do not come near to providing a complete answer. Certain points stand out clearly, however, as a guide to future investigations.

The most significant finding is that the point at which the formed blood elements are most likely to adhere, *i.e.*, the point of lowest surface tension, is the intercellular cement line. After injury to the vessel wall, the "stickiness" of the cement substance appears to increase and platelets become adherent in great numbers.

It should be emphasized that these changes represent alterations in the physiology of the endothelium and occur in the absence of demonstrable anatomical change in the endothelial cell. This is in accord with the dynamic concept of the endothelial cells, as advanced by Chambers and Zweifach.[6, 7] It appears that the endothelium of all blood vessels is able to react to injury or a change in the environment without gross structural changes appearing.

The only observations recorded in the literature which approximate in any way the findings presented here, are those of Klemensiewicz[23] on the blood vessels of amphibia. He reported that the endothelial cells, when injured, secreted a jelly-like fibrin membrane which trapped spindle cells and initiated an agglutination thrombus. This author did not mention the intercellular lines.

A number of investigators have speculated on the role of the intercellular cement in the inception of thrombosis. Fremont-Smith,[13] at the 1948 Coagulation Conference, urged investigation of the relation of Chambers' findings to thrombosis. In a recent paper, Raeburn[34] suggests injury of the endothelium allows the sticky intercollagenous cement substance to pass through the endothelial cells to the vein surface, or that the endothelial cell, by virtue of its fibroblastic potentialities, becomes adhesive. Copley[9] speculated that the cement substance might have thromboplastic properties.

The results of supravital staining of the endothelium throws light on a number of histologic problems. The stigmata and stomata seen in endothelium were a great source of contention among classical histologists.[25, 26] It is believed that the stigmata, which they describe, are the silhouettes of platelets and platelet thrombi seen after silver nitrate staining (Fig. 3). It is prob-

able that the stomata represent a retraction of the endothelial cell edge which has been seen *in vivo* in the capillaries, and is reversible in the living state, if injury is not too severe.[2]

As the new technics are used *in vitro,* it is impossible to see platelets leave the blood stream and become adherent to the cement. Of necessity, then, the interpretation of the pictures obtained must rest on indirect evidence. The foremost piece of evidence is the similarity in size, shape and staining of the platelets on the cement to those stained by the same technic in smears of dog blood. In addition, these observations are completely in accord with the *in vivo* observations of earlier workers who saw platelets adhere to injured endothelium. No other bodies, which could conceivably be platelets or platelet thrombi, appear anywhere on the endothelium, except on the intercellular cement lines. Differences in size, which appear in individual platelet thrombi from unaltered platelets, have been described in detail by Ferguson[11] in studies of the changes in the platelets, occurring when it adheres to a wettable surface *in vitro,* the size of the platelet increasing to four times that of the original. These changes have been described by other authors.[28, 45] The tendency of the platelets to coalesce into platelet thrombi is also well established.[45] Zucker[47] reported that she was unable to see fibrin on the platelet thrombi formed in hemostasis. Some platelet thrombi have been observed without a fibrin coating, but it appears on the surface of the platelets very soon after their formation. Platelets and platelet thrombi appear occasionally on the surface of normal endothelium, but are seen in great numbers when the endothelium is injured. It is possible that the intercellular cement enters into the production of platelet thrombi, as metachromic areas can be seen in certain platelet thrombi, and this phenomenon is hard to explain. The ability of platelets to lower surface tension has been reported.[8] Indirect evidence of platelet deposition is offered by the results of Fontaine's work.[12] He reported a drop in the platelet count in veins subjected to experimental operations when compared with the platelet number on the unoperated side.

In four of the entire series of treated veins, gross thrombi appeared (Fig. 25). One was a strongly adherent mixed thrombus. The others were typical loosely adherent red thrombi but, in general, the thrombi produced tended to remain microscopic. Jaques[20] has pointed out the difficulty of producing thrombosis experimentally in normal animals. It is paradoxical that a phenomenon, which may occur clinically despite all precautions, should be so difficult to produce intentionally. This tendency of experimentally-produced thrombi to remain limited, emphasizes the efficiency of the mechanism which checks the spread of thrombosis.

From a biological point of view, thrombosis is a means of maintaining the fluid level of the vascular compartment by plugging holes in it when they occur. The unchecked spread of thrombosis would be a catastrophe equal in magnitude to exsanguination. The physical action of the blood in diluting and washing away locally produced thrombotic factors is one of the chief detriments to the spread. Another physical factor is the absorption of thrombin on the relatively great surface of newly formed fibrin.[33] Secondary defenses included plasma anti-thrombin, tissue and blood anti-thromboplastins and possibly heparin and its plasma co-factor, circulating fibrinolysin, which may play a part in dissolving the clot, once it has formed.[37] The surprising limitation of fibrin production in stage 2 injury of the endothelium and total desquamation mentioned earlier may be related to a more rapid mobilization of clot-limiting

mechanisms or to their more rapid release through a discontinuous endothelial surface. Studies now in progress, in which a new whole mount technic is in use, reveal that the mast cells, which are regarded as the source of heparin, occur in layers 20 to 30μ below the endothelial surface. In addition, they occur in large numbers around the vasa venarum. In these locations, the rapid local release of heparin is possible.

Two eighteenth century surgeons, Charles Thackrah[41] and William Hewson,[15] produced a series of experiments on vein segments, isolated between ligatures, to which little has been added. Copley[9] recently repeated their experiments, confirm-

FIG. 19

FIG. 20

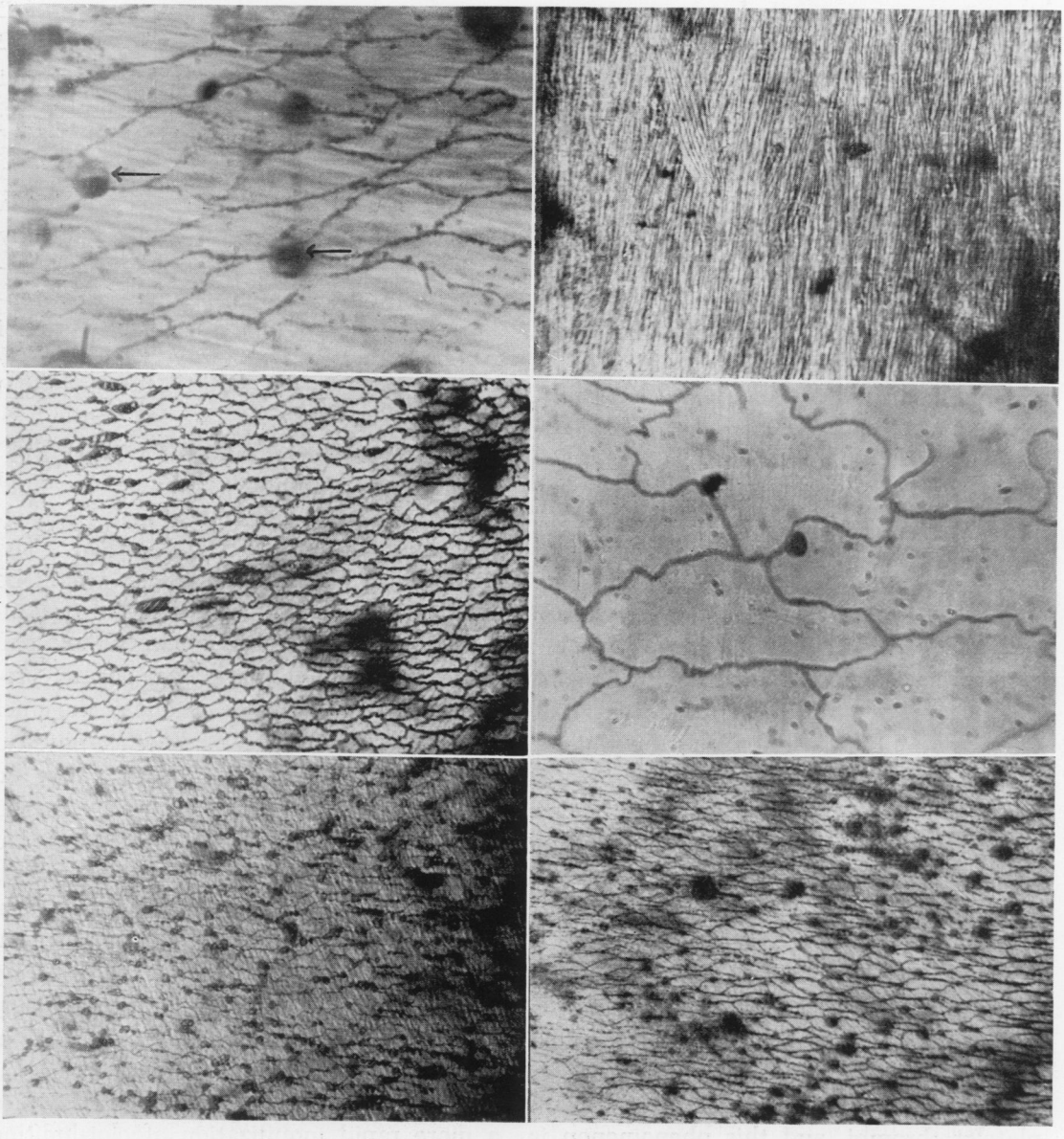

FIG. 21

FIG. 22

FIG. 23

FIG. 24

(See opposite page for legends.)

ing them. He also showed that if thromboplastin is injected into a vein segment, empty of blood, and the blood is then allowed to fill the segment, a coagulation thrombus occurs. Copley implies that this discounts any inhibitory action by the vessel wall. It is not conceivable to the author that such a situation is duplicated in the living animal.

It is well known that if a vein is doubly ligated, the blood in the enclosed segment will not clot. This is true even if the vessel wall is previously injured as shown by Copley.[9] Study of the endothelium of such injured segments by the new technic reveals that thrombosis occurs at the points of injury, but it does not spread into the completely stagnant blood. It is hoped to elucidate the modus operandi of this inhibition in future work.

These experiments pose a stumbling block to those who view stagnation as the most important factor in thrombosis.

It is commonly held that the endothelium of blood vessels draws its nutriment from the blood in contact with the surface. This concept is upheld by the results of our experiments in stripping. The desquamation of endothelium, which O'Neill ascribed to cutting off the blood supply to the endothelium by interruption of the vasa venarum, is shown to be a foreign-body reaction to the rubber dam, used in separating the blood vessel from its bed.

Our experiences with heparin support the contention of Best,[3, 4] Solandt,[40] Murray,[31] Jacques[18, 19] and numerous others that heparin is effective in preventing deposition and agglutination of platelets on the endothelial surface.[22] The affinity of heparin for intercellular cement, *in vitro,* is shown by its ability to bring about staining of that substance by toluidin blue, which is a specific stain for heparin. This tendency for heparin to combine with the cement is further evidence of the proteinous nature of the cement substance.[19]

When the blood was rendered incoagulable by a large dose of heparin, thrombi still appeared on severely injured walls. This suggests that local mechanisms, which operate to protect injured areas in the vessel wall with fibrin coats, are exceedingly powerful. It is probable that thromboplastic substances are produced in excess permitting limited fibrin production.

The observations in this paper are in accord with the clinical and pathologic

FIG. 19.—Photomicrograph of a jugular vein of a dog, stained with indigo tetrasulfonate-thionin (x 330). This section was treated with 0.2 cc. of 5 per cent Croton oil in olive oil for one hour. Arrows point to light staining circular fibrin clumps, overlying darker staining, oval platelet thrombi. Thionin staining shows that the fibrin and platelet thrombus, though associated, are separate entities.

FIG. 20.—Photomicrograph of a jugular vein of a dog, stained with indigo tetrasulfonate-methylene blue : azure A (x 75). This vein was stripped of its covering and enclosed in rubber dam for 24 hours. Total desquamation is shown.

FIG. 21.—Photomicrograph of the jugular vein of a dog, stained with indigo tetrasulfonate-methylene blue : azure A (x 75). This vein is from the opposite side from that shown in Figure 20. Treatment was identical, except that a polyethylene tube enclosed the vein. Note the almost normal appearance of the endothelium.

FIG. 22.—Photomicrograph of the endothelium of a jugular vein of a dog, stained with heparin-toluidin blue (x 675). The dog received heparin in a dose of 0.75 mg. per Kg. The vein was removed one hour after heparinization. Note the platelets appearing on the intercellular lines.

FIG. 23.—Photomicrograph of the endothelium of the jugular vein of a dog, stained with indigo tetrasulfonate-toluidin blue (x 75). This dog received heparin in a dose of 2 mg. per Kg. The vein was treated with 0.1 cc. of 5 per cent Croton oil in olive oil, 35 minutes after heparinization, and remained *in situ* 20 minutes. Blood was incoagulable during this period. Note numerous platelet thrombi. Fibrin formation is present, but is scanty.

FIG. 24.—Photomicrograph of the jugular vein of a dog, stained with indigo tetrasulfonate-methylene blue : azure A (x 75). This dog received heparin in a dose of 2 mg. per Kg. A bulldog clamp was applied twice for 30 second periods, 30 minutes after heparinization. The vein remained *in situ* 45 minutes, during which time the blood was incoagulable. Note numerous fibrin thrombi.

observations and the ideas advanced by Frykholm.[14] It is his thesis that thrombosis begins in the small intramuscular veins, draining groups of muscles, such as those of the calf and adductor group. These muscles are subject to pressure of the mattress on the limb, which in combination with a diminished venous return, results in a collapse of the vein lumen. Apposition of endothelial surface is postulated to cause injury and initiate thrombosis. Others[16, 17, 34] have confirmed Frykholm's findings though McLachlin[29] has recently contested them.

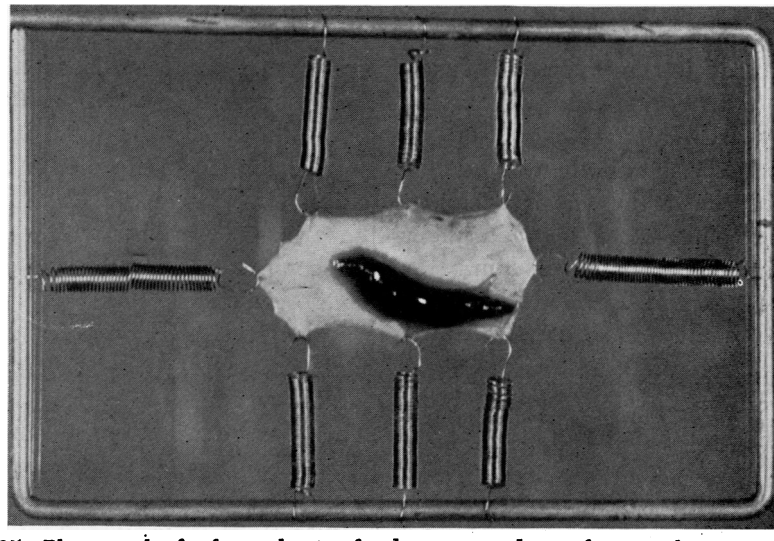

FIG. 25.—Photograph of a femoral vein of a dog, mounted on a frame. This vein was treated with 0.01 cc. of acetic acid in olive oil for 24 hours, plus a constricting ligature. Note the typical red thrombus, loosely attached to the endothelium at a few points. The adherent strands are much paler than the body of the thrombus.

The smaller veins studied in this series were peculiarly susceptible to injury, despite all technical precautions. It is felt that their tendency to collapse during dissection played a great part in the appearance of injured areas and prominent fibrin production. The fact that injury and thrombosis were materially reduced when the veins were kept distended during dissection by a limb tourniquet, supports this idea. Simpson[39] presented a statistical study which showed increased mortality from thromboembolism during a period in the last war, when large numbers of elderly people slept on deck chairs in the air raid shelters. Provision of proper sleeping facilities reduced the incidence of embolism to the usual level. He felt that injury of the limb veins resulting from prolonged pressure of the cross-bars was a major factor in the occurrence of thrombosis. Specialists[24, 36, 46] in peripheral vascular diseases report that thrombosis in the leg veins of persons sitting up for long periods in buses, trains and planes, is not uncommon. Injury of the vein wall by prolonged pressure is felt to be an important factor.

The demonstration, by Helen Wright[43] and others,[30] that an increase in platelet stickiness occurs postoperatively is given added weight when viewed in conjunction with the results of the present work. It is obvious that sticky platelets will be much more likely to adhere to a sticky, than to a non-wettable endothelial surface.

SUMMARY AND CONCLUSIONS

1. The normal histology of venous endothelium as demonstrated by the new staining technics is described.

2. The "stigmata" and "stomata" described by the classical histologists are con-

cluded to be the results of the injury to the endothelium and the subsequent thrombosis.

3. The reaction of the vein wall to injuries of a mechanical and chemical nature and the resultant inception of thrombosis on the injured venous endothelium is described.

4. The reaction to injury of the endothelium is divided into two stages: (1) minimal reaction; (2) maximal reaction. These stages are defined and differentiated.

5. A repetition of the experiments of J. F. O'Neill are presented and his observations confirmed. The changes seen are regarded as the results of a foreign-body reaction rather than the effects of interrupting the vasa venarum.

6. Results of acute and chronic congestion of veins are presented. These results are regarded as inconclusive in the present series of experiments.

7. Observations on isolated vein segments with normal and injured walls are presented. Thrombosis is shown to begin on the endothelium of injured segments and remains limited to the injured point.

8. Results of heparinization of normal dogs and those with experimentally injured veins are presented. It is concluded that heparin prevents deposition of platelets on the intercellular cement in normal veins, and that it limits but does not prevent local thrombosis from occurring on injured endothelium.

9. The results of preliminary distention of the saphenous veins, in preventing injury and thrombosis during dissection, indicate that this treatment results in a decrease of injury and thrombosis.

10. The validity of the observations and their relation to the findings of other investigators are discussed.

11. The mechanisms limiting the spread of intravascular thrombosis are discussed.

12. Correlation of clinical observations with the experimental findings are presented, and the thesis that injury to the veins of the lower limb plays an important part in the inception of thrombosis is supported.

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