FIBRINOLYTIC PROPERTIES OF VASCULAR ENDOTHELIUM

B. A. WARREN*

From the Sir William Dunn School of Pathology, Oxford

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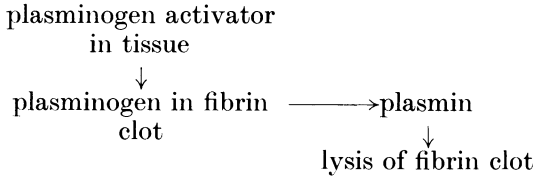
TODD (1958, 1959) has recently described a histological technique for the localisation of plasminogen activator in tissue sections. The method consists of incorporating a frozen tissue section in a fibrin clot or laying a preformed fibrin clot over the frozen section on a glass slide. The preparation is then incubated at 37° for a period of time which depends on the nature of the tissue. The presence of activator is indicated by lysis of the fibrin clot, which is seen as a circular area of liquefaction around the centre of enzyme activity.

The reaction may be represented thus:



By means of these " fibrinolysis autographs " Todd (1958) found that human thyroid, lung, uterus, ovary, prostate, adrenal, kidney, heart, muscle and spleen showed scattered foci of fibrinolytic activity when tested on bovine fibrin clot. Normal liver produced practically no lysis. Later work (Todd, 1959) showed that the plasminogen activator was concentrated around veins, venules and pulmonary arteries and appeared to be closely associated with their endothelium, and it was found that endothelial cells scraped off human inferior vena cava showed activity when incorporated in a bovine fibrin film (Todd, 1959, 1961). Endothelial cells from arteries did not show this activity with the exception of cells from the pulmonary artery and its branches.

The present study was undertaken to examine the fibrinolytic activity of a number of tissues from ox, pig, rat, mouse and rabbit by Todd's technique including the endothelium of the great vessels, which was studied by the use of frozen *häutchen*.

MATERIALS AND METHODS

Fibrinogen

Bovine fibrinogen.—Commercial fibrinogen (Bovine plasma fraction 1, Armour) was used in 2 and 4 per cent solutions in isotonic saline buffered with veronal acetate buffer (Michaelis modified by Biggs and Macfarlane, 1962) pH 7·2–7·4 and ionic strength 0·16.

Rabbit fibrinogen.—This was made by the method of Jaques (1943). A polythene catheter

* British Commonwealth Scholar.

was inserted into the left common carotid artery of an anaesthetised rabbit and the animal bled into a flask containing 10 ml. of a 3·8 per cent solution of sodium citrate. The yield of blood was usually 85–95 ml. per rabbit. This citrated blood was centrifuged and the plasma taken off. Prothrombin was removed by adsorption with 2 ml. aluminium hydroxide suspension (of a consistency just permitting pipetting) for every 50 ml. of plasma, the mixture being incubated at 37° for 15 min. The precipitate was centrifuged down and the procedure repeated once.

To prepare fibrinogen an equal volume of 2 M phosphate buffer was added to the prothrombin-free plasma, both solutions being at 6°. The precipitate was centrifuged down at 2°, washed with 50 ml. of M phosphate buffer and dissolved in 40 ml. of 0·25 M phosphate buffer. Solution of the precipitate was aided by warming the mixture to 37° in a water-bath. This method of precipitation was repeated twice. The final precipitate was made up in veronal acetate buffered saline of just sufficient volume to dissolve it. This fibrinogen solution was dialysed against the buffered saline for 12 hr., then centrifuged and any precipitate discarded. The amount of fibrinogen obtained from each animal by this method was 100–150 mg. dissolved in 15–20 ml. of veronal buffered saline.

Thrombin

One ampoule of Maw's thrombin containing 5000 NIH units was dissolved in 50 per cent glycerol to give a stock solution of 1000 units per ml. The stock solution was diluted with veronal acetate buffer to give a working solution of 25 units per ml.

Animal tissues

Bovine and pig tissues were collected from animals freshly killed by electrocution and exsanguination at a slaughter-house. Rats and mice were anaesthetised with ether and exsanguinated by cutting both femoral arteries. Rabbits were anaesthetised with Nembutal and ether and their blood collected into a flask as described above.

Frozen sections

Frozen sections were cut on the " Pearse " cold microtome (" Cryostat," South London Electrical Equipment Company Ltd.) at a thickness of between 8 and 12 μ, and transferred to glass slides. These were placed on a level plate-glass square. A clotting mixture composed of 5 drops of fibrinogen solution and 5 drops of working thrombin solution was spread evenly over the section after mixing in a Pasteur pipette.

Frozen häutchen *preparations*

Sheets of endothelial cells were removed from the aorta and inferior vena cava of ox, pig, rat and rabbit in the following manner. In the rat and rabbit the aorta from the arch to the diaphragm and the inferior vena cava from the right auricle to the diaphragm were found to be the most suitable vessel segments because of the small amount of surrounding fat and the ease with which the segments could be removed without much stretching of the vessel walls. As much fat as possible was cleaned from the outside of the vessel, in the case of the inferior vena cava while *in situ*. As soon as it had been removed and cleaned the vessel was immersed in warm veronal acetate buffered saline and opened lengthwise.

The vessel was pinned out on a cork board and the segment selected was blotted gently with " Kleenex " tissue (Kimberly-Clark Ltd.), excised and applied to a clean dry slide with the endothelial surface downward. Care was taken not to allow any air bubbles or moisture between the endothelium and the glass. A chuck, standing in a plastic beaker half-filled with a freezing mixture of alcohol and dry ice, and with a guard to prevent alcohol spitting onto the preparation, was used as a flat freezing source. The glass slide was placed on the head of the chuck with the tissue in the centre of the area in contact with the chuck. After a few seconds a wave of ice crystals traversed the thickness of the vessel wall, commonly beginning in one corner. The muscle layers of the vessel wall were immediately rolled back with forceps leaving the endothelial layer stuck to the glass slide. If there was any moisture between the endothelium and the glass slide during this procedure it formed ice crystals and only these were left on the slide. A clotting mixture was then spread evenly over the slide or preformed fibrin sheets (Todd, 1961) were placed on the preparation.

Frozen section and frozen *häutchen* preparations were put in a slide rack which was stood on end in a 1-l. beaker containing a little water, so as to keep the slides level and the fibrin clots moist. The beaker was put in an incubator at 37°.

The slides were examined at intervals of approximately ½ hr. and removed when areas of fibrinolysis were seen or in any case after 6 hr. in the initial examination of a tissue.

Serial frozen häutchen.—These were produced by repeating the above procedure with the same vessel segment. The aim was to leave a layer of the vessel wall on the slide at each " pull ". After 5 frozen *häutchen* preparations had been produced the vessel segment was usually too ragged to proceed further.

Fixation and staining

After incubation both frozen section and *häutchen* preparations were fixed in formol saline for 15 min., washed in distilled water, stained with Harris's haematoxylin and eosin and mounted in glycerine jelly.

Production of " silver " lines

If the surface of fresh endothelium is washed free of excess chloride and treated with a weak solution of silver nitrate a characteristic network of dark brown lines appears over the intercellular junctions on exposure to light or to 2 per cent cobalt nitrate (Poole, Sanders and Florey, 1958).

TABLE.—*Fibrinolytic Activity of a Number of Tissues of Ox, Pig, Rat, Mouse and Rabbit*

	Tissues of					
	Ox tested against bovine fibrin	Pig tested against bovine fibrin	Rat tested against bovine fibrin	Mouse tested against bovine fibrin	Rabbit tested against bovine fibrin	Rabbit tested against rabbit fibrin
Lung	Active*	Active	Very active	Very active	Inactive	Active after prolonged incubation
Heart	Active	Very active	Active	Pulmonary artery very active	Inactive; pulmonary artery *häutchen* inactive	Inactive; pulmonary artery *häutchen* active
Brain	Active	Very active	Active	Active	Inactive	Active
Adrenal	Diffuse lysis over medulla	Active	Active	Active	Inactive	Active
Muscle	Active	Active	Occasional area of lysis	Inactive	Inactive	Slight activity
Kidney	Lysis around glomeruli and over medulla	Lysis around glomeruli and over medulla	Lysis around large renal vessels and over medulla	Lysis over medulla and around large vessels	Lysis over medulla	Lysis over medulla
Liver	Occasional area of lysis around portal tracts	Active; lysis around portal tracts	Occasional area of lysis around vessels	Inactive	Inactive	Lysis after prolonged incubation
Spleen	Inactive	Active; lysis around small vessels	Area of lysis around hilar and some trabecular vessels	Inactive	Inactive	Inactive

The comments indicate the activity of the enzyme causing lysis around blood vessels in the tissues named as shown by the fibrinolysis autograph technique. " Prolonged " refers to incubation periods of over 6 hr.

* Calf lung is more active than ox lung.

RESULTS

The results are shown in the Table. It will be seen that areas of lysis of bovine fibrin occurred around vessels in a number of tissues of ox, pig, rat and mouse. Of tissues tested, lung, heart and brain were most active in this respect. Figs. 1, 2 and 3 illustrate the appearance of areas of lysis in these tissues. With the exception of the renal medulla, in all tissues the areas of lysis were initially discrete, and surrounded vessels (Figs. 1–4). Areas of lysis around vessels down to the size of arterioles or small venules could be distinguished in some sections. In each of the animals tested the activity of the renal medulla was diffuse, the fibrin liquefying

EXPLANATION OF PLATES

FIG. 1.—Area of lysis around branch of pulmonary artery in mouse lung. Bovine fibrin 2 per cent. Incubation 2 hr. H. and E. ×80.

FIG. 2.—Circular area of lysis around vessels in rat ventricle. Bovine fibrin 1 per cent. Incubation 3½ hr. H. and E. ×75.

FIG. 3.—Rat brain preparations: (*a*) Clear area of fibrinolysis around vessels in sulcus of rat brain. Bovine fibrin 1 per cent. Incubation 5 hr. H. and E. ×68. (*b*) Higher power view of rat brain preparation showing fibrinolysis around small vessels. Bovine fibrin 1 per cent. Incubation 2¼ hr. H. and E. ×68.

FIG. 4.—Lysis around vessel in ox muscle. Bovine fibrin 1 per cent. Incubation 4 hr. H. and E. ×80.

FIG. 5.—Frozen section of pig kidney incorporated into 1 per cent bovine fibrin clot showing circular area of fibrinolysis around glomerulus at the edge of the section. Incubation 45 min. H. and E. ×80.

FIG. 6.—Frozen *häutchen* preparation of rat inferior vena cava. The field is covered by an almost continuous sheet of endothelial cells of which only the nuclei are visible. Three areas of diffuse lysis are present ("L"). Bovine fibrin clot 2 per cent. Incubation 1½ hr. ×66.

FIG. 7.—Frozen *häutchen* reparation of rat inferior vena cava. A diffuse zone of lysis can be seen surrounding the sheet of endothelial cells. Bovine fibrin 1 per cent. Incubation 3 hr. 40 min. H. and E. ×26.

FIG. 8.—Frozen *häutchen* preparation of rat aorta. Bovine fibrin 1 per cent. Incubation 1½ hr. H. and E. (*a*) Low power view showing area of lysis around sheets of endothelial cells. ×49. (*b*) High power view of same preparation. ×270.

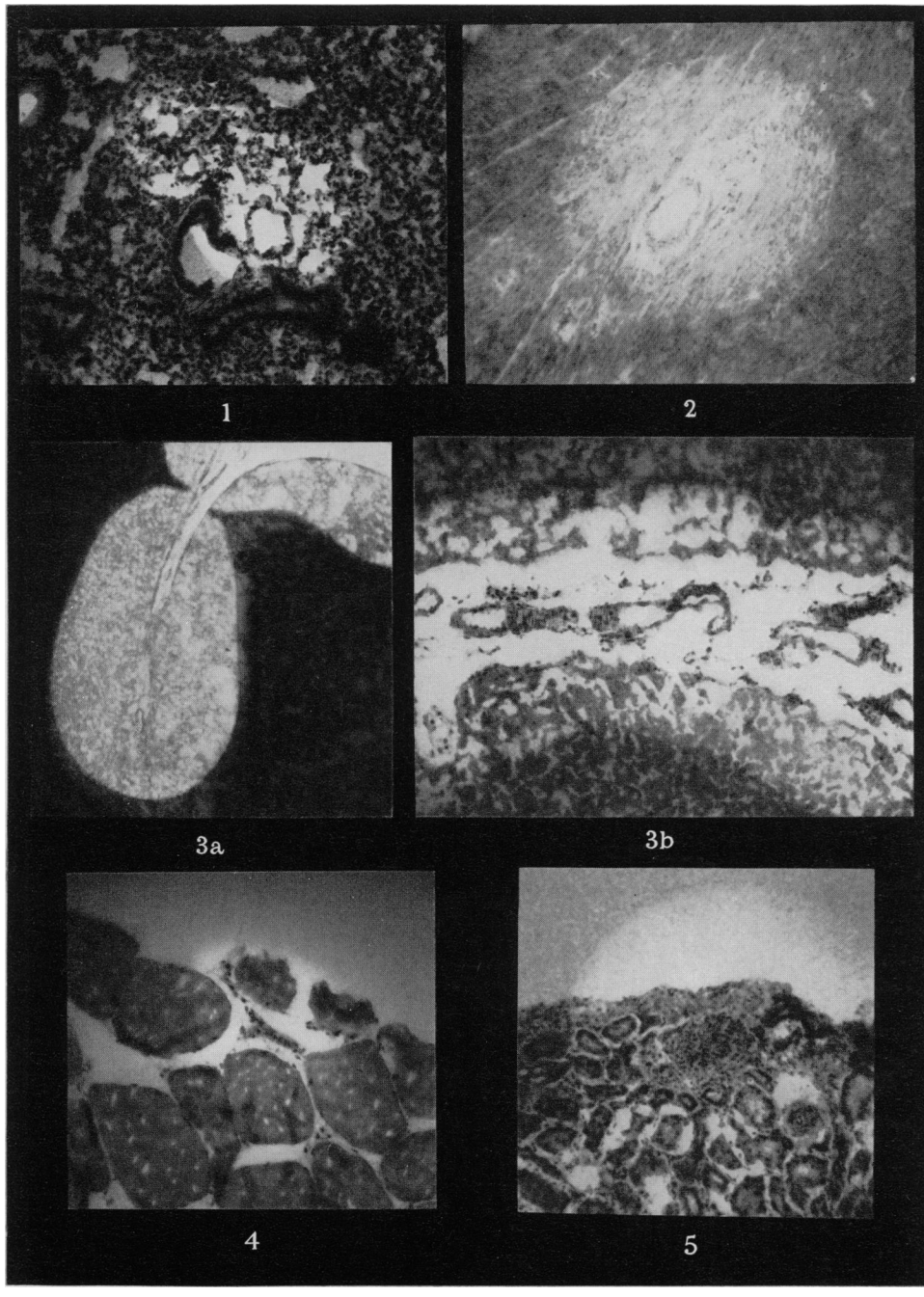
FIG. 9.—(a) Low power view of a sheet of endothelial cells from a rat inferior vena cava which had been lightly "silvered" before making a frozen *häutchen* preparation. Fibrin clot 1 per cent. Incubation 50 min. H. and E. ×80. (*b*) High power view of part of (*a*). ×455.

FIG. 10.—(*a*) Frozen *häutchen* preparation of "silvered" rat aorta. Two circular areas of fibrinolysis are seen around two small sheets of endothelial cells. Bovine fibrin 1 per cent. Incubation 3 hr. 40 min. H. and E. ×102. (*b*) High power field of Fig. 10*a*. ×255.

FIG. 11.—(*a*) Low power view of rabbit inferior vena cava *häutchen* preparation with overlying rabbit fibrin clot. Sheets of endothelial cells are present with surrounding areas of lysis. Rabbit fibrin 0·4 per cent. Incubation 40 min. H. and E. ×32. (*b*) High power view of same preparation as Fig. 11*a*. The clear area extending beyond the sheet of endothelial cells is a zone of lysis. ×300.

FIG. 12.—(*a*) Low power view of rabbit aorta frozen *häutchen* preparation with overlying 0·4 per cent rabbit fibrin clot. Clear areas of lysis can be seen around small sheets of endothelial cells. Incubation 1½ hr. H. and E. ×32. (*b*) High power view of same preparation as Fig. 12*a*. ×420.

FIG. 13.—Frozen section of ox aorta in 1 per cent bovine fibrin. Incubation 4 hr. H. and E. (*a*) Areas of lysis can be seen surrounding the vasa vasorum. The endothelium has been lost in the preparation of the section. ×22. E = Endothelial surface, A = Adventitia, V = Vasa vasorum. (*b*) Higher power view of one such vessel at the centre of an area of lysis. ×96.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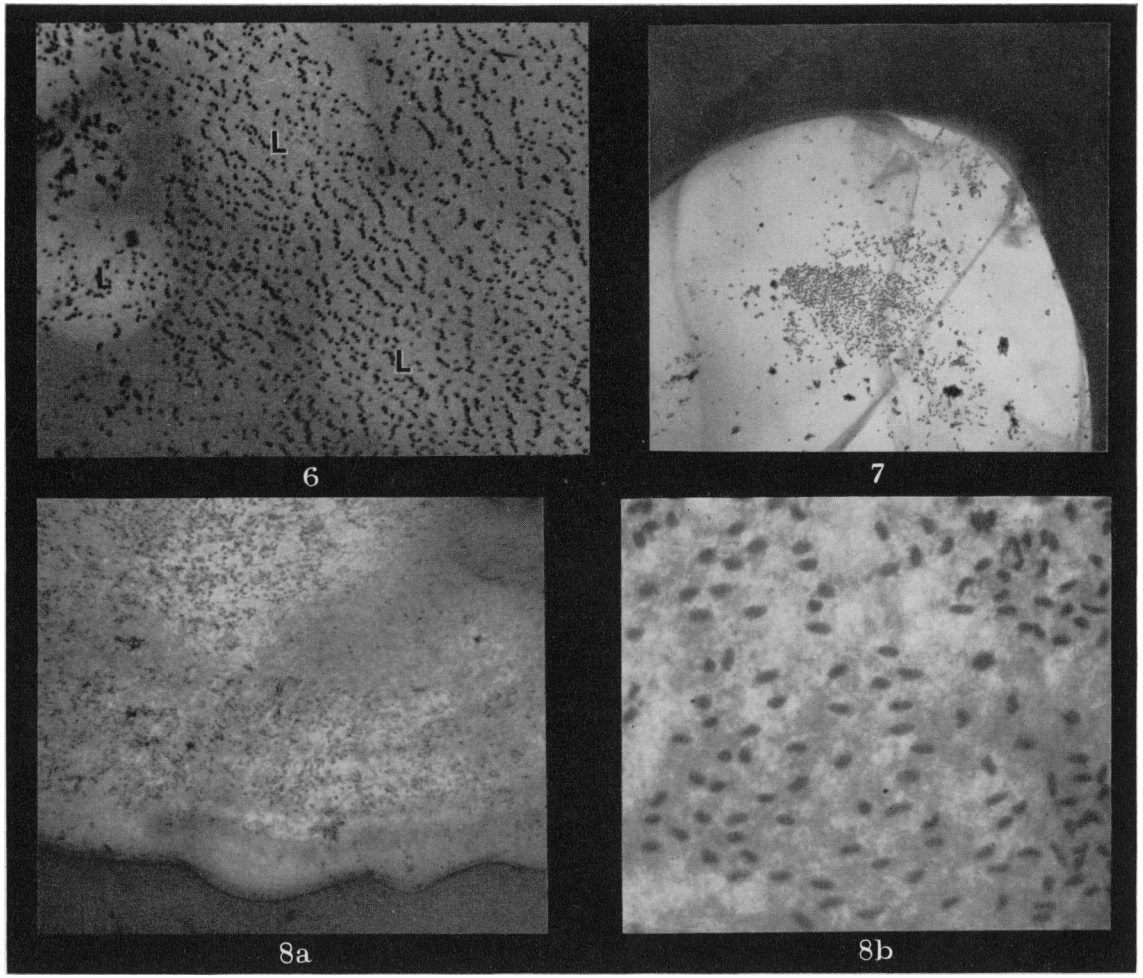

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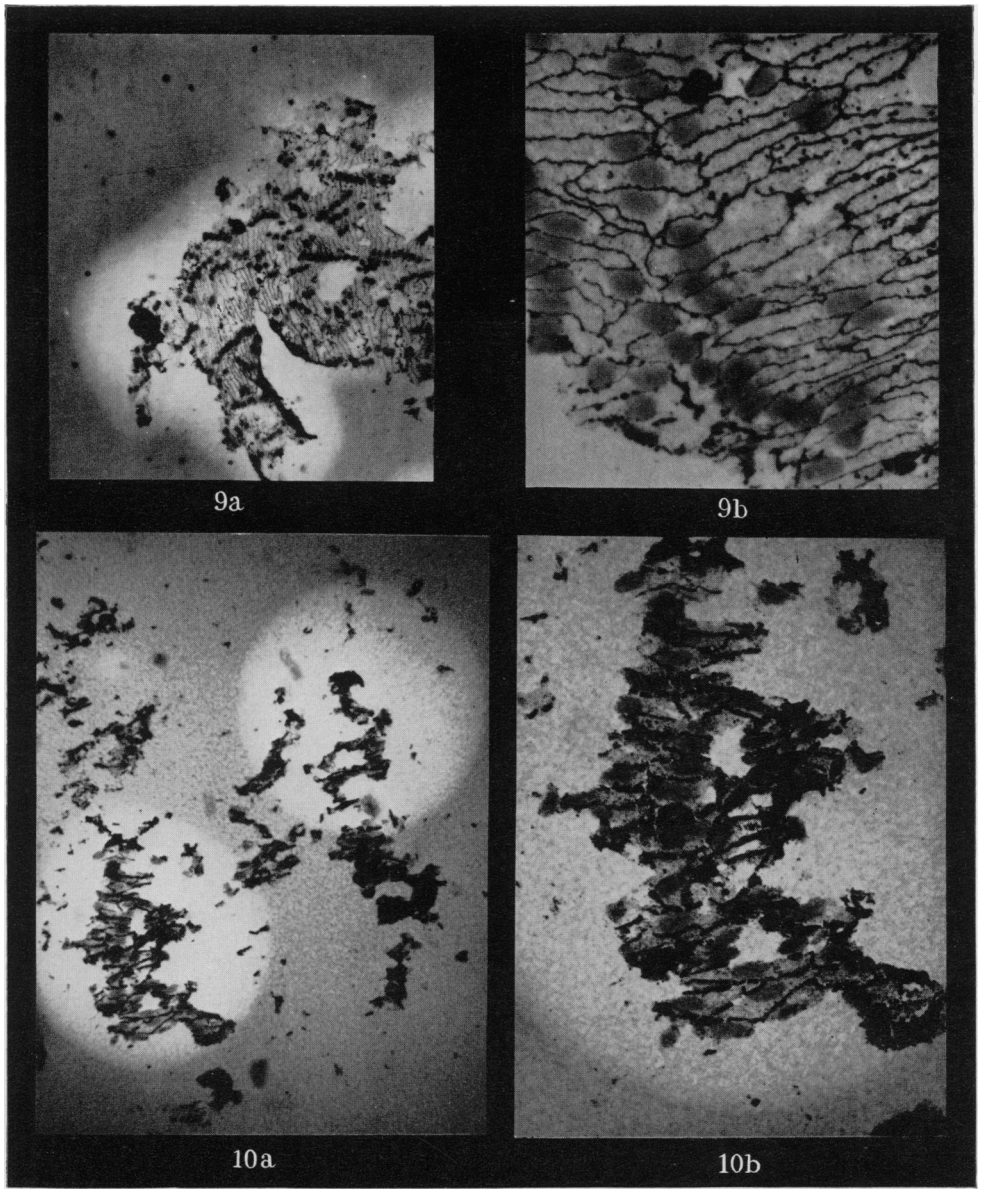
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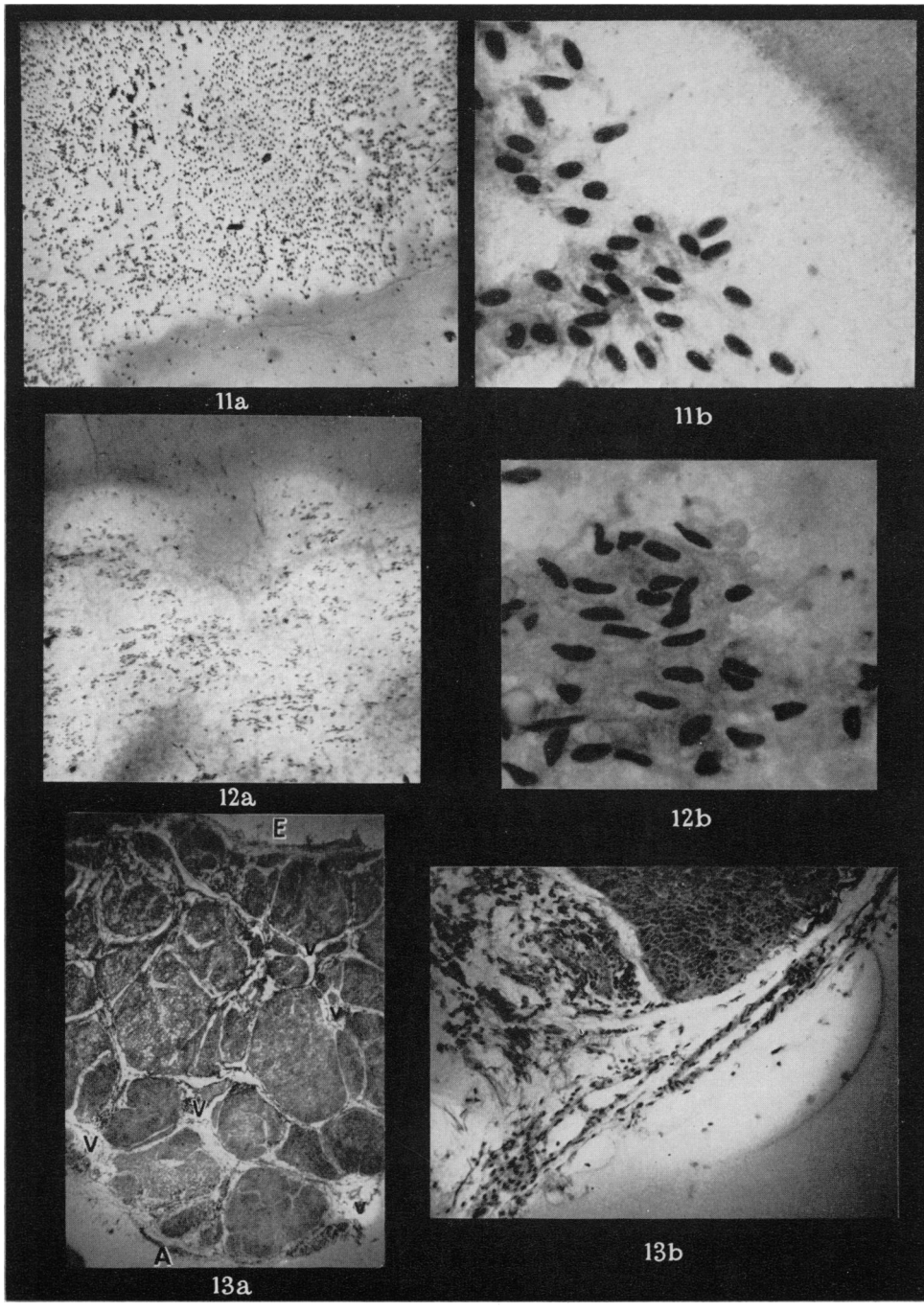
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uniformly over that part of the medulla included in the section. In addition, lysis occurred around glomeruli in ox and pig sections (Fig. 5).

A comparison was made of the induction of lysis by rabbit tissues in bovine and rabbit fibrin. Rabbit lung, heart, adrenal, muscle, liver and spleen were inactive on bovine fibrin. There was diffuse lysis over the medulla of the rabbit kidney after incubation. On rabbit fibrin, rabbit lung, brain, adrenal, liver and kidney were all active and muscle had slight activity.

Frozen *häutchen* preparations from the inferior vena cava and aorta of ox, pig, and rat tested on bovine fibrin, showed areas of lysis around endothelial cells similar to that shown in Figs. 6, 7 and 8. Endothelial cells stripped far more readily off the inferior vena cava than off the aorta by this method. Lysis of the fibrin clot around venous endothelial cells usually appeared 1 to 2 hr. after incubation (sometimes earlier), while that around aortic endothelial cells often required 3 to 5 hr. incubation to become evident macroscopically. After an incubation period of $1\frac{1}{2}$ hr. the preparation of rat inferior vena cava in Fig. 6 shows 3 pale areas representing commencing areas of lysis (marked " L "). Fig. 7 is a similar preparation incubated for 3 hr. 40 min. and shows a wide zone of lysis around a sheet of endothelial cells. As can be seen from comparison of Figs. 6 and 7 prolonged incubation of active preparations leads to widespread lysis. In such sections the endothelial sheets at the centre of the lysed areas are liable to be washed away during fixation and staining. Fig. 8 demonstrates lysis around rat aortic endothelial cells in a 1 per cent bovine fibrin clot. By lightly " silvering " the intima before pulling the frozen *häutchen*, " cement " lines, characteristic of endothelium, can be shown in the sheets of cells which produce lysis. Fig. 9*a* shows at low magnification a sheet of endothelial cells from rat inferior vena cava treated in such a way.

The black strands extending from the main sheet are parts of the endothelial layer rolled up on themselves. The characteristic mosaic of endothelial " cement " lines is evident and the whole sheet is surrounded by a clear zone of lysis. Fig. 9*b* shows details of the " cement " lines, and several rows of oval nuclei stained with haematoxylin. Figs. 10*a* and 10*b* are photomicrographs of " silvered " rat aorta *häutchen* and lysis around two sheets of 30–40 endothelial cells is seen. " Cement " lines are present. Sheets of cells from rat aorta prepared by the frozen *häutchen* technique were much smaller than those from rat inferior vena cava.

Preparations of rabbit inferior vena cava and aorta did not produce lysis of bovine fibrin even after 12 hr. incubation, but they lysed rabbit fibrin in 1–2 hr. (Figs. 11 and 12). Figs. 11*a* and 12*a* show at low magnification *häutchen* of rabbit inferior vena cava and aorta in rabbit fibrin. Lysis is seen as a pale region around the endothelial cells, whose nuclei only are easily discernible at this magnification. Fig. 11*b* shows the edge of the sheet of venous endothelium and the clear zone of lysis extending from it. Fig. 12*b* shows detail of the aortic endothelial cells in the centre of an area of lysis.

In an attempt to remove the layers of the vessel wall seriatim to determine their fibrinolytic activity several *häutchen* preparations were made successively from the one piece of tissue. These were produced from rabbit inferior vena cava by 5 consecutive " pulls " starting with the endothelial layer, and were half covered by rabbit fibrin and half by bovine fibrin. After incubating for 2 hr. 20 min. at 37°, lysis of the rabbit fibrin was seen in slides 1 and 2 but not in 3, 4 or 5. The bovine fibrin was not lysed in any of the slides. Smooth muscle cells

and other elements in the vessel wall both by this method and in histological section were inactive except for the vasa vasorum, and the activity of these was probably due to their own endothelium. The aorta does not readily lend itself to the making of serial *häutchen* partly because of the thickness of the wall and partly because the media tears unevenly, but it gave similar results.

DISCUSSION

In 1957, Albrechtsen, using a potassium thiocyanate method, extracted tissue activator from the tissues of a number of species and estimated its amount by measuring areas of lysis on standard bovine fibrin plates. Those tissues which Albrechtsen found to have the highest activator content, *i.e.*, rat and mouse lung and pig heart were found in this investigation to be very active (Fig. 1). Tissues which he placed in the intermediate range of activity (that is, those which produced areas of clearing on standard bovine fibrin plates of 20–100 sq.mm./g. fresh tissue) all showed development of lytic areas around their blood vessels in the present study. These were ox heart, brain and adrenal ; pig brain, adrenal and muscle ; rat heart (Fig. 2), brain (Fig. 3) and adrenal. Ox lung and muscle (Fig. 4) and pig lung developed areas of lysis around vessels although by Albrechtsen's method they were inactive. Other constituents of these tissues may make extraction of activator by potassium thiocyanate difficult or the origin of the lytic areas in these tissues may be different.

The fibrinolytic system of plasma has been reported to show some degree of specificity by Fischer (1946) and Cliffton and Downie (1950). Fischer, working on chicken Rous sarcoma cells, was hindered in cultivating permanent strains of these cells by liquefaction of chicken plasma medium, but he could grow the cells through many passages without liquefaction in a medium composed of rabbit plasma and chick embryo tissue. When the strain was transferred again to chicken plasma medium, liquefaction took place. Cliffton and Downie found that a euglobulin fraction of serum or plasma from animals and man contained a fibrinolytic precursor or active enzyme. They were able to show that the activation patterns of the enzyme precursor of man and monkey were different from that of dog, guinea-pig and rabbit. It appears from the results presented here that the rabbit endothelial fibrinolytic system differs from that of ox, pig and rat in that it does not lyse bovine clot (with the exception of renal medulla). However, rabbit tissue sections and *häutchen* lyse rabbit fibrin producing a similar appearance to that resulting from the action of bovine, pig and rat preparations on bovine fibrin (Figs. 11 and 12).

As can be seen from Figs. 6, 7 and 8 there was variation in the activity of endothelial cells in some frozen *häutchen* preparations. Rabbit inferior vena cava and aorta *häutchen* tested on rabbit fibrin showed the least variation and in some slides it was absent. After lightly " silvering " the vessel the variation of activity in endothelial sheets seems to be due, at least in part, to variable contact with the substances involved in this process, since overstained material does not lyse fibrin. Consideration of the variation in frozen *häutchen* preparations of " unsilvered " vessels raises the following possibilities. There may be differences in the freeze-thaw damage inflicted on the cells in the endothelial layer as it is peeled from the vessel wall, reflected in varying degrees of denaturation of cell enzymes. Alternatively, this difference may be a real variation in activity and not an artefact.

Minor differences of enzyme structural change produced by freeze denaturation may be magnified by the heterologous nature of many of the preparations. Or, perhaps greater activity may be present in some endothelial cells than in others, similar to the recently reported (Romanul and Bannister, 1962) localised alkaline phosphatase activity in the endothelium of small arteries and arterioles at their origin from larger vessels, though this does not seem likely.

Todd (1961) found that streptokinase was required in the medium to evoke lysis around endothelial cells of human systemic arteries in bovine fibrin. He deduced from this that these cells contained proactivator in contrast to the venous endothelial cells and the pulmonary artery endothelial cells which contained activator. In this investigation frozen *häutchen* of ox, pig and rat aortas showed direct activity on bovine fibrin, without the addition of streptokinase, though their activity was less than that of the venous endothelium from the same species.

Lieberman and Kellog (1961) examined the fibrinolytic activity of arterial tissues and found that plasminogen activator was concentrated mainly in the adventitia of human arteries. The results of serial *häutchen* and the appearance of the aorta in section (Fig. 13) suggest that the concentration of activator in the adventitia of arteries is due to the presence of the vasa vasorum there. In turn, the activator content of the vasa vasorum is probably concentrated in their endothelium.

SUMMARY

The fibrinolytic activity of a number of tissues from ox, pig, rat, mouse and rabbit was examined by the fibrinolytic autograph technique. Of the tissues tested rat and mouse lung, and pig heart and brain were found to be the most active on bovine fibrin. The lysis of fibrin was always clearly associated with blood vessels except over the renal medulla.

Häutchen preparations of endothelial cells from the inferior vena cava and aorta from ox, pig and rat induced fibrinolysis in bovine fibrin clot. Preparations from the inferior vena cava were more active than those from the aorta of the same species.

Häutchen preparations from rabbit inferior vena cava and aorta induced fibrinolysis in rabbit fibrin but not in bovine fibrin. Omitting consideration of the renal medulla, the same difference held good for the blood vessels in frozen sections from other organs of the rabbit.

When the intima was lightly " silvered " the sheets of cells in frozen *häutchen* preparations that produced lysis exhibited the characteristic " cement " lines of endothelium. Serial frozen *häutchen* of rabbit inferior vena cava and aorta showed lysis only associated with endothelial cells and around fragments of vasa vasorum.

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