PRODUCTION OF INFLAMMATORY CHANGES IN THE MICROCIRCULATION BY CATIONIC PROTEINS EXTRACTED FROM LYSOSOMES*

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The identity of those mechanisms which initiate and regulate inflammatory changes in injured tissues is still largely unknown. A number of agents have been suggested as mediators of increased permeability of small blood vessels, but few of these have been implicated in the production of leucocyte sticking and emigration, the *sine qua non* of the inflammatory reaction. In the present study, the authors have attempted to reproduce these latter phenomena using protein materials extracted from lysosomes.

It was de Duve who originally suggested that lysosomal components could play a causal role in the development of tissue damage (1). Attracted by this hypothesis, many investigators have sought to implicate lysosomes and their products in the pathogenesis of tissue-injury reactions (2–7). However, direct tests of the capacity of lysosomes to induce such reactions were not undertaken until the discovery of Cohn and Hirsch (8) that the specific cytoplasmic granules of rabbit peritoneal exudate polymorphonuclear (PMN) leucocytes are lysosomal in nature. Following their demonstration of a simple and reliable technique for the collection of large quantities of these granules in relatively pure form, the direct production of inflammatory changes in tissues was accomplished by Weissmann and Thomas (9) using either PMN leucocyte granules lysed by freezing and thawing or agents which readily disrupt these particles. At the same time, Thomas (10) substituted intact leucocyte granules for the preparatory dose of bacterial endotoxin and succeeded in producing Shwartzman-like lesions in rabbit skin prepared in this manner and then challenged with intradermal epinephrine or intravenous endotoxin. Thomas' observations were later confirmed by Halpern (11), who also found that proteolytic enzyme inhibitors blocked the Shwartzman-like lesions produced by PMN granules, thus suggesting the importance of lysosomal acid proteases in the reaction. Finally, Golub and Spitznagel (12) reported observations on dermal lesions induced in rabbits by homologous PMN lysosomes and suggested that the granules of leucocytes are potent sources of tissue damage in the Arthus reaction.

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In addition to the probable role of lysosomal proteases and other hydrolytic enzymes in the pathogenesis of tissue injury, as conceived by de Duve and documented by the studies referred to above, a complementary approach to the study of lysosomes in tissue injury has developed as a result of further delineation of the properties of PMN leucocyte lysosomes. Polycationic proteins, having the capacity to agglutinate and kill bacteria, were recently extracted from these granules (guinea pig, rabbit) by Zeya and Spitznagel (13). Shortly thereafter, Horn and Spicer (14) reported the presence of basic proteins in association with sulfated mucopolysaccharides in the azurophil granules of rabbit neutrophil PMN leucocytes, especially in the immature, myeloid neutrophils. Spitznagel and Chi (15) had already shown that these cationic proteins are released from PMN leucocytes following emigration of such cells into perivascular spaces in response to infection (guinea pig and rabbit). These observations seemed especially significant when viewed in the light of Frimmer and Hegner's (16) finding that polycationic polypeptides extracted from calf thymus exert leucotactic and permeability-increasing effects upon the microcirculation of the rat mesentery.

It thus seemed reasonable, to us, that the cationic proteins present in PMN leucocyte lysosomes would likewise accelerate the development of inflammation by promoting increased emigration of leucocytes and increased permeability of small blood vessels following the discharge of such proteins from the initial granulocytes appearing at injured sites in tissues. In order to test this proposal, we undertook a study of the effects of cationic proteins extracted from exudate PMN leucocyte lysosomes (rabbit) upon the microcirculation of autologous, homologous, and heterologous tissue (mesentery) observed in the living state. A preliminary description of the results of this study has been published elsewhere (17). The present report confirms and extends this work.

Materials and Methods

Rabbit peritoneal exudate leucocytes were collected in heparinized saline 4 hours after the intraperitoneal administration of 0.1 per cent glycogen to the animals. Total and differential white cell counts were performed on samples of each exudate and the cell suspensions from all animals were then pooled. After washing in sucrose, the leucocytes (95 to 100 per cent PMN cells) were lysed in 0.34 M sucrose by the technique of Cohn and Hirsch (8). The lysed cells were centrifuged at low-speed (400 *G* for 10 minutes), after which the floating layer of lipids and the sediment consisting mainly of nuclei and unbroken cells were both discarded. The balance of the supernate was then centrifuged at 8500 *G* for 15 minutes in order to sediment the granules. The post–8500 *G* supernatant fraction (cell sap plus microsomes) was used in control tests. The granules were washed in sucrose, recentrifuged, and employed in the following preparations.

Whole Granule Preparations.—Washed leucocyte granules were resuspended in saline buffered to pH 7.0 and ruptured by repeated, rapid freezing and thawing in acetone–carbon dioxide freezing mixture (seven freeze-thaw cycles). The suspension of lysed granules was clarified by high-speed centrifugation (17,000 *G* for 20 minutes) and the supernate was then dialyzed in the cold against buffered saline at pH 7.0, before testing. The granule-free, post–8500 *G* supernatant fraction of the sucrose-lysed PMN leucocytes was also frozen-thawed and dialyzed, before use as a control.

Preparation of Cationic Protein Fractions of the Granules.—As an alternative to the preparation of whole granule lysate, extraction of the cationic proteins of these particles was carried out according to the technique of Ui (18) as modified by Zeya and Spitznagel (13). Washed granules were repeatedly extracted with ice-cold, 0.2 N sulfuric acid. The viscous acid extracts were pooled and clarified by high-speed centrifugation. Two protein fractions were then separated from the acid extract by precipitation with ethanol. One fraction was collected at a 20 per cent concentration of ethanol (3 hours at −5°C); a second fraction was collected at 45 per cent ethanol concentration (overnight at −10°C). Each precipitate was dissolved in 3.0 ml of 0.005 M hydrochloric acid and sulfate ions were removed by addition of 0.2 N barium chloride to the protein solutions. After centrifuging and discarding the precipitate of barium sulfate, the protein-chloride solutions were dialyzed in the cold against changes of 0.15 M saline at pH 3.6 until barium could no longer be detected in the dialysate (three, 2 hour dialyses against 100 volumes of acidified saline). The dialyzed materials were either tested immediately or were lyophilized and stored under vacuum at 4°C.

Tests on the Microcirculation.—Mesenteries of rats and rabbits were exteriorized and maintained under physiologic conditions, suitable for microscopic observation, by previously described techniques (19). All materials to be tested were made up in isotonic saline adjusted to pH 7.0. In the case of rat mesentery, the Ringer gelatin drip which is normally used to keep the preparation moist and at 37°C was interrupted and the test material (or control) was applied topically and allowed to remain on the tissue for selected time intervals. In the case of rabbit mesentery, the vessels of which are more susceptible to injury, the test solution was infused into the tubing through which the Ringer gelatin flowed onto the mesentery, and the drip was kept continuously running. Tissues were observed at magnifications of 120 or 200 for periods up to 1 hour. Photomicrographs of the preparations in the living state were taken at intervals before and after application of test materials. In selected instances, test animals were killed at the end of the period of observation and the mesenteries were fixed *in situ* with Zenker's formalin and then stained with Harris' hematoxylin and eosin mixture.

Effects on Leucocytes and Platelets in Vitro.—Rabbit blood was withdrawn by cardiac puncture into siliconized syringes moistened with heparin. Final heparin concentration was adjusted to 10 units per ml blood. Centrifugation at 1000 RPM (PR-2 International Refrigerated centrifuge, International Centrifuge, Boston) for 10 minutes provided a platelet-rich supernate. Leucocytes were collected either from buffy coat or after dextran sedimentation of red cells. Known concentrations of the protein fractions to be tested were added directly to the platelet or leucocyte suspensions in siliconized glass tubes and incubated at 37°C. Observations were carried out with Zeiss phase objectives.

Effects on Isolated Liver Mitochondria.—Rat and rabbit livers were homogenized in 0.25 M sucrose, and mitochondrial fractions were collected by differential high-speed centrifugation of the homogenates. Washed mitochondria were suspended in 0.25 M sucrose containing 0.02 M tris buffer at pH 7.4. Following the addition of protein fractions to these suspensions, swelling of the liver particles at 22°C was monitored in a Beckman DB spectrophotometer as optical density change at 509 mμ. Control rates of swelling were similarly determined in the absence of added protein.

Physical-Chemical Properties of the 20 Per Cent Ethanol-Precipitated Fraction.—UV absorption spectral analyses of dialyzed samples of the 20 per cent ethanol-precipitated fraction were carried out using different concentrations of protein in 0.1 M saline at pH 3.6. Recordings were obtained with a Beckman DB spectrophotometer and Photovolt recorder (varicord model 43, Photovolt Corporation, New York). Starch gel electrophoresis of the 20 per cent ethanol fraction was carried out in pH 4.0 acetate buffer (0.1 ionic strength diluted 1:2 in the gel) for 2½ hours at room temperature and at a potential gradient of 4 volts per cm. Proteins in the gel were then stained with nigrosin or with naphthalene black B.

Biochemical Properties of the Protein Fractions.—Per cent of ribonucleic acid present in the fractions was determined by the orcinol reaction (20). The fractions were also assayed for activity of the following enzymes: acid phenolphthalein phosphatase (21), beta glucuronidase (22), acid ribonuclease (23), lysozyme (24), and catalase (25). Acid cathepsins were not assayed in our experiments, but identical fractions prepared from rabbit PMN leucocyte granules have recently been tested for catheptic activity by others (26) (see Discussion).

Pyrogen Assay.—Pyrogenicity of the 20 per cent ethanol fraction was tested in endotoxin-tolerant and conventional rabbits by an established technique (27) (see acknowledgment).

Assay of Pharmacologic (Musculotropic) Properties.—Tests for kinin-like activity of the 20 per cent ethanol fraction were carried out by measuring contractile effects of the material on the isolated rabbit aortic strip (28) and guinea pig ileum (29), and by measuring effects of the material on mean carotid blood pressure of anesthetized rats.

RESULTS

Effects of Lysed Whole Granules on the Microcirculation of Rats and Rabbits.—Leucocyte granules were disrupted by freezing-thawing and samples of the dialyzed supernate (50 μg of protein) were applied topically onto the rat mesentery. Within 2 or 3 minutes, increasing numbers of leucocytes began to adhere to the endothelial surface of capillaries (8 to 12 μ in diameter) as well as collecting venules (25 to 40 μ diameter). In the latter, adhesion of white cells gradually increased until a layer of cells imbedded in an amorphous material could be seen covering the entire inner surface of the vessels. At 10 minutes, emigration of leucocytes into the perivascular tissue could be observed. In some vessels, palisading of cells was so extensive that blood flow was reduced even to the point of complete stasis. After 20 to 30 minutes, occasional petechial hemorrhages were observed, marked by extravasation of red cells through defects in the walls of both the capillaries and collecting venules. When intact leucocyte granules were applied to the surface of the rat mesoappendix, a similar but significantly milder and more slowly developing set of reactions was observed. On the other hand, the granule-free, post–8500 *G* supernatant fraction of the lysed PMN leucocytes had no significant effect on the mesenteric vessels of the rat.

The effects obtained with granules of exudate leucocytes harvested from rabbits after a single treatment with glycogen were equivalent to those obtained with granules collected from rabbits repeatedly primed with this agent. In addition, rabbit granule preparations produced an identical injury in mesenteric vessels of the homologous species (rabbit). Even more significant was the fact that lysed granules produced the same inflammatory changes when tested on autologous tissue; that is, when applied to mesenteries of leucocyte donor rabbits (tested 1 month after collection of exudate leucocytes).

Effects of Cationic Proteins of the Granules on the Microcirculation of Rats and Rabbits.—Protein fractions, prepared as described under Materials and Methods and adjusted to pH 7.0, were similarly tested on exteriorized mesenteries of rats and rabbits. The 20 per cent ethanol fraction produced all of the aforementioned injury reactions (leucocyte sticking, emigration, stasis, and hemorrhage) in the rat and rabbit mesentery with greater intensity than that pre-

viously described for lysates of whole granules. As little as 20 μg of protein of this fraction produced an effect equivalent to, or greater than, that produced by 50 μg of protein of lysed whole granules. On the other hand, equal amounts of the 45 per cent ethanol-precipitated protein fraction were essentially inactive in these tests.

It was frequently observed during these tests that the initial phase of leucocyte sticking produced by the cationic protein could be reversed by washing the material from the mesentery. Longer application of the agent, to the point of emigration of white cells, produced lasting changes, although even these could be modified in degree by removal of the protein.

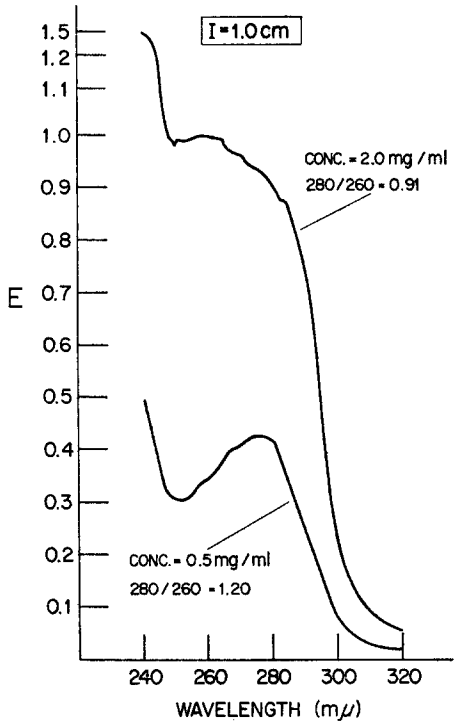
Typical effects of the 20 per cent ethanol fraction on rat mesenteric vessels photographed in the living state are shown in Figs. 1 to 4 and on rabbit mesenteric vessels in Figs. 5 and 6. Representative stained preparations of rabbit mesenteries treated with this fraction are shown in Figs. 9 to 16. Photographs of fixed and stained tissue show that polymorphonuclear cells comprised the major type of leucocyte involved in the sticking and emigration response. In addition, platelet thrombi were not infrequently found in association with damaged vessels (see Fig. 9).

Effects of Cationic Proteins Extracted from Leucocyte Granules on Rabbit Platelets and Leucocytes in Vitro.—Both the 20 per cent and the 45 per cent ethanol-precipitated protein fractions produced aggregation of platelets within a few minutes when incubated with these blood elements *in vitro*. The rate of clumping was proportional to the concentration of test protein used in these tests. These effects were observed under phase microscopy (see Figs. 7 and 8). The reaction was not inhibited by heparin at a concentration of 0.1 mg per ml, but failed to occur (even with concentrations of test protein as high as 400 μg per ml) when the protein was added to platelet suspensions prepared by sedimenting the erythrocytes with dextran. Rabbit leucocytes were not directly affected by incubation with test proteins, and even those cells which had been incidentally trapped within platelet clumps could be observed to migrate free after relatively short intervals.

Effects of the 20 Per Cent Ethanol Fraction on Liver Mitochondria.—Frimmer and Hegner (16), in their studies dealing with the inflammatory action of cationic proteins extracted from calf thymus, reported general membrane effects of their materials including production of mitochondrial swelling. We therefore tested the leucocyte cationic proteins under similar conditions. At a concentration of 10 μg of protein per ml of particle suspension, a 2½-fold increase in the rate of mitochondrial swelling occurred in most experiments. However, the effect was not observed consistently and the interpretation of results was further complicated by the fact that higher concentrations of the test protein caused aggregation of the liver particles and markedly decreased the rate of swelling under these circumstances.

Physical-Chemical and Biochemical Properties of the Cationic Proteins.—Text-

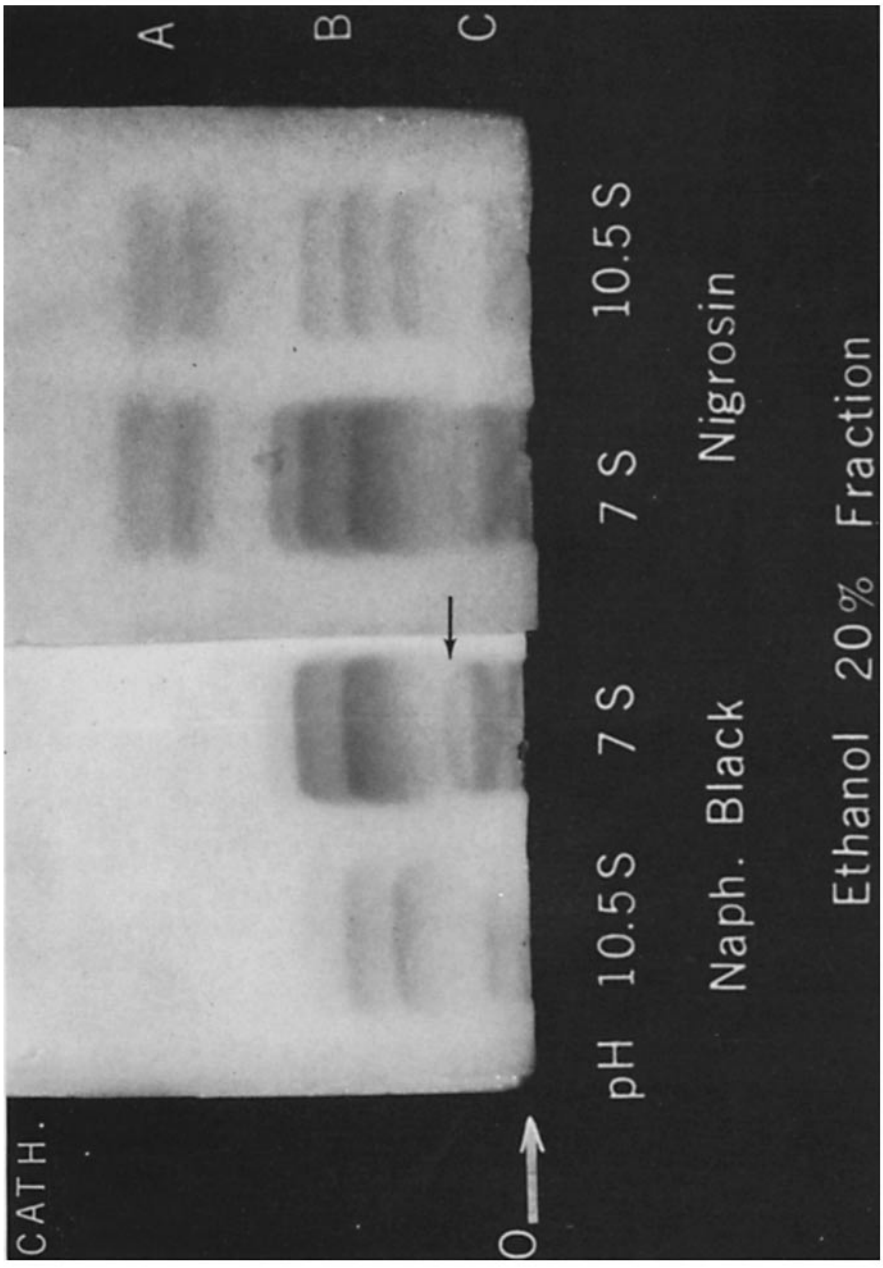
fig. 1 shows the UV absorption spectrum obtained with the 20 per cent ethanol–precipitated fraction at two concentrations of protein. At the highest concentration employed in this test (2 mg per ml) the 280/260 absorbance ratio indicated a 4 per cent contamination of the fraction with nucleic acid. Assays for ribose (orcinol reaction) also showed the presence of 4 per cent (by weight) of



TEXT-FIG. 1. Ultraviolet absorption spectrum of the cationic protein fraction (of rabbit exudate PMN lysosomes) precipitated by 20 per cent ethanol. Protein was dissolved in 0.1 M NaCl, pH 3.7.

ribonucleic acid. A likely source of contamination with RNA could have been microsomal elements released during cell lysis and incompletely removed from the granule pellet during subsequent washing. The small amount of RNA present in the 20 per cent ethanol fraction was not considered to contribute significantly to its biologic effects on the microcirculation, in view of the failure of the RNA-rich, post–8500 *G* supernate to produce inflammation in the mesentery.

Text-fig. 2 shows the pattern obtained when samples of the 20 per cent ethanol fraction were subjected to electrophoresis in starch gel and were stained by the methods described earlier. The three groups of bands marked *A*, *B*,



TEXT-FIG. 2. Starch gel electrophoresis pattern of cationic protein fraction precipitated by 20 per cent ethanol. Cathode is at top of figure. 7 S, whole protein solution; 10.5S, protein solution after titration with NaOH as described in Results. Note absence of staining of A bands with naphthalene black, and loss of one of the C bands (arrow) from the 10.5S material.

and *C* in the photograph probably represent distinct protein species with different charge properties, while the individual bands within each of the three major groups may be artifacts resulting from polymerization of protein molecules. Zeya and Spitznagel (13) also reported the separation into three bands of a closely related cationic protein fraction (obtained from guinea pig PMN leucocyte granules) after electrophoresis in pH 4.0 acetate buffer on oxoid strip. Examination of Text-fig. 2 shows a distinct difference between the most rapidly migrating component (A bands) and the other proteins, in that the former material failed to stain with naphthalene black. Another feature of the electrophoretic pattern is worthy of mention. Titration of the acidified solution

TABLE I

Enzymatic Properties of Ethanol-Precipitated Protein Fractions of Rabbit PMN Lysosomes

Fraction	Recovery of total granule protein	Enzymes				
		Acid phosph.	Beta-gluc.	Acid RNAase	Lysozyme	Catalase
	per cent	μg*	μg*	*OD*‡	μg§	
Lysed granule supernate	25	2.9	2.5	0.065	2100	0
20 per cent ethanol fraction	9	0	0	0	30	0
45 per cent ethanol fraction	8	0.1	0.005	0.068	500	0

* Acid phosphatase and beta glucuronidase activities expressed as μg phenolphthalein released per 100 μg protein per hour at 37°C.

‡ Acid ribonuclease activity expressed as increment in optical density measured at 2600 A per 100 μg protein/hour/37°C.

§ Lysozyme activity expressed as microgram equivalents of crystalline egg white lysozyme per milliliter of fraction.

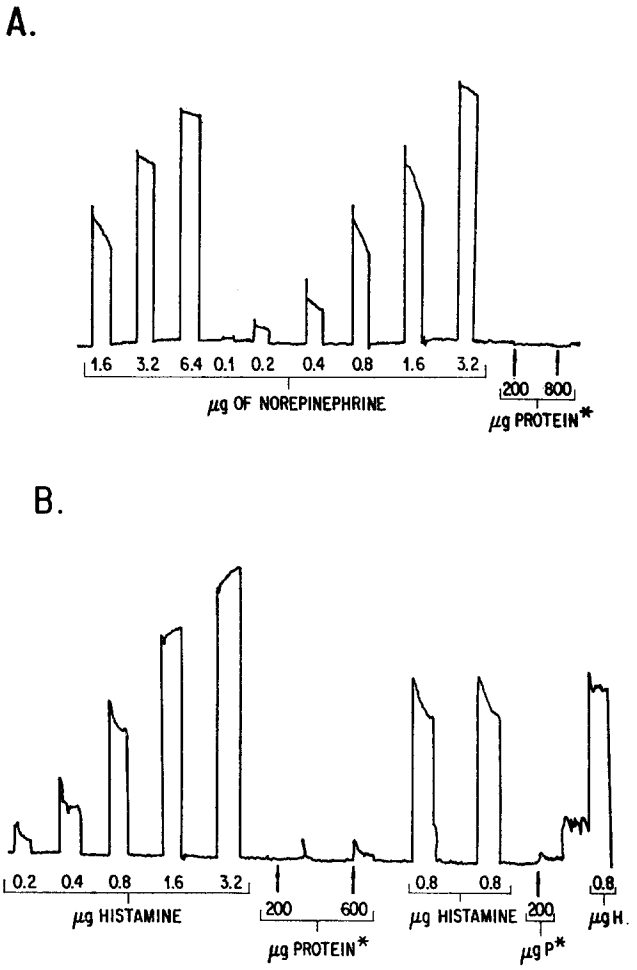
of the 20 per cent ethanol fraction to pH 10.5 was found to precipitate half of the protein present initially. When this supernate was reacidified and subjected to electrophoresis under the same conditions as before, the resultant pattern (identified as 10.5 S in Text-fig. 2) showed an expected over-all reduction in the quantity of protein present in all three major bands, and a distinct qualitative change in the C band group, as indicated by the complete absence of one of its sub-bands (marked by the arrow in Text-fig. 2). Moreover, the pH 10.5 supernate, when neutralized and retested on the rat mesentery showed a significantly greater inflammatory reaction per unit of protein. The topical application of as little as 3 μg of this material produced a prominent sticking reaction in the microvessels equivalent to that produced by 5 to 10 times as much of the parent 20 per cent ethanol protein fraction. The reason for the increased biologic activity following removal of one of the C band proteins is currently under investigation.

Finally, the enzymatic properties of the various granule fractions employed in these experiments are summarized in Table I. The data conclusively demonstrate that the 20 per cent ethanol fraction, while possessing all of the inflammatory activity of the whole granules, contained little activity of any of the lysosomal enzymes for which it was tested. On the other hand, the 45 per cent ethanol fraction, which (as reported in preceding sections) was relatively inactive in mesentery tests, contained appreciable ribonuclease and lysozyme activity. The interpretation of these results will be discussed in a later section (see Discussion).

Pyrogen Tests.—In view of Berlin and Wood's (30) recent demonstration that phagocytosis is accompanied by release of endogenous pyrogen from peritoneal exudate leucocytes of rabbits, and the earlier observations of Hirsch and Cohn (31) relating phagocytic activity of these cells to degranulation of their cytoplasm, there is good reason to suspect that leucocytic pyrogen may be related to one or more of the components of the lysosomes of these cells. It seemed especially appropriate to test the proinflammatory cationic protein fraction of the PMN leucocyte granules for pyrogenicity, on the basis of the reported presence of basic amino acids such as histidine and arginine in endogenous pyrogen (32). However, tests of the material revealed no pyrogenic response in either conventional or endotoxin-tolerant rabbits following intravenous injection of this material.[1]

Pharmacologic Tests.—The inflammatory effect of the 20 per cent ethanol fraction was not associated with any discernible musculotropic activity. *In vitro*, even large concentrations of this protein did not contract the isolated guinea pig ileum or influence the contractile response of the ileum to histamine (Text-fig. 3 *B*). There was no evidence of epinephrine-like activity when this protein was tested on vascular smooth muscle *in vitro* (rabbit aortic strip, see Text-fig. 3 *A*). Although a transient fall in blood pressure was observed in the rat after injection of 150 μg of test protein, the vasodepressor effect was not as pronounced as that induced by only 5 μg of bradykinin (Text-fig. 3 *C*). When the protein was injected into rats pretreated with a blocking dose of an antihistamine (phenergan hydrochloride), no vasodepressor response occurred. This suggests that an endogenous histamine release mechanism may be involved in the production of transient hypotension by these cationic proteins.

[1] Failure to induce a febrile response is especially significant in view of the recently reported production of a permeability-increasing and leucocyte-migration promoting substance by viable granulocytes *in vitro*, which substance closely resembles leucocytic pyrogen in many respects. (See Moses, J. M., Ebert, R. H., Graham, R. C., and Brine, K. L., Pathogenesis of inflammation. I. The production of an inflammatory substance from rabbit granulocytes *in vitro* and its relationship to leucocyte pyrogen, *J. Exp. Med.*, 1964, **120**, 57.) The absence of pyrogenicity in lysosomal cationic protein and marked differences between the kinetics of vascular permeability changes induced by this protein and Moses' "granulocytic substance" (to be reported elsewhere), clearly differentiate these two principles.



TEXT-FIGS. 3 *A* to 3 *C*. Pharmacologic tests for smooth muscle contracting and vasoactive properties of leucocyte granule cationic protein (20 per cent ethanol fraction).

TEXT-FIG. 3 *A*. Effect on rabbit isolated aortic strip; Kymographic tracing.

TEXT-FIG. 3 *B*. Effect on guinea pig isolated ileum; Kymographic tracing.

TEXT-FIG. 3 *C*. Effect on mean arterial blood pressure (rat). 320 gm rat; nembutal anesthesia. M.A.B.P., mean arterial blood pressure.

DISCUSSION

Mediator of Inflammation.—Frimmer and Hegner (16), on the basis of their isolation of a basic polypeptide with leucotactic and permeability-increasing properties from calf thymus, suggested that the release of histone-like materials from nuclei of damaged cells might constitute a leucotactic stimulus in inflammation. On the other hand, the data presented in this paper demonstrate that cationic proteins with the property of stimulating leucocyte adhesion and emigration are also present in the lysosomal granules of exudate polymorphonuclear leucocytes, and it therefore seems equally likely that the release of such agents from the lysosomes of damaged cells may represent the major leucotactic stimulus during the development of inflammatory reactions. The demonstration of these basic proteins in the very same cells which are a prominent feature of the early stages of the inflammatory response lends further credence to the importance of such protein factors in the development of the reaction. The belief that disintegration of polymorphs at the site of injury might yield a substance which exacerbates the altered vascular permeability and leucocytic emigration has received support from the work of Stetson and Good (33), Page and Good (34), and Humphrey (35, 36), all of whom have shown that experimentally induced leucopenia considerably reduces the intensity of local tissue injury reactions. In addition, Hurley and Spector (37) found that "of saline extracts prepared from many different tissues only that obtained from granulocytes themselves fulfilled the criterion of a specific leucocyte migration-promoting substance." Their leucocyte emigration factor was described as a non-dialyzable, heat-labile, and trypsin-inactivated agent. These authors also suggested that production of "chemotactic" agents required the interaction of serum with factors released from damaged tissues, but again they found that granulocytes were especially potent in inducing formation of chemotactic substances under these circumstances (37, 38).

Phase of Action.—It should be emphasized that the present authors believe leucocytic cationic protein is not to be considered as an initiating factor in the development of inflammation but, rather, an exacerbating influence in the progression of cell emigration to peak numbers. This interpretation is in agreement with observed facts since one can distinguish, during inflammation, two phases of cell diapedesis. The initial phase of emigration is marked by rapidly developing but slight diapedesis of white cells immediately after injury and has been considered to result from early changes in the walls of injured vessels (39). The second phase, which develops later on, involves much more extensive leucocyte migration and is thought to result from the appearance of chemotactic stimuli in the tissue, possibly derived from those granulocytes which were involved in the initial phase of cell migration (39). However, the possibility exists that cationic proteins are found in lysosomes of other cell types, including endothelial cells, in which case even the initiation of leucocyte sticking and emigration could be the result of the release of a basic protein. Furthermore, in instances where damage to endothelial cell nuclei occurs immediately after injury, cationic polypeptides of the type described by Frimmer and Hegner (16) could be responsible for the initial phase of leucocyte diapedesis.

Mechanism of Action.—Among the mechanisms by which polycationic proteins could affect the microcirculation is the release of histamine from mast cells. The fact that these proteins caused transient vasodepression in rats which could be inhibited by pretreatment with an antihistamine clearly supports this possibility. On the other hand, when rats were pretreated with 15 mg per kg of phenergan hydrochloride, adhesion and emigration of leucocytes in the capillaries and venules continued to be manifest after the 20 per cent ethanol fraction was applied to the mesentery even though dilatation of small blood vessels was suppressed. It is thus doubtful whether release of mast cell histamine plays a significant role in the action of the cationic protein. More work is needed before we can adequately evaluate the possibility that the strongly basic character of this protein, *per se,* is in some way responsible for its inflammatory effect. This aspect of the problem may be approached by considering the forces which normally prevent leucocytes from sticking to endothelium and by considering whether such forces might be overcome through the action of cationic protein. Bangham and Pethica (40), for example, suggested that the polymorph and endothelial cell do not adhere to one another, at physiologic pH, because of mutual repulsion of like negative charges on their membranes, although weaker forces of attraction based on cation bridging between surface carboxyl groups were also present, according to these authors. Bangham and Pethica's view is that the energy of electrostatic repulsion between cells becomes diminished during inflammation as a result of changes in the shape of the cell surface. On the other hand, the findings presented here suggest that, in inflammation, the energy of cell attraction is increased as a result of the availability of cationic protein molecules which can act as ligands between negative radicals.

Charge Effects vs. Enzyme Action.—It should be stressed that the failure to correlate inflammatory activity with the presence of acid phenolphthalein phosphatase, beta glucuronidase, acid ribonuclease, lysozyme, or catalase activity in the 20 per cent ethanol fraction, does not preclude the possibility that some as yet undetected acid hydrolase is responsible for the effect of this fraction upon the microcirculation. However, the data rule out the possibility that these enzymes *per se* are involved in the reaction. Furthermore, in an extension of their studies on the role of PMN lysosomes in the Arthus reaction, Golub and Spitznagel recently presented preliminary results (26) which showed that protein extracted from rabbit PMN granules with weak acid, and precipitated by 20 per cent ethanol was able to reproduce the tissue injury normally evoked in rabbit skin by the whole granule fraction, whereas a 45 per cent ethanol–precipitated fraction of this material was not effective. Of greater significance to this discussion is the fact that the active cationic protein fraction was reported by these workers to show no cathepsin (acid protease) activity.

Effect on Platelets.—In dealing with the mechanisms by which the active protein produced its effects, attention should also be called to the fact that this protein causes platelet aggregation *in vitro.* While this observation is not sur-

prising (in view of a similar effect with other polycationic materials), the occasional appearance of platelet thrombi within vessels of rabbit mesentery after exposure to this protein (see Fig. 9), tends to implicate platelet agglutination in the *in vivo* leucocyte sticking reaction produced by the 20 per cent ethanol fraction. This interpretation is weakened, however, by the observation that PMN cationic protein precipitated by 45 per cent ethanol also causes platelet clumping *in vitro*, but fails to initiate significant leucocyte sticking reactions in the mesentery.

Conclusions.—It is perhaps interesting to note that the cationic protein under discussion is probably an integral component of the local defense reaction of host tissues against bacterial infection, for not only does this material stimulate emigration of defensive, phagocytic cells from the blood stream into areas of bacterial lodgment, but it is also, by itself, a potent bactericidal agent (13). In addition, the present studies and those of Spitznagel and Chi (15) suggest that the chemotactic property of bacterial cells in infected tissues (a property which is not always present when bacteria are tested *in vitro*), may be due to the adsorption of leucocyte cationic protein onto bacterial surfaces.

In conclusion, the foregoing data amply demonstrate that a component of the cationic proteins present in the lysosomes of rabbit exudate PMN leucocytes, is capable of inducing sticking and emigration of leucocytes in homologous and autologous mesenteric tissue. Since leucocyte adhesion and emigration is a cardinal feature of the inflammatory response, these findings offer fresh support for the presumed role of lysosomal particles (at least those of PMN leucocytes) in the pathogenesis of tissue injury.

SUMMARY

Lysosomal granules of rabbit exudate polymorphonuclear (PMN) leucocytes were isolated and then lysed by freezing-thawing. Topical application of this material to rat and rabbit mesentery produced sticking and emigration of leucocytes, stasis of blood flow, and petechial hemorrhage. The granule-free, supernatant fraction of the homogenized leucocytes failed to produce any of these reactions.

Cationic proteins extracted from these granules by weak acid and precipitated by ethanol at concentrations of 20 and 45 per cent, were also tested on heterologous, homologous, and autologous mesenteric vessels. The 20 per cent ethanol–precipitated fraction produced all of the afore-mentioned injury reactions, whereas the 45 per cent fraction was inactive. The intensity of inflammatory changes produced by the active cationic protein fraction was greater than that produced by lysed whole granules.

Both the 20 per cent and 45 per cent ethanol fractions of cationic protein induced clumping of rabbit platelets, *in vitro*.

The 20 per cent ethanol fraction also caused a slight acceleration in rate of swelling of isolated rabbit liver mitochondria.

The active material proved to be non-pyrogenic in rabbits.

This material exhibited no kinin-like effects when tested on isolated smooth muscle preparations (rabbit aorta and guinea pig ileum). In the rat, the protein produced a transient vasodepression which was inhibited by pretreatment of the animal with an antihistamine.

Ultraviolet absorption data and ribose assays showed that the 20 per cent ethanol fraction contained only 4 per cent or less of ribonucleic acid. Upon electrophoresis in starch gel, using acid buffer, this fraction separated into at least three major components which migrated towards the cathode.

Precipitation of one of the slowly migrating components by titration of the fraction to pH 10.5 greatly increased the inflammatory activity of the material.

The inflammatory basic protein fraction was essentially devoid of acid phosphatase, beta glucuronidase, acid ribonuclease, lysozyme, and catalase activity. The non-inflammatory basic protein fraction contained appreciable quantities of acid ribonuclease and lysozyme.

The foregoing data demonstrate that certain of the cationic proteins present in lysosomes of rabbit exudate PMN leucocytes can reproduce one of the cardinal features of the inflammatory response; namely, adhesion and emigration of leucocytes in the microcirculation. These findings offer fresh support for the role of lysosomes in the pathogenesis of tissue injury, and may help to account for the propagation of leucocyte emigration to peak numbers during inflammatory reactions.

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EXPLANATION OF PLATES

PLATE 72

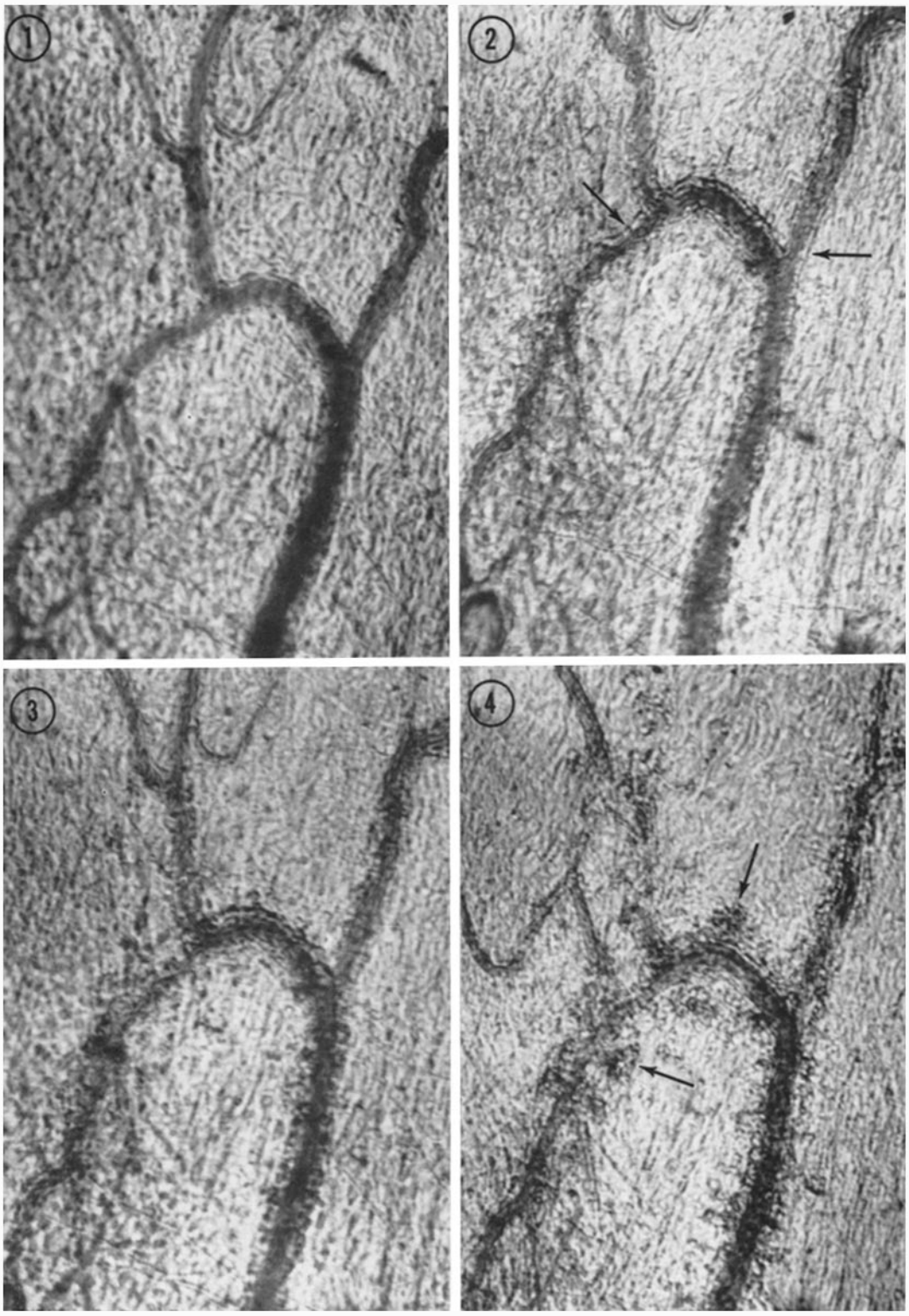
FIGS. 1 to 4. Effects of lysosomal cationic protein on the microcirculation of the rat mesentery. Living preparations. × 120.

FIG. 1. Collecting venule prior to application of test protein.

FIG. 2. Same vessel 13 minutes after application of protein. Note amorphous layer adherent to endothelium (arrows) with beginning of leucocyte sticking in this layer.

FIG. 3. Same vessel at 18 minutes. Extensive palisading of leucocytes is present, and blood flow is restricted to the center of the vessel.

FIG. 4. Same vessel at 25 minutes. Emigration of white cells is present along the vessel's entire length, and several petechial hemorrhages are visible (arrows).



(Janoff and Zweifach: Cationic proteins in inflammation)

PLATE 73

FIGS. 5 and 6. Effects of lysosomal cationic protein on the microcirculation of the rabbit mesentery. Living preparation. × 200.

FIG. 5. Normal appearance of venule in rabbit mesentery immediately before application of test protein.

FIG. 6. Same vessel 25 minutes after application of test protein. Leucocytes are emigrating into perivascular tissue (arrows). Flow in vessel is sluggish and individual cells are discernible along borders of vessel (compare with Fig. 5).

FIGS. 7 and 8. Effect of lysosomal cationic protein on rabbit platelets *in vitro*. Phase microscopy. × 256.

FIG. 7. Suspension of platelets in heparinized plasma after 20 minutes incubation at 37°C with 400 μg per ml of test protein.

FIG. 8. Same suspension of platelets incubated for 20 minutes without the protein.

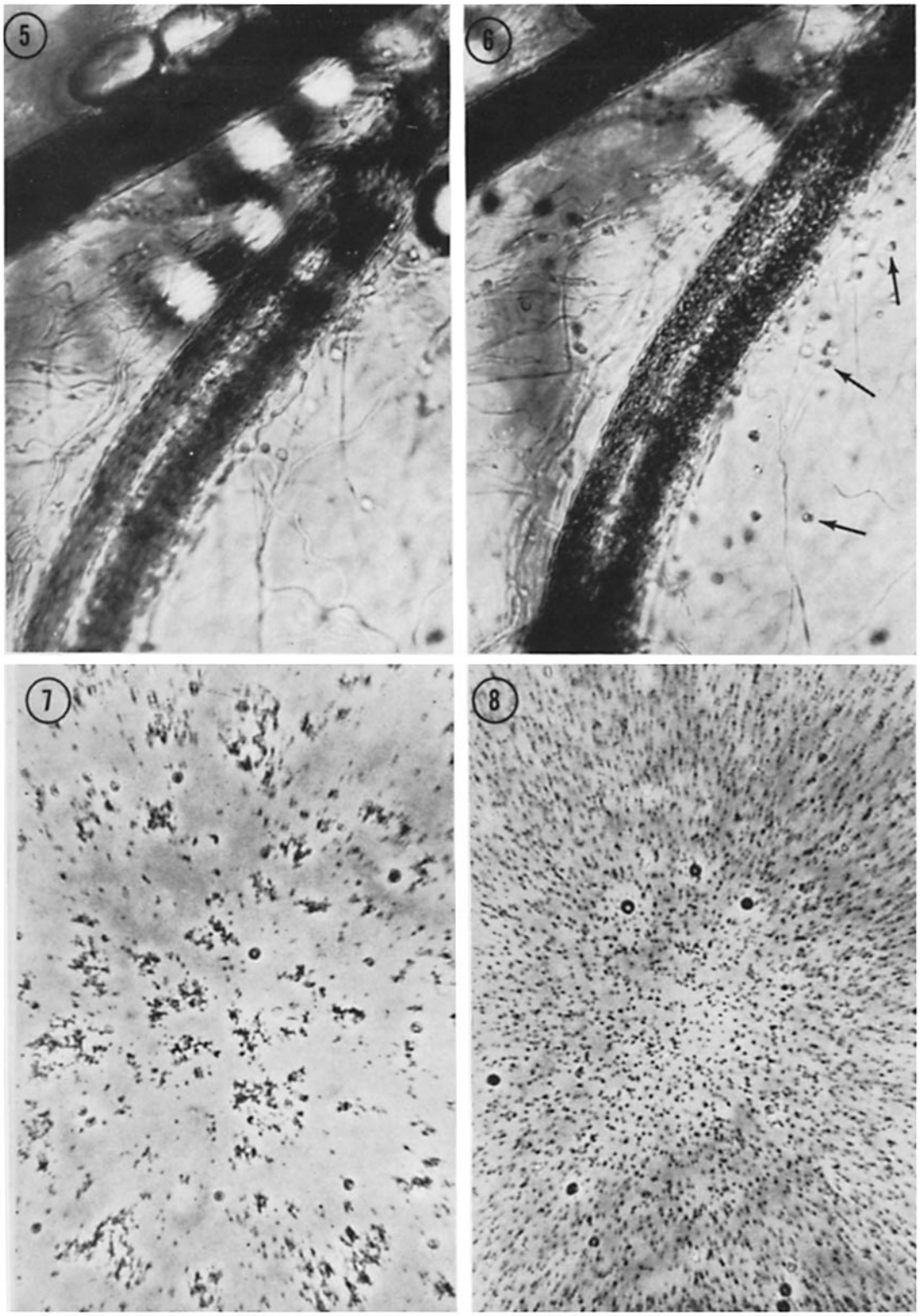


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PLATE 74

FIGS. 9 to 12. Effects of lysosomal cationic protein on the microcirculation of the rabbit mesentery. Tissues taken after 1 hour of continuous application of test protein (see Materials and Methods). Preparations fixed with Zenker's formalin and stained with Harris' hematoxylin and eosin.

FIG. 9. A portion of mesentery showing dense aggregates with the appearance of platelet thrombi containing trapped red cells. × 256.

FIG. 10. Venules showing extensive margination of leucocytes. × 256.

FIG. 11. High-power view of a part of the blood vessel shown in Fig. 10. Polymorphonuclear leucocytes are visible at numerous sites within the vessels or adherent to the vessel walls. × 640.

FIG. 12. High-power view of another part of the same vessel showing several polymorphonuclear leucocytes in the process of emerging into the perivascular tissue (arrow). × 640.

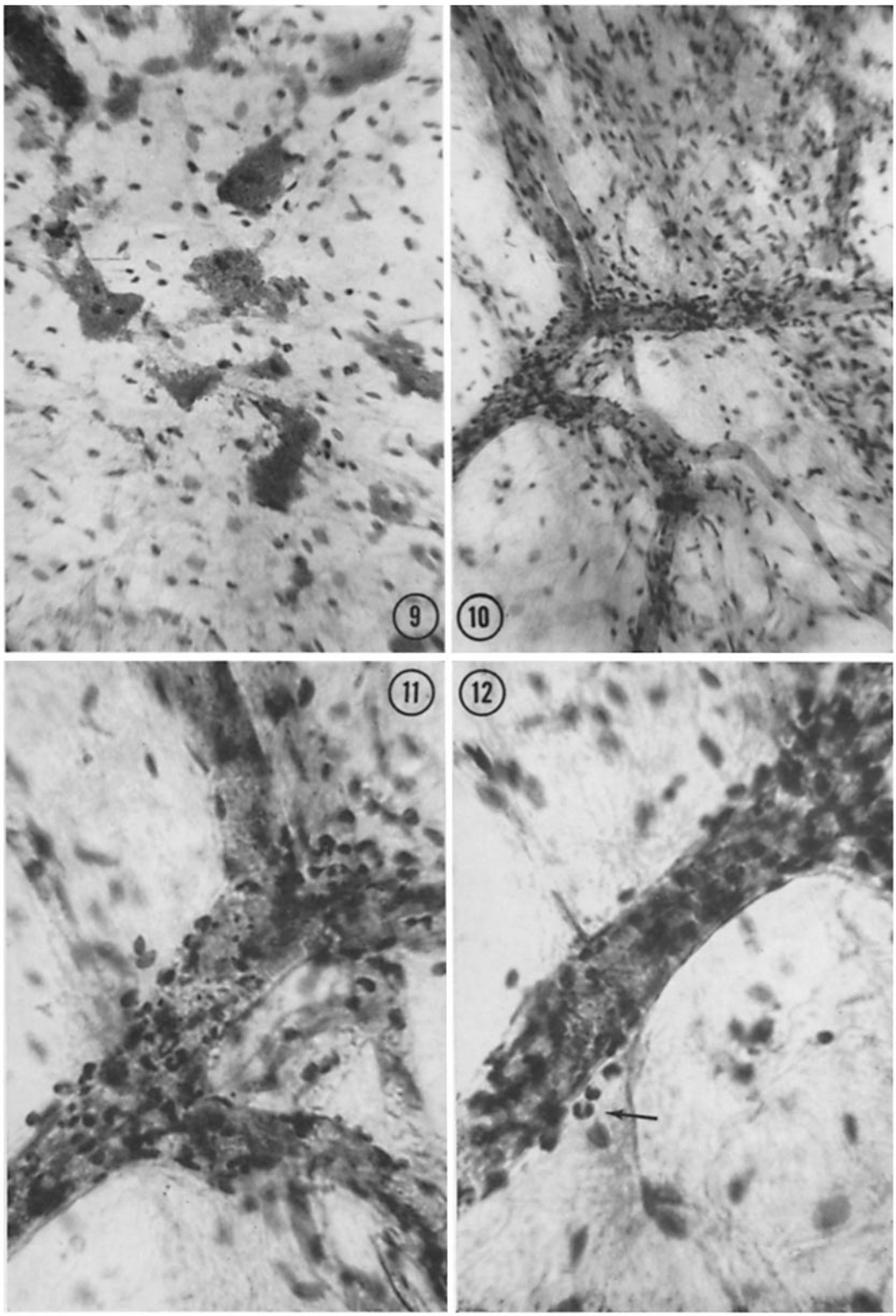

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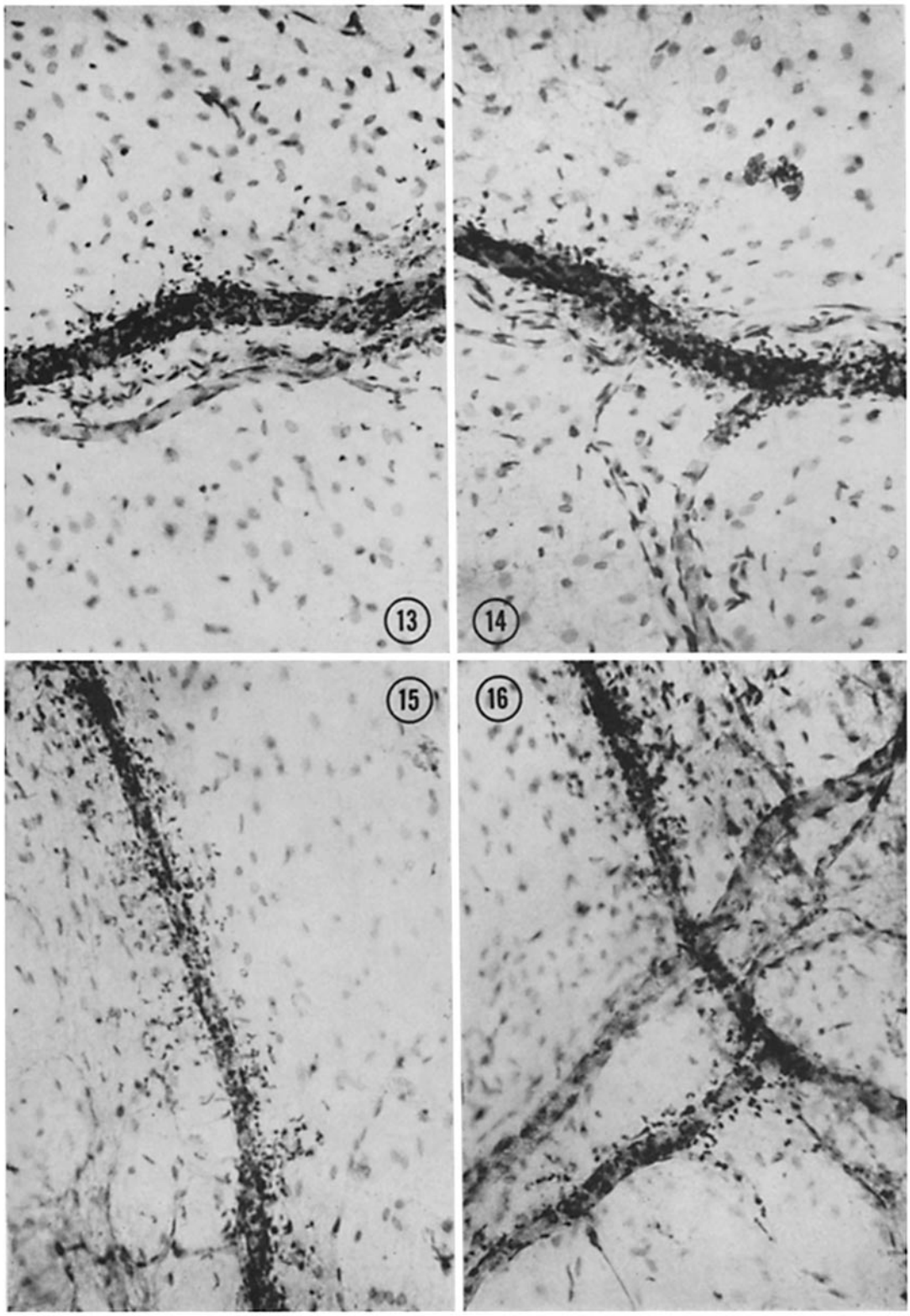
PLATE 75

FIGS. 13 to 16. Effects of lysosomal cationic protein on the microcirculat rabbit mesentery. Additional fixed and stained preparations. × 256.

FIGS. 13 and 14. Margination of leucocytes.

FIGS. 15 and 16. Emigration of leucocytes.



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