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21. In addition to *Anacystis montana* and *Botryococcus braunii* a green alga *Chlorella pyrenoidosa* and a marine alga from Hawaii, thought to be of the order Fucales, were analyzed (*2*) and found to contain a C_{17} alkene (76.9 percent) and a C_{17} alkane (100 percent), respectively.
22. After completion of our work we learned about the recent analysis of four species of algae (*Nostoc, Anacystis, Spirogyra*, and *Chlorella*) by another group of investigators [J. Han, E. D. McCarthy, W. Van Hoeven, M. Calvin, W. H. Bradley, *Proc. Natl. Acad. Sci. U.S.* **59**, 29 (1968)]. According to this report the C_{17} alkane is the major component in the molecular weight range C_{15} to C_{20}. These results, including the high content of heptadecene in *Chlorella*, agree with our own findings (*2, 3*). However, with the exception of *Spirogyra*, the algae analyzed by them did not contain any appreciable amount of hydrocarbons of high molecular weight. Recently K. Stransky, M. Streibl, and F. Šorm [*Collection Czecho. Chem. Commun.* **33**, 416 (1968)] have reported, in agreement with our results, the presence of alkanes and alkenes of high molecular weight in *Scenedesmus quadricauda*.

23. Parts of this work were presented at the International Summer School, Biology in Space, Cambridge, England, 8–20 July 1967 and at the meeting of the Geological Society of America, New Orleans, 20–22 Nov. 1967. Supported in part by research grants NSG 257 and NGR 44-005-020 from NASA. H.J.S. was partially supported by NDEA Title IV Fellowship No. 66-8250. We thank Dr. M. Rodriguez-Lopez, Centro de Investigaciones Biologicas, Instituto Marañon, Madrid, Spain, for supplying us with a sample of *Anacystis montana*. *Botryococcus braunii* was obtained from Dr. R. C. Starr, Indiana University.

29 April 1968

Vascular Injury and Lysis of Basement Membrane in vitro by Neutral Protease of Human Leukocytes

Abstract. *Frozen and thawed granules of human, peripheral-blood leukocytes rapidly produce hemorrhage when injected into animal tissues. The effect is blocked by inhibitors of proteolysis. The granule extract can digest vascular basement membrane in vitro at neutral* p*H. In addition, basement membranes of blood vessels damaged in vivo by the leukocyte fraction are found to be attenuated when examined by electron microscopy. The proteases of human leukocyte granules differ in several important respects from known lysosomal cathepsins and trypsin-like esterases. Polymorphonuclear neutrophils are a major source of the neutral proteases present in circulating white cells, and release these enzymes during phagocytosis of immune complexes.*

Recently the role of leukocyte proteases in inflammation has been reexamined (*1*). Attention has been focused on the acid cathepsins of these cells as a result of studies in rabbits indicating that such granule-bound enzymes participate in immunological vascular injury of the Arthus type (*2, 3*). For example, cathepsins D and E degrade vascular basement membrane in vitro (*3*) at the *p*H optima (2.5 to 3.0) of these enzymes. However, injection of such proteases into rabbit tissues fails to induce comparable vascular injury, probably because proteolysis is prevented by the neutral *p*H along endothelial surfaces. Therefore vessel damage mediated by acid cathepsins requires that tissue *p*H be considerably lowered in order for these hydrolases to act. The concentration of acid needed for intracellular functions of cathepsins can be attained within phagocytic vacuoles, but it is not certain that *p*H at the blood-tissue interface can be sufficiently lowered during inflammation to permit cathepsins D and E to degrade basement membrane or other vascular components extracellularly.

On the other hand, significant differences exist between the proteolytic enzymes of rabbit and human white blood cells, including the presence in the latter of trypsin-like enzyme (leukoprotease) which is active at physiological *p*H (*4*). This enzyme is found in polymorphonuclear leukocytes (PMN) of many species in addition to man; some of its properties and possible actions in infection have been reviewed (*4, 5*). Our experiments deal with lysosomal proteases of human leukocytes as potential mediators of vascular damage. Our observations not only confirm the presence of marked neutral proteolytic activity in human PMN, but show that this activity is also present in the lysosomal fraction of these cells and that it is released during phagocytosis. With regard to inflammation, our experiments show that human PMN granules can degrade vascular basement membrane in vitro at neutral *p*H and can rapidly produce vascular injury when injected into normal animal tissues (an effect which is blocked by inhibitors of proteolytic enzymes. Studies with synthetic substrates and with inhibitors of serine and thiol-dependent enzymes reveal important differences between human leukocyte granule proteases and the known cathepsins and trypsin-like esterases. A preliminary report of these findings has been presented (*6*).

Human white cells were obtained from freshly drawn, citrated venous blood of normal male subjects after sedimentation of erythrocytes with dextran. The washed leukocytes were homogenized, and their cytoplasmic granules were obtained essentially as described before (*7*). Granule fractions were then resuspended in 0.15*M* sodium phosphate buffer (*p*H 8.0) and disrupted by freezing and subsequent thawing. Membranes of the ruptured granules were sedimented by high-speed centrifugation; the clarified supernatants were dialyzed against cold, normal saline buffered with 0.01*M* phosphate to *p*H 7.4; the final extracts were stored at −60°C until used.

Granule protein (50 to 100 μg) was injected into the skin or bowel wall of rabbits or passed via tracheal catheter into the lungs of mice. As markers of vessel leakage, the animals were given intravenous Evans blue (4 ml of a 1 percent solution in isotonic NaCl per kilogram of body weight) and carbon suspension (Pelikan C11/1431a, 1 ml per kilogram of body weight). Accumulation of both labels was visible within the injected tissues 30 minutes after administration of the extract. The lesions reached maximum size by 1 to 2 hours, becoming 1.5 to 2.0 cm in

diameter and developing numerous, confluent petechial hemorrhages. In contrast, injection of the granule-free cell-sap fraction of the leukocyte homogenate (100 μg of protein) produced only a mild (less than 5 mm) transient blueing of skin tissue without gross signs of hemorrhage or carbon extravasation. The hemorrhagic component of the skin reaction was completely suppressed by local admixture of trypsin inhibitor from soybean (20 μg) or salivary kallikrein inhibitor (200 units) with the granule extract, whereas previous systemic medication of the test animal with heparin (1000 unit/kg) aggravated the response. Serum samples from rabbits with injections in the skin did not give precipitin reactions when tested by double diffusion in agar against the granule extract at concentrations ranging from 6.25 to 250 μg of protein N per milliliter. Microscopic examination of tissues at 30 minutes to 1 hour revealed perivascular accumulations of red cells indicating severe damage to blood vessels. In the skin of animals given injections of carbon suspension, many vessels were observed surrounded by zones of extravasated carbon without any signs of overt hemorrhage having yet developed. Very few of the test animal's own leukocytes were present at reaction sites in bowel and skin at these early times. Control tissues (injected with buffer) contained carbon in the lumen of blood vessels, but only rarely showed signs of extravascular carbon passage. Extravasated erythrocytes were never found. Identical observations were made on blood vessels of skin that had been injected with mixtures of granule extract and inhibitors and proteolytic enzymes.

Other samples of bowel wall were biopsied 1 hour after injection of the granule extract or control vehicle and were immediately transferred to 4 percent glutaraldehyde buffered to *p*H 7.4 with 0.1*M* sodium phosphate. Tissues were fixed at 4°C for 18 hours. After treatment with 2 percent osmium tetroxide for ½ hour, the tissues were embedded in Epon 812, sectioned with glass knives, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM2A electron microscope. Vessels exposed to the action of the granule extract showed various degrees of attenuation of endothelial cell and pericyte basement membranes, in contrast to the controls (Fig. 1).

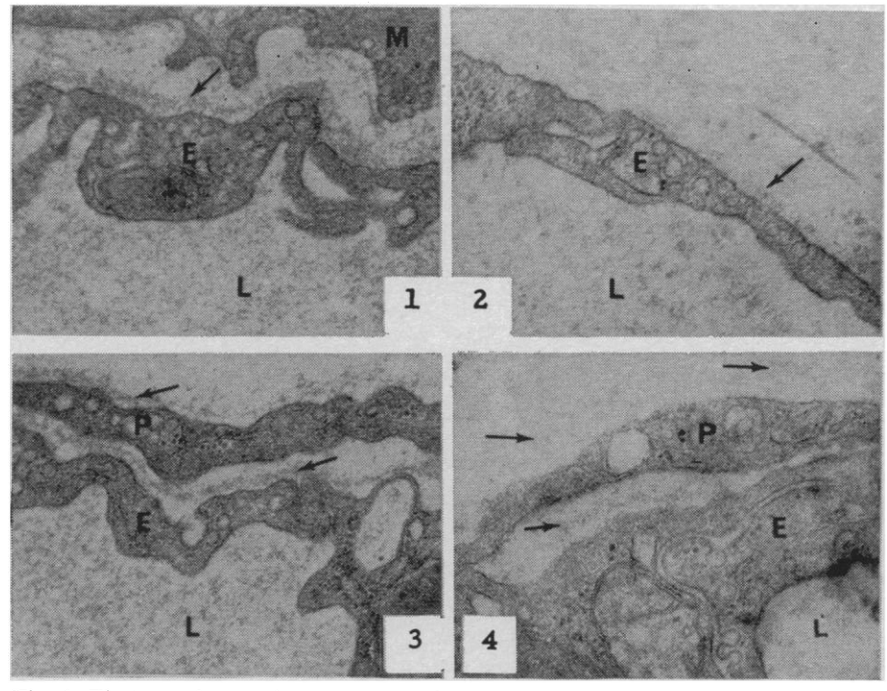

Fig. 1. Electron-microscopic appearance of vascular endothelium in rabbit bowel wall 1 hour after injection of human leukocyte granule extract. Parts 1 and 3 of the figure show portions of control vessels (× 45,000). Parts 2 and 4 are from vessels exposed to the granule extract (× 45,000). Basement membranes are marked with arrows. *M*, smooth muscles cell; *E*, endothelial cell; *P*, pericythe; and *L*, lumen of blood vessels.

We next studied the character of the proteolytic activity present in these granules and the action of the granules on vascular basement membrane in vitro. Figure 2 shows an activity curve of proteolysis by the granule extract with denatured hemoglobin as substrate as a function of *p*H. The reactions were assayed according to the method of Anson (*8*), as modified by Press (*9*). Buffer solutions were: *p*H 2 (HCl and KCl), *p*H 3 and 4 (citric acid and sodium citrate), *p*H 5 (citric acid and sodium phosphate), *p*H 6 to 8 (sodium phosphate), *p*H 9 and 10 (tris and HCl). The final buffer concentration in all cases was 0.06*M*. Fifty micrograms of granule protein was used throughout, and incubation was at 37°C for 30 minutes. The *p*H optimum was at neutrality with a smaller peak of activity at *p*H 3. The former activity is 10 times greater than the latter. The neutral proteolytic activity given by an equal amount of cell-sap protein is clearly less than that of the granule extract (Fig. 2).

Other properties of the neutral protease of human leukocyte granules are the following: The tissue hemorrhagic action is heat-sensitive, 90 percent of this activity being lost after 60 minutes exposure to 60°C and complete loss occurring after 10 minutes at 80°C. Digestion of hemoglobin is completely inhibited by 0.01*M* diisopropyl phosphofluoridate (DFP) but is not appreciably altered by 0.001*M* iodoacetamide. In these tests, crystalline trypsin and papain were always used as controls for proteases sensitive to DFP and iodoacetamide. These enzymes were appropriately inhibited. From these observations and from the curve of activity as a function of *p*H, it is clear that human leukocyte granules contain a protease (or proteases) radically different from the known cathepsins. None of the latter is inhibited by DFP, whereas those cathepsins with *p*H optimums approaching neutrality are SH-dependent and strongly inhibited by iodoacetamide. The granule protease (or proteases) also failed to hydrolyze a number of synthetic trypsin substrates at *p*H 7 (benzoyl-arginine-*p*-nitroanalide, benzoyl-arginine naphthylamide, poly-L-lysine, and poly-L-arginine). Assays in which 0.01*M* tritium-labeled tosylarginine methyl ester (TAME) was used as substrate (*10*) revealed weak trypsin-like esterase activity in the granule extract; but this activity was less than 0.1 percent of the activity of crystalline trypsin itself (on an equal weight basis). Moreover,

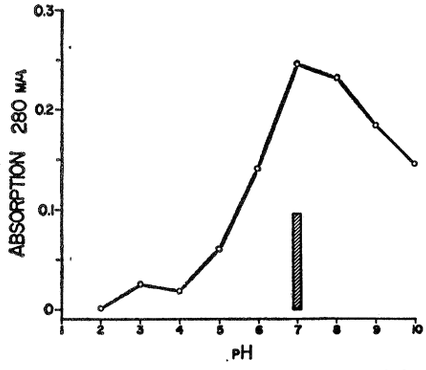

Fig. 2. Effect of *p*H on protease activity of human leukocyte granule extract with denatured hemoglobin as substrate. Fifty micrograms of granule protein were incubated at 37°C for 30 minutes at all *p*H values. The bar represents the hydrolysis given by 50 μg of granule-free cell-sap protein of the same leukocyte preparation.

0.025*M* TAME failed to inhibit the digestion of hemoglobin by the granule extract, whereas this concentration of TAME did inhibit 56 percent of the proteolytic action of trypsin on hemoglobin under the same experimental conditions. Thus, the major neutral protease of human leukocyte granules is not a trypsin-like esterase.

Analysis of the effect of granule extract upon vascular basement membrane in vitro was carried out as follows. Eight milligrams of lyophilized renal vascular basement membrane (rabbit) (*10*) was incubated in 0.15*M* phosphate buffer (*p*H 7) together with

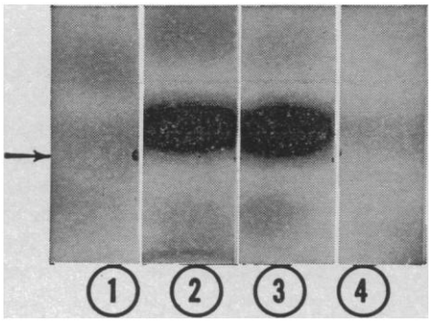

Fig. 3. Paper-strip electrophoresis of digestion supernatants of renal basement membrane (RBM) incubated with leukocyte granule extracts. Electrophoresis was carried out for 4½ hours at room temperature at a constant current of 3 ma per 10 cm bridge-gap. (1) RBM alone; (2) RBM plus trypsin at *p*H 7; (3) RBM plus granule extract at *p*H 7; (4) RBM plus granule extract at *p*H 2.8. Supernatants of trypsin and of granule extracts incubated alone were negative (no staining of electrophoresis strips). The arrow indicates the origin; cathode is at top of figure.

50 μg of crystalline trypsin or 500 μg of granule protein. Incubation mixtures of basement membrane and enzymes were also prepared in citrate buffer at *p*H 2.8. Controls were incubated in the absence of added enzyme. After incubation for 17 hours at 37°C with constant shaking, samples of the digestion supernatants were clarified by high-speed centrifugation and analyzed for products of basement-membrane hydrolysis. Paper-strip electrophoresis was performed on cellulose acetate at *p*H 8.6 (0.024*M* barbital buffer) with 20 μl of the supernatant fractions. The strips were dried at 60°C and stained with 0.2 percent ninhydrin in *N*-butanol (Fig. 3). The granule extract caused extensive digestion of basement membrane at neutral *p*H; this digestion was indistinguishable from that produced by trypsin under identical conditions. Neither enzyme was appreciably active at *p*H 2.8. In addition, the digestion supernatants were analyzed by Folin protein measurements and by immunodiffusion against sheep antiserum to rabbit renal basement membrane (*3, 10*). Both procedures confirmed the results obtained by electrophoretic analysis.

To demonstrate that the neutrophil was the principal source of the protease obtained from circulating human leukocytes, we first passed blood through a nylon-wool column to selectively remove PMN. A sample of the resulting neutrophil-poor preparation containing 17.3×10^6 total leukocytes (20 percent PMN) gave a yield of granule neutral protease equivalent to that obtained from 4.5×10^6 leukocytes of normal blood (71 percent PMN). Thus, a good correlation exists between enzyme yield and numbers of neutrophils in the starting blood.

In a final experiment, we incubated human blood leukocytes with an immune precipitate to observe the effect of phagocytosis on protease release. The precipitate consisted of complexes of bovine serum albumin (BSA) and antiserum to BSA, exposed to fresh guinea pig serum as a source of complement. An amount of precipitate containing 0.44 mg of antibody N was incubated 1½ hours at 37°C with 1 ml of leukocyte suspension containing 10^8 PMN. The incubation medium consisted of bicarbonate-buffered Ringer-Locke balanced salt solution (*p*H 7.4) with 0.004*M* Ca^{2+} and Mg^{2+}. Under these conditions, the leukocytes remained viable (dye exclusion) but became degranulated (Wright's stain) as a result of phagocytosis of the precipitates. In conjunction with the degranulation of the cells, a threefold increase in release of neutral protease into the incubation medium was observed over that which occurred when cells were incubated alone.

In conclusion, the foregoing results suggest that in man immunological vascular injury mediated by PMN may be due in part to the action of neutral protease (or proteases) contained in the lysosomes of these cells. Although responsible enzyme (or enzymes) remain to be identified, our observations lend further support to the view that proteases from neutrophils can injure vascular basement membranes.

Note added in proof: Since this paper was submitted for publication, neutral proteases have been purified from Arthus skin lesions of rabbits (*11*) and a collagenase active at neutral *p*H has been identified in the lysosomes of human polymorphonuclear leukocytes (*12*). An elastinolytic enzyme, which may participate in digestion of elastic lamina during arteritis, is also present in human leukocyte lysosomes (*13*).

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