under constant medical and nursing supervision throughout the experiment.

6. Tritiated 11-OH-Δ^9THC (specific activity, 24.6 μc/mg) labeled in the 2, 4, 8, and 10 positions was enzymatically synthesized at the Research Triangle Institute, Research Triangle Park, N.C., and supplied by Dr. M. C. Braude, National Institute of Mental Health. The material (7 mg) initially contained 80 percent 11-OH-Δ^9THC. After purification by extraction and thin-layer chromatography, 4 mg of 11-OH-Δ^9THC were obtained. On thin-layer chromatography the material contained one radioactive peak having the same chromatographic behavior as authenic 11-OH-Δ^9THC. The purified 11-OH-Δ^9THC was prepared for intravenous administration by dissolving 1 mg of the drug in absolute alcohol (parenteral administration grade). Each dose was prepared under aseptic conditions and placed in an ampoule. A portion of the 11-OH-Δ^9THC solution was analyzed by mass spectrometry and shown to contain essentially compounds having the same molecular weight and fragmentation characteristics as 11-OH-Δ^9THC. The possibility exists that minor percentages of other monohydroxylated tetrahydrocannabinols were present in the solution.

7. L. Lemberger, J. Axelrod, I. J. Kopin, *Ann. N.Y. Acad. Sci.* **191**, 142 (1971).
8. S. Agurell, I. M. Nilsson, J. L. G. Nilsson, A. Ohlsson, M. Widman, K. Leander, *Acta Pharm. Suec.* **8**, 698 (1971).
9. S. Agurell, I. M. Nilsson, A. Ohlsson, F. Sandberg, *Biochem. Pharmacol.* **19**, 1333 (1970).

10. We thank Drs. B. L. Martz, R. B. Forney, and G. F. Kiplinger for their very helpful suggestions, Miss T. Syester for her excellent technical assistance, and Dr. Allen Fites for preparing the 11-OH-Δ^9THC in ampoules.

20 March 1972; revised 12 April 1972

Collagenase: Localization in Polymorphonuclear Leukocyte Granules in the Rabbit

Abstract. *Polymorphonuclear leukocyte granules were submitted to zonal fractionation through a discontinuous sucrose gradient. Azurophilic and specific granules were enzymatically characterized by peroxidase and alkaline phosphatase activity, respectively. The enzymes formed modal distributions like those reported by others. Collagenase activity was consistently associated with the specific granules containing alkaline phosphatase.*

A collagenase, active against undenatured collagen fibrils at neutral and alkaline *p*H, has been extracted from the granule fraction of human polymorphonuclear (PMN) leukocytes (*1*, *2*). The leukocyte collagenase cleaved collagen into distinctive products characteristic of other animal collagenases. The enzyme differed from other tissue collagenases in that activity was demonstrated after extraction from PMN leukocyte granules and did not require tissue culture for detection. Collagenase from PMN leukocytes was inhibited by ethylenediaminetetraacetic acid, cysteine, and reduced glutathione but not by human serum.

Cytochemical and electron microscopic studies of human marrow (*3*, *4*) indicated that neutrophilic leukocytes contained at least two granule types, azurophils and specifics, which differed in morphology, time of formation, and enzymatic activity. Baggiolini *et al.* (*5*) and Zeya and Spitznagel (*6*) isolated azurophils, specifics, and a third, less well-defined group of particles from rabbit PMN leukocytes by sucrose gradient centrifugation. Large, relatively dense azurophilic or primary granules contained essentially all of the myeloperoxidase activity, a third of the lysozyme activity, and high relative concentrations of characteristic lysosomal acid hydrolases. The smaller, less dense group of specific or secondary granules contained the majority of alkaline phosphatase activity. The third group of granules were morphologically ambiguous, and their nature and composition is at present unresolved (*4*, *7*).

Two enzymatically heterogenous populations of rabbit PMN leukocyte granules, azurophils (A) and specifics (B), have been described, the former characterized by peroxidase and the latter by alkaline phosphatase. We sought to determine whether collagenase was also localized in a particular granule fraction of rabbit PMN leukocytes.

Polymorphonuclear leukocytes were obtained from rabbit peritoneal exudates induced aseptically with 0.5 percent glycogen in saline essentially as described by Hirsch (*8*). The exudates were submitted to differential cell count in stained smears and averaged 96 to 98 percent PMN leukocytes. Exudates with less than 5×10^6 PMN cells per milliliter or with gross erythrocyte contamination or abnormally high mononuclear leukocyte concentrations were discarded. The cells were exposed to brief hemolysis with hypotonic saline, washed in 0.34*M* sucrose, and resuspended in 20 ml of 0.34*M* sucrose for homogenization. Cells were lightly homogenized for 2 minutes with a Teflon pestle. Granules were isolated from nuclei, residual erythrocytes, debris, and intact cells by centrifugation for 10 minutes at 400*g*. These initial preparation and homogenization procedures were critical for reproducible distribution of enzymes in the gradient. All steps were followed by phase microscopy.

Table 1. Relative enzyme concentration of granule pellets after centrifugation of fractions pooled as shown in Fig. 1a. Alkaline phosphatase and collagenase were found predominantly in the region containing B particles.

Fraction	Enzyme (percent recovery)		
	Alkaline phosphatase	Collagenase	Peroxidase
Soluble phase	9.6	9.8	12.2
C	8.2	14.1	2.6
CB	4.5	0	3.2
B	41.7	59.0	11.0
BA	12.0	7.2	16.1
A	5.0	9.2	42.3

The 20-ml 400*g* supernatant represented the starting sample for zonal centrifugation procedures. Fractionation was accomplished by procedures outlined by Cline and Ryel (*9*). A discontinuous sucrose gradient consisting of 17, 28, 42 and 55 percent sucrose was loaded into a spinning B-XIV Spinco zonal rotor (2000 rev/min) in order of increasing density. The sample was layered over the gradient and centrifuged at 20,000 rev/min for 18 minutes. Following centrifugation, the gradient containing the fractionated components was displaced from the spinning rotor (2000 rev/min) with 60 percent sucrose. The effluent was continuously monitored with a Beckman DB-GT spectrophotometer at 280 nm, and collected in 20-ml fractions. The sucrose concentration of individual fractions was determined with a Bausch and Lomb 3-L refractometer. All granule preparation and centrifugation operations were conducted at 4°C.

Alkaline phosphatase was assayed by a modification of the method of Babson *et al.* (*10*) with phenolphthalein monophosphate as substrate. Myeloperoxidase activity was assayed as the rate of hydrogen peroxide decomposition with *O*-dianisidine as hydrogen donor. Protein content was estimated by the method of Lowry *et al.* (*11*). Specific collagenolytic activity was measured with the [^{14}C]glycine-labeled fibril assay described by Lazarus *et al.* (*2*) with modifications described by Robertson *et al.* (*12*). All assays were conducted at 37°C in the presence of 0.005*M* $CaCl_2$, 0.02*M* NaCl, and 0.3*M* tris(hydroxymethyl)aminomethane, *p*H 7.5 (*13*). Collagen substrates were highly resistant to nonspecific protease diges-

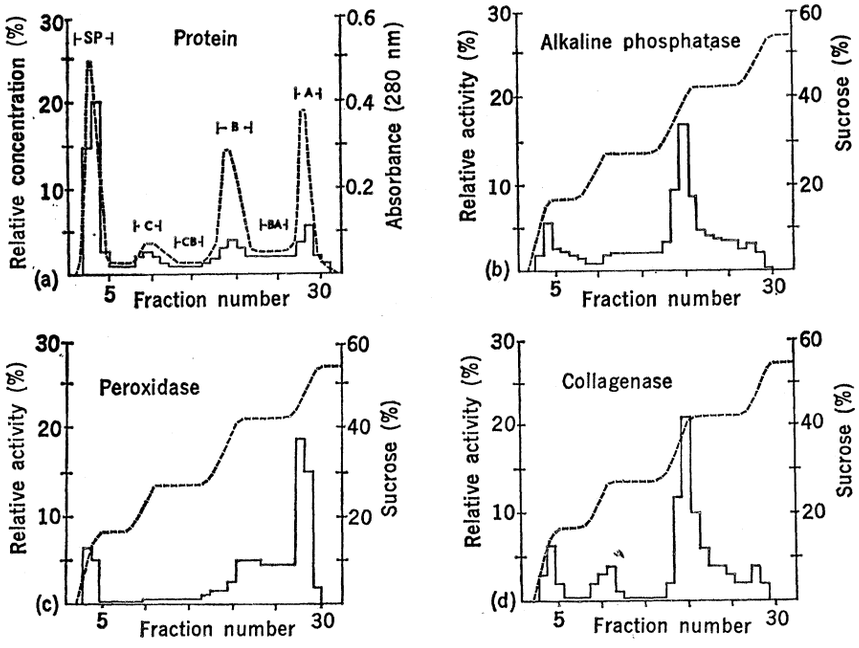

Fig. 1. Fractionation of leukocyte granules through a discontinuous sucrose gradient at 20,000 rev/min for 18 minutes. Absorbance was corrected for sucrose concentration. Protein was determined by the method of Lowry *et al.* (*11*). Peroxidase (c) was found at the heavy end of the gradient, and alkaline phosphatase (b) and collagenase (d) were consistently found together in the same region of the gradient. *SP*, Soluble phase; A, B, and C, regions of particle peaks; BA and CB, regions between peaks.

tion, which never exceeded 3 percent in reaction mixtures containing 0.5 μg of trypsin per microgram of collagen. A number of detergent preparations designed to release latent enzyme activity were tested and found to interfere with the collagenase assay. Freezing and thawing repeated ten times was used, therefore, to release granule enzyme activity before all assay procedures. Assays were performed either on individual 20-ml fractions collected from the rotor or on granule pellets resulting from centrifugation of fractions pooled as shown in Fig. 1a.

Fractionation of rabbit PMN leukocyte granules yielded the distribution patterns in Fig. 1. Intact granules were demonstrated in areas C, B, and A by phase microscopy. A few particles were noted in area BA. Myeloperoxidase and alkaline phosphatase assumed modal distributions consistent with those reported elsewhere (*5, 6*). Collagenase activity was consistently associated with the alkaline phosphatase–containing B granules (Fig. 1, area B). Assays of granule pellets from pooled fractions shown in Table 1 confirmed the strong correlation between collagenase and alkaline phosphatase activity.

Granules in PMN leukocytes appear to possess many of the properties of lysosomes (*14*), and leukocyte granules are responsible for intracellular digestion of materials engulfed by these cells (*14*). Free extracellular granules have also been described in inflamed connective tissue (*15*), wound healing sites (*16*), tuberculin sensitvity reactions (*17*), and delayed hypersensitivity and arthus reactions (*18*). We report the presence of two alkaline hydrolases, alkaline phosphatase and collagenase, in a well-defined cytoplasmic body characteristic of lysosomes. The collagenase derived from PMN leukocyte granules of man manifests a broad *p*H optimum of 7 to 9.5 (*2*). In the case of polymorphonuclear leukocytes, therefore, particular lysosomal-type granules are associated with enzymatic degradation of constituents at an acid *p*H, and other types appear to be concerned with degradation of constituents at neutral to alkaline *p*H.

P. B. Robertson
R. B. Ryel, R. E. Taylor
K. W. Shyu, H. M. Fullmer
Institute of Dental Research, School of Dentistry, University of Alabama, Birmingham 35233

References and Notes

1. G. S. Lazarus, R. S. Brown, J. R. Daniels, H. M. Fullmer, *Science* **159**, 1483 (1968).
2. G. S. Lazarus, J. R. Daniels, R. S. Brown, H. A. Bladen, H. M. Fullmer, *J. Clin. Invest.* **47**, 2622 (1968).
3. D. F. Bainton and M. G. Farquhar, *J. Cell Biol.* **39**, 299 (1968); J. L. Ullyot, D. F. Bainton, M. G. Farquhar, in *Program of Histochemical Society, 21st Annual Meeting* (1970), abstract 24; D. C. Pease, *Biood* **11**, 501 (1956).
4. B. K. Wetzel, S. S. Spicer, R. G. Horn, *J. Histochem. Cytochem.* **15**, 311 (1967).
5. M. Baggiolini, J. G. Hirsch, C. deDuve, *J. Cell Biol.* **40**, 529 (1969).
6. H. I. Zeya and J. K. Spitznagel, *Science* **163**, 1069 (1969); *Lab. Invest.* **24**, 229 (1971).
7. M. G. Farquhar, D. F. Bainton, M. Baggiolini, C. deDuve, in *American Society of Cell Biology, 11th Annual Meeting* (1971), abstract 172.
8. J. G. Hirsch, *J. Exp. Med.* **103**, 589 (1956).
9. G. B. Cline and R. B. Ryel, *Methods Enzymol.* **22**, 168 (1971).
10. A. L. Babson, S. J. Greeley, C. M. Coleman, G. E. Phillips, *Clin. Chem.* **12**, 482 (1966).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
12. P. B. Robertson, R. E. Taylor, K. W. Shyu, H. M. Fullmer, in *Abstract of Papers, International Association of Dental Research, 50th General Session* (1972), abstract 786.
13. H. M. Fullmer, R. E. Taylor, R. W. Guthrie, *J. Dent. Res.* **51**, 349 (1972).
14. Z. A. Cohen and J. G. Hirsch, *J. Exp. Med.* **112**, 983 (1960).
15. H. L. Freedman, M. A. Listgarten, N. S. Taichman, *J. Periodontal Res.* **3**, 313 (1968); P. R. Garant and J. E. Muluihill, *ibid.* **6**, 266 (1971).
16. R. Ross, *J. Dent. Res.* **45**, 449 (1966).
17. J. R. Wiener, G. Lattes, D. Spiro, *Amer. J. Pathol.* **50**, 485 (1967).
18. L. Grant, M. H. Ross, J. Moses, P. Prose, B. W. Zwiefach, R. H. Ebert, *Z. Zellforsch. Microsk. Anat.* **77**, 554 (1967).

10 March 1972

Ascorbate-Nitrite Reaction: Possible Means of Blocking the Formation of Carcinogenic N-Nitroso Compounds

Abstract. *The formation of carcinogenic* N-*nitroso compounds by the chemical reaction between nitrous acid and oxytetracycline, morpholine, piperazine,* N-*methylaniline, methylurea, and (in some experiments) dimethylamine was blocked by ascorbic acid. The extent of blocking depended on the compound nitrosated and on the experimental conditions. Urea and ammonium sulfamate were less effective as blocking agents. The possibility of in vivo formation of carcinogenic* N-*nitroso compounds from drugs could be lessened by the combination of such drugs with the ascorbic acid.*

Most *N*-nitroso derivatives of secondary amines and amides are powerful carcinogens, which can act in several species (*1*). These derivatives might possibly be produced in the human stomach by the acid-catalyzed reaction between nitrite (*2*), which is present in some foods, and *N*-nitrosatable compounds,