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Isolation and Properties of Human Leukocyte Lysosomes *in vitro*.* (30091)

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Cohn and Hirsch(1) have isolated the cytoplasmic granules of rabbit polymorphonuclear (PMN) leukocytes, demonstrating that in composition and behavior they resembled lysosomes of rat liver described by De Duve (2). These granules, like lysosomes, were rich in acid hydrolases (cathepsins, DNAase, acid phosphatase and beta glucuronidase), but also contained the basic anti-bacterial substances lysozyme and phagocytin. Enzymes associated with the granules could be released from their limiting membranes by mechanical trauma, hypotonic or acid media or by surface active agents. Subsequent studies on optically homogeneous suspensions of such granules have shown that other agents which labilize lysosomal membranes (lysolecithin, streptolysins, ultraviolet irradiation, vitamin A(3) and etiocholanolone(4) also release enzymes from PMN granules *in vitro*.

Heretofore it has not been possible to study isolated human PMN granules in similar fashion, mainly because methods employed for isolation of the relatively larger granules of rabbits damaged the smaller leukocyte lysosomes of human cells. The experiments to be described below outline a general method for isolation of human PMN granules in good yield from 25 ml of peripheral venous blood and describe some of their properties *in vitro*.

Materials and methods. Separation procedure. Twenty-five ml of blood, drawn in a heparinized ('liquaemin' Organon, 5,000 U/ml) syringe, were allowed to sediment 1-1½ hours at 37°C in an 18 × 125 mm screw cap tube. Approximately 6 ml of plasma, containing 40-60 million cells, 60-75% of which were PMN's, were removed with a Pasteur pipette inserted near the red cell-plasma interface and centrifuged at 110 *g* for 5 minutes in a 10 ml conical centrifuge tube. The cellular pellet was resuspended by slowly swirling cells off the pellet with fluid ejected from a rubber-bulbed Pasteur pipette and washed, first in 5 ml Earle's Balanced Salt Solution, then in 3 ml 0.34 M sucrose and finally very slowly resuspended, so as to avoid clumps, in 5 ml of 0.34 M sucrose with 0.5 ml heparin. The cell suspension (in an ice water bath) was then pipetted with rapid, sudden ejection of the suspension through a Pasteur pipette; a considerable increase in the viscosity of the solution was observed. Pipetting was discontinued just before the viscosity began to drop (approximately 15-20 times). A measured aliquot (original volume) of the suspension was centrifuged in 5 ml lusteroid tubes in a Servall Automatic Superspeed Centrifuge at 2,000 *g* for 10 minutes and the supernatant carefully poured off into another lusteroid tube. The remaining 'nuclear' pellet was washed in 2 ml of 0.34 M sucrose and the supernatant was added to the first. The combined supernatants were centrifuged at 27,000 *g* for 20 minutes, resulting in a white pellet with a viscous supernatant in which there was a thin, fluffy, strand of material. The supernatant and strand were carefully poured off

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and the 'granular' pellet washed in 1 ml of 0.34 M sucrose. The final supernatant and wash were combined and will be referred to as the post-granule supernatant. Each of the pellets (nuclear and granular) was diluted to original volume.

Chemical analysis. Protein content was determined by the method of Lowry *et al* (5). Beta glucuronidase activity was assayed with phenolphthalein glucuronidate (Sigma Chemical Co.) as substrate, by the method of Fishman *et al*(6) and acid phosphatase activity according to the method of Huggins and Talalay(7) against standards of acid phosphatase (Worthington Biochemical Corp.). The enzymes were incubated at 37°C for 3 to 4 hours.

Lysozyme was assayed using suspensions of *M. lysodeicticus* (Worthington Biochemical Corp.) in 0.15 M phosphate buffer, pH 6.3, adjusted to an absorbance (A) of .420 at 420 mμ. Two-tenths of 1 ml of material was added to 2 ml of suspension, mixed, and the linear decrease at 420 mμ in absorbance was recorded for one minute on a Photovolt Corp. Linear/Log Varicord Model 43 Continuous Recorder at 25°C. There was a linear relationship between concentrations of crystalline egg white lysozyme and rate of decrease of absorbance at one minute.

All fractions were frozen and thawed 6 times in dry ice-acetone before assay.

Disruption studies. 'Granule' fractions were suspended in 0.34 M sucrose and added V/V to 25 mM tris-HCl buffer in 0.3 M sucrose at pH 7.4(3). Streptolysin S (SLS) (a gift of A. W. Bernheimer) lysolecithin, (Mann Research Chemicals) and etiocholanolone (Steraloids, Inc.) were added to final concentrations of 1,000 HU/ml and 5×10^{-4} M respectively. The absorbance (A) at 520 mμ was followed over time at 25°C for SLS and lysolecithin and at 37°C for etiocholanolone. Appropriate diluents (10% normal saline, 10-20% H_2O, 1% absolute alcohol) were added to control samples. The samples were then centrifuged at 27,000 *g* for 20 minutes, the supernatants removed and the pellets resuspended to original volume. Beta glucuronidase, acid phosphatase activity and protein content were determined on the supernatant and pellet. The same experiments were also performed in 0.34 M sucrose alone, but without recording changes in absorbance.

Results. Distribution of enzymes. Analyses of the total lysate, 'nuclear' fraction, 'granule' fraction and post-granule supernatant for acid phosphatase, beta glucuronidase, lysozyme and protein, as summarized in Fig. 1, demonstrated that 66% of the total recovered beta glucuronidase activity, 58% of the acid phosphatase activity and 50% of the lysozyme activity was found in the 'granule' fraction. The major portion of the protein (63%) was in the post granule supernatant. The specific activity (mg reaction product/mg protein/hour) of each of these enzymes was increased by a factor of 2 to 3½ over the specific activity of the total lysate (Fig. 1).

Phase microscopy. Phase microscopy revealed occasional intact cells in the total lysate, but predominantly swollen nuclei and granules in Brownian motion. The nuclear fraction showed nuclei and granules which characteristically appeared to be agglutinated onto circular membrane fragments. The granular fraction showed phase-dense granules in Brownian motion, occasionally in small clumps.

Properties of granules (disruption studies). When 'granule' fractions were incubated either in 0.34 M sucrose alone, or mixed V/V with tris-HCl buffer, less than 6% of the total enzyme was released into the supernatant. However, in the presence of lysolecithin (5×10^{-4} M), 94% of total acid phosphatase and 73% of total beta glucuronidase were released into the supernatant in a non-sedimentable form. Addition of SLS at room temperature released 52% of the total enzyme activity. Incubation at 37°C was necessary to demonstrate an effect of etiocholanolone, a finding in keeping with the effect of the hormone on rabbit leukocyte granules(4). Under these conditions, the steroid released over 32% of the total enzyme activity into the supernatant, whereas 15% or less was released from control samples containing 1% absolute alcohol (Table I).

Reduction in the absorbance at 520 mμ under these conditions paralleled enzyme re-

lease (Fig. 2). However, lysolecithin, which released almost all of the enzymes into the supernatant, did not reduce the absorbance as drastically as might be expected if all of the absorbance were due to structures susceptible to lysis by lysolecithin.

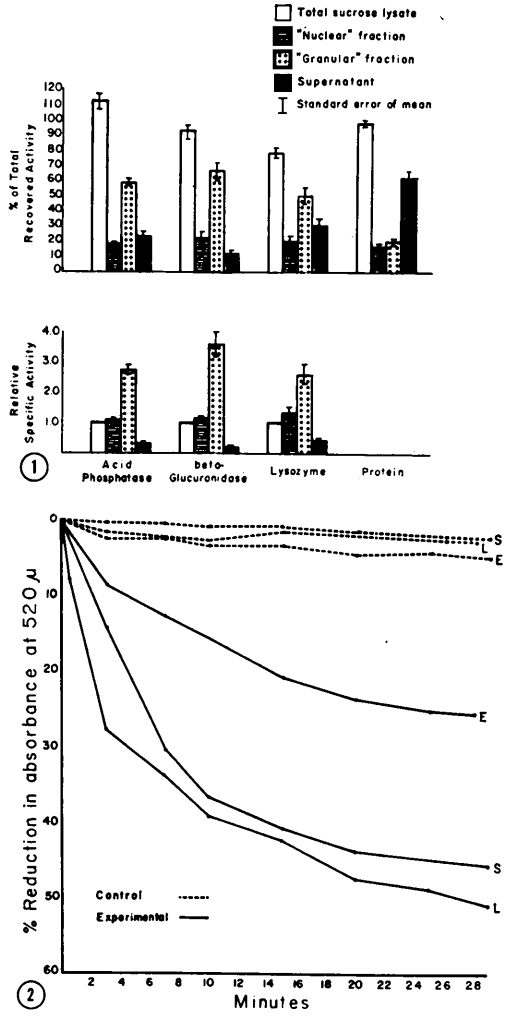

FIG. 1. Activities of acid hydrolases, and lysozyme in fractions derived from human PMNs disrupted in 0.34M sucrose. Relative specific activity = specific activity of fraction / specific activity of total lysate. See text for details of preparation.

FIG. 2. Reductions in apparent absorbance (average initial absorbance = 0.400) of human leukocyte lysosomes suspended in 0.3M sucrose (pH 7.4 0.025M tris buffer). Experimental (solid lines): S = Streptolysin S, 1,000 H.U./ml, 25°C. L = Lysolecithin, 5×10^{-4}M, 25°C. E = Etiocholanolone, 5×10^{-4}M, 37°C. Controls (hatched lines): S = 10% V/V normal saline, 25°C. L = 20% V/V H_2O, 25°C. E = 1% V/V absolute alcohol, 37°C.

TABLE I. % of Total Enzyme Released into Non-Sedimentable Form.

	Acid phosphatase*	Beta glucuronidase†
Control	3.7	6.1
Lysolecithin	94	73
Control	10	6.2
Streptolysin S	52	52
Control	15	8.5
Etiocholanolone	44	32

* Total enzyme activities averaged 0.51 μg acid phosphatase/μg protein/hr.

† Total enzyme activities averaged 0.08 μg phenolphthalein/μg protein/hr.

Discussion. A convenient method has been developed for isolation of human PMN granules from small quantities of blood. The conditions for their separation were originally determined by monitoring % of total enzyme/% of total protein in fractions obtained. The first difficulty encountered was breakage of the granules simultaneously with disruption of the cells. This was avoided by washing the cells in balanced salt solution before applying the sucrose-disruption technique of Cohn and Hirsch(1). Once intact granules in a cell lysate were obtained, they tended to agglutinate onto cell fragments and sediment with the nuclear fraction. This was avoided by addition of heparin to the final suspension before cell lysis and by gentle resuspension and washing to minimize cell fragment formation prior to final suspension.

The final granule fractions were visually free from apparent contamination by nuclei and cell debris, as determined by phase contrast microscopy. This fraction is undoubtedly contaminated with mitochondria, especially since the original cell population was not entirely composed of PMNs. However, the low centrifugal force ($110 \times g \times 5'$) used during washing led to a preferential loss of lymphocytes, while the higher centrifugal forces ($2{,}000 \times g \times 10'$) used to separate the nuclear pellet tended to sediment mitochondria. Under the conditions described, the method was reproducible and made possible the study of human granules from small quantities of blood.

The distribution of enzymes in the fractions and the relative specific activities are

similar to those obtained in the fractionation of rabbit leukocytes. However, the specific activity of beta glucuronidase is greater in relation to acid phosphatase in human granules than in rabbit granules. The significance of this is difficult to evaluate in view of the known artifacts of these relatively crude separative procedures.

Once granules were obtained, it was possible to study conditions under which the membrane-bound acid hydrolases were released into a non-sedimentable form. It is possible that in pathologic conditions, such as the rheumatic diseases, Chediak-Higashi disease, or juvenile amaurotic idiocy (Spielmeyer-Vogt disease) abnormalities in content or release of enzymes may be found. A specific abnormality in the enzyme content of liver lysosomes has been described by Hers (8) in one form of juvenile glycogen storage disease. It should therefore now be possible to investigate abnormalities of content, or variations in the isozyme pattern, of enzymes contained in human leukocyte lysosomes.

Summary. A method has been described for isolation of human polymorphonuclear leukocyte lysosomes from 25 ml of peripheral venous blood. These granules resembled in content and behavior the granules of rabbit white cells and rat and rabbit liver. The "latent" acid hydrolases could be released from the granules by agents which have been shown to disrupt lysosomes from other tissues: streptolysins, lysolecithin, and etiocholanolone.

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Isosorbide, a New Oral Osmotic Diuretic. (30092)

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The osmotic diuretic properties of hexitols when given intravenously have been known for some time. The diuretic properties of sorbitol were reported by West and Burget (1), by Krantz and Carr(2) and by Leimdorfer(3). A large portion of mannitol given intravenously in man was recovered in the urine by Smith *et al*(4). The use of mannitol as an intravenous osmotic diuretic is mentioned by Krantz and Carr(2). Mazze and Barry(5) have refocused the attention of the clinician in the use of intravenous mannitol as an osmotic diuretic.

Hexitols such as mannitol are not clinically useful osmotic diuretics when given orally. This is because hexitols given orally do not provide the necessary blood level due to (a) slow absorption and (b) exposure to liver oxidation(6,7).

Considering other hexitols and their anhydrides, Smith *et al*(4) observed that the renal clearance in dogs of isomannide and isosorbide (sorbide) was about one-half of that of creatinine, sorbitol, mannitol, dulcitol and sorbitan. However, their report included only renal clearance data on intravenous isosorbide in the dog.

Diuretic activity of isomannide when given orally has been reported(8,9,10). During studies relating to the structure of hexitols