STUDIES ON LYSOSOMES

XI. Characterization of a Hydrolase-Rich Fraction from Human Lymphocytes

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ABSTRACT

Pure suspensions of human lymphocytes were separated from peripheral blood by means of nylon wool, homogenized in 0.34 M sucrose–0.01 M EDTA solution, and fractionated by differential centrifugation. The bulk of acid hydrolase activity was found to be concentrated in a 20,000 $g \times 20$ min granular fraction, whereas nuclear, debris, and supernatant fractions contained lesser concentrations of hydrolases. Acid hydrolase activity present in the granular fraction showed appropriate "latency" as judged by its dose-dependent release into the 20,000 $g \times 20$ min supernatant after exposure to membrane-disruptive agents such as streptolysin S, filipin, and lysolecithin. Heparin proved to be necessary in the suspending medium so that reproducible homogenization and cell fractionation could be obtained. Even excessive contamination of lymphocyte suspensions with platelets did not appreciably alter the acid hydrolase activity of lymphocyte homogenates or the distribution of enzymes in subcellular fractions. Discontinuous density-gradient centrifugation of a 500 $g \times 10$ min supernatant, containing both acid hydrolase–rich organelles and mitochondria, resulted in partial resolution of hydrolase-rich organelles from mitochondria. Fine structural studies of the intact lymphocytes showed the presence of acid phosphatase–positive, membrane-bounded organelles. Electron microscopy of the "large granule" (20,000 $g \times 20$ min) fraction of such lymphocytes demonstrated 80–90% mitochondria, 5–10% platelets, and 5–10% membrane-bounded acid phosphatase–positive structures. The data indicate the presence in human peripheral blood lymphocytes of acid hydrolase–rich granules which possess many of the biochemical and structural characteristics of lysosomes in other tissues.

INTRODUCTION

Lysosomes have been described in most mammalian cells, although criteria for the identification of these organelles are by no means uniform (1). Biochemically, their identification rests upon isolation of subcellular granules, the sedimentation properties of which differ significantly from those of mitochondria, microsomes, and peroxisomes (2). Lysosomal acid hydrolases possess latency, i.e. their activity upon substrates in isotonic media can be increased considerably by measures designed to disrupt biological membranes. Furthermore, disruption of membranes leads to release of the several hydrolytic enzymes from granules in parallel fashion. Morphologically,

their structure is usually that of a single, or occasionally double, membrane-bounded organelle containing one, or preferably more than one, hydrolytic enzyme identified by electron microscopic cytochemistry (3). By light microscopic, cytochemical techniques, latency of hydrolases can also be demonstrated (4).

Despite the ubiquity of lysosomes, their existence in lymphocytes has not been unequivocally demonstrated. At the electron microscopic level several investigators have demonstrated a few acid phosphatase–staining granules in freshly obtained (12), cultured (13), and column-separated (14) lymphocytes, a finding consistent with the paucity of both cytoplasm and other cytoplasmic organelles. Indeed, Allison and Mallucci (5) have shown a degree of latency in these structures by cytochemical techniques. Bowers and de Duve (6–8), working with rat spleen homogenates, described a class of organelles which contained an incomplete complement of acid hydrolases. Since they disappeared after cortisol administration, the granules were considered to represent lysosomes of lymphocytes. We have previously suggested (9) that labilization of lymphocyte lysosomes might accompany the widespread gene activation which precedes transformation of lymphocytes into actively synthetic and dividing cells, a suggestion circumstantially supported by the observations of Allison and Mallucci (5). Since workers in this (10, 11) and in other (5, 13, 14) laboratories have already demonstrated that lymphocytes *late* in transformation (shortly before mitosis) become filled with newly formed granules rich in acid hydrolases, it was necessary to document the existence of such organelles in the *resting*, or untransformed, lymphocyte.

Therefore we undertook to determine the distribution and properties of acid hydrolases in relatively pure preparations of human peripheral blood lymphocytes. These studies were combined with ultrastructural examinations of intact cells and subcellular fractions. The data presented below indicate that a group of organelles may indeed be isolated from such preparations which fulfill the biochemical and morphological criteria for lysosomes.

MATERIALS AND METHODS

Separative Procedures

Human peripheral blood lymphocytes were obtained under sterile conditions from units of freshly drawn, heparinized blood (2,000 U heparin per 450 ml blood). The whole blood was allowed to sediment for 2–2½ hr at 37°C in screw-top culture tubes (15 ml), and the plasma was separated from the sedimented red cells. For removal of the majority of platelets, adenosine-5′-diphosphate (ADP, Grade 1, sodium salt from Sigma Chemical Co., St. Louis, Mo.) dissolved in minimum essential medium Eagle, Spinner, (MEM-S from Grand Island Biological Co., Grand Island, N. Y.) was added to the plasma at a final concentration of 10–15 μg/ml plasma. The resulting platelet clumps and agglomerates were removed by a combination of decantation and filtration through a disposable plastic filter set (Fenwal Laboratories, Inc. Morton Grove, Ill.). Separation of polymorphonuclear leukocytes from lymphocytes was achieved by passage through a column of 8–9 g of nylon wool packed into a separatory funnel (60 ml). Since previous experience had shown that use of unwashed nylon wool obtained from Leuko-Pak (Fenwal Laboratories, Inc.) was accompanied by an increase in the number of nonviable cells, the wool was washed six to eight times in demineralized water over a period of at least 1 wk. After preincubation for 30 min in a 37°C incubator, the plasma was allowed to run from a disposable plastic bag (Transfer Pack, Fenwal Laboratories, Inc.) dropwise through the autoclaved, dried, and prewarmed column over a period of 60–90 min at 37°C. The column was then washed with ½–1 volume of MEM-S for another 60–90 min at 37°C. The plasma was collected in a screw-top bottle, and 5,000 U heparin per 100 ml plasma or MEM-S–plasma mixture were added. The cells were next washed three times in MEM-S and centrifuged at 1,000 rpm (=224 *g* at tip of tube) in 35-ml round-bottom, screw-top glass centrifuge tubes, in an International Universal Model UV Centrifuge. The final pellet was resuspended in 20 ml MEM-S containing 20% fetal calf serum, 1% 200 mM L-glutamine, 100 U penicillin and 100 μg streptomycin per milliliter (Grand Island Biological Co.), and allowed to stand overnight at room temperature.

Cell Counts and Viability

White blood cell counts were determined with a 1:20 or 1:1 dilution of cell suspension with appropriately diluted counting fluid. Purity of cell suspensions was measured by differential counts on sediments which had been smeared and stained with McNeal's tetrachrome stain. Platelet counts were performed by standard methods, with procaine solution as dilution fluid. Cell viability was determined on a 1:1 dilution of cell suspension with erythrosin B (Fisher Scientific Company, Fair Lawn, N. J.) at a final concentration of 0.067%. The dye was dissolved

in phosphate-buffered saline and cells were incubated with erythrosin B for 5 min at room temperature.

Culture Procedure

Cell suspensions were transferred to 50-ml screw-top, shallow conical glass centrifuge tubes (Bellco Glass, Inc., Vineland, N. Y.) and adjusted to a cell concentration of 1.5–2.5 × 10^6 per milliliter in a final volume of 40 ml of complete medium. The cells were cultured for 210 min in a 37°C incubator, and 30 min before harvesting 0.1 ml 0.9% NaCl solution was added per 5.0 ml of culture medium.

After being harvested, cell suspensions were cooled in ice water, and 10–15 ml of ice-cold MEM-S medium was added. The cells were then centrifuged at 4°C in a refrigerated International model PR-2 centrifuge at 1800 rpm (= 724 *g* at tip of tube) for 15 min. For lysis of the contaminating red cells by hypotonicity, the cells were resuspended in 20.0 ml of ice-cold 0.35% NaCl solution for 1 min. After readjustment of isotonic conditions by addition of 2.4 ml of 5% NaCl solution, cell suspensions were centrifuged in the refrigerated centrifuge at 1800 rpm for 10 min. For complete lysis of erythrocytes, usually a twofold repetition of the hypotonic shock was necessary. Only one-half the volume of both NaCl solutions was used terminally. The final white pellet was resuspended in 3.7–4.0 ml of ice-cold 0.34 M sucrose containing 0.01 M disodium ethylenediamine tetraacetate (EDTA; Matheson, Coleman, and Bell, East Rutherford, N.J.) adjusted to pH 7.0 with 1 N NaOH. For satisfactory results of fractionation and enzyme recovery, it was necessary to add heparin (50 U/ml) to the sucrose-EDTA solution. However, a series of experiments was performed without addition of heparin.

Homogenization

Homogenization was carried out with a motor-driven (variable speed motor with flexible drive shaft and adjusted chuck; Tri-R Instruments, Jamaica, N. Y.) all-glass tissue grinder with a piston-type ground pestle, and a working capacity of 15 ml (No. 7725 from Corning Glass Works, Corning, N. Y.). Identical results were obtained with an all-glass tissue grinder produced by Kontes Glass Co. in Vineland, N. J. (size B catalog No. K-88550). Before use, grinding surfaces were smoothed by several hundred up-and-down strokes with 5 ml 0.34 M sucrose in the grinder. During homogenization, the tissue grinder was chilled with ice water. Cell lysis was followed microscopically.

Fractionation by Differential Centrifugation

The experiments were performed at 4°C. A 2.0 ml aliquot of whole homogenate was transferred to a conical centrifuge tube and centrifuged in the refrigerated centrifuge at 500 rpm (= 56 *g* at tip of tube) for 1 min. The supernatant was carefully removed by means of a fine-tipped Pasteur pipette and transferred to 5 ml lusteroid centrifuge tubes. The initial low-speed pellet represented the "debris" fraction. The supernatant was then centrifuged in an angle-head Sorvall Superspeed Model SS-3 Centrifuge at 500 *g* for 10 min with heparinized sucrose-EDTA. This step resulted in an opalescent supernatant fluid which was viscous and usually somewhat cloudy owing to finely dispersed floating material. The supernatant was carefully pipetted off with a fine-tipped Pasteur pipette and transferred to 5-ml lusteroid centrifuge tubes. The remnant pellet constituted the "nuclear" fraction. The supernatant was centrifuged at 20,000 *g* for 20 min in the Sorvall centrifuge. This step resulted in an opalescent "postgranular" supernatant which was carefully removed by means of a fine-tipped Pasteur pipette, and a thin, well-packed pellet which was considered as "granular" fraction. All pellets were then taken to original volume with 0.34 M sucrose containing EDTA and heparin as described above, and resuspended by using an all-glass tissue grinder as used for initial homogenization. All fractions were stored at −18°C.

For release of enzyme activity from the particulate phases, whole homogenates and subcellular fractions were treated with Triton X-100 (Rohm & Haas, Philadelphia, Pa.) at a final concentration of 0.1%. After 15 min of standing at 4°C, all preparations were centrifuged at 20,000 *g* for 20 min, and the supernatants were removed with a fine-tipped Pasteur pipette and used for biochemical analyses.

Fractionation by Density-Gradient Isopycnic Centrifugation

Discontinuous sucrose gradients were prepared at 4°C with 10-ml aliquots of 1.8, 1.7, 1.6, 1.5, and 1.4 M sucrose. 5 ml of "postnuclear" supernatant were layered above the gradient, and the tube was centrifuged at 22,500 rpm (= 100,000 *g* at tip of tube) and 5°C for 180 min in the SW 25.2 swinging-bucket rotor of the Beckman Model L-2 preparative Ultracentrifuge. After centrifugation, 18–19 fractions (each 3.1–2.9 ml) were collected through a pin hole in the bottom of the tube. The preparation of such gradients has been discussed in detail elsewhere (15).

Biochemical Analyses

ENZYME ASSAYS: *Beta glucuronidase* activity was assayed by a modification of the method of Talalay et al. (16), with 0.2 ml homogenate, 0.6 ml acetate buffer, 0.1 M, pH 4.6, and 0.1 ml substrate (phenolphthalein-glucuronic acid, 0.01 M, pH 7.0; Sigma Chemical Co.). After 6 hr of incubation at 37°C, the

reaction was stopped by addition of 2.0 ml ice-cold glycine buffer, 0.2 M, in 0.2 M NaCl, pH 10.4, and the optical density was read at 550 mμ. The enzyme activity was expressed as micromoles of phenolphthalein released from substrate per 100 mg protein per hour.

Acid phosphatase activity was determined by a modification of the method of Valentine and Beck (17) described in a previous paper (11), with 0.2 ml homogenate and 1.8 ml 0.052 M D,L-beta-glycerophosphate disodium salt pentahydrate (Grade 1 from Sigma Chemical Co.; content of the alpha isomer less than 0.1%) in acetate buffer, 0.05 M pH 5.0, as substrate. After 5–6 hr of incubation at 37°C, the reaction was stopped by addition of 0.4 ml ice-cold 35% trichloracetic acid, and the mixture was filtered (Whatman paper No. 42). The protein-free filtrate was then analyzed for inorganic phosphorus by a modification of the method of Chen et al. (18) so that 0.3 ml of filtrate added to 0.7 ml of assay solution yielded the same final concentrations as described in the original method. The optical density was read at 660 mμ, and the enzyme activity was indicated as micromoles phosphorus released from substrate per milligram protein per hour.

Acid ribonuclease activity was also assayed. Initial attempts to assay the activity of this enzyme with the method described by de Duve et al. (19) showed that in lymphocyte homogenates only little acid ribonuclease activity was detectable even after an incubation period of 6 hours at 37° C. Using bovine ribonuclease and ribonucleic acid derived from yeast, Kalnitsky et al. (20) demonstrated that addition of urea in rather high concentrations to ribonuclease-substrate mixtures was capable of increasing the formation of acid-soluble oligonucleotides by the enzyme. By assay of lymphocyte homogenates, it could be shown that the concentration of oligonucleotides liberated by acid ribonuclease was 5–10 times higher if the enzyme-substrate mixture contained urea. The best results were obtained by adding 0.5 ml lymphocyte homogenate to 0.3 ml substrate-urea mixture. The final enzyme-substrate-urea mixture contained 8.4 mg sRNA.Y (Schwarz Bio Reasearch Inc., Organgeburg, N.Y.), 2.4 mmoles urea, 0.04 mmoles acetate buffer, pH 5, and 0.27 mmole sucrose. A variety of ribonucleic acid preparations were used as substrate. Most showed a high and variable increase of acid-soluble oligonucleotide concentration in the reagent blank after an incubation period of 6 hr at 37°C which was apparently owing to a varying contamination of these ribonucleic acid preparations with ribonuclease and/or a spontaneous hydrolysis of ribonucleic acid during the assay period. However, reagent blanks prepared with the sRNA preparation supplied by Schwarz Bio Research revealed only a slight and constant increase of acid-soluble oligonucleotide concentration during incubation. After 6 hr of incubation at 37°C, the reaction was stopped by addition of 0.8 ml ice-cold 10% perchloric acid containing 0.25% uranyl acetate. After 1 hr of standing at 4°C, the mixture was filtered (Whatman paper No. 42), aliquots of the filtrate were diluted appropriately with distilled water, and the optical density was read at 260 mμ in a Beckman Model DU spectrophotometer. The enzyme activity was expressed as micromoles of mononucleotides released per milligram protein per hour. Mononucleotide concentration was estimated, as described by de Duve et al. (19), by using an extinction coefficient of ϵ 260 = 8.5 × 10^3.

Malate dehydrogenase activity was determined by the method of Mehler et al. (21), by using 0.03–0.05 ml homogenate, 0.3 ml phosphate buffer (0.25 M, pH 7.4), 0.1 ml $NADH_2$ (1.5 mM, pH 8.5–9.0, adjusted with NaOH), and 2.4 ml distilled water. The reaction was started by addition of 0.1 ml oxalacetate (7.6 mM, pH 7.4, adjusted with NaOH), and the initial rate of change of absorbancy per minute at 340 mμ and room temperature was recorded. The enzyme activity was expressed as micromoles $NADH_2$ oxidized per milligram protein per hour.

Protein was determined by the method of Lowry et al. (22) against a standard of crystalline human serum albumin (National Biochemical Co., Cleveland, O.). After addition of the Folin-Ciocalteau reagent, the assay mixture became cloudy owing to a finely dispersed precipitate caused by the presence of Triton X-100 in the homogenate. This precipitate could be eliminated by centrifugation at 20,000 *g* for 10 min without changing the final reading in the supernatant at 660 mμ. Because of a dose-dependent inhibition of chromogen formation by EDTA, the standard curves were determined with albumin in sucrose-EDTA containing heparin.

Release of Sedimentable Hydrolase Activity

The experiments were carried out with postnuclear supernatants, since mechanical resuspension of isolated granules frequently resulted in release of their enzymes. Samples (0.04 ml) of a solution containing the test compound were added to 0.76 ml aliquots of postnuclear supernatants. The applicability of these procedures to studies of lysosomes from liver and leukocytes has been previously documented (23, 24). The mixture was incubated for 60 min at 37°C and then centrifuged at 20,000 *g* for 20 min in the Sorvall centrifuge at 4°C. Both supernatants and pellets (the latter resuspended to original volume with 0.34 M sucrose containing 0.01 M EDTA, pH 7.0, 50 U/ml heparin and 0.1% Triton X-100) were assayed for beta glucuronidase and acid phosphatase activity. The following test substances were used: streptolysin S (obtained from Dr. A. W. Bernheimer, New York University School of Medicine, New York) dissolved in phosphate buffer, 0.1 M, 7.4; filipin (The Upjohn Co., Kalamazoo, Mich.) dissolved in dimethyl sulf-

oxide (DMSO; Sigma Chemical Co.) lysolecithin (Mann Research Labs Inc., New York) dissolved in 50% ethanol.

Electron Microscopy: Whole Cells

The whole cell pellets were fixed in 1.5% redistilled glutaraldehyde in 0.05 M sodium cacodylate containing 7.5% sucrose at 4°C for 90 min (25). The cell buttons were then washed overnight in 0.08 M sodium cacodylate. The cells were postfixed in Millonig's phosphate-buffered osmium tetroxide (26) for 1 hr. Cells were dehydrated in graded concentrations of ethyl alcohol and in propylene oxide and embedded in Epon 812 epoxy resin (27).

Thin sections were cut with an LKB or Huxley microtome and placed on carbonized copper grids. The sections were stained with 1% aqueous uranyl acetate (28), lead citrate (29), or a combination of these stains. The material was examined in an RCA-EMU-3G electron microscope at primary magnifications 3,000–40,000.

Subcellular Fractions

Pellets were harvested in 0.34 M sucrose containing 0.01 M disodium ethylenediaminetetraacetate and heparin (50 U/ml). The fractions were then embedded in 1.0% agar to facilitate handling. Fractions were fixed in 1.5% redistilled glutaraldehyde in 0.05 M sodium cacodylate containing 7.5% sucrose at 4°C for 90 min. They were washed overnight in 0.08 M sodium cacodylate, postfixed in Millonig's osmium tetroxide, dehydrated, embedded, and studied in a manner similar to that for studying the whole cells. Subcellular fractions from a total of four experiments were examined.

Electron Microscopic Histochemistry

Whole cells and subcellular fractions were fixed for 5 min at 4°C in 1.5% glutaraldehyde in 0.05 M sodium cacodylate containing 7.5% sucrose. This brief period of fixation has been demonstrated to permit retention of acid phosphatase activity (30). The pellets were

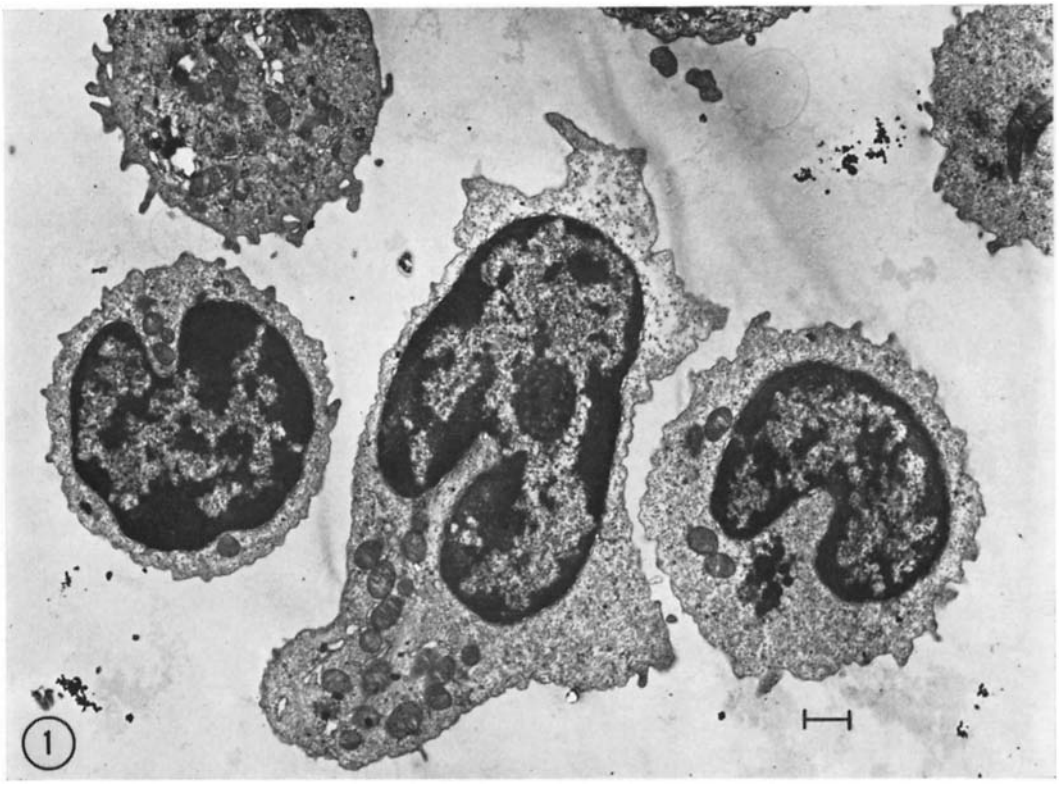

FIGURE 1 Human peripheral blood lymphocytes separated on nylon wool column. Low-magnification electron micrograph showing two small lymphocytes and medium-sized lymphocyte in center. ×6300. As in all illustrations cells are fixed in glutaraldehyde-osmium tetroxide and embedded in Epon. Sections are stained with uranyl acetate-lead citrate. Scale, 1 μ.

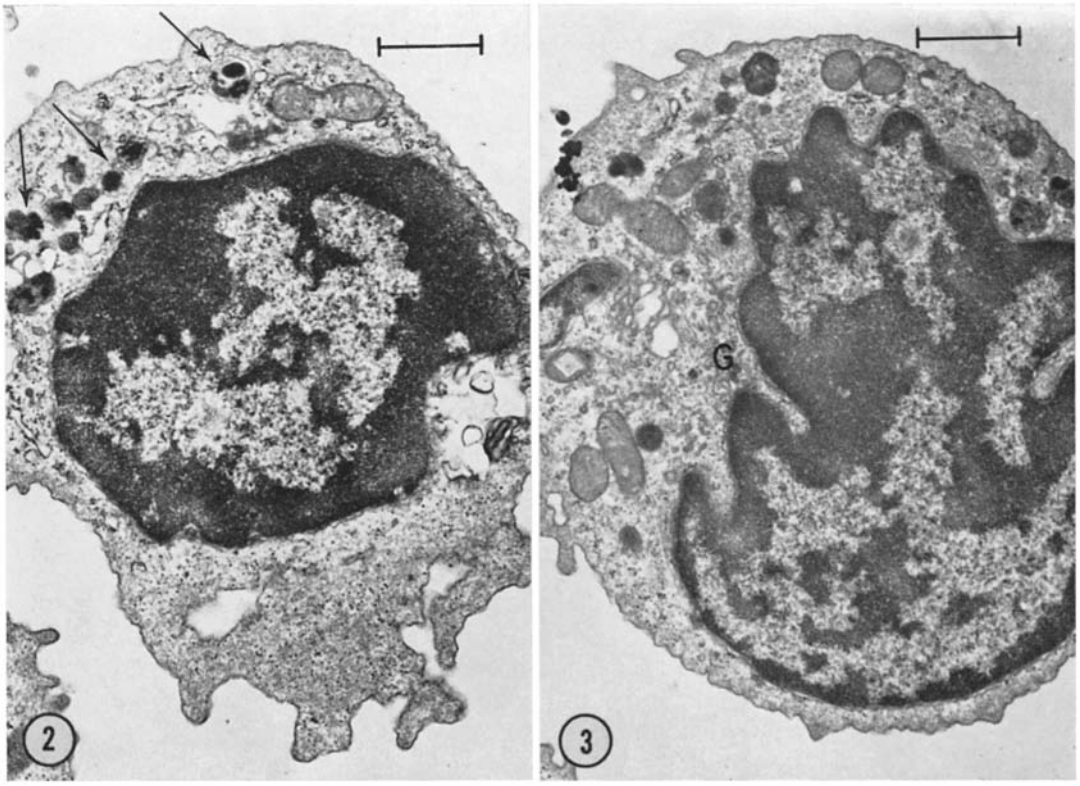

FIGURE 2 Column-separated small lymphocyte showing several lysosome-like bodies (arrows). ×13,000.

FIGURE 3 Intact small lymphocyte. A Golgi zone is seen (*G*). ×13,000.

then washed six times (10 min each) in cold 0.08 M sodium cacodylate. Following this procedure the pellets were rapidly frozen for 2 min in a mixture of dry ice and ethanol and thawed at room temperature. The pellets were then incubated for 30 min at room temperature in a modified Gomori substrate mixture (31). As previously noted by Ericsson and Trump (32), the intensity of the reaction product varied with incubation time; however, the most specific localization was found with 30 min incubation. The substrate was prepared as follows. 0.42 g of lead nitrate was dissolved in 300 ml of 0.05 M acetate buffer (pH 5.0). To 50 ml of this solution was added 5 ml of 0.1 M sodium beta glycerophosphate. This mixture was filtered prior to use. Following incubation the pellets were washed six times (15 min each) in 0.05 M acetate (pH 5.0) containing 7.5% sucrose and 4% formaldehyde. The pellets were allowed to be rinsed overnight in 0.05 M acetate buffer (pH 5.0) and then were post-fixed, dehydrated, embedded, and examined as described. Controls consisted of incubations in which glycerophosphate substrate was omitted or 0.01 M sodium fluoride was added to the media.

RESULTS

Separative Procedures and Cellular Viability

The separative method used resulted in cell suspensions containing 99.8% ± 0.2 (N = 26) pure mononuclear cells, as determined from tetrachrome-stained smears. The mononuclear cells consisted almost exclusively of what appeared to be small lymphocytes. Only occasionally, a few large lymphocytes or monocyte-like cells were observed. By addition of ADP and subsequent filtration of plasma, the majority of platelets could be removed. Small, macroscopically visible clumps of platelets which passed through both filter and the nylon wool column during the separative process usually became adherent to the wall of the non-siliconized glass centrifuge tubes during the wash

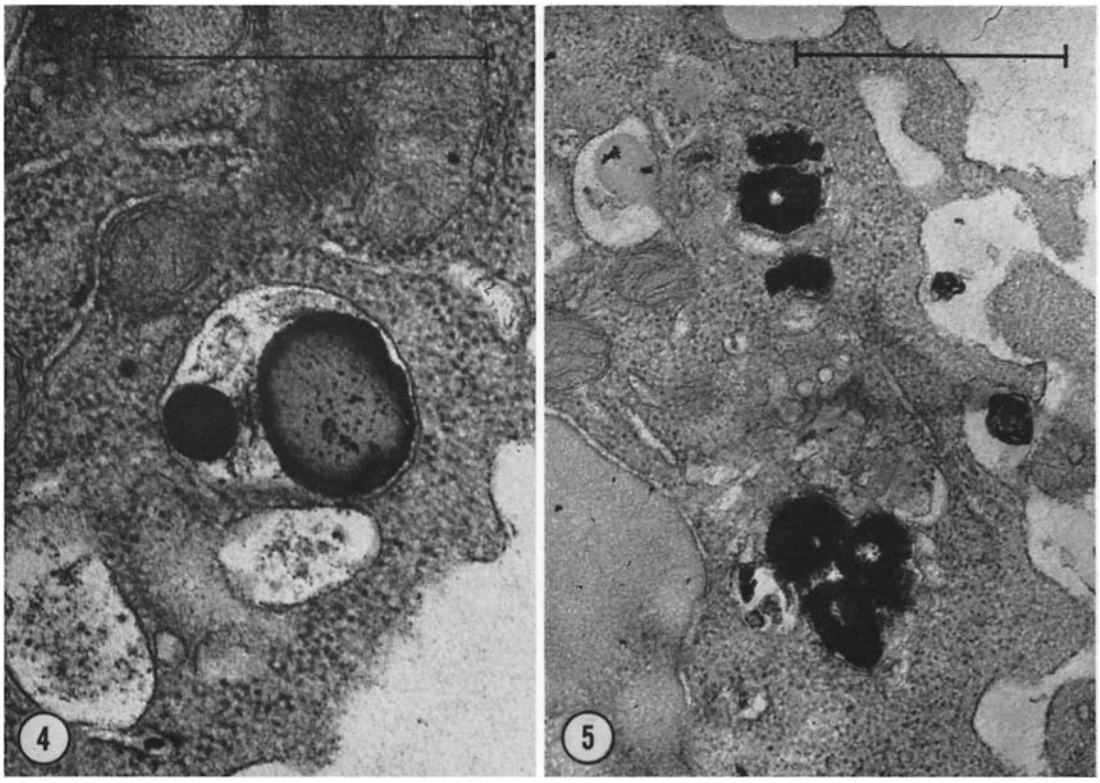

FIGURE 4 Acid phosphatase–positive organelle in an intact lymphocyte incubated in Gomori reaction mixture. A single membrane surrounds two acid phosphatase–positive structures. ×52,500.

FIGURE 5 Acid phosphatase–positive bodies in small lymphocytes. Note dense peripheral staining of these granules. ×36,000.

procedure. These were eliminated simply by changing the centrifuge tube. However, a small, variable percentage of platelets was still present in the final cell suspension. The average contamination with platelets at this stage was 250 × 10^6 per 40 ml culture medium (range: 140–410 × 10^6 per 40 ml). The viability of the lymphocytes (judged by the percentage of cells which excluded erythrosin B) averaged 95.4% ± 0.3 (N = 67) 15 hr after separation and suspension at room temperature.

Ultrastructure and Histochemistry of Whole Cells

By electron microscopy the final cell population was seen to consist of 99% small lymphocytes with occasional medium-sized cells (Fig. 1). Intact platelets were infrequently observed. The cells displayed cytoarchitectural features which were not distinguishable from those described previously, by workers from this laboratory, for the resting lymphocyte (14, 33) and, by others, for normal human peripheral blood (34, 35) (Figs. 2, 3). In electron micrographs the cells ranged from 5 to 8 μ in diameter. Nuclei were heterochromatic and nucleoli were observed in about 40% of cells. The cytoplasm contained several mitochondria, many free ribosomes, and sparse endoplasmic reticulum. The Golgi apparatus was poorly developed in these resting lymphocytes and rarely contained acid phosphatase–positive bodies (Fig. 3).

The cytoplasm also contained varying numbers, usually two to six, of heterogeneous electron-opaque membrane-bounded bodies. These organelles measured 0.3–0.6 μ in diameter. They were usually round or elliptical, and bounded by a single membrane. These organelles often contained granular, filamentous, and at times lamellar

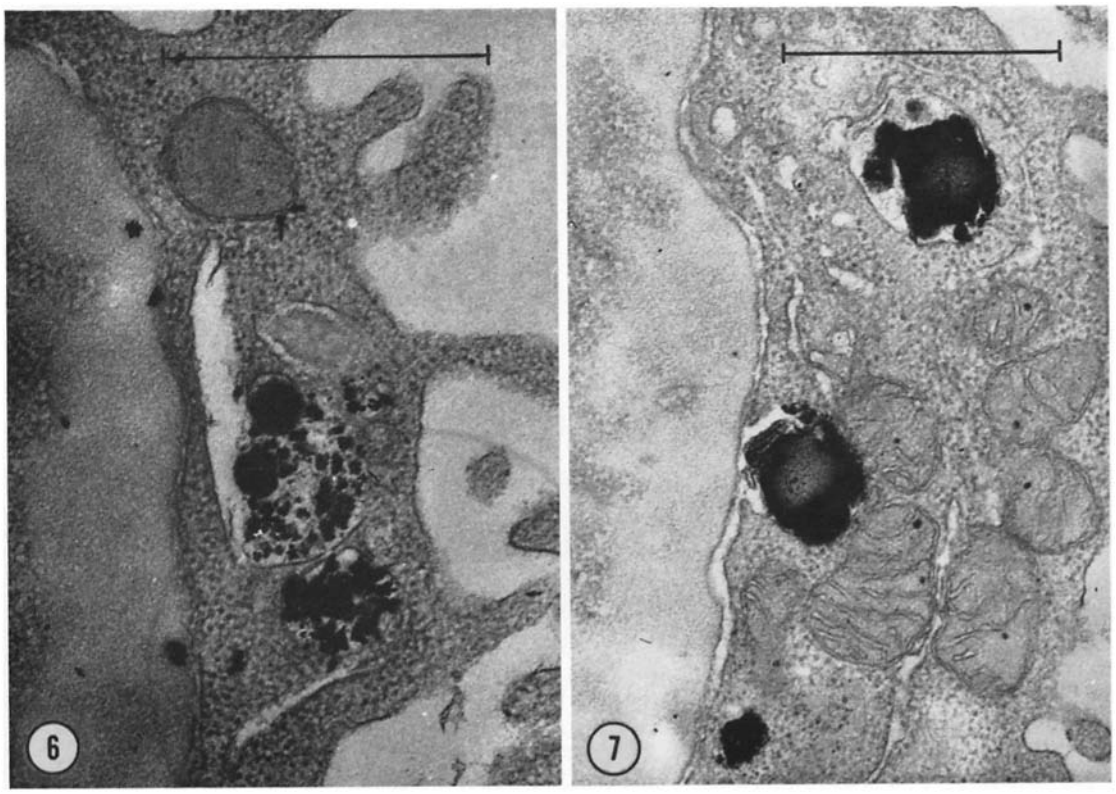

FIGURE 6 Lead reaction product present in small discrete particles within acid phosphatase–positive organelles. ×43,000.

FIGURE 7 Acid phosphatase–positive organelles. Note the absence of reaction product in other cytoplasmic structures. ×38,000.

structures. When intact cells were incubated in the Gomori reaction mixture for acid phosphatase, several types of deposition of reaction product were observed (Figs. 4–7). The reaction product showed variation in intensity and site of distribution. In some instances (Figs. 4, 5) individual organelles appeared to represent a fusion of several smaller membrane-bounded bodies which were acid phosphatase–positive. Some of these bodies (Fig. 6) showed multiple granular deposits of the reaction product. No significant deposition of the lead reaction product was observed in other cytoplasmic organelles, or in the nucleus.

Kinetics of Enzymes

The activity of three acid hydrolases, beta glucuronidase, acid phosphatase, and ribonuclease, increased in linear fashion with increasing concentration of homogenate (Fig. 8), after 6 hr of incubation at 37°C. The kinetics of malate dehydrogenase activity (assayed over 2–3 min) were linear with homogenate concentration and time of assay. The activity of beta glucuronidase, acid phosphatase, and malate dehydrogenase remained unaffected by addition of heparin (final concentration 50 U/ml) to the homogenate. However, as has been observed for a variety of ribonucleases (36, 37), heparin caused a potent inhibition of acid ribonuclease activity. Enzyme activity dropped to 24% of the initial level after addition of 12.5 U heparin per milliliter homogenate (Fig. 9), and to 5% after addition of 50 U heparin per milliliter homogenate. It was not possible to abolish the inhibitory effect of heparin by addition of protamine sulfate either to the homogenate or to the assay mixture. Therefore, in experiments in which acid ribonuclease activity was determined, homogenization and fractionation were carried out with-

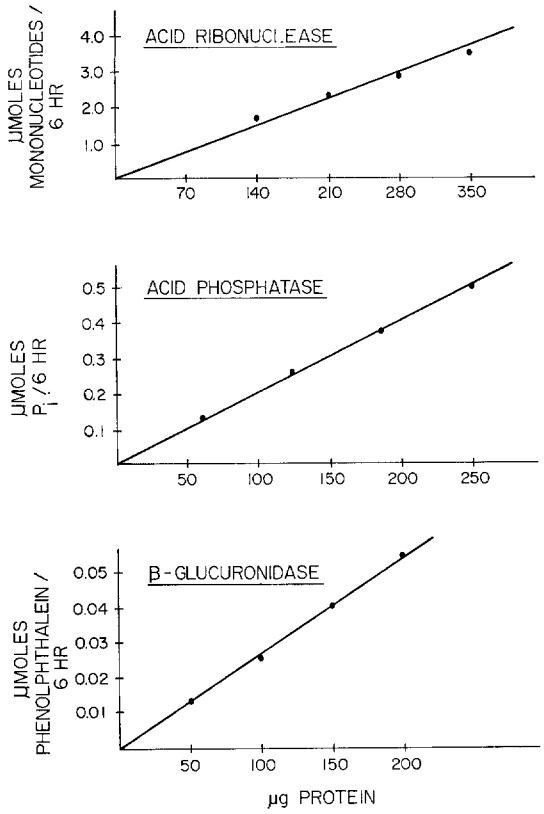

FIGURE 8. Kinetics of enzymes. Concentration of homogenate was varied with a constant incubation time of 6 hr. Conditions of assay as described under Materials and Methods.

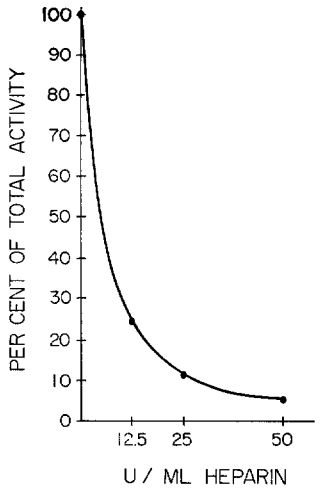

FIGURE 9. The effect of heparin on acid ribonuclease activity of lymphocyte homogenates. Conditions of assay as described under Materials and Methods.

TABLE I

Enzyme Activity in Heparinized and Heparin-Free Whole Homogenates of Human Peripheral Blood Lymphocytes

Enzyme	No. of experiments	Activity units ± SEM
(*a*)Heparinized homogenates		
Beta glucuronidase	15	5.3 ± 0.5
Acid phosphatase	4	0.4 ± 0.1
Malate dehydrogenase	4	54.7 ± 3.5
(*b*) Heparin-free homogenates		
Beta glucuronidase	4	8.0 ± 1.1
Acid ribonuclease	3	1.9 ± 0.2

Activity units: beta glucuronidase, μmoles phenolphthalein released/100 mg protein/1 hr; acid phosphatase, μmoles phosphorus released/1 mg protein/1 hr; acid ribonuclease, μmoles mononucleotides released/1 mg protein/1 hr; malate dehydrogenase, μmoles $NADH_2$ oxidized/1 mg protein/1 hr.

out heparin. In fractions from sucrose-density gradients, the heparin was sufficiently diluted and acid ribonuclease activity could be assayed directly. At concentrations used for release of enzyme activity from the granules, streptolysin S, filipin, and lysolecithin did not themselves affect the enzyme assays.

Homogenization

Satisfactory homogenization of lymphocytes suspended in heparinized (50 U/ml) sucrose-EDTA solution was obtained with a motor speed of 6.5–7.0 and 11–12 up-and-down strokes of the tissue grinder. Microscopic examination revealed that few cells were left intact in the homogenate. During the homogenization procedure, a loss of cells was unavoidable. This loss, however, was considerably higher if heparin-free sucrose-EDTA solution was used. Under these conditions, macroscopically visible clumps of cellular material became adherent to the glass surfaces and could not be removed by several manipulations. No macroscopically detectable material remained adherent to the glass when heparinized sucrose-EDTA was used.

Total Enzyme Activity and Distribution among Subcellular Fractions

HEPARINIZED HOMOGENATES: The activities of acid hydrolases and malate dehydrogenase

TABLE II

Distribution of Acid Hydrolase and Malate Dehydrogenase Activities and Protein Content Among Subcellular Fractions Derived from Heparinized and Heparin-free Homogenates of Human Peripheral Blood Lymphocytes

Values represent per cent of the total recovered activity ±SEM.

Enzyme	No. of experiments	Fraction			
		Debris	Nuclei	Granules	Supernatant
(*a*) Heparinized homogenates					
Beta glucuronidase	14	5.2 ± 0.4	25.9 ± 4.5	54.1 ± 4.2	14.8 ± 1.2
Acid phosphatase	4	0.0 ± 0.0	26.8 ± 4.0	47.7 ± 3.5	25.5 ± 1.7
Malate dehydrogenase	4	2.2 ± 0.6	16.7 ± 1.1	13.4 ± 0.7	67.7 ± 1.3
Protein	14	6.6 ± 1.1	13.1 ± 2.0	14.2 ± 1.0	66.1 ± 2.3
(*b*) Heparin-free homogenates			Pellet		
Beta glucuronidase	4		92.2		7.8 ± 2.3
Acid ribonuclease	4		91.0		9.0 ± 1.7
Malate dehydrogenase	4		65.8		34.2 ± 8.9
Protein	4		77.4		22.6 ± 4.5

present in whole homogenates are given in Table I. In preliminary experiments, it was found that satisfactory fractionation of lymphocyte homogenates as well as recovery of enzyme activities in the subcellular fractions could be obtained only by the addition of heparin to the sucrose-EDTA solution. At a concentration of heparin of 50 U/ml, the fractionation procedure proved to be quite reproducible (Table II). In Fig. 10, the relative specific activity (percentage of recovered enzyme activity over percentage of recovered protein) of beta glucuronidase, acid phosphatase, and malate dehydrogenase in each fraction is plotted against the percentage of recovered protein present in each fraction. The acid hydrolases revealed the highest relative specific activity in the granular fraction which sedimented at 20,000 *g* for 20 min. This fraction (Table II) also contained the highest percentage of the total recovered acid hydrolase activity. Considerable acid hydrolase activity was also present in the nuclear fraction. Although some organelles rich in acid hydrolases may have been trapped by nuclear material, consequently to appear in this fraction, the possibility cannot be excluded that part of the acid hydrolase activity in the nuclear fraction was due to the presence of unbroken cells not sedimented at 56 *g*. It is also likely that beta glucuronidase activity associated with the debris fraction originated from nonlysed cells. Under these conditions, the content of acid hydrolases in supernatant fractions did not exceed 14.8 ± 1.2% of recovered activity for beta glucuronidase, and 25.5 ± 1.7% of recovered activity for acid phosphatase. The distribution pattern of malate dehydrogenase activity differed considerably from that of the acid hydrolases, with 67.7 ± 1.3% of malate dehydrogenase in a nonsedimentable form. This finding would agree with the recent studies of Dioguardi et al. (38) who found that 40–45% of total malate dehydrogenase activity in purified human peripheral blood lymphocytes was due to the cytoplasmic isozyme. In the present studies, the sedimentable portion of malate dehydrogenase activity was distributed equally over the nuclear and granular fractions. These data suggest that the mitochondria sedimented not only with the granular but also with the nuclear pellet. The malate dehydrogenase activity present in the nuclear fraction could, however, be due in part to unbroken cells not sedimented at 56 *g*.

EFFECT OF VARYING CONDITIONS: Lymphocytes could not be fractionated to any acceptable degree unless heparin was present. In the absence of heparin the granular fraction contained virtually no acid hydrolase activity and the enzyme activity was distributed between the nuclear and debris fractions. Therefore, heparin-free homogenates were split into a pellet fraction sedimenting at 20,000 *g* for 20 min, and the corresponding "postpellet" supernatant. Compared to heparinized homogenates, such supernatants (Table II) were found to show significant decreases both in enzyme activity and in protein content, possibly reflecting incomplete cell lysis. In addition, recovery of total enzyme activities

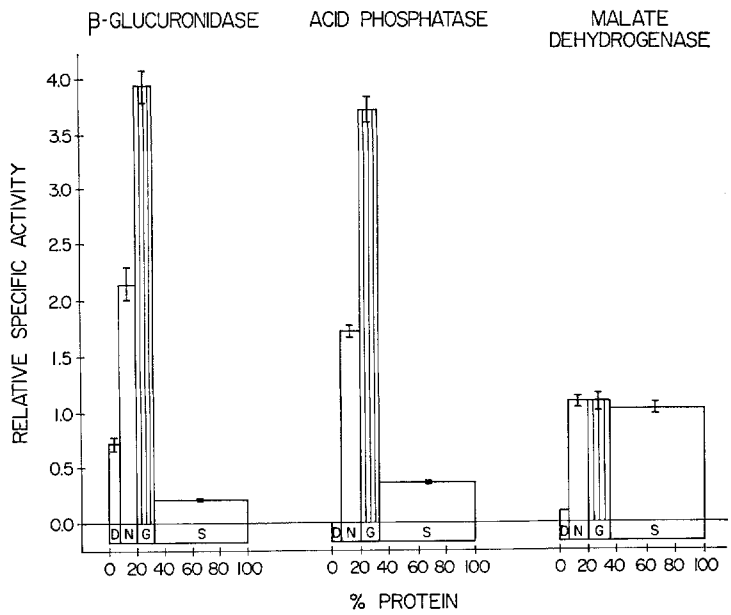

FIGURE 10 Distribution of enzyme activities in subcellular fraction from human lymphocytes. Distribution pattern of lymphocyte enzymes as established by differential centrifugation. Fractions are plotted in order of separation: debris (*D*), nuclei (*N*), granular (*G*) and supernatant (*S*). Conditions of isolation for each fraction are given in the text. The relative specific activity (per cent of total recovered activity/per cent of total recovered proteins) in each fraction is plotted on the ordinate. The abscissa represents the per cent of total protein in each fraction.

was considerably diminished in heparin-free homogenates, amounting to 39.0 ± 2.1% for beta glucuronidase and 77.1 ± 5.7% for malate dehydrogenase whereas protein recovery remained high (129.4 ± 14.1%). In contrast, in heparinized preparations, enzyme recovery approximated 90%, amounting to 89.9 ± 1.6% for beta glucuronidase, 88.4 ± 6.0% for acid phosphatase, 94.5 ± 6.2% for malate dehydrogenase. The protein recovery was 117.5 ± 3.4%. The total recovery of acid ribonuclease (assayed only in heparin-free homogenates) was 82.2 ± 4.2% with 9.0 ± 1.7% in the 20,000 *g* supernatant.

The effect was also examined of different homogenizers on the subcellular distribution of beta glucuronidase and protein. The use of an all-Plexiglas tissue-grinder (Bellco Glass, Inc.) with a smaller clearance between pestle and tube (Table III) resulted in a higher activity of beta glucuronidase in the supernatant (42.1% as opposed to 15.6%); this indicates that under these conditions more hydrolase-containing organelles were injured. In contrast, distribution of protein content between the granular fraction and the 20,000 *g* supernatant did not change. Similar results were obtained by increasing time, or intensity, of homogenization in the all-glass tissue grinder.

Influence of Platelet Contamination

Despite addition of ADP to the plasma it was impossible to obtain lymphocytes entirely free of platelets. Since platelets contain hydrolase-rich organelles (40), the effect of platelet contamination was studied, with respect to both total enzyme activity and its subcellular distribution. After addition of ADP, platelets were withdrawn from the plasma as described above, resuspended in 10 ml MEM-S medium, centrifuged at 1500 rpm (=503 *g* at tip of tube) for 10 min in the International universal model UV centrifuge, and the supernatant was discarded. 25 ml of "complete" MEM-S medium were then added to the pellet, the platelets resuspended as completely as possible, and the platelet-rich supernatant was decanted. After 15 hr of standing at room temperature, the supernatant was centrifuged at 600 rpm (=80 *g* at tip of tube) for 10 min for removal of platelet clumps and contaminating

TABLE III

Influence of Homogenization with Different Types of Tissue Grinder upon Distribution of Beta Glucuronidase Activity and Protein Content Among Subcellular Fractions Derived from Human Peripheral Blood Lymphocytes Cultured in Vitro

	All-glass grinder	All-Plexiglass grinder
Beta glucuronidase (% recovered activity)		
Debris	5.0	3.2
Nuclei	28.0	16.6
Granules	51.4	38.1
Supernatant	15.6	42.1
Protein (% of recovery)		
Debris	0.1	3.9
Nuclei	18.2	10.7
Granules	14.4	16.3
Supernatant	67.3	69.1

white cells. The resulting platelet-rich supernatant was decanted, and an aliquot of this preparation was added to an aliquot of a platelet-poor suspension of lymphocytes derived from the same unit of blood. This mixture was adjusted to 40 ml with complete MEM-S medium. Another aliquot of the platelet-poor suspension of lymphocytes was adjusted to 40 ml with complete MEM-S medium alone. Both platelet-rich and platelet-poor cultures were incubated for 5 hr at 37°C, and processed as described for routine cultures. Despite an almost 10-fold increase in platelets (Table IV), the homogenates derived from the platelet-rich cultures yielded only minimal increases of protein and beta glucuronidase content. Addition of platelets had little effect upon the subcellular distribution of enzyme activity, with little increase of beta glucuronidase activity in the granular fraction. Similar results were obtained for acid phosphatase activity. Indeed, no relationship could be found between the factor by which the platelet number was increased and the factors by which the enzyme activities rose. Addition of 142 × 10^6 platelets[1] directly to the granular fraction resulted in an expected increase of both beta glucuronidase activity and protein content. However, the relative specific activity of beta glucuronidase in this granular fraction *decreased* from 7.6 to 3.1 Therefore, it would appear that extensive contamination of the granular fraction with platelets could not explain the high relative specific activity found in fractionation studies. These results suggest that the acid hydrolase activities determined in lymphocyte cultures originate almost exclusively from enzyme-rich organelles present in the lymphocytes themselves.

[1] This approaches the entire platelet population present in the original volume of blood and obviously exceeds the maximum platelet contamination possible.

TABLE IV

Beta Glucuronidase Activity in Homogenates and Subcellular Fractions Derived from Lymphocyte Cultures Contaminated with and without Platelets

	Platelet-poor	Platelet-rich	Increase over platelet-poor
Platelets/culture (×10^6)	250	2367	9.47
Lymphocytes/culture (× 10^6)	89.2	89.2	—
Protein/1.0 ml whole homogenate, *mg*	0.94	1.19	1.27
Protein, Recovered/1.0 ml homogenate, *mg*	1.05	1.38	1.31
Beta glucuronidase, activity/ 1.0 ml whole homogenate	0.28	0.34	1.21
Beta glucuronidase, recovered activity/1.0 ml homogenate	0.25	0.29	1.16
Distribution among subcellular fractions (% recovered activity)			
Debris	2.30	1.81	—
Nuclei	12.03	11.56	—
Granules	67.24	69.92	1.04
Supernatant	18.44	16.72	—

Activity units, μmoles phenolphthalein released per 6 hr. Mean of three experiments.

Electron Microscopic Studies of the Large Granule Fraction

Fine structural study of the large granule (20,000 *g* × 20 min) pellet revealed that this fraction was composed predominantly of mitochondria, occupying about 80–90% of the total surface area. Mitochondria demonstrated the two types of swelling characteristic of isolation in sucrose (39). The first type involved swelling of the intermembranous compartment and was the more common. The second type, observed in about

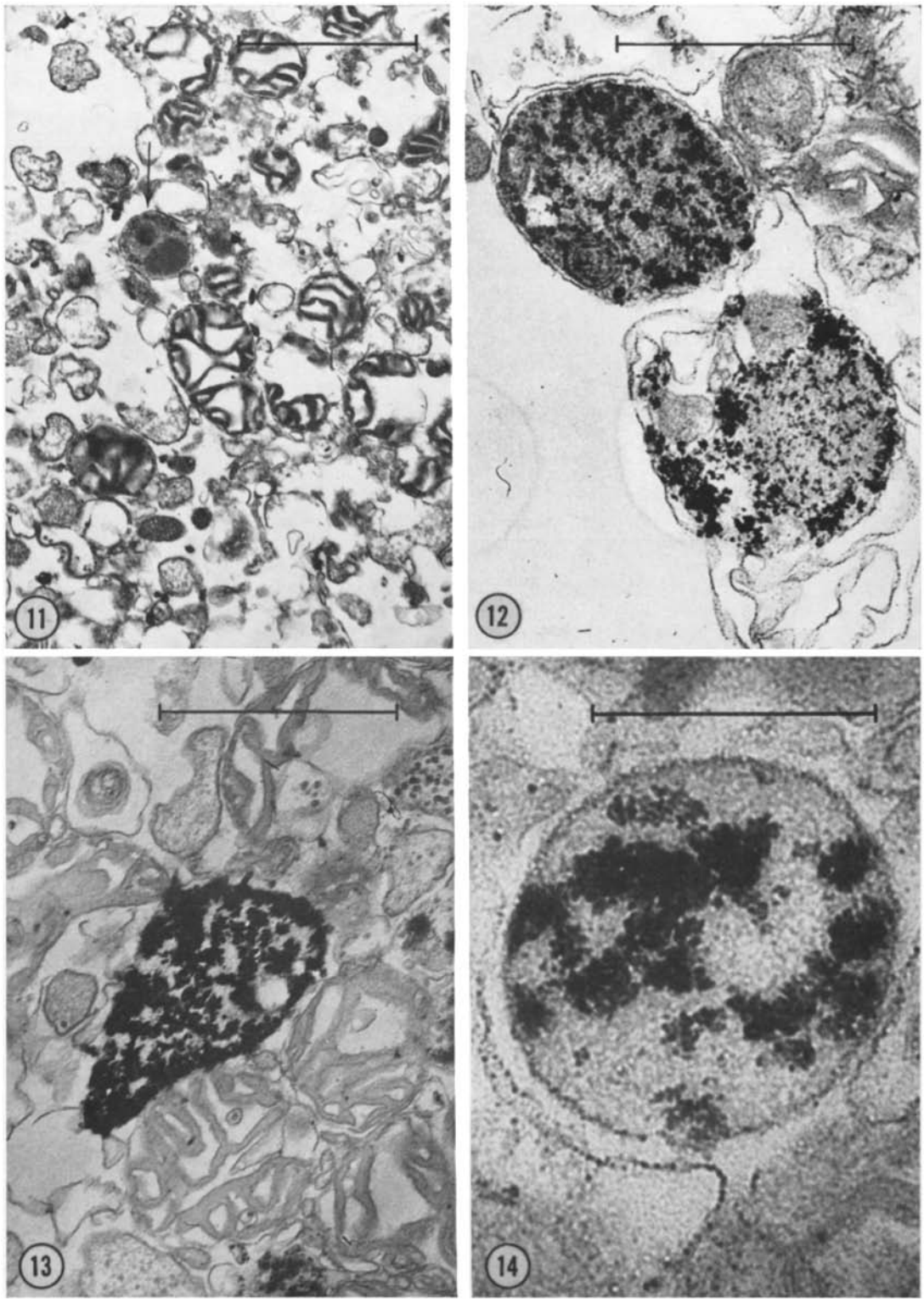

FIGURE 11 Acid phosphatase control. Lymphocyte granule fraction incubated in absence of substrate. Note the electron-opaque lysosome-like organelle (arrow) and mitochondria showing swelling of the intermembranous compartment. No lead reaction product is seen. ×23,500.

FIGURES 12–14 Lymphocyte large granule fraction incubated in Gomori reaction mixture. Acid-phosphatase lead reaction product is demonstrated. Figs. 12 and 13, ×38,000; Fig. 14, ×43,500.

10% of mitochondria, was swelling of the mitochondrial matrix compartment.

About 5–10% of the surface area of the granule fraction was comprised of intact platelets which contained typical platelet granules. Nuclei, cell debris, and intact cells were not observed in this fraction.

The remaining 5–10% of the surface area of the granule fraction was comprised of heterogeneous, membrane-bounded vacuolar structures. These organelles measured 0.4–0.9 μ in diameter. Many showed intense lead precipitates when incubated in the Gomori reaction mixture (Figs. 12–14). Some of these organelles contained fragments of mitochondria and endoplasmic reticulum and thus corresponded to autophagic vacuoles (Fig. 12). Others showed a spectrum in the sites and distribution of reaction product, some staining more intensely than others. No deposition of reaction product was found in sections incubated without substrates or with 0.01 M sodium fluoride (Fig. 11). The fractions incubated in the Gomori mixture showed some, however slight, nonspecific deposition of reaction product on mitochondrial membranes. No intramitochondrial reaction product was observed.

Separation of Acid Hydrolase–Containing Organelles by Density-Gradient Isopycnic Centrifugation

From the results obtained with differential centrifugation, it became evident that the granular fraction which sedimented at 20,000 *g* for 20 min contained a mixture of both acid hydrolase–rich organelles and mitochondria. Both acid hydrolases and the mitochondrial marker enzyme, malate dehydrogenase, were present in the granular pellet. For separation of these elements and for determination of whether the acid hydrolases would sediment together under more discriminating conditions of separation, studies were performed with centrifugation of the postnuclear supernatant in a discontinuous sucrose-density gradient. Fractions of four gradients were assayed for distribution of beta glucuronidase, acid phosphatase, acid ribonuclease and malate dehydrogenase activities; protein content was also determined. Although some variation in the distribution of enzyme activities was found, the procedure was fairly reproducible. In Fig. 15, a representative distribution pattern is shown. A quantitative separation of acid hydrolase–containing organelles from mitochondria could not be obtained with this method, as the majority of the recovered activities of all four enzymes and the bulk of protein formed a high peak in the upper third of the gradient (fraction 13). However, (unshaded area of Fig. 15) a considerable portion of the recovered activities of all three acid hydrolases was found in the densest layers. In this portion of the gradient, malate dehydrogenase activity was absent or very low; indicating that these fractions were enriched in hydrolase–containing organelles, which had become separated from mitochondria. Table V lists the relative specific activities of acid phosphatase, acid ribonuclease, beta glucuronidase, and malate dehydrogenase in five fractions of the gradient shown in Fig. 15. In sucrose layers corresponding to 1.8, 1.7, and 1.6 M, the relative specific activities of the three acid hydrolases far exceeded those of the mitochondrial marker enzyme.

TABLE V

Distribution of Enzymes in Discontinuous Sucrose-Density Gradients of Postgranular Fractions of Human Lymphocytes

Fraction no.	Relative specific activities *(RSA)				
	Sucrose	Acid phosphatase	Acid RNase	β-Glucuronidase	Malate dehydrogenase
	M				
1–4	1.8	4.0	1.41	1.02	0.47
5–8	1.7	4.2	16.2	3.01	1.03
9–12	1.6	3.72	6.52	4.56	0.97
13–16	1.5	1.02	0.55	1.20	1.36
17–19	1.4	0.80	0.46	0.53	0.81

$$*\text{RSA} = \frac{\text{per cent enzyme in fraction}}{\text{per cent protein in fraction}}$$

and represents value for each fractional peak.

Release of Sedimentable Acid Hydrolase Activity

Whether the sedimentable acid hydrolase activity present in lymphocyte homogenates could be shown to be associated with membrane-bounded organelles was determined by obtaining evidence of latency. Since it was not possible to resuspend the granular pellet without vigorous measures which injured the particles, the postnuclear supernatant was employed. Postnuclear supernates were incubated with and without agents known to be capable of causing membrane lysis. Following such treatment, the enzyme activity

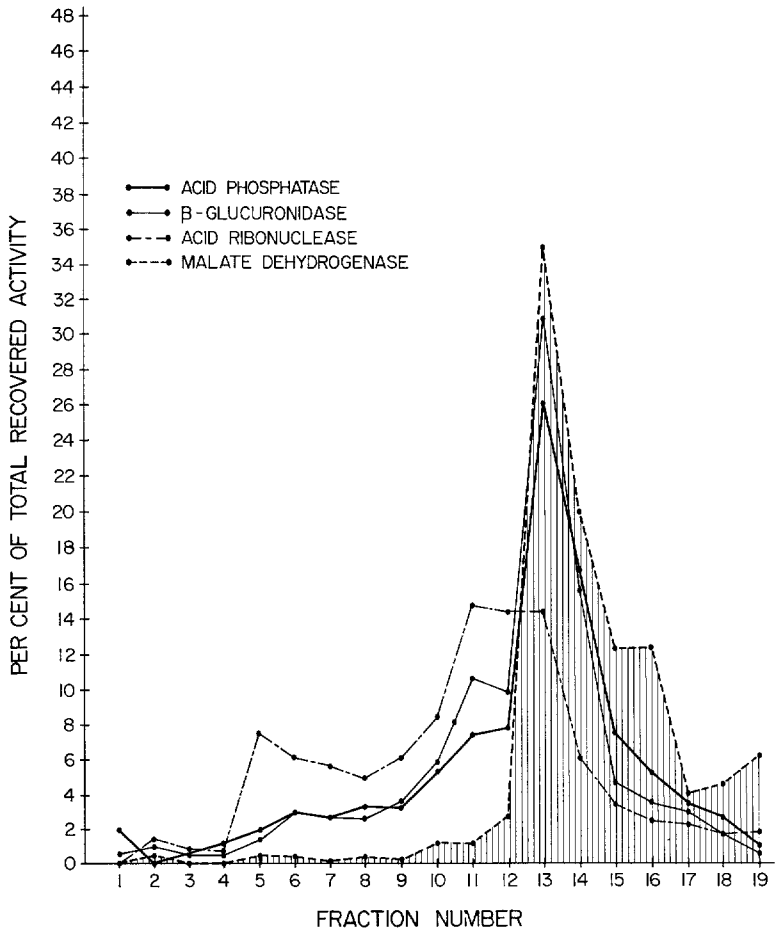

FIGURE 15 Distribution patterns of enzymes in a postnuclear supernatant of lymphocytes as determined in a discontinuous sucrose-density gradient. Centrifugation at 22,500 rpm for 3 hr, (100,000 *g* at tip of tube). (Fractions 1–4, 1.8 M sucrose; 5–8, 1.7 M sucrose; 9–12, 1.6 M sucrose; 13–16, 1.5 M sucrose; 17–19, 1.4 M sucrose). Protein distribution paralleled malate dehydrogenase and is omitted (as is load volume) for clarity.

rendered nonsedimentable at 20,000 *g*, as well as that remaining in the pellet, was determined. Triton X-100 (0.1%) released 98% of total enzyme activities from sedimentable form, as judged by assays of enzyme activities in both the 20,000 *g* supernatant and the corresponding resuspended pellet. Therefore all enzyme activities were calculated as a percentage of the enzyme activities released by Triton X-100 (15, 23, 24). This nonsedimentable enzyme activity was composed of both the soluble enzyme activity present in the postnuclear supernatant before incubation with the agent, and the enzyme activity released by the action of the added compound. Addition of streptolysin S in concentrations ranging from 16.6 to 416 HU per milliliter produced a dose-dependent release of beta glucuronidase activity from a sedimentable to a nonsedimentable form, reaching 88.4% of the total at a concentration of 416 HU per ml (Fig. 16). Incubation of the postnuclear supernatant with streptolysin S also resulted in release of malate dehydrogenase activity (23, 24). The membrane-active, polyene, antibiotic filipin (final concentrations ranging from 5×10^{-7} to 5×10^{-4} M) also rendered beta glucuronidase activity unsedimentable. Acid phosphatase activity was released in parallel with beta glucuronidase activity, an observation which clearly suggests their association with a single group of organelles. In contrast, filipin did not liberate malate dehy-

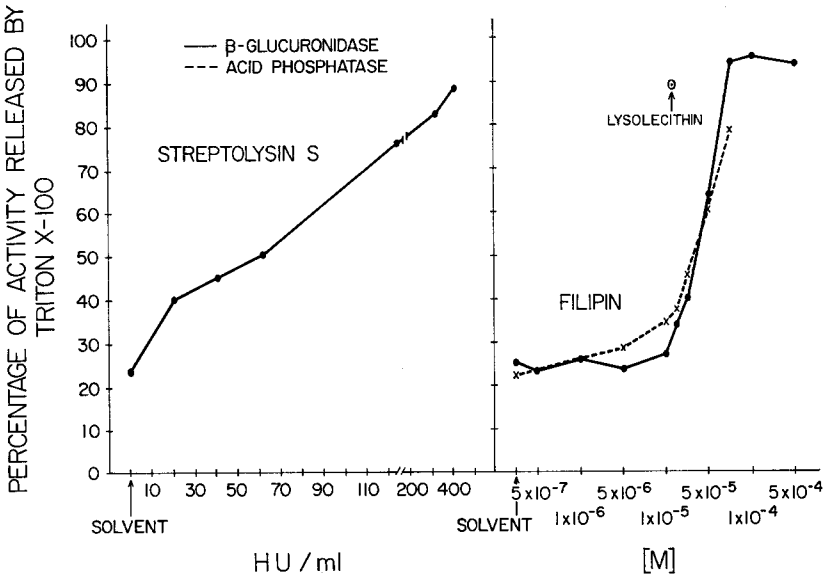

FIGURE 16 Release of sedimentable acid hydrolase activity from postnuclear fraction of lymphocytes. Postnuclear fractions were incubated with varying concentrations of streptolysin S and filipin for 60 min at 37°C. The enzyme activity no longer sedimentable at 20,000 *g* × 20 min after such treatment was then calculated as per cent of the total enzyme activity (per cent of activity released by Triton X-100; see text).

drogenase activity; a selective effect of filipin on lysosomes has been previously described (41). Release of beta glucuronidase activity by lysolecithin (final concentration of 1.5×10^{-5} M) reached 89.1%. In the experiments described, enzyme activities remaining in the pellet were complementary to those recovered in the 20,000 *g* supernatant at all concentrations of agent; this indicates that increases of enzyme activity in the supernatant were due to release rather than activation of enzymes.

DISCUSSION

In electron photomicrographs of resting peripheral blood lymphocytes (see Introduction and references 12–14) relatively few structures identifiable as lysosomes have been demonstrated. Nor were many acid phosphatase–positive organelles observed in column-separated lymphocytes. Consistent with this paucity of organelles, we have found low activities of three acid hydrolytic enzymes, compared with levels found in polymorphonuclear leukocytes (42, 11) and macrophages (43). The acid hydrolases of lymphocytes behaved as though they were associated with lysosomes, in that they were concentrated in a subcellular, granular fraction which sedimented between 500 and 20,000 *g* in 0.34 M sucrose. They could be partially separated from mitochondrial enzymes in such fractions by means of discontinuous sucrose-density gradients which yielded subfractions rich in three acid hydrolases of high relative specific activity. Furthermore, the acid hydrolases were shown to behave as though they were membrane-bounded, since they could be rendered nonsedimentable by agents active on membranes, such as streptolysin S, filipin, and lysolecithin.

Because of the disparity between the activities of hydrolases in lymphocytes and in other formed blood elements, it was critical that all cell suspensions be free of contaminating elements. Negligible (average 0.2%) contamination by cellular elements other than lymphocytes was found, as determined by tetrachrome smears and survey electron micrographs. Although platelets could not be completely eliminated, experiments in which these elements were added to lymphocyte preparations showed that these did not influence the levels of acid hydrolases under the experimental conditions used.

It was considered possible that the fractionation procedures led to the formation of membrane fragments which might fortuitously have absorbed hydrolases from the cytoplasm or other

structures, subsequently to sediment as clumps of artifactual origin. This possibility is unlikely since electron microscopic histochemistry showed the presence of membrane-bounded, acid phosphatase-rich organelles both in the starting preparations and in the granular fractions themselves. Furthermore, the response of hydrolases to specific "labilizers" of lysosomes, e.g. filipin, would suggest that the enzymes were indeed bounded by membranes which behaved like those of lysosomes from other tissues.

The possibility cannot be excluded that one or another of the two morphologically distinct types of lysosomes arose as a consequence of the separative procedure itself. Thus, the secondary lysosomes (autophagic vacuoles and residual bodies) seen in the starting material may have developed because of cellular responses to ADP, the nylon-wool column, or the suspending medium. However, both primary and secondary lysosomes can be identified in peripheral blood lymphocytes (14, 33) which have not undergone vigorous handling. Finally, the sparsity of acid phosphatase-positive organelles in hydrolase-rich fractions (approximately 5%) compares with similar results obtained by Baudhuin et al. for large granule fractions from liver (44).

Thus, there appear to be organelles in lymphocytes which, although few in number, look and behave like lysosomes in other tissues. It will be of interest to determine whether these organelles in human lymphocytes contain the complete complement of acid hydrolases or correspond to the L15 population of lysosomes in spleen homogenates described by Bowers and de Duve (6–8) who suggest that the organelles are derived from lymphocytes. The relative paucity of lysosomes in resting lymphocytes may be directly related to the few digestive or degradative functions which these "repressed" cells subserve. When, however, lymphocytes become stimulated to undergo metabolic and structural rearrangements (as under the influence of phytohemagglutinin) lysosomes become quite prominent and may indeed contribute to remodeling of the cells (11).

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