THE ISOLATION AND PROPERTIES OF THE SPECIFIC CYTOPLASMIC GRANULES OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES*

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The role of the polymorphonuclear leucocyte in host defense reactions is intimately related to its ability to engulf and destroy a wide variety of microorganisms. Although the process of ingestion has been extensively studied, little is known concerning the chain of reactions leading to the intracellular inactivation and subsequent degradation of bacteria. The initial bactericidal event which presumably occurs in the cytoplasm of the leucocyte may be mediated by a number of intrinsic antimicrobial agents. These substances, *e.g.* phagocytin, lysozyme, and lactic acid, are present within the leucocyte in high concentrations and have potent activities *in vitro*. The possible role of each of these materials has recently been reviewed (1). In addition, leucocytes contain a variety of hydrolytic enzymes which, although not directly responsible for the inactivation of bacteria, may play a synergistic role in the "digestion" of engulfed particles. Many important aspects of intraleucocytic events still remain unknown. Among these are (*a*) the localization of biologically active materials within the leucocyte, (*b*) the mechanisms by which such substances interact with bacteria and (*c*) the influence of the ingested particle on the activities of the phagocyte.

One of the most striking morphological properties of the cytoplasm of adult polymorphonuclear leucocytes is the presence of numerous characteristically staining granules. These granules have been known since the studies of Ehrlich (2) and are important in the morphological identification of blood cells. Little is known, however, regarding the composition and function of these entities. Since the granule is the major particulate element of leucocyte cytoplasm, it seemed of interest to investigate its nature and properties. This report will describe methods for the fractionation of subcellular particles from rabbit polymorphonuclear leucocytes by differential centrifugation. A granule-rich fraction has been obtained which appears morphologically homogeneous. The distribution of selected antimicrobial agents and enzymes in these leucocyte fractions will be presented.

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Materials and Methods

Peritoneal Exudates.—The procedures employed for the preparation of peritoneal exudates have been described in detail in previous publications (3). Adult New Zealand rabbits were injected with 200 ml. of 0.1 per cent glycogen in pyrogen-free saline. Four hours later 100 ml. of heparinized saline were introduced into the peritoneal cavity and the exudate withdrawn by gravity drainage. The rabbits employed were carefully selected for their ability to produce large numbers of exudate cells. These animals had been repeatedly primed with glycogen-saline prior to their use in these experiments. All of the exudates employed contained 8 to 13 $\times$ 10^6 PMN leucocytes per ml. with a total fluid yield of 150 to 250 ml. Stained smears and wet mount differential counts revealed 99 per cent or more polymorphonuclear leucocytes. Exudates contaminated with large numbers of erythrocytes were discarded. Immediately after collection the exudates were screened through four thicknesses of cheese cloth to remove clumps of cells and debris and duplicate cell counts were performed. The subsequent processing of the cells will be dealt with in the section given to results.

Chemical Analysis.—Total nitrogen was determined colorimetrically with Nesslers reagent after digestion with 5 N sulfuric acid, copper selenite, and hydrogen peroxide (4). Inorganic phosphorus was determined by the method of Fiske and SubbaRow (5), and total phosphorus after digestion with 10 N sulfuric acid. The analysis of protein was performed by a modified Lowry procedure (6). The colored complex was read at 650 mμ against a standard of crystalline egg white lysozyme and expressed as protein equivalents.

The separation of phospholipid, ribonucleic and deoxyribonucleic acids was performed by the method of Schneider (7). Phospholipid-phosphorus was determined on the ether-alcohol extracts after digestion with 10 N sulfuric acid. Ribonucleic acid was determined on the hot trichloracetic acid extract by the method of Mejbaum (8), employing a purified preparation of yeast RNA as a standard. Deoxyribonucleic acid was determined colorimetrically with diphenylamine (9). A highly purified preparation of calf thymus DNA, supplied by Dr. Philip Siekevitz of The Rockefeller Institute, was used as a standard.

Enzymatic Analysis.—Alkaline phosphatase was determined by a modification of the procedure of Valentine and Beck (10). The reaction mixture contained 0.02 M sodium β-glycerophosphate (Eastman), and 0.001 M $MgCl_2$ in a final volume of 5.0 ml. at pH 9.9. Reactions were allowed to proceed at 38°C. for 30 to 60 minutes and stopped by the addition of 1.0 ml. of 30 per cent trichloracetic acid. Inorganic phosphorous was determined on the protein-free filtrate.

Acid Phosphatase was determined with sodium β-glycerophosphate at a final pH of 5.0. The same procedure was employed as for alkaline phosphatase. Magnesium ion had no enhancing effect and was usually omitted from the reaction mixture. The results of both acid and alkaline phosphatase are expressed as milligrams of inorganic phosphorus liberated per 60 minutes of incubation and related to milligrams of enzyme nitrogen.

Cathepsin was assayed by a modification of the procedures of Adams and Smith (11) and Anson (12). The substrate was 2 per cent denatured hemoglobin (Nutritional Biochemical Co.), dissolved in 0.05 M acetate buffer and adjusted to a final pH of 3.8. The reaction mixture contained 1.0 ml. of substrate, enzyme and acetate buffer to a final volume of 2.0 ml. Following incubation at 38°C. for 30 to 120 minutes, the reaction was stopped with 2.0 ml. of 5 per cent trichloracetic acid. Aliquots of the deproteinized filtrate were then assayed for chromogen by the method of Lowry *et al.* (6). The results are expressed as protein equivalents liberated/60 minutes/mg. of enzyme nitrogen. Attempts to demonstrate an alkaline reactive protease in the fractions was unsuccessful when determined at pH 8.0 against denatured hemoglobin substrate. The absence of "leukoprotease" in the rabbit polymorphonuclear leucocyte has been reported previously.

Nucleotidase activity was assayed according to the method of Swendseid *et al.* (13). Ad-

enosine-5-phosphate (Schwarz) was employed as substrate and the pH maintained at 4.0. The reaction was carried out at 38°C. for 60 to 120 minutes and stopped by the addition of 0.5 ml. of 10 per cent trichloracetic acid. Protein-free filtrates were then analyzed for inorganic phosphorus. A small number of experiments employing adenosine-3-phosphate as substrate gave distributions similar to adenosine-5-phosphate but the amount of inorganic phosphorus liberated from this nucleotide was 50 to 80 per cent higher.

Ribonuclease was measured by a modification of the method of Schneider and Hogeboom (14). The reaction mixture was made up to a total volume of 2.0 ml. and contained 0.05 M sodium succinate buffer, 0.005 M magnesium sulfate and 1.0 mg. of ribonucleic acid at pH 5.0. The RNA was a commercial preparation (Schwarz) which had been dialyzed 48 hours against distilled water and lyophilized. After incubation at 38°C. for 30 to 120 minutes the reaction was stopped with 1.0 ml. of 10 per cent perchloric acid containing 0.25 per cent uranyl acetate. After 2 hours at 4°C. the mixture was centrifuged and the supernate read in a model DU Beckman spectrophotometer at 260 mμ. The results are expressed as 260 mμ absorbing units released by the enzyme in 60 minutes.

Deoxyribonuclease was assayed by the method of Schneider and Hogeboom (14). A sample of highly purified calf thymus DNA was employed as substrate. Following incubation at 38°C. for 60 to 120 minutes the reaction was stopped with 12 per cent perchloric acid, and the optical density of the supernate read at 260 mμ.

Beta glucuronidase activity was assayed by the procedures of Follette *et al.* (15) and Fishman (16). Phenophthalein-β-glucuronide was obtained from Sigma Chemical Co., St. Louis, as a 0.01 M solution. Reactions were carried out at pH 4.5 for 60 to 180 minutes at 38°C.

Lysozyme was assayed by a modification of the procedure of Shugar (17). *M. leisodykticus* was grown on penassay agar slants, washed three times with physiological saline and suspended in 0.1 M phosphate buffer at pH 6.5 to an O.D. of 0.30 at 450 mμ. Enzyme (0.2 ml.) was added to 1.8 ml. of leisodykticus substrate, mixed, and the optical density followed at 450 mμ in a Junior Coleman spectrophotometer at 25°C. A linear decrease in optical density over a 3 minute period was obtained with enzyme concentrations ranging from 0.5 to 8.0 micrograms. Readings were taken at 1 minute intervals for 3 minutes and the slopes compared to standards of crystalline egg white lysozyme.

Each of the enzymatic assays described gave linear responses with variations in enzyme concentration and time of incubation. Both substrate and enzyme blanks on each of the leucocyte fractions were included in each determination. Duplicate determinations were performed on each fraction and often at two concentrations. The enhancement of enzyme activity by surface-active agents, repeated freezing and thawing, etc., will be described in the results.

Bactericidal Assays.—The assay of bactericidal factors in leucocyte fractions was performed by the method of Hirsch (18). Prior to use, each of the fractions was extracted with 0.009 M citric acid at 4°C. for 2 hours or more. After incubation the mixtures were centrifuged at 10,000 *g* for 30 minutes and the supernatant fluid was employed in the bactericidal assay. Results of such assays are expressed as the reciprocal of the highest dilution which killed 50 per cent or more of the inoculum after incubation at 37°C. for 2 hours. The various microorganisms under test were used as 18 hour penassay broth cultures.

Microscopy.—The morphological characteristics of leucocyte fractions were evaluated by a variety of techniques. Smears were prepared as for blood films and stained with Wright's stain, and wet mounts were evaluated by phase microscopy.

Electron microscopy of the granule fraction was kindly performed by Dr. David J. L. Luck of The Rockefeller Institute. Granules were centrifuged at 8,000 *g* in lusteroid tubes to form a firm pellet. The supernate was decanted and 1 per cent OsO_4 in saline was added to the tube. After fixation overnight at 4°C. the pellet was washed with 70 per cent ethanol, cut

into small fragments, and dehydrated. The fragments were then embedded in methacrylate, sectioned on a Porter-Blum microtome, lead-stained, and examined with an RCA EMU electron microscope. Orientation was maintained and it was possible to evaluate the homogeneity and morphology at various levels in the pellet.

Intact polymorphonuclear leucocytes were obtained directly from the peritoneal cavity and allowed to drip into a chilled tube containing 1 per cent buffered OsO_4. The cells were then centrifuged and rapidly dehydrated and embedded. Thin sections and staining were performed as for the granules.

RESULTS

Preliminary Observations on the Disruption and Fractionation of Leucocytes

Although polymorphonuclear leucocytes appear to be fragile cells which react to a variety of mechanical stimuli, they are difficult to disrupt in a controlled manner. Prior to a study of their cytoplasmic contents it was necessary to devise methods which would allow efficient disruption of the cell and still maintain the integrity of the particulate elements. Initial studies which employed homogenization were unsuccessful for a number of reasons. It was found that homogenization with a motor driven teflon pestle, in neutral buffer solutions, resulted in inefficient cell breakage unless the cells were carefully washed free of exudate fluid protein and were maintained at concentrations of 80 to 160 $\times$ 10^6/ml. Under these conditions, disruption would occur after 3 to 5 minutes of homogenization, but the cytoplasmic components were severely agglutinated and sedimented completely at 100 *g*. Further experiments revealed that less agglutination occurred if the cells were homogenized in isotonic sucrose solutions. Such homogenates still showed considerable agglutination of the granules and the majority of these elements sedimented through a discontinuous sucrose gradient (10 to 60 per cent) to form a pellet with the nuclei. This was thought to be related to residual salt, so sucrose-washed cells were then employed.

When exudate leucocytes were washed once in cold 0.34 M sucrose and then resuspended in the same medium without homogenization, an abrupt increase occurred in the viscosity of the suspension. Microscopic examination of such suspensions showed that the majority of the leucocytes had disrupted under these conditions liberating their cytoplasmic granules. The granules showed no evidence of agglutination and were present as monodispersed particles. The effects of various media were then evaluated in regard to leucocyte disruption and the agglutination of granules. Table I summarizes the results of these studies.

1×10^8 screened peritoneal exudate leucocytes were dispensed to 50 ml. conical centrifuge tubes and sedimented at 200 *g* for 5 minutes. The supernatant fluid was removed by suction and the cells resuspended in 5.0 ml. of ice cold media by gentle pipetting. The cells were again sedimented and the pellet resuspended in the same media. After the second resuspension they were vigorously pipetted for 1 minute. Following this treatment the suspension was

centrifuged at 200 *g* for 5 minutes and the gross and microscopic appearance of the resulting sediment and supernate was evaluated.

The nature of the medium was found to influence both the disruption of the cells and the state of the granules. Treatment with phosphate buffered saline did not result in cell disruption. When various salt solutions were added to sucrose a portion of the cells disrupted, but the granules were agglutinated and sedimented at low centrifugal forces. A similar result was obtained when 1×10^{-3} M $CaCl_2$ was combined with sucrose. When a non-ionic medium was employed, *i.e.* sucrose alone, cell breakage was maximal and the granules

TABLE I

The Influence of the Medium on the Disruption of Polymorphonuclear Leucocytes

Medium	Characteristics of centrifuged suspensions*	
	Supernatant fluid‡	Pellet§
1. 0.9 per cent NaCl, 0.01 M PO_4 pH 7.4	Clear	4+ intact cells
2. 0.9 per cent NaCl, 0.34 M sucrose	"	3+ intact cells, agglut. granules
3. 0.3 per cent " 0.34 M "	Slightly opalescent	" " " "
4. 0.005 M PO_4 pH 7.4, 0.34 M sucrose	" "	" " " "
5. 0.01 M KCl, 0.34 M sucrose	" "	" " " "
6. 0.001 M $CaCl_2$, 0.34 M sucrose	Clear	2+ intact cells, agglut. granules
7. 0.34 M sucrose	Opalescent 4+	0 intact cells, swollen nuclei
8. 0.88 M "	" "	" " " "
9. 1.02 M "	" "	" " " "

* Leucocytes were washed once and resuspended in described media by vigorous pipetting. The suspensions were then centrifuged at 1,000 R.P.M/5′ and evaluated for leucocyte disruption.

‡ Visual estimate.

§ Phase microscopy.

remained in the supernate. The liberated granules could then be collected by centrifugation at higher speeds. Subsequent experiments showed that a concentration of 0.34 M sucrose was optimal for cell breakage and the subsequent centrifugal separation of the granules.

Procedure for the Separation of Leucocyte Granules

The sucrose lysis procedure was then employed for the separation of granules from other cellular elements. The method to be described was used in all the studies on the localization and activity of specific factors presented later in this report.

Between 0.8 and 1.5×10^9 leucocytes were dispensed in chilled 50 ml. screw-capped tubes and centrifuged at 250 *g* for 5 minutes. The clear supernate was removed by suction and the

tubes briefly drained on filter paper to eliminate traces of exudate fluid. The cells were then pooled, gently resuspended in 50 ml. of ice cold 0.34 M sucrose and immediately centrifuged at 250 *g* for 5 minutes. This procedure constituted the initial sucrose wash. Supernatant fluids from this wash were usually clear but occasionally were opalescent and contained discrete granules. Prolonged exposure of the leucocytes to sucrose during this step resulted in an increasing number of disrupted cells.

The cell pellet resulting from the sucrose wash was drained and 15 to 20 ml. of cold 0.34 M sucrose was added rapidly. Resuspension of the cells was then accomplished by vigorous pipetting until a smooth suspension resulted. Upon adding the second aliquot of sucrose a prompt increase in viscosity occurred and the fluid became increasingly turbid. Occasionally, small gross clumps of cells were present after pipetting. Under these circumstances 2 to 3 strokes with a chilled teflon pestle were required to disperse the cells. Pipetting was then continued for 60 to 90 seconds until efficient disruption of the leucocytes had taken place. The completeness of cell breakage was followed during this process by phase microscopy.

After disruption had taken place, the lysate was dispensed to chilled 15.0 ml. conical centrifuge tubes and spun for 10 minutes at 400 *g*. This resulted in the sedimentation of intact nuclei, erythrocytes, and a portion of the larger granules. The supernate was milky white in appearance and contained large numbers of discrete granules. A thin lipoid film was usually present at the surface and was carefully lifted off and discarded. The granule-rich supernate (S_1) was then removed by means of a curved Pasteur pipette and stored at 0°C. until further processing.

The 400 *g* or "nuclear" pellet which contained a portion of the granules was gently resuspended in 3.0 ml. of cold sucrose and recentrifuged at 400 *g* for another 10 minute period. The resulting supernate (S_2) was combined with (S_1) and the nuclear pellet resuspended to known volume with 0.34 M sucrose.

The combined supernatant fluids (S_1 and S_2) were then transferred to chilled 16 × 95 mm. lusteroid tubes and centrifuged at 8,200 *g* for 15 minutes at 4°C. in a high speed centrifuge (Lourdes, model AB). This resulted in a firmly packed white pellet and a slightly opalescent supernate (S_3). Occasionally, a small quantity of fluffy material was found on top of the pellet. When present this was removed and combined with the post-granule supernate (S_3).

The 8,200 *g* pellet which contained the granules was gently resuspended in sucrose by means of a teflon pestle and again centrifuged at 8,200 *g* for 15 minutes. Both the washed granule pellet and post-granule supernate were then brought to known volume with 0.34 M sucrose and employed for further studies.

Morphological and Chemical Properties of Leucocyte Fractions

Each step of the centrifugal separation of leucocyte components was followed by both phase microscopy and stained smears. Following sucrose lysis there was less then 1 per cent intact cells as evaluated by direct count in a hemocytometer. Phase microscopy of the lysate showed numerous dispersed granules as well as spherical nuclei which did not have their usual lobed appearance. After centrifugation at 400 *g* the "nuclear" pellet contained swollen nuclei, strands of nuclear membrane, erythrocytes, and granules which adhered to the surface of the nuclear structures. The 400 *g* supernate contained well dispersed granules without evidence of other morphological entities. After washing the nuclear pellet with sucrose, fewer granules were present although considerable numbers still adhered to the agglutinated nuclei. These granules

seemed somewhat larger than those present in the 400 *g* supernate. Under phase contrast the granules appeared as round dense structures which exhibited Brownian motion. Wright's stains on smears of the granules in both the 400 *g* pellet and supernate showed discrete pink-staining structures which were similar to those seen in intact leucocytes.

Electron micrographs of the 8,200 *g* pellet (Fig. 1) revealed that the majority of the formed elements were granules. These showed a well defined limiting membrane surrounding an electron-dense internal matrix. An occasional mitochondrion could be identified by its internal cristae but these accounted for less than 10 per cent of the particles. Nuclei were not present and there was no significant contamination with smaller elements. Examination of vertical sections through the pellet suggested that the larger granules were concentrated

TABLE II

The Distribution of Nucleic acids and Phospholipid in Leucocyte Fractions

Leucocyte fraction	Nitrogen	RNA			DNA			Phospholipid-phosphorus			Mg. RNA / Mg. DNA
		Total	Per cent fraction	Mg. RNA / Mg. N_2	Total	Per cent fraction	Mg. DNA / Mg. N_2	Total	Per cent fraction	μg. PLP-P / mg. N_2	
	mg.	*mg.*			*mg.*			*µg.*			
Total sucrose lysate...	8.47	1.17	(100)	0.138	4.57	(100)	0.539	176.9	(100)	20.88	0.25
400 *g* pellet (nuclear)..	2.63	0.445	36.4	0.169	2.62	52.5	0.996	41.9	23	15.93	0.169
8.200 *g* pellet (granules)	1.62	0.012	0.98	0.0074	0.014	0.28	0.008	32.4	18	20.00	—
Postgranule supernate.	4.30	0.771	63.1	0.180	2.36	47.3	0.548	105.8	58	24.60	0.33
Recovery, *per cent*.....	101	104			108			102			

in the base although no clear cut structural difference was noted. Thin sections through intact leucocytes (Fig. 2) did, however, reveal that the larger granules were somewhat more electron-dense than the smaller ones.

It was apparent that at the time of cell lysis a considerable portion of the nuclei had also disrupted. The swollen state of the nuclei in the 400 *g* pellet as well as the presence of empty nuclear membranes suggested this possibility. Since the nuclei were agglutinated it was not possible to estimate directly the number which had broken during the lysis procedure. This was indirectly evaluated by examining the distribution of deoxyribonucleic acid in the various fractions. A representative experiment is presented in Table II. It can be seen that 50 per cent of the deoxyribonucleic acid was present in the postgranule supernate, suggesting the solubilization of approximately one-half the nuclei in the original preparation. The granule pellet contained only trace amounts of nucleic acids.

Small amounts of phospholipid were present in the granule fraction, amount-

ing to 20 per cent of the total lysate. The postgranule supernate contained the highest percentage and highest specific activity of phospholipid, indicating a high lipid content of the soluble and smaller cytoplasmic elements.

The nitrogen content of the various fractions was relatively constant over 20 separate fractionations. The majority of the preparations employed had a total nitrogen content of 7 to 12 mg. Of this total, approximately 30 per cent was present in the "nuclear" pellet, 20 per cent in the granule pellet and the remainder in the postgranule supernate.

Properties of Leucocyte Granules

Prior to high speed centrifugation the granules remained monodispersed for periods up to 36 hours when stored at 4°C. Following high speed centrifugation, gentle homogenization was required to resuspend the particles, and after this treatment small clumps of 4 to 5 granules were still present. Repeated washing of the granules resulted in increased stickiness so that they sedimented along the entire length of the tube. Because of this property the majority of the experiments were conducted with granules washed once.

Early observations on the stability of granules revealed that treatment with weak acids resulted in a prompt and striking reduction in optical density. A more careful study was then conducted on the conditions resulting in granule lysis. Table III represents an experiment on the influence of tonicity, acids, and surface-active agents.

Granules were isolated according to the methods previously described. The final granule pellet was resuspended in ice cold 0.34 M sucrose and maintained in an ice bath. The optical density of this suspension was 3.7 at 450 mμ. At prescribed intervals a 0.2 ml. aliquot was placed in 19 × 74 mm. Coleman cuvettes which contained 1.8 ml. of the particular medium at 25°C. The tubes were sealed with rubber stoppers and inverted to mix the contents. After 5 and 15 minutes of incubation at 25°C. the tubes were again mixed and the optical density read at 450 mμ against a 0.34 M sucrose blank. The changes in optical density are expressed in terms of the control tube which contained 0.34 M sucrose and which exhibited no change in density during the course of the experiment.

In the presence of sucrose alone, little or no change occurred in the density of the suspensions except in hypotonic solutions. At 0.063 and 0.034 M concentrations some clearing occurred, and this was not related to the aggregation of particles. When the granules were suspended in acid media, either in the presence or absence of sucrose, a prompt clearing of the suspension took place. This occurred with either hydrochloric, citric or lactic acids and resulted in a 95 per cent reduction in density within the first few minutes of incubation. The nonionic detergent triton when tested at a final concentration of 0.5 per cent had but little effect on the granules. In contrast, saponin (Merck) had a pronounced lytic influence and was maximally active at a concentration of 1 per cent. A similar effect of saponin was noted in the presence of sucrose.

Examination of granule suspensions lysed with acid revealed small numbers of pale spherical bodies of the same size as intact granules but without internal phase contrast material. These bodies did not stain pink with Wright's stain and probably represented empty granule membranes. After treatment with 1 per cent saponin, granule morphology was destroyed and amorphous debris was seen on both stained smears and phase contrast examination. It is of in-

TABLE III

The Influence of Tonicity, pH and Surface-Active Agents on the Lysis of Purified Granule Suspensions

Medium, final concentration	O.D. 450 mμ at 25°C. for*		Per cent reduction in O.D. 450 mμ at 25°C. for 15 min.
	5 min.	15 min.	
0.34 M sucrose	0.35	0.35	0
0.25 M "	0.35	0.35	0
0.125 M "	0.35	0.32	9
0.063 M "	0.32	0.28	20
0.034 M "	0.30	0.26	26
0.1 N HCl	0.018	0.012	96
0.01 N HCl	0.017	0.015	96
0.005 N HCl	0.015	0.014	96
0.001 N HCl	0.021	0.017	95
0.005 N HCl, 0.34 M sucrose	0.013	0.013	96
0.001 M citric acid	0.020	0.014	96
0.001 M citric acid, 0.34 M sucrose	0.018	0.015	96
0.001 M lactic acid	0.017	0.014	96
0.5 per cent triton	0.34	0.33	6
0.1 " " saponin	0.29	0.25	24
0.5 " " "	0.23	0.15	58
1.0 " " "	0.12	0.09	74

* Zero time O.D. 450 mμ in 0.34 M sucrose = 0.35.

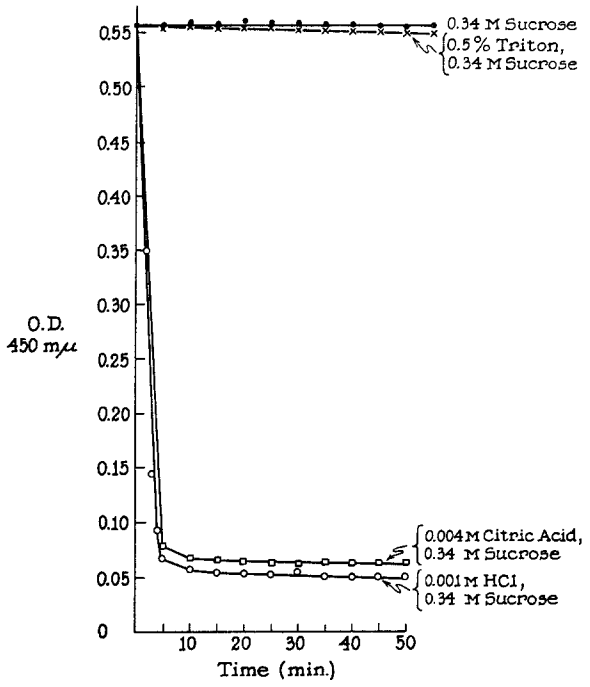
terest that acid treatment of air-dried leucocyte smears resulted in the disappearance of granules from the cytoplasm.

The time course of granule lysis in acid is plotted in Text-fig. 1. The reaction began almost immediately and was completed within 10 minutes. In contrast, granules in sucrose solution alone retained their morphological and tinctorial properties and appeared intact after 50 minutes of incubation at 25°C.

In view of the marked sensitivity of granules to acid media it was of interest to study the influence of pH *per se* on the lysis reaction.

Aliquots of a granule suspension were placed in small colorimeter tubes containing various buffers made up in 0.25 M sucrose. The following buffers (0.05 M) were prepared to study the

range between pH 2.0 and 9.0 at one-half pH unit increments: phthalate, pH 2.0–3.5; lactate, pH 4.0–5.0; acetate, pH 5.0–6.0; phosphate, 6.5–7.5; glycylglycine, pH 8.0–9.0. Each of these solutions was checked with a glass electrode and the final pH was accurate within 0.2 pH units. The tubes were then stoppered and incubated at 25°C. for 30 minutes with occasional mixing. Following incubation the optical density was read at 450 mμ and compared to a 0.25 M sucrose control. The optical density of the sucrose control (0.42) remained constant during the 30 minutes of incubation.



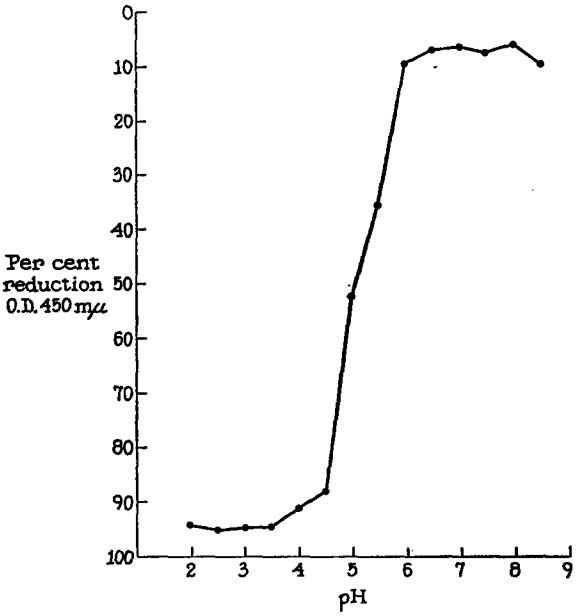
TEXT-FIG. 1. The time course of acid induced granule lysis.

In the neutral and slightly alkaline range (Text-fig. 2) the granules remained stable and there was only a slight reduction in optical density, probably related to agglutination of the particles in the ionic environments. When the pH of the medium fell below 6.0, lysis began, and was completed at pH 4.5. Definite lysis occurred at pH 5.5 and a 50 per cent reduction in optical density took place at pH 5.0. At pH 4.0 and below there was maximal clearing of the suspension, similar to the previous results employing unbuffered acids.

The Localization and Properties of the Bactericidal Factors in Leucocyte Fractions

Previous experiments (19) have demonstrated that polymorphonuclear leucocytes from mammalian sources contain large amounts of a bactericidal agent

which has been termed phagocytin. This material, in contrast to other naturally occurring bactericidins, *e.g.* histone, (*a*) is found only in polymorphonuclear leucocytes, (*b*) has an *in vitro* spectrum of activity which includes both Gram-positive and Gram-negative bacteria and (*c*) is resistant to the action of proteolytic enzymes (19). Phagocytin can be extracted from leucocytes by means of weak acids and is thought to be present in the cytoplasm of the cell. These properties suggested that phagocytin might be present in the granule and prompted the investigation of bactericidal factors in leucocyte fractions prepared by sucrose lysis.



TEXT-FIG. 2. The influence of pH on the optical density of granule suspensions.

Leucocytes were disrupted and centrifuged according to the methods described above Each of the four fractions, *i.e.* (*a*) total sucrose lysate, (*b*) 400 *g* pellet (nuclear), (*c*) 8,200 *g* pellet (granules) and (*d*) the postgranule supernate was assayed for both bactericidal activity and total nitrogen. Aliquots of the fractions, usually 0.1 ml., were extracted in 0.9 ml. of 0.009 M citric acid at 4°C. for 2 or more hours. The extract was clarified by centrifugation at 8,000 *g* for 20 minutes and the clear supernate was collected for the bactericidal assays, employing *Escherichia coli* K-12 as the test microbe.

Table IV presents the results of two experiments performed on different lots of leucocytes. It is apparent that the majority of the bactericidal activity of the total lysate was contained in the granule fraction. Very little activity was found in the postgranule supernate although this fraction contained the largest portion

of cell nitrogen. In seven such experiments an appreciable amount of activity (12 to 26 per cent) was associated with the nuclear fraction. As stated previously this fraction was heterogeneous and contained variable numbers of granules, which could account for this finding. The localization of phagocytin activity in the granule fraction was further indicated by a specific activity which was 2.5 to 3.0-fold greater than that of the unfractionated lysate.

Further studies were performed to establish that the bactericidin present in the granules was, in fact, phagocytin. The antibacterial spectrum was tested on a number of Gram-positive and Gram-negative organisms including *Salmonella typhimurium* RIA and SR11, *Klebsiella pneumoniae*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Staphylococcus albus*, and *Staphylococcus aureus*. Each of these organisms was killed by the granule extract, the relative activity being similar to that described previously for phagocytin (19).

TABLE IV

The Distribution of Phagocytin in Leucocyte Fractions

Fraction	Nitrogen	Phagocytin		Specific activity	
	mg.	*units*	*per cent*	*units/μg. N_2*	*μg. N_2/unit*
Experiment No. 1					
Total sucrose lysate	10.44	1,280,000	(100)	122	0.0082
400 *g* pellet (nuclear)	2.57	220,000	23	85	0.011
8,200 *g* pellet (granules)	2.45	704,000	75	290	0.0034
Postgranule supernate	5.32	20,000	2	3.7	0.27
Experiment No. 2					
Total sucrose lysate	4.03	640,000	(100)	158	0.0063
400 *g* pellet (nuclear)	0.73	80,000	14	109	0.0092
8,200 *g* pellet (granules)	1.02	480,000	85	471	0.0021
Postgranule supernate	2.21	5,000	1	2.3	0.434

The resistance of phagocytin to pepsin digestion was employed as another criterion for the identification of the granule-associated bactericidin.

Aliquots of each of the four fractions were extracted with 0.009 M citric acid as described previously. The extracts were then digested for 2 hours at 37°C. with 2.0 μg./ml. of crystalline pepsin. The digests as well as the untreated control extracts were then tested for bactericidal activity against *Escherichia coli* K-12.

The bactericidin present in the citric acid extracts of the granule fraction was relatively unaffected by the action of pepsin. Several experiments showed that there was either no change in the bactericidal endpoint or at most a twofold reduction. Similar findings were obtained with the total sucrose lysate and the 400 *g* pellet. In contrast, pepsin digestion of the postgranule supernate resulted

in an 8 to 16-fold reduction in activity. This suggested that the postgranule supernate contained bactericidal factors (probably histones) other than phagocytin.

Since the granules were quite sensitive to changes in hydrogen ion concentration, it was of interest to examine the influence of pH on the liberation of both phagocytin and protein from granule suspensions.

Aliquots of a purified granule suspension were added to 0.05 M buffers made up in 0.25 M sucrose. The same buffer systems were employed as for the lysis experiments. After incubation

TABLE V

The Effect of pH and Saponin on the Release of Protein and Phagocytin from Leucocyte Granules

pH	Material released into granule supernate after incubation at 25°C. for 30 min.				Material remaining in granule pellet after incubation at 25°C. for 30 min.*			
	Protein	Per cent released	Phagocytin	Per cent released	Protein	Per cent remaining	Phagocytin	Per cent remaining
	μg./ml.		*units/ml.*		*μg/ml.*		*units/ml.*	
2.0	370	99	12,800	>90	4.0	1.0	<100	<1
3.0	374	97	12,800	>90	8.0	3	<100	<1
4.0	248	94	12,800	>90	22.0	6	100	<1
5.0	182	51	3,200	33	174	49	6,400	66
6.0	100	28	200	2	254	72	12,800	>90
7.0	70	20	200	2	284	80	12,800	>90
8.0	50	13	100	<1	326	87	12,800	>90
0.34 M sucrose alone	48	13	<100	<1	316	87	12,800	>90
1 per cent saponin	346	92	12,800	>90	30	8	200	<10

* Pellet extracted with 0.009 M citric acid.

at 25°C. for 30 minutes the suspensions were centrifuged at 9,000 *g* for 20 minutes. The supernatant fluid was carefully removed and stored for the analysis of protein and phagocytin. The pellet was then extracted with 0.009 M citric acid, clarified by centrifugation, and the supernatant fluid assayed for protein and phagocytin.

The results of one such experiment are presented in Table V. At pH values from 6.0 to 8.0, a range in which granule lysis did not occur by optical methods, essentially all the protein and phagocytin were present in the pellet and extractable with citric acid. At pH 5.0, granule lysis began to occur with the solubilization of 50 per cent of the total protein and one-third of the phagocytin activity. When the pH was maintained at 4.0 or lower, all the protein and phagocytin were solubilized, and this was accompanied by a 95 per cent decrease in the optical density of the preparation. In sucrose alone, the granules remained in-

tact, whereas 1 per cent saponin solubilized both protein and bactericidal activity. These results indicated that granule lysis was accompanied by the concomitant liberation of both protein and phagocytin. Phagocytin displayed full activity when liberated from the granules by either saponin or controlled pH lysis and did not require further acid treatment for antimicrobial activity.

The Distribution and Activity of Selected Polymorphonuclear Leucocyte Enzymes

The examination of the enzymatic composition of leucocyte fractions was primarily directed towards hydrolytic enzymes which might play a role in the degradation of ingested particles. A number of hydrolases had been previously reported to be present in rabbit polymorphonuclear leucocytes. These included

TABLE VI

Activation of Enzymic Activity in Granule Suspensions

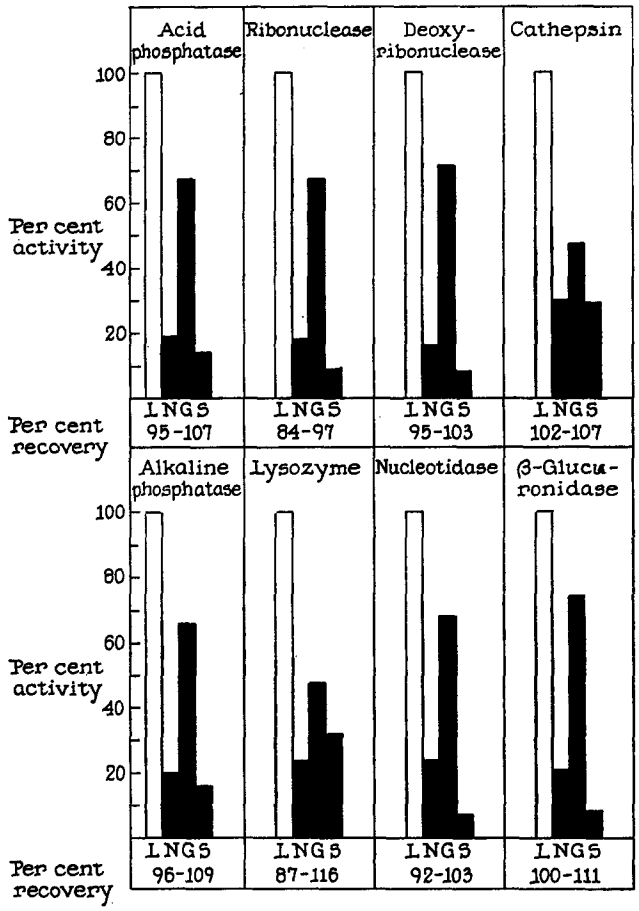
Enzyme	Treatment of granules	Increase in activity over untreated granules
		per cent
Alkaline phosphatase	Saponin	87
Acid phosphatase	"	96
Ribonuclease	Freeze-thaw 6 times	70
Deoxyribonuclease	" " 6 "	66
Nucleotidase	Saponin	62
β-Glucuronidase	"	84
Lysozyme	Freeze-thaw, 6 times	92
Cathepsin	Saponin	68

ribonuclease (20), deoxyribonuclease (21), alkaline and acid phosphomonoesterase (22), protease (23), beta glucuronidase (24), and lysozyme (25).

Initial experiments with a variety of these enzymes gave erratic results in terms of both recovery and specific activity of the enzymes. Previous reports by Rossiter (26) had emphasized that the enzymatic activity of rabbit leucocytes could be increased considerably by the use of surface-active agents. This treatment was associated with the "solubilization" of cytoplasmic components and was thought to be related to the release of enzymes from structural elements. A similar procedure was employed on fractions obtained from leucocytes lysed with sucrose. This resulted in major increases in enzymatic activity and reasonably good recoveries.

Sucrose lysates were separated by centrifugation as described previously. The entire procedure took approximately 120 minutes and the fractions were maintained in an ice bath until the onset of the analysis. Prior to the actual assay, each of the fractions was pretreated with either saponin or repeated freezing and thawing to obtain maximal activity. The type of pretreatment employed varied according to the specific enzyme under study, since in some

instances saponin was found to inhibit the enzyme. Activation with saponin was performed by incorporating this material into the incubation mixture at a final concentration of 0.2 per cent, whereas freezing and thawing was carried out on the fractions prior to the assay. Each of the fractions was frozen and thawed six times in an alcohol-dry ice bath.



TEXT-FIG. 3. The distribution and recovery of selected enzymes in leucocyte fractions. The letters under each column stand for the following fractions: *L*, total sucrose lysate; *N*, 400 *g* "nuclear" pellet; *G*, 8200 *g* "granule" pellet; *S*, 8200 *g* supernate.

Following this type of treatment the enzymatic activity of certain of the fractions increased as much as twofold. This was particularly true of the total sucrose lysate and the 8,200 *g* pellet, whereas little if any increase in activity occurred in the postgranule supernate. Table VI shows the results of an experiment which demonstrates the influence of pretreatment on the activity of enzymes present in the granule fraction. In this case, each of the assays was con-

ducted for 20 minutes at 38°C. and the activity compared to untreated control preparations. It was noted that considerable enhancement occurred in untreated preparations if the incubation was continued for longer periods of time. This may be related to the gradual disruption of granules which occurred at the acid pH employed for certain of the enzymes. The same methods of pretreatment (Table VI) were then applied to all fractions and the distribution of enzymes examined.

TABLE VII

*Specific Activities of Selected Enzymes in Leucocyte Fractions**

Leucocyte fraction	Acid‡ phosphatase	Ribonuclease§	Deoxyribonuclease‖	Cathepsin¶	Alkaline** phosphatase	Lysozyme‡‡	Nucleotidase§§	β-Glucuronidase‖‖
Total sucrose lysate	0.684	7.67	2.70	2.18	5.52	52.1	0.220	0.524
400 *g* pellet (nuclear)	0.727	5.98	2.01	2.08	6.20	36.1	0.200	0.499
8,200 *g* pellet (granules)	1.82	20.8	7.12	3.41	16.8	111.7	0.529	1.25
Postgranule supernate	0.190	1.30	0.431	1.54	1.14	39.6	0.036	0.076
Specific activity 8,200 *g* pellet / Specific activity total lysate	2.66	2.71	2.63	1.56	3.04	2.14	2.40	2.38

* Mean values of 3 experiments.

‡ Milligrams of phosphorus liberated, pH 5.0/hour/mg. nitrogen.

§ O.D. 260 mμ units released, pH 5.0/hour/mg. nitrogen.

‖ O.D. 260 mμ units released, pH 5.0/hour/mg. nitrogen.

¶ Protein equivalents liberated, pH 3.8/hour/mg. nitrogen.

** Milligrams of phosphorus liberated, pH 9.9/hour/mg. nitrogen.

‡‡ Micrograms of crystalline egg white lysozyme equivalents/mg. nitrogen.

§§ Milligrams of phosphorus liberated, pH 4.0/hour/mg. nitrogen.

‖‖ Milligrams of phenophthalein liberated, pH 4.5/hour/mg. nitrogen.

Text-fig. 3 shows the mean values of three experiments in which the per cent distribution and recovery of eight enzymes was determined. In general, six of the enzymes, *i.e.* acid and alkaline phosphomonoesterase, ribonuclease, deoxyribonuclease, beta glucuronidase, and 5′-nucleotidase were predominantly present in the granule fraction. Only small amounts of activity were present in the postgranule supernate and the remainder in the 400 *g* pellet. Since the "nuclear" pellet contained a considerable number of granules, it is possible that these elements were in part responsible for its activity.

Both lysozyme and cathepsin exhibited a somewhat different pattern of distribution with a larger percentage of activity in the 400 *g* pellet and in the postgranule supernate. Approximately 50 per cent of the total activity of each of these two enzymes was associated with the granule fraction.

The specific activities of the enzymes were calculated from the same experi-

ments shown in Text-fig. 3 and are presented in Table VII. Each of the enzymes localized in the 8,200 *g* (granule) pellet showed specific activities which were considerably greater than in the total sucrose lysate. As indicated in the lowest line of Table VII, the specific activity of the granule fraction was 2 to 3-fold greater than that of the unfractionated lysate.

It was next of interest to determine whether the granule-associated enzymes could be liberated in a soluble form following disruption of the particles.

TABLE VIII

The Influence of Granule Disruption on the Liberation of Selected Granule-Associated Enzymes

Pretreatment of granules	Enzyme	Activity	
		Per cent in 15,000 *g* pellet	Per cent in 15,000 *g* supernate
+	Acid phosphatase	12	88
0	" "	98	2
+	Ribonuclease	24	76
0	"	95	5
+	β-Glucuronidase	18	82
0	"	96	4
+	Alkaline phosphatase	26	74
0	" "	94	6
+	Cathepsin	8	92
0	"	97	3
+	Protein	9	91
0	"	98	2

Purified granules were suspended in cold 0.34 M sucrose and frozen and thawed six times in a dry ice-alcohol bath. Following this treatment the suspension was homogenized for three 1-minute periods in a chilled Waring blendor. The suspension was then dispensed to lusteroid tubes and centrifuged at 15,000 *g* for 20 minutes at 4°C. The supernatant fluid was carefully removed and the minute pellet resuspended in sucrose. A control non-treated preparation was carried through the identical centrifugation procedure and both the disrupted and control preparations assayed for protein and enzymatic activity. The results are expressed as the per cent of protein and enzyme activity present in the supernatant fluid in a non-sedimentable form.

Table VIII represents one such experiment and demonstrates the release of the majority of the granule-associated enzymes and protein following the physical disruption of the particle. Somewhat similar results have been obtained

through the use of controlled acid lysis of the granules at pH 3.5–4.5. Under the latter conditions, however, 30 to 60 per cent of enzymatic activity was lost, presumably because of the prolonged exposure to the low pH. This differs from the results obtained with phagocytin in that this bactericidal substance was quite stable and recoverable in acid media.

DISCUSSION

The successful isolation and study of granules from rabbit polymorphonuclear leucocytes was dependant upon a number of methodological factors including; (*a*) the availability of large uniform populations of polymorphonuclear leucocytes, (*b*) the preponderance of granules as the major cytoplasmic component in these cells, and (*c*) the efficient but relatively non-traumatic disruption of the leucocytes by sucrose lysis. Each of these factors was of importance in obtaining good yields of intact, homogeneous, specific granules.

The mechanism of lysis by sucrose is not clear at this time. Two factors may be of importance in this phenomenon. These are the non-ionic environment which may damage the leucocyte envelope and/or an osmotic effect produced by the penetration of a non-utilizable molecule. In addition, sucrose treatment had a pronounced effect on the nuclei and resulted in the breakage and solubilization of a considerable proportion of nuclear contents. This finding was substantiated by the presence of approximately one-half the deoxyribonucleic acid in the postgranule supernatant fluid.

Once lysis of the cell had occurred, the separation of the granules from other cellular constituents was simply achieved by differential centrifugation. Morphologically, the only leucocyte fraction which appeared relatively homogeneous was the 8,200 *g* pellet. Studies of this fraction by means of both light and electron microscopy showed the presence of intact granules with occasional mitochondria. Both the 400 *g* pellet and the postgranule supernate were grossly heterogeneous, making the biochemical and bactericidal studies of these fractions difficult to interpret.

Although the 8,200 *g* pellet was comprised mainly of granules, it is important to consider the possibility that more than one type of granule exists in the cytoplasm of the leucocyte. This was suggested by the variation in size and electron density of the granules,—particularly as visualized in the intact leucocyte. This variation could represent either stages in the maturation of the granule, or separate entities with distinct enzymatic and chemical properties. Additional fractionation studies are required before this question can be answered. In any event the studies presented appear to be representative of the total granule population of the rabbit leucocyte.

Morphologically, the isolated granules are discrete elements exhibiting a limiting membrane which surrounds an amorphous internal matrix. The dis-

ruption of the granule as well as the solubilization of its contents can be achieved by a variety of techniques. These procedures result in a marked reduction in the optical density of granule suspensions and in the concomitant liberation of functionally active materials. Particularly interesting is the sensitivity of the granules to acid media, since this may represent one possible mechanism for the release of their contents *in situ*. The recent observations of Becker *et al.* (27) and Cohn and Morse (28) demonstrating an increase in lactic acid production by phagocyting leucocytes are pertinent in this regard.

Although the chemical composition of the granules has not been completely elucidated, it is apparent that they contain protein as well as small amounts of phospholipid. The trace amounts of nucleic acids present in the granule pellet most likely represent nuclear contamination. There are, however, a number of biologically active materials associated with the granule pellet. Included in this category is an antimicrobial agent with the properties of phagocytin. Approximately 80 per cent of this material is present in the granule fraction in a bound form and can be completely liberated by disrupting the granules with acid or saponin.

In addition, the granule pellet contains the largest amounts of several hydrolases, the majority of which are active under acid conditions. These include acid phosphatase, nucleotidase, ribonuclease, deoxyribonuclease, and beta glucuronidase. Lysozyme and cathepsin are distributed somewhat differently with 50 per cent of the total activity present in the granule pellet. Although this difference remains unexplained, the activity or availability of both enzymes appears to be enhanced by procedures which disrupt the granule. It must be recognized that the data obtained on the distribution of enzymes are in a sense preliminary. This is the result of the many known artefacts which occur in cell fractionation procedures (29) and which in this case may include the liberation and redistribution of enzymes following sucrose lysis of the leucocytes.

The preceding results on the nature and localization of enzymes appear to be analogous to those described by DeDuve (30) for the lysosomes of rat liver. These particles are considered to represent sacs of acid hydrolases which sediment with mitochondria in the usual sucrose fractionation schemes. Intact lysosomes exhibit low enzymatic activity, presumably because of a limiting membrane which is impermeable to substrates. Exposure to hypotonic or acid media, surface-active agents, and mechanical trauma results in large increases in enzymatic activity. These particles represent only a small portion of the mitochondrial fraction and have not yet been separated in a homogeneous state. Although their function has not been established, it is presumably connected with the disposal of exogenous materials and/or with catabolic events in cellular metabolism.

From the data available thus far it appears that the rabbit polymorphonuclear leucocyte contains a variety of potentially important substances which are

compartmented within membrane systems and thereby excluded from other elements in the cytoplasm. Since the granules form such a large part of the cytoplasm of this highly specialized cell, it seems likely that they are in some way related to the destruction and degradation of ingested particles. If this hypothesis is correct, then a mechanism is required to deliver granule contents to the vicinity of the phagocyted particle. This would necessitate the disruption of the granule in response to the ingestion of foreign materials. This subject will be discussed more fully in the following reports.

SUMMARY

A method has been described for isolation of the specific cytoplasmic granules of rabbit polymorphonuclear leucocytes. Homogeneous suspensions of leucocytes were disrupted by lysis in 0.34 M sucrose. This procedure liberated the cytoplasmic contents of the cell and dissolved a considerable proportion of the nuclei. Following disruption, the sucrose lysate was separated into three fractions by differential centrifugation, *i.e.* 400 *g* or nuclear pellet, 8,200 *g* or granule pellet and the postgranule supernate. Microscopic examination revealed that the 8,200 *g* pellet was composed of intact granules as well as occasional mitochondria. The other two fractions were morphologically heterogeneous.

Studies with isolated granules demonstrated their lysis by a variety of weak acids and surface-active agents. When buffered solutions were employed between the ranges of pH 2.0 and 9.0, granule lysis began at pH 5.5 and was complete at pH 4.0.

Chemical analysis disclosed that the granule pellet contained protein and phospholipid with only traces of nucleic acids.

Approximately 70 to 80 per cent of the total cellular antimicrobial agent phagocytin was present in the granule fraction. This material was liberated from the granules by acid (pH 5.0 or lower).

Studies on selected enzymes showed that acid phosphatase, alkaline phosphatase, nucleotidase, ribonuclease, deoxyribonuclease, and beta glucuronidase were predominantly localized in the granule fraction. Approximately 50 per cent of total cellular lysozyme and cathepsin were also present in the 8,200 *g* pellet. Disruption of the granules was associated with the release of the majority of granule protein and enzymes in a non-sedimentable form. The properties and composition of rabbit polymorphonuclear leucocyte granules seem to be analogous to those of liver lysosomes.

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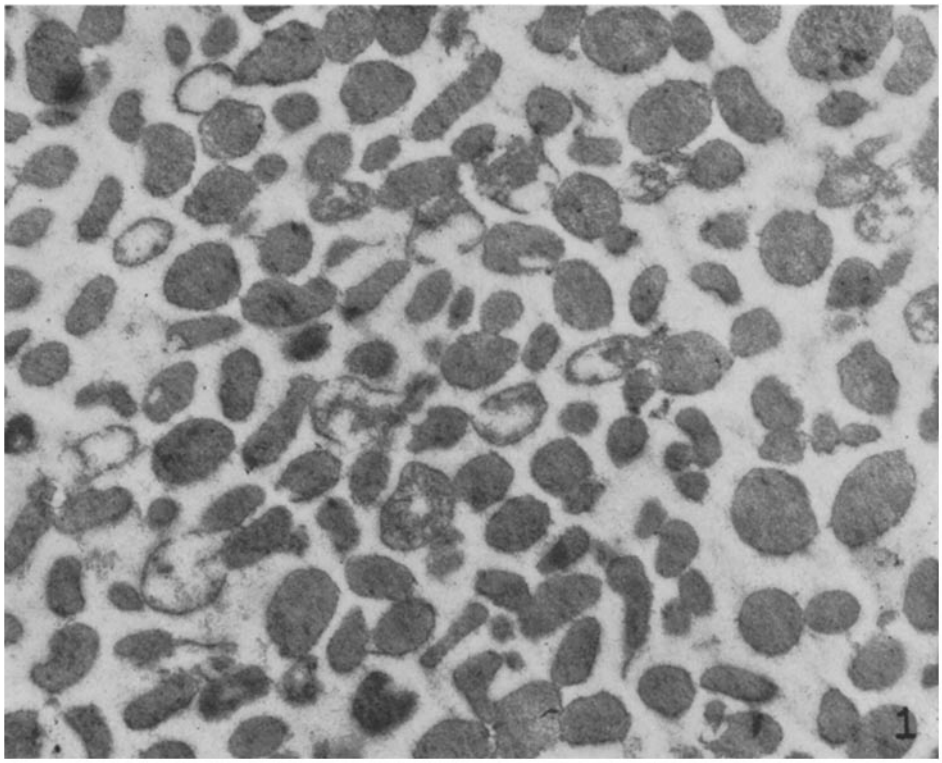
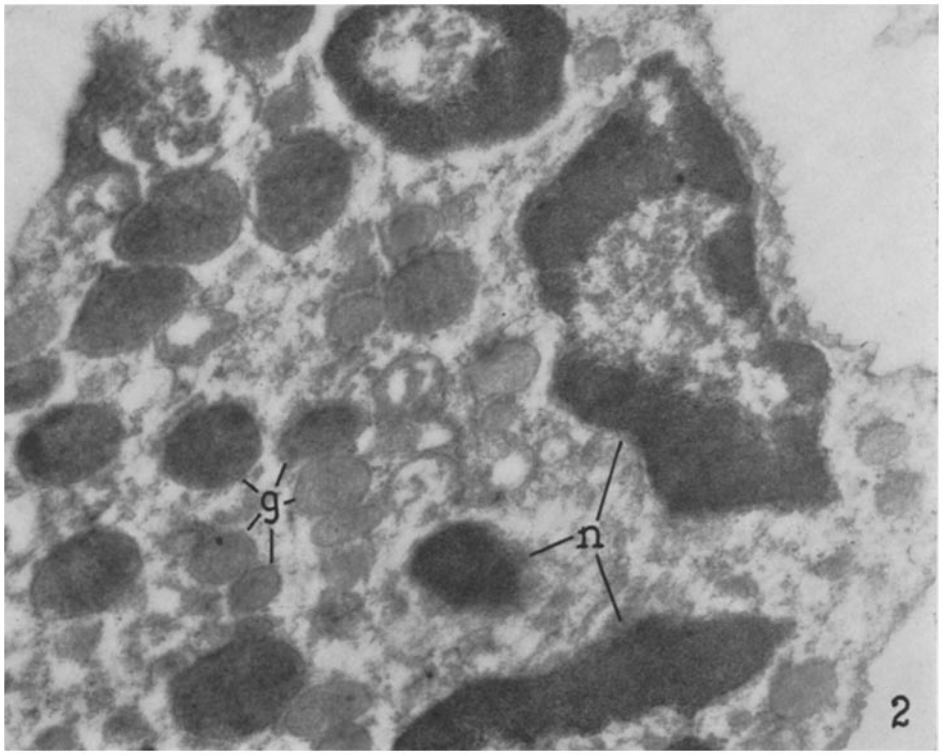
EXPLANATION OF PLATE 85

FIG. 1. A representative field taken from a sectioned granule pellet. × 23,000.

FIG. 2. A portion of a rabbit polymorphonuclear leucocyte obtained from the peritoneal cavity. Segments of the nucleus appear at *n* and granules of different size and density at *g*. × 23,000.





(Cohn and Hirsch: Properties of leucocyte granules)