Biochem. J. (1970) **116**, 207–216
Printed in Great Britain

The Distributions of some Granule-Associated Enzymes in Guinea-Pig Polymorphonuclear Leucocytes

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(*Received* 16 *September* 1969)

1. Homogenates of guinea-pig polymorphonuclear leucocytes were separated by differential centrifugation into six particulate fractions and a soluble fraction. 2. The distributions in these fractions of protein, DNA, succinate dehydrogenase, β-glucuronidase, peroxidase, alkaline phosphatase, acid phosphatase (against *p*-nitrophenyl phosphate and β-glycerophosphate), cathepsin, and catalase were compared. 3. Almost all of the DNA sedimented in the first two pellets, indicating that the nuclei were relatively intact. 4. The four hydrolases and peroxidase showed different distribution patterns, although these activities were previously reported to be localized mainly in the single 'granule' fraction isolated from leucocytes. 5. The particles containing peroxidase, acid phosphatase and alkaline phosphatase all exhibited latency. Maximum activity for each enzyme was obtained at roughly similar concentrations of Triton X-100. 6. The acid phosphatase of these cells was distributed between two populations of particles that differed in both sedimentation characteristics and density. The acid phosphatase(s) of the two populations showed slightly different substrate specificities. This bimodal distribution was not an artifact of the procedure used to elicit the cells. 7. Catalase was recovered almost entirely in the soluble fraction and showed no latency in freshly prepared homogenates. No urate oxidase was detected. 8. We conclude that the 'granule' fraction of the polymorphonuclear leucocyte, as isolated by previous workers, contains at least three, probably more, populations of particles with different enzyme contents, and that these cells probably do not contain peroxisomes.

The isolation and characterization of a subcellular fraction containing the typical intracellular granules of the rabbit polymorphonuclear leucocyte (Cohn & Hirsch, 1960*a*,*b*; Hirsch & Cohn, 1960) constituted one of the first confirmations of the presence in other cells of the particles analogous to the lysosomes of liver (de Duve, 1964). The critical role of these granules in the intracellular events of phagocytosis was also demonstrated.

Cohn & Hirsch (1960*a*) noted little morphological heterogeneity in their isolated granule fractions, and many subsequent workers have treated leucocyte granule fractions derived from a variety of species as relatively homogenous preparations. However, the heterogeneity of these particles is indicated by morphological and histochemical evidence (Pease, 1956; Watanabe, 1957; Yoshida, 1960; Florey, 1962; Wetzel, Horn & Spicer, 1963; Dannenberg, Burstone & Walter, 1963; Zucker-Franklin & Hirsch, 1964; Horn, Spicer & Wetzel, 1964; Enomoto & Kitani, 1966; Takikawa & Ohta, 1966; Bainton & Farquhar, 1966; Wetzel, Horn & Spicer, 1967*a*; Wetzel, Spicer & Horn, 1967*b*; Watanabe, Donahue & Hoggatt, 1967; Bainton & Farquhar, 1968*a*,*b*), by the distribution of materials after cell fractionation (Vercauteren, 1964; Lutzner, 1964; Yu, Kummerow & Nishida, 1966; Vercauteren, Roels-De Schrijver & Decleir, 1967; Baggiolini, Hirsch & de Duve, 1969), and by the differential release of cellular materials under specified conditions (Woodin, 1962; Woodin, French & Marchesi, 1963; Woodin & Wieneke, 1964; Pruzansky & Patterson, 1967). Several different species and types of cell preparation were used in the studies cited, and some of the observations seemed difficult to reconcile. We have therefore investigated the distribution of several cellular components in a single type of cell, the PMN† of sterile peritoneal exudates from the guinea pig. Recent studies of the cytoplasmic components of rabbit PMN have given, in part, similar results (Baggiolini *et al.* 1969).

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† Abbreviations: PMN, Polymorphonuclear leucocyte(s); S1–S6 and PFS, sediments 1–6 and particle-free supernatant respectively, from the subcellular fractionation scheme described.

MATERIALS AND METHODS

Subcellular fractionation

Isolation of PMN. The PMN were elicited with sodium caseinate solution as described previously (Michell, Pancake, Noseworthy & Karnovsky, 1969). They were harvested in 0.9% NaCl, washed once with Krebs–Ringer phosphate medium, pH 7.4 (Umbreit, Burris & Stauffer, 1957), and once with approximately 10 vol. of 0.34 M-sucrose (adjusted to pH 7.4 with sodium bicarbonate).

Preparation of homogenates. A medium of freshly-prepared chilled 0.34 M-sucrose, adjusted to pH 7.4 with sodium bicarbonate ('sucrose solution'), was used to prepare homogenates and separate the particulate fractions.

The washed cells (see above) were gently dispersed in sucrose solution by pipetting and the final volume was made 8–10 ml/ml of packed cells. The suspension was filtered through cotton gauze and homogenized for 30–45 s in a chilled Potter–Elvehjem homogenizer with a mechanically driven Teflon pestle (type 4288-B, Size C, diam. 0.993 in and the radial clearance 0.003–0.0045 in; supplied by A. H. Thomas Co., Philadelphia, Pa., U.S.A.).

Fractionation of the homogenates by differential centrifugation. The refrigerated centrifuges used were the International model B-20, with the 8 × 50 ml (type 870) angle rotor, and the Beckman model L-2, with the type 40 angle rotor. All manipulations before the sedimentation of sediment 6 were achieved by using the International centrifuge. The values of centrifugal field quoted are calculated for the centre of the tube. In the isolations of sediments 1–4 the loosely packed surface layers of the pellets were carefully retained with the pellets. Volumes quoted are expressed per ml of original packed cells.

The homogenate was centrifuged at 1800*g* for 10 min, the pellet was homogenized in 4 vol. of sucrose solution and the suspension centrifuged at 1800*g* for 10 min. The pellet was homogenized twice more in 2 vol. of sucrose solution and sedimented at 1800*g* to give sediment 1 (S1). The combined supernatants were centrifuged at 3100*g* for 10 min. The pellet was resuspended in 2.5 vol. of sucrose solution and sedimented again at 3100*g* to give sediment 2 (S2). Two further fractions were sedimented and washed once each as described above for S2, except that the centrifugal fields used were: sediment 3 (S3), 4600*g* for 10 min; sediment 4 (S4), 7200*g* for 10 min. Sediment 5 (S5) was sedimented at 26000*g* for 10 min and was not washed. The supernatant fluid and the loose superficial layer of the pellet were decanted and thoroughly mixed and sediment 6 (S6) was sedimented at 105000*g* for 60 min. The supernatant fluids were decanted and combined to give a particle-free supernatant (PFS), and the pellets (S6) were drained.

Resuspension and storage of fractions. Each pellet was resuspended in sucrose solution by gentle homogenization and the volumes were noted. The protein concentrations of the samples at this stage were between 1 and 12 mg/ml. The components of the fractions were assayed when fresh or after storage at −20°C for 5 days or less.

Separation of particles by isopycnic density-gradient centrifugation. Linear gradients were generated from solutions of sucrose in water or in heavy water (from the New England Nuclear Corp., Boston, Mass., U.S.A.) with devices based on the principles described by Kuff, Hogeboom & Dalton (1956), by Bock & Ling (1954) and by Lakshmanan & Lieberman (1954), as discussed by de Duve, Berthet & Beaufay (1959).

The sample of cellular material to be studied was added to the top of the gradient and centrifuged at 0°C on the Beckman L-2 centrifuge in either the SW 50L rotor for 1.5 h at 204000*g* or in the SW 25.1 rotor for 7 h at 63500*g*. Gradients were sampled either by slicing the tubes (when the SW 50L was used) or by aspiration from the bottom of the tubes. The fractions were immediately diluted with a medium containing 1 mM-$NaHCO_3$, 1 mM-EDTA and 0.01% Triton X-100 at pH 7.4 (Beaufay *et al.* 1964).

Preparation of samples for electron microscopy. Samples of the resuspended pellets (0.2 ml of S3, S4 and S5) and of the supernatant from the sedimentation of S5 (1.0 ml of S6 + PFS) were layered under sucrose solution in centrifuge tubes and were centrifuged at 100000*g* for 30 min. The drained pellets were fixed *in situ* by the methods of Bainton & Farquhar (1966) for intact leucocytes. Primary fixation was for 1 h at 4°C in 1.5% glutaraldehyde, buffered at pH 7.4 with 0.067 M-cacodylate containing 1.0% sucrose. The pellets were then washed overnight in the buffer at 4°C. Small blocks cut from the pellets were then post-fixed for 1 h at 4°C with 1.0% osmium tetroxide in 0.05 M-acetate–veronal buffered at pH 7.4 and containing 5% sucrose. The blocks were stained for 15 min at room temperature in 0.5% uranyl acetate buffered at pH 5.0 with veronal–acetate, and were dehydrated and embedded in Araldite by routine methods for electron microscopy. The blocks were cut from the pellets and embedded so as to ensure that thin sections cut from them would contain both the upper and lower surfaces of the original pellet. Thin sections were stained with alkaline lead solutions and examined in an AEI EM6 electron microscope.

Enzyme assays

All assays, with the exception of catalase which shows first-order kinetics, gave rates of reaction that were constant throughout the specified periods of incubation. Enzyme activities were proportional to the protein concentrations used within the specified ranges.

Succinate dehydrogenase (*EC* 1.3.99.1). This was assayed in the presence of 2 mM-EDTA by a modification (Porteous & Clark, 1965) of the method of Pennington (1961). Incubations were in a total volume of 1 ml containing 0.1–0.5 mg of protein, for up to 45 min at 37°C. Samples of the ethyl acetate extract containing the formazan were clarified by the addition of one-tenth of a volume of methanol and their extinctions measured at 490 nm (E^{1cm}_{490} 20.1×10^3; Pennington, 1961).

Peroxidase (*EC* 1.11.1.7). The method was a modification of the method of Maehly (1954). To each of two spectrophotometer cuvettes were added: 0.2 ml of 0.1 M-sodium phosphate buffer (pH 7.0), 1.0 ml of 20 mM-guaiacol, 0.2 ml of 1% Triton X-100, 0.02–0.1 mg of enzyme protein and water to a total volume of 3 ml. The cuvettes were incubated at 37°C in the Perkin–Elmer Model 202 spectrophotometer for 2 min. Hydrogen peroxide (final concentration approximately 0.6 mM) was added to the test cell, both cuvettes were mixed and the appearance of tetraguaiacol was followed at 470 nm. The initial reaction rate was calculated by using E^{1cm}_{470}

26.6×10^3 for tetraguaiacol (George, 1953). In the assay described here the initial rate of tetraguaiacol production by a peroxidase-rich particulate fraction (S4) was not changed by the addition of a large excess of soluble material (PFS) (cf. Vercauteren, 1964).

Uricase (*EC* 1.7.3.3). This was measured by the method of Baudhuin *et al.* (1964).

Catalase (*EC* 1.11.1.6). The assay method was that of Baudhuin *et al.* (1964) except that the incubation temperature was 25°C.

β-Glucuronidase (*EC* 3.2.1.31). This was usually assayed with *p*-nitrophenyl β-D-glucuronide (Sigma Chemical Co., St Louis, Mo., U.S.A.). The assay contained: 50mM-sodium acetate buffer (pH5.0), 0.1% (w/v) Triton X-100, 1mM-*p*-nitrophenyl glucuronide and 0.02–0.2mg of enzyme protein. Incubations were in a total volume of 1ml for up to 3h at 37°C. The reaction was stopped by the addition of 2ml of 0.1M-NaOH, and the extinction determined at 410nm. Results were calculated by using $E_{410}^{1\,cm}$ 1.84×10^4 (Kezdy & Bender, 1962). When 1mM-phenolphthalein β-D-glucuronide was used the reaction was stopped and activities were calculated as described by Gianetto & de Duve (1955). Activity with *p*-nitrophenyl β-D-glucuronide was about 2.5 times that with phenolphthalein β-D-glucuronide.

Acid phosphatase (p-*nitrophenyl phosphate*) (*EC* 3.1.3.2). The assay mixture contained: 50mM-sodium acetate buffer (pH5.0), 0.1% (w/v) Triton X-100, 5mM-*p*-nitrophenyl phosphate and 0.01–0.1mg of enzyme protein. Incubations were in a total volume of 1ml for 10–30min at 37°C. The reaction was stopped and activity measured as described for β-glucuronidase.

Alkaline phosphatase (*EC* 3.1.3.1). This was measured similarly, except that the buffer was 50mM-glycine–NaOH (pH10) and 10mM-$MgCl_2$ was included in the incubation medium.

Acid phosphatase (*β-glycerophosphate*). 50mM-β-Glycerophosphate (adjusted to pH5.0 with NaOH) was substituted for *p*-nitrophenyl phosphate and incubated for 1h at 37°C. The reaction was stopped with 5ml of cold 8% trichloroacetic acid, the mixture filtered and P_i assayed on a sample of the filtrate (Fiske & Subbarow, 1925).

Cathepsin (*EC* 3.4.4.23). The assay was based on the method of Anson (1937). The assays contained: 2% haemoglobin (bovine type II, Sigma Chemical Co.), 0.1% (w/v) Triton X-100 and 0.165M-acetic acid. This mixture was incubated at 37°C for 30min and 0.05–0.2mg of enzyme protein was then added to give a final assay volume of 1ml. Incubation was continued at 37°C for 10min (control) or 130min (test). The reaction was stopped by the addition of 1ml of ice-cold 8.2% trichloroacetic acid. Non-precipitable protein degradation products in the filtrate were measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Chemical methods

Extraction and assay of nucleic acids. Samples of the fractions, containing 2–12mg of protein, were precipitated and washed with cold trichloroacetic acid and extracted with ethanol; nucleic acids were extracted into hot 5% trichloroacetic acid as described by Schneider (1957). DNA was measured in the extracts by the method of Burton (1956), except that the colour was developed overnight at 30°C or 37°C. Purified calf thymus DNA (Sigma, type V) was used as standard.

Protein assay. The method of Lowry *et al.* (1951) was used, with human serum albumin as the standard.

^{125}I-labelled casein. This was prepared as described by Reif (1966).

RESULTS

Effects of variation in the homogenization procedure. In a study of the effects caused by varying the method of homogenization, two variables were examined. (*a*) The proportion of cellular acid phosphatase released in a form not sedimentable at 100*g* for 5min indicated the completeness of cell rupture. (*b*) The proportion of the released acid phosphatase that was latent before treatment with Triton X-100 indicated the intactness of granules. Conditions chosen for routine use gave a 70% release of phosphatase with the first homogenization, combined with 80% latency. With an increased period of homogenization decreased degrees of latency were observed with little improvement in cell breakage. Less vigorous homogenization decreased cell breakage, with a slight increase in latency.

Effect on β-glucuronidase content of washing the 'nuclear' (S1) pellet. A homogenate was centrifuged at 1150*g* for 10min. The pellet was washed three times, as described above for S1, except that centrifugation was for 10min at 1150*g* each time. Under these conditions the final sediment contained 80–90% of the material usually isolated as S1. The original material, the final pellet and the four supernatant fractions were each analysed for β-glucuronidase activity. This enzyme was chosen because, with peroxidase, it occurred to a greater extent in the fractions containing the nuclei than did the other enzymes studied. Removal of β-glucuronidase was assumed therefore to parallel or to follow the removal of the other enzymes. About half of the total β-glucuronidase of the homogenate initially sedimented in this fraction. This was decreased to approximately one quarter after three washes. The pattern of removal showed that further washing would be unlikely to remove most of the activity remaining in this sediment.

In a study of the preparation of a granule fraction from human leucocytes it was reported (Hirschhorn & Weissmann, 1965) that addition of heparin to fractionation media was a useful method for decreasing the 'stickiness' of the particles and the consequent sedimentation of large quantities of granules with the nuclei. This was attempted with the guinea-pig cells, but the pellets obtained were extremely bulky and viscous and difficult to handle.

Table 1. *Composition of subcellular fractions prepared from guinea-pig polymorphonuclear leucocytes*

The absolute values for all entities except DNA and cathepsin are given as means ± S.D. for the numbers of measurements given in parentheses. For DNA and cathepsin the range of values is given because only two measurements were made. Protein content is expressed in mg/ml of homogenate; DNA in μg/mg of protein; succinate dehydrogenase and β-glucuronidase in nmol of product produced/min per mg of protein; acid phosphatase and alkaline phosphatase in μmol of *p*-nitrophenol released/min per mg of protein; peroxidase in μmol of tetraguaiacol synthesized/min per mg of protein (4 μmol of H_2O_2 were utilized/μmol of tetraguaiacol); cathepsin in μg of albumin equivalent released/min per mg of protein; catalase in units/mg of protein (see the Materials and Methods section). The percentage figures are averages based on total recovered activity; the recoveries and the number of fractionations involved are given in the final column.

	Absolute composition of the homogenate	Percentage values							
		Sediment no.							
		1	2	3	4	5	6	PFS	Recovery
Protein	10.8 ± 1.48 (5)	31.2	6.6	3.7	3.7	5.2	11.7	37.9	97.6 (5)
DNA	158 ± 21 (2)	78.6	10.9	2.4	1.0	0.2	6.6	0.3	107.7 (2)
Succinate dehydrogenase	2.16 ± 0.67 (5)	23.2	22.1	16.6	14.5	13.6	9.9	0	76.2 (2)
β-glucuronidase	4.58 ± 1.43 (6)	35.1	13.7	12.7	8.3	7.9	8.8	13.2	88.7 (4)
Peroxidase	0.261 ± 0.081 (5)	31.2	16.6	15.9	13.0	15.6	8.1	0	99.7 (4)
Alkaline phosphatase	0.240 ± 0.029 (5)	7.9	7.4	9.5	13.7	28.1	29.6	3.3	89.4 (2)
Acid phosphatase	0.202 ± 0.065 (5)	19.6	9.2	7.8	6.5	13.5	33.9	10.4	97.7 (4)
Cathepsin	180.0 ± 1 (2)	27.7	11.7	7.5	5.7	7.7	13.7	27.0	92.7 (2)
Catalase	0.172 ± 0,034 (4)	2.5	1.0	0.8	0.9	2.6	3.4	89.0	125.7 (2)

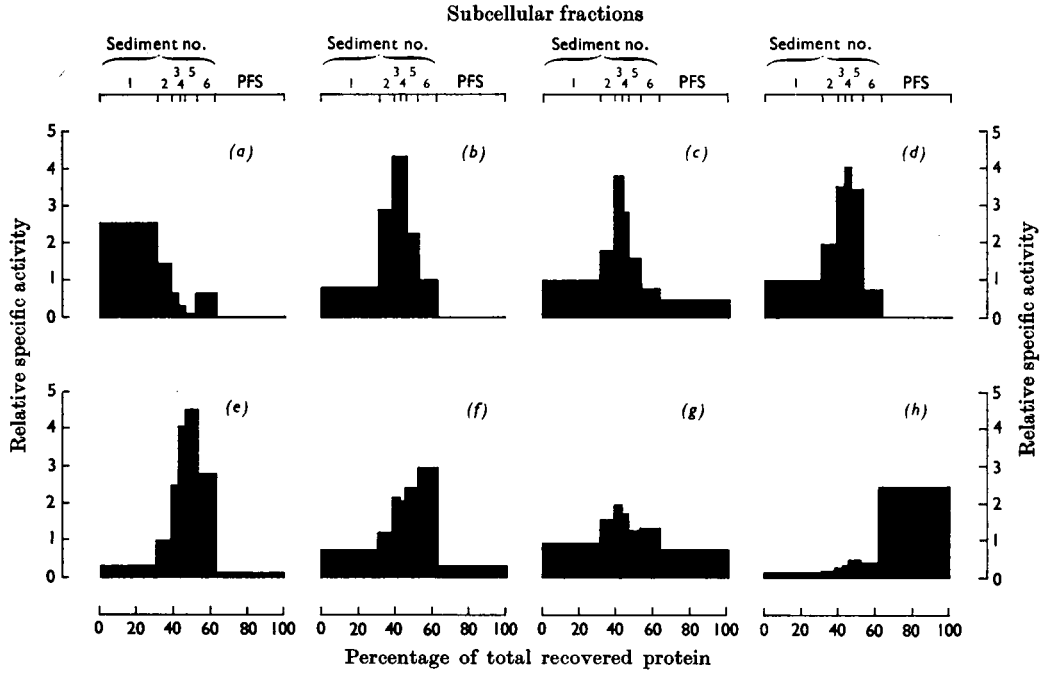


Fig. 1. Distribution patterns of enzymes and DNA. (*a*) DNA; (*b*) succinate dehydrogenase; (*c*) β-glucuronidase; (*d*) peroxidase; (*e*) alkaline phosphatase; (*f*) acid phosphatase (*p*-nitrophenyl phosphate); (*g*) cathepsin; (*h*) catalase. Fractions are represented in the order of their isolation from left to right, S1–S6 and PFS. Each fraction is represented separately on the ordinate scale by its relative specific activity (percentage of total recovered activity/percentage of total recovered protein). On the abscissa scale each fraction is represented (cumulatively from left to right) by its protein content, expressed as a percentage of total recovered protein. The results are the means from two experiments.

Distribution of cellular components after differential centrifugation. The gravitational fields used for the preparation of S1 to S6 were chosen to reveal differences in distribution between the cell components being studied. The total concentrations or activities and the distributions of nine cellular components in the seven isolated fractions are shown in Table 1. Results from two fractionations in which most of these quantities were measured on the same preparations of subcellular fractions are shown in Fig. 1.

DNA was located mainly in the first two sediments, showing that most of the nuclei sedimented in these fractions. However, a second small peak of DNA in sediment 6 probably represents material released from a small number of broken nuclei and adsorbed on membrane fragments. Succinate dehydrogenase, a mitochondrial enzyme, showed a single peak of activity in sediments 3 and 4. As was expected in these cells, which contain only a small number of mitochondria, the total activity of this enzyme was low.

The catalase of the guinea-pig polymorph was recovered almost entirely in soluble form. A test for latency of catalase in freshly prepared homogenates, assayed in the presence and absence of 1% Triton X-100, proved negative, as did a search for urate oxidase.

The other five enzymes studied (β-glucuronidase, peroxidase, cathepsin, acid phosphatase and alkaline phosphatase) are all members of the group of components assigned in rabbit PMN to a location in the 'granule' fraction (Cohn & Hirsch, 1960*a*). However, in the present study each of these enzymes showed a distribution that was clearly different from that of the others. The peak specific activities usually occurred respectively in sediment 3 (β-glucuronidase), sediment 4 (peroxidase), sediment 5 (alkaline phosphatase) and sediment 6 (acid phosphatase). Cathepsin showed an extremely heterogeneous distribution, not being clearly restricted to any of the separated fractions. The least clearly defined of these distinctions was that between β-glucuronidase and peroxidase. However, the validity of the difference is illustrated in Fig. 2(*a*), which was derived in the following way. The (percentage of total recovered peroxidase activity)/(percentage of total recovered β-glucuronidase activity) ratio was determined for each of the separated fractions from four experiments and the average values for each equivalent group of fractions were plotted. The relative activities of the two enzymes differed in each fraction. However, the observed relationship, with a decreased peroxidase activity/glucuronidase activity ratio in sediment 6 after a steady increase through the previous fractions, is not that which would be expected if the particles containing each activity

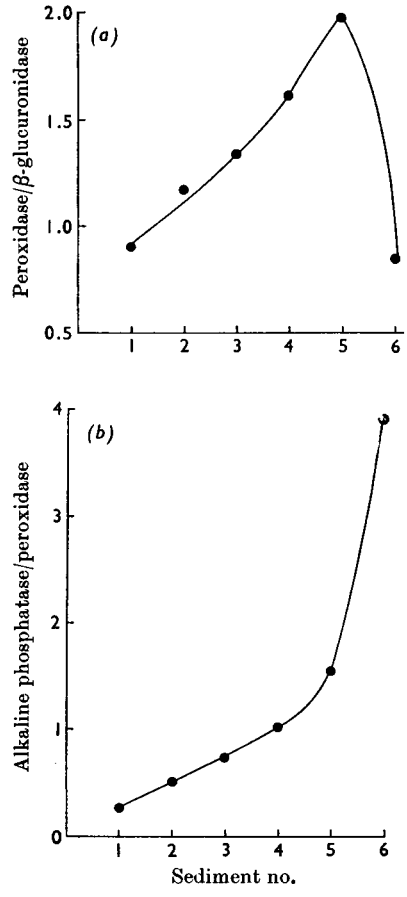


Fig. 2. Ratios of enzyme activities in the particulate fractions S1–S6. (*a*) Peroxidase/β-glucuronidase; (*b*) alkaline phosphatase/peroxidase. Further details are given in the text.

sedimented as separate and single populations with different sedimentation rates. What is probably a relationship of this type is shown in Fig. 2(*b*), which records alkaline phosphatase activity/peroxidase activity ratios averaged from two experiments (those shown in Fig. 1). In this case the ratio increased continuously throughout the fractionation. The fall in peroxidase activity/glucuronidase activity ratio in sediment 6 may be indicative of a second species of particle containing a small proportion of the total glucuronidase activity and sedimenting in this fraction, possibly the particle containing slowly sedimenting acid phosphatase (see below).

Particles containing acid phosphatase. The presence of a large proportion of the acid phosphatase of the homogenate in sediment 6, a 'microsomal' fraction by the usual criteria, is most unusual and

Table 2. *Substrate specificity of acid phosphatase in different subcellular fractions*

Homogenates were fractionated by the usual procedure, except that material corresponding to sediments 2, 3 and 4 was collected at 7200*g* for 10 min as a single fraction and washed once. The distributions are expressed as in Table 1 as percentages of total recovered activity and are averages from two experiments. Recoveries were between 90 and 105% for protein and between 85 and 110% for phosphatase.

	Sediment no.				
	1	2+3+4	5	6	PFS
p-Nitrophenyl phosphatase	19.5	18.9	14.7	34.4	12.6
β-Glycerophosphatase	20.4	24.4	14.5	31.3	9.5
Protein	33.9	13.5	5.3	10.8	36.5

was investigated further. The distribution was very different from that found by Cohn & Hirsch (1960*a*). This difference might have been due to the difference between the substrates used. A comparison of the distributions of acid *p*-nitrophenyl phosphatase and acid β-glycerophosphatase showed only a slight relative enrichment of the former in the slowly sedimenting fraction and of the latter in the rapidly sedimenting material (Table 2).

Another possibility (Fig. 1) was that acid phosphatase might be present in two different populations of particles. There was a small but reproducible difference in specificity towards the two different substrates in different fractions. This was investigated further by isopycnic density-gradient studies of isolated particulate fractions and of a cell 'low-speed supernatant' (the supernatant fraction recovered after the sedimentation and washing of S1 and, in our terminology, consisting of a mixture of S2–S6 and PFS). The results of such a study are shown in Fig. 3, which shows the distribution of acid *p*-nitrophenyl phosphatase in S6 and in a sediment (2+3+4), prepared as described in the legend to Table 2. It is clear that S6 contained a single population of particles containing acid phosphatase (density approximately 1.17) and sediment (2+3+4) contains a different and denser population (density approximately 1.21). Further, the distribution of this more dense component corresponds fairly closely to that of both peroxidase and β-glucuronidase. The large quantity of soluble phosphatase at the top of the S6 gradient is probably a result of the rather vigorous conditions required to suspend this pellet in an homogeneous fashion.

Graham, Karnovsky, Shafer, Glass & Karnovsky (1967) reported that guinea-pig PMN elicited with polyvinylpyrrolidone 'appeared to be in a better state of preservation' than those elicited with casein: the casein-elicited cells used in that study were replete with large vacuoles. This suggested

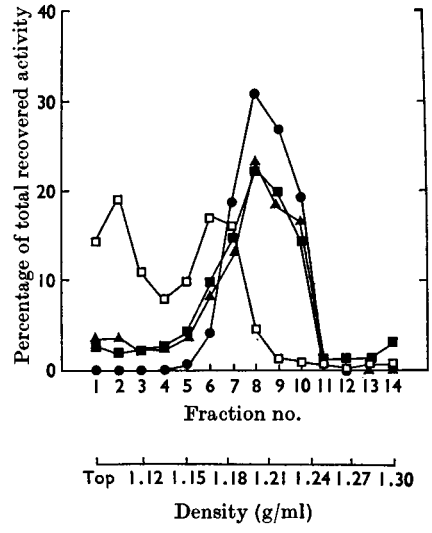


Fig. 3. Distributions of enzyme activities after isopycnic density-gradient separations of S(2+3+4) and of S6. From S(2+3+4): ●, peroxidase; ▲, β-glucuronidase; ■, acid phosphatase. □, Acid phosphatase of S6. 'Top' indicates soluble material.

that the cells might be ingesting the eliciting agent. This, after degranulation, could lead to the appearance of a new group of intracellular particles containing lysosomal enzymes. Cells were therefore isolated from guinea pigs that had received an injection of ^{125}I-labelled casein (1.12×10^8 c.p.m.). Of the injected label 55%, was recovered in the peritoneal washings that contained the PMN. After the cells had been sedimented and washed once each with Krebs–Ringer phosphate buffer and with sucrose, only 0.12% of the injected label remained associated with them. They were then homogenized and fractionated to obtain a twice-washed S1 and a low-speed supernatant as described above. These fractions were subjected to isopycnic density-gradient centrifugation. Of the 57% of the total cell-associated activity that was found in the low-speed supernatant, almost all was at the top of the gradient with the soluble components of the cell. On the other hand, 75% of the activity in the S1 fraction equilibrated in the gradient at a fairly high density, with the peak activity at a higher density than that of the more dense of the two acid phosphatase peaks. Much of this radioactivity was associated with a very viscous and turbid region of the gradient that contained the nuclei. Some activity was also present in a rather loosely packed pellet. From these results it seems that the isolated cells contained about 3% of their protein content as casein. Of this, half was in 'soluble' and half in

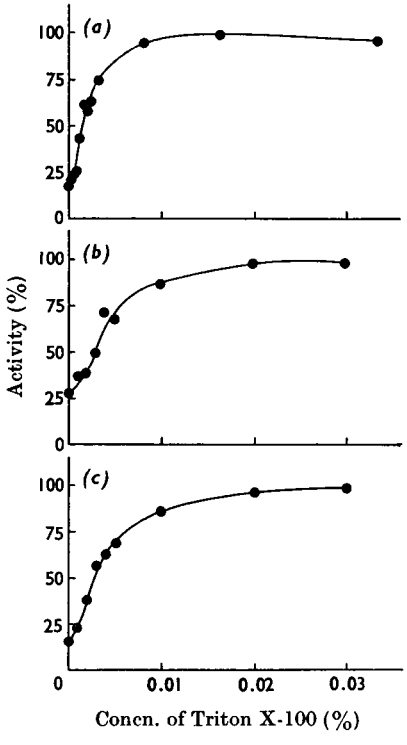


Fig. 4. Latency of enzymes. (*a*) Peroxidase; (*b*) alkaline phosphatase; (*c*) acid phosphatase. Activities were assayed after incubation with various concentrations of Triton X-100. Experimental details are given in the text below.

particulate fractions. The labelled particulate material did not distribute in the same way as either of the particles containing acid phosphatase. Thus both of these particles represent normal components of the cells rather than artifacts resulting from the caseinate injection used to elicit the cells. In all of the radioactive fractions examined (casein, sediment 1 and the low-speed supernatant) the ^{125}I was present in a form of which 65–85% was precipitable by 5% trichloroacetic acid; i.e. the casein had not been significantly degraded to small fragments.

Latency of the particulate enzymes. In view of the differences in distribution observed with several of the enzymes considered typical of the leucocyte granule fraction, it was important to check the latency of these enzymes. For the latency measurements all assays contained 0.25M-sucrose and the following other modifications were used: (*a*) peroxidase was preincubated with Triton X-100 for 2min at 37°C before the addition of H_2O_2; (*b*) other enzymes were incubated with Triton X-100 for 10min before the addition of substrate and assays were incubated for only 10min. The measurements were made with either homogenates or an unwashed mixed-granule fraction that was a mixture of sediments 1 to 5.

Peroxidase, alkaline phosphatase, and acid phosphatase all showed striking latency (Figs. 4*a*, 4*b* and 4*c*) and in each case the latent enzyme was released by low concentrations of Triton X-100 similar to those that activate the enzymes of lysosomes, rather than those of mitochondria or peroxisomes.

Observations in the electron microscope. S3 contained nuclei, fragments of cells, mitochondria and cellular debris, with interspersed electron-opaque granules in various proportions. These granules predominated in the upper portion of pellet S3, the whole of S4 and the lower portion of S5 (Plate 1*a*). The granules were bounded by a single membrane, were round, oval or elongated, and their matrices varied in electron-opacity, although this variation in staining was not seen in all experiments. It was not possible to differentiate azurophil from other types of granules morphologically. The upper part of S5 and the whole of S6 were also homogeneous, but here the material consisted largely of membrane-bounded vesicles of various sizes, and with electron-lucent interiors (Plate 1*b*). A small proportion of these vesicles represented rough endoplasmic reticulum.

DISCUSSION

The materials whose distributions and behaviour were investigated were chosen for the following reasons: (*a*) DNA and succinate dehydrogenase were markers for nuclear material and mitochondria respectively; (*b*) the four hydrolases and peroxidase have all been previously characterized as components of the 'granule' fraction of PMN; (*c*) catalase and urate oxidase were selected as probable components of peroxisomes (de Duve & Baudhuin, 1966), should particles of this type be present; (*d*) catalase was also studied because it is an enzyme for which conflicting data exist from previous work (Vercauteren, 1962; Vercauteren *et al.* 1967; Evans & Rechcigl, 1967).

Methods. The homogenization and fractionation methods used in this study were considered adequate for the following reasons. (i) The nuclei were recovered largely intact, as judged from the high proportion of DNA recovered in S1 and S2. This is in marked contrast with the findings of Cohn & Hirsch (1960*a*) with rabbit PMN and a different homogenization procedure where only about 50% of the cellular DNA sedimented with relatively low gravitational fields and the remainder was recovered unsedimented in the 'postgranule supernatant'. (ii) Only quite small quantities of the enzymes

previously characterized as 'granule-associated', with the exception of cathepsin, occurred in the final supernatant after centrifugation at $6 \times 10^6 g$-min. (iii) A large proportion of the granules were reasonably intact, judged from the high degree of latency shown by three enzymes. (iv) Absence of catalase from the particulate fractions indicated essentially quantitative cell breakage.

Enzyme distributions

Acid phosphatase. Previous studies of leucocytes have produced conflicting results on the localization of this activity. Reasons for this confusion probably include differences in the choice of substrate for enzyme assays, the presence in PMN of at least two, and probably three, particle populations all containing acid phosphatase activity, and differences between cells from different species. Previously, with β-glycerophosphate, activity was found in a single population of rapidly sedimenting granules from rabbit and guinea-pig PMN (Cohn & Hirsch, 1960*a*; Woodin, 1962; Cagan & Karnovsky, 1964). Recently however, a considerable amount of slowly sedimenting β-glycerophosphatase has been identified (Table 2; Baggiolini *et al.* 1969).

Experiments with *p*-nitrophenyl phosphate or phenyl phosphate have shown a large amount of slowly sedimenting activity in horse, rabbit and guinea-pig cells (Vercauteren, 1964; Baggiolini *et al.* 1969; Table 1 and Fig. 1) that is probably the same as the unsedimented activity from rat cells in the experiments of Yu *et al.* (1966). Our experiments did not establish whether the localization of this activity is different from that of slowly sedimenting β-glycerophosphatase, as found in the rabbit by Baggiolini *et al.* (1969). In cells from the guinea pig, human and rat there is also a considerable quantity of rapidly sedimenting *p*-nitrophenyl phosphatase (Table 1 and Fig. 1; Schultz, Corlin, Oddi, Kaminker & Jones, 1965; Yu *et al.* 1966), which is almost absent from rabbit PMN (Baggiolini *et al.*, 1969). The presence of two types of particle containing *p*-nitrophenyl phosphatase activity in the guinea-pig cells was confirmed in our studies by isopycnic density-gradient centrifugation, which showed that the denser peak of activity was also associated with the peroxidase and β-glucuronidase activity.

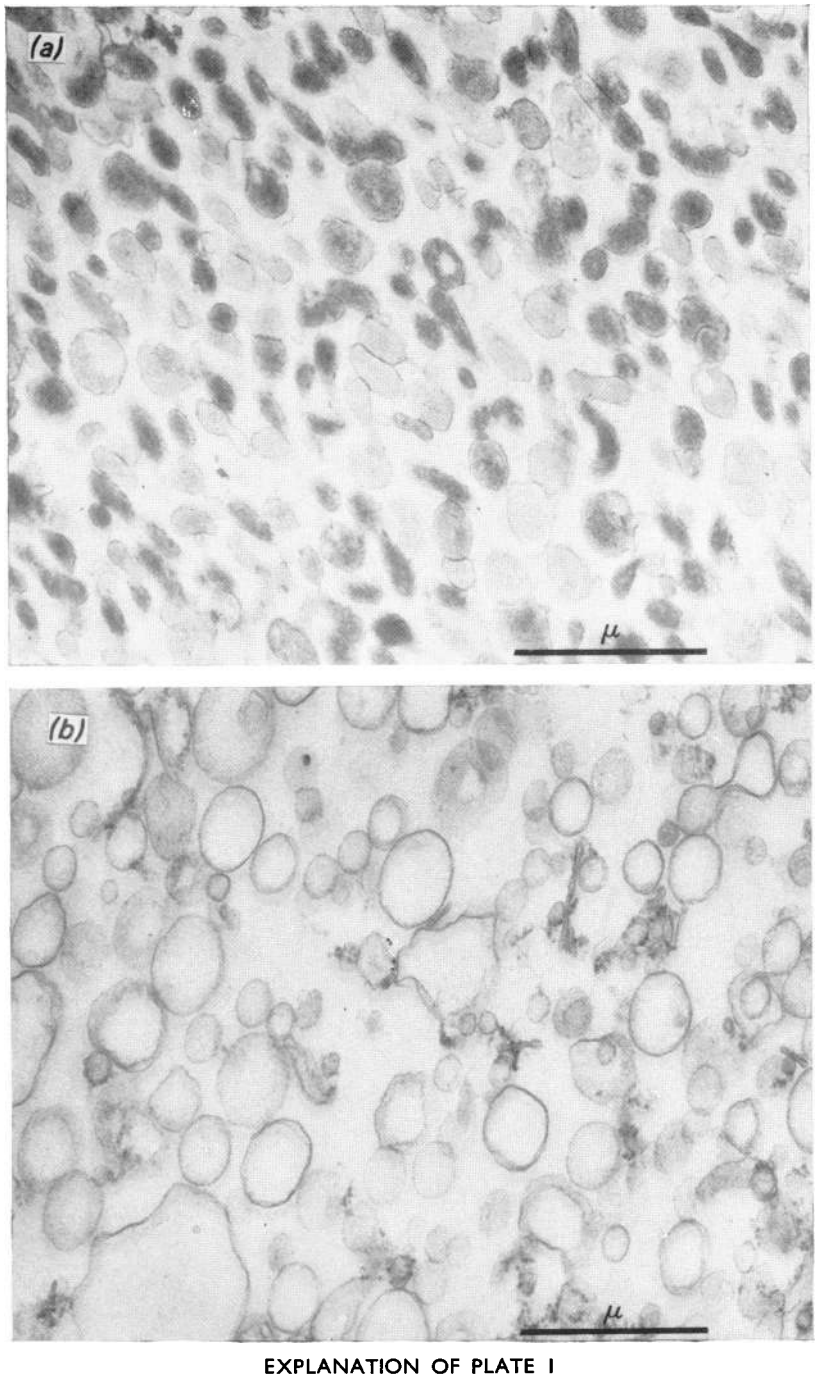
Wetzel *et al.* (1967*b*) studied rabbit bone-marrow PMN histochemically and found that acid phosphatase was detectable in both the largest granules (azurophil or primary) and the smallest (tertiary) of the three types of cytoplasmic organelles that they identified by electron microscopy. In contrast, Bainton & Farquhar (1968*a*,*b*) located acid phosphatase in the azurophil granules of cells from the same site, and found no evidence for phosphatase-containing 'tertiary' granules. Although in our studies the rapidly sedimenting acid phosphatase may be associated with azurophil granules, it is clear that the slowly sedimenting (S6) activity cannot be, as such structures do not appear in this fraction. Further, S6 contains no particles similar to the dense pleomorphic tertiary granules described by Wetzel *et al.* (1967*a*).

Alkaline phosphatase. The intermediate sedimentation rate observed for this enzyme, between those of peroxidase and glucuronidase and that of most of the acid phosphatase, is again consistent with data from experiments on horse blood leucocytes (Vercauteren, 1964) and with those of Baggiolini *et al.* (1969), obtained with rabbit PMN. Wetzel *et al.* (1967*b*) and Bainton & Farquhar (1968*a*,*b*) showed clearly by histochemical and cytochemical methods that alkaline phosphatase was restricted to specific granules in mature PMN of rabbit bone marrow.

Peroxidase and β-glucuronidase. The relatively rapid sedimentation of particles containing these activities is in agreement with the results of previous work (Cohn & Hirsch, 1960*a*; Schultz & Kaminker, 1962; Vercauteren, 1964; Cagan & Karnovsky, 1964; Weissmann, Becher & Thomas, 1964; Schultz *et al.* 1965; Hirschhorn & Weissmann, 1965; Yu *et al.* 1966; Pruzansky & Patterson, 1967). However, separation of these two activities by differential centrifugation has not been described previously, although their separation into two particle populations after density-gradient centrifugation of guinea-pig PMN preparations has been reported (Lutzner, 1964). We did not confirm the reported difference in density between the particles containing these enzymes but we did find small, but consistent, differences in sedimentation behaviour (Fig. 2*a*). Other differences between these enzymes have been observed previously. (*a*) Under certain conditions treatment of rabbit PMN with leucocidin causes the release from the cells of either peroxidase and β-glucuronidase or of glucuronidase alone (Woodin & Wieneke, 1964). (*b*) Baehner, Karnovsky & Karnovsky (1969) found that although the subcellular distributions of these enzymes in normal human leucocytes are similar, their distributions in cells from patients suffering from chronic granulomatous disease are markedly different.

Recent evidence from both subcellular fractionation and histochemical techniques has pointed strongly to the localization of both peroxidase and β-glucuronidase in the azurophil granules of rabbit PMN (Bainton & Farquhar, 1968*a*; Baggiolini *et al.* 1969). This work, however, does not rule out the possibility of a certain amount of heterogeneity within this group of particles.

Cathepsin. The distribution of this activity reported previously (Cohn & Hirsch, 1960*a*) was not typical. It seems from our results that this difference was due to a very general distribution of activity.



EXPLANATION OF PLATE 1

(*a*) Representative field typical of S4 and the bottom of S5, showing granules bounded by a single membrane, and of various shapes and electron-opacities. Magnification: ×25000. (*b*) Representative field typical of the top of S5 and of S6, showing electron-lucent vesicles bounded by a single membrane and of various sizes. In addition, some fragments of rough endoplasmic reticulum and flattened plates are present in small proportion. Magnification: ×25000.

This diffuse distribution probably reflected activity by more than one enzyme species under the assay conditions used.

Catalase and urate oxidase. The finding that the catalase of PMN was almost entirely soluble confirmed the results of Evans & Rechcigl (1967). This, the lack of detectable latency for catalase and the absence of any detectable urate oxidase activity all indicate that peroxisomes are probably absent from these cells.

General conclusion

Much of the previous work on the heterogeneity of leucocyte granules has been based on ultrastructure and enzyme histochemistry. The conclusions derived from that work, namely that acid phosphatase, alkaline phosphatase and β-glucuronidase reside in distinct particle populations, and that acid phosphatase activity occurs in two different particle populations, have been confirmed by a biochemical approach. Further, electron microscopy of the fractions gave clear morphological evidence of differences. Whether peroxidase and β-glucuronidase occur in different particle populations was not fully resolved. Recent experiments on rabbit PMN, using zonal centrifugation, have given partially similar results (Baggiolini *et al.* 1969). These observations put a limitation on the interpretation of studies of leucocyte granules and their functions when only one or two enzymes are studied. However, they also open the way to studies of the behaviour of the individual particle populations during leucocyte function; the first information of this type is already available (Pruzansky & Patterson, 1967). It may soon be possible to study the kinetics of interaction of each type of particle with the phagocytic vacuole, although definitive studies of this problem will probably await development of methods for isolation of intact phagocytic vacuoles.

This research was supported by grants from the United States Public Health Service, numbers AI-03260 and HE09125. R.H.M. received a travel grant from the Wellcome Trust.

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