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Subcellular Distribution of Glycosidases in Human Polymorphonuclear Leucocytes

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The subcellular distribution of nine glycosidases were studied in fractions of homogenized human polymorphonuclear leucocytes (neutrophils) obtained by isopycnic centrifugation through linear sucrose density gradients. The substrates were 4-methylumbelliferyl glycosides. All nine glycosides were hydrolysed by enzymes in neutrophil cytosol fractions, and by enzymes in at least one granule population. α-Glucosidase activity sedimented in sucrose density gradients to a point ($\rho = 1.180$ g/ml) just above the specific granules, possibly the 'tertiary' granule population. The peak corresponding to α-glucosidase did not co-sediment with, but considerably overlapped, the peak corresponding to lactoferrin, a marker for specific granules ($\rho = 1.187$ g/ml). α-Galactosidase activity was found primarily in heavy azurophil granules ($\rho = 1.222$ g/ml). α-Mannosidase activity was found primarily in light azurophil granules ($\rho = 1.206$ g/ml), following the distribution of myeloperoxidase, the commonly used azurophil granule marker. β-Glucosidase activity was concentrated in mitochondrial fractions ($\rho = 1.160$ g/ml). All other glycosidases presented complex distributions, with activities not restricted to one granule class. Granule-associated glycosidase activities were increased 2–38 times when measured in the presence of 0.05% Triton X-100, indicating latency of the enzymes within granules.

The polymorphonuclear leucocyte (neutrophil) is the hallmark of acute inflammation and is of central importance in determining the outcome of the initial interactions between man and invading micro-organisms. Without properly functioning neutrophils, man dies owing to overwhelming infection (Bridges *et al.*, 1959; Hersh *et al.*, 1965). A few laboratories have investigated the neutrophil on a biochemical and substructural level to understand more clearly how it functions (Bretz & Baggiolini, 1974; Spitznagel *et al.*, 1974; West *et al.*, 1974; Kane & Peters, 1975). The most successful attempts at subcellular fractionation of neutrophils have used isopycnic centrifugation of neutrophil homogenates through linear sucrose density gradients, and similar results have been obtained by most investigators. Neutrophils contain three major types of granules, each of which contains a unique marker protein. The primary or azurophil granules contain myeloperoxidase (Bainton & Farquar, 1968) and are separable into two populations with different densities and quantitative enzyme composition, the light azurophils ($\rho = 1.206$ g/ml) having more myeloperoxidase than the heavy azurophils ($\rho = 1.222$ g/ml). The secondary or specific granules ($\rho = 1.187$ g/ml) contain lactoferrin and vitamin B_{12}-binding proteins (Leffell & Spitznagel, 1972; Kane & Peters, 1975). The last population is an ill-defined granule population, the 'tertiary' granules, and contains no known unique markers (Kane & Peters, 1975).

We have a continuing interest in more precisely defining the subcellular structures of neutrophils to understand the neutrophil's overall function. In more precisely knowing the granule location and biochemistry of neutrophil glycosidases, we can better understand and more accurately predict the mechanisms of intraleucocytic bactericidal and degradative processes.

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Materials and Methods

Subcellular fractionation

Neutrophils were isolated from freshly drawn venous blood by Plasmagel and Ficoll–Hypaque sedimentation, as described by Spitznagel *et al.* (1974) and Rest & Spitznagel (1977). All donors signed informed-consent forms approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina. Purified-neutrophil suspensions contained 90–96% neutrophils, 0–2% lymphocytes, 0–2% monocytes and 3–8% eosinophils. Subcellular fractions were obtained as

described previously (Rest & Spitznagel, 1977). Briefly, supernatant fluids (from centrifugation at 126*g* for 15min) of homogenized neutrophils [in 25% (w/w) sucrose] were layered over linear 30–53% (w/w) unbuffered sucrose gradients in Beckman SW 25.2 rotor tubes. Gradients were centrifuged at 2000rev./min for 15min, then accelerated to 21000rev./min and centrifuged for an additional 120min at 21°C. Gradients were collected in 59 fractions of volume 1ml, and A_{450} of these fractions was measured to locate subcellular organelles.

Glycosidase assay

Glycosidases were assayed in a total volume of 100μl in glass tubes (12mm×75mm) containing the appropriate 4-methylumbelliferyl glycosides (Research Products International Corp., Elk Grove Village, IL, U.S.A.) added as 50-fold concentrated solutions (2μl) in dimethyl sulphoxide (Sigma Chemical Co., St. Louis, MO, U.S.A.), 0.1M-sodium acetate or 0.1M-sodium citrate buffer, 0.05% Triton X-100, and 0.1–5mg of sample protein. Assays were carried out at 37°C in a reciprocal shaking water bath and were stopped by the addition of 2.0ml of 50mM-glycine/NaOH buffer containing 5mM-EDTA (sodium salt), pH10.5. Fluorescence of samples was read within 1h with a Turner model 430 spectrofluorimeter, with an excitation wavelength of 366nm and an emission wavelength of 446nm. Table 1 shows the glycosides used and their assay concentration. Results are expressed as nmol of substrate hydrolysed/min per mg of protein.

Other enzyme and protein assays

Myeloperoxidase (EC 1.11.1.7) activity was measured by the method described in the *Worthington Enzyme Manual* (1972). Myeloperoxidase standard from human leukaemic neutrophils was purified as described previously (Rest & Spitznagel, 1977). One unit of myeloperoxidase causes an increase in A_{460} of 0.001 through a 1cm light-path in a 1ml reaction mixture with *o*-dianisidine as the hydrogen donor. Protein was determined by the method of Lowry *et al.* (1951) with egg-white lysozyme (Sigma) as standard. Lactoferrin was measured by the radial-immunodiffusion method of Mancini *et al.* (1965). Lactoferrin standard was purified from human milk by the method of Querinjean *et al.* (1971).

Results

Isopycnic fractionation of subcellular organelles

Although a complete description of this separation technique has been given previously (Spitznagel *et al.*, 1974; Rest & Spitznagel, 1977) a short explanation will aid understanding the data in the present paper. Isopycnic centrifugation of postnuclear supernatants of neutrophil homogenates through linear sucrose density gradients yields five visible bands. In order of ascending density (from the top of the tube), the bands consist of membrane fragments ($\rho = 1.136$g/ml), mitochondria ($\rho = 1.160$g/ml), specific granules ($\rho = 1.187$g/ml), light azurophil granules ($\rho = 1.206$g/ml) and heavy azurophil granules ($\rho = 1.222$g/ml).

pH optima of enzyme activity

pH optima were measured in 0.1M-sodium acetate or 0.1M-sodium citrate buffers (Table 2). Samples were from sucrose-density-gradient fractions of highest specific activity for the respective glycosidase. pH optima of the cytosol-associated α- and β-galactosidases and α-mannosidase were consistently lower than the granule-associated glycosidases when tested in sodium acetate buffer. These differences were not observed with sodium citrate buffer. Specific activities of *N*-acetyl-β-D-glucosaminidase, α-D-mannosidase and β-D-galactosidase were highest when measured in sodium citrate buffer, whereas the

Table 1. *Assay conditions and specific activity of* 4-*methylumbelliferyl glycosidases used in this study*

Glycosides were added to reaction mixtures as 50-fold-concentrated solutions in dimethyl sulphoxide. The postnuclear supernatant was from centrifugation of homogenized neutrophils at 126*g* for 15min. For further details see under 'Glycosidase assay'.

4-Methylumbelliferyl glycoside	EC no. of enzyme involved	Concn. (μM)	Buffer, pH	Specific activity in postnuclear supernatant (nmol/min per mg of protein)
α-D-Glucoside	EC 3.2.1.20	1.0	Acetate, 5.5	5.125
β-D-Glucoside	EC 3.2.1.21	5.0	Citrate, 4.5	0.035
α-D-Galactoside	EC 3.2.1.22	2.0	Citrate, 4.5	1.417
β-D-Galactoside	EC 3.2.1.23	1.0	Acetate, 3.5	3.125
β-D-Glucuronide	EC 3.2.1.31	2.0	Citrate, 4.5	2.375
N-Acetyl-β-D-glucosaminide	EC 3.2.1.30	2.0	Citrate, 4.5	17.500
N-Acetyl-β-D-galactosaminide	EC 3.2.1.53	1.0	Citrate, 4.5	3.500
α-D-Mannoside	EC 3.2.1.24	3.5	Acetate, 4.5	13.250
α-L-Fucoside	EC 3.2.1.51	1.0	Citrate, 5.0	1.500
α-L-Arabinoside	EC 3.2.1.55	1.0	Acetate, 4.5	0.467

Table 2. *Optimal pH values for activities of granule-associated glycosidases*

Reactions were for 20 min as described in the Materials and Methods section, with 5–20 μl (0.1–2.0 μg of protein) of each of the indicated sucrose-density-gradient fractions. The results are from a representative gradient divided into 59 fractions of volume 1 ml. Sources of the fractions shown are: cytosol, fraction 2; specific granules, fraction 28; light-azurophil granules, fraction 37; heavy azurophil granules, fraction 45. Further details of the subcellular fractionation are described in the Materials and Methods section.

	pH for optimal activity in:	
Glycosidase and fraction assayed	0.05 M-Sodium acetate	0.05 M-Sodium citrate
α-D-Glucosidase		
126*g* supernatant	5.5	6
Cytosol	6–6.5	6
Specific granules	5.5–6.5	6
α-D-Galactosidase		
126*g* supernatant	5	4.5
Cytosol	⩽3	4.5
Light azurophil granules	4.5	4.5
Heavy azurophil granules	4 and 5.5	4.5
β-D-Galactosidase		
126*g* supernatant	4	4
Cytosol	4	4
Specific granules	5	4
Light azurophil granules	4–4.5	4
β-D-Glucuronidase		
126*g* supernatant	4	5
Cytosol	4	5
Specific granules	4	5
Light azurophil granules	4	5
N-Acetyl-β-D-glucosaminidase		
126*g* supernatant	5.5	4.5
Cytosol	6	4.5
Light azurophil granules	5.5	4.5
Heavy azurophil granules	5–5.5	4.5
N-Acetyl-β-D-galactosaminidase		
126*g* supernatant	5	4.5
Cytosol	5	4.5
Light azurophil granules	4.5–5	4–4.4
Heavy azurophil granules	5	4.5
α-D-Mannosidase		
126*g* supernatant	⩽3	4
Cytosol	⩽3	4
Light azurophil granules	3.4–4	4
Heavy azurophil granules	4.5	4
α-L-Fucosidase		
126*g* supernatant	5–6.5	5–5.5
Cytosol	5 and 6	5
Specific granules	None observed	5.5
Light azurophil granules	6	5

specific activity of β-D-glucuronidase was highest when tested in sodium acetate buffer. The other glycosidases had similar specific activities in both buffers (data not shown).

Linearity of glycosidase assays

All of the glycosidase activities measured were linear with respect to time (up to 1 h) and sample protein concentration (up to 4 μg/100 μl). Assays were therefore performed well within these limits, usually for 15–30 min, with 0.1–1.0 μg of sample protein.

Subcellular distribution of the glycosidases

The distribution of the nine different glycosidases was determined by assaying the 59 individual sucrose-density-gradient fractions (Fig. 1). The location of glycosidase activities within a gradient was compared with the absorbance profile of gradient fractions at A_{450} and with the location of known granule marker proteins, i.e. myeloperoxidase for the two azurophil granules and lactoferrin for the specific granules. All glycosidases were located in the cytosol (8–54%; Table 3) and in at least one additional location. Glycosidase activity in cytosol fractions was probably not due to glycosidases released from granules during homogenization, since only 3.6 and 5.4% of myeloperoxidase and lactoferrin respectively were released into the cytosol fractions. Activity in fractions 53–59 is attributable to eosinophil granules (for a further explanation of this eosinophil peak see Spitznagel *et al.*, 1974).

A few glycosidases had relatively simple distributions (it should be remembered that all the glycosidases had additional activity in the cytosol fractions). α-Mannosidase and α-galactosidase activities were located solely in azurophil granules. α-Mannosidase was distributed similarly to myelo-

Table 3. *Percentages of glycosidase activities in cytosol fractions* (1–4) *of sucrose density gradients*

Values are calculated as 100×(total activity in fractions 1–4/total activity in 59 fractions of the sucrose density gradient). For further details see under 'Subcellular distribution of glycosidases'.

Enzyme	Activity in cytosol (%)
α-D-Glucosidase	22.8
β-D-Glucosidase	53.9
α-D-Galactosidase	20.3
β-D-Galactosidase	17.7
β-D-Glucuronidase	8.0
N-Acetyl-β-D-glucosidase	12.2
N-Acetyl-β-D-galactosidase	12.6
α-D-Mannosidase	14.8
α-L-Fucosidase	17.9
Myeloperoxidase	3.6
Lactoferrin	5.4

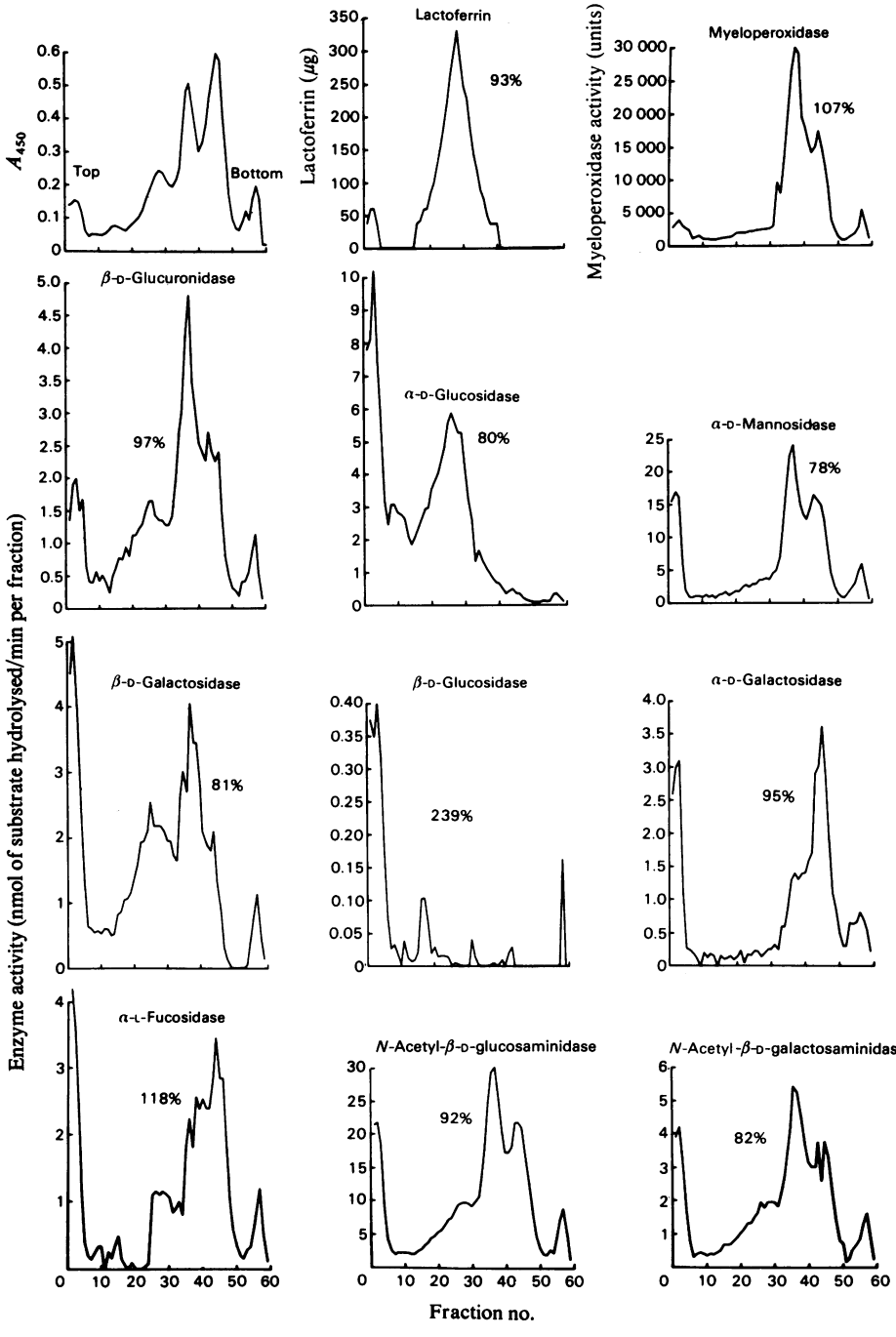

Fig. 1. *Distribution of glycosidase activity in isopycnically separated neutrophil homogenates*
The linear sucrose gradient was collected in 59 fractions of volume 1 ml, and a part of each fraction (1–20 μl, 0.1–2.0 μg) was assayed as described in the Materials and Methods section. The A_{450} profile is included as an indicator of granule subpopulations. Lactoferrin and myeloperoxidase distributions are included as a measure of specific and azurophil granules respectively. Percentages refer to recovery of protein or enzyme activity as follows: 100×(total activity in 59 fractions/total activity in postnuclear supernatant loaded on sucrose density gradient).

Table 4. *Latency of granule-associated glycosidases*

Glycosidase activities were measured in freshly isolated granule fractions with and without 0.05% Triton X-100. For further details see under 'Latency of glycosidase activity within granule fractions'. Latency (%) is defined as as 100 × {[(activity with Triton) − (activity without Triton)]/(activity with Triton)}.

Glycosidase	Activity (nmol/min per fraction) −Triton	+Triton	Latency (%)	Granule fraction assayed
α-D-Glucosidase	1.1	4.2	74	Specific
α-D-Galactosidase	3.0	6.8	56	Heavy azurophil
β-D-Galactosidase	0.8	6.6	88	Light azurophil
N-Acetyl-β-D-glucosaminidase	1.8	17.2	90	Light azurophil
N-Acetyl-β-D-galactosaminidase	1.0	6.2	84	Light azurophil
β-D-Glucuronidase	1.0	38	97	Light azurophil
α-D-Mannosidase	6.2	15.4	60	Light azurophil

peroxidase between light (60%) and heavy (40%) azurophil granules. α-Galactosidase was distributed in an opposite manner, i.e. approx. 35% in light and 65% in heavy azurophil granules, which more closely resembled the distribution of lysozyme within the azurophil granules (Spitznagel *et al.*, 1974). α-Glucosidase activity gave a peak at fraction 26 of the representative sucrose gradient shown in Fig. 1 with a shoulder at fractions 28–29. Lactoferrin, a specific-granule marker, gave a peak at fraction 28. The difference in the peak activities of these two proteins was seen consistently in other gradients. This might be the location of the 'tertiary' granules. There is a minor peak of α-glucosidase activity at fractions 8–9, which are possibly microsomal fractions ($\rho = 1.140$ g/ml), such as those described by Kane & Peters (1975).

The other glycosidases showed complex distributions in sucrose gradients. *N*-Acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase sedimented with azurophil granules in a manner similar to myeloperoxidase, with minor activity sedimenting with the specific granules. α-L-Fucosidase activity was also distributed between the specific and azurophil granules; however, there was more activity in the heavy azurophils than in the light azurophils.

β-D-Galactosidase and β-D-glucuronidase presented the most complex distributions. Both sedimented in 'tertiary' granules (fractions 25–26) and specific granules (fraction 28), both having higher specific activities in the former. Both also sedimented, in a very complex fashion, in and around the azurophil-granule peaks marked by myeloperoxidase (fractions 37 and 45). β-D-Galactosidase activity had obvious peaks at fractions 35, 37 and 44, with a shoulder at fractions 38 and 39. β-D-Glucuronidase gave peaks at fractions 37, 43 and 46. This suggested subpopulations for both the light and heavy azurophils. β-Glucosidase was least active of all the glycosidases tested, and the most difficult to measure. The only major peak observed for this glycosidase co-sedimented with the mitochondria (fraction 15).

Latency of glycosidase activity within granule fractions

To measure the latency of glycosidase activity within granules, sucrose-gradient fractions (collected less than 2h before assay) were assayed for enzyme activity in the presence and in the absence of 0.05% Triton X-100, in solutions that were all 0.34M with respect to sucrose. Latency ranged from 56 to 97% (Table 4).

Discussion

Several investigations have been concerned with the separation of subcellular organelles of human neutrophils and with the distribution of enzymes within these organelles (Bretz & Baggiolini, 1974; Spitznagel *et al.*, 1974; West *et al.*, 1974; Kane & Peters, 1975). Surprisingly few discrepancies exist between the results of these authors despite the numerous techniques used. There is general agreement that there are three major granule populations separable by isopycnic centrifugation: one population of specific or secondary granules ($\rho = 1.18$–1.19 g/ml) and two populations of azurophil of primary granules ($\rho = 1.20$–1.21 and 1.22 g/ml respectively). Each of these populations appears to be heterogeneous by electron-microscopic and biochemical analyses (Bretz & Baggiolini, 1974; Spitznagel *et al.*, 1974; West *et al.*, 1974). Our investigation of the subcellular distribution of several glycosidases was to define more precisely the enzymic heterogeneity of the different granule subpopulations.

One of the most striking observations of this research was the complex distributions of many of the glycosidases. One would think that glycosidases might be neatly packaged in one or two granule populations in some orderly manner. Indeed several investigators have used glycosidases as markers of azurophil granules (Avila & Convit, 1973*a*,*b*, 1974). Of the nine glycosidases studied, possibly one was confined to a single granule population, and all nine showed some degree of activity in cytosol fractions. In studies such as those involving

neutrophil degranulation, myeloperoxidase and lactoferrin are used as markers for primary and secondary granules respectively (Bainton & Farquar, 1968; Leffell & Spitznagel, 1972, 1975). Lysozyme and β-glucuronidase, found in both granule populations, are often used as general lysosomal markers (White & Estensen, 1974; Wright *et al.*, 1977). None of the glycosidases that we studied was present solely in the specific granules. α-Glucosidase sedimented with what we believe to be the 'tertiary' granules also described by Kane & Peters (1975). This fraction appeared in the sucrose density gradients at a point just above (lighter than) the specific granules. Since there are no known markers for the tertiary granules and since they are not clearly separated from the specific granules by the techniques used in this study, it is not possible to be certain that the α-glucosidase activity was actually within this population. It is possible that this broad peak corresponding to α-glucosidase activity represents a subpopulation of specific granules. Indeed the 'tertiary' granules might be a subpopulation of specific granules. As shown in Fig. 1, lactoferrin and α-glucosidase activity significantly overlap. We know that phorbal myristate acetate, a specific-granule mobilizer (White & Estensen, 1974), causes the release, along with lactoferrin, of a portion of the α-glucosidase activity from viable neutrophils (P. Wang-Iverson, R. F. Rest & J. K. Spitznagel, unpublished work).

Two glucosidases were, apart from their cytosol activity, totally confined to the azurophil granules. α-Mannosidase activity followed the distribution of myeloperoxidase, with approx. 70% of its activity in the light azurophil granules. In contrast, α-galactosidase activity was found mostly (approx. 60%) in the heavy azurophil granules. This is the first time that an azurophil-granule-associated enzyme has been observed to be located mainly in the heavy azurophil granules. This information supports and extends that of others suggesting the heterogeneity of the two major populations of azurophil granules (West *et al.*, 1974; Kane & Peters, 1975). It indicates that the two azurophil-granule populations are truly distinct, i.e. that the heavy-azurophil-granule population does not simply represent aggregates of light azurophil granules, or that light azurophils are simply pieces of heavy azurophils. Since lysozyme is distributed between the specific and heavy azurophil granules, and lysozyme has been shown to be associated with lactoferrin in the specific granules (Leffell & Spitznagel, 1972), then lysozyme distridistribution in the heavy azurophils is similar to the distribution of α-galactosidase.

The data suggest that it is an oversimplification to generalize that most acid hydrolases are located in the azurophil granules. Glycosidases, as a group of acid hydrolases, are distributed throughout the different populations and subpopulations of both azurophil and specific granules. β-Glucuronidase, β-galactosidase, β-fucosidase, *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase are all located primarily in the azurophil granules, but also show measurable activity in the regions of the 'tertiary' and specific granules. For instance, when one closely studies the distribution of myeloperoxidase and *N*-acetylgalactosaminidase, an asymmetry is observed in the light-azurophil-granule peak. When one looks at the distribution of *N*-acetylglucosaminidase, α-fucosidase, β-glucuronidase and α-galactosidase, a double peak (or an asymmetry) is seen in the heavy azurophil granules. Thus not only are there two isopycnically distinct azurophil-granule populations, but each of these populations appears to be biochemically and physically heterogeneous.

The glycosidase activities in the cytosol fractions were probably not due to enzymes released from the granules for at least two reasons. In the same representative gradient (Fig. 1) less than 5% of lactoferrin and myeloperoxidase was released into the cytosol fractions, whereas 8–54% of the glycosidase activities was found in the cytosol fractions. Also, differences were observed in pH–activity profiles between granule- and cytosol-associated glycosidases when measured in sodium acetate buffer (Table 2), indicating possible differences in the enzymes themselves.

The results presented here can lead to a more complete interpretation of previous studies that used glycosidases such as β-glucuronidase, β-galactosidase or α-fucosidase as lysosomal markers. The fluorimetric determination of glycosidases is a rapid sensitive micro-method. Combined with the data and techniques presented here, glycosidase determinations can be used as accurate specific probes in studies involving enzymic composition, fate and function of neutrophil granules.

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