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STUDIES ON HUMAN POLYMORPHONUCLEAR LEUKOCYTE ENZYMES

II. COMPARATIVE STUDY OF THE PHYSICAL PROPERTIES OF PRIMARY AND SPECIFIC GRANULES

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SUMMARY

1. Seven distinct acid hydrolase activities present in cytoplasmic extracts from human polymorphonuclear leukocytes occur in latent form to the extent of 14–30% of their total activity, depending on the enzyme. This latency can be decreased or suppressed by exposure to Triton X-100 or to media of low osmotic pressure, by treatment in a Waring blender, by very acid pH and by freezing and thawing, but not by increasing the substrate concentration in the assay medium up to 16-fold the Michaelis constant of the enzymes. In preparations subjected to graded activating treatments, acid hydrolases are released in closely parallel fashion, suggesting that they are associated with particles possessing similar properties.

2. Acid phenylphosphatase and cyanide-insensitive NADH oxidase exhibited no latency under the conditions of the present experiments. It is concluded that, with the exception of acid phenylphosphatase activity, the acid hydrolases studied are associated with lysosome-like particles.

3. Alkaline phosphatase (EC 3.1.3.1) is also partly (44%) latent in cytoplasmic extracts of human polymorphonuclear leukocytes. Latent alkaline phosphatase can be released by some of the treatments that suppress the latency of the lysosomal enzymes, but differs from the latter by a complete insensitivity to exposure to media of low osmotic pressure and by an absolute requirement of an ionic media in order to maintain its latency. It is concluded from these results that the alkaline phosphatase-containing granules have some physical properties different from lysosomes.

INTRODUCTION

It has long been known that heterophil leukocytes contain two main groups of granules which differ from each other by their size and staining properties, also by their mode of formation and by the time at which they appear during the maturation of the cells[1,2]. Both types of granules have been separated in rabbit[3], guinea pig[4] and

in human polymorphonuclear leukocytes[5]. According to these results, the primary granules contain substantial amounts of acid hydrolases, which are characteristic components of lysosomes in numerous other cell types. Thus they may be considered true lysosomes. They contain also significant amounts of lysozyme (EC 3.2.1.17), peroxidase (EC 1.11.1.7) and bacteriocidal agents. The specific granules contain (at least in rabbit polymorphonuclear leukocytes[3]) essentially all the alkaline phosphatase (EC 3.1.3.1) activity and lactoferrin[6] of the preparation and considerable amounts of lysozyme. In the case of mature human polymorphonuclear leukocytes, it has been demonstrated by histochemical and cytochemical methods that alkaline phosphatase activity is restricted to the specific granules[7]. However, these granules are devoid of acid hydrolases and therefore do not qualify as lysosomes. Furthermore, their contents and function remain largely unknown.

The results described in the present paper show that specific granules exhibit the structure-linked latency characteristic of lysosomes.

MATERIALS AND METHODS

Unless otherwise stated, cytoplasmic extracts of particulate fractions of human polymorphonuclear leukocytes separated by previously described methods, were prepared in ice-cold 0.34 M sucrose–50 mM KCl–100 units heparin/ml by centrifuging a total homogenate at 250 × *g* (10 min). The resulting supernatant was separated with a Pasteur pipette and employed for further studies.

Enzyme assays

All enzymes were assayed according to the techniques described by Avila and Convit[8], at pH 5.0 for acid hydrolases, pH 6.5 for lysozyme, pH 6.0 for cyanide-insensitive NADH oxidase and pH 9.9 for alkaline phosphatase and alkaline cathepsin. However, due to their very low activities, α- and β-galactosidase (EC 3.2.1.22 and EC 3.2.1.23) were measured by fluorimetric techniques with 0.5 mM 4-methylumbelliferyl α-D-galactoside or β-D-galactoside in 100 mM acetate buffer (pH 5.0) in a final volume of 0.2 ml and for 60 min at 37 °C. The reaction was stopped with 3 ml of 133 mM glycine buffer (pH 10.7) and the fluorescence measured by the method of Ockerman[9].

Alkaline cathepsin. This was determined in a total volume of 1 ml of 50 mM carbonate buffer (pH 9.9) with urea-denatured haemoglobin as substrate. The reaction was stopped with 1 ml ice-cold 8% (w/v) trichloroacetic acid and then processed exactly as for acid cathepsin[8].

Free activities were measured in the presence of 0.34 M sucrose at 37 °C and total activities were assayed similarly but using an enzyme preparation homogenized in a Waring blender homogenizer at maximum speed for 80 s. This procedure gave similar results to those obtained with 0.1% Triton X-100.

The incubation time was 1 h, because it was found that polymorphonuclear leukocyte particles remain stable for this length of time in cytoplasmic extracts, under the assay conditions.

In other experiments, a large granular fraction was isolated by centrifuging a cytoplasmic extract at 59 000 × *g* for 10 min at 4 °C in a Beckman L2-65B centrifuge. The pellet which contained the granules was washed once and resuspended in a small quantity of cold medium. Samples of this suspension were suitably diluted with

media of given composition and incubated. At various times after the beginning of the experiment samples of the diluted suspension were centrifuged at 59 000 × *g* for 10 min. Part of the supernatant was collected and kept for enzyme assay. Another portion of the granules was treated for 80 s in a Waring Blender for measurement of total activities. When all the samples had been collected, their enzymic activities were determined, according to the techniques described previously[8], to provide an index of the amount of soluble enzyme present at the time the suspension was centrifuged.

RESULTS

Effect of the homogenizing medium

Various tests were performed in order to find out in which homogenizing medium the highest proportion of bound enzyme could be recovered. Several experiments were usually carried out with the same preparation. In preliminary experiments 0.34 M sucrose–100 units heparin/ml was used as homogenizing medium[8]. When subcellular fractionations were carried out in this medium about 68% of the alkaline phosphatase activity was found in the supernatant, while only 28% and 14% of the β-glucuronidase (EC 3.2.1.31) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) remained unsedimentable under the same conditions. This low proportion of the two acid hydrolases in the supernatant argues against a mechanical damage of the cytoplasmic granules during the homogenization or sonication procedures. The simplest explanation for the high alkaline phosphatase activity in the supernatant is that 0.34 M sucrose is in some way inducing the release of the enzyme from the granules with which it is associated. In order to study this phenomenon, we studied the intracellular distribution of alkaline phosphatase activity in the presence of several media (Table I).

It was found that when 0.15 M KCl was used only 31% of the alkaline phospha-

TABLE I

EFFECT OF NON-IONIC AND IONIC MEDIA ON THE PERCENTAGE OF β-GLUCURONIDASE, β-*N*-ACETYLGLUCOSAMINIDASE AND ALKALINE PHOSPHATASE ACTIVITIES PRESENT IN THE SUPERNATANT SEPARATED FROM A HUMAN POLYMORPHONUCLEAR LEUKOCYTE HOMOGENATE BY CENTRIFUGATION AT 59 000 × *g* (10 min)

The number in parentheses refer to numbers of experiments. Average percentage recovery was 97% for alkaline phosphatase, 96% for β-glucuronidase and 93% for β-*N*-acetylglucosaminidase

Media	*% in the supernatant*		
	Alkaline Phosphatase	*β-Glucuronidase*	*β-N-Acetyl-glucosaminidase*
0.25 M sucrose (3)	53	20	22
0.25 M sucrose–100 units heparin/ml (4)	53	21	23
0.34 M sucrose (8)	60	26	20
0.34 M sucrose–100 units heparin/ml (42)	68	28	14
0.15 M KCl (8)	31	25	25
0.15 M KCl–100 units heparin/ml (3)	34	19	20
0.34 M sucrose–50 mM KCl–100 units heparin/ml (6)	28	13	19

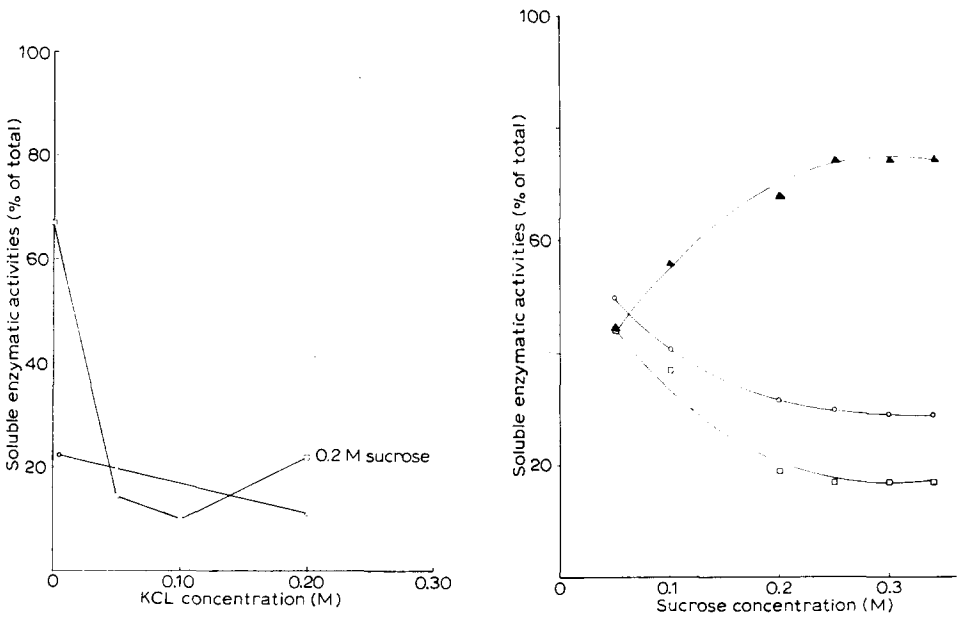


Fig. 1. Effect of an ionic media on soluble alkaline phosphatase activity. Cytoplasmic extracts were prepared either (a) in KCl only at the different molarities indicated in abscissa (○—○) or, (b) in a constant sucrose molarity (0.20 M) with changes in KCl molarities as indicated on the abscissa (□—□). After an incubation at 4 °C for 10 min the cytoplasmic extract was centrifuged at 59 600 × *g* (10 min). Results are expressed as the percentage of the total activity present in the supernatant after centrifugation.

Fig. 2. Influence of a non-ionic media on soluble enzymatic activities. Cytoplasmic extracts were prepared in different sucrose molarities and centrifuged at 59 600 × *g* (10 min). Results are expressed as the percentage of the total activity present in the supernatant after centrifugation. ▲—▲, alkaline phosphatase; ○—○, β-*N*-acetylglucosaminidase; □—□, β-glucuronidase.

TABLE II

LATENCY OF ENZYMES IN CYTOPLASMIC EXTRACT

Free activities, measured in 1-h assays, except for lysozyme (6-min assays) are expressed as percentages of the total activities measured in the same extract. Statistics refer to the means and S.D. The numbers of individual determinations are given in parentheses. Assays were carried out in 0.34 M sucrose–50 mM KCl–100 units heparin/ml.

Enzyme	*Free activity (% of total)*
β-Glucuronidase	23 ± 5 (13)
α-Galactosidase	25 ± 4 (4)
β-Galactosidase	30 ± 6 (4)
β-*N*-Acetylglucosaminidase	23 ± 5 (13)
Acid β-glycerophosphatase	22 ± 5 (6)
Acid cathepsin	20 ± 3 (4)
Lysozyme	14 ± 2 (6)
Alkaline cathepsin	32 ± 4 (5)
Alkaline phosphatase	44 ± 8 (14)
Acid phenylphosphatase	90 ± 6 (5)
NADH oxidase (cyanide-insensitive)	93 ± 4 (4)

tase, but about 25% of the β-glucuronidase and β-*N*-acetylglucosaminidase were found in the supernatant. Moreover, when different concentrations of KCl were used in combination with 0.20 M sucrose it was found that even 5 mM KCl was able to decrease the proportion of alkaline phosphatase activity in the supernatant (Fig. 1), suggesting either a protection by KCl against a sucrose-induced breakage of the secondary granules or an adsorption artifact. Evidence for the former possibility was the fact that when the free alkaline phosphatase activity was measured in a 0.34 M sucrose–50 mM KCl medium it was found that about 44% of the total activity was latent (Table II).

In order to know if sucrose or the non-ionic medium is responsible for the breakage of the specific granules, mannitol and maltose at the same concentrations were used. It was found that they are also able to induce the breakage of the specific granules (Table III).

These results give support to the concept of the absolute necessity of the specific

TABLE III

EFFECT OF NON-IONIC AND IONIC MEDIA ON THE PERCENTAGE OF β-GLUCURONIDASE, β-*N*-ACETYLGLUCOSAMINIDASE AND ALKALINE PHOSPHATASE ACTIVITIES PRESENT IN THE SUPERNATANT SEPARATED FROM A HUMAN POLYMORPHONUCLEAR LEUKOCYTE HOMOGENATE BY CENTRIFUGATION AT 59 000 × *g* (10 min)

The numbers in parentheses refer to numbers of experiments. Average percentage recovery was 90% for alkaline phosphatase, 88% for β-glucuronidase and 87% for β-*N*-acetylglucosaminidase.

Media	*% in the supernatant*		
	Alkaline phosphatase	*β-Glucuronidase*	*β-N-Acetyl-glucosaminidase*
0.25 M sucrose (3)	53	20	22
0.25 M maltose (2)	50	23	26
0.25 M mannitol (2)	51	26	24
0.15 M KCl (9)	31	25	25
0.25 M sucrose–50 mM KCl (2)	32	26	21
0.25 M maltose–50 mM KCl (2)	34	23	20
0.25 M mannitol–50 mM KCl (2)	33	22	24
0.34 M sucrose (3)	60	26	20
0.34 M sucrose–50 mM NaCl (2)	34	23	22
0.34 M sucrose–50 mM KCl (2)	26	24	24
0.34 M sucrose–50 mM sodium succinate (2)	31	20	22
0.34 M sucrose–50 mM Mg SO_4 (2)	31	21	21
0.34 M sucrose–50 mM $MgCl_2$ (2)	24	23	20
0.34 M sucrose–50 mM sodium acetate (3)	25	25	21

granules for an ionic medium in order to maintain their physical integrity and consequently their latency (see below).

In order to know if the protective effect of KCl on the integrity of secondary granules is a specific one, several ions were tested for their activities against secondary granules, always in the presence of 0.34 M sucrose (Table III). It was found that no specific ion is necessary to afford protection to the secondary granules.

Latency of enzymes

The values listed in Table II show that, except for acid phenylphosphatase, all

acid hydrolases under study displayed considerable latency in cytoplasmic extracts. No significant differences were observed between the free activities of the various enzymes. The free activity of acid phenylphosphatase and NADH oxidase was not significantly different from its total activity. Alkaline phosphatase also showed latency, though to a smaller degree than the acid hydrolases.

Effect of the osmotic pressure of the medium on the intracellular distribution of alkaline phosphatase, β-glucuronidase and β-N-acetylglucosaminidase

Fig. 2 shows that increasing the tonicity of the homogenization medium decreased the percentage of the β-glucuronidase and β-*N*-acetylglucosaminidase

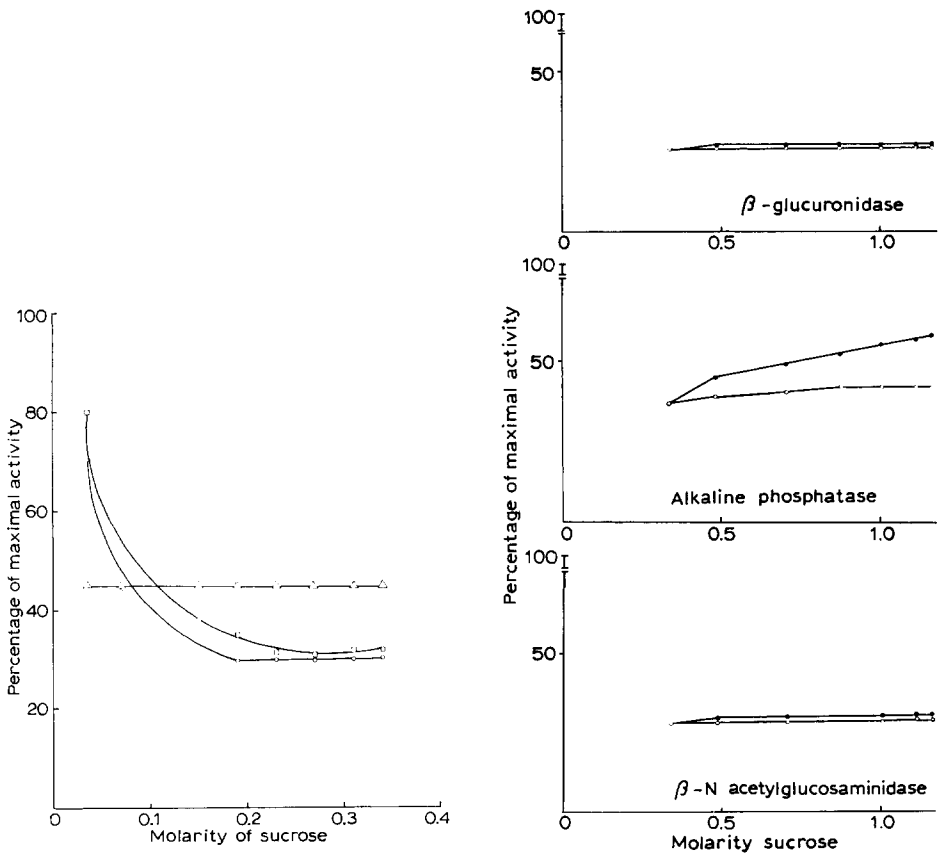


Fig. 3. Influence of hypotonic media on free activities. Sucrose solutions were prepared in 50 mM KCl instead of water. Cytoplasmic extracts were prepared in different sucrose molarities and incubated at pH 5.0 (acid hydrolases) and pH 9.9 (alkaline phosphatase) at 37 °C for 1 h in the presence of the substrates. Total activities were measured on blender-treated preparations. △—△, alkaline phosphatase; ○—○, β-glucuronidase; □--□, β-*N*-acetylglucosaminidase.

Fig. 4. Influence of sucrose concentration on free alkaline phosphatase, β-glucuronidase and β-*N*-acetylglucosaminidase activities of a human polymorphonuclear leukocyte cytoplasmic extract. Particles were exposed to the sucrose concentrations indicated on the abscissa for 15 min at 2 °C. After this treatment they were either kept at the same sucrose concentration (○—○); or returned to 0.34 M sucrose (●—●).

present in the supernatant, suggesting that sucrose affords an osmotic protection to primary granules. Fig. 3 shows the same results when measuring free activities. The acid hydrolases all exhibit a progressive loss of latency as the concentration of sucrose is decreased. Moreover, their osmotic activation curves run roughly parallel with each other. On the other hand, an entirely different behaviour was found in the case of alkaline phosphatase-bearing granules. High molarities of sucrose induced a release of the enzyme to the supernatant, showing that as the molarity of the non-ionic medium increases there is a simultaneous increase in the percentage of the alkaline phosphatase present in the supernatant. However, when we measured free activity in different molarities of sucrose, but always in the presence of 50 mM KCl, it was found that secondary granules are very resistant to hypotonic shock, remaining unaffected by the low sucrose molarities used in this experiment.

The above experiments do not mean that the concentration of sucrose can be widely changed without any effect on the specific granules. As shown in Fig. 4, an increase in free alkaline phosphatase is obtained when specific granules of a cytoplasmic extract are exposed to very high hypertonic sucrose for 15 min at 4 °C. Moreover, the activation of alkaline phosphatase is markedly increased if the granules are brought back in a 0.34 M medium after exposure to hypertonic sucrose. These results are easily explained if we admit that specific granules are freely permeable to sucrose. The increase in free alkaline phosphatase of particulates suspended in hypertonic sucrose upon dilution of the medium could be due to differences in the diffusion rates of sucrose and of water through the specific granule membrane.

It can also be seen from Table I and Table III that in 0.34 M sucrose–50 mM KCl–100 units heparin/ml the percentage of the unsedimentable activity of the different enzymes studied roughly parallels the percentage of free activity of these enzymes, suggesting that there is little, if any, secondary adsorption on the granules under our working conditions. This fact is due, perhaps, to the presence of heparin in our homogenization medium.

Influence of substrate concentration

In these experiments the free and total activity of a specific granule enzyme (alkaline phosphatase) and, for comparison, of a lysosomal enzyme (acid phosphatase (EC 3.1.3.2), was measured over a range of substrate concentrations extending from below the K_m to several times the K_m. The rationale for this type of experiment was explained by de Duve[10] and the method has been used to study the latency of rat spleen lysosomal β-glucuronidase[11], rat liver α-glucosidase (EC 3.2.1.20)[12], and bone acid hydrolases[13].

Experiments were carried out at the pH values 5.0 and 9.9 for acid and alkaline phosphatase, respectively, which correspond to the pH optima of the enzymes. Raising the concentration of β-glycerophosphate, even within the range where the enzyme is saturated, hardly causes a greater proportion of the total activity to appear as free enzyme. This fact is strong evidence of the essential impermeability of the primary and secondary granules to β-glycerophosphate. However, the average percentage of free activity is about 39% for alkaline phosphatase but of 20% for acid phosphatase (Table IV).

TABLE IV

INFLUENCE OF SUBSTRATE CONCENTRATION ON FREE ACTIVITY OF ALKALINE AND ACID PHOSPHATASES

Free activities measured on cytoplasmic extracts, incubated 1 h at 37 °C in 0.34 M sucrose and 50 mM acetate buffer (pH 5.0) for acid β-glycerophosphatase or 50 mM carbonate buffer (pH 9.9) for alkaline phosphatase; total activities measured similarly on blender-treated cytoplasmic extract. Results are expressed as percentages of the total activities measured in the same extract.

β-Glycerophosphate (mM)	*Free activity (% of total activity)*	
	Alkaline	*Acid*
10	37	20
20	39	21
40	42	20
60	41	19
80	38	21
100	38	22
120	36	20
160	42	20

Effect of detergents and effect of Triton X-100

Acid hydrolases were released in a closely parallel fashion, the free activity approaching the total activity at a detergent concentration of about 0.01%. Higher concentrations of Triton X-100 up to about 0.02% were not inhibitory to the enzymes, and the shape of the curves indicated clearly that the detergent concentration of 0.01%

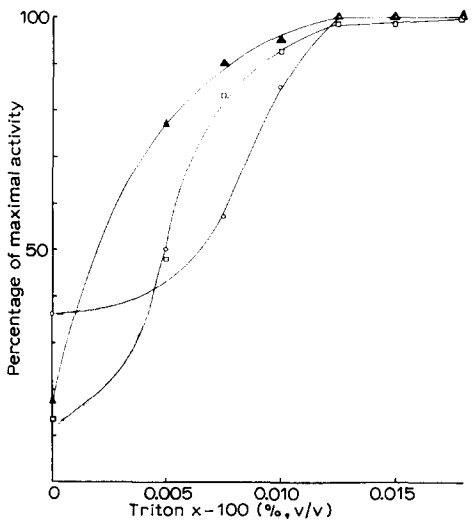


Fig. 5. Influence of Triton X-100 concentration in incubation medium on free activities. ○—○, alkaline phosphatase; ▲—▲, β-glucuronidase; □—□, β-*N*-acetylglucosaminidase. Free activities were measured in 1-h assays in the presence of Triton X-100 at the concentration shown on the abscissa. The amounts of cytoplasmic extract added corresponded to 15.3 and 3 mg of original polymorphonuclear leukocyte per ml of incubation medium for alkaline phosphatase, β-glucuronidase and β-*N*-acetylglucosaminidase, respectively. Results are expressed as percentage of the highest observed activities.

chosen for the assays of total activity in some of our experiments was amply sufficient to unmask the latent hydrolases completely. From these results it can be seen also that human polymorphonuclear leukocyte lysosomes are very sensitive to Triton X-100, because liver[14] and bone lysosomes[13] require at least 0.04% and 0.03% of Triton X-100, respectively, to obtain total activation. However, human polymorphonuclear leukocyte lysosomes are similar to guinea pig polymorphonuclear leukocyte lysosomes which are totally activated at 0.01% Triton X-100[4]. Specific granules behaved similarly to primary granules showing total activation at 0.01% Triton X-100 (Fig. 5). Similar results have also been reported for guinea pig specific granules[4].

Effect of digitonin

When digitonin in the range 0–1 mg/ml was used to activate primary and specific granules, it was found that in the case of specific granules 100% activation was obtained at 0.1 mg/ml digitonin, while at this concentration 70–80% of the β-*N*-acetylglucosaminidase and β-glucuronidase activity was released (Fig. 6).

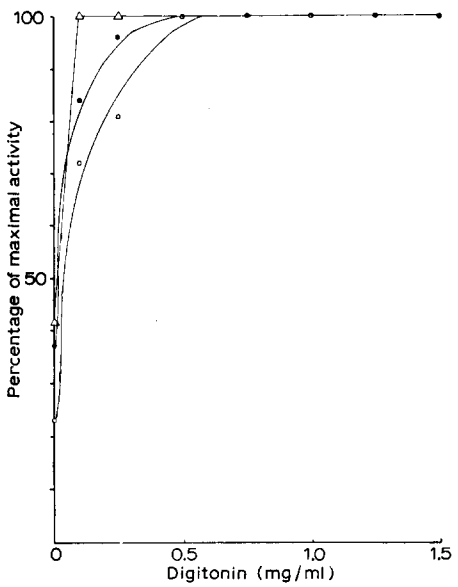
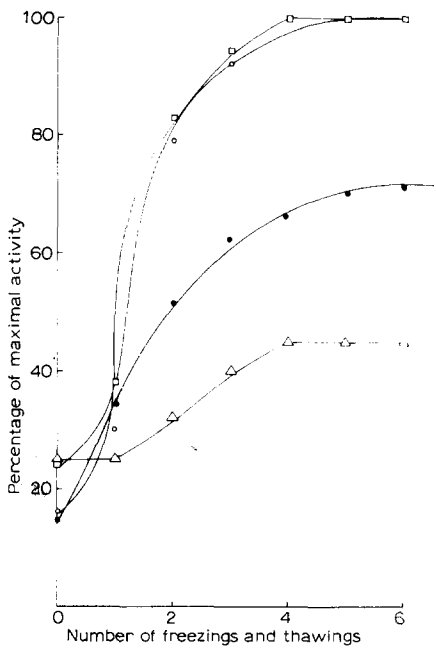


Fig. 6. Release of acid hydrolases and alkaline phosphatase by increasing concentration of digitonin. Free activities were measured in the presence of digitonin at the concentrations indicated, with an amount of cytoplasmic extract corresponding to 6 mg of original polymorphonuclear leukocyte per ml. Results are expressed as percentage of highest observed activity △—△, alkaline phosphatase; ○—○, β-glucuronidase; ●—●, β-*N*-acetylglucosaminidase.

Freezing and thawing

Fig. 7 shows that a similar parallelism obtained when repeated freezing and thawing was used to release β-*N*-acetylglucosaminidase and β-glucuronidase. After four cycles of freezing and thawing about 100% of the former enzymes were released, but only 44% of the alkaline phosphatase activity. Lysozyme which, at least in rabbit polymorphonuclear leukocytes, is present in both types of granules[15], was released in



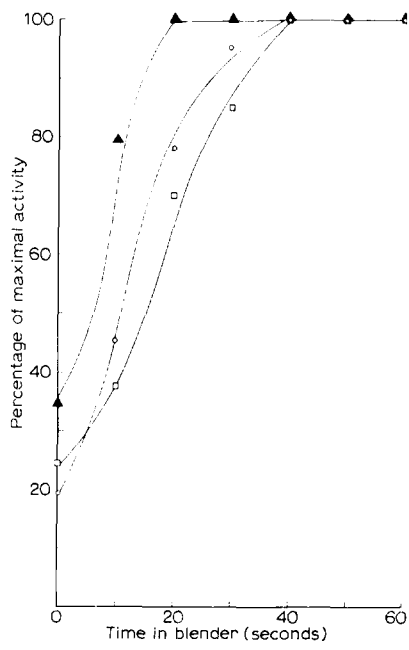


Fig. 7. Activation of acid hydrolases and alkaline phosphatase by repeated freezing and thawing. Cytoplasmic extracts in 0.34 M sucrose–50 mM KCl were frozen and thawed an increasing number of times. △—△, alkaline phosphatase; ○—○, β-*N*-acetylglucosaminidase; □—□, β-glucuronidase; ●—●, lysozyme.

Fig. 8. Activation of acid hydrolases and alkaline phosphatase by disintegration of particles in a Waring blender. Cytoplasmic extracts in 0.34 M sucrose–50 mM KCl were treated in an Ultra-Turrax running at 20 000 rev./min. for increasing periods of time. ▲—▲, alkaline phosphatase; ○—○, β-*N*-acetylglucosaminidase; □—□, β-glucuronidase.

TABLE V

INFLUENCE OF TREATMENT IN THE WARING BLENDER ON SEDIMENTABILITY OF ACID HYDROLASES AND ALKALINE PHOSPHATASE

The cytoplasmic extract was treated for 80 s in a cooled Waring blender. A sample of this preparation was centrifuged at 300 000 × *g* (10 min). The supernatant was assayed for the total enzyme activities: these are given as percentages of the corresponding activities measured on non-centrifuged samples. 10–15% losses were recorded for all enzymes in these experiments.

Enzyme	*Unsedimentable activity (% of total)*	
	Control	*Treatment in Waring blender*
β-Galactosidase	12	58
α-Galactosidase	19	64
β-Glucuronidase	23	54
N-Acetyl-β-glucosaminidase	22	63
Alkaline phosphatase	40	78
Acid β-glycerophosphatase	26	59
Alkaline cathepsin	15	38

an intermediate form, between the acid hydrolases pattern and the alkaline phosphatase.

Mechanical disruption

Treatment of a cytoplasmic extract in a Waring blender for increasing lengths of time led to a progressive loss of latency of the two acid hydrolases measured and of alkaline phosphatase (Fig. 8) and their partial release in soluble form (Table V). In this experiment alkaline phosphatase was more readily activated than the lysosomal hydrolases.

Influence of temperature

Fig. 9 (a) illustrates the influence on the free activity of the acid hydrolases and alkaline phosphatase of the incubation at different temperatures of a cytoplasmic extract in the presence of the enzyme substrates and at pH values 5.0 and 9.9, respectively. It is clear that the latency phenomenon for both types of granules is highly

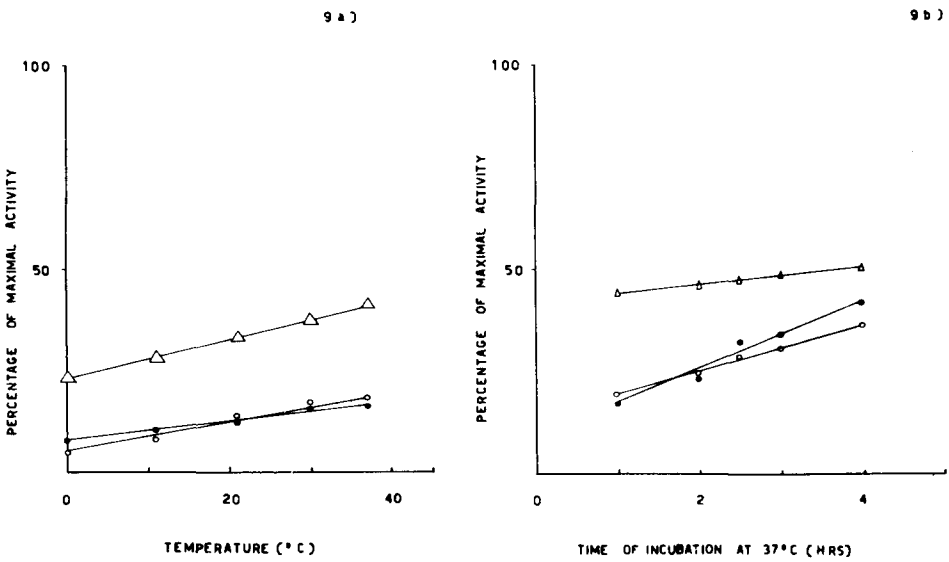


Fig. 9. (a) Influence of the preincubation at different temperatures on free activities measured at pH 5.0 (acid hydrolases) and pH 9.9 (alkaline phosphatase). (b) Influence of time of incubation on free activities of β-glucuronidase (○—○); β-*N*-acetylglucosaminidase (●—●); alkaline phosphatase (△—△); in cytoplasmic extract of human polymorphonuclear leukocytes. Free activity was measured on intact granules incubated in 0.34 M sucrose–10 mM KCl. Total activities were measured similarly on blender-treated granules.

sensitive to an increase in temperature and that the thermal release of the acid hydrolases occurs in a parallel fashion. Regarding the effect of the incubation time on free activities, it can also be seen that acid hydrolase and alkaline phosphatase activities suffered a discrete loss of latency during incubation at pH values 5.0 and 9.9, respectively, and 37 °C (Fig. 9 (b)).

However, human polymorphonuclear leukocyte lysosomes appear to differ from liver lysosomes by a greater stability at pH 5.0 and 37 °C. They resemble those of rat spleen and thymus[16] and bone lysosomes[13] in this respect.

Influence of pH on free and total activities

Equal quantities of a cytoplasmic extract were incubated at 37 °C in individual test tubes containing 0.34 M sucrose and either 0.1 M glycine–NaOH buffer (range 8.4–10.6) or 0.1 M acetate buffer (range 3.3–6.0). The free activity was measured by incubating the enzyme preparation with the substrates for 1 h. Similar tests were made on blender-treated preparations. As is shown in Fig. 10, a difference between free and total enzyme becomes evident when the pH is varied. As a result in the case of acid hydrolases the proportion of total enzyme estimated as free activity is not constant, but varies from 100%, in the pH range 3.3–4.4, to 23–31%, at pH 6.0. An increase in the rate of destruction of lysosomes during the assay can easily account for

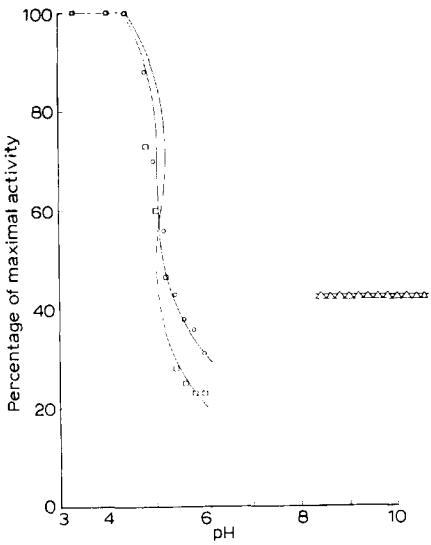


Fig. 10. Influence of pH on free activities. For experimental conditions see text. ○—○, β-*N*-acetylglucosaminidase; □—□, β-glucuronidase; △—△, alkaline phosphatase.

these results, since a less acid pH value should have a protective effect on lysosomes. Additional evidence for this possibility was given by the preceding experiments, in which it was demonstrated that the proportion of free activities of the acid hydrolases is directly related to the temperature of incubation of the granules.

Surprising are the results for alkaline phosphatase, assayed in the pH range 8.4–10.6. It was found that for this enzyme the proportion of free activity in the pH range 8.4–10.6 remains constant at about 46%. If, as we have suggested for primary granules, the loss of the latency of acid hydrolases could be attributed to autolytic processes, we must postulate either that there is no alkaline hydrolytic activity at this pH range in a human polymorphonuclear leukocyte homogenate or that specific granule membranes are strongly resistant to autolytic processes, at least under our working conditions. Evidence against the former hypothesis are the findings of alkaline cathepsin activity (see below) and of phospholipase activity in human polymorphonuclear leukocytes[17], both enzymes with optimum pH values in the range used in our experiments. Furthermore, our experiments on the effect of temperature

on free activities suggest that there must exist some autolytic processes at alkaline pHs in order to explain the increase in alkaline phosphatase free activities. Concerning the second hypothesis, experiments are actually in progress in order to test this possibility.

DISCUSSION

The present study of human polymorphonuclear leukocyte granules describes different experimental conditions designed to gain insight into the physical properties of human polymorphonuclear leukocyte primary and specific granules. In general our results show that: (a) seven distinct acid hydrolase activities and alkaline phosphatase and alkaline cathepsin occur in partly latent form in cytoplasmic extracts of human polymorphonuclear leukocytes and that latency belongs exclusively to the particle-bound form of the enzyme, (b) they suggest a lysosome-like structure for the specific granules, with some characteristic properties of the specific granule membrane, and (c) great similarities in the response of the membranes of both types of granules under the same experimental conditions suggesting that some chemical similarities must exist between them. These facts perhaps can give a biochemical explanation of the participation of both types of granules in the phagocytic process, in which they tend to fuse with the same phagocytic vacuole.

The latency of alkaline phosphatase and acid hydrolases provided a method for investigating some of the properties of specific granules and lysosomes in suspension by following the activation of the enzymes when the granules were exposed to various experimental conditions. Such experiments do not require purified fractions and permit comparisons between specific granules and lysosomes.

In their response to the various treatments that suppress enzyme latency, primary granules behave very much like typical lysosomes. In particular they were susceptible to osmotic disruption and are sensitive to treatments known to affect the integrity of lipoproteins.

In order to study the permeability characteristics of specific granules, alkaline phosphatase was used as marker enzyme for these granules. This enzyme showed latency, although less than those found for lysosomal enzymes.

Different activating procedures showed similarities between lysosomes and specific granules; for example specific granules were also activated by detergents, freezing and thawing and mechanical disruption in a similar fashion to lysosomes. However specific granules were characterized by their absolute requirement of an ionic medium in order to maintain membrane integrity, as shown by solubilization and a decrease in the latency of alkaline phosphatase in non-ionic media. Specific granules were also found to be freely permeable to sucrose and to be sensitive to damage of the external membrane by high osmotic pressure, the primary granules being impermeable to sucrose and unaffected by high osmotic pressure.

From the comparative analysis of human polymorphonuclear leukocyte primary and specific granules it seems that our findings are consistent with a first approximation model of specific granules as a bag-like structure whose membrane is impermeable to substrates, having no osmotic space impermeable to sucrose. However, when subjected to similar conditions, in some cases the specific granules mem-

brane showed a quite different behaviour from that of primary granule membrane, suggesting a quite different biochemical composition. These results are perhaps due to the fact that primary granules arise from the concave face of the Golgi complex during the promyelocyte stage, while specific granules are produced later, during the myelocyte stage arising from the opposite or convex face of the Golgi complex. The demonstration of distinct enzymic contents between primary and specific granules, coupled with the persistence of both types of granules in mature polymorphonuclear leukocytes suggests that the different granules may have independent functions[7].

In our experiments no latency for cyanide-insensitive NADH oxidase activity was found. This fact could be easily explained by the subcellular localization of this enzyme, which has been shown to be loosely attached to the outer side of granule membranes[8]. No latency was found either for acid phenylphosphatase activity, which has been shown to be located in membranes of very low density when subjected to density gradient centrifugation (Avila, J. L. and Convit, J., unpublished). Finally, concerning alkaline cathepsin we have found latency for this enzyme activity. In preliminary experiments, we have found that, when submitted to subcellular fractionation procedures, the alkaline cathepsin pattern does not fit the distribution pattern of alkaline phosphatase, suggesting a different subcellular localization. A logical conclusion of our experiments is that in order to prepare a human polymorphonuclear leukocyte homogenate suitable for subcellular fractionation studies, an ionic medium must be used to protect specific granules.

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