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STUDIES ON HUMAN POLYMORPHONUCLEAR LEUKOCYTE ENZYMES

I. ASSAY OF ACID HYDROLASES AND OTHER ENZYMES

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SUMMARY

Eight acid hydrolases, peroxidase (EC 1.11.1.7), cyanide-insensitive NADH oxidase and alkaline phosphatase (EC 3.1.3.1) were demonstrated in homogenates of human polymorphonuclear leukocytes. The main kinetic characters of these enzymes have been studied and methods for their quantitative assay have been developed. The activities present in human leukocytes are given and compared with those found in rabbit heterophil leukocytes. Homogenates were separated by differential centrifugation into two particulate fractions and a soluble fraction. The influence of the homogenization medium on the distribution of protein, DNA, cyanide-insensitive NADH oxidase and acid β-glycerophosphatase in these fractions was studied.

INTRODUCTION

The circulating white blood cell is a convenient system for biochemical study in humans due to its availability for analysis. Improved techniques in the isolation of human white cells from peripheral blood have stimulated a search for differences between leukocytes in health and disease[1–3].

The role of the polymorphonuclear leukocytes in host defense reactions is intimately related to their ability to engulf and destroy a wide variety of microorganisms. Leukocytes contain a variety of hydrolytic enzymes and substances which are directly responsible for the inactivation and digestion of bacteria and other engulfed particles. On the other hand, leukocytes represent one of the richest sources of many of the acid hydrolases known to be associated with lysosomes in other tissues, but thus far data about hydrolytic enzymes in human polymorphonuclear leukocytes have been limited to only a small number of enzymes[4–7].

Our investigations were initiated with the aim of obtaining more complete, accurate, information concerning the existence, properties and function of lysosomes in human polymorphonuclear leukocytes.

METHODS

Isolation of polymorphonuclear leukocytes

500 ml of venous blood were taken from fasting normal subjects in acid-citrate-dextrose (Formula A), and 10–20 min later dextran (mol. wt 208 000, Pharmacia, Uppsala, Sweden) was added to a concentration of 2%, and the cells were allowed to sediment for 45 min at 37 °C. The leukocyte-rich supernatant was separated from the bulk of the sedimented erythrocytes, and centrifuged for 10 min at 150 × *g* in a refrigerated International PR-6 centrifuge, using rotor 259. Remaining red blood cells were removed by hypotonic lysis with 0.2% NaCl followed in 30 s by the same volume of 1.61% NaCl. The leukocytes were separated from the haemolyzed material by centrifugation at 150 × *g* for 10 min at 4 °C, followed by aspiration of any red cell ghosts remaining on the surface of the white cell layer. This procedure was repeated twice, giving finally about 1.0–1.4 g of leukocytes (wet wt). The isolated cells consisted of approximately 90–95% polymorphonuclear leukocytes, the rest were mononuclear cells (with less than 0.6% of monocytes).

Enzyme assays and analytical procedures

The enzyme assays were carried out at 37 °C for all the enzymes, except for peroxidase (EC 1.11.1.7) and lysozyme (EC 3.2.1.17), which were determined at 25 °C. In the present experiments, which deal exclusively with the measurements of total enzymic activities, Triton X-100 (0.1%) was added in all assays of these enzymes, except peroxidase.

Appropiate blanks, in which the enzyme (or for acid β-glycerophosphatase and alkaline β-glycerophosphatase, the substrate) was added to the incubation flasks only after the enzymic reaction was stopped, were always run in parallel to the tests and subtracted from the observed values.

Acid phosphatase (EC 3.1.3.2). Determinations of acid phosphatase activity were done by two different methods. In one, the amount of phosphate liberated from β-glycerophosphate (β-glycerophosphatase activity) was measured. This activity was assayed in a total volume of 1 ml in the presence of 50 mM β-glycerophosphate and 50 mM acetate buffer (pH 5.0). The reaction was stopped by the addition of 5 ml of 8% (w/v) trichloroacetic acid. Inorganic phosphate was determined in 1–2 ml of filtrate by the method of Chen *et al.*[8]. Acid phenylphosphatase activity was measured in 50 mM acetate buffer (pH 5.0) with 50 mM phenylphosphate in a total incubation volume of 1 ml. The reaction was stopped by the addition of 0.5 ml of 0.5 M NaOH. Free phenol was determined by its reaction with aminoantipyrine by the method of Kind and King[9]. The absorbance of the final coloured product was read at the maximum absorption wavelength, 510 nm, exactly 5 min after addition of the ferricyanide, since the final colour appeared to fade with time.

β-Galactosidase (EC 3.2.1.23); was determined in a total volume of 0.8 ml of 125 mM acetate buffer (pH 5.0), with 3.1 mM *o*-nitrophenyl β-D-galactoside as substrate. The reaction was stopped by the addition of 3 ml of 3.2% trichloroacetic acid and the *o*-nitrophenol was measured as reported by Sellinger *et al.*[10]. The colour exhibited by *o*-nitrophenol was stable for at least 2 h; it was read at 420 nm, the maximal absorption wavelength.

α-Galactosidase (EC 3.2.1.22): was assayed in a total volume of 0.8 ml of 25 mM

acetate buffer (pH 5.0), with 5 mM *o*-nitrophenyl-α-D-galactoside as substrate. The reaction was stopped as described for β-glucuronidase. The mixture was then filtered and its *o*-nitrophenol content was determined at 420 nm.

Cathepsin. This activity was assayed at pH 3.6 in a total volume of 1 ml of 0.37 mM (or 2.5% w/v) denatured haemoglobin as substrate in 0.1 M acetic acid. The reaction was stopped by the addition of 1 ml of ice-cold 8% (w/v) trichloroacetic acid; the mixture was immediately cooled in ice and filtered in the cold room. Aromatic degradation products were measured in 0.5 ml of filtrate by the protein method of Lowry *et al.*[11]. Tyrosine was used as standard and the molarity of the products of cathepsin action was expressed conventionally in terms of tyrosine equivalents of the colour developed with the Folin–Ciocalteu reagent.

β-Glucuronidase (EC 3.2.1.31): was measured in a total volume of 1 ml of 50 mM acetate buffer (pH 5.0), with 1.0 mM phenolphthalein glucuronidate as substrate. The reaction was stopped by the addition of 3 ml of a solution containing glycine (133 mM), NaCl (67 mM) and Na_2CO_3 (83 mM), pH 10.7.

The mixture was filtered and its phenolphthalein content was determined at 540 nm. All readings were made at the same time after the addition of the glycine–carbonate buffer, since the colour appeared to fade slowly with time (about 10% loss in 1 h).

β-N-Acetylglucosaminidase (EC 3.2.1.30). This activity was determined in a total volume of 1 ml of acetate buffer (pH 5.0), with 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate. The reaction was stopped as for β-glucuronidase and readings were taken at 400 nm.

Lysozyme: was assayed with *Micrococcus lysodeikticus* as substrate at a final concentration of 20%, according to the method of Prasad and Litwack[12] in a total volume of 3.4 ml.

Peroxidase. The assay was based on the method of Baggiolini *et al.*[13]. The substrate solution contained 10 ml of 0.1 M citric acid–sodium citrate buffer (pH 5.0), 1 ml of 0.1% *o*-tolidine in ethanol and 1 ml of 1.5 mM H_2O_2. 3 ml of the substrate solution was pipetted into a spectrophotometer cuvette, to which 0.4 ml of the enzyme preparation was added. Transmission at 440 nm was recorded on log paper for 3 min.

Alkaline phosphatase (EC 3.1.3.1.). Assays of this activity were done by using two different substrates. In one the amount of phosphate liberated from β-glycerophosphate (alkaline β-glycerophosphatase) was measured. This activity was assayed in a total volume of 1 ml in the presence of 0.1 M β-glycerophosphate and 50 mM carbonate buffer (pH 9.9). The reaction was stopped by the addition of 5 ml of 8% (w/v) trichloroacetic acid. Inorganic phosphate was determined as for acid β-glycerophosphatase. When phenyl phosphate was used as substrate, this activity (alkaline phenylphosphatase) was assayed, like acid phenylphosphatase, in 1 ml of incubation mixture containing 8 mM phenyl phosphate in 50 mM carbonate buffer (pH 9.9). The reaction was stopped by the addition of 0.5 ml of 3.2% trichloroacetic acid.

NADH oxidase (cyanide-insensitive): was measured in 50 mM acetate buffer (pH 6.0), with 1 mM NADH, 1 mM KCN and 1 mM nitroblue tetrazolium in a total volume of 1 ml. The reaction was stopped by adding 1 ml of 2 M HCl. The blue-coloured formazan was extracted with 2 ml of pyridine and the absorbance determined at 560 nm. A standard curve was constructed from known amounts of for-

mazan prepared by treatment of nitroblue tetrazolium with alkaline sodium ascorbate. In preliminary experiments we used phosphate buffer (pH 6.0), but due to the high control blanks we prefered to use acetate buffer.

Chemical determinations. DNA was determined by the method of Schneider[14] and protein by the method of Lowry *et al.*[11], with bovine serum albumin as the standard.

Preparation of homogenates

The cell pellet resulting from the last hypotonic osmotic shock was drained and enough cold homogenization medium added rapidly to obtain a 4% (w/v) homogenate. Resuspension of the cells was then accomplished by vigorous pipetting of the homogenization medium and stirring with a plastic rod until a smooth suspension resulted. Occasionally, small gross clumps of cells were present after pipetting and stirring. Under these circumstances 2 to 3 strokes with a chilled teflon pestle were required to disperse the cells. The suspension was then sonicated for 20 s with a Branson sonifier set in position 3 (60 W), with the standard probe. This procedure was applied irrespective of the degree of cell disruption achieved during resuspension.

Simplified subcellular fractionation procedure

In order to study the effects of the composition of the homogenization medium on the intracellular distribution of some substances and enzymes, we have devised the following fractionation scheme: after cell disruption had taken place, the lysate was homogenized 3 times (30–45 s each) with a motor-driven all-glass tissue grinder (type 4228-B; supplied by A. H. Thomas Co., Philadelphia, Pa., U.S.A.). Then the homogenate was dispensed into chilled 25-ml centrifuge tubes and centrifuged for 20 min at 250 × *g* in an International PR-6 centrifuge (rotor 259). This resulted in the sedimentation of intact nuclei and a portion of the larger granules, depending on the medium composition (see Results). The supernatant was pale green in appearance and contained large numbers of discrete granules. The granule-rich supernatant was then removed by decantation and the nuclear pellet resuspended to known volume with the homogenization medium. The resulting supernatant was then transferred to chilled tubes and centrifuged at 59 000 × *g* for 10 min at 4 °C in a Beckman L2-65 B centrifuge. This resulted in a firmly packed pale green-white pellet and clear post-granular supernatant. Occasionally a small quantity of fluffy reddish material was found on top of the pellet. When present it was added to the granule fraction. The 59 000 × *g* pellet which contained the granules was gently resuspended in a known volume of medium and employed for further studies.

RESULTS

Observations on the disruption of the leukocytes

Human polymorphonuclear leukocytes are difficult to disrupt in a controlled manner. Conventional homogenization with a motor-driven all-glass homogenizer caused inadequate cell breakage, whether in 0.25 or 0.34 M sucrose or in 0.15 M KCl. On the other hand, sonication under the conditions described above produced virtually 100% lysis, as measured by direct haemocytometer counting techniques.

When the leukocytes were resuspended in 0.34 (or 0.25) M sucrose containing

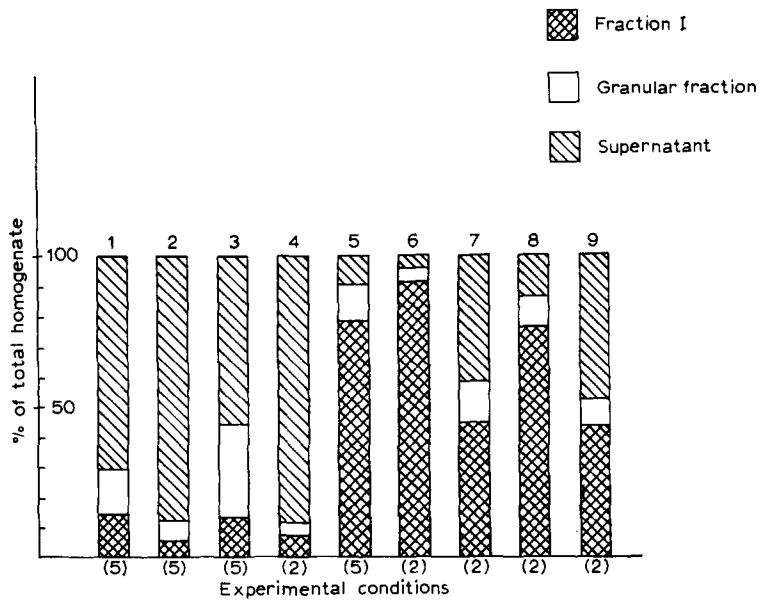

Fig. 1. Influence of different homogenization media on the distribution of proteins between subcellular fractions separated from a human polymorphonuclear leukocyte homogenate. The separation procedure was described in Experimental under *Simplified subcellular fractionation procedure*. The different experimental conditions studied were: (1) 0.34 M sucrose; (2) 0.34 M sucrose–100 units/ml heparin; (3) 0.25 M sucrose; (4) 0.25 M sucrose–100 units/ml heparin; (5) 0.15 M KCl; (6) 0.15 M KCl–10 mM imidazole buffer (pH 7.0); (7) 0.34 M sucrose–1 mM EDTA; (8) 0.34 M sucrose–3 mM EDTA (pH 7.0); (9) 0.34 M sucrose–10 mM imidazole (pH 6.8); (10) 0.15 M KCl–100 units/ml heparin. The number in parentheses under each bar refer to the number of experiments. Average recovery was 98%.

100 units of heparin per ml an abrupt increase occurred in the viscosity of the suspension. Microscopic examination of such suspensions showed that the majority of the leukocytes were disrupted, liberating their cytoplasmic granules. The granules were well dispersed and showed no evidence of agglutination.

Effect of the medium composition on the subcellular distribution of total proteins, DNA, NADH oxidase and acid β-glycerophosphatase

Figs. 1–4 show the distributions observed for protein, DNA, NADH oxidase and acid β-glycerophosphatase in different homogenization media. It appears from the DNA distribution, as well as from microscopic observations, that the nuclei were largely disrupted in sucrose solutions of low ionic strength; they were better preserved in 0.34 M sucrose containing EDTA or imidazole buffer; even better so in 0.15 M KCl. However, the conditions giving better preservation of nuclei also caused significantly larger amounts of protein, NADH oxidase and acid phosphatase to sediment with Fraction I, which showed clear evidence of agglutination. When heparin was present in the medium, Fraction I contained only small quantities of all four components tested and the supernatant fraction was particularly rich in them, except for acid phosphatase; the granule fraction had a particularly high specific acid phosphatase activity and low DNA content, and it also contained the

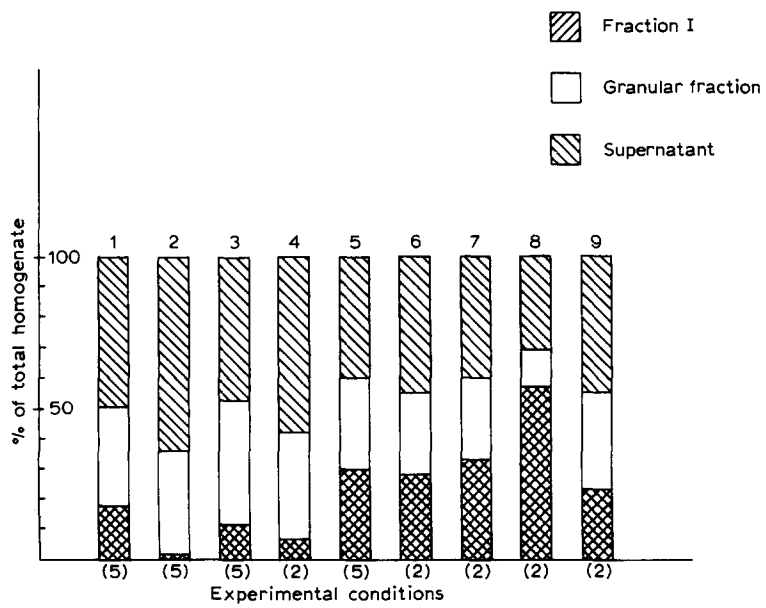

Fig. 2. Effect of different homogenization media on the distribution of DNA between subcellular fractions separated from a human polymorphonuclear leukocyte homogenate. For more details concerning experimental conditions see legend of Fig. 1. Average recovery was 96%.

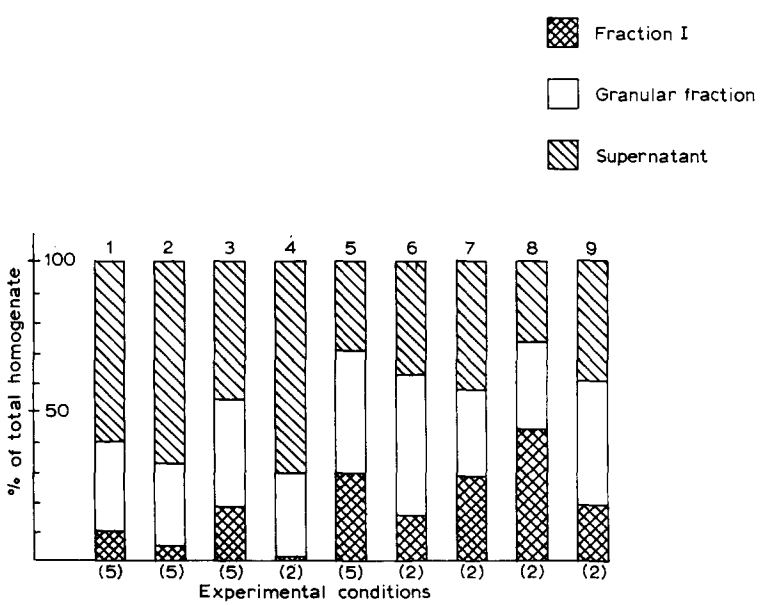

Fig. 3. Effect of different homogenization media on the distribution of cyanide-insensitive NADH oxidase between subcellular fractions separated from a human polymorphonuclear leukocyte homogenate. For more details concerning experimental conditions see legend of Fig. 1. Average recovery was 102%.

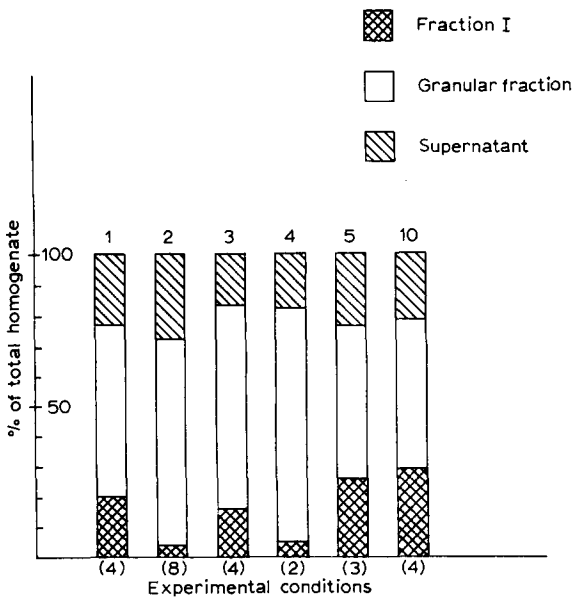

Fig. 4. Effect of different homogenization media on the distribution of acid β-glycerophosphatase activity between subcellular fractions separated from a human polymorphonuclear leukocyte homogenate. For more details concerning experimental conditions see legend of Fig. 1. Average recovery was 99%.

highest yield of acid β-glycerophosphatase (almost 80% in 0.34 M sucrose containing 100 units of heparin per ml).

Kinetics and assay of enzymes

Effect of pH. All eight hydrolases investigated displayed significant activity in the acid pH range (Fig. 5). Optimum activity was obtained between pH 4.5 and 5.5 for β-glucuronidase, cathepsin, α-galactosidase, β-galactosidase, acid β-glycerophosphatase and acid phenylphosphatase; and between pH 5.5 and 6.2 for β-*N*-acetylglucosaminidase and lysozyme. However, α-galactosidase activity showed a smaller peak of activity at pH 3.6. The phosphatase activities show a second peak near pH 10.0 and an intermediate one around pH 7.0. The optimum pH is 5.6 for peroxidase and 6.5 for NADH oxidase.

Activations and inhibitions. Alkaline β-glycerophosphatase was strongly inhibited by 0.3 M *meso*-tartrate but no inhibition was found in the presence of 0.3 M NaCl. Fluoride did not affect this enzyme activity. When a mechanically disrupted polymorphonuclear leucocyte granule preparation was assayed for NADH oxidase and alkaline β-glycerophosphatase activities and incubated in the presence of different concentrations of Triton X-100, a strong stimulation of both enzyme activities was found at 0.03% Triton X-100. The other enzymes studied were not affected by Triton X-100.

Effect of heparin. 100 units/ml heparin induced an irreversible inhibition of *N*-acetylglucosaminidase (53% of control), β-glucuronidase (52%), peroxidase (84%), acid β-glycerophosphatase (70%) and acid phenylphosphatase (56%).

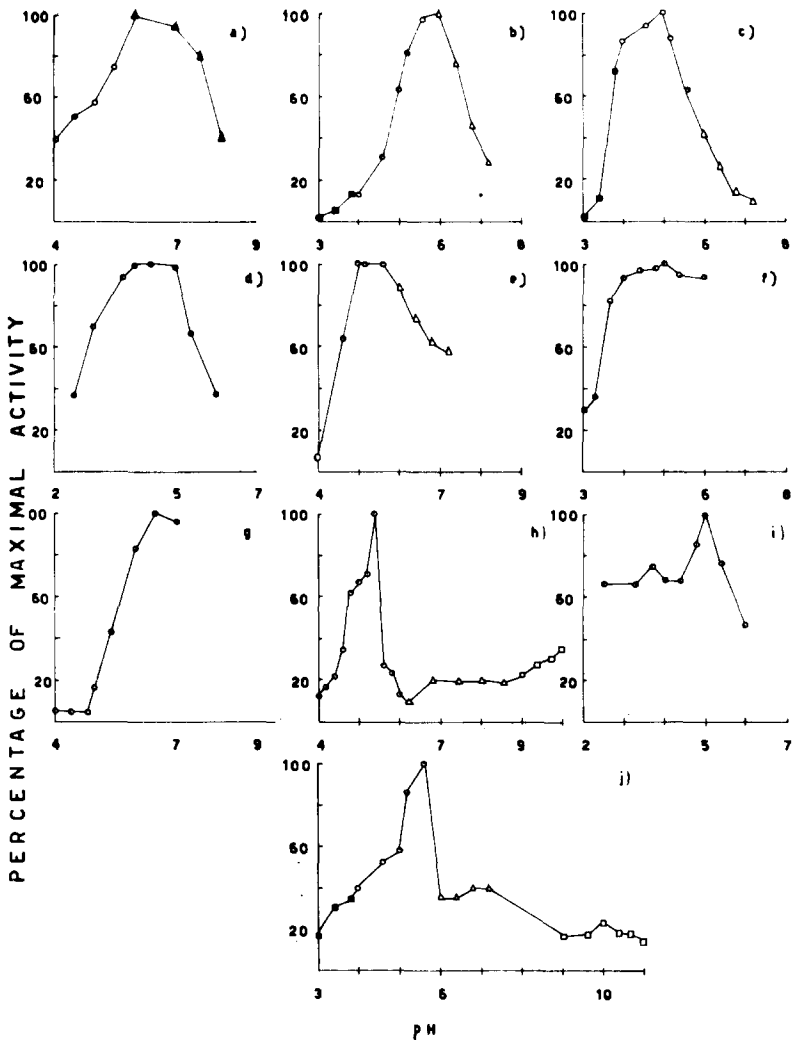

Fig. 5. Influence of pH on hydrolytic activities in leukocyte homogenate: (a) lysozyme; (b) β-*N*-acetylglucosaminidase; (c) β-glucuronidase; (d) β-galactosidase; (e) peroxidase; (f) cathepsin; (g) NADH oxidase (cyanide-insensitive); (h) phenylphosphatase; (i) α-galactosidase; (j) β-glycerophosphatase. Incubations were carried out in 50 mM or 100 mM concentrations of the following buffers: sodium citrate–citric acid (■); sodium cacodylate–HCl (△); sodium acetate–acetic acid (○); sodium bicarbonate–NaOH (□); Na_2HPO_4–Na_3PO_4 (▲); citric acid–Na_2HPO_4 (●). The haemoglobin used in the cathepsin assays was first denatured by heating for 30 min at 37 °C at pH 3.6 and then brought to the required pH.

Comparison of α- and β-galactosidase. β-Galactosidase activity was inhibited by 80% in the presence of 0.15 mM Hg^{2+}; α-galactosidase was not affected by Hg^{2+} or other thiol-blocking agents. On the other hand, α-galactosidase and β-galactosidase were irreversibly inhibited by 100 units/ml heparin (66% and 50% of control, respectively). In other experiments 20 mM galactose produced a decrease of 28% in α-galactosidase activity and no effect on β-galactosidase activity. Finally, α- and β-

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE β-GLYCEROPHOSPHATASE AND PHENYLPHOSPHATASE ACTIVITIES OF HUMAN POLYMORPHONUCLEAR LEUKOCYTE HOMOGENATE

Assays were carried out at 37 °C with either 50 mM phenylphosphate for 1 h or 50 mM β-glycerophosphate for 4 h in 50 mM acetate buffer (pH 5.0). Values are means of 7 experiments.

Addition	*Activity as % of control*	
	Phenylphosphatase	*β-Glycerophosphatase*
None	100	100
meso-Tartrate 0.3 M	167	27
Potassium citrate 0.3 M	207	61
Sodium citrate 0.3 M	171	78
Hg^{2+} 0.15 mM	9	39
Fluoride 15 mM	93	16

galactosidases were not affected by the ionic strength of the incubation mixture.

Comparison of acid phenylphosphatase and acid β-glycerophosphatase. As shown in Table I, 0.3 M DL-tartrate and 0.3 M citrate strongly increased acid phenylphosphatase activity. The reverse was true for acid β-glycerophosphatase activity. Fluoride was much more inhibitory to acid β-glycerophosphatase activity than to acid phenylphosphatase. Hg^{2+} were about equally inhibitory to the acid phosphatase activity with both substrates.

Effect of substrate concentration. In these studies, the conditions, except for substrate concentration, were those described under Experimental. All hydrolases were found to display typical Michaelis–Menten kinetics, with linear reciprocal plots of activity and enzyme concentration, from which the following K_m values were derived: 0.32 mM for cathepsin, 0.23 mM for β-*N*-acetylglucosaminidase; 16% for lysozyme; 0.9 mM for β-glucuronidase; 20 mM for acid β-glycerophosphatase; 50 mM for acid phenylphosphatase; 1.74 mM for β-galactosidase; 0.015 mM for α-galactosidase, and 29.3 mM for alkaline β-glycerophosphatase.

TABLE II

RANGE OF VALIDITY OF ENZYME ASSAYS

The values given represent maximum amounts of tissue and maximum incubation times for which linearity with respect to both variables was observed. When followed by "at least", the limits indicated correspond to the highest values tested experimentally.

Enzyme	*Leukocytes (mg)*	*Duration of incubation*
Acid β-glycerophosphatase	2.5	8 h
Acid phenylphosphatase	0.006	6 h
β-Galactosidase	30	6 h
Cathepsin	40	4 h
β-Glucuronidase	1.3	9 h
β-*N*-Acetylglucosaminidase	1.3	6 h
Lysozyme	2.8	6 min at least
Peroxidase	3.2	3 min at least
Alkaline β-glycerophosphatase	3.0	9 h
α-Galactosidase	60	4 h
NADH oxidase (cyanide-insensitive)	18	90 min
Alkaline phenylphosphatase	0.06	6 h

TABLE III

TOTAL ENZYME ACTIVITIES IN HOMOGENATES OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES

The absolute values for all entities are given as means ± S.D. for the number of measurements given in parentheses. Protein and DNA content are expressed as mg/g of leukocytes. For the enzymes tested, values give mean spec. act. in munits per mg of protein ± S.D. When not specified 1 unit of activity is defined as the amount of enzyme necessary to split 1 μmole of substrate in 1 min under the conditions used. The unit of lysozyme and peroxidase activities are those defined by Baggiolini *et al.*[13]. In the case of NADH oxidase values are expressed as ΔA/mg of protein per min. Alkaline phosphatase was assayed with β-glycerophosphate as substrate.

Enzyme	*Human spec. act.*	*Rabbit spec. act.*	*Spec. act human polymorphonuclear leucocytes* / *Spec. act. rabbit polymorphonuclear leucocytes*
Acid β-glycerophosphatase (57)	10.50 ± 4.30	8.40 ± 1.00	1.25
Acid phenylphosphatase (21)	85.10 ± 10.10	65.60 ± 9.00	1.30
β-Galactosidase (49)	2.28 ± 0.60	—	—
α-Galactosidase (12)	1.56 ± 0.31	—	—
Cathepsin (7)	3.90 ± 0.56	—	—
β-Glucuronidase (22)	1.95 ± 0.50	1.00 ± 0.40	1.95
β-*N*-Acetylglucosaminidase (22)	0.33 ± 0.07	42.00 ± 6.50	0.01
Lysozyme (45)	0.07 ± 0.02	0.12 ± 0.03	0.60
Peroxidase (39)	0.16 ± 0.06	2.45 ± 0.22	0.07
Alkaline phosphatase (33)	2.35 ± 0.95	—	—
NADH oxidase (20)	20.49 ± 3.18	—	—
Protein (28)	139.00 ± 15.00	—	—
DNA (20)	22.00 ± 4.00	—	—

In most of our assays, the substrate concentration had, for practical reasons, to be kept at a level not much higher than the K_m of the enzyme. However, there was always a sufficient excess of substrate to ensure near-zero order kinetics.

Quantitative assay and stability of enzymes. Linearity with enzyme concentration and incubation time was observed for all enzymes, up to the limits listed in Table II. When incubation was prolonged beyond the times given in Table II, the measured activities declined progressively, presumably owing to slow inactivation of the enzymes.

Enzyme activities. Considerable amounts of acid hydrolases and peroxidase are present in human polymorphonuclear leukocytes (Table III). Compared with rabbit polymorphonuclear leukocytes, peroxidase and β-*N*-acetylglucosaminidase activities were higher in rabbit leukocytes than in human polymorphonuclear leukocytes. On the other hand, β-glucuronidase activity is less in rabbit leukocytes than in human polymorphonuclear leukocytes.

DISCUSSION

By establishing valid assay conditions for eight human polymorphonuclear leukocytes hydrolases known or suspected to be associated with lysosomes in other tissues, and for alkaline phosphatase which is located in specific granules, the results described in the present work have paved the way to a comprehensive biochemical investigation of these granules in human polymorphonuclear leukocytes. We have

also looked for valid conditions for the peroxidase and NADH oxidase assays, because both enzymes are intimately connected with the bactericidal activity of polymorphonuclear leukocytes lysosomes.

In order to find a good medium for human polymorphonuclear leukocytes leukocytes homogenization and subcellular fractionation, we have assayed several homogenization media. The preparation of suitable homogenates proved very difficult and no condition was found that was entirely satisfactory. Preservation of nuclei was obtained only in media of relatively high ionic strength, or in the presence of EDTA. But there was considerable agglutination in such media and the granule fractions were poor. Almost exactly the opposite results were obtained in sucrose solutions containing 100 units/ml heparin. The nuclei were almost entirely destroyed, but the granule fractions were clean, well dispersed and isolated in good yield. Without heparin, there was much more agglutination, with equal damage to the nuclei. On the basis of these results, we chose 0.34 M sucrose containing 100 units/ml heparin as homogenization medium for all our subsequent fractionation work. This medium had the additional advantage of rendering the cells very fragile, so that their disruption could be achieved without sonication. It proved to be the only one among those tested in which a satisfactory resolution could be achieved. Its main drawback is that the heparin inhibits some of the enzymes; but this effect could be taken into account.

Regarding the subcellular distribution of DNA, we have found that in human polymorphonuclear leukocytes, the homogenization in 0.34 M sucrose alone produces 80% solubilization of DNA. In this respect our results are different from those of Michell *et al.*[15]. These authors, using guinea pig polymorphonuclear leukocytes homogenized in 0.34 M sucrose, have found only 0.3% of the DNA in the supernatant fraction. More recently, Stossel *et al.*[16] have reported for guinea pig polymorphonuclear leukocytes, using 0.34 M sucrose/500 units heparin per ml, 81% of the DNA in the supernatant. It seems that, at least in the case of human polymorphonuclear leukocytes, the solubilization of the DNA is due to the non-ionic medium used for the homogenization procedure and not to the heparin present in it, although heparin could increase the solubilization of nuclear proteins.

According to our results, the cyanide-insensitive NADH oxidase activity is mainly localized in the supernatant fraction; however, again the composition of the homogenization medium has a strong influence on the subcellular distribution. In the presence of 0.34 M sucrose about 60% of the homogenate activity is in the soluble fraction, but when homogenization is done in 0.15 M KCl, only 32% of the NADH oxidase is present in the supernatant. These results are quite similar to those reported for human polymorphonuclear leukocytes[17] and also for guinea pig polymorphonuclear leukocytes[16].

Our results for the NADH oxidase optimum pH are very different from previous results for the NADH oxidase of human polymorphonuclear leukocytes[17] and guinea pig polymorphonuclear leukocytes[18]. These authors have found an optimum pH of 4.5 for both enzymes. If, as our results have shown, cyanide-insensitive NADH oxidase is a cytoplasmic enzyme, we cannot understand the results of other authors concerning the optimum pH of this enzyme. An optimum of activity at about pH 6.5 seems more logical considering the subcellular localization of the enzyme.

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