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SUBCELLULAR LOCALIZATION AND PROPERTIES OF PYRIDOXAL PHOSPHATE PHOSPHATASES OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES AND THEIR RELATIONSHIP TO ACID AND ALKALINE PHOSPHATASE

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Using a novel fluorimetric assay for pyridoxal phosphate phosphatase, human polymorphonuclear leucocytes were found to exhibit both acid and alkaline activities. The neutrophils were homogenised in isotonic sucrose and subjected to analytical subcellular fractionation by sucrose density gradient centrifugation. The alkaline pyridoxal phosphate phosphatase showed a very similar distribution to alkaline phosphatase and was located solely to the phosphasome granules. Fractionation experiments on neutrophils treated with isotonic sucrose containing digitonin and inhibitor studies with diazotised sulphanilic acid and levamisole further confirmed that both enzyme activities had similar locations and properties. Acid pyridoxal phosphate phosphatase activity was located primarily to the tertiary granule with a partial azurophil distribution. Fractionation studies on neutrophils homogenised in isotonic sucrose containing digitonin and specific inhibitor studies showed that acid pyridoxal phosphate phosphatase and acid phosphatase were not the result of a single enzyme activity. Neutrophils were isolated from control subjects, patients with chronic granulocytic leukaemia and patients in the third trimester of pregnancy. The specific activities (munits/mg protein) of alkaline pyridoxal phosphate phosphatase and alkaline phosphatase varied widely in the three groups and the alterations occurred in a parallel manner. The specific activities of acid pyridoxal phosphate phosphatase and of acid phosphatase were similar in the three groups. These results, together with the fractionation experiments and inhibition studies strongly suggest that pyridoxal phosphate is a physiological substrate for neutrophil alkaline phosphatase.

Introduction

Normal human polymorphonuclear leukocytes are a rich source of alkaline phosphatase and the levels of activity vary considerably in certain neutrophil disorders [1,2]. Recently this alkaline phosphatase activity has been localised in a hitherto undescribed organelle (phosphasome) of the neutrophil [3,4], the function of which is as yet unknown. In these studies alkaline phosphatase was assayed with 4-methyl umbelliferyl phosphate, a synthetic, fluorogenic substrate. We are therefore trying to determine physiological substrates for neutrophil alkaline phosphatase.

Previous studies have clearly shown that neutrophil alkaline phosphatase cannot hydrolyse AMP [2,5], ADP [6], ATP [7], cyclic AMP [8], histone phosphate [9], glucose 6-phosphate (Smith, G.P., unpublished data), or inorganic pyrophosphate (Raja, K., Smith, G.P. and Peters, T.J., unpublished data). These results indicate that, contrary to alkaline phosphatase from other tissues, the neutrophil enzyme has a high degree of substrate specificity.

Pyridoxal 5′-phosphate is the main coenzyme form of pyridoxine (vitamin B-6) and plays an important role in numerous biochemical reactions. The pyridoxal content of tissues is regulated by plasma membrane transport [10], by phosphorylation of free pyridoxal [11] and by binding of the coenzyme to

apoprotein. It has also been shown that cellular phosphatases (EC 3.1.3.-) play an important role in controlling tissue pyridoxal phosphate levels [12]. When the amount of pyridoxal phosphate exceeds the binding capacity of the protein the free pyridoxal phosphate is rapidly hydrolysed and the released free pyridoxal can readily traverse the cell membranes. Although the importance of pyridoxal phosphate phosphatase is well established very little is known of its properties or its involvement in the pathogenesis of disease.

The present study determines the subcellular localization of pyridoxal phosphate phosphatase in human neutrophils using analytical subcellular fractionation techniques, examines certain properties of the enzymes and compares the neutrophil activities in leukocytic disorders known to exhibit striking alterations in alkaline phosphatase levels with those from control subjects.

Methods

Isolation and homogenization of neutrophils. Polymorphonuclear leukocytes were isolated by sedimentation and Ficoll-Hypaque centrifugation as previously described [2]. The cells were pelleted in 4 ml 0.2 mol/l sucrose/1 mmol/l Na_2 · EDTA, pH 7.2/5000 units heparin/l, and disrupted with a Dounce homogeniser (Kontes Glass Co., Vineland, NJ, U.S.A.) by 30 strokes of a tight fitting (Type B) pestle. An aliquot of the homogenate was retained for analysis and a portion centrifuged at 800 × *g* for 10 min in a Coolspin 4 × 100 ml swing out rotor (MSE Scientific Instruments, Crawley, Sussex). The post-nuclear supernatant was removed, stored on ice and subjected to analytical subcellular fractionation and enzyme analysis as described previously [2].

Analytical subcellular fractionation. Approx. 5 ml of the post-nuclear supernatant were layered onto a 28 ml sucrose-density gradient extending linearly with respect to volume, from a density of 1.05 g · cm^{-3} to one of 1.28 g · cm^{-3} and resting on a 6 ml cushion of density 1.32 g · cm^{-3} in a Beaufay automatic zonal rotor. All gradient solutions contained 1 mmol/l Na_2 · EDTA, pH 7.2 and 5000 units heparin/l. The rotor was run at 35 000 rev./min for 35 min and, after slowing to 8000 rev./min, some 15 fractions were collected, weighed and their density determined as previously described [13].

Organelle marker enzymes. The gradient fractions were assayed for marker enzymes for the principal subcellular organelles. The enzymes assayed, with the organelle, shown between parentheses, were: alkaline phosphatase, EC 3.1.3.1. (phosphasome); 5′-nucleotidase, EC 3.1.3.5. (plasma membrane); myeloperoxidase, EC 1.11.1.7 (azurophil granule); *N*-acetyl-β-glucosaminidase, EC 3.2.1.30, acid phosphatase, EC 3.1.3.2. (tertiary granule); lactate dehydrogenase, EC 1.1.1.27 (cytosol); malate dehydrogenase, EC 1.1.1.37 (mitochondria) and neutral α-glucosidase, EC 3.2.1.20 (endoplasmic reticulum). Conditions for the assays were as described previously [3]. Unsaturated vitamin B-12 binding capacity (specific granule) was determined by the charcoal radioassay [14]. Protein was estimated by a modification [15] of the method of Lowry et al. [16] with bovine serum albumin as standard.

Pyridoxal phosphate phosphatase determination. The homogenates and gradient fractions were assayed for acid and alkaline pyridoxal phosphate phosphatase activities by a method based on the isolation and fluorimetric determination of pyridoxal described by Takanashi et al. [17]. In standard assay conditions, 0.1 ml of suitably diluted homogenate or sucrose gradient fraction was incubated at 37°C for up to 60 min with 0.4 ml 2 mmol/l pyridoxal phosphate dissolved in either 0.1 mol/l sodium acetate buffer, pH 5.0 or 0.1 mol/l diethanolamine-HCl buffer, pH 9.3/20 mmol/l $MgCl_2$/0.1% Triton X-100. After termination of the assay, the pyridoxal and pyridoxal phosphate were separated by ion-exchange chromatography. The released pyridoxal was oxidised with alkaline cyanide to 4-pyridoxolactone, a highly fluorescent product. In assays with inhibitors the appropriate concentration was included in the buffer system, and appropriate controls were performed to ensure that they did not interfere with the separation of pyridoxal and pyridoxal phosphate or with the fluorigenic reaction. The effect of diazotised sulphanilic acid on leukocyte alkaline phosphatase and alkaline pyridoxal phosphate phosphatase activities in intact and disrupted cells was investigated by the method of Smolen and Weissmann [18].

Results

Optimization of pyridoxal phosphate phosphatase assay

Kinetic studies were carried out to optimise the pyridoxal phosphate phosphatase assay with human neutrophils as enzyme source. The variation in the rate of the reaction with change in pH was investigated (Fig. 1). The rate was maximal at pH 5.0, although there was a second peak at pH 9.3. Further optimization of the acid and alkaline pyridoxal phosphate phosphatase assays showed few differences between the activities. When variation in reaction rate with change in Mg^{2+} concentration was investigated it was found that neither activity was Mg^{2+}-dependant but that both were increased by up to 50% in the presence of Mg^{2+}. A final concentration of 16 mmol/l $MgCl_2$ was included in the standard assay media. Under these conditions the Michaelis constants of the enzymes for pyridoxal phosphate were determined. When the data were fitted to the points using parameters calculated from direct linear plots [19] the K_m for the acid and alkaline activities was found to be similar (0.26 mmol/l and 0.16 mmol/l, respectively). When suitable dilutions of neutrophil homogenate were assayed, pyridoxal phosphate phosphatase activity was proportional to the concentration of protein used and was linear over incubation times up to 120 min.

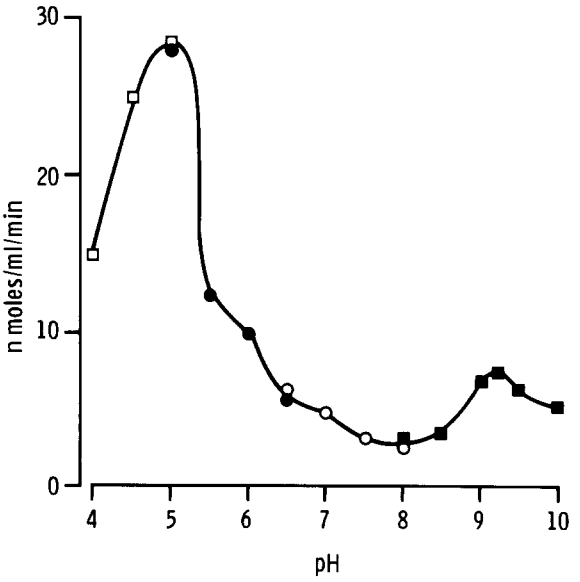

Fig. 1. Variation in the rate of pyridoxal phosphate phosphatase activity with pH, using the following buffers: - 0.1 mol/l sodium acetate (pH 4–5; □———□). Sodium-2(morpholino)ethane sulfonic acid (Mes) (pH 5–6.5; •———•), Tris-HCl (pH 6.5–8; ○———○), or diethanolamine-HCl (pH 8–10; ■———■). All assays were carried out with 1.6 mmol/l pyridoxal phosphate, 16 mmol/l $MgCl_2$ and 0.1% Triton X-100 at 37°C.

Analytical subcellular fractionation of human neutrophils

Fig. 2. shows the averaged density gradient distri-

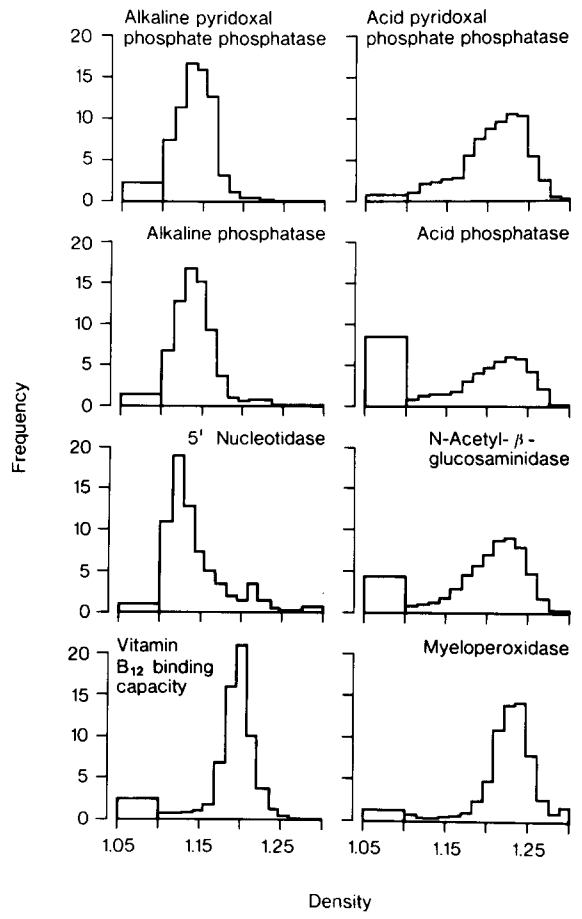

Fig. 2. Isopycnic centrifugation of post-nuclear supernatant prepared from neutrophil leukocyte homogenates. Results show mean distributions from three experiments. Frequency is defined as the fraction of total recovered activity present in the gradient fraction divided by the density span covered. The activity present over the density span 1.05–1.10 represents enzyme remaining in the sample layer. Recovered activities range from 80–105%.

butions for acid and alkaline pyridoxal phosphate phosphatase activities and some of the principal organelle marker enzymes for human neutrophils.

Localization of alkaline pyridoxal phosphate phosphatase

Alkaline pyridoxal phosphate phosphatase has a unimodal distribution with a modal density of 1.14 g · cm^{-3} (Fig. 2). The distribution profile and modal density are identical to those of alkaline phosphatase. However, 5′nucleotidase, the plasma membrane marker, exhibits a distinct distribution profile with a modal density of 1.15 g · cm^{-3}.

In order to confirm the localization of alkaline pyridoxal phosphate phosphatase to the phosphasome subcellular fractionation experiments were carried out on neutrophils which had been suspended in isotonic sucrose containing 17 or 34 μmol/l digitonin and then washed in digitonin-free sucrose. Digitonin is a selective membrane perturbant which binds to cholesterol in plasma membrane, selectively increasing the density of this organelle [20–22]. Intracellular organelles which are inaccessible to the digitonin are unaffected by this treatment. As seen in Fig. 3 the distribution of the plasma membrane marker 5′-nucleotidase is markedly affected by the digitonin treatment. After treatment with 17 μmol/l digitonin, most of the activity moved to a density of 1.16 g · cm^{-3}. After treatment with 34 μmol/l digitonin this shift was increased to a density of 1.18 g · cm^{-3}. In contrast the distributions of alkaline pyridoxal phosphate phosphatase and alkaline phosphatase were unaffected by the digitonin treatment.

Further evidence in support of an intracellular localization of alkaline pyridoxal phosphate phosphatase is shown in Fig. 4. Diazotised sulphanilic acid is a poorly permeant reagent which has been used to selectively inactivate ectoenzymes [18,23]. In intact cells (A) 5′-nucleotidase, an enzyme located to the

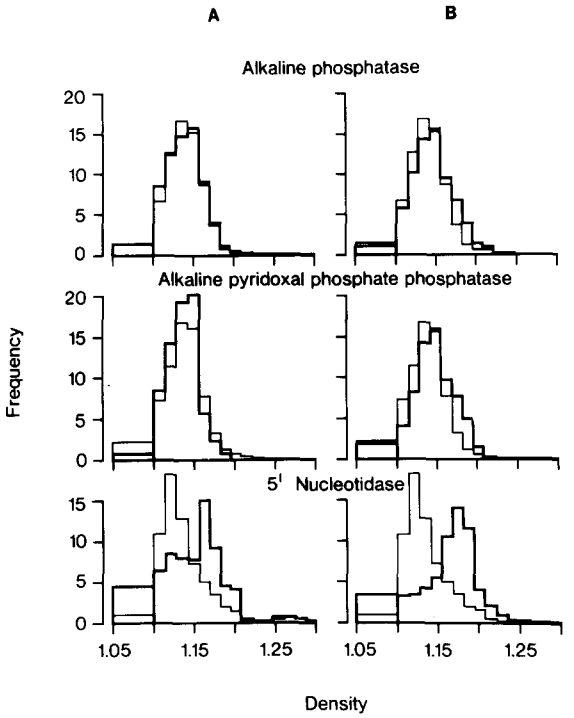

Fig. 3. Isopycnic centrifugation of post-nuclear supernatant from control (———) and digitonin-treated (———) neutrophils. Prior to homogenization the leukocytes were resuspended in 23 ml of 0.34 mol/l sucrose containing either no digitonin (control), (A) 17 μmol/l digitonin or (B) 34 μmol/l digitonin, centrifuged at 120 × *g* and washed in digitonin-free 0.2 mol/l sucrose. The cells were then homogenised and fractionated. Averaged control data were taken from Fig. 2. Data from digitonin-treated cells are from a single representative experiment.

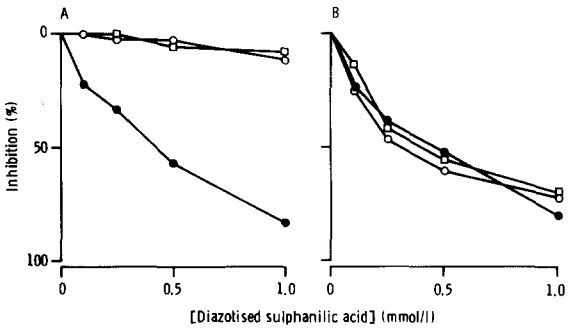

Fig. 4. A. Intact polymorphonuclear leukocytes, suspended in phosphate-buffered saline, were incubated for 15 min at 37°C with various concentrations of diazotised sulphanilic acid. The cells were then washed twice with ice-cold phosphate-buffered saline, sonicated for 30 s, and assayed for alkaline phosphatase (○———○), alkaline pyridoxal phosphate phosphatase (□———□) and 5′-nucleotidase (●———●). B. Polymorphonuclear leukocytes were suspended in phosphate-buffered saline, sonicated for 30 s and then incubated for 15 min at 37°C with various concentrations of diazotised sulphanilic acid. The treated sonicates were then assayed for alkaline phosphatase (○———○), alkaline pyridoxal phosphate phosphatase (□———□) and 5′-nucleotidase (●———●). In both studies enzyme activities are expressed as percentages of those measured for untreated cells.

external surface of the plasma membrane [5] shows a progressive and almost complete inhibition by this reagent, whilst alkaline phosphatase and alkaline pyridoxal phosphate phosphatase were both unaffected by this reagent. With sonicated cells (B) the three enzymes were inhibited by diazotised sulphanilic acid to a similar extent. These results confirm that alkaline pyridoxal phosphate phosphatase and alkaline phosphatase have intracellular localizations in the human neutrophil.

Localization of acid pyridoxal phosphate phosphatase

In Fig. 2 acid pyridoxal phosphate phosphatase is shown to have a broad distribution in the sucrose-density gradient with a modal density of 1.23 g · cm^{-3}. The modal density is similar to those of *N*-acetyl-β-glucosaminidase and acid phosphatase, the marker enzymes for the tertiary granules, and to myeloperoxidase, marker for the azurophil granules. The individual distribution profiles for these enzymes in the gradient, however, differ significantly from that of acid pyridoxal phosphate phosphatase. In particular, there is negligible soluble acid pyridoxal phosphate phosphatase.

In order to further investigate the localization of acid pyridoxal phosphate phosphatase subcellular fractionation was performed on neutrophils homogenised in isotonic sucrose containing 0.12 mmol/l digitonin. In this situation all organelles and membranes were accessible to the digitonin. Under these conditions not only does digitonin increase the equilibrium density of cholesterol-containing membranes, it also exhibits a detergent effect on some organelles, causing the release of enzymes into the soluble fraction, these results are shown in Fig. 5. Acid pyridoxal phosphate phosphatase, *N*-acetyl-β-glucosaminidase and myeloperoxidase show a marked reduction in particulate activity. However, the distribution of acid phosphatase is similar in the control and digitonin-treated neutrophils.

This evidence suggests that acid pyridoxal phosphate phosphatase is primarily located to the tertiary granules but that some may be found in the azurophil granules. Acid pyridoxal phosphate phosphatase and acid phosphatase are not located to the same subcellular organelle.

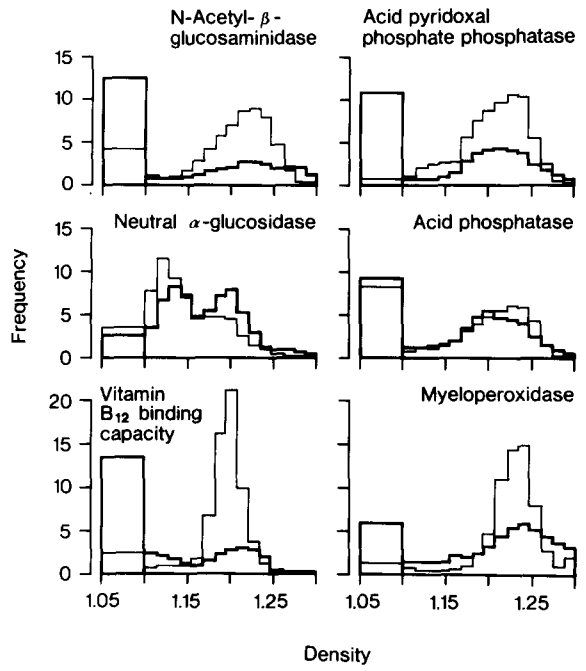

Fig. 5. Isopycnic centrifugation of post-nuclear supernatant from control (———) and digitonin-treated (———) neutrophil leukocyte homogenate. Leukocytes were homogenised in 0.2 mol/l sucrose containing either no (control) or 0.12 mol/l digitonin and fractionated. Averaged control data were taken from Fig. 2. Digitonin data show the mean distributions from two experiments.

Inhibition studies on alkaline and acid pyridoxal phosphate phosphatases

In order to further investigate acid and alkaline pyridoxal phosphate phosphatase activities certain specific inhibitor studies were performed. The effect of levamisole, a potent inhibitor of alkaline phosphatase, is shown in Fig. 6. It is clear that levamisole has an identical inhibitory effect on both enzyme activities.

The effect of sodium fluoride and *N*-ethylmaleimide on acid phosphatase and acid pyridoxal phosphate phosphatase is compared in Fig. 7. Whilst sodium fluoride strongly inhibited acid pyridoxal phosphate phosphatase it had little effect against acid phosphatase. In contrast, *N*-ethylmaleimide had no effect on acid pyridoxal phosphate phosphatase, but strongly inhibited acid phosphatase. This is further evidence that these two activities are not reflections of a single enzyme protein.

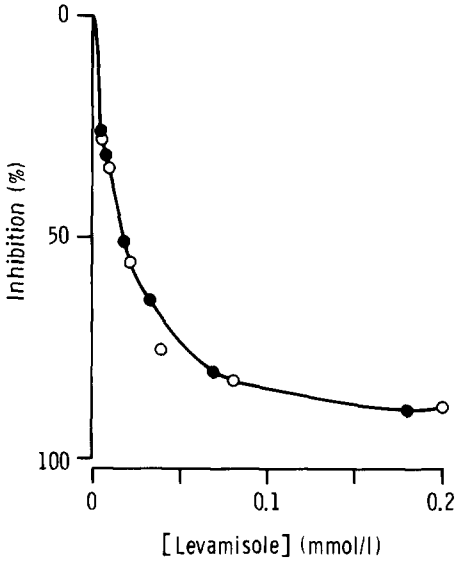

Fig. 6. Inhibition of alkaline phosphatase (•) and alkaline pyridoxal phosphate phosphatase (○) by levamisole. A neutrophil homogenate was incubated under usual assay conditions for each enzyme in the presence of various concentrations of levamisole. The plot shows the % inhibition of each enzyme.

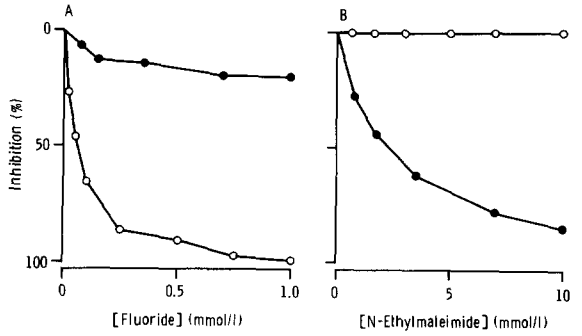

Fig. 7. Differential inhibition of acid phosphatase (•———•) and acid pyridoxal phosphate phosphatase (○———○) by (A) sodium fluoride and (B) *N*-ethylmaleimide. Neutrophil homogenate was incubated under standard assay conditions for each enzyme in the presence of either inhibitor. The plot shows the % inhibition of acid phosphatase and acid pyridoxal phosphate phosphatase by the respective inhibitors.

Phosphatase activities in patient groups

Table I shows a comparison of the specific activity of acid and alkaline pyridoxal phosphate phosphatase with acid and alkaline phosphatase in neutrophil homogenates from normal subjects, from women in the third trimester of pregnancy and patients with chronic granulocytic leukaemia. Both alkaline phosphatase and alkaline pyridoxal phosphate phosphatase showed marked variation in the groups. The specific activities of both enzymes were increased 8-fold above normal in neutrophils from the pregnant women and reduced to 13% of the control level in patients with chronic granulocytic leukaemia. Neither acid phosphatase nor acid pyridoxal phosphate phosphatase showed any significant variations in the three groups.

Discussion

The presence of a phosphatase-hydrolysing pyridoxal phosphate has been reported in brain tissue [24–26], rat liver [27] and rat pineal gland [28]. The present study has demonstrated that human polymorphonuclear leukocytes also have significant amounts of pyridoxal phosphate phosphatase activity

TABLE I

NEUTROPHIL HOMOGENATE PHOSPHATASE ACTIVITIES

Results are shown as mean values ± S.E. with the number of preparations assayed in duplicate shown between parentheses. Enzyme activities are expressed as munits/mg protein.

	Chronic granulocytic leukaemia	Control subjects	Pregnant patients
Alkaline phosphatase	0.477 ± 0.100 [a] (8)	3.23 ± 0.342 (10)	26.9 ± 3.18 [a] (8)
Alkaline pyridoxal phosphate phosphatase	0.204 ± 0.046 [a] (8)	1.71 ± 0.119 (11)	12.7 ± 1.79 [a] (10)
Acid phosphatase	19.1 ± 3.49 (7)	24.4 ± 1.25 (12)	24.2 ± 1.81 (10)
Acid pyridoxal phosphate phosphatase	13.3 ± 2.37 (8)	7.28 ± 0.600 (12)	8.87 ± 0.815 (10)

[a] Significant differences from control subjects analyzed by Student's *t*-test, $P < 0.001$.

which can be reliably measured by a novel fluorimetric assay. The change in the rate of reaction with pH indicated that there are both acid and alkaline activities in human neutrophils. This is in contrast to studies in other tissues where only alkaline pyridoxal phosphate phosphatase activity has been reported.

In agreement with previous reports [24,29], it was found that although alkaline pyridoxal phosphate phosphatase was not Mg^{2+}-dependant, it was required for maximal activity. The apparent K_m for pyridoxal phosphate phosphatase at pH 9.3 was 0.16 mmol/l which was in fairly good agreement with values reported by Lumeng and Li [29] for rat liver (0.055 mmol/l), by Bishayee and Bachhawat [26] for rat brain (0.135 mmol/l) and by Ebadi and Govitrapong [28] for rat pineal gland (0.07 mmol/l). Results of analytical subcellular fractionation have shown that that alkaline pyridoxal phosphate phosphatase is located solely in the low density phosphasome granule. This has been confirmed by recent cytochemical studies (Wilson, P.D., unpublished data). This is the first report of a possible physiological substrate for neutrophil alkaline phosphatase. Further studies to compare alkaline pyridoxal phosphate phosphatase and alkaline phosphatase showed striking similarities between the two activities. In addition to having the same subcellular localization they both showed identical inhibition curves with levamisole, a specific inhibitor of alkaline phosphatase, and similar variations in neutrophil disorders. These results are in agreement with studies on rat liver [29] and human brain [24], where it was shown that alkaline phosphatase was the principal enzyme responsible for hydrolysis of pyridoxal phosphate.

Analytical subcellular fractionation studies have shown that acid pyridoxal phosphate phosphatase was found primarily in the tertiary granules, with some azurophil granule activity. Further fractionation experiments on neutrophils homogenised in digitonin indicate that acid pyridoxal phosphate phosphatase and acid phosphatase are not located to the same subcellular organelle. Inhibitor studies using sodium fluoride and *N*-ethylmaleimide clearly show that acid pyridoxal phosphate phosphatase and acid phosphatase are distinct activities, and not the result of a single enzyme activity. This is in agreement with the results of Begum and Bachhawat [30] who showed that pyridoxal phosphate is not a substrate for brain acid phosphatase.

Although the importance of pyridoxal phosphate in metabolism is well established very little is known of its involvement in disease. A deficiency of pyridoxal phosphate has been implicated in such diverse diseases as arteriosclerosis [31], convulsions [32,33], sideroblastic anaemia [34,35], toxaemia of pregnancy [36,37] and liver disease [38]. However, more interesting from the view of this report is that certain population groups, including pregnant women [39, 40], women taking oral contraceptives [41] and assorted leukaemia patients [42] have been shown to have low levels of plasma or leucocyte pyridoxal phosphate. Further studies in these patient groups should indicate whether neutrophil alkaline phosphatase is responsible for regulating the level of phosphorylated pyridoxine in these cells. In addition they may further elucidate the physiological and pathological role of this enigmatic enzyme.

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