Protein Kinase and Phosphatases from Human Polymorphonuclear Leucocytes

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Purified preparations of human polymorphonuclear leucocytes contain a protein kinase in the cytosol which is stimulated by cyclic AMP and cyclic IMP but not by other cyclic nucleotides. The holoenzyme had a molecular weight of 66000 estimated by gel filtration; when it was incubated with histone or cyclic AMP, it dissociated into two smaller subunits of molecular weight 45000 and 30000; the former remained cyclic AMP-sensitive, whereas the latter had become independent of added cyclic AMP. By means of substrate-affinity chromatography on histone–Sephadex 4B, cyclic [^3H]AMP-binding activity (regulatory or R subunit) could be resolved into two peaks of enzyme activity, one again independent of added cyclic AMP, with a molecular weight of 30000 (catalytic or C subunit). Also by means of substrate-affinity chromatography it was possible to resolve 'specific' polymorphonuclear leucocyte histone phosphatases from 'non-specific' phosphomonoesterases capable of dephosphorylating histone previously phosphorylated by the protein kinase. Specific histone phosphatase displayed greatest affinity for histone–Sephadex 4B, followed by acid *p*-nitrophenyl phosphatase, and the unretained acid β -glycerophosphatase. Polymorphonuclear leucocyte histone phosphatase, purified approx. 40-fold, was further resolved from the other phosphatases by gel filtration on Sephadex G-150 from which it was eluted with apparent molecular weights of 45000 and 18700. The apparent K_m values for dephosphorylation of histone are $4.3 \times 10^{-6}\text{M}$ and $3.6 \times 10^{-6}\text{M}$. Most (69%) of cytoplasmic histone phosphatase was found in the cell sap, whereas 20% remained tightly associated with polymorphonuclear leucocyte lysosomes from which it could not be solubilized by treatments (Triton X-100, freeze–thawing) that released approx. 70% of lysosomal β -glucuronidase or acid phosphatases. Although both soluble and particulate enzymes required 5–10mM- Mn^{2+} for maximal activation, and showed a pH maximum of 6.5–7.0, only the particulate enzyme was partly inhibited by ammonium molybdate. Polymorphonuclear leucocyte histone phosphatases were neither inhibited nor stimulated by those cyclic nucleotides that greatly stimulate the protein kinase of the same subcellular fraction.

Several functions of polymorphonuclear leucocytes, such as lysosomal enzyme release (Weissmann *et al.*, 1971; Zurier *et al.*, 1973a), chemotaxis (Estensen *et al.*, 1973) and phagocytosis (Weissmann *et al.*, 1972), are influenced by cyclic nucleotides. In general, cyclic AMP or agents that cause its intracellular accumulation inhibit whereas cyclic GMP or agents that lead to its intracellular accumulation enhance these functions (Estensen *et al.*, 1973; Zurier *et al.*, 1974). It is, however, at present unclear by what means these effects of cyclic nucleotides are exerted, or which enzymes of the polymorphonuclear leucocytes are susceptible to their regulation. We have previously isolated and characterized a cyclic AMP-sensitive protein kinase from these leucocytes and suggested that, as in other cells, the effects of cyclic AMP may be mediated by the phosphorylation of an,

as yet, unidentified substrate (Tsung *et al.*, 1972; Zurier *et al.*, 1973a,b). The studies reported here concern the further purification, properties and subunit structure of this enzyme, and it is suggested that it is preferentially stimulated by cyclic AMP rather than cyclic GMP. For such an enzyme (which was assayed with histone as its preferred substrate) to serve a regulatory role, histone phosphatases are also required, and it is the purpose of this report to describe also the properties of polymorphonuclear leucocyte enzymes which can dephosphorylate protein kinase-phosphorylated histone.

Lysosomal (Vreven *et al.*, 1973) as well as non-lysosomal (Shibko & Tappel, 1963; Meisler & Langan, 1969), or bicompartamental (Paigen & Griffiths, 1959), localization of phosphoprotein phosphatases has been reported in other cell types,

and, moreover, purified phosphoprotein phosphatases have been reported to display activity towards phosphomonoesters of phenol and *p*-nitrophenol (Singer & Fruton, 1957; Sundararajan & Sarma, 1959; Glomset, 1959; Revel & Racker, 1960). Since polymorphonuclear leucocytes contain both lysosomal and non-lysosomal phosphomonoesterases (Baggiolini *et al.*, 1969, 1970; Farquhar *et al.*, 1972) it was important to resolve 'specific' phosphoprotein phosphatases from these other enzymes, and to establish the subcellular distribution of the former. The present report describes the separation of histone phosphatase from non-specific phosphomonoesterases of the polymorphonuclear leucocyte by means of affinity chromatography on histone-Sepharose; it will also describe the properties of the lysosomal and non-lysosomal enzymes.

Materials and Methods

Polymorphonuclear leucocytes were obtained from freshly drawn heparinized human peripheral blood as described by Böyum (1968) by the use of Hypaque-Ficoll density gradients. The polymorphonuclear leucocytes (96–97% pure) were stored in a freezer at -20°C until purification was started, or used fresh for subcellular fractionation. Freeze-dried calf thymus, *p*-nitro phenylphosphate, β -glycerophosphate and phenolphthalein gluconate were obtained from Sigma, St. Louis, Mo., U.S.A. Sephadex and Sepharose 4B were obtained from Pharmacia, Piscataway, N.J., U.S.A. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Triton X-100 were purchased from New England Nuclear Corp., Boston, Mass., U.S.A.

Assay for protein kinase activity

Protein kinase activity was determined by measuring the amount of $\gamma\text{-}^{32}\text{P}$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ incorporated into histone (Tsung *et al.*, 1972). The standard assay mixture, unless otherwise indicated, contained in a final volume of 0.2 ml, 1 μmol of potassium phosphate buffer (pH 6.5), 2 μmol of MgCl_2 , 100 μg of histone, 1 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 0.4 μmol of theophylline, 28 μg of enzyme protein and 5 μM -cyclic nucleotide where appropriate. The mixture was incubated at 37°C for 5 min and the reaction was terminated by the addition of 0.2 ml of 10% (w/v) trichloroacetic acid. The protein precipitate was collected on Millipore filters and counted for radioactivity in toluene-Permafluor (Packard Instrument Co., La Grange, Ill. U.S.A.). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Preparation of protein kinase

Frozen polymorphonuclear leucocytes obtained from 3 litres of human blood were thawed and

homogenized with 4 vol. of neutral 4 mM-EDTA solution for 1 min at 0°C with a VirTis homogenizer at maximal speed. The homogenate was centrifuged at 27000g for 30 min at 4°C . Solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly with stirring to the supernatant fraction to 50% saturation. After at least 30 min of stirring, the precipitate was collected by centrifugation at 16000g for 20 min and dissolved in 6% of the crude extract volume of 5 mM-potassium phosphate buffer, pH 7.0, containing 6 mM-2-mercaptoethanol and 10% glycerol (PMG buffer). The resulting solution was dialysed against 20 vol. of the same buffer with two changes of buffer within 14 h of dialysis at 4°C . After dialysis, the solution was centrifuged at 27000g for 30 min, and the resulting precipitate was discarded. The enzyme solution was applied to a column (2 cm $\times$ 12 cm) of DEAE-cellulose which had been equilibrated with PMG buffer. The flow rate was 2 ml/min. Enzyme activity was eluted with a potassium phosphate buffer gradient from 5 to 200 mM in a total volume of 300 ml. The eluate of the column was collected in 5 ml fractions. Protein kinase activity was determined as described in the previous section. The peak fractions of protein kinase activity were pooled, concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, dialysed overnight against PMG buffer, and then stored at -20°C until used. Except where otherwise indicated, this enzyme preparation was used for all studies reported here.

Preparation of histone-Sepharose

The histone-Sepharose 4B was prepared as described by Cuatrecasas (1970); 5 g of solid CNBr was added to 40 ml of a suspension of Sepharose 4B in water (20 ml of water + 20 ml of settled Sepharose). The solution was maintained at pH 11 with 4 M-NaOH, and ice was added periodically to keep the temperature at 25°C . The reaction was complete in about 35 min. After the addition of more ice, the suspension was transferred to a Buchner funnel and washed with 1500 ml of cold 0.1 M- NaHCO_3 (pH 9.0). Histone (90 mg) in 20 ml of the same buffer was added to the treated Sepharose and the mixture was incubated for 20 h at 4°C with slow stirring and then washed with 20 vol. of 10 mM-Mes buffer [2-(*N*-morpholino)-ethanesulphonic acid], pH 6.5. Over 70% of added histone was linked to Sepharose, judged by protein analysis of effluents.

Preparation of labelled ^{32}P -histone

^{32}P -labelled histone was prepared by incubating histone with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of cyclic AMP-sensitive protein kinase, the kinase having been partially purified from bovine heart by column chromatography on DEAE-cellulose as described previously (Tsung *et al.*, 1972). The phosphorylation

reaction was terminated by the addition of the same volume of 50% (w/v) trichloroacetic acid. The resulting precipitate was centrifuged, washed twice, by dissolving in water and reprecipitating with the same volume of 50% trichloroacetic acid, and then dialysed against distilled water for 18 h.

Enzyme assays

Histone phosphatase activity was assayed by measuring the release of radioactive orthophosphate from ^{32}P -labelled histone. For routine assays, the reaction mixture contained, in a final volume of 0.2 ml, 50 mM-Mes buffer, pH 6.5, 1 mM-dithiothreitol, 10 mM- MnCl_2 , 10 μM - ^{32}P -labelled histone (based on the ^{32}P content of the labelled protein) and 1–40 μg of enzyme protein. The incubation was performed at 37°C for 10 min and terminated by the addition of 0.8 ml of 25% trichloroacetic acid. After the addition of 0.2 ml of 5 mg of bovine serum albumin/ml as a carrier for the precipitation, protein was removed by centrifugation. Orthophosphate was extracted from the deproteinized supernatant by a modification of the method of Plant (1963). To 0.8 ml of deproteinized supernatant were added 0.1 ml of 10 mM- KH_2PO_4 and 0.3 ml of 5% ammonium molybdate. The resulting phosphomolybdate complex was extracted with 1 ml of isobutyl alcohol, and the radioactivity of the isobutyl alcohol extract was measured. The amount of histone labelled with ^{32}P and the amount of phosphate released from the substrate histone have been calculated from the specific radioactivity of the labelled ATP used as precursor in the histone-phosphorylation reaction, neglecting the phosphate present in the histone before its phosphorylation by radioactive ATP.

Acid *p*-nitrophenyl phosphatase activity was determined by incubating samples for 10 min at 37°C with sodium acetate buffer, pH 5.0, (0.15 M) and *p*-nitrophenyl phosphate (2.0 mM) in a final volume of 1.0 ml. The reaction was started by the addition of substrate and stopped with 0.1 ml of 1 M-NaOH. The extinction of the liberated *p*-nitrophenol was read at 420 nm (Neil & Horner, 1964). Acid β -glycerophosphatase activity and β -glucuronidase activity were assayed by the method of Gianetto & de Duve (1955).

Isolation of histone phosphatase from human polymorphonuclear leucocytes by histone-Sepharose affinity chromatography

Frozen polymorphonuclear leucocytes which were prepared from 1 litre of human blood were thawed and homogenized with 5 vol. of 10 mM-Mes buffer (pH 6.5)–1 mM-dithiothreitol at 0°C with a VirTis homogenizer at high speed for 1 min. The homogenate

was centrifuged at 27000g for 30 min at 4°C. Solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly with stirring to the supernatant fraction to 70% saturation. After at least 30 min of stirring, the precipitate was collected by centrifugation at 16000g for 20 min and dissolved in 6% of the crude extract volume of the same buffer. The resulting solution was dialysed against 500 vol. of the same buffer with two changes of buffer during approx. 18 h. After dialysis, the enzyme solution was applied to a column (0.9 cm $\times$ 10 cm) of histone-Sepharose 4B equilibrated with 10 mM-Mes buffer (pH 6.5)–1 mM-dithiothreitol. This was followed by the addition of the same volume of buffer as contained the applied sample, and then by 10 ml of the same buffer. The column was eluted with a linear gradient of 0–0.3 M-NaCl in the same buffer in a total volume of 100 ml. No enzyme activity was observed above 0.3 M-NaCl concentration. The flow rate was 2 ml/min. The eluates of the column were collected in 2 ml fractions.

Gel filtration on Sephadex G-150

The fractions of peak histone phosphatase activity obtained by histone-Sepharose 4B chromatography and from the $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction from the 27000g supernatant were applied to a column (0.9 cm $\times$ 70 cm) equilibrated with 10 mM-Mes buffer, pH 6.5, containing 1 mM-dithiothreitol and 0.05 M-NaCl. The flow rate was 1 ml/4 min. The eluates of the column were collected in 1 ml fractions.

Preparation of subcellular fractions

Subcellular fractions of purified human polymorphonuclear leucocytes were prepared essentially as described by Tsung & Weissmann (1973): lysosomal granules were sedimented at 27000g for 20 min, and a small granule fraction was sedimented at 105000g for 2 h. Homogenization was carried out in 3 vol. of 0.25 M-sucrose containing 10 mM-Mes buffer, pH 6.5, and 1 mM-dithiothreitol. To avoid contamination of final supernatants by lysosomal materials, only those preparations were used in which over 90% of total β -glucuronidase and acid β -glycerophosphatase were sedimentable: this accounted for the presence of unruptured cells in the 800g nuclei and debris' fractions. Enzyme activity of all particulate fractions was assayed in the presence of 0.1% Triton X-100 to unmask latent enzyme activity.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Human polymorphonuclear leucocyte protein kinase

In experiments not reported in detail here, human polymorphonuclear leucocyte protein kinase had an apparent K_m for histone of $20\mu\text{g/ml}$ and K_m values of 62 and 88nm for cyclic AMP and cyclic IMP respectively. Both in the presence and absence of cyclic AMP the enzyme had an apparent K_m for ATP of approx. $10\mu\text{M}$. The kinase required 10mM-Mg^{2+} , had a pH optimum of 6.5 and an isoelectric point of 4.9. Activities sensitive to cyclic IMP and cyclic AMP could not be dissociated from each other by a variety of isolation procedures and this lack of discrimination was not due to contaminating deaminases or transaminases.

Sephadex G-75 chromatography of protein kinase

Molecular-sieve chromatography on a Sephadex G-75 column ($1\text{cm}\times 54\text{cm}$; equilibrated with PMG buffer) was done on the $(\text{NH}_4)_2\text{SO}_4$ precipitate of the 27000g supernatant. This precipitate was purified 10-fold in activity over that of the 27000g supernatant. Activity of the holoenzyme (over 85% of total activity) equally stimulated by cyclic AMP and cyclic IMP (Fig. 1a), was eluted at the same position as bovine serum albumin and therefore was approx. 66000 daltons in size, calculated as described by

Whitaker (1963). Peak fractions were purified 35-fold in activity over that of the 27000g supernatant.

Dissociation of protein kinase by histone and cyclic nucleotides

To determine whether the holoenzyme could be dissociated further to subunits of lower molecular weight, pooled fractions from the Sephadex G-75 peak were incubated with $50\mu\text{M}$ -cyclic AMP and histone (1mg/ml) for 1 h at 0°C before rechromatography. Preliminary experiments in which these fractions were eluted with PMG buffer, demonstrated that over 60% of recovered activity remained in the holoenzyme form. An additional new peak (approx. mol.wt. 45000) appeared, with a shoulder at approx. 30000. Activity of the first two peaks remained cyclic AMP-sensitive. Dissociation was demonstrated in clearer fashion when protein kinase was eluted in 10mM-Mes buffer, pH6.5. With this eluent [after dissociation by $50\mu\text{M}$ -cyclic AMP (not shown), or by $50\mu\text{M}$ -cyclic AMP and 1mg of histone/ml (Fig. 1b)] protein kinase activity was shifted from fractions in the holoenzyme position to fractions in two other peaks, one at a molecular weight of 45000 and another at a molecular weight of approx. 30000. Whereas the activity in the former peak remained cyclic AMP-sensitive, the activity in the latter fractions was largely independent of added cyclic AMP.

Fig. 1. Sephadex G-75 column chromatography of $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction of a 27000g supernatant of polymorphonuclear leucocytes

(a) A column ($1\text{cm}\times 54\text{cm}$) was equilibrated with PMG buffer (pH7.0) containing 0.5M-NaCl ; 1ml (16mg) of the $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction was applied to the column at a flow rate of 15ml/h , and 1ml fractions were collected. The kinase activity was determined in the presence or absence of $5\mu\text{M}$ -cyclic nucleotides as described in the Materials and Methods section. (b) A column ($1\text{cm}\times 54\text{cm}$) was equilibrated with 10mM-Mes buffer (pH6.5) containing 0.5M-NaCl ; 3mg of enzyme protein eluted from Sephadex G-75 (a) was incubated for 1 h at 0°C with 0.1ml of $50\mu\text{M}$ -cyclic AMP and 1mg of histone/ml. After incubation, the mixture was eluted from the column at a flow rate of 15ml/h ; 0.5ml fractions were collected. The kinase activity was determined in the presence of $5\mu\text{M}$ -cyclic nucleotides as described in the Materials and Methods section. Molecular weight was calculated as described by Whitaker (1963), with reference proteins as indicated by the arrows. $\circ$, + cyclic AMP; $\blacktriangle$, cyclic IMP; $\bullet$, no cyclic nucleotide present. BSA, bovine serum albumin.

Separation of human polymorphonuclear leucocyte protein kinase into catalytic and regulatory subunit fractions: histone-Sepharose 4B affinity chromatography

Cyclic AMP-sensitive protein kinases can be separated into regulatory (R), or cyclic AMP-binding, and catalytic (C), or cyclic-independent, subunits by substrate-affinity chromatography on a histone-Sepharose 4B column in the presence of cyclic [^3H]AMP (Whitaker, 1963; Miyamoto *et al.*, 1969; Cuatrecasas, 1970; Corbin *et al.*, 1972). The fractions (Fig. 1a) of peak protein kinase activity, obtained by Sephadex G-75 chromatography (approx. 35-fold purification over 27000g supernatants) were applied to a column equilibrated with 10mM-Mes buffer, pH 6.5. Fig. 2 shows the results of such an experiment: cyclic [^3H]AMP emerged before the NaCl gradient and at low NaCl concentration, and this peak of radioactivity (protein-bound as determined by gel filtration which retarded free cyclic [^3H]AMP) was clearly separated from the peaks of protein kinase activity. Two peaks emerged; the peak eluted at higher ionic strength was now largely independent of added cyclic AMP. Both its elution profile from histone-Sepharose 4B and its cyclic nucleotide independence identify this activity as that usually associated with the C subunit (Reimann *et al.*, 1971; Corbin *et al.*, 1972).

Fig. 2. Substrate-affinity chromatography of the protein kinase ($(\text{NH}_4)_2\text{SO}_4$ precipitate of 27000g supernatant after dissociation with cyclic AMP

To 0.9ml of the enzyme solution (14g of protein) was added 0.1ml of 0.5mM-cyclic [^3H]AMP. After a 1h incubation at 0°C, the solution was applied to a column (0.9cm x 9cm) equilibrated with 10mM-Mes buffer, pH 6.5. This was followed by the addition of 7.2ml of the same buffer containing 0.1ml of 0.5mM-cyclic [^3H]AMP and then by 3ml of buffer without cyclic AMP. The column was then eluted with a linear gradient of 0–0.5M-NaCl in the same buffer with no added cyclic AMP. Fractions (3ml) were assayed for protein kinase activity with histone as substrate in the presence (○) or absence (●) of cyclic AMP; 0.05ml portions were analysed for cyclic [^3H]AMP (▲) by radioactivity counting. —, NaCl gradient.

Gel filtration of the C subunit

Fractions under the peak eluted at high ionic strength (C subunit), subjected to gel filtration on Sephadex G-75 yielded an estimated molecular weight of 30000. The cyclic [^3H]AMP-sensitive (R) subunit (first peak in Fig. 2) was also applied to Sephadex G-75, both directly and after $(\text{NH}_4)_2\text{SO}_4$ precipitation. In neither circumstance, and despite numerous attempts, was it possible to recover a discrete protein peak, although protein-free cyclic [^3H]AMP could be resolved from the fractions. Difficulties with recoveries of the R subunit have been described by Corbin *et al.* (1972) and these reflect not only the lability of the binding subunit to aging but also its susceptibility to degradation by proteinases such as trypsin.

Isolation of histone phosphatase by substrate-affinity chromatography

The elution profile on histone-Sepharose is shown in Fig. 3. Two peaks were obtained from a linear gradient of 0–0.3M-NaCl in the 10mM-MES buffer (pH 6.4)–1mM-dithiothreitol. The 'histone-specific' phosphatase, peak II, was eluted at a NaCl concentration of 0.1M, and contained 69% of total recovered activity. Peak I, which displayed activities against both acid *p*-nitrophenyl phosphate and histone phosphate, was eluted at a NaCl concentration of 0.07M. Most (70%) of the acid β -glycerophosphatase activity was eluted before the column was eluted with a linear gradient of 0–0.3M-NaCl in the same buffer. On the other hand, 85% of acid *p*-nitrophenyl phosphatase activity was eluted at a NaCl concentration of 0.07M with the histone phosphatase peak I.

Gel filtration of histone phosphatase on Sephadex G-150 and estimation of molecular weight

To determine elution patterns of the two peak fractions of histone phosphatase activity and to test the possibility that dissociation of the enzyme into subunits had been induced by substrate-affinity chromatography, gel-filtration experiments were carried out on a calibrated Sephadex G-150 column. Histone phosphatase activity of peak I obtained from substrate-affinity chromatography could be resolved into two major peaks B, C and one minor peak A. Although the two major peaks (B and C) were clearly separate from acid *p*-nitrophenyl phosphatase activity, the minor peak still displayed activity against *p*-nitrophenyl phosphate (Fig. 4a). Peak II obtained from substrate affinity chromatography could be resolved into two peaks which corresponded (judged by the elution profile) to peaks B and C of peak I (Fig. 4b).

Fig. 3. Substrate-affinity chromatography of histone phosphatase on histone-Sepharose column

A column (0.9cm×10cm) was equilibrated with 10mM-Mes buffer, pH6.5, containing 1mM-dithiothreitol; 19mg of $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction in 2ml of the same buffer solution was applied to the column. This was followed by 12ml of the same buffer and then the column was eluted with a linear gradient of 0–0.3 M-NaCl in the same buffer in a total volume of 100ml. The flow rate was 1 ml/min. The eluates of the column were collected in 2ml fractions. ●, Histone phosphatase; ○, acid *p*-nitrophenyl phosphatase; ▲, acid β -glycerophosphatase. —, NaCl gradient.

Fig. 4. Gel filtration of histone phosphatase on Sephadex G-150

The column (1cm×69cm) was equilibrated with 10mM-Mes buffer, pH6.5, containing 1mM-dithiothreitol and 50mM-NaCl. The flow rate was 1 ml/4min. The eluates were collected in 1ml fractions. (a) 740 μ g of peak-I material from the histone-Sepharose column; (b) 360 μ g of peak-II material from the histone-Sepharose column; (c) 4mg of the $(\text{NH}_4)_2\text{SO}_4$ fraction. ●, Histone phosphatase; ○, acid *p*-nitrophenyl phosphatase; ▲, acid β -glycerophosphatase.

To determine whether histone phosphatase had dissociated into subunits as a result of substrate-affinity chromatography, the $(\text{NH}_4)_2\text{SO}_4$ precipitate of the 27000g supernatant was applied directly to the Sephadex G-150 column. The same two major peaks (B and C) and one minor peak (A) were observed (Fig. 4c), indicating that these did not result from enzyme dissociation into subunits by the substrate-affinity chromatography. The apparent molecular weights of histone phosphatase and the two acid phosphatases were estimated as described by Whitaker (1963), after the elution volumes of the phosphatase activities had been compared with protein markers of known molecular weight (Fig. 5). Acid β -glycerophosphatase and acid *p*-nitrophenyl phosphatase were eluted in the molecular-weight regions of 162000 and 125000 respectively. Assuming that the histone phosphatase activity at the molecular weight region 125000 was due to non-specific phosphatase activity, then the apparent molecular weights of 'specific' histone phosphatases are 45000 and 18700.

A summary of the purification of histone phosphatase from human polymorphonuclear leucocytes by substrate-affinity chromatography and gel filtration on Sephadex G-150 is shown in Table 1. The 23-fold increase in enzyme activity obtained by the affinity-chromatographic step is almost equivalent to the purification of liver histone phosphatase through the calcium phosphate gel and DEAE-cellulose-chromatographic steps described by Meisler & Langan (1969). The more than 200% recovery in the $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction may reflect the presence of an inhibitor in the homogenate such as that described by Kato & Bishop (1972). The protein

concentrations of material from peaks B and C of the eluate from Sephadex G-150 were too low to be reliably detected by the method used. In any case, since histone phosphatase activity is displayed by other (phosphomonoesterase) enzymes, the 'purification' achieved is grossly underestimated.

Histone phosphatase activity in subcellular fractions

Human polymorphonuclear leucocytes were subjected to subcellular fractionation as previously described (Tsung & Weissmann, 1973), with β -

Fig. 5. Estimation of the molecular weights of human polymorphonuclear leucocyte histone phosphatase and acid phosphatases by gel filtration on Sephadex G-150

Data were plotted by the method of Whitaker (1963). The nomenclature used for designating histone phosphatase fractions is given in Fig. 4. The column void volume was 19 ml. Marker proteins in column effluents were detected by absorption at 280 nm, and 420 nm for cytochrome *c*. V_e , elution volume; V_0 , void volume. (1) cytochrome *c* (mol. wt. 12 500); (2) ovalbumin (45 000); (3) bovine serum albumin (67 000); (4) glyceraldehyde 3-phosphate dehydrogenase (140 000); (5) histone phosphatase peak A; (6) histone phosphatase peak B; (7) histone phosphatase peak C; (8) acid *p*-nitrophenyl phosphatase; (9) acid β -glycerophosphatase.

glucuronidase as a marker for primary or azurophilic granules (true lysosomes). When activity of the 800 g pellet was neglected, since unbroken cells remained in this fraction (judged by electron micrographs and the specific activity of β -glucuronidase), twice as much histone phosphatase remained in the cytosol as in the sedimentable fractions, i.e. total activity of 1553 units (see Table 1 for definition of unit) compared with 783 units, or 66% compared with 33% of cytoplasmic activity respectively. In contrast, this fraction contained only 7% of β -glucuronidase activity, a finding compatible with inadequate homogenization and lack of disruption of polymorphonuclear leucocyte granules *in vitro*. Since only the large-granule (27 000 g) fraction of human polymorphonuclear leucocytes has been adequately characterized as lysosomal (Kato & Bishop, 1972; Tsung & Weissman, 1973), attention was paid to the properties of the lysosomal and cytoplasmic histone phosphatases.

Ammonium molybdate effect on cytosol and lysosomal histone phosphatase

Paigen & Griffiths (1959) have reported that lysosomal phosphoprotein phosphatase can be distinguished from the soluble enzyme by virtue of the relative susceptibility of the former enzyme to ammonium molybdate inhibition. The inhibition by ammonium molybdate of lysosomal histone phosphatase activity was observed (Table 2). However, the cytosol histone phosphatase was insensitive to ammonium molybdate up to a concentration of 0.1 mM. The results correlated well with the observations of Paigen & Griffiths (1959).

Substrate-affinity chromatography of cytosol fraction

To determine whether the soluble histone phosphatase of the cytosol could be separated from non-specific phosphatases by means of affinity to the substrate, the cytosol fraction was applied to a histone-Sepharose column. The elution profile of

Table 1. Purification of histone phosphatase from human polymorphonuclear leucocytes

The purification procedure is described in the Materials and Methods section. One unit of enzyme is the amount that catalyses the release of 1 pmol of orthophosphate per 10 min from phosphorylated histone.

Fraction	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Recovery (%)	Purification (-fold)
Homogenate	57	128	7296		
27 000g supernatant	25	362	9050	124	2.8
(NH ₄) ₂ SO ₄ precipitate	19	1087	20653	283	8.5
Histone-Sepharose eluate (peak II of Fig. 3)	0.48	2911	931	13	22.7
Sephadex G-150 eluate (peak B of Fig. 4)	0.004	5220	21	0.2	40.8
Sephadex G-150 eluate (peak C of Fig. 4)	0.004	4000	16	0.2	31.2

Table 2. *Effect of ammonium molybdate on cytosol and lysosomal histone phosphates*

The assay was performed as described in the Materials and Methods section except that 0.1% (v/v) of Triton X-100 was added to the assay system when the lysosomal fraction was used; 48 μ g of lysosomal and 50 μ g of the cytosol fractions were used.

[Ammonium molybdate] (M)	Histone phosphatase activity			
	Lysosomal fraction (c.p.m.)	% inhibition	Cytosol fraction (c.p.m.)	% inhibition
—	2318		5312	
10^{-5}	1714	35	5284	0.5
10^{-7}	1076	59	5302	0.2
10^{-3}	608	77	3856	27

Fig. 6. *Substrate-affinity chromatography of 105000g supernatant fraction on histone-Sepharose column*

Conditions were identical with those described for Fig. 3; 2 ml (4 mg) of the 105000g supernatant was applied to the column. ●, Histone phosphatase; ○, acid *p*-nitrophenyl phosphatase; —, NaCl gradient.

Table 3. *Solubilization of lysosomal phosphatases by Triton X-100*

Lysosomal fraction (15 mg) suspended in 2 ml of 0.25M-sucrose containing 10mM-Mes buffer, pH 6.5, and 1 mM-dithiothreitol was incubated with 0.1% Triton X-100 for 30 min at 0°C, and then centrifuged at 105000g for 1 h. The precipitate was suspended mechanically in the same medium by means of a glass-Teflon homogenizer. Enzyme activities were assayed as described in the Materials and Methods section except that the incubation time was 30 min for histone phosphatase and β -glycerophosphatase assays.

	Histone phosphatase		β -Glycerophosphatase		<i>p</i> -Nitrophenyl phosphatase		β -Glucuronidase	
	Total activity (pmol)	% activity	Total activity (nmol)	% activity	Total activity (nmol)	% activity	Total activity (nmol)	% activity
Soluble form	126	2.5	21000	62.5	4515	67.4	6122	70
Particulate form	4826	97.5	12600	37.5	2183	32.6	2623	30

cytosol histone phosphatase on histone-Sepharose showed a single peak at the position of peak II of Fig. 3 which was eluted at a NaCl concentration of 0.1M, and this 'specific' histone phosphatase activity

peak could be clearly separated from the peak of acid *p*-nitrophenyl phosphatase activity (Fig. 6). When the cytosol fraction was rechromatographed on Sephadex G-150 (not shown) activity appeared only in the

molecular-weight regions of 45000 and 18700. These results suggest that the bulk of the 'specific' histone phosphatase of human polymorphonuclear leucocytes is located in the cytosol fraction as in rat (Shibko & Tappel, 1963; Meisler & Langan, 1969) and mouse liver (Paigen & Griffiths, 1959), and that most of the histone phosphatase activity of peak II of the chromatogram in Fig. 3 is due to a non-cytosol enzyme with *p*-nitrophenyl phosphatase activity.

Solubilization of lysosomal fraction by Triton X-100

In order to compare lysosomal with cytosol histone phosphatase, solubilization of the lysosomal enzyme was attempted, with little success. Acid β -glycerophosphatase and acid *p*-nitrophenyl phosphatase were examined simultaneously. Table 3 shows the result of treatment with 0.1% Triton X-100. Less than 3% of total histone phosphatase activity was released from the lysosomal fraction despite the solubilization of 62.5 and 67.3% of total acid β -glycerophosphatase and acid *p*-nitrophenyl phosphatase activity respectively. Release of 70% of total β -glucuronidase activity was observed in this experiment. Even when the Triton X-100 concentration was increased to 10% (v/v), over 80% of the total activity of histone phosphatase still remained in the particulate fraction. In other experiments, results identical with those obtained by treatment with 0.1% Triton X-100 were obtained after freezing and thawing 10 times. Owing to the small amount and lability of the solubilized histone phosphatase activity, we were unable to elute assayable activity of this enzyme by means of

substrate-affinity or Sephadex G-150 chromatography. Nevertheless, these results clearly demonstrate the presence of a lysosomal particulate histone phosphatase which contributes roughly 20% of the total cytoplasmic activity.

Effect of substrate concentration on histone phosphatase activity

Since the solubilization of histone phosphatase from the lysosomal fraction was unsuccessful, the apparent K_m values for dephosphorylation of histone of the material from peak B or peak C from Fig. 4(a) was studied; they did not differ significantly, being 4.3×10^{-6} M and 3.6×10^{-6} M respectively (Fig. 7). These results are in concordance with the elution profile of the histone phosphatase after substrate-affinity chromatography in which 'histone-specific' phosphatase appeared as a single peak (Fig. 3, peak II).

pH optimum of histone phosphatase activity

The optimum pH for cytosol histone phosphatase activity was between 6.5 and 7.0 (Fig. 8), a range intermediate between that of bovine spleen (Revel & Racker, 1960) and mouse liver (Paigen, 1958) phosphoprotein phosphatase, pH 5.8–6.0, and that of liver histone phosphatase (Meisler & Langan, 1969) and phosphoprotein phosphatase from cerebral cortex (Maeno & Greengard, 1972), pH 7.2–7.5. Similar results were obtained with the lysosomal fractions described above (Fig. 8).

Fig. 7. Activity of histone phosphatase as a function of the concentration of ^{32}P -labelled histone (a)

Substrate concentrations are expressed as concentration of the ^{32}P content of the labelled histone. The reaction mixture containing 11 μg of peak-B material (●) or peak-C material (○) plus the indicated concentrations of ^{32}P -labelled histone were incubated at 37°C for 5 min. The K_m value for peak-B material was 4.3×10^{-6} M (b), and that for peak-C material was 3.6×10^{-6} M (c).

Fig. 8. pH-dependence of histone phosphatase of human polymorphonuclear leucocytes

Assays were performed as described in the Materials and Methods section; 37 μ g of $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction was used. Buffers: 0.01 M-sodium acetate buffer was used for pH 4.0–5.6; 50 mM-Mes buffer was used for pH 6.0–7.0; and 0.1 M-Tris-HCl buffer was used for pH 7.2–9.0; 0.1% Triton X-100 was added when lysosomal fraction (100 μ g) was used. ○, Cytosol fraction; ●, lysosomal fraction.

Effect of bivalent cations on the activity of histone phosphatase

The effect of various bivalent cations on the activity of human polymorphonuclear leucocyte histone phosphatase is shown in Table 4. Similar results were observed in the cytosol and lysosomal fractions. At the concentrations tested (5 and 10 mM), MnCl_2 stimulated the enzyme activity to about 5-fold, whereas MgCl_2 yielded less than 2-fold stimulation compared with the control. ZnSO_4 , CuSO_4 and FeCl_2 exhibited inhibitory effects, and CaCl_2 and $\text{Co}(\text{NO}_3)_2$ had no significant effect on the activity. The cerebral cortex phosphoprotein phosphatase studied by Kato & Bishop (1972) showed similar bivalent-cation dependency. However, Paigen & Griffiths (1959) and Revel & Racker (1960) found that Fe^{2+} was the most effective activator of mouse liver and bovine spleen phosphoprotein phosphatase.

Effect of various cyclic nucleotides on the activity of histone phosphatase

No cyclic nucleotide dependency (at 10 μ M) was observed with the histone phosphatase partially purified by substrate-affinity chromatography. Also, no significant effect of cyclic nucleotides on the histone phosphatase activity of the cytosol fraction or the lysosomal fraction was observed.

Table 4. Effect of bivalent cations on the activity of human polymorphonuclear leucocyte histone phosphatase

Histone phosphatase activity was determined with 10 μ M- ^{32}P -labelled histone as substrate under the standard assay conditions in the presence of different metal concentrations. $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction (37 μ g) was used in the enzyme activity assay at 37°C for 10 min. Activity is expressed as pmol of [^{32}P]phosphate released per 10 min.

Metal	Histone phosphatase activity at two different concentrations of metal ions	
	5 mM	10 mM
MnCl_2	19.8	20.8
CaCl_2	2.9	3.4
MgCl_2	6.8	6.0
$\text{Co}(\text{NO}_3)_2$	3.5	4.4
ZnSO_4	0.9	3.2
CuSO_4	0.8	2.8
FeCl_2	0.8	2.6
–Metal	3.7	

Discussion

In general, the properties of human polymorphonuclear leucocyte protein kinase resemble those of several cyclic AMP-sensitive protein kinases derived from other tissues with respect to apparent K_m values for ATP and histone, K_a values for cyclic AMP and cyclic IMP, pH optimum, isoelectric point, substrate specificity and requirements for bivalent cations (Miyamoto *et al.*, 1969; Tao *et al.*, 1970; Jergil & Dixon, 1970; Kuo *et al.*, 1971; Reimann *et al.*, 1971; Rubin *et al.*, 1972). Our demonstration that protein kinase activity is maximally stimulated by cyclic AMP and cyclic IMP, but not by cyclic GMP, unlike an enzyme from lobster muscle (Kuo *et al.*, 1971), suggests that cyclic AMP-mediated inhibition of leucocyte function (Weissmann *et al.*, 1971; Zurier *et al.*, 1973a,b; Goldstein *et al.*, 1973) may result from the phosphorylation of an unknown substrate by the polymorphonuclear leucocyte enzyme.

The present study also indicates that human polymorphonuclear leucocytes contain several enzymes capable of dephosphorylating histone previously phosphorylated by protein kinase. The bulk (69%) of 'specific' histone phosphatase of the cytoplasm was recovered in the cytosol, displayed strong affinity for histone-Sepharose, had molecular weights of 45000 and 18700 measured by exclusion chromatography, and was unaffected by ammonium molybdate. A minor part (20%) remained particulate in the lysosomal fraction from whence it could not be solubilized under conditions that liberated most of the lysosomal enzymes (e.g. β -glucuronidase); this activity was partially inhibited by ammonium molybdate.

In contrast, acid *p*-nitrophenyl phosphatase, which was also capable of hydrolysing histone phosphate, displayed a lesser affinity for histone-Sepharose, had an apparent molecular weight of 125000 measured by exclusion chromatography, and could be largely solubilized from lysosomes, a property shared with acid β -glycerolphosphatase, which showed no affinity for histone-Sepharose.

Consequently, it appears that the cytosol of human polymorphonuclear leucocytes contains both a cyclic AMP-sensitive protein kinase (Tsung *et al.*, 1972; Tsung & Weissmann, 1973) and a phosphoprotein phosphatase capable of phosphorylating and dephosphorylating histone respectively. Since cyclic AMP and cyclic GMP exert opposing effects on leucocyte functions (Estensen *et al.*, 1973; Zurier *et al.*, 1974) it would have been remarkable had the phosphatase proved capable of responding to these cyclic nucleotides. Since only the presence of Mn^{2+} ions stimulated the activity of histone phosphatase to any significant degree, it will be important to study the effects of cyclic GMP on the translocation of Mn^{2+} in these cells. Indeed, the specific effect of Mn^{2+} on the function of sarcoma I cells has already aroused interest (Rabinovitch & DeStefano, 1973). Nevertheless, no data are available that would permit one to frame a hypothesis as to which of several potential substrates for phosphorylation and dephosphorylation is critical for cyclic nucleotide-mediated effects on polymorphonuclear leucocytes: microtubules, contractile proteins, membranes, etc. It is quite unlikely, however, that they utilize the histone kinase-histone phosphatase system for the regulation of histone phosphorylation during gene activation as has been postulated in other cell types (Louis & Dixon, 1973), since these short-lived end-stage cells neither divide nor synthesize RNA.

The cytosol enzyme, with respect to pH optimum, bivalent-cation requirement and lack of cyclic-nucleotide response resembles other phosphoprotein phosphatases described (Paigen & Griffiths, 1959; Meisler & Langan, 1969; Paigen, 1958; Maeno & Greengard, 1972). The lysosomal enzyme of human polymorphonuclear leucocytes differs significantly, however, from the lysosomal phosphoprotein phosphatases of bone (Vreven *et al.*, 1973) and mouse liver (Shibko & Tappel, 1963). The latter, not resolved by techniques of affinity chromatography from other phosphomonoesterases, have more acid pH optima and are readily solubilized, together with their companion hydrolases, by detergents. It is impossible to exclude the idea that the particulate lysosomal histone phosphatase of polymorphonuclear leucocytes is in some fashion derived from the cytosol enzyme from which it differed only with respect to subcellular localization, solubility and partial sensitivity to ammonium molybdate. It is, however, unlikely that the cytosol enzyme had escaped from lysosomes

during homogenization, since the absence of β -glucuronidase from the cytosol serves as proof that the granules remained intact during preparation. Although approx. 15% of cytoplasmic histone phosphatase was found in granules sedimenting between 27000g and 105000g, it was not considered worthwhile to analyse enzymes in these fractions, since proper techniques for the resolution of secondary and tertiary granules of human (Hirschhorn & Weissmann, 1965), as opposed to rabbit (Baggiolini *et al.*, 1969, 1970; Farquhar *et al.*, 1972), polymorphonuclear leucocytes have not been devised.

Finally, it is probably noteworthy that, in addition to this histone phosphatase of near-neutral pH optimum, lysosomes of human and rabbit polymorphonuclear leucocytes contain several other enzymes that are active at neutral pH: these include an elastase (Janoff & Zeligs, 1968), a histonase (Davies *et al.*, 1971; Weissmann *et al.*, 1972; Louie & Dixon, 1973), lysozyme (Baggiolini *et al.*, 1969, 1970) and myeloperoxidase (Singer & Fruton, 1957). It is entirely conceivable that lysosomal enzymes which display a relatively acid pH optimum against one artificial substrate (e.g. *p*-nitrophenyl phosphate) may act at near-neutral pH on another (histone phosphate).

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