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The Isolation, Purification and some Properties of the Alkaline Phosphatase of Human Leucocytes

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Valentine & Beck (1951), Wachstein (1946), Wiltshaw & Moloney (1958) and Williams & Mendel (1954) have reported significant alterations in the alkaline phosphatase of the leucocyte in some maladies. Large increases in the content of this enzyme in the human leucocyte have been found in infection, polycythaemia vera and in leukaemoid states, and sharp decreases have been noted in chronic myelocytic leukaemia. These changes have been sufficiently well documented by both biochemical and histochemical techniques to justify their use as diagnostic aids in the study of

some blood dyscrasias (Koler, Seaman, Osgood & Vanbellinghen, 1958). However, little is known of the physiological role of this enzyme in the leucocyte nor of the part it plays in this cell's response to stress and disease.

This investigation was therefore undertaken in an attempt to elucidate the specific action of the alkaline phosphatase of the human leucocyte. A purified preparation of the enzyme from human leucocytes was prepared and was studied for pH optimum, metal requirements and substrate specificity.

MATERIALS AND METHODS

The methods used in the separation of leucocytes from whole blood have been described by Trubowitz, Feldman, Benante & Kirman (1957). Approximately 50 l. of freshly drawn human blood was processed in 5 l. batches. The washed separated leucocytes obtained from each batch were suspended in 200 ml. of water, homogenized in a glass homogenizer at 0–5° for 5 min. and stored at −20° until required.

During the course of the purification procedure, alkaline phosphatase was determined by incubating 0·3 ml. of the enzyme preparation, 9·0 ml. of sodium glycerol 2-phosphate (0·026 M), buffered at pH 9·9, with 0·1 M-sodium hydroxide and 0·02 M-sodium diethyl barbiturate, 0·2 ml. of 0·05 M-$MgCl_2$ and 0·5 ml. of 0·9% NaCl solution. All determinations were done in duplicate. The reaction was stopped after 1 hr. at 37° by the addition of 2 ml. of 30% trichloroacetic acid. Precipitated material was removed by filtration. Sample blanks consisted of mixtures to which the trichloroacetic acid was added before the enzyme and reagent blanks contained 0·3 ml. of 0·9% NaCl in place of the enzyme solution. Phosphorus was determined in 3 ml. portions of the filtrates by the method of Fiske & Subbarow (1925); after colour development, extinctions were measured at 660 mμ. Enzyme activity is reported as μg. of phosphorus liberated/hr. under these conditions. Specific activity is expressed as μg. of phosphorus liberated/hr./mg. of enzyme nitrogen. Nitrogen values were determined by the micro-Kjeldahl method. In the determination of enzyme activity on the various phosphorylated substrates, a final substrate concentration of 2 mM was employed. This concentration was chosen in view of the poor solubility of several of the substrates.

RESULTS

Purification procedure

Butanol extraction and acetone precipitation. Liberation of the enzyme from lipids and phospholipids with subsequent solubilization was achieved by extraction with butan-1-ol by the method of Morton (1950). Approximately 200 ml. of homogenized leucocytes, representing the cells from 5 l. of blood, was extracted at −10° with 0·4 vol. of butan-1-ol with stirring for 5 min. The mixture was then warmed to 37°, stirred for 5 min. and centrifuged at 0–5° for 15 min. at 900 g. This operation produced three layers: a butan-1-ol phase, an interface pellet and a lower aqueous phase. The butan-1-ol was removed by aspiration and discarded. The interface pellet, together with the aqueous phase, were filtered by suction. The pellet was washed twice with portions (10 ml.) of water; the washings were added to the aqueous phase and the pellet was discarded. The aqueous phase was then subjected to two further butan-1-ol extractions as described above. In the next step, acetone was added to the aqueous layer to a final concentration of 60% (v/v) at −10°. After 12 hr. at −20°, the precipitate was removed by centrifuging at 0–5° for 15 min. at 900 g, and the supernatant was discarded. Excess of acetone was removed from the precipitate by a stream of nitrogen.

The precipitate was redissolved either in approx. 100 ml. of water and freeze-dried (the freeze-dried preparation was stable indefinitely at −20°) or in approx. 60 ml. of 0·1 M-sodium hydrogen carbonate, and used for the next step, i.e. ammonium sulphate precipitation. Specific activity was increased from 16·2 to 402 with no significant loss of total enzyme activity. The yield of precipitate varied between 800 mg. and 1 g. of powder for each 5 l. of blood.

Ammonium sulphate fractionation. Pooled freeze-dried powder obtained in the previous series of steps was dissolved in 0·1 M-sodium hydrogen carbonate to a concentration of approx. 1 mg. of nitrogen/ml. and centrifuged at 0–5° for 15 min. at 900 g to remove traces of insoluble material. Saturated ammonium sulphate was added to 60% of saturation. The solution was centrifuged at 0–5° for 20 min. at 900 g and the supernatant discarded. The precipitate was redissolved in approx. 40 ml. of 0·1 M-sodium hydrogen carbonate and the solution was dialysed against 0·1 M-sodium hydrogen carbonate at 0–5° until free of sulphate. The specific activity was increased from 304 to 738 by this fractionation. Centrifuging the solution of the freeze-dried powder in sodium hydrogen carbonate before ammonium sulphate treatment resulted in a 100% recovery of activity with removal of 7% of the total nitrogen.

Acetone precipitation, chloroform extraction and ethanol fractionation. Acetone was added to the solution after dialysis to a final concentration of 60% (v/v). Precipitation was allowed to take place for 12 hr. at −20° and the preparation was centrifuged at 0–5° for 20 min. at 900 g. The supernatant was discarded, excess of acetone removed by means of a stream of nitrogen and the precipitate redissolved in 55 ml. of 0·1 M-sodium hydrogen carbonate. Chloroform (1 vol.) was added and the mixture was stirred vigorously at room temperature for 10 min. The preparation was again centrifuged at 0–5° for 15 min. at 900 g, and the chloroform and interface residue were discarded. Abso-

Table 1. *Specific activity and average percentage yields at major steps during the course of purification of leucocyte alkaline phosphatase*

Assays were performed with 9·0 ml. of 0·026M-sodium β-glycerol 2-phosphate (buffered at pH 9·9 with 0·02M-veronal), 0·02 ml. of 0·05M-$MgCl_2$, 0·5 ml. of 0·9% NaCl and 0·3 ml. of enzyme preparation. The range of specific activities (μg. of P liberated/hr./mg. of enzyme N) represents the values obtained in the course of the purification of ten individual batches of leucocytes. Average percentage yield refers to each individual step. Incubations were at 37° and enzyme activity was stopped by the addition of 2 ml. of 30% trichloroacetic acid.

Operation	Specific activity (μg. of P/hr./mg. of N)	Average yield (%)
Isolation and homogenization of cells	6·6–18·6	—
Butanol extraction (three times)	250–267	96
Precipitation with 60% (v/v) acetone	272–515	95
Ammonium sulphate fractionation (0–60%) and dialysis	738–953	81
Precipitation with 60% (v/v) acetone	1359–1562	65
Chloroform extraction	1936–2050	82
Ethanol fractionation (0–65%, v/v)	3025–4075	66

lute ethanol at −20° was added to 33 ml. of the aqueous solution to a final concentration of 65% (v/v). The preparation was left overnight at −20° and then centrifuged at 0–5° for 20 min. at 900*g*. The precipitate was dissolved in 22 ml. of 0·1M-sodium hydrogen carbonate, yielding a clear colourless solution. The specific activity in the best preparation was increased in this procedure from 738 to 4075. The average yields and specific activity for several batches of enzyme are summarized in Table 1.

Activators. (i) Amino acids. Abul-Fadl & King (1949) and Mathies (1958) have reported on the activation of kidney and intestinal alkaline phosphatase by preliminary incubation with amino acids such as alanine and various other compounds.

In our studies, at several stages of purification, no increase in alkaline-phosphatase activity could be demonstrated by incubation with DL-alanine under identical conditions.

(ii) Metals. Many investigators have reported the existence of metal activators for the alkaline phosphatases. Qualitative emission spectrographic analysis by Mathies (1958) of purified swine-kidney alkaline phosphatase revealed the presence of ten ions after dialysis of his best preparations. Of these, the major metals present in the order listed were zinc, copper, aluminium and iron.

As previously reported by Trubowitz *et al.* (1957), incubation of isolated leucocytes with ethylenediaminetetra-acetic acid (EDTA) resulted in marked inhibition of alkaline-phosphatase activity. Upon addition of zinc at a final concentration of 0·25 mM, phosphatase activity was restored to the original value. Mathies (1958) indicated the possibility that swine-kidney alkaline phosphatase is a zinc metalloenzyme. He found that dialysis of his preparation against EDTA caused an inactivation that was accompanied by a proportional loss in zinc. Furthermore, a linear relationship was obtained between enzyme purity and zinc concentration. Analysis of our preparations indicated an increasing zinc content during purification. Zinc content/mg. of enzyme nitrogen in the purified preparation was 33 times that found in the homogenized cells. Results are shown in Table 2. Zinc was determined by titration with potassium ferrocyanide with diphenylamine as indicator (Scott, 1939).

Table 2. *Change in zinc content with purification of leucocyte alkaline phosphatase*

Zinc determinations were performed by Dr Carl Tiedke, Laboratory of Microchemistry, Teaneck, New Jersey.

State	Zinc (μg./mg. of N)	Enzymic activity (μg. of P/hr./mg. of N)
Cells	1·34	16·2
Acetone-dried powder	5·15	402
Purified preparation	44·1	4075

Incubation of a 30-fold purified preparation with a final concentration of 0·055% sodium EDTA in water for 1 hr. resulted in 95% loss of activity as tested under the usual conditions in the presence of mM-Mg^{2+} ions. The addition of other cations at concentrations of 1–100 μM restored up to about 50% of the original activity (Table 3). Higher concentrations markedly inhibited the activity in both the chelated and unchelated series. These findings are in direct contrast with the studies with leucocytes, where the addition of zinc after EDTA-chelation resulted in complete restoration of activity (Trubowitz *et al.* 1957).

Overnight dialysis of purified enzyme against either water or sodium hydrogen carbonate (0·1M), at 0–5°, resulted in a loss of a third of the total activity. This finding might indicate, as suggested by Abul-Fadl & King (1949), that there is a specific dialysable organic group or groups which

Table 3. *Effect of metal ions on reactivation of purified leucocyte alkaline phosphatase after treatment with ethylenediaminetetra-acetic acid*

Enzyme preparation (1·0 ml.) was preincubated for 1 hr. at 37° with EDTA (sodium salt) at a final concentration of 0·055%. For assay of alkaline-phosphatase activity, 4·55 ml. of sodium β-glycerol 2-phosphate (final concn. 2 mM), buffered at pH 9·9 with 0·02M-veronal, was mixed with 0·1 ml. of 0·05M-$MgCl_2$, 0·1 ml. of the ion being tested and 0·25 ml. of enzyme preparation containing 0·30 mg. of N, and was incubated at 37° for 1 hr. If no metal ion was added after chelation, the amount of inorganic phosphorus liberated was 3·0 μg. of P/hr. In all cases the chloride salt of the metal was used.

Concn. of ion (μM)	100 (μg. of P/hr.)		10 (μg. of P/hr.)		1 (μg. of P/hr.)	
Metal ion	EDTA-treated	Not treated	EDTA-treated	Not treated	EDTA-treated	Not treated
Al^{3+}	26·0	60·6	24·2	59·8	28·9	62·9
Mn^{2+}	16·4	59·8	17·5	59·8	22·9	56·8
Cd^{2+}	5·8	52·8	6·2	56·6	14·9	62·1
Ni^{2+}	7·9	62·3	12·7	62·4	29·6	62·3
Cr^{3+}	24·4	66·8	17·7	63·0	18·5	60·4
Cu^{2+}	29·1	61·9	22·9	61·2	22·6	66·8
Fe^{2+}	16·6	50·5	22·4	67·7	21·6	61·0
Co^{2+}	22·5	66·5	20·8	63·1	18·7	62·8
Zn^{2+}	2·5	60·0	34·2	61·0	21·5	61·0

Table 4. *Relative specific activity of a 30-fold purified leucocyte alkaline-phosphatase preparation on phosphorylated substrates*

The incubation mixture consisted of 4·65 ml. of substrate (final concn. 2 mM) buffered at pH 9·9 with 0·02M-veronal, 0·1 ml. of 0·05M-$MgCl_2$ and 0·25 ml. of enzyme equivalent to 0·25 mg. of N. Incubation was at 37° for 1 hr. and enzyme activity was stopped by the addition of 1 ml. of 30% trichloroacetic acid at 5°.

Substrate	Phosphorus liberated (μg./hr.)	Substrate	Phosphorus liberated (μg./hr.)
2:3-Phosphoglyceric acid	1·0	Adenosine tetraphosphate	5·1
3-Phosphoglyceric acid	3·6	Adenosine triphosphate*	6·8
Tetrose 4-phosphate	13·0	Adenosine diphosphate	27·0
Phosphoenolpyruvic acid	13·2	5′-Adenosine monophosphate	41·7
Sodium β-glycerol 2-phosphate	30·0	3′-Adenosine monophosphate	32·3
Ribose 5-phosphate	27·8	Uridine triphosphate	10·3
Fructose 6-phosphate	9·3	Uridine diphosphate	31·1
Mannose 6-phosphate	10·2	Uridine monophosphate	41·9
Galactose 6-phosphate	10·2	Guanosine triphosphate	12·5
Glucose 6-phosphate	16·8	Guanosine diphosphate	22·2
Glucose 1-phosphate	25·9	Guanosine monophosphate	30·4
6-Phosphogluconic acid	15·4	Cytidine triphosphate	9·2
2-Deoxyglucose 6-phosphate	14·9	Cytidine diphosphate	22·1
Fructose 1:6-diphosphate*	31·7	Cytidine monophosphate	31·3
Sedoheptulose 7-phosphate	17·5	Thymidine monophosphate	48·0
Cytidine diphosphocholine	3·1	Inosine monophosphate	29·2

* High blanks were obtained.

are indispensable for activity as well as the specific protein and metal. The action of the metal may depend upon the presence of such a group. However, since the final concentration of protein in our purified preparation was very low (0·066 mg. of nitrogen/ml.), the observed loss of activity may merely be due to lability of the protein in dilute solutions.

Action on phosphorylated substrates. Table 4 summarizes the results obtained with the 30-fold-purified enzyme incubated with a number of phosphorylated substrates. As can be seen, the preparation is capable of liberating inorganic phosphorus from a wide variety of substrates including nucleotides. The results obtained with the phosphorylated sugars indicate that when the phosphorus is substituted on the end carbon atom, e.g. 3-phosphoglyceric acid, tetrose 4-phosphate, ribose 5-phosphate, glucose 6-phosphate and sedoheptulose 7-phosphate, the length of the carbon chain seems to be important. The C_5 chain is the most labile to the action of the enzyme and the series runs $3 < 4 < 5 > 6 = 7$.

Since sufficient purified material was not available, most of the substrate studies were carried out with an acetone preparation which represented a 30-fold purification of the enzyme. For several of the substrates (see Table 5) the ratio of phosphorus liberated from the substrate to that liberated from sodium β-glycerol 2-phosphate was the same with the acetone precipitate or the 250-fold-purified enzyme, which was the best preparation obtained.

Kinetic studies and K_m *values.* The purified enzyme exhibited classical first-order kinetics with all substrates used. Sufficient enzyme was not available to determine Michaelis constants on all substrates, but Table 6 shows the results obtained on five substrates. Values were obtained by plots of reciprocal velocity/reciprocal substrate concentration according to the method of Lineweaver & Burk (1934).

Competitive studies. To determine whether the phosphatase activity was due to a single enzyme or to multiple enzymes, competitive studies were undertaken. The action of the purified enzyme on a combination of sodium β-glycerol 2-phosphate and 5′-adenosine monophosphate as well as on sodium β-glycerol 2-phosphate and uridine monophosphate was compared with the results obtained when the enzyme was incubated with each substrate individually. As can be seen in Table 7, liberation of phosphate was not additive.

pH *optimum.* pH optima were investigated with several substrates. Below pH 9, activity fell off very rapidly and the optimum pH for all substrates was between 9·0 and 9·9. With sodium β-glycerol 2-phosphate specific activity fell from 1212 at pH 9·9 to 48 at pH 7·0 and with 5′-adenosine monophosphate from 1742 at pH 9·9 to 156 at pH 7·0. In all cases sodium hydroxide–0·02M-veronal buffers were employed.

Physical properties. The physical properties of the most purified preparation obtained are now being studied. Preliminary ultracentrifugal studies indicate the presence of at least two components in the preparation, with the heavier component containing approx. 85 % of the original activity.

DISCUSSION

Trubowitz *et al.* (1957) suggested, from inhibition studies with EDTA, the existence of multiple alkaline phosphatases in the leucocyte. Meislin, Lee & Wasserman (1959) arrived at similar conclusions from a study dealing with the phosphatase activity of leucocytes in myeloproliferative disorders. Follette, Valentine & Reynolds (1959) concluded from their studies on the ability of leucocytes to split sodium β-glycerol 2-phosphate and 5′-adenosine monophosphate that a single enzyme was involved. The data obtained in this

Table 5. *Comparison of activity of 30-fold-purified alkaline phosphatase with 250-fold-purified preparation*

The incubation mixture consisted of 4·65 ml. of substrate (final concn. 2 mM) buffered at pH 9·9 with 0·02M-veronal, 0·1 ml. of 0·05M-$MgCl_2$ and 0·25 ml. of enzyme equivalent to 0·25 mg. of N. Incubation was at 37° for 1 hr. and enzyme activity was stopped by the addition of 1 ml. of 30 % trichloroacetic acid at 5°. Ratio = μg. of P liberated from substrate/μg. of P liberated from β-glycerol 2-phosphate.

	Ratio	
Substrate	30-Fold-purified acetone ppt.	250-Fold-purified preparation
Fructose 1:6-diphosphate	0·28	0·32
Glucose 6-phosphate	0·27	0·29
Fructose 6-phosphate	0·36	0·33
Glucose 1-phosphate	0·77	0·78
5′-Adenosine monophosphate	1·39	1·43

Table 6. *Michaelis constants for purified leucocyte alkaline phosphatase*

The incubation mixture consisted of 4·65 ml. of substrate (final concn. 2 mM–0·024M) buffered at pH 9·9 with 0·02M-veronal, 0·1 ml. of 0·05M-$MgCl_2$ and 0·25 ml. of enzyme equivalent to 0·25 mg. of N. Incubation was at 37° for 1 hr. and enzyme activity was stopped by the addition of 1 ml. of 30 % trichloroacetic acid which had been cooled to 5°.

Substrate	K_m (mM)
5′-Adenosine monophosphate	6·1
3′-Adenosine monophosphate	7·3
Sodium β-glycerol 2-phosphate	15·9
Ribose 5-phosphate	17·5
Fructose 6-phosphate	20·0
Glucose 6-phosphate	22·2

Table 7. *Action of alkaline phosphatase on mixtures of substrates*

The incubation mixture consisted of 4·65 ml. of substrate (final concn. 2 mM) buffered at pH 9·9 with 0·02M-veronal, 0·1 ml. of 0·05M-$MgCl_2$ and 0·25 ml. of enzyme equivalent to 0·25 mg. of N. Incubation was at 37° for 1 hr. and enzyme activity was stopped by the addition of 1 ml. of 30 % trichloroacetic acid at 5°. When a combination of substrates was used, each substrate had a final concentration of 2 mM.

	P liberated (μg./hr./mg. of enzyme N)	
Substrate	30 min.	60 min.
Sodium β-glycerol 2-phosphate	584	1075
5′-Adenosine monophosphate	813	1523
Uridine monophosphate	—	1119
Sodium β-glycerol 2-phosphate + 5′-adenosine monophosphate	889	1652
Sodium β-glycerol 2-phosphate + uridine monophosphate	—	1251

present study also suggest that the activity of the leucocyte on a variety of phosphorylated compounds may reside in a single enzyme although the preparation was not homogeneous in the ultracentrifuge. Good correlation was observed for the activity of a 30-fold-purified preparation and a 250-fold-purified enzyme on a number of different and representative substrates (see Table 5). However, limitation of material precluded further comparative assays with a larger number of substrates, and it may well be that had such a study been carried out, differences in activity on some substrates might have been detected. Further evidence for the existence of a single enzyme was furnished by the lack of additive effect when the enzyme was allowed to act on mixtures of substrates (see Table 7).

The physiological significance of the demonstrated action of the enzyme on the nucleotides is not clear. The action of the leucocyte alkaline phosphatase against the nucleotides may be merely an expression of its apparent preference for the C_5 compounds.

The leucocyte has also been shown to be rich in zinc and a zinc–protein has been isolated whose concentration has been found to be low in leukaemia and high in infection (Vallee, 1949, 1959). These facts are relevant in the light of the data obtained in this study, which showed an increase in the zinc content of the enzyme with purification. Zinc has been shown to be an activator of this enzyme and indeed may be an integral part of the molecule. Thus the changes observed in the zinc concentration of the leucocyte in leukaemia and infection may be due, in part, to the corresponding changes in the alkaline phosphatase.

SUMMARY

1. The isolation and partial purification of the alkaline phosphatase of normal human leucocytes has been described. A 250-fold purification was attained with the best preparation, having a specific activity of 4075 μg. of phosphorus/mg. of nitrogen/hr. The purified enzyme contained 44·1 μg. of zinc/mg. of nitrogen.

2. The less-purified preparation was capable of liberating inorganic phosphorus from a wide variety of substrates, including mono-, di- and triphosphorylated nucleotides. The monophosphorylated nucleotides were apparently most susceptible to enzyme attack. Of the sugar intermediates, the C_5 chain with phosphorus substituted on the end carbon atom appeared to be most labile.

3. The purified enzyme exhibited first-order kinetics with the substrates tested and competitive data suggest that the liberation of inorganic phosphorus may be due to a single enzyme rather than to a multiple enzyme system, provided that no inhibition occurs.

4. No specific physiological role can as yet be ascribed to the leucocyte alkaline phosphatase.

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