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Comparative study of alkaline phosphatase activity in lymphocytes, mitogen-induced blasts, lymphoblastoid cell lines, acute myeloid leukemia, and chronic lymphatic leukemia cells

(N-alkaline phosphatase/thiophosphoric acid thioesters)

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ABSTRACT **Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline pH optimum), EC 3.1.3.1] purified from a Burkitt lymphoma cell line (Daudi) and Moloney-virus-induced murine leukemia (YAC) showed unique catalytic properties in substrate specificity and inhibition by cysteamine-*S*-phosphate. It migrated on polyacrylamide gel electrophoresis in a single activity band. Alkaline phosphatase with similar properties was found in several human lymphoblastoid cell lines, in chronic lymphatic leukemic cells, in organs of leukemic mice, and in sera of patients with certain lymphoproliferative disorders.**

The unique kinetic properties of this enzyme were established using two kinds of substrate, namely, the monoesters of orthophosphoric acid (Type I) and the *S*-substituted monoesters of thiophosphoric acid (Type II). The enzyme catalyzed the hydrolysis of Type I, but failed to hydrolyze Type II. The enzyme probably interacts directly either in catalysis or in binding with the —P—O— or —P—S— bonds. Thus, in these respects it differs from the established enzymatic mechanism of other known alkaline phosphatases, and therefore can be considered a new enzyme. This enzyme was designated N-alkaline phosphatase.

N-alkaline phosphatase was absent in untreated and mitogen-stimulated human blood lymphocytes, in murine lymph nodes, and pig thymus (even after mitogen stimulation), and in blast cells of acute myeloid leukemic patients.

The appearance of N-alkaline phosphatase only in certain types of proliferating lymphocytes and in the sera of patients with lymphoproliferative disorders may be exploited as a cell marker and as a diagnostic tool.

Concomitantly with neoplastic transformation, alterations of certain enzyme activities are known to occur (1, 2). In spite of the fact that the biological significance of these alterations in most cases is unknown, they can be exploited as convenient markers because they can be revealed in minute amounts and measured with great accuracy.

Increased alkaline phosphatase activity [APase; orthophosphoric-monoester phosphohydrolase (alkaline pH optimum), EC 3.1.3.1] with unique catalytic properties has been described in mouse organs infiltrated with lymphoma cells (3–6). Chronological studies in mice revealed that this enzyme appears in measurable amounts in the thymus prior to the diagnosis of the disease (6). APase with the same kinetic properties was also demonstrated in the sera of patients with lymphoid malignancies and infectious mononucleosis (7–9). This enzyme was designated N-alkaline phosphatase, N-APase.

Studies on the occurrence of the enzyme, its purification from human and murine cells, and its properties are described in the present paper.

Abbreviations: APase, alkaline phosphatase; N-APase, N-alkaline phosphatase; *p*-NPP, *p*-nitrophenyl phosphate; CASP, cysteamine-*S*-phosphate; AE-Cl, aminoethanol-Cl; AML, acute myeloid leukemia; CLL, chronic lymphatic leukemia; Emulphogene, BC 720, a nonionic detergent with the chemical composition alkoxypoly(ethylenoxy)-ethanol.

MATERIALS AND METHODS

Human Blood Lymphocytes. Lymphocytes were isolated from blood of normal human donors. Lymphocyte cultures were set up in 4 ml of RPMI 1640 medium containing 12.5% autologous plasma at a cell concentration of 0.5×10^6/ml. Blast transformation was by phytohemagglutinin at 5 μg/ml. After 72 hr incubation, 0.2 μCi of [2-^{14}C]thymidine was added and the cultures were harvested 16 hr later for the determination of [^{14}C]-thymidine incorporation into DNA. In each experiment lymphocyte culture of 10 donors, comprising 12 to 52×10^6 cells, were pooled for enzymatic assays.

Rat Lymphocytes. Cultures of rat lymph node cells (Wistar rats) were kindly supplied by Dr. A. Novogradsky (10).

Pig Thymocytes. Pig thymus cells prepared according to Allen and Crompton (11) were donated by Dr. B. Schechter.

Murine Lymphoma Cell Line. The YAC ascites cell line (12), a Moloney-virus-induced lymphoma, was carried in syngeneic mice.

Human Lymphoblastoid Cell Lines. Cell lines derived from Burkitt lymphomas Namalwa (13), Raji (14), Daudi (15), Maku (16), Seraphina (17); nasopharyngeal carcinoma LY28 (18); infectious mononucleosis Kaplan (19); chronic myeloid leukemia K562 (20); and acute lymphoblastic leukemia 1301 (21) were kept in RPMI medium supplemented with 10% fetal calf serum as stationary suspension cultures.

White Cells from Blood of Chronic Lymphocytic Leukemia (CLL) and Acute Myeloid Leukemia (AML) Patients. After sedimentation of erythrocytes of the blood, the plasma was collected and the white cells were centrifuged and washed three times. Six of the AML cell samples were frozen and stored in dimethylsulfoxide. The ampoules were thawed at 37° and washed three times in RPMI containing 10% fetal calf serum.

Chemicals. *p*-Nitrophenyl phosphate (*p*-NPP) and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. and Aldrich Chemical Co., respectively. DEAE-cellulose (DE-23) was purchased from Whatman Co.; Sephadex and Sepharose, from Pharmacia Fine Chemicals; Emulphogene, the detergent alkoxypoly(ethylenoxy)ethanol, from General Aniline and Film Corp.; Tris, from Sigma Co. Aminoethanol-*O*-phosphate was purchased from British Drug Houses; *O*-phosphoryl-L-serine (Lot 60622), from Calbiochem.

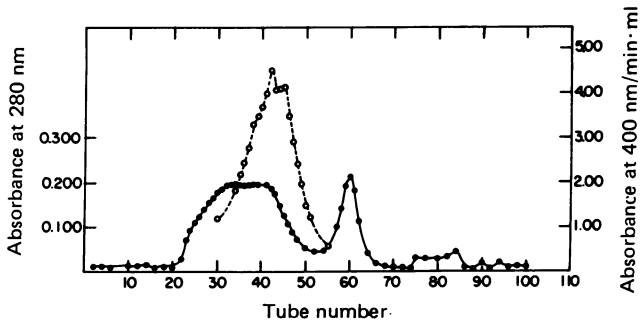


FIG. 1. DEAE-cellulose chromatography of 1% Emulphogene extract of Daudi cells. The column was equilibrated and eluted with 0.5 M AE-Cl buffer, pH 8.0, at a flow rate of 2.0 ml/20 min. A_{280} (●—●); enzymatic activity (O- - - -O).

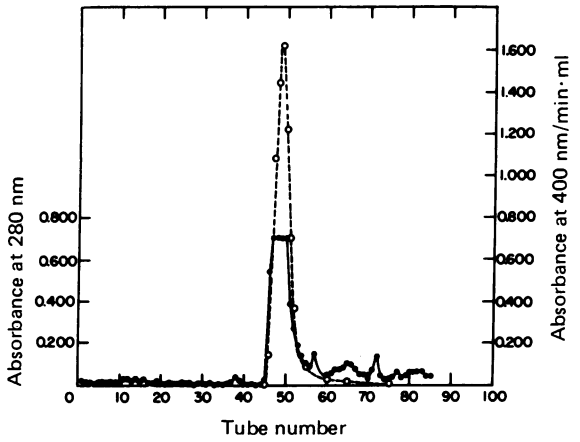


FIG. 2. From the pool of tubes 47, 48, 49, and 50 obtained from a DEAE-cellulose column (Fig. 1) 7.4 ml was concentrated with Aquacid to 2 ml and subjected to Sepharose 4B gel filtration. Elution of 2.0 ml per tube was with 0.05 M AE-Cl buffer, pH 8.0, at a flow rate of 2.0 ml/10 min. A_{280} (●—●); enzymatic activity (O- - - -O).

Aminoethanol was distilled freshly before preparing the buffers. Cysteamine-*S*-phosphate (CASP) was prepared according to Akerfeldt (22). All other compounds used were of analytical grade.

Preparation of the Cell Extracts. The number of cells extracted ranged from 10^6 to 10^8. They were washed three times with 0.1 M Tris·HCl, pH 8.0, and homogenized in the same buffer in a Sorvall microhomogenizer for 5 min at 4°. The soluble proteins were separated by ultracentrifugation at 100,000 × *g* for 30 min. The residual pellet was subjected either to 1-butanol:water extraction (0.3 ml:1.0 ml) by the addition of 0.1 ml of 1-butanol to the aqueous pellet suspension at 5 min intervals or to extraction by water containing Emulphogene. After stirring for 15 min at room temperature the extracts were centrifuged at 100,000 × *g* for 30 min.

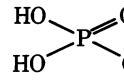
Enzymatic Assays. APase activity measurements (23) were carried out using 0.01–0.10 ml extracts in 1.0 ml of 0.5 M Tris·HCl buffer, pH 9.0, containing 1.0 mM *p*-NPP as substrate.

The hydrolysis of *p*-NPP was followed spectrophotometrically at 400 nm for 10–60 min, taking measurements every minute. The absorbance was plotted versus time, and the velocity, *v*, of *p*-nitrophenol production was calculated from the linear part of the curve. The molar absorbance of *p*-nitrophenol at 400 nm is 18,000. Activity with CASP as substrate was assayed under similar conditions. The amount of cysteamine released by hydrolysis of CASP was measured with the aid of dithionitrobenzoate (0.4 mM) in the reaction mixture, according to the method of Ellman (24). The release of 5-thio-2-nitrobenzoic acid was measured spectrophotometrically at 412 nm at 2 min intervals up to 60 min. The absorbance was plotted versus time, and from the linear part of the curve the velocity, *v*, was calculated. The molar absorbance of 5-thio-2-nitrobenzoic acid at 412 nm is 13,600. All enzymatic assays were carried out at room temperature (22°).

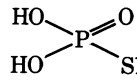
Aminoethanol-*O*-phosphate or phosphoserine was also used as a substrate in some experiments. Activity measurements of the extracts were carried out in 0.1 M aminoethanol-Cl buffer, pH 9.0, or 0.05 M barbital–Cl buffer, pH 9.0. Inorganic phosphate, one of the reaction products, was determined by the method of King (25). Aliquots (1.0 ml) were taken every minute, the amounts of inorganic phosphate were plotted versus time, and the velocity, *v*, was calculated from the linear part of the curve. Aminoethanol-*O*-phosphate and phosphoserine were hydrolyzed by all extracts with similar efficiency and the results therefore are not included in this paper. In contrast to CASP, aminoethanol-*O*-phosphate was hydrolyzed by N-APase.

Calculation of the Amount of N-APase. The known APase catalyzes the hydrolysis of monoesters of orthophosphoric acid (substrate Type I) and *S*-substituted monoesters of thiophosphoric acid (substrate Type II) with similar efficiencies (3–9).

Substrate Type I



Substrate Type II



The moieties R for substrate Type I used were: —⬡—NO_2, —CH_2—CH_2—NH_2, and NH_2—CH(—CH_2—)—COOH.

For known APases the mean value of the ratio *R*, for the rate of hydrolysis, *p*-NPP/CASP, was found to vary between 1.1 and 1.9, and was never greater than 2.0.

The amount of N-APase was calculated from the ratio of the rate of hydrolysis of the two substrates *p*-NPP and CASP.

The relative activity of N-APase was expressed according to the equation:

$$\% \text{ of N-APase activity} = (v_{p\text{-NPP}} - 1.6\, v_{\text{CASP}}),$$

where ordinary APase = $1.6\, v_{\text{CASP}}$, and $v_{p\text{-NPP}}$ is the total APase activity. $v_{p\text{-NPP}}$ and v_{CASP} are the velocities of hydrolysis of the two substrates. The factor 1.6 was chosen on the basis of variation of the *R* value 1.1–1.9 for the ordinary APase.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in 5% crosslinked polyacrylamide vertical gel columns at pH 9.5 at constant amperage of 3 mA per tube for 15–20 min at room temperature. Samples were applied to the gel in sucrose. The APase band was made visible with β-naphthyl phosphate and diazonium salt Fast Blue as described by Smith *et al.* (26). Corresponding bands on duplicate samples were eluted and analyzed.

Column Chromatography. DE-23 (Whatman) was pretreated according to Peterson and Chiazze (27). The DE-23 (2 × 60 cm) column was equilibrated with 0.5 M aminoethanol-Cl (AE-Cl), pH 8.0, buffer. A Sephadex G-200 (or Sepharose 4B, or 6B) column (1.5 × 100 cm) was used for further purification of the APase separated by DE-23 columns. The gel column was washed and eluted with 0.015 M, AE-Cl buffer, pH 8.0, collecting 1.5 ml/20 min. The tubes were assayed for APase activity and absorbance at 280 nm was used to determine

Table 1. Results of the purification of APase from Daudi and YAC cells by column chromatography

Cell designation	Total cells in 8 ml	A_{280}	Specific activity*			Final purification factor
			First extract	DEAE	Gel filtration	
Daudi	2.4×10^8	35.00	0.006	0.125	1.150	191
YAC	5.0×10^8	12.16	0.003	0.042	3.120	1040

* Specific activity is expressed as μmol of substrate (*p*-NPP) hydrolyzed per 1 mg of protein/ml per min.

protein content, assuming that at this wavelength 1 mg/ml of protein produces an *A* of 1.0 (1 cm light path).

RESULTS

Purification of N-APase from a Cell Line (Daudi) and Moloney-Virus-Induced Murine Leukemia Cells (YAC). Water containing 1.0% (wt/vol) Emulphogene extracts from the cells amounts of enzymic activity similar to those extracted with 1-butanol:water, but is preferable for chromatography because it has only *one phase*. Furthermore, the enzyme is more stable in this extract. Probably denaturation of proteins occurs in the interface of butanol:water during storage. Therefore, for all the column work we used water–emulphogene extraction. We tried several other emulsifiers (sodium dodecyl sulfate, Triton, etc.), but the results were poor.

N-APase was found to have high affinity for several ion exchange materials. For separation of the highly negatively charged compounds present in the homogenate 5.0 mg of poly(L-lysine) at pH 8.0 was added to the extracts. The amount of poly(L-lysine) added was chosen so that no APase was precipitated. After centrifugation the supernatant was applied to a DE-23 column preequilibrated with 0.5 M AE-Cl buffer. It was necessary to use this high concentration of AE-Cl buffer because at lower concentration N-APase stuck to the column.

Extracts of 3–5 ml with known APase activity were applied to the DEAE-cellulose column. Absorbance at 280 nm and APase activity using *p*-NPP were measured and plotted versus tube number. Typical curves for Daudi and YAC cells are given in Figs. 1 and 3. The eluates did not hydrolyze CASP, or hydrolyzed it only to a very small extent. Tubes containing the highest APase activity were concentrated with Aquacid and reapplied to a Sephadex G-200 or Sepharose 4B column for further purification.

Protein content and APase activity of each tube were measured (Table 1, Figs. 2 and 4). Again, CASP was not hydrolyzed by these eluates.

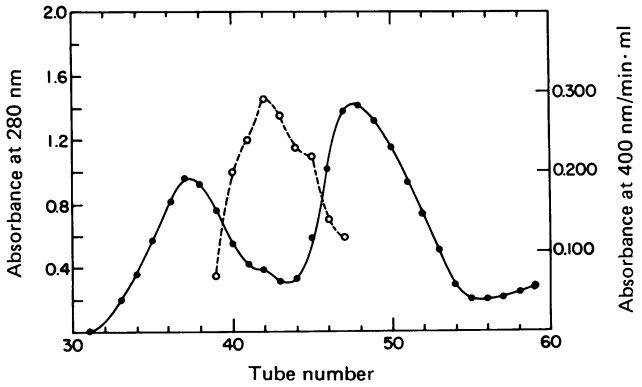


FIG. 3. DEAE-cellulose chromatography of YAC cell extract. The extraction was performed in one step with 0.1 M Tris-HCl buffer, pH 8.0, containing 1.0% Emulphogene. Elution of 2.5 ml per tube was with 0.5 M AE-Cl buffer at a flow rate of 2.5 ml/20 min. A_{280} (●—●); enzymatic activity (O- - - -O).

It is apparent that a 100- to 1000-fold purification was achieved by this procedure. The contents of the peak tubes were subjected to polyacrylamide gel electrophoresis (both in the presence and absence of Emulphogene in the gel). After the gels were stained for APase activity, only one band appeared (Fig. 5).

APase Activity in Lymphoblastoid Cell Lines and Cells of CLL and AML Patients. The identification of N-APase was based on: (*a*) the ratio, *R*, of the rate of hydrolysis of *p*-NPP versus CASP under identical conditions; (*b*) electrophoretic mobility of APase activity in polyacrylamide gel electrophoresis. In previous studies it was shown that the ratio *R* of the rate of hydrolysis of two types of substrate (Type I and Type II) from organs of normal mice and sera of normal individuals does not exceed 1.9 (3–9). The ratio *R* for human lymphocytes was found to be 1.20 ± 0.1 (calculated from Table 2); for murine lymphocytes, 1.10 ± 0.18; and for pig thymocytes, 1.18 ± 0.20.

Table 2. Hydrolysis of *p*-NPP and CASP by butanol extracts of normal and mitogen-stimulated human blood lymphocytes, human lymphoblastoid cell lines, and a murine lymphoma induced by Moloney virus

Cell*	Specific activity†		*R*‡	% N-APase
	p-NPP	CASP		
Lymphocytes	0.290	0.234	1.24	0
Lymphocytes	0.212	0.180	1.18	0
MS-lymphocytes	0.325	0.307	1.06	0
MS-lymphocytes	0.408	0.400	1.02	0
Human lymphoblastoid cell lines				
K-562	63.0	42.0	1.50	0
K-562	32.0	31.0	1.03	0
Daudi	11.0	1.0	11.00	77
Daudi	12.0	0.2	60.00	97
Raji	60.0	1.2	50.00	98
Raji	360.0	1.5	240.00	99
Seraphina	14.0	1.0	14.00	89
Namalva	12.8	0.2	64.00	98
Kaplan	60.0	0		100
Kaplan	7.0	0		100
LY 28	5.7	0.6	9.50	83
LY 28	1.2	0.2	6.00	73
1301	10.0	0.2	50.00	97
Murine lymphoma				
YAC	132.0	22.0	6.00	73
YAC	120.0	26.2	4.58	35

* Each experiment with normal human blood lymphocytes and mitogen-stimulated (MS-) lymphocytes represents a pool of several individual donors.

† Specific activity expressed as nmol of substrate hydrolyzed/min per 10^6 cells under the experimental conditions specified in the *text*.

‡ $R = v_{p\text{-NPP}}/v_{CASP}$, where $v_{p\text{-NPP}}$ = nmol of *p*-NPP hydrolyzed/min per 10^6 cells and v_{CASP} = nmol CASP hydrolyzed/min per 10^6 cells.

Table 3. Hydrolysis of *p*-NPP and CASP by butanol extracts of peripheral leukocytes of leukemia patients

Patient	Specific activity *p*-NPP	Specific activity CASP	*R*	% N-APase
1. AML	98.60	86.00	1.1	0
2. AML	2.51	2.60	1.0	0
3. AML	510.24	296.90	1.7	0
4. AML	8.40	8.00	1.0	0
5. AML	4.20	3.10	1.3	0
6. AML	0.70	0.50	1.4	0
7. AML	0.20	0.15	1.4	0
8. AML	7.00	3.50	2.0	0
9. AML	7.00	6.20	1.2	0
10. AML	5.00	3.50	1.4	0
1. CLL	1.60	0.40	4.0	47
2. CLL	0.60	0.10	6.0	73
3. CLL	2.40	0.07	34.0	97
4. CLL	1.60	0.20	8.0	80
5. CLL	0.60	0.10	6.0	73
6. CLL	12.00	1.80	6.6	79
7. CLL	0.70	0.20	3.5	54
8. CLL	1.30	0.40	3.2	50
9. CLL	0.40	0.10	4.0	60
10. CLL	2.90	0.50	5.8	72
11. CLL	0.60	0.10	6.0	73
12. CLL	0.40	0.10	4.0	60
13. CLL	0.60	0.10	6.0	73

Specific activity and *R* are as for Table 2.

Essentially the same ratio was obtained for mitogen-stimulated cells.

The APase activities of normal and mitogen-stimulated lymphocytes, of human, murine, or porcine origin migrated identically. The mobility remained the same in the presence of Emulphogene in the gel. Thus we concluded that N-APase is absent in these cells and is not induced by mitogen-caused proliferation.

In murine lymphoma cells (YAC) and lymphoblastoid cell lines derived from Burkitt lymphomas (Daudi, Raji, Maku, Seraphina, and Namalva) N-APase comprises 50–90% of total APase activity (Table 2).

N-APase was found also in two nonlymphoma-derived lymphoblastoid lines: Kaplan and LY28. The N-APase in the Kaplan cells was nearly 100% of the total APase activity. On the other hand, N-APase could not be detected in the K562 cell line (Table 2). This cell line was established from a chronic myeloid leukemia patient.

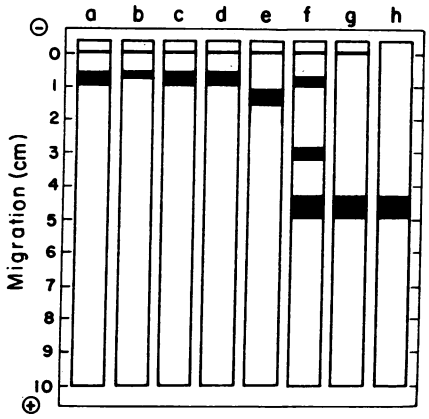


FIG. 5. Polyacrylamide gel electrophoresis of samples (5–100 μl) of the 1-butanol:water or water:1% Emulphogene extracts. The electrophoresis was performed for 15 min at 3 mA and the gels were stained for APase activity. (a) Human blood lymphocytes; (b) human blood lymphocytes plus Emulphogene; (c) mitogen-stimulated lymphocytes; (d) mitogen-stimulated lymphocytes plus Emulphogene; (e) pig lymphocytes; (f) YAC; (g) purified Daudi (Sepharose 4B column, tube 50; (h) purified YAC (Sephadex G-200 column, tube 31. Electrophoretograms performed in the presence of 0.1% Emulphogene in the gel gave similar results.

The incidence of N-APase in peripheral leukocytes of leukemia patients is shown in Table 3.

The electrophoretic mobility of the enzyme derived from these lines differed from that of normal lymphocyte APase (Fig. 5). From YAC cells a band was eluted which contained only N-APase, as the eluate hydrolyzed only Type I substrates. This was the fastest moving band among three with APase activity. The eluate of the two slower moving bands hydrolyzed both substrates with *R* values 0.98 ± 0.2 and 1.90 ± 0.2.

The APase from the Kaplan cells migrated with a single band corresponding to the electrophoretic mobility of the N-APase of YAC (and Daudi). This is in line with the kinetic measurements, according to which 100% of the activity was N-APase.

DISCUSSION

APase (EC 3.1.3.1)—the designation includes its isoenzymes—catalyzes the hydrolysis of monoesters of orthophosphoric acid, and *S*-substituted monoesters of thiophosphoric acid with similar efficiency. The nearly complete analogy between the reaction of the two types of compounds with APase means that the enzyme does not interact directly with the linking oxygen or sulfur in a rate-determining step. Since sulfur and oxygen have different van der Waals radii, polarizabilities, and basicities in these compounds, any interaction with them either in

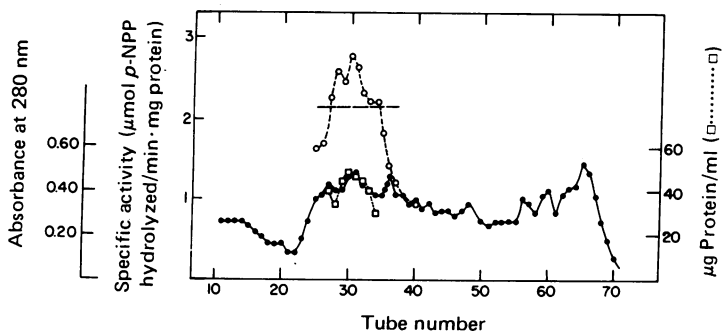


FIG. 4. The pool of tubes 41, 42, and 43 from the DEAE-cellulose column (Fig. 3) was subjected to Sephadex G-200 gel filtration. Elution of 1.9 ml per tube was with 0.01 M AE-Cl buffer, pH 8.0, at a flow rate of 1.9 ml/30 min. Absorbance at 280 nm, the protein content in μg of protein per ml as well, and APase activity are presented. A_{280} (●—●); protein μg/ml (□ · · · · · □); specific activity (○- - - -○).

binding or in catalysis (i.e., protonation) would be expected to give major differences in the catalytic hydrolysis. The lack of such differences is of significance in considering the mechanism of APase action (28–30).

The appearance of an enzyme with distinct catalytic properties and electrophoretic mobility does not *a priori* involve the synthesis of a new protein species with different primary sequence. It may be the result of conformational changes, or different assembly of the subunits, through dissociation–association, allosteric effects, or change in the metal ion(s).

Occurrence of N-APase in certain diseases is not the consequence of the high lymphocyte number in the blood or increased enzyme production in proliferating lymphocytes, but may be connected with the special type of blast transformation, as it was not detected in normal lymphocytes, and mitogen-induced blast cells.

Cells with both bone-marrow-derived (B) and thymus-derived (T) lymphocyte origin were found to produce N-APase. Burkitt lymphoma lines are B cells and so are the CLL cells. On the other hand, murine lymphoma YAC is a T cell line. Preliminary experiments also showed the presence of N-APase in a human T cell line Molt-4 (31). An important finding which may be of practical importance is that the presence of N-APase distinguished cells of lymphoid origin, as it is absent in *myeloid blasts*.

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