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PURIFICATION AND PROPERTIES OF BRAIN ALKALINE PHOSPHATASE

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Abstract—Alkaline phosphatase from sheep brain has been purified to homogeneity. The method includes butanol extraction, fractional ethanol precipitation, ion-exchange chromatography on DEAE-cellulose, and on DEAE-Sephadex followed by Sephadex G-200 filtration. By these steps, the enzyme is purified 22,920-fold with 15% recovery. The homogeneous enzyme is shown to be a sialoglycoprotein in nature. Neuraminidase treatment reduces the electrophoretic mobility of the enzyme. The enzyme shows pyridoxal phosphate phosphatase activity along with *p*-nitrophenylphosphate phosphatase activity. Both these compounds behave as mutual alternate competitive substrates. The general properties of the enzyme are described.

PYRIDOXAL phosphate plays a crucial role in the metabolism of the CNS. Its importance is evidenced not only by the presence of pyridoxal phosphate dependent enzyme like glutamate decarboxylase and glutamate transaminase in this tissue, but also by observation that reagents like isonicotinic acid hydrazide (INH), which forms complexes with the coenzyme, produce seizures, which can be cured by the administration of the vitamin (TOWER, 1958). BEGUM & BACHHAWAT (1960) observed that reagents like INH produced an enhanced dephosphorylation of the coenzyme in the crude brain extract. This observation led to a detailed investigation of the phosphatases in brain with special reference to pyridoxal phosphate phosphatase. An alkaline phosphatase was partially purified from our laboratory using pyridoxal phosphate as substrate (SARASWATHI & BACHHAWAT, 1963). During the studies on the heterogeneity of alkaline phosphatase in brain, it was observed that the two alkaline phosphatase fractions (I & II) from sheep brain which could be separated by ion-exchange chromatography on DEAE-cellulose were associated with different concentrations of sialic acid (SARASWATHI & BACHHAWAT, 1968). It was also shown that the neuraminidase (EC 3.2.1.18) treatment of the alkaline phosphatase fraction which contains the higher concentration of NeuNAc (Enzyme II) makes it chromatographically similar to enzyme I which has a lower concentration of NANA and that the difference in the kinetic properties of the two alkaline phosphatase fractions were not altered (SARASWATHI & BACHHAWAT, 1970). Subcellular distribution and age dependent variation of the enzyme in rat brain were also reported (BISHAYEE & BACHHAWAT, 1972). A detailed investigation was undertaken to further our studies on the brain alkaline phosphatases, in order to elucidate the nature of the isoenzymes and their properties in their membrane-bound state. The present study describes the purification of one of the isoenzymes of alkaline phosphatase (Enzyme II) from sheep brain to homogeneity and its properties. The purified preparation is shown to be a sialoglycoprotein in nature.

MATERIALS AND METHODS

p-Nitrophenyl phosphate (PNPP), pyridoxal-5′-phosphate (PALPO), β-naphthyl acid phosphate, fast Blue BB, adenosine-5′-triphosphate, adenosine-5′-monophosphate, glucose-6-phosphate, pyridoxamine-5′-phosphate, DL-α-glycerophosphate, crystalline bovine serum albumin, cytochrome *c*, ovomucoid trypsin inhibitor, γ-globulin and imidazole were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium β-glycerophosphate and sodium pyrophosphate were from E. Merck, AG Darmstadt. *O*-Phosphorylethanolamine was from Nutritional Biochemical Corporation, Cleveland, OH, U.S.A. Ovalbumin was obtained from Centron Research Laboratories, Bombay, India. DEAE-cellulose was purchased from Carl Schleicher and Schuell Co., NH, U.S.A. DEAE-Sephadex A-50, Sephadex G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. L-Phenylalanine and L-histidine were from CalBiochem., U.S.A. Glucose, galactose and mannose were from Pfanstiehl Laboratories, Waukegan, IL, U.S.A. Sodium dodecyl sulphate was obtained from British Drug Houses, Poole, England. Acrylamide and *N*-*N*′-bisacrylamide were obtained from Eastman Organic Chemicals, U.S.A. All other chemicals used were of analytical grade. Sheep brains were obtained from the local slaughter house and transported in ice within half an hour after the animals were killed.

The enzyme was asayed by using *p*-nitrophenylphosphate as substrate. The assay mixture obtained 0.5 μmol

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Abbreviations used: PNPP, *p*-nitrophenylphosphate; PALPO, pyridoxal-5′-phosphate; NeuNAc; *N*-acetylneuraminic acid; INH, isonicotinic acid hydrazide.

of the substrate, 50 μmol of Tris–HCl buffer pH 9.0, 0.5 μmol of $MgCl_2$ and the enzyme in a total volume of 0.5 ml. Incubation was done at 37°C for 15 min. The reaction was stopped by adding 2.5 ml of 1 M-NaOH. The yellow colour formed due to the liberated *p*-nitrophenol was measured in the Klett–Summerson colorimeter using a No. 42 filter. One unit of the enzyme is defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol from *p*-nitrophenylphosphate in 15 min under the assay conditions. The specific activity was expressed as units per mg protein.

The method of WADA & SNELL (1961) was employed when pyridoxal phosphate was used as the substrate for the enzyme. The incubation mixture contained 50 μmol of Tris–HCl buffer pH 9.0, 0.5 μmol of $MgCl_2$, 0.5 μmol of pyridoxal phosphate and the enzyme in a total volume of 0.5 ml. After incubation at 37°C for 30 min, 0.5 ml of the colour reagent (2% solution of phenylhydrazine hydrochloride in 18 N-sulphuric acid) was added and the volume was made up to 10 ml with water at 0–4°C. The yellow colour due to the formation of pyridoxal phosphate hydrazone was developed for 10 min at 0–4°C. The intensity of the colour was measured in a Klett–Summerson colorimeter using a No. 42 filter. The enzyme activity was linear under the assay conditions for a period of 45 min. One unit of pyridoxal phosphate phosphatase activity was defined as the amount of enzyme required to hydrolyse 1 μmol of pyridoxal phosphate in 30 min under the assay conditions.

Protein estimation

Protein was determined by the method of LOWRY *et al.* (1951) using crystalline bovine serum albumin as standard.

Gel electrophoresis

Polyacrylamide gel electrophoresis with 7.5% gels were carried out as described by DAVIS (1964) in 0.1 M-borate buffer, pH 8.0, using a Canalco electrophoretic apparatus for 2 h 30 min using 3 mA/tube. The gels were stained for protein with Coomassie Brilliant Blue as described by CHRAMBACH *et al.* (1967). The enzyme staining was done at pH 9.0 using β-naphthyl acid phosphate and Fast Blue BB salt, according to the method of SMITH *et al.* (1968). Sodium dodecylsulphate gel electrophoresis was done according to the method of WEBER & OSBORN (1969) using 10% gels, and 0.15 M-sodium phosphate buffer pH 7.0. The gels were stained for protein with Coomassie Brilliant Blue and for enzyme with β-naphthyl acid phosphate and fast blue BB salt as described above.

Molecular weight determination

The molecular weight of alkaline phosphatase was determined by gel filtration according to the method of ANDREWS (1965) using Sephadex G-200. The column was equilibrated with 0.05 M-Tris–HCl buffer pH 7.4 containing 0.1 M-NaCl. A flow rate of 12 ml/h was maintained and fractions of 2 ml were collected. The column was calibrated at 25°C with the following standards: bovine serum albumin, cytochrome *c*, γ-globulin, ovomucoid trypsin inhibitor and ovalbumin.

Carbohydrate analysis of the enzyme

The neutral sugar content of the enzyme was estimated by the phenol sulphuric acid method of DUBOIS *et al.* (1965), using glucose as standard. For the identification of neutral sugar the enzyme (350 μg protein) was hydrolysed at 100°C with 1 M-HCl for 5 h in a sealed tube and processed according to the method of SPIRO (1966). The sugars were identified by paper chromatography using the solvent system of MUKHERJEE & SRIRAM (1964). After drying the paper, the reducing sugar spots were located by staining with alkaline silver nitrate. The sialic acid content of the enzyme was determined according to the thiobarbituric acid method of WARREN (1959) as modified by SAIFER & GERSTENFELD (1962). Prior to sugar estimation and sugar analysis, any sugar possibly leached from the Sephadex G-200 column was removed from the enzyme protein, by dialysing the enzyme solution against saturated ammonium sulphate at 0°C to precipitate the protein alone. The precipitate was collected by centrifugation at 10,000 *g* for 30 min and it was dissolved in 0.01 M-Tris–HCl buffer pH 7.4 and dialysed exhaustively with the same buffer.

Purification of the enzyme from sheep brain

All operations were carried out at 0–4°C unless otherwise indicated.

Step I: Fresh sheep brain was freed from the adhering meninges and blood and the grey matter was removed by gross dissection. The grey matter tissue (700 g) was minced into small pieces and homogenized with 1400 ml of 0.15 M-NaCl, in a Waring blender for 1 min. The crude homogenate (2100 ml) contained 52.8 g protein. It was centrifuged at 1000 *g* for 10 min and the crude nuclear pellet was collected.

Step II: The nuclear fraction was suspended in 2 vol of 0.1 M-acetate buffer pH 5.0, *n*-butanol was added to a final concentration of 20% (v/v) and alkaline phosphatase from the nuclear fraction was extracted according to the method of MORTON (1954) as described by SARASWATHI & BACHHAWAT (1966).

Step III: To the aqueous butanol layer, distilled absolute alcohol at −15°C was added dropwise to a final concentration of 40% with constant stirring. The mixture was kept overnight, at 4°C, after which it was centrifuged at 12,000 *g* for 30 min. The supernatant, which contained the alkaline phosphatase activity was collected. Alcohol concentration in the supernatant was increased from 40% to 50% and the mixture was kept at 4°C for 3 h. The precipitate formed during this period was collected by centrifugation of the mixture at 12,000 *g* for 30 min and dissolved in a minimum volume of 0.05 M-Tris–HCl buffer pH 7.4.

Step IV: The enzyme solution from the previous step containing 120 mg protein was passed through a DEAE-cellulose column (1 × 15 cm) previously equilibrated with 0.05 M-Tris–HCl buffer pH 7.4, at a flow rate of 15 ml/h, and fractions of 5 ml were collected. The column was washed with 30 ml of the same buffer, and gradient elution was performed with an increasing concentration of sodium chloride in 0.05 M-Tris buffer, pH 7.4. The active fractions (Nos. 18–27, designated as enzyme II) were pooled and dialysed against 20 vol of 0.05 M-Tris–HCl buffer pH 7.4 with two changes.

Step V: The dialysed enzyme preparation from the above step was subjected to ion-exchange chromatography on a DEAE-Sephadex A-50 column (8 × 0.6 cm), previously equilibrated with 0.05 M-Tris–HCl buffer, pH 7.4. The column was washed with 2 bed volumes of the same buffer and the enzyme was eluted with a linear gradient of NaCl. Fractions of 2 ml were collected and the flow rate was maintained at 8 ml/h. The active fractions (pooled

volume 16 ml) were concentrated to 1 ml by using aquacide, and dialysed against 100 vol of 0.05 M Tris–HCl buffer pH 7.4 containing 0.1 M-NaCl.

Step VI: The enzyme from the previous step was placed on a column of Sephadex G-200 (50 × 1.5 cm) previously equilibrated with 0.05 M-Tris–HCl buffer pH 7.4 containing 0.1 M-NaCl. A flow rate of 12 ml/h was maintained and fractions of 2 ml were collected. The enzyme appeared in the effluent, immediately after the void volume, and the fractions from tubes 16–22 were pooled and concentrated by aquacide. The concentrated enzyme was dialysed against 0.05 M-Tris–HCl buffer pH 7.4.

RESULTS AND DISCUSSION

Purified alkaline phosphatase was obtained in 15% yield after 22,920-fold purification from sheep brain, using the conventional procedures. The different steps involved in the purification are shown in Table 1. Reproducible results were obtained for the purification when this was repeated a number of times. The pyridoxal phosphate phosphatase activity of the enzyme during the various purification steps was followed using the method of WADA & SNELL (1961). The hydrolysis ratio of pyridoxal phosphate to *p*-nitrophenylphosphate (PALPO/PNPP) was found to be nearly a constant as shown in the Table. The following features of the purification procedure were of interest. The enzyme is firmly associated with membranes, from which it can be released efficiently by butanol treatment. This step brings the enzyme into the aqueous layer, with 86% recovery and 15-fold purification. It is worthwhile to mention at this stage that our earlier work on the extraction of alkaline phosphatase from sheep brain (SARASWATHI & BACHHAWAT, 1966) has been misinterpreted in some recent studies (LUMENG & LI, 1975) as the enzyme being present in the soluble fractions of the sheep brain. The fractional ethanol precipitation was done using the undialysed butanol extract and this proved to be a simple method of removing a large amount of contaminating proteins with a further 13-fold purification. The chromatographic separation of the alkaline phosphatase isoenzymes from sheep brain has already been reported by our earlier work. The first peak, designated as Enzyme I, is collected in the fractions, essentially unadsorbed by the column. The second major peak, designated as enzyme II, is eluted from the column between a linear gradient of 0.05–0.1 M-NaCl.

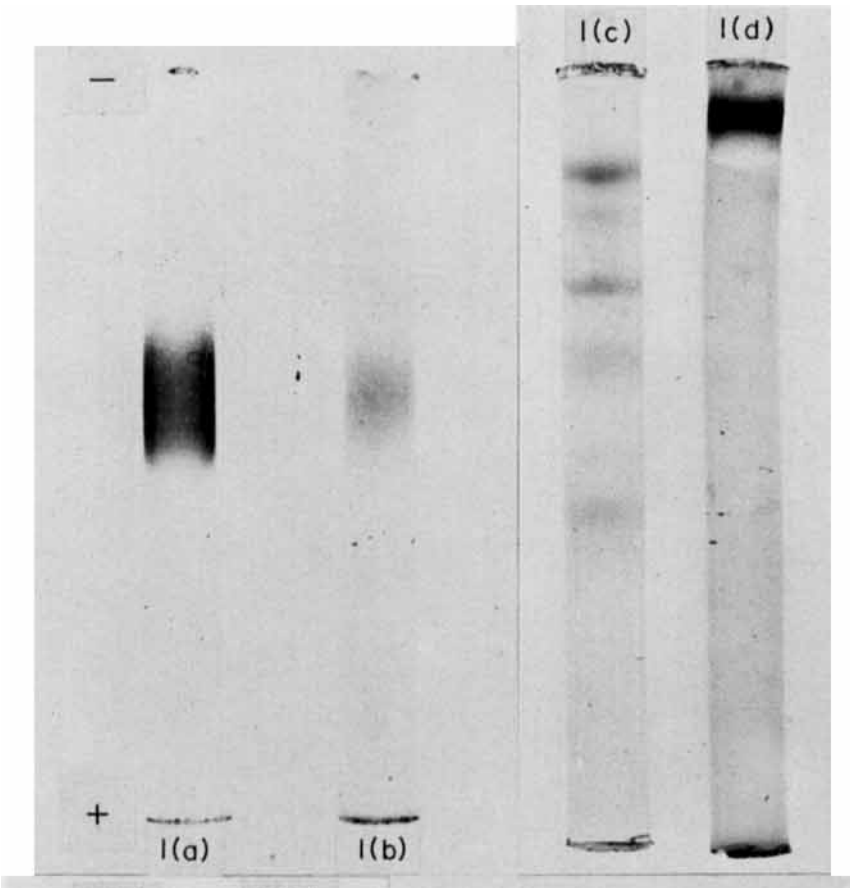
Figure 1a and 1b shows the pattern of enzyme and protein stain of the final preparation of sheep brain alkaline phosphatase, in 7.5% polyacrylamide gel at alkaline pH. The only protein band present was found to coincide with the enzyme band, suggesting that the preparation is essentially homogeneous. The diffuse appearance of the band probably reflects the possible microheterogeneity present, in common with other mammalian alkaline phosphatases (BUTTERWORTH & MOSS, 1966; ROBINSON & PIERCE, 1964). Figure 1c and 1d represents the gel electrophoretic pattern of the purified alkaline phosphatase in the presence of 0.1% sodium dodecylsulphate and 0.1% β-mercaptoethanol. It is interesting to note that sheep brain alkaline phosphatase gives the enzyme stain even after incubation with SDS and electrophoresis in phosphate buffer, according to the method of WEBER & OSBORN (1969). Stability of some bacterial and mammalian enzymes in sodium dodecyl sulphate is reported in the literature (ERICKSON, 1974; NAKAMURA & HAYASHI; 1974; KEENAN, 1974). As it can be seen from the figure, the protein stain for the enzyme in SDS shows 5 bands and the enzyme stain shows a single band, which stays very near to the origin. The anamolous behaviour of sialoglycoproteins in SDS gel electrophoresis is well documented in the literature (SEGREST & JACKSON, 1972). This is mainly due to a decreased binding and partial dissociation of these proteins by SDS. From the behaviour of the sheep brain alkaline phosphatase in SDS gel electrophoresis, it appears that the purified enzyme is partially dissociated by sodium dodecyl sulphate which gives rise to the multiple bands. At the moment, the possibility that the enzyme may not be completely homogeneous at this stage cannot be excluded.

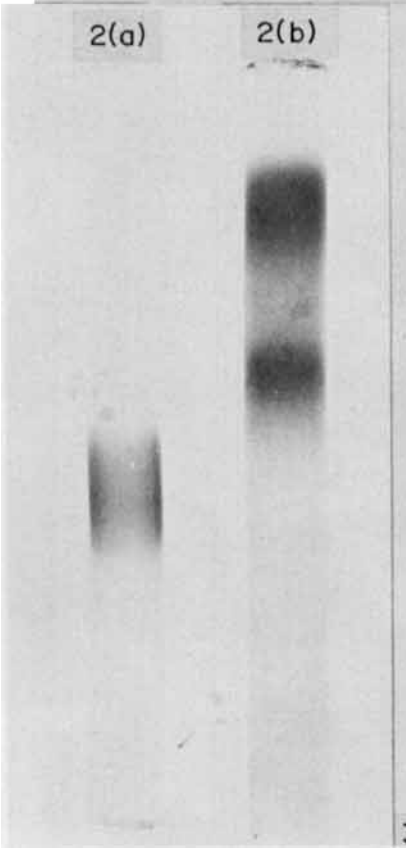
The neutral carbohydrate content of the purified enzyme was estimated to be 18%. An estimation of the sialic acid content of the enzyme showed that one molecule of the enzyme contains 11.7 molecules of sialic acid. These observations prove that the alkaline phosphates from sheep brain is sialoglycoprotein in nature. Similar conclusions were arrived at by GHOSH *et al.* (1974) who showed that the human placental alkaline phosphatase is sialoglycoprotein in nature by GLC. The sialoprotein nature of the brain enzyme is also substantiated by the electrophoretic pattern of the purified native enzyme and the neuraminidase treated enzyme. Figure 2 shows the enzyme stain obtained before and after the neuraminidase treatment. The native enzyme shows a fast moving single band, whereas the neuraminidase treated enzyme showed a complete disappearance of the faster moving band, with the appearance of two slow moving bands. A glycoprotein stain for the alkaline phosphatase could not be obtained in the borate buffer system employed in the electrophoresis procedure. Identification of neutral sugars by paper chromatography as shown in Fig. 3 suggested that glucose is the major component present in sheep brain alkaline phosphatase, the minor component being mannose. The presence of large concentrations of glucose in glycoprotein enzymes was first reported from this laboratory (SARASWATHI & BACHHAWAT, 1971). It should be mentioned at this stage that while glucose is not an universal constituent of sialoglycoproteins, many membraneous glycoproteins have been reported to contain glucose. SHIMIZU & FUNAKOSHI (1970) have reported the presence of glucose in the plasma membranes of rat ascites hepatoma. Glycoproteins of basement membranes of human and bovine kidney have

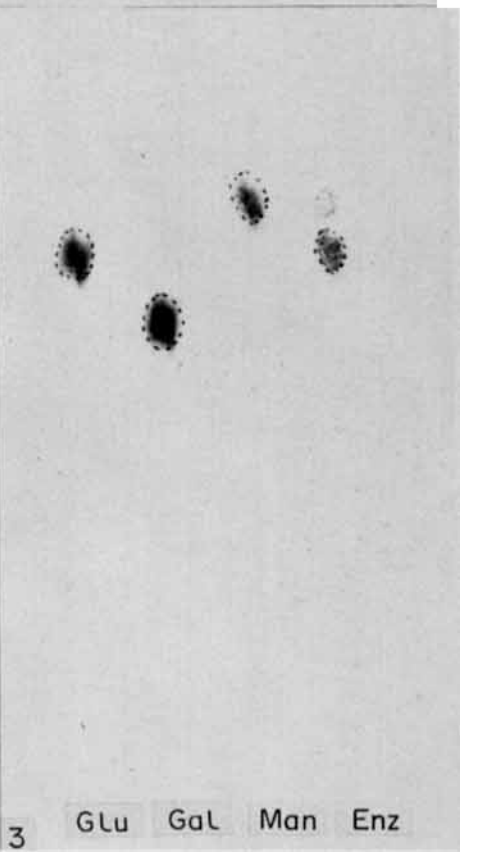
FIG. 1. Gel electrophoretic pattern of the purified alkaline phosphatase at pH 8.0. The conditions of the electrophoresis were as described in the methods. a and b indicate the enzyme and protein stain respectively. c and d: gel electrophoretic pattern of the purified alkaline phosphatase in the presence of SDS. The conditions of the electrophoresis were as described in the methods. c and d represent the protein and enzyme pattern after SDS electrophoresis.

FIG. 2. a and b. Gel electrophoretic pattern of alkaline phosphatase at pH 8.0, before (a) and after (b) neuraminidase treatment. The conditions of the neuraminidase treatment and electrophoresis were as described in the text.

FIG. 3. Paper chromatographic pattern of neutral sugars of the purified alkaline phosphatase. The details of the experiment were as described in the Methods. ENZ indicates alkaline phosphatase. Glucose, mannose and galactose are indicated by Glu, Man and Gal respectively.


—
+
1(a)
1(b)
1(c)
1(d)


2(a)
2(b)


3
Glu
Gal
Man
Enz

been shown to contain the disaccharide glucose-galactose-hydroxylysine in addition to the sialooligosaccharide chain. VAN NIEUW AMERONGEN *et al.* (1972) have isolated a soluble glucose containing sialoglycoprotein from the cortical grey matter of the calf brain. Recent work from our laboratory revealed the presence of glucose in a number of lysosomal acid hydrolases partially purified from sheep brain (BISHAYEE & BACHHAWAT, 1974) and in the highly purified hexosaminidase from normal human urine (BANERJEE & BASU, 1975). A homogeneous preparation of arylsulphatase A from sheep brain (BALASUBRAMANIAN & BACHHAWAT 1975), a homogeneous preparation of arylsulphatase A from chicken brain (AHMAD, 1976) and hexosaminidase A from monkey brain (ARUNA & BASU, 1975) also revealed the presence of glucose in the pure glycoproteins. At present, we have no knowledge regarding the mode of linkage of glucose in the brain alkaline phosphatase, the sequence of monosaccharide residues and the mode of linkage of oligosaccharide to protein.

Properties of the enzyme

The general properties of the alkaline phosphatase from sheep brain are given in Table 2. The highest affinity for the enzyme is shown by *p*-nitrophenyl phosphate which has a K_m value of 0.1 mM. The K_m values of pyridoxal phosphate and 5'-AMP were found to be 0.34 mM and 0.4 mM respectively. These values closely agree with those found by SARASWATHI & BACHHAWAT (1968) for their partially purified alkaline phosphatase II. Inorganic phosphate is shown to be the competitive inhibitor for the brain alkaline phosphatase. The K_i values for inorganic phosphate using *p*-nitrophenylphosphate and pyridoxal phosphate were estimated to be 1.15 mM and 0.45 mM respectively. The molecular weight of the enzyme as determined by gel filtration in 140,000. The possible interaction of the glycoprotein enzyme with the Sephadex was minimized by using NaCl at a concentration of 0.1 M. The enzyme has an optimal pH of 9.0. The results in Table 3 show that Tris–HCl buffer is essential for the maximum enzyme activity. Veronol, borate, bicarbonate and glycine buffers at the same pH of 9.0 produced marked inhibition of the alkaline phosphatase activity towards PALPO and 5'-AMP. Inability of a highly purified alkaline phosphatase

TABLE 1. PURIFICATION OF ALKALINE PHOSPHATASE

Purification step	Total volume (ml)	Total protein (g)	Total enzyme units × 10^{-3}		Specific activity	PALPO/PNPP	Recovery (%)	Fold purification
			PNPP	PALPO				
Homogenate	2100	52.8	11.9	9.5	0.22	0.80	100	—
Nuclear fraction	1050	41.3	9.9	7.5	0.24	0.76	83	1.1
Butanol extract		2.1	8.3	5.8	3.9	0.70	70	17
Ethanol precipitate	20	0.12	6.2	4.6	52.1	0.74	52.5	231
DEAE-Cellulose	51	0.008	4.5	3.5	560	0.78	37.5	2495
DEAE-Sephadex A-50	16	0.00087	2.5	2.0	3365	0.81	25	14940
Sephadex G-200	15	0.00036	1.8	1.4	5182	0.77	15	22920

The assay conditions for the *p*-nitrophenyl phosphate phosphatase activity and the pyridoxal phosphate phosphatase activity were the same as described in the text.

TABLE 2. PROPERTIES OF THE ENZYME

Optimum pH	9.0
K_m value for	
p-Nitrophenyl phosphate	0.1 mM
Pyridoxal phosphate	0.34 mM
5'-AMP	0.4 mM
K_i value for P_i using	
p-Nitrophenyl phosphate	1.15 mM
Pyridoxal phosphate	0.45 mM
Molecular weight	140,000
Turnover number	4.86×10^4
Neutral sugars	18%
Sialic acid	2.58%

TABLE 3. EFFECT OF VARIOUS BUFFERS ON THE ACTIVITY OF ALKALINE PHOSPHATASE

Buffer	(mmol of P_i produced/mg of protein) PNPP	PALPO	5'-AMP
Tris–HCl pH 9.0	10.4	3.2	7.5
Borate pH 9.0	7.0	2.6	3.9
Veronal–HCl pH 9.0	6.5	2.7	2.0
Glycine–NaOH pH 9.0	6.7	2.2	3.2
Bicarbonate pH 9.0	6.2	1.2	1.1
Bicarbonate pH 10.0	1.5	0	0

The incubation mixture contained 50 μmol of each buffer under investigation, 0.5 μmol of Mg^{2+}, 0.5 μmol of the substrate and 13 ng of the enzyme in a total volume of 0.5 ml. After 30 min incubation at 37°C, the inorganic phosphate liberated was estimated by the method of FISKE & SUBBAROW (1925).

from bovine brain to cleave PALPO has been reported by BRUNEL & CATHALA (1969). The present results confirm our earlier observation that PALPO was an effective substrate for the sheep brain alkaline phosphatase. Inability of bovine brain alkaline phosphatase to cleave PALPO may be attributed to the different buffer system employed by BRUNEL & CATHALA (1969) since bicarbonate buffer at pH 10.0 was found to inhibit completely the alkaline phosphatase activity using PALPO as substrate. Our results are supported by the observations of LUMENG & LI (1975) who showed that rat liver alkaline phosphatase possessed pyridoxal-phosphate phosphatase activity.

The inhibitory effects of a few amino acids and imidazole on the purified brain alkaline phosphatase was studied. In the presence of 1 mM-Mg^{2+}, 5 mM-L-phenylalanine inhibits the enzyme by 27%. In the presence of the same concentration of Mg^{2+}, 5 mM-L-histidine and 5 mM-imidazole inhibited the enzyme by 56 and 50% respectively. The inhibitory effects of imidazole and histidine are consistent with the findings of BRUNEL & CATHALA (1972) who found that imidazole was an inhibitor of L-phenylalanine insensitive alkaline phosphatases of tissues other than intestine and placenta. However, the possibility that these inhibitory effects are due to the chelation of available Mg^{2+} in the system cannot be excluded.

The substrate specificity of the purified brain alkaline phosphatase was studied using various monophosphate esters, ATP and sodium pyrophosphate, as shown in Table 4. Besides *p*-nitrophenylphosphate, the purified enzyme cleaves PALPO, 5'-AMP, pyrodoxamine phosphate effectively. The enzyme has much less activity towards α-glycerophosphate, ATP and glucose-6-phosphate.

The most characteristic feature of alkaline phosphatase is the way in which the pH optimum changes with increasing substrate concentration, as described by MORTON (1957). This type of behaviour has been demonstrated for a number of phosphomonoesters and with alkaline phosphatases from various sources (FERNLEY, 1971). A typical set of curves obtained for the purified sheep brain alkaline phosphatase using *p*-nitrophenylphosphate as substrate is given in Fig. 4. It can be seen from the figure that there is no substrate inhibition by *p*-nitrophenyl phosphate. The optimum pH shifted to higher pH values with increasing concentrations of substrate. It is interesting to note that at a concentration of 10 μM-*p*-nitrophenyl phosphate, the pH optimum for the brain alkaline phosphatase was near neutrality.

To discern whether or not pyridoxal-phosphate binds to the same substrate binding site as that of *p*-nitrophenyl phosphate, experiments were performed with mutual alternate substrates, namely *p*-nitrophenyl phosphate and pyridoxal phosphate. The Lineweaver–Burk plots of the results are shown in Fig. 5. Pyridoxal phosphate behaves as a competitive inhibitor for *p*-nitrophenyl phosphate hydrolysis by the brain alkaline phosphatase. In a separate experiment, when both PNPP and PALPO were present at 1 mM concentration each, the amount of P_i produced was found to be 8.3 mmol per mg protein in 30 min. This, when compared with the individual values of P_i liberated for PNPP (10.4 mmol) and

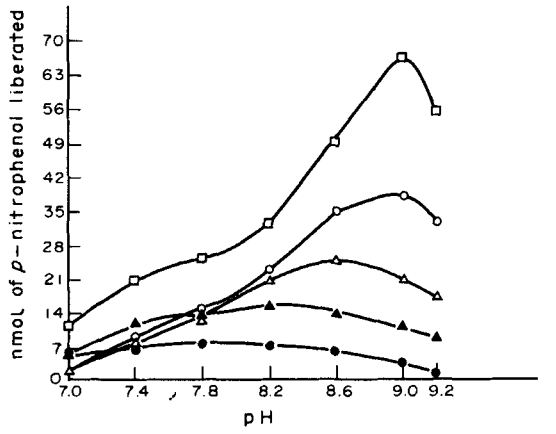


FIG. 4. Effect of substrate concentration on the pH optimum for *p*-nitrophenyl phosphate hydrolysis by the purified alkaline phosphatase. The incubation mixtures contained 50 μmol of Tris–HCl buffer (pH 7.0–9.2), 0.5 μmol of $MgCl_2$, 14 ng of the enzyme and varying concentrations of *p*-nitrophenyl phosphate in a total volume of 0.5 ml. The assay method was the same as described in the text. PNPP concentrations: ●——●, 10 μM; ▲——▲, 25 μM; △——△, 50 μM; ○——○, 100 μM; □——□, 1000 μM.

TABLE 4. SUBSTRATE SPECIFICITY

Substrate	(mmol of P_i produced per mg of enzyme)
PNPP	10.2
PALPO	3.2
5'-AMP	7.5
Pyridoxamine phosphate	5.6
β-Glycerophosphate	4.0
α-Glycerophosphate	2.9
Glucose-6-phosphate	2.4
ATP	1.3
Phosphorylethanolamine	1.7
Sodium pyrophosphate (Mg:PPi = 1:2)	4.8

The incubation mixture contained 100 μmol of Tris–HCl buffer pH 9.0, 1 μmol of Mg^{2+}, 0.5 μmol of the substrate under investigation and 29 ng of the enzyme in a total volume of 0.5 ml. Incubation was done for 30 min at 37°C. The inorganic phosphate liberated was estimated as described in the Methods. PALPO = Pyridoxal phosphate.

PALPO (3.2 mmol) under the same condition, shows that the two substrates compete with each other for the same enzyme. The K_i value for the competitive inhibition of pyridoxal phosphate was found to be 0.3 mM which agrees well with the K_m value for pyridoxal phosphate. The pyridoxal phosphate phosphatase activity of the brain alkaline phosphatase has already been worked out in this laboratory. In their later work, Brunel and his coworkers (BRUNEL & CATHALA, 1969) commented that our preparation might contain an additional alkaline phosphatase specific for pyridoxal phosphate. Our findings with the purified alkaline phosphatase from sheep brain confirm the fact that both *p*-nitrophenylphosphate phosphatase and pyridoxal phosphate phosphatase activities were catalysed by the same enzyme and they behave as mutual alternate, competitive substrates.

Glycoproteins containing acids are widely distributed in animal tissues and fluids and represent a variety of compounds of biological interest. In the nervous tissue, the sialoglycoproteins constitute 3.6–12% of the total proteins (BRUNNGRABER, 1970) and these have been implicated in such vital neural processes as brain development (WARECKA & BAUER, 1967) and information storage (BOGOCH, 1968). Extensive studies on the anatomical and subcellular distribution of the sialoglycoproteins in brain has been carried out by Brunngraber and his coworkers (DEKIRMENJIAN & BRUNNGRABER, 1969; BRUNNGRABER & JAVAID, 1975) and these investigators have demonstrated that sialoglycoproteins are located mainly in the membraneous elements of synaptosomes, axons and oligodendroglial cells. The sialoglycoprotein nature of sheep brain alkaline phosphate was of interest since it facilitated the purification of this group of glycoproteins using the specific enzyme activity as the parameter. Thus the present work describes the purification, properties and the glycoprotein nature of sheep brain alkaline phosphatase. Further work is necessary for more information regarding the function of the enzyme on the brain cell membrane.

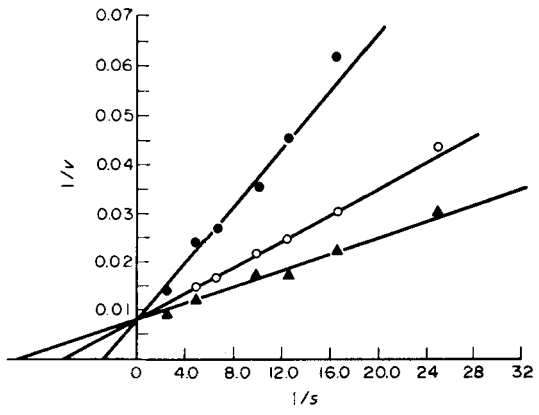


FIG. 5. Competitive inhibition of *p*-nitrophenyl phosphate hydrolysis by PALPO. The assay medium for *p*-nitrophenylphosphate hydrolysis was the same as described in the text. ▲—▲, No PALPO; ○—○, 0.2 mM PALPO; ●—●, 0.7 mM PALPO.

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