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PURIFICATION AND SOME MOLECULAR PROPERTIES OF HORSE LIVER ACYL PHOSPHATASE

G. RAMPONI, P. NASSI, G. CAPPUGI, C. TREVES AND G. MANAO
Institute of Biochemistry, University of Florence, Florence (Italy)

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SUMMARY

1. Acyl phosphatase (acyl phosphate phosphohydrolase, EC 3.6.1.7) has been purified from horse liver. The purification procedure consisted of an acetic acid extraction, an isoelectric precipitation of foreign proteins and three subsequent ion-exchange chromatographies.

2. The molecular weight was determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis and by Sephadex G-75 gel filtration and the amino acid composition by ion-exchange chromatography.

3. The above properties and the substrate specificity were compared with those found for horse muscle acyl phosphatase.

INTRODUCTION

Acyl phosphatase (acyl phosphate phosphohydrolase, EC 3.6.1.7) is one of the smallest enzymes on record. The molecular weight of horse muscle acyl phosphatase, determined by the sedimentation and the Archibald procedures, is about 9400[1], while brain acyl phosphatase has a molecular weight of 8732 calculated from the amino acid composition[2].

In 1954, Grisolia and Marshall[3] described a carbamyl phosphate phosphatase, which was subsequently shown to be the same enzyme as acyl phosphatase. The hydrolytic activity of acyl phosphatase on carbamyl phosphate was confirmed and studied by the same author[3–5]. The hydrolysis of 3-phosphoglyceryl phosphate by muscle acyl phosphatase was studied by Harary[6], Krimsky[7] and finally by Ramponi *et al.*[8], who used a highly purified enzyme preparation.

Carbamyl phosphate and 3-phosphoglyceryl phosphate, on the other hand, are naturally occurring substrates for acyl phosphatase in mammalian metabolism; so it is possible to allow some suggestions about the physiological role of this enzyme, which, by hydrolyzing these compounds, could regulate the glycolytic pathway, ureogenesis and pyrimidine biosynthesis.

Rakitzis and Mills[9], who purified acyl phosphatase from human erythrocytes, suggest that, in the presence of elevated levels of 3-phosphoglyceryl phosphate, the

enzyme would act as a "safety valve" and prevent excessive accumulation of such substrate.

It has also been demonstrated that 3-phosphoglyceryl phosphate and carbamyl phosphate can, respectively, acylate and carbamylate some proteins[10,11]. Acyl phosphatase could prevent such acylation and carbamylation[11], exerting a regulatory role on the intracellular concentration of 3-phosphoglyceryl phosphate and carbamyl phosphate.

Non-enzymatic modifications, such as acylation and carbamylation, are named elastoplastic[12] and may represent a mechanism of control of the physiological activity of enzymatic and non-enzymatic proteins.

So it seemed interesting to isolate and study acyl phosphatase from different tissues. There are two acyl phosphatase activities present in liver, one labile and the other acid, heat stable[6,13].

In this paper we report the complete purification from horse liver of the stable one and a study of some of its structural and functional properties.

Some comparative properties of muscle and liver acyl phosphatase are discussed.

EXPERIMENTAL PROCEDURES

Materials

Benzoyl phosphate and *p*-nitrobenzoyl phosphate were synthesized as by Ramponi *et al.*[14]. Acetyl phosphate, phosvitin, carbamyl phosphate and ATP were purchased from Sigma Chemical Co. 3-Phosphoglyceryl phosphate was synthesized, with slight modification[8], according to Negelein and Brömel[15]. Acetyl-AMP was synthesized by the method of Berg[16]. *p*-Nitrophenyl phosphate was obtained from Fluka AG, phosphocreatine and phosphoenolpyruvate from Boehringer & Soehne GmbH, Mannheim, Germany. CM-Sephadex C-25 and Sephadex G-75 were obtained from Pharmacia (Uppsala, Sweden), acrylamide from Eastman Organic Chemicals, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine from Serva (Heidelberg, Germany). Sodium dodecylsulfate was purchased from B.D.H. Chemicals Ltd; all other reagents were pure chemicals.

Determination of protein

Protein concentrations were estimated by the method of Beisenherz *et al.*[17] or by ultraviolet absorption.

Standard determination of activity

Acyl phosphatase activity was tested by a continuous optical method at 283 nm, using benzoyl phosphate as substrate[18]. The unit of activity is defined as the amount of enzyme which liberates from benzoyl phosphate 1 μmole of benzoate/min at 25 °C and pH 5.3. Specific activity is defined as units/mg protein.

Activity on other substrates

When substrates other than benzoyl phosphate were used, the determination of acyl phosphatase hydrolytic activity was carried out on acetyl phosphate and acetyl-AMP by determining the residual substrate after a suitable time, using the

hydroxylamine–ferric chloride method of Lipmann and Tuttle[19]; on *p*-nitrobenzoyl phosphate, by a continuous-spectrophotometric optical test at 320 nm, assuming a value of 0.500 $mM^{-1} \cdot cm^{-1}$ for the extinction change coefficient; on carbamyl phosphate by measuring the phosphate released according to the method of Baginski *et al.*[20]; on phosvitin and pyrophosphate by measuring the phosphate released by the method of Lowry and Lopez[21] and of Wöltgens and Ahsmann[22], respectively; on 3-phosphoglyceryl phosphate and phosphocreatine, by the determination of residual substrates after a suitable time with enzymatic optical tests[23,24]; on *p*-nitrophenyl phosphate at pH 5.3 as by Lowry[25] and at pH 10.4 by a continuous optical test[26]; on ATP and phosphoenolpyruvate, by measuring the release of ADP and pyruvate, respectively, by optical tests[27,28].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Reisfeld *et al.*[29]. The glass tubes were 10 cm long with an inner diameter of 0.5 cm. A 15% gel (6 cm high) was used. The sample was directly layered onto the upper surface of the separating gel. The electrophoresis was run at 3.5 mA per tube for 150 min in a cold room. The gels were stained with 0.5% amido black solution in methanol–acetic acid–water (50:10:50, by vol.) and destained with the same solvent.

Molecular weight determination

The molecular weight of the purified enzyme was determined by the sodium dodecylsulfate–polyacrylamide gel electrophoresis procedure of Dunker and Rueckert[30] and also by Sephadex G-75 gel filtration according to the method of Andrews[31]. For the sodium dodecylsulfate method, glass tubes (0.5 inner diameter × 10 cm long) with a gel height of 6 cm using a 15% gel and 0.1 M phosphate buffer pH 7.2 containing 1% sodium dodecylsulfate were employed. The acrylamide to methylenebisacrylamide ratio was 29:1 (w/w). Samples, at a concentration of about 2 mg/ml in 1% 2-mercaptoethanol (v/v), 4 M urea and 1% sodium dodecylsulfate, were incubated at 45° C for 45 min. Acyl phosphatase samples were prepared with and without mercaptoethanol. The electrophoresis was run at 8 mA/tube for 7 h at room temperature. The gels were stained with 0.5% amido black in methanol–acetic acid–water (50:10:50, by vol.) for 2 h and then destained with the same solvent. Every tube contained chymotrypsinogen as marker to correct ramdom errors. The position of the bands was determined by scanning the gels with a Gilford 2400 spectrophotometer equipped with 2410 linear transport apparatus. The conversion of migration distances to relative mobilities was accomplished by dividing each migration distance by that of chymotrypsinogen. For the gel filtration method, a column, with an internal diameter of 1 cm and a length of 60 cm, was packed to a height of 54 cm with Sephadex G-75 equilibrated with 0.05 M Tris–hydrochloride buffer, pH 7.5, containing 0.1 M KCl. The proteins used as standards were used in various combinations, usually three at a time, to calibrate the column. The quantities employed for each protein were of about 0.2–0.3 mg in a total volume of 0.15 ml. Alcohol dehydrogenase was present in all the mixtures and its elution volume was taken as the void volume (V_0). A flow rate of 3.3 ml/h was maintained by a LKB peristaltic pump and the absorbance of the effluent at 230 nm was monitored by a Gilford 2400 spectrophotometer equipped with a flow cell.

Amino acid analysis

Analysis was performed according to the procedure of Spackman *et al.*[32], in a Beckman Unichrom amino acid analyzer, equipped with a microcuvet. Protein samples were hydrolyzed at 110 °C for 20–70 h in 6 M HCl. Cystine *plus* cysteine was independently determined as cysteic acid in a performic acid oxidized protein sample[33]. Values for serine, threonine and tyrosine were corrected according to Moore and Stein[34]. Tryptophan was determined by the method of Matsubara and Sasaki[35].

N-terminal analysis

The NH_2-terminal residues of acyl phosphatase were determined by the dansyl procedure reported by Gray[36] and by the Edman degradation technique according to Schroeder[37]. Dansyl-amino acids were separated by thin-layer chromatography according to the method of Nedkov and Genov[38] and phenylthiohydantoin-amino acids by thin-layer chromatography as described by Brenner *et al.*[39].

C-terminal analysis

C-terminal analysis was performed by selective tritiation method of Matsuo *et al.*[40], which is based on oxazolone formation at the carboxy-end of the protein by the action of acetic anhydride. The oxazolones contain an active hydrogen and incorporate tritium when treated with ^3H_2O and pyridine. Protein or peptide was dissolved in 0.1 ml (100 mCi) of tritiated water; 0.2 ml of pyridine and 0.05 ml of acetic anhydride were added and the mixture was kept at room temperature for 5 h. After evaporation *in vacuo* at 40 °C, the residue was washed 10 times with distilled water and subjected to hydrolysis in 6 M HCl for 24 h. The identification of labeled amino acids was carried out by an improved procedure devised in our laboratory, consisting in the use of the amino acid analyzer[41]. The exit of the flow colorimeter was connected to a fraction collector; 2-ml fractions were collected. A 0.8-ml portion of every fraction was introduced into a counting vial and successively 0.5 ml of distilled water and 10 ml of scintillation solution (one-third parts of Triton X-100 and two-thirds parts of Liquifluor diluted 1:10 with toluene) were added.

Determination of chloride

This was carried out according to the method of Volhard.

RESULTS

Purification of the enzyme

25 kg of tissue were homogenized in 100 l of 1.5 M acetic acid. After centrifugation for 60 min at 2000 × *g* the supernatant was brought to pH 4.9. The precipitate was discarded off and the supernatant was dialyzed against tap water for 40 h. The above supernatant was chromatographed on a CM-Sephadex C-25 column (15 cm × 80 cm) equilibrated with 0.05 M acetate buffer, pH 5.8. The column was eluted by a salt concentration gradient from 0 to 1 M NaCl. Fig. 1 shows the elution diagram of this chromatography. Fractions 280–320 were pooled; these correspond to the major component with acyl phosphatase activity. The next purification must be referred to these fractions. The above fractions were rechromatographed on a CM-Sephadex

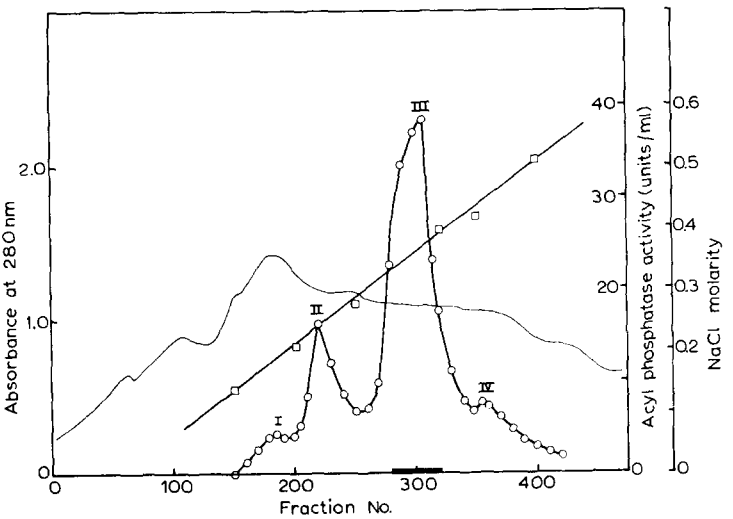

Fig. 1. The elution profile of enzyme from a 15 cm × 80 cm column of CM-Sephadex C-25 (Step 3). Experimental details are given in the text. ———, absorbance at 280 nm; ○—○, acyl phosphatase activity (units/ml); □—□, NaCl molarity. The solid bar indicates the fractions pooled for the next step.

C-25 column (5 cm × 55 cm) equilibrated with 0.05 M acetate buffer, pH 4.8. The elution was performed with a salt concentration gradient from 0 to 1 M NaCl. The elution diagram is reported in Fig. 2. The Tubes 410–438 were pooled.

One fifth of the above fraction was rechromatographed again on a CM-Sephadex C-25 column. (1 cm × 20 cm) equilibrated with 0.05 M phosphate buffer, pH 5.8.

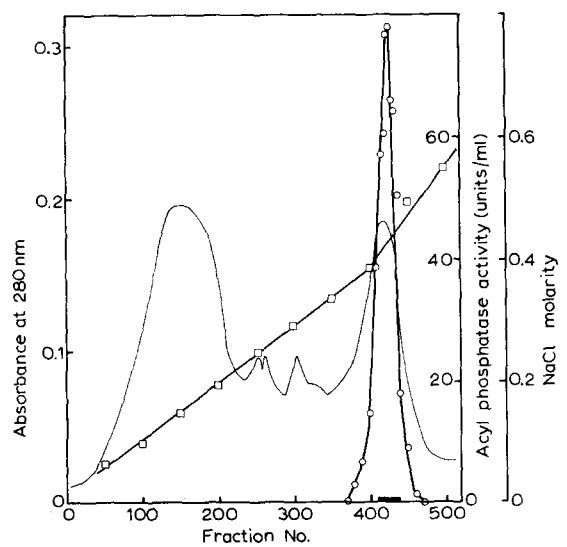

Fig. 2. The elution diagram of acyl phosphatase obtained on rechromatographying pooled fractions of Step 3 on a 5 cm × 55 cm column of CM-Sephadex C-25 (Step 4). Details are given in the text. ———, absorbance at 280 nm; ○—○, acyl phosphatase activity; □—□, NaCl molarity. Solid bar indicates the fractions pooled and used in Step 5.

TABLE I

ENZYME PURIFICATION

Step	*Fraction*	*Volume (ml)*	*Protein (mg)*	*Specific activity (units/mg)*	*Total activity (units)*	*Yield (%)*	*Purification (-fold)*
1	Acetic acid extract	93 000	1848 780	0.17	319 700	100	—
2	Foreign-proteins precipitation	98 000	56 840	2.76	157 000	49.1	16
3	First CM-Sephadex chromatography	1 900	1 805	36.6	66 000	20.6	215
4	Second CM-Sephadex chromatography	800	116	386	44 800	14.0	2 270
5	CM-Sephadex chromatography (specific elution)	102.5	7.5	2800	21 500	6.7	16 470

Phosphate, a well known inhibitor of acyl phosphatase activity[42–44,7,45,46] was chosen to attempt a specific elution of the enzyme. The column was eluted by a salt concentration gradient from 0 to 0.3 M NaCl dissolved in the above phosphate buffer. Fig. 3 shows the result of this chromatography. Fractions 100–110 were pooled; this solution had a specific activity of 2800 which indicates a 16 000-fold purification. This procedure was repeated for all the preparation. In Table I the steps of the purification procedure are summarized.

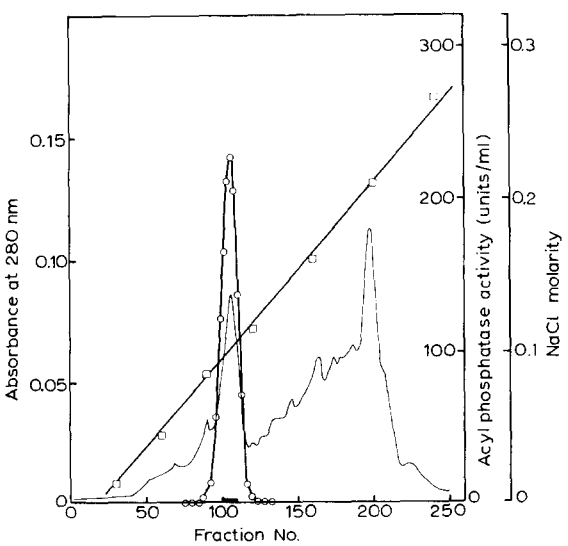

Fig. 3. Specific phosphate elution of enzyme on a 1 cm × 20 cm. CM-Sephadex C-25 column; details are given in the text. ———, absorbance at 280 nm; ○—○, acyl phosphatase activity; □—□, NaCl molarity. The solid bar indicates the portion of the eluted solution containing the purified enzyme.

Properties of the purified enzyme

Polyacrylamide gel electrophoresis. Fig. 4A shows the pattern of liver acyl phosphatase at the fifth step of the purification procedure. It can be seen that an intense

TABLE II

COMPARISON OF AMINO ACID COMPOSITION OF HORSE LIVER AND HORSE MUSCLE ACYL PHOSPHATASE

The values reported for horse muscle acyl phosphatase are from Ramponi *et al.*[1].

Amino acid	*Amino acid residue (%)*		*Residues/molecule of protein*[a]	
	Liver	*Muscle*	*Liver*	*Muscle*
Lysine	11.60	11.29	8	8
Histidine	1.05	0.81	1	1
Arginine	8.22	8.61	5	5
Aspartic acid	8.27	7.85	7	6
Threonine	5.65	5.58	5	5
Serine	12.29	9.07	13	10
Glutamic acid	13.95	12.80	10	9
Proline	2.98	3.57	3	3
Glycine	5.66	4.20	9	7
Alanine	3.04	2.53	4	3
Cystine (half)	1.41	1.87	1	2
Valine	8.75	8.89	8	8
Methionine	1.58	2.23	1	2
Isoleucine	3.16	2.96	3	2
Leucine	3.29	3.85	3	3
Tyrosine	4.32	4.88	2	3
Phenylalanine	4.76	4.96	3	3
Tryptophan	0	(3.35)	0	1[b]

[a] Nearest integral number, calculated on the basis of a molecular weight of 9400.
[b] Corrected value obtained from the complete enzymic hydrolysis[47].

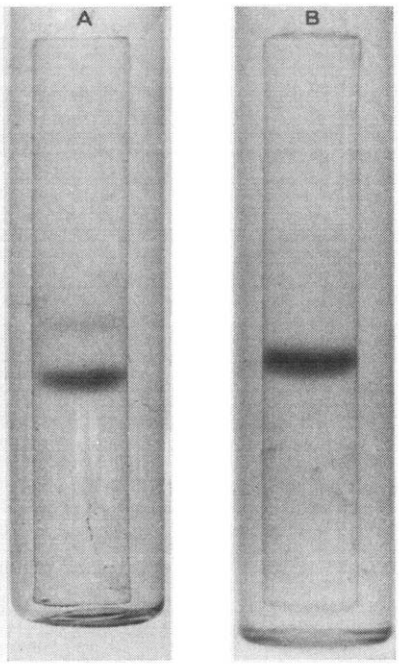

Fig. 4. Polyacrylamide gel electrophoresis patterns of acyl phosphatase at the fifth step of purification (A) and after rechromatography (B).

band is present accounting for about 90% of proteins, with one other very small impurity. Fig. 4B shows the pattern of the enzyme rechromatographed and used for amino acid analysis.

Molecular weight. Fig. 5A shows the plot obtained with Sephadex G-75 gel filtration method. From this plot an apparent molecular weight of 8300 was found for liver acyl phosphatase. The plot obtained with the sodium dodecylsulfate–polyacrylamide method is reported in Fig. 5B. It can be seen that the relative mobility of liver acyl phosphatase overlaps the mobility of muscle acyl phosphatase.

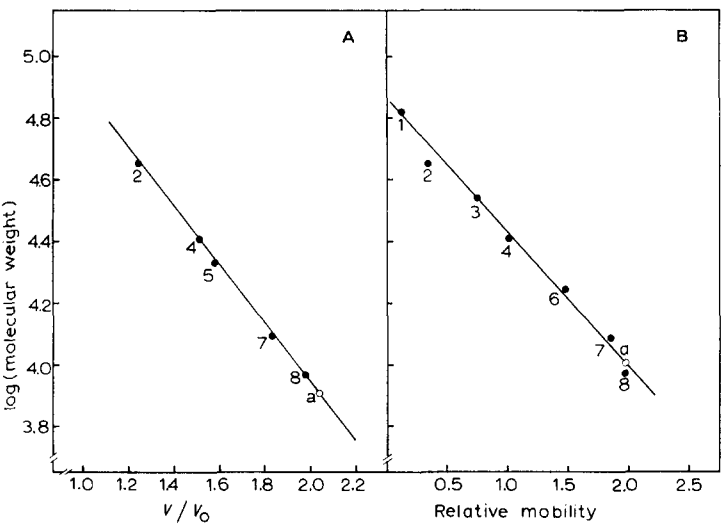

Fig. 5. Molecular weight determination of purified protein. (A) plot obtained with Sephadex G-75 gel filtration method, expressed as V/V_0 against log (mol. wt). (B) plot obtained with sodium dodecylsulfate–polyacrylamide method, expressed as relative mobility against log (mol. wt). 1, bovine serum albumin; 2, ovalbumin; 3, carboxypeptidase A; 4, chymotrypsinogen; 5, soya-bean trypsin inhibitor; 6, myoglobin; 7, cytochrome *c*; 8,horse muscle acyl phosphatase. "a" indicates the horse liver acyl phosphatase. Experimental details are given in the text.

Amino acid composition. For the amino acid analysis, the enzyme from Step 5 was purified again by another ion-exchange chromatography on CM-Sephadex C-25, equilibrated with 0.05 M phosphate buffer, pH 5.8, and then eluted with a phosphate buffer gradient, pH 5.8 (from 0.05 M to 0.2 M). In Table II the amino acid composition of the purified liver enzyme is reported and compared with that of the muscle enzyme. It can be seen that there are some small differences in the amino acid composition of the two enzymes.

NH_2-terminal amino acid. The examination of dansyl protein hydrolysate by thin-layer chromatography on silica gel revealed only ε-dansyl lysine, dansyl NH_2 and small amounts of by-product. The inability to detect any phenylthiohydantoin-amino acid by the Edman procedure, support the hypothesis that the NH_2-terminal amino acid is substituted.

COOH-terminal amino acid. Table III shows the results obtained by selective tritiation experiments. It is evident that tyrosine is the C-terminal amino acid.

Substrate specificity. The specificity of liver acyl phosphatase toward various

TABLE III

SELECTIVE TRITIATION OF HORSE LIVER ACYL PHOSPHATASE

Amino acid	*cpm**
Lysine	120
Histidine	120
Arginine	0
Aspartic acid	230
Threonine	120
Serine	380
Glutamic acid	480
Proline	40
Glycine	300
Alanine	150
Cystine	0
Valine	45
Methionine	180
Isoleucine	110
Leucine	170
Tyrosine	1130
Phenylalanine	390

* Expressed as the sum of cpm of all fractions of each amino acid.

TABLE IV

ACTIVITY OF LIVER ACYL PHOSPHATASE TOWARD A NUMBER OF COMPOUNDS

Compound	*Relative rate of enzymic hydrolysis**
p-Nitrobenzoyl phosphate**	22.5
Benzoyl phosphate**	7.5
3-Phosphoglyceryl phosphate**	0.7
Acetyl phosphate**	1
Carbamyl phosphate**	0.1
Phosphocreatine**	0
Adenosine 5′-triphosphate**	0
Pyrophosphate**	0
p-Nitrophenyl phosphate**	0
p-Nitrophenyl phosphate***	0
Acetyl-AMP**	0
Phosphoenolpyruvate**	0
Phosvitin**	0

* The rate with acetyl phosphate is taken as unity.
** Incubation was performed in 0.1 M acetate buffer, pH 5.3.
*** Incubation was performed in 0.1 M glycine–NaOH buffer, pH 10.4.

compounds is very similar to that found for muscle acyl phosphatase. The relative hydrolysis rates are reported in Table IV.

DISCUSSION

The purification method of liver acyl phosphatase described here gives a highly purified enzyme. The enzyme purified from liver shows properties similar to those

found for the enzyme purified from muscle, heart and brain. As regards amino acid analysis, the liver enzyme shows only small differences in comparison with those obtained with horse muscle acyl phosphatase.

It is interesting to note that the presence of only one residue of cysteine and the absence of a carboxyterminal glycine can be related to the absence of glutathione, which in muscle acyl phosphatase is bound by an S–S bridge to the enzyme. Furthermore from Fig. 6 it can be seen that liver acyl phosphatase does not overlap the muscle enzyme in 15% polyacrylamide gel electrophoresis carried out according to the method of Reisfeld *et al.*[29].

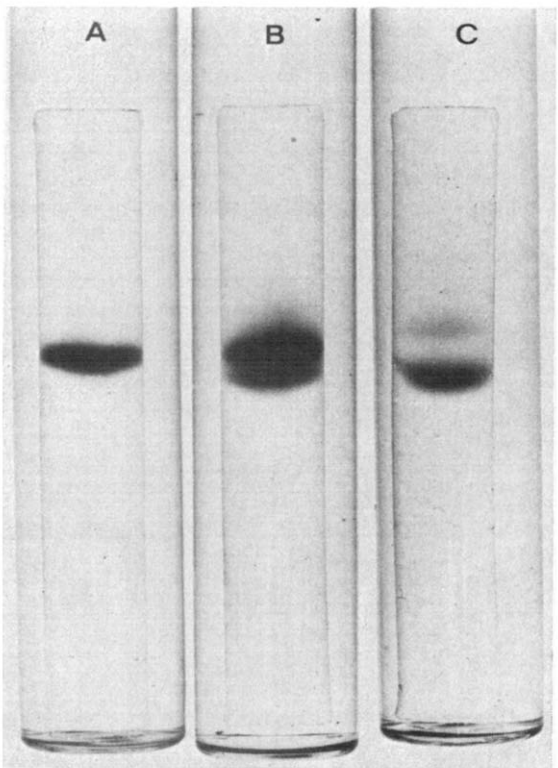

Fig. 6. 15% Polyacrylamide gel electrophoresis patterns: (A) Horse muscle acyl phosphatase. (B) Mixture of horse muscle and horse liver acyl phosphatase. (C) Horse liver acyl phosphatase.

As concerns the terminal amino acids, the carboxyterminal, tyrosine, is the same as that found for muscle acyl phosphatase, while the inability to detect an NH_2-terminal amino acid may indicate that the NH_2 group is substituted; this was also supposed by Diederich and Grisolia[2] for brain acyl phosphatase.

The molecular weight determined by sodium dodecylsulfate-polyacrylamide electrophoresis is identical to that of muscle acyl phosphatase, while the value obtained by the Sephadex G-75 procedure is lower; this fact could be explained by a different conformation of the enzyme, which consequently causes a retardation along the Sephadex column.

The specificity for various substrates is rather similar for muscle and liver enzyme.

As regards its physiological role, it is interesting to note the activity of the liver enzyme towards carbamyl phosphate. This compound can easily reach high concentrations in liver and its level may therefore be partly controlled by acyl phosphatase.

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