Isolation and Characterization of Enolase from the Rhesus Monkey (*Macaca mulatta*)†

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ABSTRACT: Enolase has been purified from the skeletal muscle of the rhesus monkey. The purified enzyme was homogeneous in the ultracentrifuge and had a molecular weight of 82,000. An absolute requirement of divalent metal ions was found for enzymatic activity. Activation occurred with Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} in decreasing order of efficacy. The kinetic properties were essentially identical to those of rabbit muscle enolase. Carboxy-terminus analyses of the monkey enolase have shown that the 82,000 molecular weight active enzyme contains two polypeptide chains. Lysine was identified as the carboxy-terminal residue in both chains by hydrazinolysis and carboxypeptidase digestion. All properties determined were very similar or identical with those of rabbit muscle enolase.

Enolase (2-phospho-D-glycerate hydro-lyase, 4.2.1.11) has been purified from a number of sources including yeast, rabbit muscle, beef brain, and trout (Warburg and Christian, 1942; Holt and Wold, 1961; Wood, 1964; Cory and Wold, 1966). Enolase from yeast and rabbit muscle has been well characterized and shown to consist of two polypeptide chains which can readily be dissociated with high concentrations of salts (Brewer and Weber, 1968; Winstead and Wold, 1965). The aim of the present investigation was to purify and characterize enolase from a primate (rhesus monkey) and to compare the catalytic and physical properties with those of rabbit muscle enolase.

Experimental Section

Assays. Enolase activity was determined by the spectrophotometric procedure described by Holt and Wold (1961) using a Beckman DU or Cary Model 15 spectrophotometer. Protein determinations were based on the 280-mμ absorbance using an extinction coefficient based on the dry weight of the purified enzyme. The barium salt of D-glyceric acid 2-phosphate (Sigma Chemical Co.) was converted to the water-soluble cyclohexylammonium salt (Winstead and Wold, 1966). Imidazole was recrystallized from benzene after treatment with activated charcoal. All other reagents used in this study were analytical reagent grade obtained from commerical sources. Glass-distilled water was used to prepare all solutions.

Physical Methods. The sedimentation velocity, diffusion, and equilibrium experiments were conducted with the Spinco Model E ultracentrifuge, using the schlieren optical system. Two methods were used for the determinations of molecular weight. In one, the diffusion coefficient was estimated from the schlieren curve formed in the valve-type synthetic-boundary cell, with the cup emptying at approximately 10,000 rpm. The first photograph was taken immediately after the boundary had formed, and at least four additional pictures were then taken at 4-min intervals for the diffusion calculations. The speed was then increased to 56,100 rpm for the determination of the sedimentation coefficient. The value of D_{app} was evaluated from the area, A, under the schlieren curve and the maximum ordinate of the gradient curve (Schachman, 1957). The value of $M_{s/D}$ was calculated by substitution of the diffusion and sedimentation coefficients into the Svedberg equation (Svedberg and Pederson, 1940).

Molecular weights were also determined by the Archibald (1947) approach to equilibrium method using a double-sector interference cell. A small volume (0.02 ml) of inert Kel-F fluorocarbon oil was added to the sample sector of the cell to facilitate the calculation of the molecular weight from the pattern at the bottom of the cell. The initial concentration, c_0, was determined by calculating the area, using the synthetic boundary data. The calculations were carried out according to Schachman (1957).

Ultracentrifuge experiments were performed at constant temperatures in the range of 19–21°. The observed sedimentation and diffusion data were corrected to standard conditions of water at 20° in the normal manner. A partial specific volume of 0.728 (Winstead and Wold, 1965) was assumed for all calculations.

Hydrolysis by Carboxypeptidases A and B. Carboxypeptidase A DFP (dialyzed and recrystallized aqueous suspension) and carboxypeptidase B DFP (frozen aqueous suspension) were obtained from Sigma Chemical Co. The carboxypeptidase digestions were conducted in 0.05 M potassium bicarbonate buffer (pH 7.6), as described by Winstead and Wold (1964). The carboxypeptidase:enolase molar ratios were 1:40 with respect to each carboxypeptidases A and B.

Hydrazinolysis. The previously described modification (Winstead and Wold, 1964) of the method of Niu and Fraenkel-Conrat (1955) was used. Anhydrous hydrazine (97%+) was obtained from Matheson, Coleman and Bell, Inc. The correction factor for lysine was obtained from hydrazinolysis of a known quantity of bovine serum albumin to which had been added a known quantity of free lysine. The recovery of lysine was 60%.

Electrophoresis. Agar gel electrophoresis was conducted according to the procedure described by Wieme (1959). The protein sample was dialyzed against the electrophoresis buffer (0.05 M Veronal, pH 8.4) for several hours before being ap-

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plied to the gel. Electrophoresis was conducted with 120 V, 60 mA, for 25 min at 4° and the gel slides were then stained with Amido Schwarz (Tsuyuki, 1963).

Chemical Methods. Amino acid analyses were performed with the Beckman–Spinco amino acid analyzer according to the method of Moore *et al.* (1958). The enzyme was hydrolyzed for 20 hr at 110° in 6 N HCl in evacuated and sealed (2 ml) vials. Correction for destruction of amino acids was made based on the recovery of a standard amino acid mixture under identical conditions. Total cysteine and cystine were determined as cysteic acid after performic acid oxidation (Hirs, 1956). Tryptophan was determined by the spectrophotometric procedure of Spies and Chambers (1949). The sulfhydryl content of the monkey enolase was determined by the method of Boyer (1954) using spectrophotometric titration with *p*-chloromercibenzoic acid in 0.1 M sodium acetate, pH 5, containing 4 M urea.

Preparation of Enzyme. The purification procedure for rabbit muscle enolase (Winstead and Wold, 1966) was used with slight modifications. Both commerical frozen muscle (obtained from Pel-Freez, Biologicals, Inc.) and muscle (obtained from monkeys anesthetized with nembutal) quick frozen with Dry Ice were used.

STEP 1. EXTRACTION. Frozen rhesus monkey muscle (900 g) was partially thawed at 4°, ground twice with a meat grinder, and homogenized with three parts (by weight) of cold EDTA solution (0.05% (w/v) of EDTA disodium dihydrogen salt) for 1 min in a Waring Blendor. The homogenate was stirred for 1 hr to allow extraction of soluble proteins. The insoluble material was removed by centrifugation at 8000*g* for 15 min at 0°. The supernatant was decanted and filtered through glass wool to remove light lipid material.

STEP 2. ACETONE FRACTIONATION. A 35–50% acetone precipitation was made according to the procedure of Winstead and Wold (1966).

STEP 3. HEAT TREATMENT. The solution of monkey enolase was heated to 55° in the present of 5% (w/v) $MgSO_4 \cdot 7H_2O$ (Winstead and Wold, 1966).

STEPS 4 AND 5. AMMONIUM SULFATE FRACTIONATION. Solid ammonium sulfate (Baker, analyzed reagent grade) was added to the supernatant from step 3 to bring the concentration to 55% (350 g/l. of supernatant).

After centrifugation (10,000*g*, 0°, 20 min) the ammonium sulfate concentration was brought to 70% saturation by further addition of solid ammonium sulfate (101 g/l. of 55% supernatant). The protein from the 55–70% ammonium sulfate fraction was suspended in 65% ammonium sulfate and stirred for 2 hr at 4°, and then centrifuged. The supernatant was discarded. The remaining precipitate was suspended in 61% ammonium sulfate, stirred for 2 hr, and centrifuged at 10,000*g* for 20 min at 0°.

STEP 6. PRECIPITATION. A saturated ammonium sulfate solution of pH 7.8 was added to the 61% ammonium sulfate supernatant until a slight turbidity occurred and it was left 10–20 hr at 4°. The precipitate was then collected by centrifugation at 14,000*g* for 15 min at 0°.

STEP 7. REPRECIPITATION. The enolase from step 6 was dissolved in a minimum amount of cold imidazole–$MgSO_4$ buffer (0.05 M imidazole containing 10^{-3} M $MgSO_4$, pH adjusted to 7.8 with HCl), and saturated ammonium sulfate solution was added until a very slight turbidity occurred. After 12–20 hr the reprecipitated enzyme was isolated by centrifugation. The enolase was stored suspended in 70% saturated ammonium sulfate solution (prepared by dilution of saturated ammonium sulfate with the imidazole buffer) at 4°. The enzyme could be stored under these conditions for several months without significant loss of activity.

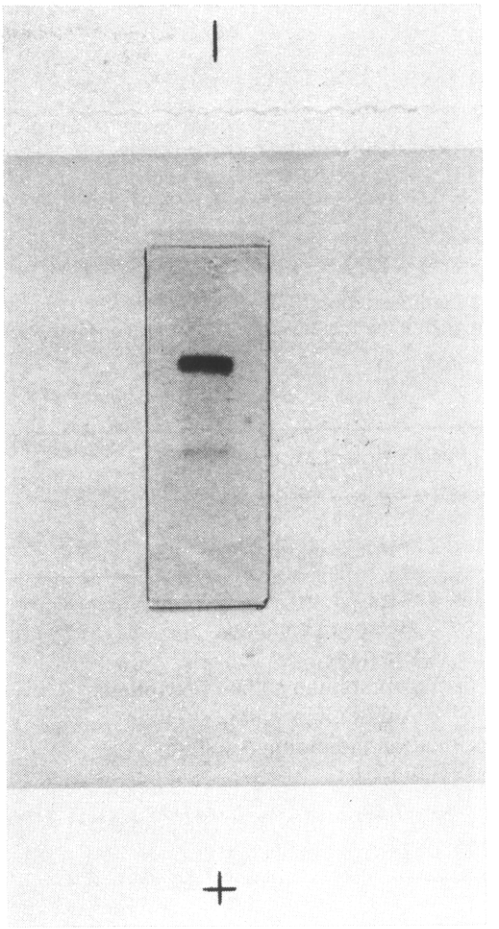

FIGURE 1: Electrophoretic pattern of monkey enolase on agar gel in 0.05 M Veronal buffer (pH 8.4) with 120 V, 60 mA, for 25 min at 4°. Slide was stained with Amido-Schwarz.

Results

The results of the rhesus muscle enolase purification are summarized in Table I. In general, better results were obtained from the muscle which was obtained from our monkey colony. This muscle was from more mature animals. The preparation was not as reproducible as that for rabbit muscle enolase, yields were poor, and little or no crystallization occurred. The electrophoretic pattern of the enzyme, indicating a slight impurity, is shown in Figure 1. Purified enolase appeared homogeneous during sedimentation (Figure 2). In 0.05 M potassium phosphate buffer (pH 7), the $s_{20,w}$ was 5.72 S for a 5.7-mg/ml sample.

The molecular weight was determined by the Archibald approach to equilibrium method and from diffusion and sedimentation data obtained using the valve-type synthetic boundary cell. The results of the two methods are in good agreement and are presented in Table II. The molecular weights calculated at the meniscus and at the bottom of the cell are consistent with the possibility of a small amount of heavy material, but probably not a significant quantity. The $D_{20,w}$ calculated from the schlieren patterns taken at low speed with the synthetic boundary cell was 6.26×10^{-7} cm²/sec.

The absorption coefficient at 280 mμ for a 1-mg/ml solution of the purified enzyme was found to be 0.880 ± 0.015

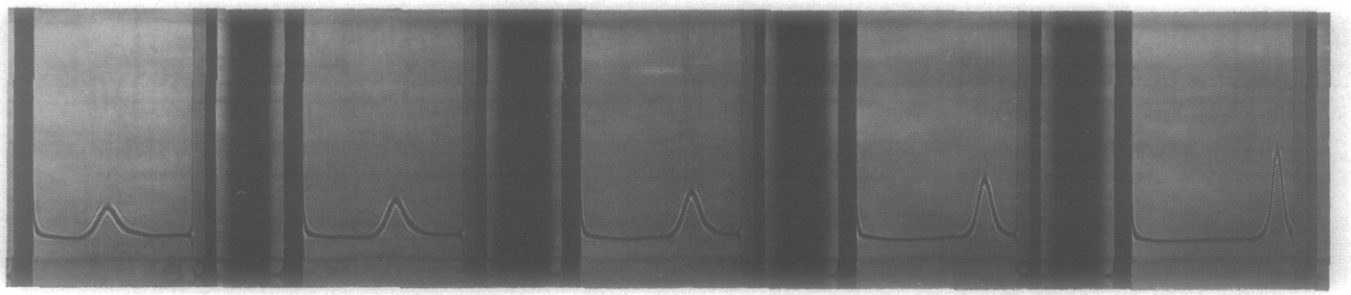

FIGURE 2: Sedimentation of monkey enolase (5.7 mg/ml) in 0.05 M phosphate buffer (pH 7) at 20°. Photographs were taken at 16-min intervals beginning 15 min after attaining constant speed (59,780 rpm). The phase-plate angle was 60°.

TABLE I: Purification of Monkey Enolase.[a]

Fractionation Step	Total Act. Units[b] (IU)[c]	Total Protein (g)	Sp Act. Units[b] (IU)[c]/mg of Protein	Recov %
Versene extract	888,000 (204,300)	74.0	12 (2.76)	100
35–50% Acetone precipitate	755,000 (173,800)	26.0	29 (6.67)	85
Heat supernatant	677,000 (155,880)	11.7	58 (13.3)	76
55–70% Ammonium sulfate precipitate	550,000 (126,600)	5.85	94 (21.6)	62
65–61% Ammonium sulfate solution	252,000 (58,000)	1.72	146 (33.6)	28
Precipitation (ammonium sulfate)	58,500 (13,464)	0.23	250 (57.54)	6.6
Reprecipitation	41,000 (9,400)	0.11	376 (86.54)	4.6

[a] From 900*g* frozen muscle. [b] The amount of enzyme which will give an absorbance change of 0.1/min at 240 mμ (Holt and Wold, 1961). [c] International enzyme units (IU) in parentheses.

cm^{-1} based on measurement of several samples dried to a constant weight.

An absolute requirement for a divalent metal ion for activation was found. Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} were found to activate the enzyme (Table III) in 5×10^{-2} M imidazole buffer with 10^{-3} M substrate and 0.4 M KCl at pH 7.0. These conditions are comparable to those of Holt and Wold (1961), who found that these ions activated the rabbit muscle enolase. Calcium ions acted as a competitive inhibitor as illustrated by the Lineweaver–Burk plot (Figure 3). The K_I was calculated to be 1.8×10^{-4} M. From the data in Figure 4, K_m was calculated to be 1.25×10^{-4} M and V_{max} was 7700 moles of substrate/min per mole of enzyme. The Michaelis constant for rabbit enolase under these assay conditions was also determined to be 1.25×10^{-4} M. Figure 4 also shows that phosphate ions inhibit the reaction competitively with a K_I of 1.7×10^{-2} M. The pH optimum and Mg^{2+} concentration optimum are indicated in Figure 5. The pH optimum is 7.0 and the Mg^{2+} concentration optimum is approximately 1.6×10^{-3} M.

The rhesus monkey enolase was found to lose activity rapidly and to precipitate in the presence of a large excess of *p*-chloromercuribenzoic acid (Figure 6); however, four to five sulfhydryl groups could be titrated without appreciable loss of activity. Total sulfhydryl content of the rhesus enolase was 11.4 moles/mole of enzyme compared to 11.3 moles for the rabbit enolase determined under identical conditions.

Preliminary studies of the amino acid content (Table IV) show similarity with the results of Holt and Wold (1961) for

TABLE II: Molecular Weight of Monkey Enolase.

Enolase Concn[a] (mg/ml)	Time of Picture (min)	Mol Wt M_m[b]	Mol Wt M_b[b]
Archibald Method (Approach to Sedimentation Equilibrium) (Speed, 12,590 rpm)			
4.8	9	82,300	81,300
	25	81,800	82,300
	41	80,800	83,100
Synthetic Boundary Method			
4.8		82,500	

[a] 0.05 M potassium phosphate buffer (pH 7.0). [b] M_m and M_b signify the molecular weights calculated from the meniscus and the bottom of the cell, respectively.

TABLE III: Metal Ion Activation of Monkey Muscle Enolase.

Metal Ion	Concn (M)[a]	Rel Act.
Mg^{2+}	1.6×10^{-3}	100
Mn^{2+}	1×10^{-4}	46
Zn^{2+}	5×10^{-4}	35
Co^{2+}	1×10^{-4}	9
None		0

[a] Optimal concentration for enzymatic activity.

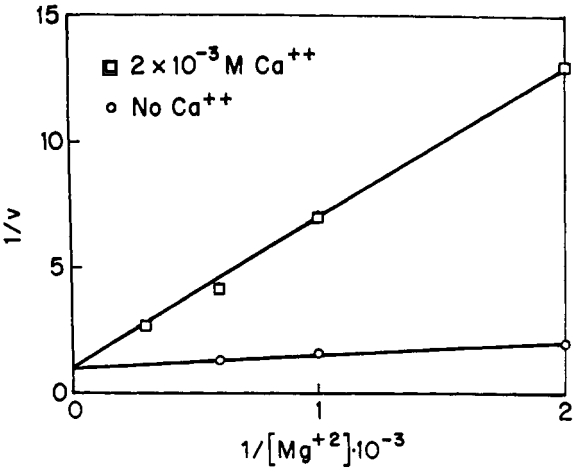

FIGURE 3: Lineweaver–Burk plots of the effect of magnesium and calcium ions on the rate of the enolase reaction. The reactions were conducted in 0.05 M imidazole buffer (pH 7.0), containing 10^{-3} M D-glyceric acid phosphate and 0.4 M KCl. (○) No Ca^{2+}; (□) in the presence of 2×10^{-3} M Ca^{2+}.

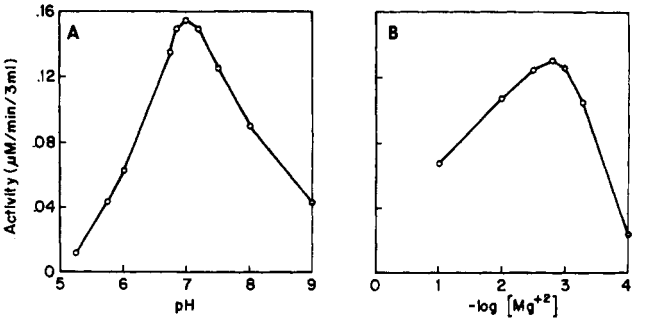

FIGURE 5: The effect of pH and magnesium ions on the rate of the enolase reaction. The observed optical density change was corrected for the effect of pH and Mg ions on the absorbance of enolpyruvic acid phosphate (Wold and Ballou, 1957). The substrate was 10^{-3} M D-glyceric acid phosphate in 0.05 M imidazole containing 0.4 M KCl. The magnesium ion concentration was held constant at 10^{-3} M in A and pH constant at 7.0 in B.

the rabbit enzyme. The half-cystine was determined as cysteic acid after performic acid oxidation. This value compares favorably with the sulfhydryl value, thus indicating no disulfide bonds in the rhesus enolase molecule. The tryptophan value was considerably lower than that previously reported for the rabbit enzyme; however, in the present study nearly identical values of tryptophan content for both enolases were obtained.

The release of amino acids from the rhesus monkey enolase upon treatment with carboxypeptidases A and B is given in Figure 7. The data are consistent with an alanyllysine C-terminal sequence for two polypeptide chains. Incubation of the protein with carboxypeptidase A for 6 hr yielded 0.28 mole of lysine and 0.25 mole of alanine per mole of enolase, and incubation with carboxypeptidase B for 6 hr yielded 1.8 moles of lysine and 0.33 mole of alanine.

In order to definitely establish that the two lysines were released from two separate alanyllysine sequences, hydrazinolysis was conducted. A corrected value of 1.95 moles of lysine was obtained on duplicate experiments. These results, in addition to the carboxypeptidase experiments, show conclusively that there are two free carboxy-terminal lysines in monkey enolase.

Discussion

Previously the only mammalian muscle enolase isolated was from rabbit. The enolase isolated from the rhesus monkey muscle is compared to that of the rabbit muscle in Table V. These data indicate nearly identical properties for the enolases from these two mammalian muscle sources.

Malmström (1962) reported activation of rabbit enolase with Mn^{2+} and Zn^{2+}, which were 46 and 27%, respectively, compared to activation with Mg^{2+}. Rhesus monkey enolase was activated 46 and 35% respectively, by Mn^{2+} and Zn^{2+}. Holt and Wold (1961) reported 8% activation of rabbit enolase with Co^{2+}. Monkey enolase is activated by 9% with Co^{2+}. Calcium ions failed to activate the monkey enolase and was found to be a competitive inhibitor; the phosphate ion also inhibits competitively.

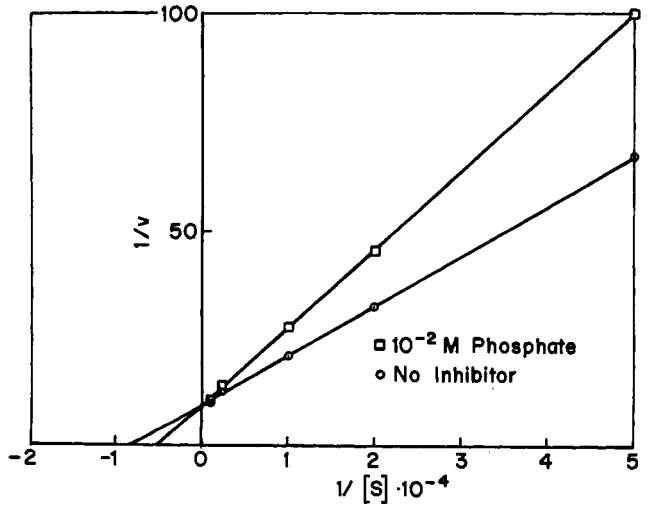

FIGURE 4: Lineweaver–Burk plots of the effect of substrate and of phosphate ions on the rate of the enolase reaction. The reactions were conducted in 0.05 M imidazole buffer (pH 7.0), containing 10^{-3} M magnesium sulfate and 0.4 M KCl. (○) No inhibitor; (□) in the presence of 10^{-2} M phosphate.

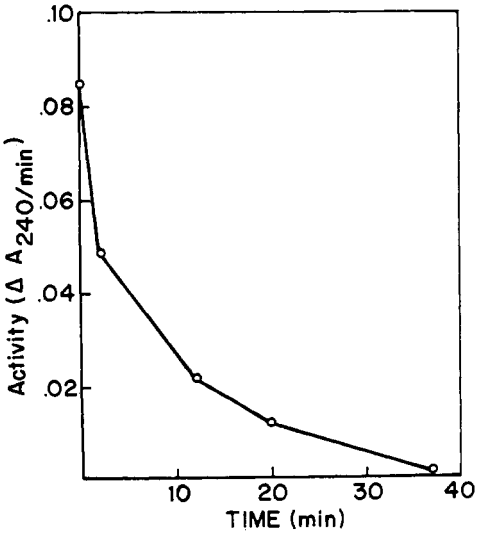

FIGURE 6: Effect of *p*-chloromercuribenzoic acid on monkey enolase activity at 25° in 0.05 M potassium phosphate (pH 7.0) with a *p*-chloromercuribenzoic acid:enolase ratio of 700:1.

TABLE IV: Amino Acid Composition of Monkey and Rabbit Muscle Enolases.

	Monkey Moles of Amino Acid/ 82,000 g of Protein	Rabbit[a] Moles of Amino Acid/ 82,000 g of Protein
Lysine	60.7	67.0
Histidine	14.2	18.7
Arginine	30.1	29.7
Aspartic acid	81.3	77.2
Threonine	29.4	33.1
Serine	36.7	33.2
Glutamic acid	71.3	67.1
Proline	32.5	25.7
Glycine	67.0	71.8
Alanine	78.4	75.6
Valine	48.2	56.6
Methionine	12.6	12.4
Isoleucine	38.9	43.2
Leucine	62.6	66.6
Tyrosine	18.2	17.6
Phenylalanine	26.8	25.0
Tryptophan	6.1	10.1
Half-cystine	9.5	6.2
Total	724.5	736.8

[a] Data of Holt and Wold corrected for 82,000 g of protein.

TABLE V: Comparison of Some Properties of Rabbit and Monkey Enolase.

Property	Enzyme: Rabbit	Enzyme: Monkey
Molecular weight	82,000[a]	82,000
$s_{20,w}$, S (5–6 mg/ml)	5.70	5.72
$D_{20,w}$ (cm/sec)	6.35×10^{-7}	6.26×10^{-7}
A_{280}/mg	0.90[b]	0.88
K_m	1.25×10^{-4}	1.25×10^{-4}
V_{max} (moles of substrate/min per mole of enzyme)	7700	7700
Specific activity (IU)	86	86
pH optimum	6.9[c]	7.0
$[Mg^{2+}]$ optimum (moles/l.)	$1–2 \times 10^{-3}$[c]	1.6×10^{-3}
Activation by other metals	Mn^{2+}, Zn^{2+};[c] Co^{2+}[b]	Mn^{2+}, Zn^{2+}, Co^{2+}
Moles of SH/ mole	11.3	11.4
Moles of C-terminal amino acid/mole	2 Lys[d]	2 Lys
Number of peptide chains/mole	2[d]	2

[a] Winstead and Wold (1965). [b] Holt and Wold (1961). [c] Malmström (1962). [d] Winstead and Wold (1964).

Although the rhesus monkey enolase lost activity rapidly in the presence of a large excess of *p*-chloromercuribenzoic acid, four to five sulfhydryl groups could be titrated without appreciable loss of activity. Malmström (1962) reported that *p*-chloromercuribenzoic acid in a concentration corresponding to less than 5 SH groups/molecule of rabbit enolase caused no turbidity and little change in catalytic activity. Malmström's titration of total SH content of rabbit enolase by *p*-chloromercuribenzoic acid indicated 12.1–12.4 SH groups/molecule of enzyme; however, in our laboratory a value of 11.3 was obtained. The present data obtained for the rhesus enolase indicate 11.4 SH groups, which is in reasonably good agreement.

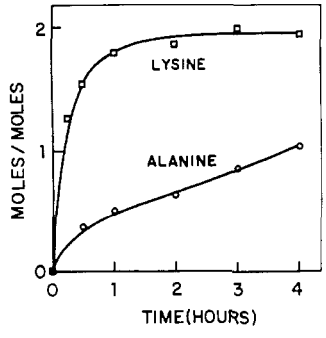

FIGURE 7: The kinetics of amino acid release from monkey muscle enolase during digestion with carboxypeptidases A and B at 25°. The carboxypeptidases:enolase molar ratios were 1:40 with respect to each carboxypeptidase A and B.

The C-terminal analysis clearly demonstrates the presence of two polypeptide chains in the rhesus enzyme with C-terminal amino acids of lysine preceded by alanine, identical with the rabbit enzyme (Winstead and Wold, 1964).

The studies performed on the enolase from rhesus monkey muscle indicate that this enzyme is very similar if not identical with the enolase from rabbit muscle. The greatest difference observed between the enolases from the two sources was the lack of crystallization of the rhesus enolase, or at least the lack of well-formed crystals which are easily obtainable from rabbit muscle preparations. The difficulty of crystallization could possibly be explained by the presence of a slight impurity not present in the rabbit muscle enzyme. Further studies are required to elucidate this difference.

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Spontaneous Reactivation of Acetylcholinesterase Inhibited with Para-Substituted Phenyl Methylphosphonochloridates*

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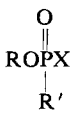
ABSTRACT: Four para-substituted phenyl methylphosphonochloridates (H, I; CH_3O, II; NO_2, III; and CN, IV) were prepared by the reaction of excess methyl phosphonodichloridate with the appropriate phenol. The rates of hydrolysis of I–IV were determined at pH 4.90 (25°, acetate buffer) and showed excellent correlation with published inductive σ values for para substituents. Spontaneous reactivation of acetylcholinesterase (AChE) inhibited with I, II, and IV exhibited a 3.6 times larger substituent effect, which is interpreted in terms of hydrophobic interactions within the active site. No significant enhancement of the substituent effect was observed in the parallel aging reaction. AChE inhibited with III showed no detectable reactivation. The pH–rate profile for the aging reaction of AChE inhibited with IV is bell shaped implicating participation by two groups within the active site having pK_a's of 6.2 and 9.0. The rate of spontaneous reactivation shows a similar pH profile. Inhibition and spontaneous reactivation results using the bis ester analogs of III and IV support the active site specificity of I–IV.

Organophosphorus compounds of the type



where X is a labile leaving group will irreversibly inhibit AChE[1] by formation of a phosphonate ester bond to the active site serine. Once phosphonylated, the enzyme may react in one of two ways (Cohen and Oosterbaan, 1963; Engelhard *et al.*, 1967): (1) it may reactivate by cleavage of the serine phosphonate bond, or (2) it may age by cleavage of the other phosphonate ester bond. The reactivation is catalyzed by strong nucleophiles such as oximes (Gilbert *et al.*, 1961) and hydroxamic acids (Franchetti *et al.*, 1970) while aging, in the case where R is an alkyl group, has been shown to be acid catalyzed and probably involves a carbonium ion intermediate (Michel *et al.*, 1967).

To date, few *in vitro* studies of the spontaneous (*i.e.*, no external catalytic species other than H_2O, OH^-, and H_3O^+) reactivation and aging of phosphorylated AChE have been done (Wilson *et al.*, 1958; Lee, 1964; Reiner and Aldridge, 1967; Aldridge, 1969). Similarly, little, if anything, is known about the reactivation–aging behavior when R is an aryl group. It is to these areas that the work in this paper is directed.

Experimental Section

Synthesis. The four phosphonochloridates used in this work were all prepared in a similar manner. The appropriate para-substituted phenol was dissolved in toluene and added to a twofold excess of methyl phosphonodichloridate also in toluene. The reaction mixture was stirred at room temperature for several hours and the toluene evaporated under reduced pressure. The addition of a tertiary amine to scavenge the HCl produced during the reaction was found to be unnecessary since aryl phosphonates are not particularly sensitive to acid attack (Bunton *et al.*, 1968). All four were purified by distillation. The physical data for each compound follow.

Phenyl methylphosphonochloridate (I), liquid at room temperature, was distilled at 82° and 0.02 mm. Petrov *et al.* (1961) reported a distillation temperature for this compound of 153–155° at 21 mm. *Anal.* Calcd for $C_7H_8ClO_2P$: C, 44.1; H, 4.2; Cl, 18.6. Found: C, 43.8; H, 3.9; Cl, 18.4. The nuclear magnetic resonance (nmr) spectrum (given in parts per million) showed (60 MHz, $CDCl_3$, Me_4Si external standard) a doublet at δ 2.09 (J = 17 Hz integrating to three protons) and a multiplet centered at δ 7.3 integrating to five protons.

p-Methoxyphenyl methylphosphonochloridate (II), liquid at room temperature, was distilled at 120–125° at 0.06 mm. *Anal.* Calcd for $C_8H_{10}ClO_3P$: C, 43.6; H, 4.6; Cl, 16.1. Found: C, 43.3; H, 4.6; Cl, 16.1. The nmr spectrum (60 MHz, $CDCl_3$, Me_4Si external standard) showed a doublet at δ 2.10 (J =

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[1] Abbreviations used are: AChE, acetylcholinesterase.