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Biochemistry

Purification of a specific reversible tyrosine-*O*-phosphate phosphatase

(L-configuration specificity/phosphate transfer/Mg^{2+} independence/Zn^{2+}, Mn^{2+} inhibition)

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ABSTRACT **A phosphatase specific for tyrosine-*O*-phosphate (Tyr-*P*) was separated from several nonspecific phosphatases present in the third instar larvae of *Drosophila melanogaster*. The enzyme hydrolyzed L-Tyr-*P*, with an apparent K_m of 0.14 mM, but not D-Tyr-*P* after being freed from hydrolytic activity toward *p*-nitrophenyl phosphate, the common phosphatase substrate. Such purified preparations also catalyzed a reversible phosphate transfer reaction from unlabeled Tyr-*P* to [^{3}H]tyrosine. The transfer activity was 1.4–14% of the hydrolytic activity, depending on the initial concentration of tyrosine (0.25–4.0 mM). The two activities coincided throughout purification. However, they differed in pH optimum, that of hydrolysis being 6.5–7 and that of phosphate transfer being 7–7.5. The two activities were also differentially inhibited by 1-*p*-bromotetramisole oxalate in the presence of EDTA and by Mn^{2+}. Addition of Mg^{2+} did not affect either hydrolysis or phosphate transfer, but 5 mM Zn^{2+} was 65% inhibitory to both. Sodium fluoride strongly inhibited both reactions, and this inhibition was reversed by EDTA, while EDTA itself had no effect. P_i had no effect and no detectable incorporation of $^{32}P_i$ into Tyr-*P* was observed, indicating that the phosphate transfer reaction is not a simple reversal of hydrolysis. No ATP-linked phosphorylation of tyrosine was found.**

The discovery that the translation product of the transforming *src* gene of Rous sarcoma virus is a phosphotransferase (1–4) with tyrosine as a specific target (5, 6) has called attention to tyrosine as a phosphate acceptor. Prior to this discovery, the only case of tyrosine as an adenosine-phosphoryl acceptor was reported by Stadtman for bacterial glutamine synthetase (7). Thus, the finding of free tyrosine-*O*-phosphate (Tyr-*P*) accumulating in reasonably large amounts during the larval stage of some flies caught our interest (8). Tyr-*P* appeared to be hydrolyzed on transition to pupation to serve as a reagent for use in cuticle staining, since apparently a phosphatase was activated at the end of the larval stage. Mitchell and Lunan (8, 9) have studied the formation and breakdown of Tyr-*P* in *Drosophila* and concluded that it is a storage product for tyrosine. This is supported by the finding that not only Tyr-*P* but, in another species of *Drosophila*, a tyrosine glucoside (10) was accumulated rather than Tyr-*P* for storage of tyrosine. On the other hand, the interesting study of Tyr-*P* in another fly, *Sarcophaga bullata* (11), indicated that Tyr-*P* here persisted throughout pupation till the phase of emergence. We were interested in exploring the synthesis of the Tyr-*P* that, in *Drosophila*, increased during the early third instar larval phase, reached a plateau concentration at the beginning of pupation, and then was rather rapidly hydrolyzed (8).

Although neither Mitchell and co-workers (8–10) nor Seligman *et al.* (11) found a clearcut explanation for the synthetic reaction, their experiments on injection of [^{14}C]tyrosine and [^{14}C]Tyr-*P* indicated a steady-state interconversion between the tyrosine and Tyr-*P* pools (11, 12). In the present study, we isolated a Tyr-*P*-specific phosphatase that catalyzed both hydrolysis and the conversion of [^{3}H]tyrosine + Tyr-*P* → [^{3}H]Tyr-*P* + tyrosine. Here, we describe the isolation and some of the properties of this *Drosophila* enzyme.

EXPERIMENTAL PROCEDURES

Materials. *D. melanogaster* (Oregon R strain, kindly provided by A. Garen, Yale University) was grown in mass culture as described by Elgin and Miller (13) using a modified larval food. It contained $CaCl_2$ (0.18 g), Na, K tartrate (2.9 g), glucose (21 g), sucrose (11 g), Difco Bacto agar (1.4 g), molasses (5 ml), phosphoric acid (85%; 1 ml), propionic acid (0.1 ml), *p*-hydroxybenzoic acid (0.01%; 5 ml), heat-inactivated brewer's yeast (100 g), and 340 ml of distilled water. Third instar larvae were harvested and kept frozen at −20°C until use. L- and D-Tyr-*P* and L-[^{3}H]Tyr-*P* were synthesized as described by Mitchell and Lunan (8). L- and D-tyrosine, *p*-nitrophenyl phosphate (NP-*P*), L-phenylalanine, L- and D-dihydroxyphenylalanine (dopa), and 1-phenyl-2-thiourea were purchased from Sigma. *p*-Aminobenzamidine dihydrochloride and 1-*p*-bromotetramisole oxalate were obtained from Aldrich, and L-[^{3}H]tyrosine (30 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was from ICN.

Assay Systems. The hydrolytic activity of Tyr-*P* phosphatase was measured as the rate of [^{3}H]tyrosine release from [^{3}H]Tyr-*P*, and phosphate transfer activity was measured as the rate of [^{3}H]Tyr-*P* formation from [^{3}H]tyrosine and unlabeled Tyr-*P*. Both assays were carried out in (total vol, 50 μl) 50 mM Tris·HCl, pH 7.2/1 mM dithiothreitol/2.5 mM Tyr-*P*/0.25 mM tyrosine containing 2 μCi of [^{3}H]Tyr-*P* (hydrolysis) or 10 μCi of [^{3}H]tyrosine (phosphate transfer reaction). Mixtures were incubated at 37°C for 30 min, and the reaction was terminated by chilling the mixture to 0°C. Five-microliter aliquots were applied to cellulose thin-layer plates (Nagel; cell 300-0.1). Tyr-*P* was separated from tyrosine by electrophoresis (500 V, 1 hr, 25°C), using acetic acid/pyridine/H_2O, 50:5:945 (pH 3.5), as described by Hunter and Sefton (5). Spots of Tyr-*P* and tyrosine were located by spraying with ninhydrin, scraped off, and assayed in a liquid scintillation counter with 10 ml of Hydrofluor. Within the enzyme concentrations used, the rates of [^{3}H]tyrosine release and [^{3}H]Tyr-*P* formation were linear with respect to the incubation time at 37°C for ≈40 min. Therefore, all experiments were based on incubation for 30 min unless otherwise indicated.

The release of tyrosine from Tyr-*P* was also measured colorimetrically by the Lowry procedure (14). The release of 15

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Abbreviations: Tyr-*P*, tyrosine-*O*-phosphate; NP-*P*, *p*-nitrophenyl phosphate; dopa, dihydroxyphenylalanine.

nmol of tyrosine per 2.25 ml of our assay mixture gave an increase in A_{750} value of 0.1. However, this assay was applied only for the most highly purified enzyme preparation, because in this case the contribution to A_{750} by the enzyme protein was negligible (<0.005).

Purification of Specific Tyr-*P* Phosphatase. The purification of Tyr-*P* phosphatase is summarized in Table 1. All procedures were carried out at 0–4°C; 100 g of frozen larvae was thawed and suspended in 300 ml of 50 mM Tris·HCl, pH 7.2/5 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol/5 mM *p*-aminobenzamidine dihydrochloride/2 mM 1-phenyl-2-thiourea (buffer A). Larvae were homogenized by using a high-pressure-nitrogen cell-disruption chamber (Parr, Moline, IL), and debris was removed by centrifugation at 10,000 × *g* for 10 min. The pellet was extracted with 100 ml of buffer A and combined with the supernatant from the centrifugation step to give 440 ml of crude extract. The crude extract (fraction 0) was clarified by centrifugation at 100,000 × *g* for 30 min, and the supernatant was fractionated by addition of ammonium sulfate. Material (fraction 1) precipitating at 50–75% $(NH_4)_2SO_4$ saturation was collected and dissolved in 50 mM Tris·HCl, pH 7.2/5 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol (buffer B), dialyzed against buffer B, and loaded onto a DEAE-cellulose column (4 × 25 cm) equilibrated with buffer B. The column was washed with buffer B and eluted with a linear gradient of 0–0.4 M NaCl in buffer B. Tyr-*P* phosphatase eluted at ≈0.05 M NaCl. The active fractions were pooled (fraction 2) and concentrated on a small column of hydroxylapatite (1.8 × 8 cm), which retained the enzyme. Elution was with 0.4 M $(NH_4)_2SO_4$ in buffer B. This enzyme fraction was concentrated by ultrafiltration using a Diaflo membrane PM 10 (Amicon). The concentrate was clarified by centrifugation and applied to a Sephadex G-200 gel filtration column (2.1 × 98 cm) washed with 0.1 M NaCl in buffer B. At this step (fraction 3), the Tyr-*P* phosphatase became virtually free of NP-*P* hydrolyzing activity (Fig. 1). Further purification was achieved by chromatofocusing and tyrosine–agarose hydrophobic interaction chromatography. Chromatofocusing with Polybuffer 94 and Polybuffer 74 (Pharmacia) was carried out according to the protocol provided by the manufacturer. A pH gradient of 7–4 was obtained, and Tyr-*P* phosphatase was eluted at pH 5.4. Active fractions were pooled (fraction 4), adjusted to 2 M $(NH_4)_2SO_4$ and applied to a tyrosine-agarose column (Sigma, 1.0 × 12.7 cm) equilibrated with 2 M $(NH_4)_2SO_4$ in buffer B. Polybuffer (from the chromatofocusing step) was removed by washing the column with 2 M $(NH_4)_2SO_4$ in buffer B. Tyr-*P* phosphatase was eluted from the tyrosine-agarose column with decreasing (2–0 M) concentrations of $(NH_4)_2SO_4$ in buffer B. The enzyme was eluted at ≈0.8 M $(NH_4)_2SO_4$ (fraction 5) and was stable at 4°C at least for 3 wk.

Other Methods. Hydrolysis of NP-*P* was assayed by the method of Garen and Levinthal (15). Gel electrofocusing was carried out as described by Wrigley (16). Protein concentration was measured by the dye-binding method described by Bradford (17), using a Bio-Rad protein assay kit.

Table 1. Purification of Tyr-*P* phosphatase

Step	Fraction	Total protein, mg	Specific activity, (nmol/min)/mg: Hydrolysis	Specific activity, (nmol/min)/mg: Phosphate transfer
Crude extract	0	4,270	3.67	0.0589
Ammonium sulfate precipitation	1	1,620	4.53	0.0778
DEAE-cellulose chromatography	2	69.8	37.0	0.687
Sephadex G-200 chromatography	3	3.34	393	7.34
Chromato-focusing	4	0.44	1,950	32.2
Tyr-agarose chromatography	5	0.025	27,500	359

Specific activities were measured in the presence of 10 mM EDTA/5 mM Na phosphate to suppress the activity of nonspecific phosphatases in crude preparations. Hydrolysis and phosphate transfer activities of Tyr-*P* phosphatase were not affected by the presence of EDTA/Na phosphate (see Table 3), while the activity of nonspecific phosphatases assayed by hydrolysis of NP-*P* was inhibited 97%.

RESULTS

Isolation of Tyr-*P* Phosphatase. Separation of the Tyr-*P*-specific phosphatase was achieved by removing the nonspecific phosphatases present in the larvae (18–21), which hydrolyze Tyr-*P* as well as the common phosphatase substrate NP-*P*. The purification procedure is summarized in Table 1; the assays were carried out in the presence of EDTA and P_i, which inhibit only the nonspecific phosphatases. Nonspecific phosphatases were assayed by hydrolysis of NP-*P* in the absence of EDTA/P_i and removed by steps 1–3 shown in Table 1. The result at the Sephadex G-200 gel filtration step (Fig. 1) indicates practically complete separation of the specific enzyme, as measured by phosphate transfer from Tyr-*P* to [^{3}H]tyrosine, from nonspecific phosphatases. Calibration of the Sephadex column (Fig. 1) indicates that the molecular weight of the Tyr-*P*-specific phosphatase is ≈170,000.

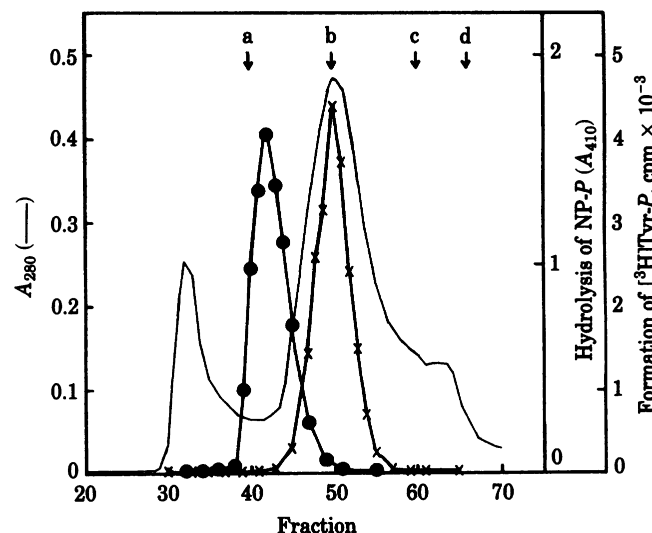

FIG. 1. Separation of Tyr-*P* phosphatase from nonspecific phosphatases by Sephadex G-200 gel filtration. The DEAE-cellulose fraction (see Table 1) was applied to a Sephadex G-200 column (2.1 × 98 cm) and eluted with 0.1 M NaCl in buffer B. Fractions of 5 ml were collected at a flow rate of 10 ml/hr. Tyr-*P* phosphatase was assayed as phosphate transfer activity by measuring the formation of [^{3}H]Tyr-*P* (●). Nonspecific phosphatase activity was assayed by hydrolysis of NP-*P* (×) according to the method of Garen and Levinthal (15). The reaction mixture (250 μl) contained 0.5 M Tris·HCl (pH 8.0), 2 mM $MgCl_2$, 8 mM NP-*P*, and 50 μl of enzyme fraction. Incubation was done at 37°C for 30 min. Reaction was terminated by addition of 0.6 ml of 0.25 M NaOH, and the absorbance of *p*-nitrophenol released was determined at 410 nm. The column was calibrated with standard proteins under similar elution conditions to estimate the molecular weight of native Tyr-*P* phosphatase. ↓, Standard proteins: a, catalase (M_r, 210,000); b, bovine serum albumin (M_r, 68,000); c, chymotrypsinogen A (M_r, 25,000); d, cytochrome *c* (M_r, 12,500).

Coincidence of Phosphate Transfer and Hydrolytic Activities. The specific activities of the hydrolysis and phosphate transfer increased in parallel throughout the purification (Table 1). The peaks of the two activities also overlapped in gel electrofocusing experiments (Fig. 2). The isoelectric point of the enzyme appears to be 5.6, a value consistent with the result obtained by chromatofocusing chromatography in which the enzyme eluted at pH 5.4.

The rate of the phosphate transfer reaction is compared with that of hydrolysis as a function of tyrosine concentration in Fig. 3. As expected, as the tyrosine concentration increased, the hydrolysis reaction was increasingly inhibited while the phosphate transfer reaction was stimulated. Thus, transfer activity was found to be 1.4–14% of hydrolytic activity, depending on the initial tyrosine concentration (0.25–4 mM).

Specificity of Tyr-*P* Phosphatase. Only L-Tyr-*P* was found to be the substrate of the enzyme and the apparent K_m of the reaction was 0.14 mM. Serine phosphate, threonine phosphate, ATP, and NP-*P* were not hydrolyzed. The preparation of D-Tyr-*P* was slightly hydrolyzed, but the hydrolysis stopped after 15 min (Fig. 4). Therefore, this limited hydrolysis is considered to be due to contamination with the L-isomer. In contrast, the D-isomer was well hydrolyzed by subsequently added *Escherichia coli* alkaline phosphatase.

Effects of Divalent Cations, Inhibitors, and pH. Addition of Mg^{2+} did not affect the rate of hydrolysis or phosphate transfer (Table 2). Ca^{2+} was slightly, and Mn^{2+} was significantly, inhibitory for both reactions; however, phosphate transfer appeared to be more sensitive than hydrolysis in both cases. Zn^{2+} was most inhibitory among the divalent cations tested, and affected the two reactions equally.

The effects of known phosphatase inhibitors are shown in Table 3. Na phosphate and EDTA did not affect either hydrolysis or phosphate transfer. However, NaF strongly inhibited both reactions. Inhibition was also observed with 1-*p*-bromotetramisole oxalate, an anthelminitic drug reported to suppress

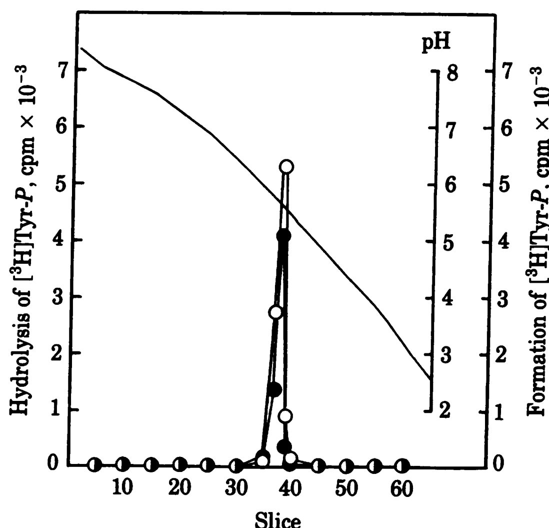

FIG. 2. Coincidence of hydrolytic and phosphate transfer activities in gel electrofocusing. Tyr-*P* phosphatase (0.5 μg) was applied to a 5% polyacrylamide disc gel (5 × 60 mm) containing 1% Ampholines (LKB) and subjected to electrofocusing at 200 V for 16 hr at 4°C. Then, the gel was sliced into 1-mm fractions, 50 μl of buffer B was added to each fraction, and the fractions were kept overnight at 4°C. Ten-microliter aliquots of each fraction were separately assayed for hydrolysis of [^{3}H]Tyr-*P* (○) and formation of [^{3}H]Tyr-*P* (●) to measure phosphate transfer activity. The pH gradient was measured as described by Wrigley (16).

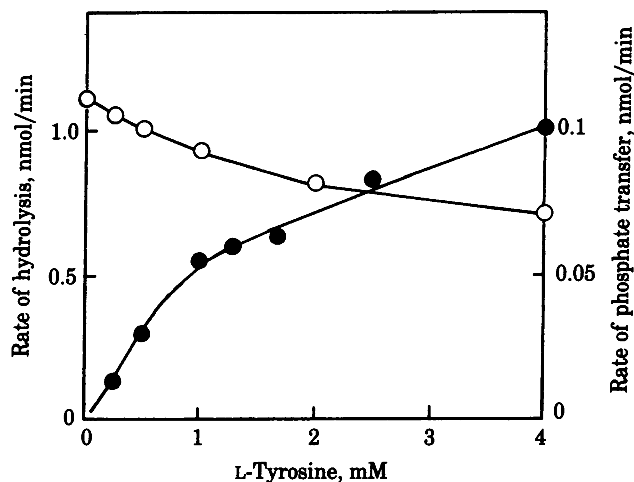

FIG. 3. Effect of tyrosine concentration on rates of hydrolysis and phosphate transfer reactions. Rate of hydrolysis (○) was measured as rate of [^{3}H]tyrosine release from [^{3}H]Tyr-*P*, and rate of phosphate transfer (●) was measured as rate of [^{3}H]Tyr-*P* formation from [^{3}H]tyrosine and unlabeled Tyr-*P*. The reaction mixture (50 μl) contained 40 ng of Tyr-*P* phosphatase, 2.5 mM Tyr-*P*, various amounts of tyrosine, and 2 μCi of [^{3}H]Tyr-*P* or 10 μCi of [^{3}H]tyrosine. Results are given as initial rates by converting the increase of radioactivity per min into nmol/min on the basis of the initial concentrations of Tyr-*P* and tyrosine.

alkaline phosphatases in rat tissues (22). EDTA had no effect by itself but it abolished NaF inhibition. Inhibition by 1-*p*-bromotetramisole oxalate was also reversed by EDTA but, in this case, for hydrolysis only. As a tyrosine analogue, L-dopa proved to be quite inhibitory and L-configuration specific and L-phenylalanine, which is known to inhibit certain mammalian phosphatases (23), also inhibited both reactions.

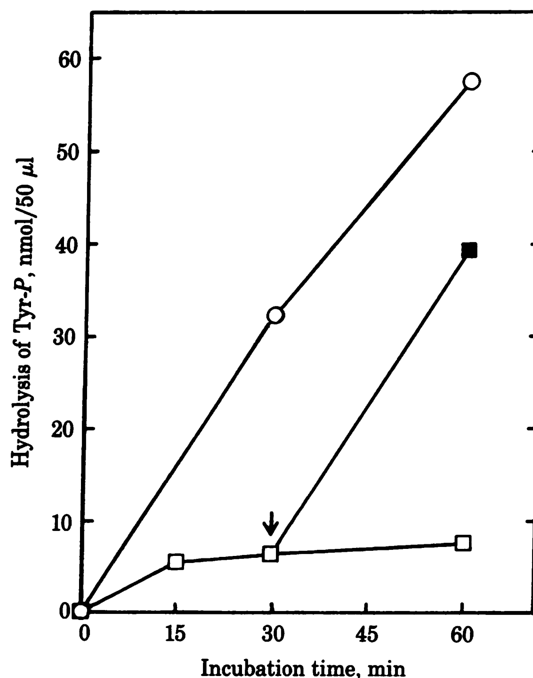

FIG. 4. Specific hydrolysis of L-Tyr-*P* by Tyr-*P* phosphatase. Reaction mixtures (50 μl) containing 50 mM Tris·HCl (pH 7.2), 40 ng of Tyr-*P* phosphatase, and 2.5 mM L-Tyr-*P* (○) or 2.5 mM D-Tyr-*P* (□) were incubated at 37°C for various times. Hydrolysis was measured colorimetrically. At 30 min (↓), *E. coli* alkaline phosphatase (0.2 μg/2 μl) was added to one (■) of the mixtures containing D-Tyr-*P*.

Table 2. Effects of divalent cations on hydrolysis and phosphate transfer reactions

Addition	Activity, %	
	Hydrolysis	Phosphate transfer
None	100	100
$MgCl_2$	100	100
$CaCl_2$	89	75
$MnCl_2$	71	42
$ZnSO_4$	35	33

Hydrolysis and phosphate transfer activities were measured by using 40 ng of Tyr-*P* phosphatase in the presence of various metal salts at 5 mM. One hundred percent values were 1.1 (nmol/min)/50 μl and 14 (pmol/min)/50 μl for hydrolysis and phosphate transfer reactions, respectively.

The effect of pH is shown in Fig. 5. Hydrolytic activity and phosphate transfer activity were comparable at neutral and alkaline pH but separated in the acidic region. The pH optimum of hydrolysis was 6.5–7.0 and that of phosphate transfer was 7.0–7.5.

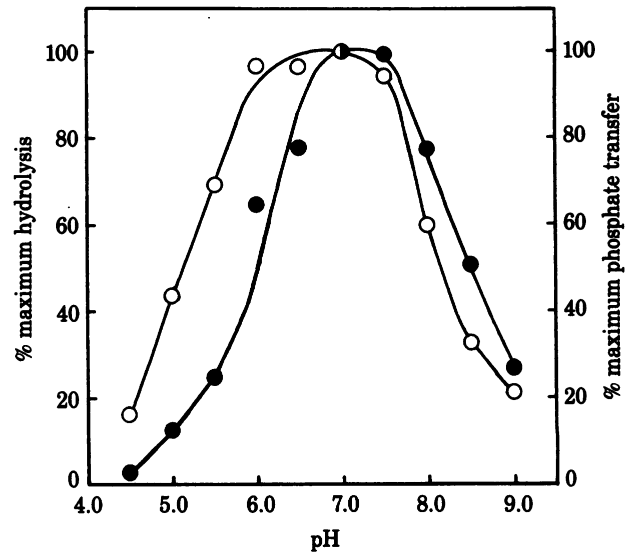

FIG. 5. Effect of pH on hydrolysis and phosphate transfer reactions. Reaction mixtures (50 μl) containing 100 mM Tris acetate buffer of the specified pH, 2.5 mM Tyr-*P*, 0.25 mM [^{3}H]tyrosine (800 μCi/mmol), and 40 ng of Tyr-*P* phosphatase were incubated at 37°C for 30 min. Reactions were terminated by chilling. Five-microliter aliquots were assayed for [^{3}H]Tyr-*P* formation to measure phosphate transfer activity (●) and the rest of the reaction mixture (45 μl) was used for colorimetric measurement of hydrolysis of Tyr-*P* (○).

DISCUSSION

In *D. melanogaster* and *S. bullata*, large amounts of Tyr-*P* are accumulated in the hemolymph as a storage form of tyrosine (9, 11). In both cases, it is suggested by [^{14}C]tyrosine injection experiments (11, 12) that the Tyr-*P* is in a steady-state interconversion with free tyrosine, the latter being used as an active metabolite by way of dopa (9). This interconversion may result from a balance between hydrolysis of Tyr-*P* and phosphorylation of free tyrosine mediated by the Tyr-*P*-specific enzyme described here. An enzyme promoting direct synthesis of Tyr-*P* by transphosphorylation from ATP or other phosphate donor could not be found. In this connection, Seligman *et al.* (11) have suggested in their study of *S. bullata* that Tyr-*P* is made from some source other than free tyrosine, such as a protein precursor by proteolysis. This seems to be supported by the finding that a strong incorporation of tyrosine into protein was observed with *Tenebrio* prior to emergence (24).

Our Tyr-*P*-specific phosphatase is remarkably similar to the serine phosphate-specific phosphatase (25–27). In both cases, the amino acid-*O*-phosphate phosphatase catalyzes both a specific phosphate transfer reaction and hydrolysis. The phosphate transfer can be considered simply as a reversible part of the hydrolysis reaction. However, simple reversal of hydrolysis is ruled out by the fact that detectable exchange of $^{32}P_i$ with the amino acid phosphate was not observed in either case (ref. 26; data not shown). In the case of the serine phosphate phosphatase, the possibility of divergent paths of hydrolysis and phosphate transfer to serine has been discussed (27), because the two reactions were shown to have different pH optima (26) and different sensitivities to certain compounds (27). In the case of the Tyr-*P* phosphatase, the phosphate transfer and hydrolytic activities could not be separated throughout purification (Table 1) or by gel electrofocusing (Fig. 2). However, the divergence between the two in pH optimum (Fig. 5), in the EDTA effect on 1-*p*-bromotetramisole oxalate inhibition (Table 2), and in Mn^{2+} inhibition (Table 3) seems to allow the possibility that the two reactions are catalyzed by different sites on the same enzyme.

Table 3. Effects of phosphatase inhibitors on hydrolysis and phosphate transfer reactions

Addition	Activity, %	
	Hydrolysis	Phosphate transfer
None	100	100
Na phosphate	102	100
EDTA	105	97
NaF	2.3	16
NaF/EDTA	87	94
BTMO	31	33
BTMO/EDTA	91	34
L-Phe	66	64
L-dopa	34	49
D-dopa	91	108

Reactions were carried out as in Table 2. Inhibitors (5 mM) were added to the assay mixture prior to addition of the enzyme, and incubations were started immediately after adding the enzyme. One hundred percent values were as shown in Table 2. BTMO, 1-*p*-bromotetramisole oxalate.

Some other properties of the Tyr-*P* phosphatase, especially its insensitivity to the addition of EDTA or P_i and inhibition by Zn^{2+}, however, make this enzyme quite different from the serine phosphate phosphatase and other known nonspecific phosphatases in *Drosophila* (18–21). Indeed, addition of 10 mM EDTA/5 mM Na phosphate strongly suppressed nonspecific phosphatases in crude preparations but did not affect the Tyr-*P* phosphatase (Table 1).

It may be added that insensitivity to EDTA and inhibition by Zn^{2+} have been observed with phosphotyrosyl-protein phosphatases in tumor cell membranes (28) and in normal rat tissues (29). The Zn^{2+} concentration (5 mM) required for partial inhibition of Tyr-*P* phosphatase (Table 2) appears quite high compared with that (0.01–0.1 mM) required for inhibition of hydrolysis of protein-bound Tyr-*P* (28, 29). Nevertheless, the analogies suggest a similarity between the free and protein-bound Tyr-*P*-specific phosphatases.

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