Glycogen Synthetase-D Phosphatase

I. SOME NEW PROPERTIES OF THE PARTIALLY PURIFIED ENZYME FROM RABBIT SKELETAL MUSCLE*

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KANEFUSA KATO† and JONATHAN S. BISHOP

From the Department of Internal Medicine and the General Clinical Research Center, College of Health Sciences, University of Minnesota, Minneapolis, Minnesota 55455

SUMMARY

Purification of the phospho-protein phosphatase of skeletal muscle which promotes the conversion of the phospho- (D or b) form to the dephospho- (I or a) form of glycogen synthetase (UDPglucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11) was aided by the use of buffers which include manganese chloride. The Mn²⁺ (5 mM) appeared to stabilize the enzyme and to induce greater recovery from DEAE-cellulose chromatography than reported previously. The enzyme was purified more than 1,000-fold from the 78,000 g supernatant of rabbit muscle homogenate. It was essentially free of phosphorylase and synthetase. Phosphatase activity was measured by two methods; (a) as the rate of conversion of glycogen synthetase-D to the I form (D to I conversion) and (b) as the rate of orthophosphate release from 32P-labeled synthetase-D (32P, release).

Inhibition of the phosphatase reaction (more than 50%) was found in the presence of F⁻ (10 mM), Na₂SO₃ (1 mM), and P, or PP, (0.2 mM). More than a 2-fold increase in phosphatase activity was found in the presence of divalent metal cations: Mn²⁺ > Ca²⁺ > Mg²⁺ (half-maximal concentration was 0.6 to 1.2 mM).

Glucose-6-P (up to 1 mM) had no effect on the rate of 32P, release, but increased about 2-fold the rate of D to I conversion (at 0.1 mM), as did galactose-6-P and glucosamine-6-P to a lesser extent. A glycogen concentration of 0.5 to 1.5 mg per ml was found to be optimal compared to the slower reaction rates found with greater or lesser concentrations.

A histone phosphatase activity found in this preparation, measured using 32P-labeled histone phosphate as substrate, had properties similar to and was copurified with glycogen synthetase-D phosphatase. However, the above carbohydrate-type effectors of synthetase and synthetase-D phosphatase reactions were without effect on the rate of histone dephosphorylation. These findings suggest that these two phosphatase activities may reside in the same enzyme protein.

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† Present address, Department of Biochemistry, School of Medicine, Nagoya University, Nagoya, Japan.

Recent knowledge has emphasized that the activities of certain enzymes, which exist in two interconvertible forms, are regulated by phosphorylation—dephosphorylation processes promoted by protein kinases and phosphoprotein phosphatases. In mammalian systems conversion of glycogen synthetase from its phosphorylated "inactive" form (synthetase D or b) to its dephosphorylated "active" form (synthetase I or a) is catalyzed by glycogen synthetase-D phosphatase. When a change in metabolic or endocrine status results in an increased rate of glycogen synthesis associated with a greater fraction of the cellular content of glycogen synthetase being in the I form, this phenomenon could be ascribed to an increased activity of the phosphatase. Because of the characteristics of the enzyme in crude preparations from various tissues, such increased activity of glycogen synthetase D phosphatase has been thought to be promoted by one of several occurrences: either (a) a decreased concentration of an inhibitor, such as glycogen (2), ATP (3), or phosphorylase a (4); or (b) an increased concentration of an activator, such as glucose-6-P (5); or even (c) a conversion of the phosphatase itself to a more active form (6, 7).

In an attempt to clarify the phenomena of the altered activity states of glycogen synthetase-D phosphatase in crude tissue extracts we now report a method of partial purification, and some new properties, of the enzyme from skeletal muscle.

METHODS

UDP-[U-14C]glucose was prepared according to Thomas et al. (8) or was obtained from New England Nuclear. UDP-glucose, ATP, ADP, AMP, 3',5' cyclic AMP, glucose-6-P, glucose-1-P, fructose-6-P, glucose-1,6-P₂, fructose-1,6-P₂, glucosamine-6-P, phosphoenolpyruvate, 3-phosphoglycerate, and crystalline bovine serum albumin and calf thymus histone (Type II-A) were obtained from Sigma. Dithiothreitol, O-phospho-L-serine, O-phospho-dL-serine, and phosphitin (from chicken egg) were from Calbiochem. The barium salt of galactose-6-P (Boehringer and Sons) was converted to the sodium salt before use. DEAE-cellulose (Selectacel, Type 20) was from the Brown Company, Berlin, N. H., and Whatman DE 52 was from Reeve Angel Co. Sephadex and Sepharose gels and Ficoll were from Pharmacia Fine Chemicals.

1 The abbreviation used is: cyclic AMP, cyclic adenosine 3',5'-monophosphate.
Rabbit liver glycogen (Sigma, Type III) was treated with a mixed ion exchange resin (Amberlite MG-3) before use; muscle phosphorylase a (1150 Cori units per mg protein, obtained from Sigma as the twice-crystallized enzyme) was treated with Noritate and recrystallized twice more before use. Lyophilized phosphorylase b (obtained from Sigma as the twice-crystallized enzyme with 1260 Cori units per mg, containing 1.0 μmole of AMP per 100 mg of protein) was used without further recrystallization. ATP, labeled with 32P, (25 mCi per mmole, New England Nuclear) was prepared essentially by the method of Glynn and Chappell (9) except that the product was absorbed on acid-washed Norite A, eluted with 50% ethanol containing 2% ammonium hydroxide, and evaporated to dryness in vaccuo. Humam salivary amylase was lyophilized and stored at -15°C after purification by Sephadex G-25 chromatography according to Shainkin and Birk (10). Protein kinase, the peak I fraction eluted at pH 7.0 by a phosphate gradient from a DE-52 cellulose column, was prepared from the supernatant of an acidified (pH 4.8) extract of rabbit skeletal muscle homogenate which was treated essentially as described by Reimann et al. (11), except that the two calcium phosphate gel steps were omitted.

Glycogen synthetase-D was purified from rabbit skeletal muscle by the method of Brown and Lamor (12) except that some products of the fourth ethanol step which showed appreciable synthetase-D phosphatase activity when assayed with manganese were further purified. The enzyme was dialyzed overnight against 50 mM Tris-Cl, pH 7.8, containing 10 mM mercaptoethanol and 5 mM EDTA, was centrifuged (27,000 × g, 10 min) to remove denatured protein, and was then put on a Sepharose 4B or 6B column (1.8 × 90 cm). The glycogen-bound synthetase, which appeared in the exclusion volume, was precipitated with 15% ethanol (at -10°C) drained, resuspended in a small volume of 10 mM Tris-Cl, pH 7.8, containing 1 mM EDTA, 5 mM diithiothreitol, and dialyzed briefly to remove traces of ethanol. The enzyme, so treated, had 17 to 22 units (micromoles of glucose transferred to glycogen from 4.45 mM UDP-glucose per min at 30°C) per mg of protein, with less than 5% synthetase-I and little or no synthetase-D phosphatase activity under the standard phosphatase assay conditions (with 5 mM MnCl2, see below). It contained 20 mg of glycogen per 50 units of synthetase. The final preparation was frozen at -20°C for 6 months without any measurable loss of activity. Prior to use, the enzyme was diluted 4- to 6-fold with 10 mM Tris-Cl, pH 7.8, containing 1 mM EDTA, 5 mM diithiothreitol, and 0.1% bovine serum albumin and was previously incubated for 30 min at 30°C.

Synthetase-D labeled with 32P was prepared in a similar manner (12) using γ-labeled [32P]ATP (3,5) for the (D to I) conversion of the synthetase obtained from the second ethanol precipitation step, which also contained endogenous synthetase-I kinase. About 100 units of this synthetase (70% D, 30% I), suspended in 1.5 ml of 50 mM Tris-Cl, pH 8.2, 30 mM mercaptoethanol, and 5 mM EDTA was brought to a total volume of 2.0 ml with the addition of a solution containing 16 μmoles of MgCl2, 20 μmoles of [32P]ATP (about 500 μCi) and 0.1 μmole of 3',5' cyclic AMP. After 3 days incubation at 5°C the I form of the synthetase was reduced to about 10% of the total enzyme activity. The enzyme was then diluted with 6.5 ml of 50 mM Tris-Cl, pH 7.8, 50 mM mercaptoethanol, and 5 mM EDTA, and was twice precipitated in 15% ethanol (−10°C). The enzyme from the fourth ethanol precipitation step was resuspended in 1.5 ml of 10 mM Tris-Cl, pH 7.0, 50 mM mercaptoethanol, 1 mM EDTA, and incubated at 4°C with 250 μg of human salivary amylose during 24 hours of dialysis against 2 liters of this same buffer. After removal of denatured protein the enzyme was brought to pH 7.8 by adding enough 500 mM Tris-Cl buffer, pH 7.8, containing 50 mM EDTA to bring the buffer concentration to 50 mM. The labeled synthetase, low in glycogen and contained in less than 2 ml of solution, was put on a Sepharose 4B column (1 × 28 cm) equilibrated and eluted with the same buffer containing 50 mM mercaptoethanol. Both the 32P bound to protein and the synthetase activity were retarded and appeared in the same eluate fractions, which were pooled. The principal active fraction of the enzyme at this stage has been shown to be virtually homogeneous when subjected to disc gel electrophoresis with sodium lauryl sulfate (13). After addition of rabbit liver glycogen (1 mg per ml) the enzyme was precipitated with 15% ethanol. The glycogen-protein pellet was resuspended, dialyzed, and stored frozen as above. A typical preparation of the labeled synthetase-D (20.7 units per mg) had 17.1 nmoles of 32P per mg of protein. Recrystomography of this enzyme preparation on Sepharose 4B showed complete correspondence of radioactivity with synthetase activity, which now appeared with glycogen in the exclusion volume.

Phospho-histone, labeled with 32P, was prepared from calf thymus histone using γ-labeled [32P]ATP according to a method which was essentially that of Meisler and Langan (14), except that 0.1 mM 3',5' cyclic AMP and 2 mM theophylline were added to the reaction mixture (5.0 ml) which contained 0.5 mg of cyclic AMP-dependent protein kinase. The washed and dried [32P]-phospho-histone was dissolved in 0.2 ml EDTA, pH 7.0, and dialyzed overnight against 1 liter of this solution before use. Of the final product only the phospho-histone which was most water soluble (yield about 40%) was used. This fraction had the greatest 32P content (35 to 40 μmoles per g) 98% of which was alkali-labile (1 N NaOH, 15 min, 100°C). The less water-soluble P-histone had less 32P bound but was also a substrate for this phosphatase. There were about 6 μmoles of alkali-labile phosphate per g in the histone as purchased.

**Assay of Glycogen Synthetase-D Phosphatase**

*Method 1. (D to I Conversion)—For the majority of the studies phosphatase activity was measured as the rate of conversion of synthetase-D to the I form in the following medium: 50 mM imidazole-Cl, pH 7.4; with 5 mM MnCl2, or without manganese but with 0.5 to 2.0 mM EDTA, 0.07% bovine serum albumin; 1.7 mM diithiothreitol, approximately 2 units per ml (0.1 mg per ml) of purified synthetase-D; with about 1 mg per ml of rabbit liver glycogen, and phosphatase suitably diluted to no more than 30% of the substrate in 4 min, i.e. 0.15 unit of phosphatase per ml. The final volume was 60 μl. The reaction was started by adding the synthetase-D (contained in 10 μl) to the other previously warmed reactants. At zero time, and after...
4 min of incubation at 30°, 10 µl of the reaction medium were transferred into 140 µl of the dilution medium, which contained 50 mM Tris-Cl, pH 7.8, 20 mM EDTA, 10 mM Na₂SO₄, 25 mM KF, 1% rabbit liver glycogen, and 0.1% bovine serum albumin. Then, within 1 min after the termination of phosphatase action, the synthetase assay was started by the transfer of 20 µl of the above diluted phosphatase reaction medium into 60 µl of the synthetase test mixture which was incubated at 30° for 17 min. The synthetase I assay was performed in duplicate by the filter paper method of Thomas et al. (8), using 4.45 mM UDP [³²Cl-]

The synthetase assay mixture which was incubated at 30° for 17 min. With each phosphatase assay total (D + I) synthetase concentration was monitored with a test mixture including 7.2 mM glucose 6-P and was always found to be unchanged during the D to I conversion. The phosphatase activity was calculated from the net increase of the I activity which occurred during the 4 min of phosphatase incubation, 1 unit of phosphatase activity being defined as that amount catalyzing the conversion of 1 unit of synthetase-D to the I form per min at 30°.

Method 2. (³²P) Release—When [³²P]-labeled synthetase-D was used (2.0 units with about 20,000 cpm per ml), a separate tube containing 80 µl of the above reaction mixture was used for each time period of the incubation. After removal of 10 µl for dilution before assay of synthetase activity, the tube was placed in ice and as soon as possible 60 µl were removed for application directly to filter paper squares (Whatman 31 ET) for estimation of [³²P] bound to protein. These filter paper squares were placed in 10% trichloracetic acid for 5 min and then washed twice in 5% trichloracetic acid at 25° and twice in 5% trichloroacetic acid at 90° for 5 min and then dried after washing with ethanol-ether; the [³²P] bound to protein was counted in a scintillation counter using 0.57; 2,5-diphenylosazole (PPO) in ethanol-ether; the [³²P]-labeled protein precipitated on the washed filter paper from 60 µl of the reaction mixture.

Histone phosphatase activity was also measured under the above conditions. The reaction was started in the same volume and composition of reaction mixture by the addition of [³²P]-labeled P-histone, about 10 µM final concentration (based on the alkali-labile [³²P] content of the labeled histone). The reaction was terminated by the addition of 90 µl of silicotungstic acid (20 µM in 0.02 N sulfuric acid). The precipitated protein was removed by centrifugation and orthophosphate was extracted from the protein-free supernatant fluid into 1-butanol as the molybdovanadophosphate complex, according to Parvin and Smith (15). Seventy-five microliter aliquots of the I-butanol supernatant layer, in duplicate and from duplicate assays, were transferred to filter paper squares (Whatman 31 ET, 2 X 2 cm), dried, and placed in vials containing 0.5% 2,5-diphenyloxazole (PPO) in toluene. With this washing procedure, control samples had 100% of the radioactivity observed if the sample was simply dried without washing. Phosphatase activity (Δ cpm per min) was considered to be the decrease per min in counts per min of the [³²P]-labeled protein precipitated on the washed filter paper from 60 µl of the reaction mixture.

Histone phosphatase activity was also measured under the above conditions. The reaction was started in the same volume and composition of reaction mixture by the addition of [³²P]-labeled histone-D, about 10 µM final concentration (based on the alkali-labile [³²P] content of the labeled histone). The reaction was terminated by the addition of 90 µl of silicotungstic acid (20 µM in 0.02 N sulfuric acid). The precipitated protein was removed by centrifugation and orthophosphate was extracted from the protein-free supernatant fluid into 1-butanol as the molybdovanadophosphate complex, according to Parvin and Smith (15). Seventy-five microliter aliquots of the 1-butanol supernatant layer, in duplicate and from duplicate assays, were transferred to filter paper squares (Whatman 31 ET, 2 X 2 cm), dried, and placed in vials containing 0.5% 2,5-diphenyloxazole in toluene for liquid scintillation counting. The amount of P1 released was determined from the counts observed, relating this value to the specific activity of the [³²P]ATP with which the histone had been phosphorylated and neglecting the unlabeled seryl-P of the histone as purchased. Unless stated otherwise, the phosphatase used, which had been purified through the Sephadex G-200 column step (Table I), contained about 0.07 mg of protein per ml and was diluted as much as 20-fold with 5 mM dithiothreitol containing 0.1% bovine serum albumin. One unit of P-histone phosphatase activity is defined as that which results in the hydrolysis of 1 nmole of [³²P]orthophosphosphate per min under the conditions specified above.

Glycogen was measured as carbohydrate by the phenol-sulfuric acid method of DuBois et al. (16). Protein was measured by the biuret (17) or by the Folin-Lowry method (18) following precipitation in 5% trichloracetic acid with bovine serum albumin as a standard. Inorganic phosphate was measured by the Fiske-SubbaRow method (19) for phosphorylase assays or by the method of Parvin and Smith (15) for hydrolysis of phosphate from organic phosphate compounds.
Phosphatase activity was eluted between 200 and 300 ml, with no protein peak distinguishable from the moderate amount of trailing protein. The most active fractions were frozen either immediately or after concentration with Ficoll. At this stage the enzyme was also stable to repeated freezing and thawing, with no loss of activity in 6 months storage at -65°. The results of a typical purification (Table I) indicate that the most active fractions after Sephadex filtration had a specific activity of 15.5 units per mg of protein. In smaller scale purification at this step, using 2 ml of enzyme applied to a Sephadex G-200 column (2 x 88 cm), trailing protein did not extend to the active fractions, and a better purification was obtained (18 units per mg of protein). Unless stated otherwise, all studies described below were carried out using phosphatase purified by Sephadex G-200 chromatography.

11. Properties of Synthetase-D Phosphatase Activity

The proportionality of activity with respect to enzyme concentration and time of incubation is shown in Fig. 1, using reaction mixtures both without and with manganese ion (5 mM). The addition of dithiothreitol (1.7 mM) and bovine serum albumin (0.07%) tended to stabilize the enzyme activity during the assay. Nevertheless, the reaction rate usually became somewhat slower after 8 to 10 min of incubation at 30°, or when more than 50% of the added synthetase-D (2.0 units per ml) was converted to the I form. The $K_m$ for glycogen synthetase-D was about 0.5 unit per ml (not shown here) as described by Villar-Palasi (2), whether assayed in the absence or in the presence of divalent cation. Heating the phosphatase for 4 min at 57° resulted in 80% loss of activity, whether measured in the absence or in the presence of manganese (5 mM).

A requirement for glycogen during the phosphatase assay is indicated in Fig. 2 using a certain synthetase-D preparation which was unusually low in glycogen content. Maximal phosphatase activity was seen with a glycogen concentration of 0.05 to 0.15% whereas inhibition was seen not only with lower concentrations but also with higher glycogen concentrations, the latter observation as reported by Villar-Palasi (2).

When pH was varied the greatest phosphatase activity was observed near neutrality, but in the presence of Mn$^{2+}$ or Ca$^{2+}$ (5 mM) the stimulated activity was greatest in a more narrow range (pH 7.0 to 7.4). (Fig. 3.)

Effect of Divalent Cations—Synthetase-D phosphatase, at all stages of purification beyond and including the first ethanol precipitation step, was activated by divalent cations: Mn$^{2+}$ > Ca$^{2+}$ > Mg$^{2+}$, each with an apparent half-maximal concentration in the range of 0.6 to 1.2 mM (Fig. 4). This relationship was confirmed by measuring the rate of $^{32}$P release from $^{32}$P-labeled synthetase-D as substrate (Fig. 5A). Because of its greater activation of the phosphatase, manganese ion was selected as the cation to be added in excess to the EDTA-containing buffers during purification beyond the first ethanol precipitation step. This practice resulted in a satisfactory elution of phosphatase activity from Sephadex G-200 columns.

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Proportionality of synthetase-D phosphatase activity with respect to time and enzyme concentration. Phosphatase (0.17 mg per ml) was added, in indicated amounts, of reaction mixtures to either: A, (-)Mn$^{2+}$; or B, (+)5 mM Mn$^{2+}$. Aliquots of the mixture (10 µl) were removed as a function of the time of incubation. The insets show the same experimental results, with velocity of the phosphatase reaction (Δ cpm per 4-min incubation) plotted as a function of the amount of enzyme added.

<p>| TABLE I | Purification of glycogen synthetase-D phosphatase from rabbit skeletal muscle |
|----------------------|---------------------------------|----------------------|----------------------|----------------------|----------------------|</p>
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Total activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>14,000 × g supernatant</td>
<td>10,700</td>
<td>0.025</td>
<td>540</td>
<td>268</td>
<td>74%</td>
</tr>
<tr>
<td>78,000 × g supernatant</td>
<td>9,310</td>
<td>0.015</td>
<td>520</td>
<td>144</td>
<td>100%</td>
</tr>
<tr>
<td>Ethanol (0 to 30%)</td>
<td>9,160</td>
<td>0.193</td>
<td>105</td>
<td>340</td>
<td>236%</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>198</td>
<td>0.985</td>
<td>131</td>
<td>195</td>
<td>135%</td>
</tr>
<tr>
<td>Ethanol (0 to 25%) (frozen-thawed)</td>
<td>81.4</td>
<td>3.53</td>
<td>5.9</td>
<td>287</td>
<td>199%</td>
</tr>
<tr>
<td>Sephadex G-200 pooled fractions peak</td>
<td>33.2</td>
<td>8.37</td>
<td>110</td>
<td>278</td>
<td>193%</td>
</tr>
<tr>
<td>6.9</td>
<td>29</td>
<td>15.5</td>
<td>6</td>
<td>107</td>
<td>75%</td>
</tr>
</tbody>
</table>

- Activity without Mn$^{2+}$, since 5 mM Mn$^{2+}$ added to assay of crude extracts results in activity as low as 20% of the activity seen in assays using 1 mM EDTA without added Mn$^{2+}$. Inorganic phosphate ion has been identified as a major inhibitory component in these crude extracts.
- Activity in the presence of 25 mM NaCl, which is an inhibitory concentration only in early stages of purification.
FIG. 2 (left). Influence of glycogen concentration on phosphatase activity. The reaction mixture contained KOH-extracted rabbit liver glycogen in the indicated final concentrations, with the lowest concentration being that which was contained in the synthetase-D preparation used. During the phosphatase reaction, the total synthetase activity was constant (at 2.0 units per ml of reaction mixture) at each concentration of glycogen.

FIG. 3 (center). Phosphatase activity as a function of pH. The reaction mixture was made from 200 mM concentrations of the following buffers at the indicated pH: with EDTA (1 mM) in 2-(N-morpholino)ethane sulfonate (O); imidazole (■); Tris (dotted circle); with CaCl₂ (5 mM) in 2-(N-morpholino)ethane sulfonate (△); imidazole (▲); Tris (dotted triangle).

FIG. 4 (right). Influence of divalent cation concentration on phosphatase activity. The Ficoll concentrated phosphatase (2.1 mg per ml), which had been dialyzed for 24 hours against EDTA (5 mM)-containing buffer, was diluted (1:15) in 0.1% bovine serum albumin and 5 mM dithiothreitol. The diluted phosphatase was added (see "Methods") to reaction mixtures which contained the indicated concentrations of: MgCl₂ (●), CaCl₂ (■), or MnCl₂ (▲).

Fig. 5. Effect of modifiers of phosphatase activity; comparison of increase of synthetase I (open bars) with 32P release (closed bars) from 32P labeled synthetase-D (see "Methods", Assay Methods 1 and 2). Phosphatase activity is expressed as percentage of activity of the control. A, phosphatase preparation was the same as in Fig. 4; B, all incubations contained Mn²⁺ (5 mM). Control phosphatase values are 0.008 and 0.097 unit per ml and 0.025 and 0.167 nmole of 32Pi released per min per ml of reaction mixture for A and B, respectively.

Effect of Other Ions and Metabolites—The effects of other ions on the activity of purified phosphatase are summarized in Table II. The marked inhibition by Pi and PPi (half-maximal concentration of 0.2 mM) was also present despite a 20-fold molar excess of manganese ion. The moderate inhibition by Na₂S0₃ (27% at 10⁻⁵ M) was unexpected in view of the finding of Hizukuri and Larner (20) that the presence of SO₃⁻ (10 mM) was optimal for the D to I conversion in various crude preparations of the enzymes from rat liver. Na₂SO₄, Na₂CO₃, NaHCO₃ (10⁻³ M), NaCl, and KCl (up to 10⁻² M) were not inhibitory. Likewise, ATP, ADP (10⁻⁴ to 10⁻³ M), AMP, theophylline, and caffeine (5 × 10⁻⁴ M) had no influence on phosphatase activity.

Glucose and galactose (10⁻³ M and 10⁻² M) and several phosphorylated carbohydrate metabolites had essentially no effect upon the phosphatase reaction at 10⁻⁴ M and 10⁻³ M concentrations: glucose-1-P, glucose-1, 6-P₂, fructose-6-P, fructose-1, 6-P₂, P-enolpyruvate, and 3-P-glycerate (Table III). Glucose-6-P (0.1 mM) increased the rate of D to I conversion more than 2 fold, but this effect upon the phosphatase reaction could not be
TABLE II

Effect of ions on glycogen synthetase-D phosphatase from rabbit skeletal muscle

Phosphatase was measured by Method 1.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentrations used</th>
<th>Synthetase-D phosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>Units/ml</td>
</tr>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.044</td>
</tr>
<tr>
<td>Pᵢ</td>
<td>1.0</td>
<td>0.022</td>
</tr>
<tr>
<td>PPᵢ</td>
<td>1.0</td>
<td>0.011</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>1.0</td>
<td>0.005</td>
</tr>
<tr>
<td>Na₂PO₄</td>
<td>1.0</td>
<td>0.045</td>
</tr>
<tr>
<td>KF</td>
<td>2.0</td>
<td>0.012</td>
</tr>
<tr>
<td>KCl</td>
<td>10.0</td>
<td>0.034</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0</td>
<td>0.038</td>
</tr>
</tbody>
</table>

TABLE III

Effect of metabolites on glycogen synthetase-D phosphatase from rabbit skeletal muscle

Phosphatase was measured by Method 1.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentrations used</th>
<th>Synthetase-D phosphatase activity + 5 mM MnCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>0.109</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>1.0</td>
<td>0.236</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>0.1</td>
<td>0.077</td>
</tr>
<tr>
<td>Fructose-1,6-P</td>
<td>0.1</td>
<td>0.094</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>0.1</td>
<td>0.109</td>
</tr>
<tr>
<td>Glucose-1-P</td>
<td>0.1</td>
<td>0.126</td>
</tr>
<tr>
<td>Glucose-1,6-P</td>
<td>0.1</td>
<td>0.094</td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>0.1</td>
<td>0.076</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>0.1</td>
<td>0.109</td>
</tr>
</tbody>
</table>

It was thought unlikely that this discrepancy was an artifact caused by an activation of synthetase-D by the glucose-6-P (which was diluted 1:45 after the phosphatase incubation), since similar results were observed when an aliquot of the phosphatase reaction mixture was treated by Sephadex G-25 gel filtration before the synthetase assay (Fig. 7A). It was found that galactose-6-P and glucosamine-6-P also stimulated D to I conversion, although to a lesser degree than glucose-6-P, with half-maximal effects being obtained at somewhat higher concentrations than that of glucose-6-P (Fig. 7B).3

3 In some experiments with phosphorylated carbohydrates, the relative potencies of these positive effectors appeared less because in the 1 min period of incubation used the limits for linearity of the assay were exceeded (see Fig. 1).
a total volume of 1.0 ml of standard reaction mixture with 5
mM MnCl₂ (synthetase-D omitted) (data not shown). No con-
version to the b form was observed when muscle phosphorylase
a (up to 1.0 mg per ml) was incubated with synthetase phos-
phatase (data not shown). However, under similar conditions
³²P-labeled phospho-histone (0.25 mg per ml) served as an al-
ternate substrate for the phosphatase, as is reported in detail
below.

Studies of inhibition of the phosphatase reaction brought
about by various phosphoproteins were difficult to interpret in
more than a qualitative way since high concentrations of some
proteins, but not others, produced either cloudiness in the pre-

cence of Mn⁴⁺ or direct inhibition of the synthetase assay. How-

ever, the following results are presented since their controls
were not grossly abnormal with respect to these problems.
Muscle phosphorylase a (up to 1.0 mg per ml) inhibited (up to
43%) the conversion of synthetase-D to I. This finding was
not considered unique for the phosphorylated form of the pro-
tein since phosphorylase b was also somewhat inhibitory (up to
25%) at these concentrations. Phospho-histone (up to 0.27
mg per ml) inhibited (up to 54%), whereas phosvitin (up to
0.05 mg per ml) had no effect on the conversion of synthetase-D
to I. At the highest concentrations tested the ratio of alkali-
labile phosphate in the phosphoproteins relative to that in the
synthetase-D of the standard reaction mixture was 1.7, 1.7,
and 20 for phosphorylase a, phos-phia, and phosvitin, re-
spectively.⁴

III. Properties of P-Histone Phosphatase Activity

Phosphorylated histone was found to serve as a substrate for
the purified synthetase-D phosphatase from rabbit skeletal
muscle as reported above. When P⁻³²P-labeled histone was
incubated with a 1000-fold purified synthetase-D phosphatase
preparation, the rate of release of inorganic phosphate was
linear with respect to the length of the incubation period and
to increasing concentrations of enzyme (data not shown), provided
less than 50% of the substrate was consumed. Manganese ion
(2 to 10 mM) produced more than a 2-fold positive effect upon,
and Cu⁴⁺, Co⁴⁺, and Mg⁴⁺ were less potent upon, whereas Cu⁴⁺
and Zn⁴⁺ exerted a negative effect upon P-histone phosphatase
(Fig. 8). ⁵ Reaction rates were optimal in the range of pH 6.4
to 7.4, as was seen with synthetase-D phosphatase. Inorganic
phosphate (5 × 10⁻⁴ M), PP₁ (5 × 10⁻⁴ M), KF (5 × 10⁻² M),
and Na₂SO₄ (10⁻² M) inhibited activity more than 70%, either
in the presence or absence of 5 mM MnCl₂. As reported for the
liver enzyme (14) sodium chloride (10⁻¹ M) activated the muscle
P-histone phosphatase (both with and without Mn⁴⁺) about
50% (data not shown), in contrast to its negative effect on syn-

tetase-D phosphatase (Table I). Glycogen up to a concentra-
tion of 2% (w/v), and glucose-6-P, galactose-6-P, and gluco-
samine-6-P (up to 0.5 mM), which modify synthetase-D phosphatase
activity vide supra, were found to have no influence upon the
rate of dephosphorylation of phospho-histone (data not shown).

⁴ Assuming 6 moles of phosphate per 100,000 g of synthetase D
(29), the standard reaction mixture (2 units = 0.1 mg of syn-

tetase-D protein per ml) has 6.7 moles of alkaline-labile phosphate
per ml. The alkaline-labile phosphate concentrations (15) of the
samples of phosvitin (commercial source) and phospho-histone
(prepared according to the method described, using unlabeled
ATP) were 2.9 moles per g and 38.4 μmoles per g, respectively.

⁵ Data for the influence of Co⁴⁺, Cu⁴⁺, and Zn⁴⁺ on synthetase-D
phosphatase are not reported because it was found that these
ions caused direct inhibition of synthetase in its separate assay
system.

![Fig. 8. Effect of divalent metal cations on histone phospho-
phatase activity. The reaction mixture, contained 0.48 μg of undi-
slyzed enzyme and the indicated concentrations of cations as their
sulfates or chlorides, was incubated as indicated in Fig. 1.](image)

![Fig. 9. Activity of phosphatase as function of P-histone con-
centration, assayed with 1 mM EDTA (dashed lines) and with 5
mM MnCl₂ (solid lines), alone (○) or with addition of purified
synthetase-D from rabbit skeletal muscle (12 μg) (△) under con-
ditions of Fig. 1.](image)

Glucose (10⁻³ M), ATP, ADP, AMP (5 × 10⁻⁴ M) and theo-

phylline (10⁻³ M), had no effect on histone phosphatase activity.

In the dephosphorylation of [³²P]P-histone the apparent Kₐ
(for [³²P]P-serine residues) was about 5 μM both in the presence
and in the absence of Mn²⁺ (Fig. 9). Synthetase-D (0.2 mg
per ml = 4 units per ml) inhibited histone phosphatase. Fur-
thermore, the type of inhibition seen when the concentration of
P-histone was increased from 0.1 to 0.6 mg per ml was usually,
but not consistently, of the competitive type (Fig. 9). Perhaps
the inconstant results were observed because of nonspecific
protein-protein interactions, as was observed with these proteins
previously (vide supra).

IV. Co-Purification of Two Phosphatase Activities

To test the possibility that the two phosphatase activities
might reside in the same protein molecule, histone phosphatase
activity was determined at all steps in the purification of syn-

thetase-D phosphatase from the 78,000 × g supernatant frac-
tion of a muscle homogenate (Table IV). The procedure was
as previously described except that the enzyme was eluted from
the DEAE-cellulose column by a gradient increase of NaCl
concentration. Histone phosphatase activity was purified to
the same degree (1,000-fold) as was synthetase-D phosphatase,
since the ratio of the specific activities of two phosphatases at each step was essentially constant. A final specific activity of 49 units (nmoles of $^{32}$P$_i$ from $[^{32}P]P$-histone per min per mg) was calculated from assay under the conditions described, which was as high as that achieved by the purification of histone phosphatase from rat liver reported by Meisler and Langan (14).

On DEAE cellulose chromatography, histone phosphatase activity was found in the same fractions as synthetase-D phosphatase, eluting with concentrations of from 25 to 200 mM NaCl (Fig. 10). The higher activity of histone phosphatase relative to that of synthetase-D phosphatase at higher concentrations of the salt was ascribed both to an activation of histone phosphatase (14) and to an inhibition of synthetase-D phosphatase by the NaCl in the eluate carried over into the assay mixtures.

The elution volume of histone phosphatase activity from a Sephadex G-200 column was identical with that of synthetase-D phosphatase (Fig. 11).

**DISCUSSION**

In the study of partially purified glycogen synthetase-D phosphatase carried out by Villar-Palasi (2), it was reported that instability to freezing and thawing and poor recovery from DEAE-cellulose chromatography resulted in only about a 10-fold purification with a 10% recovery. In the course of recent studies it was observed that highly purified synthetase preparations, both the D and I forms by various published procedures (12, 13, 21), evidenced phosphatase-like properties which were apparently totally dependent upon Mn$^{2+}$ for its activity.

**Table IV**

Co-purification of phospho-histone phosphatase activity with synthetase-D phosphatase activity from rabbit skeletal muscle

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total volume</th>
<th>P-Histone phosphatase</th>
<th>Synthetase-D phosphatase</th>
<th>Ratio of histone to synthetase-D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>ml</td>
<td>units</td>
<td>Specific activity</td>
<td>units/mg</td>
</tr>
<tr>
<td>78,000 X g supernatanta</td>
<td>4680</td>
<td>234</td>
<td>165</td>
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<td>Ethanol (0 to 30%)</td>
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<td>226</td>
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<td>DEAE-celluloseb</td>
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<td>203</td>
<td>0.61</td>
<td>80.0</td>
</tr>
<tr>
<td>Sephadex G-200c</td>
<td>2.1</td>
<td>35</td>
<td>74.0</td>
<td>35.2</td>
<td>27.9</td>
</tr>
</tbody>
</table>

a The assay mixture of this fraction did not contain MnCl$_2$, which inhibited the phosphatases in such crude extracts (see Table I).

The fractions eluting between 50 mM and 200 mM NaCl were pooled. The concentration of Na$^+$ carried over from them into the assay mixture (more than 10 mM) has been found to be inhibitory to synthetase-D phosphatase activity but stimulatory to histone phosphatase activity (see Fig. 10 and Table I).

b A half volume (1.5 ml) of the resuspended ethanol precipitate was applied onto the Sephadex G-200 column (2 X 88 cm), and 14 fractions (2.5 ml) with the most activity were pooled.

c A half volume (1.5 ml) of the resuspended second ethanol precipitate was applied onto the Sephadex G-200 column (2 X 88 cm), and 14 fractions (2.5 ml) with the most activity were pooled.

**Table IV**

Co-purification of phospho-histone phosphatase activity with synthetase-D phosphatase activity from rabbit skeletal muscle

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**Elution from DEAE Cellulose**

**Elution from Sephadex G-200**

Fig. 10 (left). Elution of the activities of synthetase-D phosphatase and P-histone phosphatase both from the same column of DEAE-cellulose. The resuspended extract of 30% ethanol precipitation from a crude extract (78,000 X g) of rabbit skeletal muscle was dialyzed overnight at 4°C against 2 liters of 50 mM Tris-Cl, pH 7.4, containing 50 mM mercaptoethanol, 1 mM EDTA, and 5 mM MnCl$_2$. After removal of denatured protein by centrifugation (27,000 X g, 15 min), the enzyme (45 ml, 1.8 g of protein) was applied on a DEAE column (2.5 X 30 cm) and washed with 1 bed volume of buffer. Elution was with an 800 ml linear gradient of NaCl (5 mM to 400 mM) added to the above buffer. Both phosphatase activities were assayed (+5 mM MnCl$_2$) using 10-ml aliquots of 10-ml fractions collected at a flow rate of 30 ml per hour.

Fig. 11 (right). Elution of the activities of synthetase-D phosphatase and P-histone phosphatase both from the same column of Sephadex G-200. The resuspended ethanol precipitate (second) of the phosphatase after DEAE chromatography (1.5 ml, 15 mg of protein) was applied to a Sephadex G-200 column (2 X 88 cm) and eluted with 50 mM Tris-Cl, pH 7.4, containing 10 mM mercaptoethanol, 1 mM EDTA, and 5 mM MnCl$_2$. Both phosphatase activities were assayed (+5 mM MnCl$_2$) (see Fig. 1) using 10-ml aliquots of 2.5-ml fractions collected at a flow rate of 12 ml per hour.
idea that manganese ion might stabilize the phosphatase during the purification procedures proved helpful as indicated in this report of a method for purification of the enzyme more than 1000-fold. The steps in the procedure where a divalent metal ion appeared to be of greatest benefit were in recovery both from DEAE-cellulose column chromatography and from subsequent storage by freezing in early stages of purification. (The most purified enzyme appeared reasonably stable to freezing even in the absence of free manganese.) It is possible that synthetase-D phosphatase may have an absolute requirement for a divalent metal ion, although we have not yet obtained a preparation which was completely inactive except in the presence of this ion.

The similarity of properties of three phospho-protein phosphohydrolases suggests a common mechanism of action, if not an identity in fact. The effect of divalent cations to stimulate and to stabilize synthetase-D and histone-phosphate phosphatase (reported here) is similar to that reported in less highly purified preparations of the phosphatase for active phosphorylase b kinase, reported by Riley et al. (22). The co-purification of the two activities reported here is evidence in the direction of a similarity of these enzyme proteins, which would be of particular interest in view of the current concept (21, 23) that a single protein kinase catalyses the opposite (phosphorylation) reaction in these three systems. By comparison, other phosphatases for P-proteins are known whose requirement for divalent cations is either not apparent despite extensive purification (that for phosphorylase α (24)), or may not have been tested (those for casein, phosphitin, and pyruvate dehydrogenase (25, 26)).

Since the rate of phosphate release from 32P-labeled synthetase-D usually corresponded with the rate of conversion of synthetase-D to the I form, the unusual lack of agreement between phosphokinase catalyses the opposite (phosphorylation) reaction and to stabilize synthetase-D and phospho-histone phosphatase may have an absolute requirement for a divalent cation to stimulate the phosphatase may have an absolute requirement for a divalent cation to increase the in vitro activity as judged by a “plus-minus MgCl2” assay. The administration of insulin to the intact animal brought about a change from one activity state to the other. It is possible that such a change in cation dependence may result from mobilization of the enzyme from the particulate fraction into the soluble fraction of liver, since such a distinction in properties has recently been described with the protein phosphatase of brain tissue (31). On the other hand, histone phosphatase of the soluble portion of rat liver extracts has been reported to be resolved by DEAE-cellulose chromatography into two fractions with different activities (14). It is apparent that there are many more interesting questions raised by the findings reported here than there are answers.

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