AN INORGANIC PYROPHOSPHATASE OF SWINE BRAIN*

BY ULYSSES S. SEAL† AND FRANCIS BINKLEY

(From the Department of Biochemistry, Division of Basic Health Sciences, Emory University, Georgia)

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In recent years, a number of reactions leading to the liberation of inorganic pyrophosphate have been described. Since the only known reaction leading to a net decrease in the concentration of inorganic pyrophosphate is that of hydrolysis, the properties of inorganic pyrophosphatases are of interest. The discovery of an inorganic pyrophosphatase in muscle was made by Lohmann (2) and one was demonstrated in mammalian nervous tissue by Gordon (3). A purification procedure for an inorganic pyrophosphatase of nervous tissue has been reported (4). This procedure resulted in a preparation hydrolyzing adenosine triphosphate as well as inorganic pyrophosphate.

The characterization of the inorganic pyrophosphatases of mammalian nervous tissue (as well as of other tissues) has been complicated by heterogeneity of the preparations used. The dependence of activity upon sulfhydryl groupings, the effect of metal ions, and the relationship of adenosinetriphosphatase and inorganic pyrophosphatase have been disputed. The enzyme described here is conveniently prepared and appears to be quite stable.

Materials and Methods

Materials—Frozen swine brains were obtained from the local slaughterhouses and stored at -20° until used. There was no decline in activity with 2 years of storage. The Tris (tris(hydroxymethyl)aminomethane) and p-chloromercuribenzoate were obtained from the Sigma Chemical Company, glutathione and adenosine triphosphate from the Schwarz Laboratories, Inc., cysteine (free base and hydrochloride) and β-glycerol phosphate from the Nutritional Biochemicals Corporation.

Methods—Inorganic and acid-labile phosphates were determined by the method of Allen (5). Nitrogen was determined by a micro-Kjeldahl procedure. For routine assays during purification, 3 ml. of 0.1 M Tris-HCl, pH 7.8, 1 ml. of 0.05 M MgCl₂, 1 ml. of 0.01 M cysteine, 1 ml. of 0.01 M EDTA (ethylendiaminetetraacetic acid), 1 ml. of 0.025 M pyrophosphate,

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† Predoctoral Fellow of the United States Public Health Service.
and 1 ml. of water were equilibrated at 37°, and then 1 ml. of suitably di-
luted enzyme was added. After 15 minutes the reaction was stopped by
the addition of 1 ml. of 50 per cent trichloroacetic acid. The mixture was
filtered, and inorganic phosphate was determined immediately on an ali-
quot. The assays on the purified material differed in that the final volume
of the incubation mixture was 10 ml.; 1 ml. aliquots were removed at var-
ious time intervals and added directly to 5 ml. of 12 per cent perchloric
acid for phosphate determination. A unit of enzyme is defined as that
amount which will liberate 1 mg. of orthophosphate P in 1 minute at 37°
under the standard assay conditions. Specific activity refers to units per
mg. of protein N. Water, redistilled from glass, was used for the prepara-
tion of all assay reagents and in the final steps of purification. Correc-
tions for total inorganic phosphate, found in both the substrate and enzyme,
were usually less than 5 per cent of the experimental values.

EXPERIMENTAL

Preparation of Enzyme. *Step I*—5 pounds of brain tissue were allowed
to thaw partially. 200 gm. portions were homogenized in a Waring blender
for 45 seconds with 500 ml. of 0.1 M MgCl₂. The homogenate was allowed
to stand for 24 hours at about 7°. It was then centrifuged for 20 minutes
at 1000 x g in an International refrigerated centrifuge, model PR-2, at 0°.
The precipitate contained about 85 per cent of the adenosinetriphos-
tase activity of the whole homogenate. The pyrophosphatase activity
remaining in the precipitate could be recovered by washing with additional
0.1 M MgCl₂. However, this was not done routinely.

*Step II*—An additional 0.1 mole of solid MgCl₂ and 500 mg. of sodium
lauryl sulfate were added to each liter of supernatant solution. The mix-
ture was then incubated for 1 hour at 37°, cooled, and centrifuged, and the
precipitate was discarded.

*Step III*—To each liter of supernatant solution were added 100 ml. of a
mixture of chloroform and octanol (95 and 5 parts respectively by volume)
and the mixture was shaken mechanically for 20 minutes. The mixture
was centrifuged and the supernatant solution was collected. To each
liter were added 15 gm. of Hyflo Super Cel (Johns-Manville) and 5 gm. of
Lloyd’s reagent. The mixture was filtered on large Büchner funnels with
gentle suction.

*Step IV*—The filtrate was collected and 75 ml. of alumina gel (6) were
added to each liter of filtrate. The suspension was mixed for 20 minutes,
centrifuged, and the supernatant solution was discarded. From this point
only glass-distilled water was used. The gel was washed once with 5 vol-
umes of water (per volume of gel), once with 5 volumes of 0.05 M pyrophos-
phate, pH 5.2, and the activity was then eluted with two portions of 2 vol-
umes each of 0.1 M pyrophosphate, pH 6.5. These eluates retained full activity for 3 to 6 months when kept refrigerated. All the nitrogen in the eluates was precipitated upon the addition of trichloroacetic acid.

**Step V**—The two eluates were fractionated separately with ammonium sulfate. 30 gm. per 100 ml. of eluate were added, the solution was allowed to stand for 30 minutes, was centrifuged, and the precipitate was discarded. 35 gm. of additional ammonium sulfate were added, the solution was allowed to stand for 30 minutes, and the precipitate was collected by centrifugation. The centrifuge bottles were allowed to drain in an inverted position and the precipitate was dissolved in 10 ml. of 0.01 M phosphate buffer (pH 6.5) per 100 ml. of eluate. Any residue remaining after 2 days was removed by centrifugation and discarded. The over-all purification was about 165-fold (Table I).

**Table I**

**Summary of Purification**

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Volume</th>
<th>Total units</th>
<th>Total protein nitrogen</th>
<th>Specific activity</th>
<th>Yield per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Homogenate</td>
<td>13,200</td>
<td>3520</td>
<td>35,244</td>
<td>0.10</td>
</tr>
<tr>
<td>I</td>
<td>Supernatant fluid</td>
<td>10,120</td>
<td>2700</td>
<td>6,776</td>
<td>0.40</td>
</tr>
<tr>
<td>II</td>
<td>Sodium lauryl sulfate</td>
<td>9,900</td>
<td>2440</td>
<td>2,970</td>
<td>0.82</td>
</tr>
<tr>
<td>III</td>
<td>Chloroform-octanol</td>
<td>9,900</td>
<td>2510</td>
<td>2,574</td>
<td>0.97</td>
</tr>
<tr>
<td>IV</td>
<td>Alumina eluate 1</td>
<td>1,470</td>
<td>1860</td>
<td>308</td>
<td>6.03</td>
</tr>
<tr>
<td>V</td>
<td>(NH₄)₂SO₄</td>
<td>132</td>
<td>1770</td>
<td>168</td>
<td>16.7</td>
</tr>
</tbody>
</table>

The material from the ammonium sulfate fractionation contained no detectable adenosinetriphosphatase, alkaline phosphatase, acid phosphatase, “acid” pyrophosphatase, or 5’-nucleotidase activity.

**pH Optimum**—The pH optimum was broad with a peak at pH 7.6 to 7.8. It was not shifted by the presence of cysteine or EDTA. The initial homogenate showed an additional peak at pH 5.0. The presence of this activity was reported by Lowry (7). This activity was retained in the precipitate of Step I. The activity of the “acid” pyrophosphatase was about 3 per cent of the “alkaline” pyrophosphatase and was not affected by 0.01 M EDTA or the absence of magnesium ions; this material is being studied further.

**Metal Ion Effects**—The activity shows an absolute requirement for magnesium ion. No activity was observed with any other polyvalent ion tested. In the presence of magnesium ion, all other polyvalent metal ions tested (except barium) decreased the activity to some degree. As may be seen in Table II, the metal ions fell into three groups with respect to the
mode of inhibition. For the first group (Cd, Hg, Ni), the presence of cysteine (or glutathione) was required to prevent or reverse the inhibition.

**Table II**

<table>
<thead>
<tr>
<th>Ion</th>
<th>Inhibition</th>
<th>Reversal with</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.005 M cysteine</td>
<td>0.001 M EDTA</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>100</td>
<td>75</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>100</td>
<td>90</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>100</td>
<td>75</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>100</td>
<td>90</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>75</td>
<td>81</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>70</td>
<td>80</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>24</td>
<td>0</td>
<td>100</td>
<td></td>
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<tr>
<td>Ca²⁺</td>
<td>22</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>50</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

The final concentration of all metal ions was 1 × 10⁻⁴ M. The mixture was preincubated for 5 minutes at 37° with the metal ion, and the reaction was started by the addition of substrate. For demonstration of reversal, either cysteine or EDTA was added 5 minutes after the metal ion, and the mixture was preincubated for 5 more minutes before the addition of substrate. The assay was as described under "Materials and methods," except that cysteine and EDTA were added only as indicated.

**Table III**

<table>
<thead>
<tr>
<th>EDTA</th>
<th>None</th>
<th>Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 × 10⁻⁴ M</td>
<td>5 × 10⁻³ M</td>
</tr>
<tr>
<td></td>
<td>γ P</td>
<td>γ P</td>
</tr>
<tr>
<td>None</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>1 × 10⁻⁴</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>1 × 10⁻³</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>1 × 10⁻²</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>2 × 10⁻²</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>5 × 10⁻³</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

The reaction was started by the addition of 1 ml. of enzyme solution containing 5 γ of protein N. The figures represent micrograms of P released in 7.5 minutes per micrograms of protein N. The assay was as described under "Materials and methods," except that cysteine and EDTA were added as indicated above.

The effect of EDTA was variable. For the second group of metal ions (Co, Fe, Zn) cysteine or glutathione and EDTA were equally effective in reversing the inhibition. For the third group of metal ions (Mn, Ca),
EDTA prevented or reversed the inhibitory effects and sulfhydryl compounds were not effective.

**Sulfhydryl Reagents**—The activating effect of cysteine (and glutathione) is shown in Table III. In these experiments, the enzyme was preincubated for 5 minutes with the sulfhydryl compound. The degree of activation afforded by the two compounds was identical. Thioglycolic acid was also effective as an activator. The activity was inhibited by p-chloromercuribenzoate (Fig. 1). At 90 per cent inhibition by \(1 \times 10^{-4}\) M p-chloromercuribenzoate, the reactivation by 0.01 M cysteine was 75 per cent. \(1 \times 10^{-3}\) M cyanide and \(1 \times 10^{-5}\) M ascorbic acid had no effect upon the activity.

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

**Fig. 1.** The incubation mixture contained \(1 \times 10^{-3}\) M Tris-maleate buffer at pH 6.8, \(5 \times 10^{-3}\) M Mg\(^{+2}\), \(1 \times 10^{-3}\) M EDTA, 100 \(\mu\) of protein nitrogen, and various concentrations of p-chloromercuribenzoate (PCMB) in a final volume of 10 ml.

Fluoride, at \(2 \times 10^{-5}\) M, inhibited 50 per cent and the inhibition was not reversed by cysteine or EDTA.

**Chelating Agents**—EDTA was effective as an activating agent in the presence and absence of sulfhydryl compounds (Table III). The maximal activation, in the absence of sulfhydryl compounds, occurred at an EDTA concentration of \(2 \times 10^{-8}\) M. In the presence of sulfhydryl compounds, the degree of activation was related to the concentration of EDTA and the sulfhydryl compound. Diethylthiocarbamate, Versene-Fe-3-specific, and \(\alpha,\alpha'\)-dipyridyl were not effective as activating agents.

**DISCUSSION**

The initial separation of the adenosinetriphosphatase and the inorganic pyrophosphatase activities of brain, with nearly quantitative recovery of
both, demonstrates the non-identity of the two activities in this tissue. The preparation described here does not hydrolyze other phosphate esters. The activation with magnesium ion is a function of the concentration of substrate and follows, in general, the same relationships of activation as the enzyme from yeast (8) and rat brain (9).

It is apparent that the activity is dependent upon sulphydryl groupings and that the inhibitions by heavy metal ions are due largely to reactions with the essential sulphydryl groupings of the enzyme. Inhibitions with heavy metal ions or with reagents more or less specific for sulphydryl groupings are fully reversible. It is of interest that a variety of sulphydryl compounds are equally effective in the activation of the enzyme and in the reversal of inhibitions. The activation with low concentrations of EDTA may be explained on the basis of binding of trace metals and the retardation of autoxidation. However, the mechanism of activation with higher concentrations of EDTA, previously observed in a rat liver preparation by Swanson (10), is obscure. Experiments with the yeast inorganic pyrophosphatase, prepared by the method of Heppel and Hilmo (11), indicate that it is activated in a similar fashion by EDTA.

It is of interest to compare the pyrophosphatase of brain tissue with that isolated by Kunitz (8) from yeast. Attempts at crystallization of the enzyme of brain tissue have not been successful; the activity of our material is similar to that of Kunitz prior to crystallization. Nevertheless, certain aspects of behavior may be contrasted. Magnesium ion was the only ion activating the hydrolysis by the enzyme from brain tissue, whereas magnesium, cobaltous, and manganous ions were activators for the yeast enzyme. The pH-activity relationships of the two enzymes are similar with the optimum in each case, depending upon the ratio of magnesium ion to substrate. The stability to temperature is similar. There appears to be no comparable data on the role of sulphydryl groupings in activity and no report of the necessity for sulphydryl compounds for optimal activity of the enzyme from yeast.

**SUMMARY**

1. An inorganic pyrophosphatase of swine brain has been purified about 165-fold. The preparation contained no detectable adenosinetriphosphatase or alkaline phosphatase activity.

2. The enzyme had a pH optimum of 7.6 to 7.8, required magnesium ions, and was activated by ethylenediaminetetraacetic acid, cysteine, glutathione, and thioglycolic acid. It was inhibited by a variety of polyvalent metal ions and p-chloromercuribenzoate. These inhibitions were reversible.
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