PARTIAL PURIFICATION AND PROPERTIES OF RENAL GLUTAMINASE

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In 1935, Krebs (1) suggested that there may be several glutaminases. A useful preparation of renal "glutaminase" was described by Archibald (2); Greenstein and Carter (3) demonstrated the existence of two types of glutaminases which were activated by α-keto acids and phosphate, respectively. The mechanism of the α-keto acid-activated glutaminases has been demonstrated by Meister and Tice (4), whereas nothing is known of the enzymatic mechanism or physiological role of the phosphate-activated enzyme in kidney. This paper reports the results of an initial attempt to purify and study the properties of the phosphate-activated glutaminase of kidney.

EXPERIMENTAL

Methods—Ammonia was determined by either direct nesslerization by using Vanselow's reagent (5) or the procedure of Brown et al. (6). Glutamic acid was determined manometrically with an acetone powder of Escherichia coli prepared by the procedure of Najjar and Fisher (7). Glutathione disappearance was determined by the method of Ball, Revel, and Cooper (8), by using the alloxan procedure of Patterson and Lazarow (9). Protein was determined by micro-Kjeldahl analysis or from the ratio of absorbance at 280 and 260 μm according to Warburg and Christian (10).

Purification Procedure—Otey, Birnbaum, and Greenstein (11) reported a soluble preparation of glutaminase from a butanol powder of kidney, and our initial studies were made with this procedure. It should be noted that the preparation so obtained contains considerable quantities of glutamic acid, as demonstrated by paper electrophoresis at pH 8.1 in 0.1 M borate and with glutamic decarboxylase. Moreover, the protein of this preparation was found to bind added glutamic acid in such fashion as to render a fraction thereof unavailable to glutamic decarboxylase. The endogenous

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glutamate could be removed by dialysis for 6 hours against phosphate buffer, but approximately 50 per cent of the enzyme activity was lost during this procedure. The observation of Speck (12) that borate stabilizes glutamine solutions and of Otey et al. (11) that borate also stabilizes renal glutaminase suggested the use of borate in dialyses of the enzyme. Complete removal of the endogenous glutamate could be accomplished by dialysis for 8 hours against 0.05 M borate at pH 8.1, with no loss in activity. In consequence, all subsequent operations were performed in 0.03 M borate unless otherwise specified. Numerous attempts to obtain partial purification of glutaminase from the extract described by Otey et al. (11) by procedures such as heat treatment, isoelectric precipitation, protamine removal of nucleic acid, fractionation with metals, ammonium sulfate, ethanol, or absorption and elution from calcium phosphate, calcium borate, alumina CY gel, Dowex 1 chloride or borate, Dowex 50, Amberlite IRC-4B, IR-400, or XE-64, hydroxyapatite or Celite columns were all of no avail. Similarly, attempts at electrophoresis on potato starch blocks and at electroconvection did not yield useful preparations.

When the distribution of the enzyme in cellular fractions was studied with the aid of conventional differential centrifugation techniques, renal glutaminase was found to be located exclusively in the mitochondria. Resuspension of mitochondria in water or in solutions of sodium lauryl sulfate, digitonin, or deoxycholate released no more than 50 per cent of the enzyme activity from the particles. Sonic vibration of isolated mitochondria did afford almost complete solubility of the enzyme, but this procedure appeared of limited value in obtaining relatively large scale preparations of the enzyme. Archibald (2) had indicated that dog kidney is perhaps the richest source of the enzyme; however, mitochondria from fresh pig kidneys were found to possess at least equal activity and were used thereafter as a starting material. Approximately 300-fold purification of the enzyme from fresh pig kidney was effected by the following procedure.

Fresh pig kidney is homogenized in 5 volumes of 8.5 per cent sucrose containing 0.02 M borate at pH 8.1. The homogenate is centrifuged at 2500 × g in the conical head of a refrigerated centrifuge (International Equipment Company, model PR-1) for 15 minutes, and the sediment is discarded. The supernatant solution is strained through two layers of cheesecloth and then centrifuged in a refrigerated Sharples centrifuge at maximal speed. The precipitate so obtained is lyophilized. 31 gm. are homogenized for 2 minutes in 1100 ml. of butanol at 0°. The butanol should be freshly distilled, or Mathieson "white label reagent" may be employed. The homogenate is poured onto a Büchner funnel and sucked almost to dryness and then rehomogenized in 500 ml. of acetone at 0°
for 30 seconds and again filtered on a Büchner funnel. Just before dryness is attained, 100 ml. of diethyl ether are added, on the funnel, and the preparation is sucked dry. The powder is stored in a vacuum desiccator. The powder so prepared contains all the enzyme activity of the original kidney and is stable for at least 6 months in the cold.

A soluble extract is prepared by stirring 5 gm. of powder in 120 ml. of 0.01 M borate, pH 8.1, in the cold for 1 hour and then centrifuging at 29,000 \( \times g \) in a refrigerated centrifuge (Lourdes Instrument Corporation, model AB). Attempts to purify the enzyme from the supernatant fluid by precipitation with metals, ammonium sulfate, ethanol, or absorption and elution from calcium phosphate, calcium borate, or alumina \( \gamma \) gel were unsuccessful, nor did protamine precipitation, isoelectric precipitation, or heat treatment prove of value at this point, whereas precipitation by sodium phosphate effected useful purification. To the supernatant fluid are added, with stirring over a 15 minute period, 50 ml. of cold 4 M phosphate, pH 8, and the suspension is stirred for an additional 15 minutes. The precipitate obtained by centrifugation for 15 minutes at 29,000 \( \times g \) is dissolved in 20 ml. of 0.01 M borate, pH 8.1, and the enzyme is reprecipitated with 10 ml. of 4 M phosphate and isolated by centrifugation. This precipitate is dissolved in 10 ml. of 0.01 M borate. Again attempts at further purification by absorption and elution from Celite, alumina \( \gamma \), calcium phosphate, hydroxyapatite, Amberlite IR-4B, IR-400, or XE-64, Dowex 1 chloride or borate, or triethylamine cellulose met with indifferent success; frequently the enzyme could not be obtained again from the column.

However, chromatography on Amberlite XE-97 proved a useful procedure. The column is prepared by washing the hydrogen form of the resin free of chloride and then stirring each pound of resin with 3 liters of 0.1 M borate, pH 8.1, for 30 minutes, and allowing to settle before addition of a second portion of borate buffer. This suspension is then poured into a 1 cm. column to a height of 30 cm. The column is washed for 2 days with 0.1 M borate, pH 8.5, until the pH of the effluent is at least 7.8. The column is then transferred to the cold room. 200 mg. samples of protein are placed on the column and then eluted with 0.15 M glycine in 0.05 M borate, pH 9, at a flow rate between 3 and 4 ml. per 25 minutes. The effluent is collected in 3 ml. samples. The amber-colored material in the protein solution appears in the first three or four tubes and the major portion of the activity in the next nine tubes. Generally, however, the total contents of Tubes 5 to 20 were collected and concentrated by dialysis against saturated polyvinyl pyrolidone in 0.05 M borate, pH 8.1. As shown in Table I, repetition of the chromatography on XE-97 resulted in doubling the specific activity of the final protein.
Properties of Enzyme—The ratio of absorption at 280 to 260 m\(\mu\) of the final preparation rarely exceeded 1, presumably indicating the presence of nucleotides which have not yet been identified. Incubation of Fraction D with ribonuclease at 30° for 30 minutes was without effect either on the absorption spectrum or on the enzymatic activity of the reisolated protein.

The possible presence of bound nucleotide in the preparation directed attention to the description by Binkley and Olson (13) of a preparation from kidney, containing an unidentified nucleotide, which exhibited glutathionase activity only in the presence of glutamine. Accordingly, the glutathionase activity of Fractions B and E were assayed by incubating equivalent glutaminase activities in Thunberg tubes containing glutathione, with and without glutamine, in 0.02 m phosphate at pH 8.2. The mixtures were degassed, placed in a nitrogen atmosphere, and incubated at 37°. Aliquots were then assayed for ammonia, glutamic acid, and glutathione.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Stage</th>
<th>Absorbance at 280 m(\mu)</th>
<th>Absorbance at 260 m(\mu)</th>
<th>Specific activity</th>
<th>Total units recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Initial extract of mitochondrial powder</td>
<td>0.82</td>
<td>8.85</td>
<td>12,400</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1st phosphate ppt.</td>
<td>1.13</td>
<td>41.9</td>
<td>12,200</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2nd “</td>
<td>1.22</td>
<td>54</td>
<td>11,900</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1st XE 07 eluate</td>
<td>1.08</td>
<td>112</td>
<td>10,540</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2nd “</td>
<td>1.03</td>
<td>223</td>
<td>10,440</td>
<td></td>
</tr>
</tbody>
</table>

As will be seen in Fig. 1, Fraction B exhibited glutathionase activity which was enhanced 5-fold by the presence of glutamine, whereas the more highly purified glutaminase exhibited no glutathionase activity even in the presence of glutamine. With both preparations, glutathione markedly inhibited glutaminase activity. It will be noted that the release of glutamate, measured as CO\(_2\) production by glutamic decarboxylase, coincides in each case with the NH\(_3\) formed rather than with glutathione which has disappeared. This suggests formation of a polyglutamate rather than hydrolysis of the glutathione. The mechanism of this glutamine-dependent “glutathionase” activity of the cruder preparation is currently under investigation.

As shown in Fig. 2, pig kidney glutaminase shows a pH optimum at 8.0; activity falls off sharply below pH 7 and above pH 8.5. The enzyme is rapidly inactivated by p-chloromercuribenzoate at 10\(^{-5}\) m and is not reactivated by glutathione or cysteine.

It had been reported (3) that anions other than phosphate can effect stimulation of glutaminase activity, and it appeared desirable to confirm...
Fig. 1. Glutathionase activity of glutaminase preparations. All incubations were conducted in a total of 5 ml. of 0.2 M sodium phosphate, pH 8.2, in a nitrogen atmosphere at 37°. Appropriate vessels contained 67.5 μmoles of glutamine or 32.5 μmoles of glutathione. A, flasks contained glutamine; B, flasks contained glutathione; C, flasks contained both glutamine and glutathione. The solid lines represent activity of 0.45 mg. of Fraction E; broken lines represent 4.5 mg. of Fraction B. Disappearance of glutathione is shown as (△), NH₃ production as (○), and appearance of glutamate as (□).

Fig. 2. Influence of pH on glutaminase activity. Each tube contained 55 μ of Fraction D and 12.5 μmoles of glutamine in 1.0 ml. of 0.4 M phosphate and was incubated at 37° for 30 minutes. All values are corrected for non-enzymatic hydrolysis of glutamine.
these observations with purified material. As will be seen from Table II “activation” was effected by all the doubly charged anions tested, whereas singly charged anions were without effect. It is of some interest that pyrophosphate was slightly more active than orthophosphate. It has been reported that the livers and kidneys of rats maintained on a vitamin B_6-deficient diet exhibit a lowered glutaminase activity (14). Preincubation of enzyme Fraction D with pyridoxal phosphate, in the presence and absence of phosphate, was without effect on enzymatic activity; however, no attempt was made to determine the pyridoxal or pyridoxamine content of the preparation as obtained from the column.

**TABLE II**

**Influence of Various Anions on Activity of Pig Kidney Glutaminase**

<table>
<thead>
<tr>
<th>Anion</th>
<th>Concentration</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>0.5 M</td>
<td>100 per cent</td>
</tr>
<tr>
<td>Sulfate</td>
<td>0.5 M</td>
<td>106 per cent</td>
</tr>
<tr>
<td>Arsenate</td>
<td>0.5 M</td>
<td>74 per cent</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>0.1 M</td>
<td>98 per cent</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)aminomethane</td>
<td>0.5 M</td>
<td>0 per cent</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.5 M</td>
<td>0 per cent</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.5 M</td>
<td>0 per cent</td>
</tr>
<tr>
<td>Cyanide</td>
<td>0.5 M</td>
<td>0 per cent</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.5 M</td>
<td>0 per cent</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.25 M</td>
<td>0 per cent</td>
</tr>
</tbody>
</table>

The incubation mixtures consisted of 2 γ of enzyme (Fraction D) and 12 μmoles of glutamine, in a total volume of 1.0 ml. of 0.03 M sodium borate, pH 8.1, at 37° for 30 minutes.

Perhaps the most unusual feature of this enzyme is the phosphate activation. As shown in Fig. 3, maximal glutaminase activity is attained at approximately 0.4 M phosphate. _K_m_ for phosphate appears to be of the order of 0.05 M. Most investigators of this enzyme have been impressed with its lability, and, in the course of the present studies, it was observed that in solutions free of doubly charged anions brief incubation of the enzyme at 37° resulted in complete inactivation. This was accomplished by dialysis of the enzyme against Dowex 1 in 0.01 M borate at 2°, followed by rapid dilution of 0.01 ml. of enzyme solution with 0.99 ml. of water at 37°. In contrast, the presence of 0.03 M borate or 0.4 M phosphate afforded complete protection against loss of activity in control incubations; whereas incubation of the enzyme with substrate, in the absence of divalent anions, resulted in complete inactivation within 5
minutes. To elucidate the role of phosphate in this system, samples of enzyme were preincubated for 5 minutes with phosphate varying in concentration from 0.01 to 0.4 M. Glutamine and sufficient phosphate to make a final concentration of 0.4 M were then added, and incubation was continued for 30 minutes. The data so obtained were identical with those shown in Fig. 3; the total enzyme activity remaining, after enzyme was preincubated at any given phosphate concentration for 5 minutes, was identical with that observed in the previous experiment in which, without preincubation, glutaminase activity was determined at each phosphate concentration of the present preincubations.

These data suggest that glutaminase must be rapidly inactivated when incubated at 37°C in the absence of appropriate anions and that the apparent phosphate "activation" is largely protection against such inactivation. As a partial check on this hypothesis, it appeared desirable to determine the velocity of the inactivation of enzyme when incubated in the absence of anions. Fig. 4 shows the result of incubating enzyme for varying time intervals at 37°C; residual enzymatic activity was assayed by the rapid addition of glutamine and sufficient orthophosphate to yield a 0.4 M solution. The enzyme preparation was Fraction D, freed of phosphate by dialysis against Dowex 1 at 2°C. It will be seen that 50 per cent of the activity disappeared within 20 seconds and that after 2 minutes the enzyme preparation was essentially inert.

In contrast to the effect of phosphate, borate appears to protect the
Fig. 4. Velocity of glutaminase inactivation in the absence of appropriate anions. Preincubation was performed by adding 0.01 ml. of enzyme solution containing 50 \(\gamma\) of Fraction D in 0.01 M borate, pH 8.1, at 0° to 1.00 ml. of water at 37°. At the time indicated, 1.0 ml. of 0.8 M phosphate, pH 8.1, containing 12.5 \(\mu\)moles of glutamine, was added, and incubation was continued for 30 minutes at 37°. All values are corrected for non-enzymatic hydrolysis of glutamine.

Fig. 5. Inhibition of glutaminase by borate at various phosphate concentrations. Each tube contained 12.5 \(\mu\)moles of glutamine and 55 \(\gamma\) of Fraction D in a total volume of 1.0 ml. and was incubated at 37° for 30 minutes. The phosphate concentrations were 0.1 M (○), 0.05 M (▲), and 0.02 M (■). All values are corrected for non-enzymatic hydrolysis of glutamine.
enzyme while preventing enzymatic activity. Thus, when enzyme is preincubated in a small volume of 0.03 M borate for 5 minutes and then added to glutamine and sufficient phosphate to yield a final concentration of 0.4 M, almost maximal activity is observed. On the assumption that, in protecting the enzyme, borate must interact with it at the same locus as that involved in the phosphate effect, it appeared likely that borate and phosphate might compete for this locus. As shown in Fig. 5, this is indeed the case. A final concentration of 0.05 M borate is sufficient to result in 50 per cent inhibition of glutaminase activity when phosphate is present at its $K_m$ concentration; the exact mechanism of the borate and phosphate effects remains obscure and will be pursued with more highly purified enzyme preparations.

**Effects of Glutamate and Ammonia**—To ascertain the effects of ammonia and glutamate on the course of the enzymatic reaction, these were incubated at varying concentrations with enzyme (E) and substrate in phosphate buffer. The data are shown in Fig. 6. $K_m$ for glutamine is approximately $5 \times 10^{-3}$ M. It will be seen that in this system ammonia acts as a competitive inhibitor, while glutamic acid is a more effective but, apparently, non-competitive inhibitor. These findings are compatible
with formulation of the glutaminase reactions as

\[
\text{Glutamine} + E \rightleftharpoons E\text{-glutamate} + \text{NH}_3 \downarrow \text{H}_2\text{O} \\
E + \text{glutamate}
\]

According to this formulation, ammonia in the medium would be expected to exchange with glutamine whereas glutamate would not, analogous to previous findings with horse liver esterase (15). Accordingly, experiments were conducted in which 300 mg. of enzyme Preparation D were incubated with 120 μmoles of glutamine, 120 μmoles of NH₃, and 120 μmoles of glutamate in 0.4 M phosphate, pH 8.1, at 37° for 30 minutes. In one series the incubation included glutamic acid-1-C¹⁴, 1.8 × 10⁵ c.p.m., and in a second series the ammonia present contained 34 atom per cent excess of N¹⁵. After the incubation period, glutamine was separated from glutamic acid by chromatography on XE-64 columns as described by Meister (16). To the glutamine so obtained were added 240 μmoles of unlabeled glutamic acid, and the chromatography was repeated. The appearance of glutamine from the column was monitored with the use of ninhydrin (17). These assays were checked by hydrolysis for 11 minutes in 1 N H₂SO₄, distillation, and determination of the ammonia by nesslerization. Radioactivity of an aliquot of the unhydrolyzed glutamine was determined in a gas flow counter.

When NH₃-N¹⁵ was employed, the reaction was stopped by decreasing the pH to 4.5 with concentrated sulfuric acid. After aliquots were taken for ammonia determination, 1 ml. of 10 M NH₃ and 3 ml. of saturated sodium borate, pH 10.6, were added, and the ammonia was distilled in 3 ml. of 5 N HCl for 15 minutes. A second 1 ml. portion of 10 M NH₃ was added, and distillation was repeated. In control tubes, this procedure was demonstrated to be adequate to remove completely the NH₃-N¹⁵ remaining from the enzymatic incubation. The solution was then made 1 N with respect to H₂SO₄ and placed in a boiling water bath for 11 minutes to hydrolyze the glutamine. Thereafter the hydrolysate was neutralized, 1.5 ml. of 10_p M NaOH were added, and distillation was performed into 3 ml. of 0.1 M HCl. The ammonium chloride so obtained was converted to N₂ with sodium hypobromite, and isotopic analysis was conducted in model 21-201 mass spectrometer of the Consolidated Instrument Company.

Under these conditions no significant incorporation of glutamate-C¹⁴ into the residual glutamine was observed. In contrast, as shown in Table III, significant reincorporation of ammonia into glutamine occurred; the extent of this reincorporation was strikingly dependent upon pH. At pH 7.1, 8.1, and 9.1, respectively, 0.135, 0.81, and 2.61 per cent of
theoretical complete equilibrium were obtained. Since the hydrolytic reaction was considerably more rapid at pH 8.1 than at pH 9.1, the effect of pH on the reincorporation of ammonia is considered to reflect the increasing concentration of NH₃ at the expense of NH₄⁺ at increased pH. It is of interest that, in the absence of added phosphate, the reincorporation of ammonia, like the hydrolytic reaction, was markedly impaired. Unfortunately, enzyme Fraction D contains a considerable quantity of phosphate which, in these experiments, was not removed by dialysis. Pre-

**Table III**

**Incorporation of N¹⁴H₃ into Glutamine by Glutaminase**

The incubation mixtures contained 120 μmoles each of glutamine, glutamate, NH₃ containing 34 atom per cent excess N¹⁴, 300 γ of Fraction D in 5.0 ml. of 0.02 M borate. The appropriate vessels contained 0.4 M phosphate. P₁ = inorganic phosphorus.

<table>
<thead>
<tr>
<th>Time</th>
<th>Conditions</th>
<th>pH 7.1</th>
<th></th>
<th>pH 8.1</th>
<th></th>
<th>pH 9.1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hydrol-ysis</td>
<td>Incorporation</td>
<td>Hydrol-ysis</td>
<td>Incorporation</td>
<td>Hydrol-ysis</td>
<td>Incorporation</td>
</tr>
<tr>
<td>min.</td>
<td>No enzyme or P₁</td>
<td>1</td>
<td>0.06</td>
<td>2</td>
<td>0.08</td>
<td>2</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>3</td>
<td>0.05</td>
<td>4</td>
<td>0.08</td>
<td>3</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>&quot; P₁</td>
<td>4</td>
<td>0.07</td>
<td>8</td>
<td>0.08</td>
<td>15</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>15</td>
<td>0.09</td>
<td>31</td>
<td>0.48</td>
<td>34</td>
<td>0.81</td>
</tr>
<tr>
<td>30</td>
<td>No enzyme or P₁</td>
<td>2</td>
<td>0.06</td>
<td>2</td>
<td>0.06</td>
<td>2</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>5</td>
<td>0.06</td>
<td>5</td>
<td>0.06</td>
<td>4</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>&quot; P₁</td>
<td>7</td>
<td>0.06</td>
<td>9</td>
<td>0.06</td>
<td>32</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>31</td>
<td>0.14</td>
<td>44</td>
<td>0.81</td>
<td>51</td>
<td>2.37</td>
</tr>
</tbody>
</table>

* Expressed as per cent hydrolysis = (NH₃ (formed))/(glutamine (initial)) X 100.
† Expressed as per cent equilibrium = (N¹⁴ atom per cent excess in final glutamine)/17 X 100.

sumably the effect of phosphate noted here would have been much more pronounced had phosphate-free enzyme been employed. These data are all in keeping with the formulation of the hydrolytic reaction shown earlier and indicate that the renal glutaminase is potentially a glutamyl-transferring enzyme.

Azaserine has been observed to inhibit a number of enzymes which catalyze transfer from the amide nitrogen of glutamine (18). When incubated with glutaminase in 0.4 M phosphate, azaserine effected approximately 50 per cent inhibition of hydrolysis when present in equimolar concentration with the substrate. In contrast, however, in each of a number of runs, azaserine effected a relatively small inhibition of the reincorporation of ammonia into glutamine. The significance of this
observation remains to be determined. The extent of this incorporation of ammonia is of the order expected from the degree of inhibition of hydrolysis of the hydrolytic reaction by ammonia at this concentration.

SUMMARY

A procedure is described for partial purification of a phosphate-activated glutaminase from pig renal mitochondria. The most highly purified preparation does not exhibit the glutamine-dependent glutathionase activity of cruder preparations. Phosphate is found to "activate" the enzyme by protecting against the rapid inactivation of the enzyme which occurs when incubated in the absence of anions. Borate similarly protects the enzyme, but also inhibits the hydrolytic reaction. Ammonia, but not glutamate, was found to be reincorporated into glutamine when incubated with enzyme, glutamine, and phosphate. Azaserine, ammonia, glutamate, and p-chloromercuribenzoate inhibited the hydrolytic reaction.

BIBLIOGRAPHY

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