RIBONUCLEASE

VII. PARTIAL PURIFICATION AND CHARACTERIZATION OF A RIBONUCLEASE INHIBITOR IN RAT LIVER SUPERNATANT FRACTION*

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In a previous report (1) some of the properties of an RNase inhibitor occurring in the supernatant fraction prepared from rat liver were investigated, and the distribution of the inhibitor in various tissues of the rat was determined. In the present work, the inhibitor has been separated from the i-RNase which also occurs in the supernatant fraction. Some of the properties of the i-RNase are considered in the following paper. A new assay system for RNase inhibitor and i-RNase has been devised which allows a more certain assessment of the amounts of these substances present in various preparations. RNase inhibitor has been partially purified, and the action of various substances on the inhibitor, as well as some further properties of it, is also examined in this report.

Materials and Methods

Preparation of Supernatant Fraction Containing RNase Inhibitor—Male Wistar strain rats weighing between 175 and 300 gm., obtained from the Wistar Institute, were used to supply the liver tissue. These rats were etherized and killed by heart puncture exsanguination, and the livers were removed, rinsed in ice-cold glass-distilled water, and weighed. They were then homogenized in 9 volumes of ice-cold glass-distilled water with

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1 The abbreviations used are RNase, ribonuclease; i-RNase, inactive ribonuclease (this term i-RNase is used for the inactive enzyme with the understanding that it may actually be a complex between alkaline RNase and RNase inhibitor); RNA, ribonucleic acid; CMB, p-chloromercuribenzoic acid; ABC buffer, acetate, borate, cacodylate buffer; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; Tris, tris(hydroxymethyl)aminomethane.

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use of a TenBroeck all-glass homogenizer which had a special close clearance between the plunger and pestle.\textsuperscript{2} It has been observed that the inhibitor titers were often quite low when a loose fitting plunger was used. This may have been due to incomplete breakage of cells. Each homogenizer was used only about twenty times. The homogenate was centrifuged in a Spinco model L preparative ultracentrifuge at 60,000 $\times$ $g$ for 65 minutes. The supernatant fraction was removed and again centrifuged at 60,000 $\times$ $g$ for an additional 35 minutes. The supernatant fluid from the second centrifugation is the supernatant fraction referred to in the following experiments.

Assay for RNase Inhibitor and i-RNase—It was recognized that the assay system previously described (1) had several limitations, principally that

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Inhibition of increasing quantities of crystalline pancreatic RNase by RNase inhibitor. For an explanation, see the text.}
\end{figure}

the range of concentrations of inhibitor that could be assessed was narrow, and, that, if an enzyme-inhibitor complex existed, erroneous conclusions as to inhibitor concentration might be reached.

The first efforts to devise a better assay system were based on attempts to titrate a standard amount of RNase inhibitor with crystalline pancreatic RNase. When this was carried out, it was observed that the relationship between the amount of RNase added and the per cent inhibition was non-linear. It was discovered, however, that, when the results were plotted on a semilogarithmic basis, the per cent inhibition on a logarithmic scale, and the quantity of crystalline RNase on a linear scale, a straight line was obtained as, for example, Curve A in Fig. 1. Furthermore, when different amounts of inhibitor were used, a family of straight lines, all of identical slope, was obtained. Another such is Curve B in Fig. 1. When the intercepts of the straight lines with an extension of the 50 per cent

\textsuperscript{2} Obtainable from the Kontes Glass Company or Ace Glass, Inc., Vineland, New Jersey.
inhibition line were dropped to intercept the abscissa, the intercepts on this scale were directly proportional to the amount of inhibitor in the solution. Thus, Curve A, showing 25 per cent inhibition of 0.1 \( \gamma \) of crystalline pancreatic RNase, contained 0.07 unit of inhibitor, while Curve B with 80 per cent inhibition of 0.1 \( \gamma \) of pancreatic RNase contained 0.12 unit of inhibitor. Numerous experiments with aliquots of the same inhibitor solution showed very good proportionality for considerable ranges of inhibitor concentration and for inhibitions ranging from approximately 10 to 95 per cent when 0.1 \( \gamma \) of crystalline pancreatic RNase was used. Since the slopes of the curves obtained with different inhibitor concentrations were constant, it was apparent that only one point was necessary to determine the intercept with the 50 per cent inhibition line, and hence the RNase inhibitor concentration. To determine i-RNase, an aliquot of the solution is treated with \( 4 \times 10^{-4} \) M CMB and the RNase activity determined compared to a control without CMB. This concentration of CMB gives the maximal activation of i-RNase.

**Directions for Assay of RNase Inhibitor and i-RNase**

**RNase Inhibitor**—Duplicate tubes containing 1 ml. of 0.1 M Veronal-acetate buffer, pH 7.8, 0.45 ml. of water, and 0.5 ml. of a solution containing 0.1 \( \gamma \) of crystalline pancreatic RNase in 0.1 per cent gelatin solution are set up (all RNase dilutions are in 0.1 per cent gelatin solution). To this is added, with thorough mixing, 0.05 ml. of RNase inhibitor preparation, followed by 1 ml. of 1 per cent RNA solution at zero time. RNA uniformly labeled with \( ^{32} \)P is generally used (2). The mixture is incubated at 37° in a water bath for 30 minutes, after which 3 ml. of 1 N HCl in 76 per cent ethanol are added and the tube is well mixed and allowed to stand for 8 minutes. The mixture is filtered with the use of 9 cm. Whatman No. 42 filter paper, the funnels being covered with watch glasses. A blank containing all of the components but the RNase inhibitor is run simultaneously as well as a blank containing only buffer, water, and RNA. The radioactivities of the filtrates are determined. If unlabeled RNA is utilized, the absorption at 260 m\( \mu \) of a 1:50 dilution of the filtrate may be measured; use of absorption measurements necessitates running tissue blanks in which all components but RNA are present. The per cent inhibition of the RNase is calculated. The inhibitor concentration may be read directly from a graph such as that in Fig. 1, or from a table, which may be constructed from the graph, relating per cent inhibition to RNase inhibitor concentration. Corrections for any RNase activity of the RNase inhibitor preparation may be made from the blank used in the i-RNase assay described below. Generally, there is no activity present in such preparations. It should be noted that the inhibitor units are actually
the micrograms of crystalline pancreatic RNase that are 50 per cent inhibited by the particular preparation being tested and, thus, they have real rather than arbitrary dimensions. For assay of unpurified rat liver supernatant fractions, an aliquot of 0.1 ml. instead of 0.05 ml. is usually employed; this is true also when i-RNase in unpurified supernatant fraction is assayed. The order of addition of the components is critical. RNA must always be added last after RNase inhibitor and crystalline pancreatic RNase have been mixed.

**i-RNase**—This assay is run simultaneously with the one for RNase inhibitor except where it is known that there is no i-RNase present. Duplicate tubes containing 1 ml. of 0.1 M Veronal-acetate buffer, pH 7.8, 0.83 ml. of water, and 0.12 ml. of 0.01 M CMB neutralized to pH 8.2 are set up, and 0.05 ml. of the same preparation used in the inhibitor assay is added. Finally, 1 ml. of 1 per cent P32-labeled RNA is added at zero time. Blanks consist of duplicate tubes with water substituted for the CMB. The tubes are incubated in a water bath at 37°C for 30 minutes, and then the unhydrolyzed RNA is precipitated and removed by filtration as above. The i-RNase activity is the difference in activity between the CMB-treated and the untreated preparation. It may be expressed in several ways, either as counts per minute in the filtrate, where relative activities suffice, or as the activity compared to that of 0.015 μg of crystalline pancreatic RNase which is assayed at the same time. Nitrogen contents of the various samples tested were determined by the micro-Kjeldahl method or by calculation after determination of protein by a modified biuret reaction. The latter method was used whenever ammonium sulfate was likely to be present. Specific activities are expressed as activity per mg. of nitrogen.

The distribution of RNase inhibitor in various tissues of the rat previously reported (1), as well as the activities of RNase inhibitor in x-irradiated (3) and carcinogen-fed rats (4), should be reexamined with the use of the new method of assay.

**Purification of RNase Inhibitor**—60 ml. of rat liver supernatant fraction prepared as described above, are treated with solid ammonium sulfate (12.54 gm.) to 35 per cent saturation. All operations are conducted at 0°C. The precipitate is removed by centrifugation at 60,000 × g for 20 minutes and discarded. Ammonium sulfate is added to the supernatant fluid to 55 per cent saturation (8.52 gm.), and the mixture is stirred and centrifuged at 60,000 × g for 20 minutes. The tan-colored precipitate contains almost all of the activity of RNase inhibitor and i-RNase of the original supernatant fraction. Salt fractionation under these conditions does not separate RNase inhibitor from i-RNase. The centrifuge tubes containing the 55 per cent precipitate are inverted to drain thoroughly,
and the precipitate is dissolved in 20 ml. of ice-cold glass-distilled water. It dissolves completely, giving a golden yellow solution. To this are added 3.6 gm. of calcium phosphate gel, and the mixture is thoroughly stirred and allowed to stand in ice for 15 minutes, after which the gel is centrifuged at 15,000 × g for 15 minutes. The supernatant fluid from the gel contains 90 to 100 per cent of the i-RNase and little or no RNase inhibitor. The RNase inhibitor, which is absorbed on the gel, is eluted by the use of 9 to 10 ml. of ice-cold 0.2 M phosphate buffer, pH 7.3. The eluted gel is removed by centrifugation; the eluate containing RNase inhibitor is also colored yellow.

The results of a typical purification experiment are shown in Table I. In a series of twenty purification experiments the specific activities of the

| Table I
<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>Purification of Ribonuclease Inhibitor</strong></td>
</tr>
<tr>
<td><strong>Fraction</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Original supernatant fraction</td>
</tr>
<tr>
<td>55% ppt.</td>
</tr>
<tr>
<td>Supernatant from calcium phosphate gel</td>
</tr>
<tr>
<td>Eluate of calcium phosphate gel</td>
</tr>
<tr>
<td>55% ppt. of eluate</td>
</tr>
</tbody>
</table>

* Calculated from protein values determined by the biuret method and assuming 16 per cent nitrogen in protein.

The eluate ranged from 20 to 50 units per mg. of N; the purification factor ranging from 5 to 10 times that of the original supernatant fraction. The data in Table I show that the separation of RNase inhibitor from i-RNase, under the conditions described, is fairly complete.

It is essential to maintain the protein concentration at a fairly high level during the purification of RNase inhibitor. Dilution with water or 0.25 M sucrose solution causes considerable loss of inhibitor activity. It may be for this reason that the inhibitor has been overlooked by other investigators, for if, in the preparation of cell fractions, the washings from the various fractions are combined to form a large volume of supernatant fraction, the dilution may become great enough to obscure completely the action of the inhibitor. The data in Table I indicate a 44 per cent recovery of RNase inhibitor in the eluate from the calcium phosphate gel. A further precipitation by 55 per cent saturation with ammonium sulfate, as illustrated in the last line of Table I, not only fails to increase the spe-

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3 Gaston de Lamirande, personal communication.
specific activity of the preparation but results in considerable further loss of inhibitor. It was observed that the elution process, as presently carried out, removes all of the protein absorbed by the gel. It is possible that solvents may be found that will selectively remove RNase inhibitor.

Results

Stability of RNase Inhibitor during Preservation—Storage of the supernatant fraction of rat liver at $-15^\circ$ in the frozen state retained inhibitor activity unchanged for at least a month. Under the same conditions the 55 per cent precipitate lost 25 to 30 per cent of its initial activity on freezing but then remained unchanged thereafter. Storage at $0^\circ$ led to a rapid decline in activity as illustrated in Fig. 2. Occasionally there was little change in the first 24 hours. Lyophilization under optimal conditions permitted retention of 60 to 75 per cent of the initial activity of RNase inhibitor in solutions of the 55 per cent precipitate. A considerable quantity of protein was rendered insoluble by this treatment. At present frozen supernatant fraction is employed for studies on RNase inhibitor. i-RNase is not appreciably affected by any of the above treatments.

Inactivation of RNase Inhibitor by Polysaccharide from Yeast and by RNA—It had been noted in some early work (1) that very low inhibitor activities were observed in certain experiments. Normally 0.1 ml. of supernatant fraction from rat liver gives 70 to 90 per cent inhibition of 0.015 $\gamma$ of crystalline pancreatic RNase, but occasionally the range of inhibition, under the same conditions, was 20 to 30 per cent, or perhaps 40 to 60 per cent. Extensive investigation showed that these inconsistencies...
were not related either to the physiological state of the animal, the method of preparation of the supernatant fraction, or the reagents used. At this time, the RNA preparations employed as substrate for the assays contained a quantity of insoluble material which varied in amount from preparation to preparation. This insoluble residue could be easily removed by centrifugation of the RNA solution at $60,000 \times g$ for 25 to 30 minutes, during which it formed a gelatinous precipitate. This precipitate could be purified by washing and recentrifugation to give a white, fluffy amorphous solid. It contained only traces of nitrogen and phosphorus, no $P_{32}$ activity, and, upon hydrolysis with dilute sulfuric acid, gave an intense color in the anthrone reaction for a reducing sugar. It seems possible that this ma-

**Table II**

*Effect of Order of Addition of Reagents and Presence of Yeast Polysaccharide on Activity of Ribonuclease Inhibitor*

<table>
<thead>
<tr>
<th>Conditions* and order of addition</th>
<th>Inhibitor unit</th>
<th>Change from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA + polysaccharide, inhibitor, 0.1 $\gamma$ RNase</td>
<td>0.002</td>
<td>-97.5</td>
</tr>
<tr>
<td>Inhibitor, 0.1 $\gamma$ RNase, RNA + polysaccharide</td>
<td>0.014</td>
<td>-82.5</td>
</tr>
<tr>
<td>&quot; RNA, 0.1 $\gamma$ RNase</td>
<td>0.007</td>
<td>-91.2</td>
</tr>
<tr>
<td>&quot; 0.1 $\gamma$ RNase, RNA (control)</td>
<td>0.080</td>
<td></td>
</tr>
</tbody>
</table>

* The RNase inhibitor used was 0.05 ml. of a solution of 55 per cent precipitate preparation. The assay procedure was the same as that described under "Materials and methods." RNA, which had been incubated with polysaccharide for over 1 week, was used in Experiments 1 and 2, while the identical preparation of RNA without the polysaccharide was used in Experiments 3 and 4. Samples were incubated for 10 minutes at room temperature before the final incubation in the water bath.

After numerous experiments, it was demonstrated that the action of RNase inhibitor could be prevented by (a) the combination of RNA with inhibitor, and (b) a slow reaction between RNA and the yeast polysaccharide. The results of a typical experiment are given in Table II in which the experimental details are also described. It may be seen that the presence of the polysaccharide, when suitably incubated with the RNA, gave 82.5 per cent inactivation of the inhibitor, whereas, when RNase inhibitor is incubated first with RNA (reverse of the normal order of addition), the inactivation of the inhibitor was 91.2 per cent. When both factors operated together, the effect was somewhat additive, resulting in 97.5 per cent inactivation of the inhibitor.
The mechanism of these actions is obscure at present. The finding that the order of addition is of such paramount importance suggests that RNA combines readily with the inhibitor, perhaps through groups that do not interfere with the subsequent degradation of the RNA by crystalline pancreatic RNase but which effectively prevent the combination of inhibitor with RNase. In this connection it would be of interest to study the interaction of modified RNA preparations or of DNA with RNase inhibitor. Although preliminary experiments have indicated that RNase inhibitor has no action on DNase, DNA, in a manner similar to RNA, may inhibit the action of RNase inhibitor. It is also possible that rat liver RNA would not react with RNase inhibitor; this is being tested. Further study of these reactions is continuing in the hope that it may lead to an understanding of the mechanisms of action, not only of RNase inhibitor but also of RNase on RNA.

Table III

<table>
<thead>
<tr>
<th>Conditions*</th>
<th>Inhibitor unit</th>
<th>Inactivation per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated RNase inhibitor (control)</td>
<td>0.081</td>
<td>-</td>
</tr>
<tr>
<td>Inhibitor + 4 \times 10^{-4} M CMB</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>&quot; + 4 \times 10^{-4} &quot; + H_2S</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>&quot; + 4 \times 10^{-4} &quot; Pb++</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>&quot; + 4 \times 10^{-4} &quot; + H_2S</td>
<td>0.078</td>
<td>4</td>
</tr>
</tbody>
</table>

* Aliquots of RNase inhibitor in the form of a solution of the 5.5 per cent precipitate were incubated with the 4 \times 10^{-4} M CMB or Pb++ for 10 minutes at 0°. Hydrogen sulfide gas was then passed through the solution for 10 minutes, followed by purified nitrogen gas, until all the hydrogen sulfide was removed. The control was treated similarly except for the addition of the sulfhydryl reactants. Hydrogen sulfide or the sulfhydryl reactants had no effect on the activity of crystalline pancreatic RNase used in the assay for RNase inhibitor.

Effect of Sulfhydryl Reactants on RNase Inhibitor—Since it has been demonstrated that certain sulfhydryl reactants release RNase from combination with RNase inhibitor (1), the action of these reagents on the inhibitor is of considerable interest.

The results of a typical experiment in which the action of lead acetate and CMB on RNase inhibitor was observed are given in Table III, and the experimental details are also described. Consideration of the data indicates that the inactivation of RNase inhibitor by CMB is not reversed when the CMB is removed by saturation with hydrogen sulfide gas. Addition of cysteine also has no effect. However, the inactivation caused by Pb++
may be nearly completely reversed by subsequent removal of the Pb$^{++}$ by hydrogen sulfide. In other experiments the reversal was not as complete as in that illustrated in Table III. The presence of i-RNase in the 55 per cent precipitate used in these experiments complicates the situation somewhat, and it would probably be advantageous to use the calcium phosphate gel eluate, which contains only RNase inhibitor, in these studies. The reversible inactivation of RNase inhibitor by sulfhydryl reactants suggests that free —SH groups are essential for its activity. This does

**Table IV**

*Effect of Various Buffers and Ionic Strength on Ribonuclease Inhibitor*

The values are the average of two separate experiments.

<table>
<thead>
<tr>
<th>Buffer*</th>
<th>Amount</th>
<th>Inhibitor unit</th>
<th>Relative RNase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veronal-acetate</td>
<td>0.10</td>
<td>0.085</td>
<td>97</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.10</td>
<td>0.093</td>
<td>100</td>
</tr>
<tr>
<td>Tris</td>
<td>0.10</td>
<td>0.073</td>
<td>99</td>
</tr>
<tr>
<td>ABC</td>
<td>0.10</td>
<td>0.065</td>
<td>69</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.01</td>
<td>0.095</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.077</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.088</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.094</td>
<td>83</td>
</tr>
<tr>
<td>Veronal-acetate</td>
<td>0.10</td>
<td>0.081</td>
<td></td>
</tr>
</tbody>
</table>

* All buffers were pH 7.80 ± 0.05. The assay system was identical with that described under "Materials and methods." A solution of the 55 per cent precipitate was used in the above experiments.

† Relative RNase activity is the activity of 0.1 y of crystalline pancreatic RNase with the different buffers on a comparative basis.

not necessarily mean that combination of inhibitor with RNase takes place through a sulfhydryl linkage; in fact, crystalline pancreatic RNase, which combines readily with the inhibitor, has been shown to contain no free —SH groups (6). Additional experiments concerning the action of sulfhydryl reactants on i-RNase are described in Paper VIII of this series.

**Effect of Various Buffers and Ionic Strength on RNase Inhibitor**—In order to determine more about the optimal conditions for the action of RNase inhibitor, the effect of various buffers and changes in ionic strength were studied (Table IV). Inspection of the data reveals that ABC buffer and phosphate buffer give the highest inhibitor titers, and Veronal-acetate and Tris give lower titers. Since the inhibitor titer is dependent on the inhibition of added crystalline pancreatic RNase, a depressing effect of the
buffer on the enzyme alone would result in a higher inhibitor titer. This is what has happened in the case of ABC buffer. With 0.1 M Veronal-acetate, phosphate, or Tris buffers, however, RNase activity is relatively unaffected by the buffer so that differences in the inhibitor titers in these three cases represent effects of the buffer on RNase inhibitor or on its reaction with RNase. Since the ionic strength of these three buffers is not the same, the changes observed may be related to ionic strength, particularly since phosphate, with the highest ionic strength, gives the highest inhibitor titer. Examination of the data in the lower half of Table IV indicates that this is not so, since with increasing ionic strength inhibitor titers fall off and then rise again. The rise that occurs with concentrations of phosphate greater than 0.1 M is occasioned, however, mostly by a detrimental effect of high ionic strength on the activity of crystalline pancreatic RNase; an increase from 0.1 to 0.2 M causes a 17 per cent drop in RNase activity. The results in Table IV suggest that the use of 0.01 M phosphate buffer, pH 7.8, would probably be preferential to the present use of 0.1 M Veronal-acetate at the same pH.

**DISCUSSION**

RNase inhibitor appears to have a widespread occurrence (1, 7), but its physiological role is unknown at present. One might conjecture that its function is to inactivate any RNase that diffuses out of the mitochondria or lysosomes during the life of the cell. This could occur since alkaline RNase of rat liver is probably a small molecule capable of diffusing through membranes under certain conditions (8). If this hypothesis is correct, one might expect a fairly steady increase in the amount of i-RNase during the aging of the organism.

It is also possible that RNase inhibitor plays a part in the changes that take place during cell division. Jacobson and Webb (9) have demonstrated histologically an RNA or ribonucleoprotein coating on chromosomes which disappears during certain stages of division. In addition, the division process coincides with a large increase in the sulfhydryl content of the cell (10). A release of RNase from inhibitor would give rise to an increase in —SH groups; the released RNase would then be available to degrade the RNA or ribonucleoprotein coating the chromosomes. Considerable additional work must be done to study RNase inhibitor and i-RNase during various physiological changes in the organism before the question of the function of these substances can be answered. The specificity of RNase inhibitor is another question of considerable interest. Different tissues from the same animal contain different RNases (11), and a variety of RNases has been isolated from plant and animal sources. Also, an RNase inhibitor has been isolated from plant sources (12) but it is a thermostable,
dialyzable substance and therefore completely different from the RNase inhibitor in rat liver. The cross-reactions of different enzymes and inhibitors are being studied.

SUMMARY

1. Ribonuclease (RNase) inhibitor in rat liver supernatant fraction has been purified up to 10-fold and separated from inactive ribonuclease (i-RNase) by a combination of salt precipitation and calcium phosphate gel treatment.

2. A new assay system for RNase inhibitor and i-RNase has been devised which gives a more certain indication of the amounts of these substances in a preparation, allowing also determination of a wider range of concentrations.

3. The stability of various inhibitor preparations to storage under different conditions has been examined.

4. RNase inhibitor was found to be inactivated when incubated with ribonucleic acid, or under certain conditions in the presence of an insoluble polysaccharide isolated from yeast.

5. The effect of p-chloromercuribenzoate and Pb⁺⁺ on RNase inhibitor was determined. Both reagents inactivate the inhibitor; only the inactivation by Pb⁺⁺ is partly reversible.

6. The effects of several buffers and changes in ionic strength on the activity of RNase inhibitor were examined. Phosphate buffer of low ionic strength was found to be most favorable to the reaction.

BIBLIOGRAPHY

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