THE ANTIGENICITY OF d-RIBONUCLEASE; THE INHIBITION OF THE ENZYME BY ITS HOMOLOGOUS IMMUNE SERUM*

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A thermostable enzyme capable of digesting d-ribose (yeast) nucleic acid was first described by W. Jones in 1920 (1). A partial purification of this enzyme with acetone was carried out by Dubos and Thompson who called the enzyme ribonuclease (2). Kunitz described the preparation and properties of a crystalline protein he isolated from beef pancreas which appeared to be the same as the ribonuclease. He provisionally called this material ribonuclease. This crystalline preparation has a molecular weight of about 15,000 (3).

Enzymes have been reported to act as antigens. However, these enzymes all have had a molecular weight of at least 35,000. It appeared of interest to determine whether an enzyme of this small molecular weight (15,000) could be antigenic. Whether the activity of the enzyme could be inhibited by its combination with specific antiserum also offered an interesting problem.

Materials Used

The d-ribonuclease preparations used in this communication were kindly supplied by Dr. M. Kunitz to whom we are greatly indebted. Five different ribonuclease preparations have been used: a crude preparation, and products crystallized respectively, three, five, six, and eight times. Methods for the preparations of the crystallized ribonuclease as well as some of its properties are given by Kunitz (3).

The d-ribose (yeast) nucleic acid used was a Pfanstiehl commercial product which was purified in this laboratory by a method using (NH₄)₂SO₄ and CHCl₃ as previously described (4) to give a phosphorus content of 9.3 per cent (King's modification (5) of Fiske and SubbaRow (6)).

EXPERIMENTAL

Immunization Experiments

Antisera against the purified crystalline preparation of ribonuclease were prepared by three different methods:

(a) Intramuscular injection of ribonuclease adsorbed on aluminum gel after the method of Hektoen and Welker (7). A single injection of 110 mg. of crystalline ribonuclease adsorbed on aluminum gel was given. The amount of ribonuclease in the

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gel was determined by nitrogen analysis (Kjeldahl method). (b) Intravenous injections of the preparation used in (a). (c) Intravenous injections of saline solutions of crystalline ribonuclease.

Antibodies were demonstrated following immunization by all of the above methods. Tests for the precipitation were set up by mixing 0.2 ml. of antigen in the proper dilution, with 0.2 ml. of antiserum. These tubes were placed in the 37°C.

**TABLE I**

<table>
<thead>
<tr>
<th>Specificity of Antiserum</th>
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</thead>
<tbody>
<tr>
<td>Antiserum</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>RN (6 X purified)</td>
</tr>
<tr>
<td>RN (crude) No. 1</td>
</tr>
<tr>
<td>RN (crude) No. 2</td>
</tr>
<tr>
<td>Cattle serum No. 1</td>
</tr>
<tr>
<td>Cattle serum No. 2</td>
</tr>
<tr>
<td>N.R.S.</td>
</tr>
</tbody>
</table>

RN = Ribonuclease. 1 = +; 2 = ++; 3 = +++; 4 = ++++.  
* All dilutions up to 62,500 are negative.

**TABLE II**

<table>
<thead>
<tr>
<th>Reactivity of Antiserum against Five Times and Eight Times Crystallised Enzyme Preparations</th>
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</thead>
<tbody>
<tr>
<td>Antiserum</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Antigen</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>5 X RN*</td>
</tr>
<tr>
<td>5 X RN*</td>
</tr>
<tr>
<td>8 X RN No. 1</td>
</tr>
<tr>
<td>8 X RN No. 2</td>
</tr>
</tbody>
</table>

RN = Ribonuclease.  
* = 5 times crystallized ribonuclease.  
† = 8 times crystallized ribonuclease.

The results obtained with the two methods of intravenous injections were the same. Antibodies were demonstrable after a course of about thirteen injections (three times weekly) totalling about 30 mg. of the purified crystalline ribonuclease. As seen from Table I the reaction was positive up to an antigen dilution of one million. Ordinarily there persisted a prozone which with some
antisera went up as high as to 1:100,000 antigen dilution. However, several antisera were prepared which did not show any antigen prozone. Also some of the antisera reacted more strongly than others. Antisera prepared against the five times crystallized enzyme preparation when tested serologically gave identical results against both the five times and eight times crystallized enzyme preparations. (See Table II.)

The crude ribonuclease was injected intravenously into rabbits, using 0.5 mg. of material at the start. Antibodies against the crude preparation could be demonstrated after a total of 9 mg. had been injected (7 injections). No reaction with this antiserum could be demonstrated against the crystalline ribonuclease. However, longer periods of immunization with the crude material gave precipitation against the crystalline preparation in dilutions up to one million. There was a definite prozone which in some cases extended to over 100,000 dilution of antigen. The crude ribonuclease, in dilutions of 2,500 to 62,500, gave precipitation against crude ribonuclease antisera. (See Table I.)

For controls, since the source of the enzymes was beef pancreas, rabbits were injected with cattle serum. Cattle antisera gave positive reactions with no prozone up to homologous antigen dilution of 31,000. No cross-reaction was given with any of the preparations of ribonuclease. The cattle serum, likewise gave no reaction against antiribonuclease sera (Table I). Normal rabbit sera when tested against the various ribonuclease preparations gave no reaction in any case.

**Action of Specific Antisera on the Enzyme Activity**

The amount of the purified enzyme preparation in the antigen-antibody precipitate was determined in the following manner: To varying amounts of the crystalline enzyme (in a volume of 0.2 ml.) was added 3 ml. of homologous antiserum. These tubes were shaken, allowed to stand in a 37°C. water bath for 1 hour, and in the refrigerator overnight. In the morning the tubes were centrifuged, the sediment washed with cold 0.85 per cent NaCl solution, and the precipitates analyzed for nitrogen. A curve was plotted using milligrams of enzyme as the abscissa and milligrams of protein precipitated as the ordinate. (See Fig. 1.) Obviously, the most satisfactory region to carry on this type of experiment would be that of antibody excess, so that no free antigen would exist. Accordingly these experiments were carried out with an antigen-antibody ratio of about 1:28 (1:40,000 antigen dilution) as shown in the following typical experiment.

0.2 mg. of enzyme in 0.2 ml. volume was added to 5 ml. of antiserum. This mixture was allowed to react in a 37°C. water bath for 1 hour, then placed in the refrigerator for 48 hours, centrifuged, and the precipitate washed once with chilled 0.85 per cent NaCl solution. The precipitate was made to 1 ml. with 0.85 per cent NaCl solution.
solution and then 1 ml. solution (10 mg.) of d-ribonucleic acid was added and the mixture stirred well to insure a uniform suspension. After the 30 minutes' time allowed for this reaction the mixture was centrifuged and the 0.5 ml. of clear supernatant was used for a phosphorus determination. An equal volume of uranium acetate reagent¹ (exactly 1.5 ml.) was added to the 1.5 ml. of the clear supernatant remaining and the mixture allowed to stand for 20 minutes. The precipitate which contained the unhydrolyzed nucleic acid was then removed by centrifugation and the amount of phosphorus was determined in 2 ml. of the supernatant which contained the hydrolyzed nucleic acid. A control was run, lacking only the antiserum.

The results of the two experiments were:
control—43 per cent hydrolysis² of the nucleic acid; with antiserum—30 per cent hydrolysis of the nucleic acid

\[
\frac{43 - 30}{45} \times 100 = 30 \text{ per cent inhibition}
\]

control—45 per cent hydrolysis of nucleic acid; with antiserum—35 per cent hydrolysis of nucleic acid

\[
\frac{45 - 35}{45} \times 100 = 22 \text{ per cent inhibition}
\]

The above results were obtained using a precipitate from an antigen-antibody combination which had been allowed to react for 48 hours.

¹ 0.25 per cent uranium acetate in 2.5 per cent trichloracetic acid (3).
² These experiments were carried out at pH 7.2 which is not the pH of optimal enzyme activity. A more alkaline pH was not used in order to avoid the dissociation of antigen-antibody combination.
Other similar experiments carried out with only 24 hour antigen-antibody incubation, showed inhibition ranging from 10 to 15 per cent.

**DISCUSSION**

The results of the present study show that crystalline d-ribonuclease is antigenic. This enzyme stimulates the formation of antibodies when injected into rabbits, using different methods of immunization, and reacts with the homologous antiserum in very high dilutions of antigen. Different preparations of the crystalline d-ribonuclease appear to exhibit different behavior in stimulating this antibody formation. Some antiserum prepared against different enzyme preparations, when tested against these enzymes in the precipitation test showed a prozone which at times went up as high as 1:100,000 antigen dilution. Other antisera, however, showed no prozone at all. Any one antiserum gave identical precipitation against all of the purified enzyme preparations. For instance, antiserum prepared against the five times crystallized enzyme gave the same precipitation titer when tested against the five times and eight times crystallized enzymes (see Table II).

Numerous papers have appeared of the inhibition effect of antisera on homologous enzyme antigens. Kirk and Sumner showed that antiserum against urease inhibited urease activity (8), Lüers and Albrecht showed that antiamylase serum inhibited amylase activity (9), and Macfarlane and Knight found that lecithinase activity of *Clostridium welchii* (*Clostridium perfringens*) toxin was inhibited by antitoxin (10).

Our attempts to determine whether the ribonuclease activity could be inhibited by its homologous antiserum were met with certain technical difficulties. The quantitative determination of the hydrolyzed nucleic acid (P determination) in systems containing the enzyme and the immune serum was not practicable because of the large amount of protein present. Methods employed to eliminate the protein by the use of precipitating agents would also precipitate the nucleic acid (4). Therefore the inhibitory effect of the specific antiserum was determined by using the washed antigen-antibody precipitate as the enzyme material. In the reaction mixture containing the nucleic acid this precipitate was finely suspended, and could be removed easily at the end of the reaction by centrifugation. The results showed that the antibody inhibited the enzyme activity from 10 to 30 per cent.

The question may be raised as to whether this reduction of the enzyme activity is really due to the blocking of active groups on the enzyme by combination with antibody or due to the physical inability of the large molecular weight nucleic acid to penetrate the suspended particles and thus come into contact with the active enzyme groups. We are unable to answer this question. However, Lüers and Albrecht observed the inhibition of amylase by its homologous antibody using starch, which is a high molecular weight substance, as substrate.
In other similar studies referred to above, this question did not arise since the substrates such as urea and lecithin are of relatively small molecular weight and can readily penetrate into the suspended particles of the antigen-antibody combination.

**SUMMARY**

The enzyme d-ribonuclease is antigenic. Antisera, prepared by three different methods, reacted against antigen dilutions up to one million.

Apparently the homologous antiserum, when combined with the d-ribonuclease inhibited the activity of the enzyme from 10 to 30 per cent.

**LITERATURE**