THE RIBONUCLEINASE OF THE SOY BEAN

I. ISOLATION OF THE ENZYME*

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The hydrolysis of nucleic acids by direct chemical methods is recognized as a rather unsatisfactory procedure for the preparation of nucleotides and nucleosides in that the mixtures obtained are difficult to separate and the yields of the desired products are low. Enzymatic hydrolysis should prove applicable to this problem and has been used, with limited success, for obtaining degradation products of both ribonucleic and deoxyribonucleic acids (1-4). Owing to the difficulties attending the preparation of the necessary enzymes, however, enzymatic methods of hydrolysis have not been widely used.

The enzymes which effect the hydrolysis of the nucleic acids and their intermediate breakdown products have been grouped together under the name nucleases and, according to Levene and Medigoreanu (5) and Bredereck (6), fall into three classifications according to the substrates upon which they act. These are, respectively, the polynucleotidases, the nucleotidases, and the nucleosidases. No attempt will be made here to review the literature concerning the nucleases. It should be pointed out, however, that there is considerable confusion in this literature concerning the specificity and the nomenclature of these enzymes. Thus, the active agent which effects the rupture of the ribonucleic acid molecule without the release of inorganic phosphate has been referred to by various workers as ribonucleodcpolymerase (7), ribonuclease (8), polynucleotidase (9), ribonucleinase (10), and even as nucleotidase (11). Some of this confusion arises naturally from the fact that the enzyme preparations were not pure and exhibited different degrees of specificity. There has been no recent and systematic investigation of the nucleases from the point of view of

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1 In this paper we have used the term nuclease activity as indicative of the presence of enzymes which promote the hydrolysis of ribonucleic acid with the formation of inorganic phosphate. The term ribonucleinase is used to designate an enzyme which brings about the formation of degradation products of ribonucleic acid in which the phosphorus is retained in organic combination. For a justification of this name see Loring and Carpenter (10).
modern enzyme and protein chemistry and, with the exception of the ribonuclease of the pancreas which has been obtained by Kunitz (8) in a crystalline form, no member of the nucleases has been prepared in a well defined state of purity.

This paper presents the results of the first of a series of studies in which it is planned to isolate and, if possible, to purify the components of a system of nucleases. The purpose of the research is twofold: to study the chemical composition and specificity of the enzymes, and to attempt their preparation in a quantity sufficient to serve for fairly large scale hydrolyses of ribonucleic acid.

The starting point of the investigation was the selection of a source of the enzymes. In 1930, Jono (12) reported that the seeds, stems, and leaves of a number of plants contained nucleases. Among the species investigated, the common soy bean exhibited a comparatively high activity in the hydrolysis of ribonucleic acid. The nuclease activity was evidenced by the increase of inorganic phosphate in the mixtures of the ribonucleic acid and extracts of the beans. Since the soy bean is readily available in quantity, it was decided to reinvestigate this reported nuclease activity. In so far as it has been possible to determine, there has been no mention in the literature of the soy bean nuclease apart from this initial report.

Our first experiments confirmed the finding that extracts of germinated soy beans were capable of promoting the hydrolysis of ribonucleic acid. A number of attempts were made to purify and to concentrate this nuclease activity but these were uniformly unsuccessful in that they led to preparations which exhibited no greater activity than did the original crude extracts. Frequently the activity was entirely destroyed by relatively mild treatment. The techniques employed in these experiments included precipitations with organic solvents, salting-out procedures, and attempts to adsorb and elute the activity with various inert adsorbents. These experiments will not be reported in detail here, as they led to little information concerning the nuclease activity.

In order to explain these failures to concentrate the nuclease activity, it was assumed that the observed breakdown of the ribonucleic acid might be due to the combined actions of more than one enzyme. Subsequent studies have proved the presence of two enzymes in the crude extracts. The first of these has been identified as a ribonuclease whose action is to convert ribonucleic acid into products which are soluble in glacial acetic acid but which retain the phosphorus of the substrate in organic combination (10). The second enzyme is responsible for the formation of the inorganic phosphate in the substrate-extract mixtures and is believed to be a phosphomonoesterase. It has not been well studied, as it is destroyed in the course of the isolation of the nucleinase.
Ribonucleinase has been isolated in a crystalline form from pancreatic tissue (8) and has been detected in blood (13), bone marrow (14), spleen (14), and Pasteurella pestis (15). We have now demonstrated its occurrence in the higher plants by the preparation of highly active solutions from soy bean seedlings. From this pattern of distribution it might be concluded that ribonucleinase is an important agent in intercellular metabolism. The products of its action upon ribonucleic acid are, however, not definitely known (15) and its physiological function within the cell must be clarified by further research.

The work reported here is primarily concerned with the proof of the occurrence and the isolation of the nucleinase. The preparation of the enzyme in larger quantities and the more detailed investigation of its reaction characteristics are in progress in this laboratory and will be the subject of a later communication.

EXPERIMENTAL

Materials—The nucleic acid used in these studies was a commercial preparation of yeast sodium nucleate. In the earlier experiments this material was used without purification. Later it was found that the preparation contained material, presumably of the nature of mononucleotides, which inhibited nucleinase activity. For all subsequent work, therefore, the yeast sodium nucleate was converted into ribonucleic acid and purified according to the procedure of Kunitz (8). After this work was completed and was being prepared for publication a report by Zittle (16) appeared which emphasizes the presence of nucleinase-inhibiting substances in some of the commercially available nucleic acids.

The soy beans were a common variety of field beans, Glycine hispida var. Manchu. The beans were sprouted under conditions which discouraged mold growth and the seedlings were macerated and extracted with a 40 per cent solution of glycerol. The following procedure has been found satisfactory for the sprouting of the beans and the preparation of the extracts.

A weighed quantity of the soy beans was washed with a 0.05 per cent solution of calcium hypochlorite. They were thoroughly rinsed with distilled water and permitted to stand in water overnight. The swollen beans were again washed with the calcium hypochlorite solution and thoroughly rinsed with distilled water. They were then placed in wide mouth reagent jars, each of which received approximately 150 gm. of the swollen beans. The jars were closed with a double layer of cheese-cloth.

We wish to express our appreciation to Standard Brands Incorporated for their gift of the yeast sodium nucleate and to the Ferry Morse Seed Company who kindly furnished the soy beans used in this investigation.
and were supported in an inverted position to facilitate drainage of excess water. The germination was carried out in a dark room at a temperature between 20-25°C. At approximately 8 hour intervals, the jars were filled with water and emptied, a procedure which served to maintain a moist atmosphere and to wash out developing bacteria. After 96 hours the sprouts were between 3 and 4 cm. in length. Under the conditions described a germination of nearly 100 per cent was obtained and no mold growths or other visible contaminations were encountered.

The sprouted beans were removed from the jars and weighed. They were then mixed with glycerol and water in proportions which were calculated in the following manner. Each 100 gm. of the dry beans were to be extracted with 750 ml. of 40 per cent glycerol. Since a considerable portion of the required water had been absorbed by the beans in the course of the germination process, the difference between the weights of the dry and germinated beans was subtracted from the calculated water requirement. The exact procedure is illustrated by a typical example.

Dry beans, 500 gm., were calculated to require 3750 ml. of extraction medium to be composed of 1500 ml. of glycerol and 2250 ml. of water. The weight of the sprouted beans was 1550 gm., of which 1050 gm. were water absorbed in the process of germination. The sprouted beans were, therefore, mixed with 1500 ml. of glycerol and 1200 ml. of water. This mass was thoroughly homogenized in a Waring blender and placed in the refrigerator for 48 hours. The fluid was then pressed out through cheesecloth and the extract was centrifuged. The heavy, cream-colored supernatant fluid was covered with toluene and stored in the refrigerator. This material is referred to as the crude extract from which all subsequent enzyme preparations were derived. When preserved as indicated, it retains its activity for many months.

Methods—The nuclease activity of the various preparations was measured by the determination of the increase of inorganic phosphate in the mixtures of the extract and ribonucleic acid. Since some of the degradation products of the ribonucleic acid react with molybdic acid, it was necessary to carry out a preliminary precipitation of the phosphate before it was determined by the Fiske-Subbarow method (17).

A 5 ml. aliquot of the enzyme-substrate mixture was pipetted into 5 ml. of a 20 per cent solution of trichloroacetic acid contained in a conical centrifuge tube. The mixture was stirred for several minutes and was centrifuged. An appropriate aliquot of the supernatant fluid, usually 5 ml., was mixed with 5 ml. of magnesia mixture (18), and the acidity of the solution was adjusted to pH 5.5 by the careful addition of concentrated ammonium hydroxide. 1 ml. of the ammonium hydroxide in excess was added and the mixture was placed in the refrigerator for 24 hours. The ammonium
magnesium phosphate was centrifuged and was washed once with 1.5 ml. of ice-cold 2 per cent ammonium hydroxide. The washed precipitate was dissolved in 1 ml. of the acid solution, Molybdate II, of Fiske and Subbarow (17) and the solution was quantitatively transferred to a 25 ml. volumetric flask. The aminonaphtholsulfonic acid solution (17) was added and the solution was made up to volume. After 5 minutes the intensity of the color was determined with a photoelectric colorimeter.

The determination of ribonuclease activity was based upon the fact that the intact nucleic acid molecule is precipitated by glacial acetic acid, whereas the products of nuclease action are soluble in this reagent (8, 15). Aliquots of the material to be analyzed were pipetted into 5 times their volume of glacial acetic acid. The mixtures were filtered and the total phosphorus in the filtrates was determined by the perchloric acid method of King (19). The increase in the total phosphorus soluble in glacial acetic acid, appropriately corrected for the controls, was a convenient measure of the ribonuclease activity.

Expression of Enzyme Activity—As no detailed study has been made of the phosphomonoesterase, the nuclease activity of the crude extracts has been expressed simply as the increase in phosphate phosphorus per ml. of the incubation mixture.

The nuclease unit employed in the later experiments is defined as that amount of the enzyme which will bring about an increase of 1 mg. of acid-soluble phosphorus in 24 hours in a mixture of the enzyme and a 1.0 per cent solution of ribonucleic acid at pH 6.2 at 30°. The total nitrogen of each enzyme solution was determined by the micro-Kjeldahl method (20) and the amount of an enzyme preparation used in a particular experiment is expressed in terms of its content of nitrogen. Willstätter and Kuhn (21) have emphasized that the expression of the concentration of an enzyme should be in terms of the number of units contained in a given amount of the preparation, a quantity which they term the enzyme value. We have calculated our results in terms of the ribonuclease value which is defined as the number of ribonuclease units per mg. of nitrogen present in the enzyme preparation. The ribonuclease value affords a convenient basis for the comparison of the activity and degree of purity of the various enzyme preparations.

Results

Nuclease Activity of Soy Bean Extracts—The selection of germinated soy beans as the better source of the nuclease activity was made upon the basis of a comparison of the activities of extracts of dormant and sprouted beans.

Two enzyme solutions were prepared. The first was a 40 per cent glycerol extract of 5 gm. of finely ground soy beans. The second was a
40 per cent glycerol extract of 5 gm. of beans which had been sprouted in the manner described above. The volumes of the two extracts were approximately equal. Duplicate experiments were set up in which 2 ml. of the appropriate extract were mixed with 40 ml. of a 1.0 per cent solution of yeast sodium nucleate in 0.02 M citrate buffer of pH 6.2. Control tubes, two of which contained only the enzyme solutions and the third of which contained the buffered substrate alone, were incubated simultaneously with the experimental mixtures at 30°. At approximately 24 hour intervals aliquots of the various solutions were withdrawn and analyzed for their content of inorganic phosphate in the manner which has been described. The results of the analyses were calculated in terms of increases in inorganic phosphorus per ml. of solution, appropriately corrected for the changes observed in the control tubes.

The results of the experiment are presented in Fig. 1. It is evident that the crude extract of the sprouted beans contained a considerably greater nuclease activity than did the extract of the dry beans. It is not implied that the enzymes present in the two extracts are identical; the experiment merely indicated that germinated beans contained a greater concentration of nuclease activity and for this reason subsequent work was carried out upon the sprouted beans.

**Demonstration of Dual Nature of Nuclease System**—It was mentioned in an earlier paragraph that the usual methods for the concentration of enzyme solutions were not applicable to the purification of the nuclease activity of the crude soy bean extracts. In the course of these attempts to purify the extracts, however, it was observed that the trichloroacetic acid filtrates prepared from the control tubes which contained only ribonucleic...
acid were usually turbid, whereas the corresponding filtrates from the experimental mixtures were frequently clear. Although the turbidity interfered in no way with the determination of the inorganic phosphate, consideration of the phenomena led to the conclusion that the turbidity was due to the precipitation of unaltered ribonucleic acid in a very finely divided form. The fact that the filtrates from mixtures which contained the enzyme preparations were frequently clear led to the speculation that the ribonucleic acid had been altered by the extracts, although nuclease activity was not evident from the results of the analyses for inorganic phosphate.

Accordingly, an experiment was set up in which each aliquot of the ribonucleic acid-soy bean extract mixture was analyzed for acid-soluble phosphorus as well as for its content of inorganic phosphate. The details of the experiment were identical with those described above for the detection of the nuclease activity of the extract of the germinated seeds. The results are presented in Fig. 2 from which it is evident that the extract was capable of forming acid-soluble phosphorus from the ribonucleic acid at a greater rate than it released inorganic phosphate. The experiment was considered as indicative of the presence of two enzymes in the crude extract, one of which was responsible for the increase in the acid-soluble phosphorus, and the second for the formation of the inorganic phosphate.

Isolation of Ribonuclease—A number of orientation experiments were carried out in an attempt to determine the best method for the separation of the two enzymes. It was found that the phosphomonoesterase activity was sensitive to acid, whereas the ribonuclease activity was not only
stable to acid but was also moderately resistant to inactivation by heat. The following procedure has served to prepare ribonuclease solutions of a good degree of activity.

The crude glycerol extract of the sprouted soy beans, 1500 ml., was diluted with an equal volume of water and the acidity adjusted to pH 4.0. The solution was placed in the refrigerator for 3 hours and was then partially neutralized by the addition of concentrated ammonium hydroxide to pH 4.8. The preparation was placed in the refrigerator for 12 hours and was then centrifuged. The precipitate was discarded. The solution was placed in cellophane bags and dialyzed against cold, running distilled water for 48 hours. The preparation was removed from the

### Table I

*Activity of Concentrated Ribonuclease*

<table>
<thead>
<tr>
<th>Incubation time (hrs.)</th>
<th>Substrate</th>
<th>Nuclease activity (inorganic phosphate)</th>
<th>Ribonuclease activity (acid-soluble phosphorus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Commercial</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0</td>
<td>Purified</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>24</td>
<td>Commercial</td>
<td>34.2</td>
<td>270</td>
</tr>
<tr>
<td>24</td>
<td>Purified</td>
<td>9.9</td>
<td>470</td>
</tr>
<tr>
<td>48</td>
<td>Commercial</td>
<td>52.5</td>
<td>292</td>
</tr>
</tbody>
</table>

dialysis sacs and was filtered from the slight sediment which had formed in the course of the dialysis. The filtrate was concentrated in vacuo to a volume of 380 ml. During the concentration the temperature was not permitted to go above 30° and the pressure varied from 12 to 17 mm. of mercury. The resulting solution has been designated the concentrated ribonuclease solution.

Solutions prepared in this manner exhibited strong ribonuclease activities and contained most of the enzyme present in the original crude extracts. The removal of the phosphomonoesterase activity was more or less complete, as evidenced by the low values which were obtained in the assays for nuclease activity.

3 The ribonuclease of the pancreas has been shown to be remarkably stable to heat inactivation (8, 22).
In the course of the determination of the activity of these concentrated ribonuclease solutions, it was observed that the commercial yeast sodium nucleate contained material which inhibited the action of the nuclease. An illustration of this inhibition is seen in Table I in which the assay of one preparation of the ribonuclease was made against the commercial substrate as well as against a sample of purified ribonucleic acid. These data not only show the inhibition of the ribonuclease but also indicate that this material, presumably of mononucleotide nature (16), leads to an enhanced value for the monophosphatase activity. This latter effect is possibly due to the fact that the presence of mononucleotides in the substrate furnished material upon which the monophosphatase acts readily. In all subsequent work the substrate of choice was the purified ribonucleic acid.

*Fractionation of Concentrated Ribonuclease by Precipitation with Ammonium Sulfate*

Concentrated nucleinase

- Make 0.5 saturated with \((\text{NH}_4)_2\text{SO}_4\); filter
- Filtrate
  - Dialyze 36 hrs.
  - Fraction B
    - Make 0.8 saturated with \((\text{NH}_4)_2\text{SO}_4\); filter; discard filtrate; dissolve ppt. in water; make 0.45 saturated with \((\text{NH}_4)_2\text{SO}_4\); filter
    - Ppt.
      - Dissolve in water; dialyze 36 hrs.; filter; discard ppt. Solution is *Fraction X*
      - Filtrate
        - Increase \((\text{NH}_4)_2\text{SO}_4\) concentration to 0.8 saturation; filter; discard ppt. Solution is *Fraction Y*
    - Filtrate
      - Dialyze 48 hrs.
      - Fraction C
        - Make 0.6 saturated with \((\text{NH}_4)_2\text{SO}_4\); filter
        - Filtrate
          - Increase \((\text{NH}_4)_2\text{SO}_4\) concentration to 0.8 saturation; filter; discard filtrate; dissolve ppt. in water; dialyze 48 hrs.; discard ppt. Solution is *Fraction E*
          - Ppt.
            - Dissolve in water; dialyze 48 hrs.; discard ppt. Filter is *Fraction E*
Further Purification of Ribonuclease—The concentrated ribonuclease solutions were further purified by fractional precipitation with ammonium sulfate. The accompanying scheme shows this fractionation which consisted of the addition of ammonium sulfate to the desired concentration, removal of the precipitated protein, dialysis of the filtrate and of the re-dissolved precipitate, and refractionation of these two solutions. All of these operations were carried out between 5–10°. The various fractions thus obtained were assayed for ribonuclease activity in the usual manner.

Table II
Distribution of Ribonuclease Activity between Fractions Obtained by Precipitation with Ammonium Sulfate

The data summarize two experiments, Nos. I and II. The fractions refer to the preparations described in the scheme. The substrate was 20 ml. of 1.0 per cent solution of purified ribonucleic acid in 0.02 M citrate buffer of pH 6.2. The enzyme was added to the substrate in quantities of 0.2 to 1.0 ml., depending upon the activity of the fraction. Enzyme concentrations are given in terms of micrograms of enzyme nitrogen per ml. of the incubation mixture. The ribonuclease activity figures are in terms of micrograms of increased acid-soluble phosphorus per ml. of incubation mixture, appropriately corrected for the controls. The incubations were carried out at 30°. The significance of the ribonuclease value is discussed in the text.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Concentrated enzyme</th>
<th>Fraction A</th>
<th>B</th>
<th>X</th>
<th>Y</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme concentration</td>
<td></td>
<td>43</td>
<td>34.5</td>
<td>8.7</td>
<td>26.4</td>
<td>50.5</td>
<td>2.9</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>Oil I</td>
<td>Oil II</td>
<td>Ribonuclease activity</td>
<td>Experiment I</td>
<td>Experiment II</td>
<td>Experiment I</td>
<td>Experiment II</td>
<td>Oil I</td>
<td>Oil II</td>
</tr>
<tr>
<td></td>
<td>470</td>
<td>231</td>
<td>296</td>
<td>139</td>
<td>104</td>
<td>315</td>
<td>453</td>
<td>460</td>
<td>604</td>
</tr>
</tbody>
</table>

The results of typical fractionations of two preparations of the concentrated nucleinase are shown in Table II. It is evident that the separation of the enzyme from inert material is not particularly sharp and that the ribonuclease activity is to be found in several of the fractions. The most active material is precipitated between the concentrations of 0.5 and 0.8 saturation with ammonium sulfate and has been designated Fraction F. One preparation of Fraction F had a nucleinase value of 239. The corresponding value for the crude extract from which this preparation was derived was not determined but calculations based upon the dry...
weights of these crude extracts indicate that their nucleinase values are less than 1.0. It is reasonable to assume that a concentration ratio of at least 240 has been effected. None of the fractions obtained by the ammonium sulfate precipitation exhibited a release of inorganic phosphate from the substrate.

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**SUMMARY**

The nuclease system of the soy bean seedling has been shown to consist of two components, a ribonuclease and a second enzyme, believed to be a phosphomonoesterase. The ribonuclease has been separated from the crude extracts and has been concentrated and partially purified.

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