THE HYDROLYSIS OF YEAST RIBONUCLEIC ACID
BY RIBONUCLEINASE*

I. THE EXTENT OF HYDROLYSIS AND THE PREPARATION OF
RIBONUCLEINASE-RESISTANT FRACTIONS AFTER
RIBONUCLEINASE TREATMENT

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The isolation of the nucleotides or their well characterized salts after
treatment of yeast ribonucleic acid with ribonucleinase has shown that the
action of the enzyme consists, in part, in the liberation of mononucleotides
(1). It has also been suggested from the work of Kunitz (2) and from the
small yields of nucleotides isolated that in addition to the formation of the
diffusible nucleotides a relatively non-diffusible fraction remains, which
unlike the mononucleotides is precipitated in acid solution in the presence
of uranium ions. It has not been clear, however, whether the non-diffusible
fraction consisted of unchanged nucleic acid or of a resistant fraction not
subject to further hydrolysis by the enzyme. Experiments by Zittle (3)
favored the former conclusion when it was demonstrated that either guany-
lic or adenylic acid or the mixture of nucleotides, produced by the action of
alkali on yeast nucleic acid, inhibited hydrolysis as measured by the amount
of glacial acetic acid-insoluble material produced. In a later publication,
however, Zittle has stated that the high polymer fraction obtained from the
digestion mixture by precipitation with alcohol was not further hydrolyzed
by ribonucleinase (4).

In the present paper the question whether or not yeast ribonucleic acid
is completely hydrolyzed by ribonucleinase has been studied by carrying
out the hydrolysis under conditions which allowed the dialysis of the ribo-
nucleotides as they were produced. Measurement of the rate of dialysis of
organic phosphorus showed that after a preliminary rapid rate, correspond-
ing to the dialysis of the mononucleotides, a slower relatively constant
dialysis rate was reached which was not affected by further addition of
enzyme. The results show that a relatively non-diffusible fraction is left
which is resistant to further hydrolysis by ribonucleinase under these con-

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1 Kunitz' ribonuclease.
This material was characterized by analyses for purine nitrogen, guanine nitrogen, and phosphorus. It differs in composition from the original nucleic acid in containing relatively larger amounts of purine nitrogen, and, when obtained by ribonucleinase treatment of commercial yeast nucleic acid can be fractionated into two parts, one insoluble in 80 per cent acetic acid and another insoluble in equal parts of 80 per cent acetic acid and alcohol.

**EXPERIMENTAL**

*Ribonucleinase*---A sample of crystalline ribonucleinase kindly furnished by Dr. M. Kunitz was used. It had been crystallized three times from ammonium sulfate solution and four times from alcohol.

*Purified Ammonium Ribonucleate*---100 gm. of yeast nucleic acid (Eastman Kodak Company) were dissolved in 200 ml. of water by the addition of sufficient ammonium hydroxide to bring the pH of the solution to about 5.8. The nucleic acid was then precipitated with 5 volumes of glacial acetic acid. After the precipitate had been washed with 50 per cent alcohol and alcohol and ether, 50 gm. of nucleic acid were obtained. It was redissolved with the aid of ammonia, and the solution placed in cellophane bags and dialyzed against 30 liters of distilled water over a period of 24 hours. The dialyzed solution was concentrated in vacuo at 40° to a volume of 100 ml., and the ammonium nucleate precipitated by the addition of 8 volumes of 95 per cent alcohol. The yield after thorough washing with alcohol and ether was 20 gm.

*Extent of Hydrolysis by Ribonucleinase* A preliminary experiment in which a solution of ammonium ribonucleate and ribonucleinase was dialyzed showed that about 66 per cent of the phosphorus had dialyzed after 27 hours. Addition of more ribonucleinase and dialysis for another 24 hours resulted in the loss of an additional 4 per cent of phosphorus. It was evident, therefore, that the rapidly diffusing constituents were largely eliminated under the conditions used after dialysis for less than 27 hours.

In order to find more accurately the per cent of nucleic acid hydrolyzed, a second experiment was performed in which the rate of dialysis of organic phosphorus per hour was determined over the first 7 hour period as well as after 24 hours. 25 ml. of ammonium nucleate solution, containing 25 mg. per ml. at pH 7.7, and 6 mg. of ribonucleinase were dialyzed at room temperature against 18 liters of distilled water over a period of 24 hours. During the first 7 hours 500 ml. of dialysate per hour were collected at hourly intervals, analyzed for organic phosphorus, and the rate of dialysis calculated as per cent of total phosphorus dialyzed per hour. The rate be-

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2 In a recent paper Carter and Greenstein (5) report that the rate of dialysis of ribonucleic acid is greatly increased in the presence of sodium chloride.
tween 7 and 24 hours was calculated from analysis of the contents of the dialysis bag after the 24 hour period. The rates plotted against time are shown in Fig. 1. It may be seen that the rate decreased rapidly after 5 hours and reached a relatively constant value after about 10 hours. The calculation of the per cent of hydrolysis on the assumption that the nucleotides are completely removed during this time gives a value of about 50 per cent of the original purified sample. A control dialysis experiment on a solution of the purified ammonium nucleate under similar conditions in the absence of ribonuclease showed that about 26 per cent of the original nucleic acid had dialyzed after 24 hours.

In order to characterize the fraction resistant to the action of ribonuclease, the material remaining after dialysis was analyzed for purine nitrogen (6), guanine nitrogen (7), and phosphorus (8). Guanine nitrogen was determined on an aliquot of the acid hydrolysate from the total purine determination after precipitation as the copper-bisulfite complex. The latter was decomposed with hydrogen sulfide and the filtrate analyzed colorimetrically with the phenol reagent (7). The values obtained were then compared with similar analyses carried out on the original nucleic acid solu-
tion of ammonium nucleate and the dialyzed control. The results, expressed as total purine nitrogen, guanine nitrogen, and adenine nitrogen to phosphorus ratios, are given in Table I. The values calculated for a tetranucleotide as given by Levene (9) are also shown. It is evident from the results that the relatively non-dialyzable fraction differs from the original nucleic acid in containing a larger concentration of purine nitrogen and in particular of guanine nitrogen. Of interest is the large discrepancy between the values for guanine to phosphorus and adenine to phosphorus ratios in either the original nucleic acid solution or the dialyzed control as compared to those required by the tetranucleotide theory. While the ratio of purine nitrogen to phosphorus is in agreement with the theory as found also by Graff and Maculla (10), the purine fraction contained approximately twice as much guanine as adenine nitrogen.

### Table I

<table>
<thead>
<tr>
<th></th>
<th>Purified ribonucleic acid</th>
<th>Purified ribonucleic acid after dialysis</th>
<th>Ribonuclease-resistant fraction</th>
<th>&quot;Statistical&quot; tetranucleotide (Levene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total purine nitrogen</td>
<td>1.13</td>
<td>1.07</td>
<td>1.47</td>
<td>1.13</td>
</tr>
<tr>
<td>Guanine nitrogen</td>
<td>0.70</td>
<td>0.71</td>
<td>1.03</td>
<td>0.565</td>
</tr>
<tr>
<td>Phosphorus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine nitrogen</td>
<td>0.43</td>
<td>0.35</td>
<td>0.44</td>
<td>0.565</td>
</tr>
<tr>
<td>Phosphorus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanine nitrogen</td>
<td>1.6</td>
<td>2.0</td>
<td>2.3</td>
<td>1</td>
</tr>
<tr>
<td>Adenine nitrogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The large amounts of guanine found with the phenol reagent (7) suggested that other constituents of nucleic acid might also give a color reaction under the conditions used. While it is known that adenine does not react, no information was available as to whether or not the pyrimidine nucleotides would give this reaction. It was logical to use the nucleotides rather than the corresponding nucleosides because it is known that the former rather than the latter are produced by mild acid hydrolysis of nucleic acid as carried out in the total purine determination. Approximately 2 mg, each of cytidylic acid and diammonium uridylate (approximately 5 times the maximum quantity of guanine used in the procedure of Hitchings) failed to give any color with the phenol reagent in the presence of sodium carbonate.

**Preparation of Ribonuclease-Resistant Fractions**—It was of interest to prepare the ribonuclease-resistant fraction in solid form to compare its general properties with the original ribonucleic acid and to determine whether
the isolated material was also resistant to further hydrolysis by ribonuclease. Approximately 76 gm. of commercial yeast nucleic acid were dissolved in 2 liters of ammonia water at pH 6. The solution was placed in a large cellophane bag equipped with a motor-driven stirrer and dialyzed against distilled water. At the start of the dialysis 76.6 mg. of ribonuclease were added in four equal portions at 6 hour intervals. Dialysis was continued for a total of 46 hours. Analysis of the solution remaining in the bag for total phosphorus showed that approximately 60 per cent of the original nucleic acid or its split-products had dialyzed during this time. The solution which remained was concentrated in vacuo at 40° to about 250 ml., filtered through Celite, and diluted with 7 volumes of glacial acetic acid. A white amorphous precipitate formed which was filtered with suction. The precipitate was washed free of acid by grinding repeatedly in a mortar with 50 per cent alcohol, 95 per cent alcohol and ether, and was air-dried. The product called Fraction A weighed 18 gm. Upon adding an equal volume of 95 per cent alcohol to the acetic acid filtrate, another precipitate separated. This was filtered, washed, and dried by the same procedure used for Fraction A, and weighed 8 gm. (Fraction B).

These fractions resemble closely the original nucleic acid in general properties. They are relatively insoluble in water, forming suspensions that are acid to Congo red paper. Unlike the original nucleic acid, whose particles coalesce under these conditions to form a sticky mass, the particles of the resistant fractions remain relatively dispersed. Fraction A contained 7.8 per cent phosphorus and 12.9 per cent moisture when dried over phosphorus pentoxide at 56°. Fraction B contained 7.9 per cent phosphorus and 12.5 per cent moisture when dried under the same conditions. Preliminary analyses for adenine and guanine indicate that these fractions differ greatly in the amounts of these two constituents present.

Effect of Ribonuclease on Resistant Fractions—Three stock solutions were prepared by dissolving 0.3 gm. samples of the two resistant fractions and the purified ammonium nucleate in three 50 ml. portions of 0.1 M acetate buffer at pH 5.0. To 4 ml. of each stock solution were added 4 ml. of an enzyme solution containing 0.6 mg. of ribonuclease per ml. in acetate buffer at pH 4.0. As a control, 4 ml. of acetate buffer at pH 4.0 were added to 4 ml. of each stock solution. All of the solutions were placed in a water bath at 25° for 30 minutes. Then, to each solution were added 8 ml. of a uranium acetate solution, made by dissolving 2.5 gm. of uranyl acetate in 100 ml. of 2.5 per cent trichloroacetic acid, in order to precipitate the unhydrolyzed nucleic acid. After standing for 30 minutes longer in the water bath, the precipitates were filtered off and filtrates were analyzed for phosphorus. The experiment was run in duplicate. The results given in Table II show that in the case of both resistant fractions there is no signifi-
cantly difference between the amounts of phosphorus unprecipitated in the enzyme experiment and in the control, whereas in the case of the ammonium nucleate much more of the phosphorus remained unprecipitated in the enzyme-treated sample than in the control. It is evident, therefore, that these resistant fractions, A and B, are not further hydrolyzed by ribonuclease. Of interest is the difference in the solubility of the two fractions in the uranyl reagent. Fraction A which was precipitated by about 87 per cent acetic acid in contrast to Fraction B was also less soluble in the presence of uranium ions and trichloroacetic acid.

### Table II

<table>
<thead>
<tr>
<th></th>
<th>Total P concentration</th>
<th>Soluble P</th>
<th></th>
<th>Control</th>
<th>After ribonuclease</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Fraction A</td>
<td>0.113</td>
<td>0.0229</td>
<td>0.0208</td>
<td></td>
<td>0.019</td>
<td>19.3</td>
</tr>
<tr>
<td>&quot; B</td>
<td>0.12</td>
<td>0.0279</td>
<td>0.0293</td>
<td></td>
<td>0.0294</td>
<td>23.8</td>
</tr>
<tr>
<td>Purified nucleic acid</td>
<td>0.112</td>
<td>0.0876</td>
<td>0.0876</td>
<td></td>
<td>0.0089</td>
<td>78.5</td>
</tr>
</tbody>
</table>

* Average of Experiments 1 and 2.

### DISCUSSION

The experiments presented above show that even under conditions in which ribonucleotides are removed as they are formed after treatment of yeast nucleic acid with ribonuclease, about 50 per cent of the original nucleic acid remains as a relatively non-dialyzable fraction which is not subject to further hydrolysis by the enzyme. As this material would be removed from the original commercial nucleic acid by the purification procedures employed, it must be formed as a result of ribonuclease action and must represent a portion of the nucleic acid molecule resistant to further hydrolysis by this enzyme under the conditions used. When commercial nucleic acid was hydrolyzed, the non-dialyzable portion could be further fractionated into at least two parts, one relatively insoluble in 85 per cent acetic acid and another precipitated from the filtrate from the first by an equal volume of alcohol. As no attempt was made to fractionate the resistant fraction from purified nucleic acid by the same procedure, it was not determined whether two fractions are also produced in this case.

The analyses of the original purified nucleic acid for purine nitrogen, gua-
nine nitrogen, and phosphorus show that about twice as much guanine as adenine is present in this preparation. Whereas the phenol reagent as used by Hitchings is not highly specific, guanine is the only compound known to be present in nucleic acid that gives a positive reaction. These results emphasize the fact reported by several workers previously, including Levene (11–14), that all preparations of yeast nucleic acid purified by precipitation with acetic acid do not conform to the "statistical" tetranucleotide theory as proposed originally by Levene and as generally accepted by most workers. The reason for the differences is not clear. It is probably related to actual differences in ribonucleic acid samples as well as to differences in methods of hydrolysis and in analytical procedures employed. Differences in the rate of hydrolysis of different yeast nucleic acid samples by alkali have recently been reported by Zittle (4).

The analyses of the enzyme-resistant fraction for purine nitrogen, guanine nitrogen, and phosphorus show a concentration of guanine in this fraction and suggest that ribonuclease action is concerned to a greater extent with the liberation of pyrimidine than of purine nucleotides.

**SUMMARY**

Under conditions in which the liberated nucleotides are removed by dialysis, purified yeast ribonucleic acid is converted to a relatively non-dialyzable fraction which is resistant to further action by ribonuclease. The resistant fraction obtained from commercial nucleic acid could be fractionated into two parts, one precipitated from 85 per cent acetic acid and the other precipitated from the filtrate of the first by an equal volume of alcohol. Analyses of the original purified nucleic acid for purine nitrogen, guanine nitrogen, and phosphorus show about twice as much guanine as adenine, instead of the equimolar ratio required by the "statistical" tetranucleotide theory. The total purine nitrogen to phosphorus ratio was in agreement with the theory. After treatment with ribonuclease the resistant fraction showed an increase in the amount of guanine nitrogen present.

**BIBLIOGRAPHY**


*The term statistical tetranucleotide has been used by Gulland et al. (15) to indicate the presence of the four mononucleotides in equimolecular proportion in yeast ribonucleic acid.*

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