THE NUCLEASE ACTIVITY OF BACILLUS SUBTILIS

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Nuclease activity was dissociated from proteolytic activity when Sachs confirmed the observations of Milroy that an agent which attacked nucleic acid to liberate inorganic phosphoric acid could not liquefy gelatin and that proteolytic enzymes liquefied gelatin but could not attack nucleic acid (1). Henceforth the rate of separation of inorganic phosphoric acid from nucleic acid became the exclusive index of nuclease activity (2-4). Activity so defined was shown by a variety of microorganisms, such as yeast (5), Penicillium glaucum, and Aspergillus niger (6), and by Bacillus coli, Bacillus proteus, and staphylococcus (7).

Convincing evidence of bacterial disintegration of nucleic acid other than the slow separation of phosphoric acid has been lacking despite the discovery of a series of enzymes which can attack the different components of nucleic acid: thus there are polynucleotidases which subdivide nucleic acid to nucleotides,1 nucleotidases which split mononucleotides to phosphoric acid and nucleosides, nucleosidases which disjoin the sugar and nitrogenous base, and phosphatases which split phosphoric acid esters of ribose to free acid and free sugar ((8) pp. 311-316). Furthermore, the accepted structural formula of yeast nucleic acid, that elucidated by Levene and Bass ((8) p. 274, 286) indicates that the separation of more than one-quarter of the phosphoric acid in nucleic acid requires the breaking of the ester bonds between its component nucleotides. It is apparent also that these ester bonds may be broken without the separation of phosphoric acid from any of the four nucleotides

1 Levene and Dillon use the term polynucleotidase activity to describe the liberation of an arbitrary amount of phosphoric acid from nucleic acid in unit time by an enzyme from intestinal juice (3).
and that the study of nuclease activity must encompass more than
the determination of inorganic phosphoric acid.

Substantiation for the foregoing argument is to be found by
inspection of the structural formula of yeast nucleic acid and of
the convenient condensed forms presented with the accompanying
scheme representing the disintegration of nucleic acid. In this

\[
\begin{align*}
\text{Adenylic acid} & : \quad O=\overset{\text{P}}{\underset{\text{HO}}{\text{P}}}O-C_\text{H}_2\text{O}_2-C_\text{H}_4\text{N}_5 \\
\text{Uridylic acid} & : \quad O=\overset{\text{P}}{\underset{\text{HO}}{\text{P}}}O-C_\text{H}_2\text{O}_2-C_\text{H}_2\text{N}_2\text{O}_2 \\
\text{Guanylic acid} & : \quad O=\overset{\text{P}}{\underset{\text{HO}}{\text{P}}}O-C_\text{H}_2\text{O}_2-C_\text{H}_4\text{N}_0 \\
\text{Cytidylic acid} & : \quad O=\overset{\text{P}}{\underset{\text{HO}}{\text{P}}}C_\text{H}_6\text{O}_3-C_\text{H}_4\text{N}_2\text{O}
\end{align*}
\]

scheme P represents phosphoric acid, R represents ribose, and N
represents nitrogenous base.

The rate and process of disintegration of yeast nucleic acid may
be deduced from the nitrogen and phosphorus in three fractions
obtained by the methods described in this report. When these
methods were applied to cultures of bacteria in solutions of yeast
nucleic acid, the results revealed the rapid, intensive decomposi-
tion of nucleic acid by Bacillus mesentericus, Bacillus megatherium,
and Bacillus subtilis.

**Methods**

The methods used in the following experiments were extensions
of methods of previous investigators. Levene and Bass had used
neutral lead acetate to separate nucleosides from phosphoric derivatives of nucleic acid ((8) p. 167), and Buell and Perkins had used uranyl nitrate to precipitate quantitatively the purine nucleotides from tungstic acid filtrates of defibrinated blood (9). I found that yeast nucleic acid was precipitated completely from dilute solutions of uranyl chloride in trichloroacetic acid at pH 1.5 to 2.0, and that under similar conditions the purine nucleotides and cytidyllic acid were precipitated only in the range of pH 3.0 to 7.0, whereas uridylic acid and the less complex derivatives of nucleic acid were soluble at any pH tried from 1.5 to 9.0. The complete precipitation of nucleic acid is demonstrated in Table II, which shows that 94 per cent of the material in the control solutions was precipitated and that the remaining 6 per cent represented nucleic acid which had been decomposed before the culture mixtures were prepared; the phosphorus-containing substance not precipitated was found to be present in the same amount whether the supernatant solution was analyzed after or without digestion with strong acid.

The following detailed description includes the procedure for the preparation of cultures of bacteria in solutions of yeast nucleic acid and embraces the methods of analysis of these culture mixtures.

Preparation of Culture Mixtures—Agar slopes were seeded with bacteria and incubated for 18 to 24 hours at 37.5°. A suspension of living bacteria was prepared by emulsification of these colonies with 5 cc. of distilled water. The substrate was prepared by dissolving 4 gm. of sodium nucleinate in 100 cc. of buffer solution at a desired pH. 4 cc. samples of this solution were distributed in culture tubes which were stoppered with cotton and autoclaved. 1 cc. of bacterial suspension or 1 cc. of distilled water, as control, was mixed with 4 cc. of autoclaved substrate, in tubes stoppered with sterile rubber caps, and then incubated at 37.5° for 48 hours.

For a range of pH 7.0 to 9.6 the buffers used, in concentration of 0.1 M, were the sodium veronal-hydrochloric acid mixtures prepared as described by Michaelis (10), and for a range of pH 5.0 to 7.0 the buffers used, in concentration of 0.2 M, were the sodium citrate-sodium hydroxide mixtures, according to Clark (11).

Method for Separation of Nucleic Acid from Culture Mixtures—The contents, 5 cc., of each culture tube were washed with water...
into large centrifuge tubes of 50 cc. capacity and the volume made to 15 cc. with distilled water and then to 25 cc. with the acid-precipitating reagent. A flocculent precipitate which began to settle in a few seconds was stirred thoroughly twice during a 10 minute interval. By spinning the mixture in a centrifuge at 900 R.P.M. for 3 minutes it yielded a supernatant fluid, which was then decanted into a 50 cc. volumetric flask, and a precipitate, which was broken up by stirring with 10 cc. of dilute reagent and again separated by spinning in a centrifuge at 900 R.P.M. for 3 minutes. This operation was repeated once more to redissolve any adherent material less complex than nucleic acid. The supernatant fluids were pooled and made up to 50 cc. with dilute reagent, and the precipitate was dissolved in 25 cc. of 0.5 N sodium carbonate.

The acid-precipitating reagent was a solution of 1.25 gm. of uranyl chloride in 100 cc. of 10 per cent trichloroacetic acid. The dilute reagent was prepared by diluting this to 2.5 volumes with distilled water. Neither of the buffer systems used, in concentrations up to 0.2 M, was precipitated by these reagents.

Method for Precipitation of Nucleotides from Uranyl Chloride Supernatant Liquids—10 cc. of uranyl chloride supernatant fluid were pipetted into a centrifuge tube and neutralized by adding about 2 cc. of N sodium carbonate, drop by drop, until the pH of the mixture was 6.8 to phenol red (as an outside indicator). During this adjustment a precipitate forms, beginning at pH 3.0, reaching a maximum size at about pH 4.3, and dissolving in greater part at pH 6.8. Drops of a solution of 10 per cent neutral lead acetate were added to this slightly acid mixture until no more precipitate could be produced by adding 10 drops more to the supernatant fluid previously cleared by spinning in a centrifuge at 900 R.P.M. for 3 minutes. Hydrogen sulfide was passed through the supernatant fluid to precipitate any excess reagent, which was then separated by spinning the mixture in a centrifuge at 1200 R.P.M. for 5 minutes. This supernatant fluid was aerated to remove excess hydrogen sulfide and then decanted into a volumetric flask where the volume was made up to 25 cc. with distilled water. The sodium veronal-hydrochloric acid buffers are completely precipitated by this procedure.

Methods of Analysis for Nitrogen and Phosphorus—The foregoing methods give three fractions of the culture mixtures, the
carbonate solution of the uranyl chloride precipitate (Fraction 1),
the uranyl chloride supernatant fluid (Fraction 2), and the super-
natant fluid after treatment with neutral lead acetate (Fraction
3). The unfractionated culture mixture is labeled Fraction 4.

Small samples, 1 to 5 cc., were taken from each of the three
fractions and analyzed for total nitrogen by means of the micro-
Kjeldahl method of Van Slyke (12). Inorganic phosphorus (i.e.
phosphoric acid not bound to organic substance), in 2 to 5 cc. of
Fraction 2, was determined either by Tisdall’s (13) or Leiboff’s
(14) colorimetric method. Total phosphorus, in 2 to 5 cc. samples
of Fractions 1 and 2, was determined as inorganic phosphorus
after the following procedure. The sample chosen was boiled with
exactly 0.5 cc. of concentrated sulfuric acid until white fumes
appeared; it was then cooled and boiled with 1 cc. of distilled water
and no more than 0.5 gm. of potassium persulfate until white
fumes again appeared. Then it was cooled, diluted with 3 cc.
of distilled water, and boiled for 10 minutes to decompose any
excess persulfate. After cooling to room temperature, this solu-
tion was ready for analysis by the method of Leiboff. Standard
phosphate solutions, with and without the prescribed treatment,
usually matched; when they failed to do so, the attending results
were rejected.

The foregoing methods are applicable to solutions containing
uranyl ions in amounts equivalent to the samples mentioned.
For the determination of phosphorus the method of Leiboff is
preferred, because it is quick and convenient for a large number of
analyses, while it gives the same results as the method of Tisdall,
as shown in Table I.

In the ensuing experiments the hydrogen ion concentration was
estimated colorimetrically by the use of Clark and Lub’s series of
indicators (11).

Results

The agents of disintegration of yeast nucleic acid were living
bacteria; specifically they were members of the aerobic, spore-
bearing group of bacilli. The three strains isolated were Bacillus
mesentericus vulgatus, Bacillus megatherium, and Bacillus subtilis.
Since the results for each strain were very much the same, those
obtained with *Bacillus subtilis* were chosen as representative of group behavior.

*Bacillus subtilis* in its vegetative form grows rapidly on simple media, is a facultative anaerobe, and finds refuge in spores which resist boiling for hours. In spite of their rapid growth the amounts of total nitrogen and of total phosphorus contributed to the medium by the bacilli were very small compared to the amounts representing breakdown products of nucleic acid. This conclusion is demonstrated in Table II. In Tables II to VI each number represents the difference of results of analysis of a control mixture from those of a mixture containing bacteria.

**Intensity of Nuclease Activity of Bacillus Subtilis**—The most favorable pH for the nuclease activity of *Bacillus subtilis*, 6.6, is demonstrated in Table III, which presents results obtained from

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Leiboff method</th>
<th>Tisdall method</th>
<th>Sample No.</th>
<th>Leiboff method</th>
<th>Tisdall method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td></td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>1</td>
<td>2.143</td>
<td>2.128</td>
<td>5</td>
<td>1.428</td>
<td>1.412</td>
</tr>
<tr>
<td>2</td>
<td>2.801</td>
<td>2.907</td>
<td>6</td>
<td>2.856</td>
<td>2.824</td>
</tr>
<tr>
<td>3</td>
<td>4.784</td>
<td>4.762</td>
<td>7</td>
<td>4.100</td>
<td>4.149</td>
</tr>
<tr>
<td>4</td>
<td>5.434</td>
<td>5.494</td>
<td>8</td>
<td>4.000</td>
<td>4.067</td>
</tr>
</tbody>
</table>

An environment of hydrogen, though it seems to accelerate phosphorus metabolism, is without influence on the nuclease activity of *Bacillus subtilis*. Substantiation for this statement is found in Table IV. One set of culture mixtures and one set of control mixtures, in tubes without stoppers, were exposed to
D. A. MacFadyen

hydrogen in a 2 liter glass jar from which oxygen had been extracted by a vacuum pump and by oxidation of an electrically heated copper wire attached to the lead cap sealing the jar and in which atmospheric pressure had been restored by intake of hydrogen. Duplicate sets of culture mixtures and of control mixtures were exposed to air at atmospheric pressure in a twin jar. Both jars were sealed and placed in an incubator at 37.5° for 18 hours.

Table IV shows, except for total and inorganic phosphorus at

Table II
Contribution of Living Bacteria to Nitrogen and Phosphorus of Culture Mixtures of Bacillus subtilis and Yeast Nucleic Acid

<table>
<thead>
<tr>
<th>Culture mixtures</th>
<th>Total N</th>
<th>Total P</th>
<th>Bacterial N*</th>
<th>Bacterial P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frac-</td>
<td>Frac-</td>
<td>Frac-</td>
<td>Frac-</td>
</tr>
<tr>
<td></td>
<td>tion 2</td>
<td>tion 1</td>
<td>tion 4</td>
<td>tion 2</td>
</tr>
<tr>
<td>Bacterial</td>
<td>12.54</td>
<td>5.95</td>
<td>18.49</td>
<td>7.41</td>
</tr>
<tr>
<td>“</td>
<td>14.88</td>
<td>4.07</td>
<td>18.95</td>
<td>8.79</td>
</tr>
<tr>
<td>Control</td>
<td>1.05</td>
<td>16.35</td>
<td>17.40</td>
<td>0.62†</td>
</tr>
<tr>
<td>“</td>
<td>1.01</td>
<td>16.59</td>
<td>17.60</td>
<td>0.62†</td>
</tr>
</tbody>
</table>

Fraction 1, carbonate solution of the uranyl chloride precipitate; Fraction 2, the uranyl chloride supernatant fluid; Fraction 3, the supernatant fluid after treatment with neutral lead acetate; Fraction 4, the unfractionated culture mixture.

* Bacterial N equals 18.49 - 17.50 and 18.95 - 17.50; bacterial P equals 10.54 - 10.35 and 10.64 - 10.35.
† Inorganic phosphorus.

pH 6.0 and 6.6, that the nuclease activities of Bacillus subtilis were the same in an atmosphere of low oxygen tension as in an atmosphere of air. The discrepancies must have represented inorganic phosphorus, since the total nitrogen and organic phosphorus were the same for each environment; they were accounted for by examination of the uranyl chloride precipitates which showed that the total phosphorus of the anaerobic precipitate (Fraction 1) at pH 6.0 and 6.6 was greater than that of the aerobic precipitate of the same fraction by almost the exact amounts of the discrepancies, and further that no phosphorus could be
detected without preliminary treatment of the precipitate with strong acid. These results suggest that the discrepancies of inorganic phosphorus represent substance ingested by the bacilli.

**Table III**

*Optimum Hydrogen Ion Concentration for Disintegration of Yeast Nucleic Acid by Cultures of Bacillus subtilis at 37.5° for 48 Hours*

Nucleic acid, 3.2 per cent; pH as shown; time of incubation, 48 hours.

<table>
<thead>
<tr>
<th>pH</th>
<th>Fraction 1 (a)</th>
<th>Fraction 2 (b)</th>
<th>Nuclease activity (c)</th>
<th>Total P</th>
<th>Nuclease activity (f)</th>
<th>P: N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>per cent</td>
<td>mg.</td>
<td>mg.</td>
<td>per cent</td>
</tr>
<tr>
<td>6.0</td>
<td>12.9</td>
<td>14.9</td>
<td>53.6</td>
<td>7.18</td>
<td>8.72</td>
<td>54.9</td>
</tr>
<tr>
<td>6.5</td>
<td>5.8</td>
<td>22.9</td>
<td>80.0</td>
<td>2.48</td>
<td>13.52</td>
<td>84.5</td>
</tr>
<tr>
<td>7.2</td>
<td>7.1</td>
<td>20.8</td>
<td>71.0</td>
<td>3.54</td>
<td>12.46</td>
<td>78.0</td>
</tr>
<tr>
<td>7.8</td>
<td>12.7</td>
<td>14.7</td>
<td>53.6</td>
<td>7.42</td>
<td>8.51</td>
<td>53.4</td>
</tr>
<tr>
<td>8.2</td>
<td>17.9</td>
<td>9.4</td>
<td>34.4</td>
<td>10.51</td>
<td>5.43</td>
<td>54.1</td>
</tr>
</tbody>
</table>

\[ c = \frac{b}{(b + a)} \times 100; f = \frac{e}{(d + e)} \times 100. \]

See note below Table II for explanation of fractions.

**Table IV**

*Nuclease Activity of Bacillus subtilis under Aerobic and Anaerobic Conditions*

Nucleic acid, 3.2 per cent; pH as shown; time of incubation, 48 hours.

<table>
<thead>
<tr>
<th>pH</th>
<th>Total N</th>
<th>Total P</th>
<th>Inorganic P</th>
<th>Organic P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>6.0</td>
<td>10.06</td>
<td>9.86</td>
<td>5.92</td>
<td>4.75</td>
</tr>
<tr>
<td>6.6</td>
<td>15.62</td>
<td>15.45</td>
<td>9.19</td>
<td>8.17</td>
</tr>
<tr>
<td>7.6</td>
<td>12.99</td>
<td>12.75</td>
<td>7.64</td>
<td>7.49</td>
</tr>
<tr>
<td>8.2</td>
<td>9.44</td>
<td>9.40</td>
<td>5.43</td>
<td>5.47</td>
</tr>
</tbody>
</table>

*Bacillus subtilis* continues to disintegrate yeast nucleic acid equally well, whether it be subcultured from solutions of nucleic acid or from agar slopes. From colonies on an agar slope bacilli were transferred to a 4 per cent solution of yeast nucleic acid and
thereafter kept vigorous at $37.5^\circ$ by forty-five daily subcultures from each medium. The last generations were transferred to agar slopes, incubated at $37.5^\circ$ for 24 hours, and from them culture mixtures with yeast nucleic acid were made in the way described for pH 6.6. Each result in Table V was obtained from a separate culture mixture and so this table emphasizes the accuracy of the methods of fractionation as well as the reduplication of results.

Since yeast nucleic acid contains 16.3 per cent nitrogen and 9.63 per cent phosphorus, according to Levene and Bass ((8) pp. 274, 286), then the intensity of disintegration of nucleic acid by Bacillus subtilis may be stated in terms of mg. of nucleic acid broken down in 48 hours by multiplying the total nitrogen in the uranyl chloride supernatant fluid by 6.13 and by multiplying the total phosphorus therein by 10.38. When these calculations are made from values in Table III, it will be seen that about 130 mg. of nucleic acid were disintegrated in 48 hours and that the 130 mg. represented 80 per cent of the available nucleic acid.

**Table V**

<table>
<thead>
<tr>
<th>Time of incubation at $37^\circ$, days</th>
<th>Agent of nucleic acid disintegration</th>
<th>Inorganic P in uranyl chloride supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45th subculture from agar slopes</td>
<td>45th subculture from 4% yeast nucleic acid acid</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>0</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>3</td>
<td>2.388</td>
<td>2.382</td>
</tr>
<tr>
<td>6</td>
<td>4.088</td>
<td>3.840</td>
</tr>
<tr>
<td>9</td>
<td>4.580</td>
<td>4.680</td>
</tr>
<tr>
<td>Average at 9 days</td>
<td></td>
<td>4.653</td>
</tr>
</tbody>
</table>

Extent and Quality of Disintegration of Yeast Nucleic Acid by Bacillus subtilis—Since of the nitrogenous derivatives of nucleic acid, neutral lead acetate precipitates only nucleotides, the ratio of total nitrogen in Fraction 3 to that in Fraction 2 will indicate the proportion of nucleotides broken down after separation from nucleic acid. The breakdown of nucleotides can occur in
three ways: (1) PRN → P + RN, (2) PRN → P + R + N, (3) PRN → PR + N. All three ways are included in the foregoing calculation.

It will be seen that processes (1) and (2) necessitate the separation of phosphoric acid, and therefore the ratio of inorganic phosphorus in Fraction 2 to total phosphorus in the same fraction will indicate the proportion of nucleotides broken down in both these ways. The other process (3) can then be calculated as the difference of per cent hydrolysis estimated from values for phosphorus from that estimated from values for nitrogen. The results of these calculations are presented in Table VI. Comparison of columns (f) and (g) in Table VI shows that the nucleotides were disintegrated mainly to phosphoric acid ester of ribose and nitrogenous bases. This is particularly striking at alkaline pH values: at pH 8.2 the nucleotides were decomposed in this way only. It is quite possible that part of or all the inorganic phosphorus liberated at acid and neutral pH values may have been due to the hydrogen ion concentration independently of the bacteria, because phosphoric acid esters of ribose decompose

### Table VI

**Extent and Quality of Disintegration of Nucleotides by Bacillus subtilis**

Nucleic acid, 3.2 per cent; pH as shown; time of incubation, 48 hours.

<table>
<thead>
<tr>
<th>pH</th>
<th>Total N</th>
<th>Nucleotide hydrolysis</th>
<th>Total P</th>
<th>Inorganic P</th>
<th>Nucleotide hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction 2</td>
<td>Fraction 3</td>
<td>(a)</td>
<td>per cent</td>
<td>mg.</td>
</tr>
<tr>
<td>6.0</td>
<td>14.9</td>
<td>11.5</td>
<td>77</td>
<td>8.72</td>
<td>2.67</td>
</tr>
<tr>
<td>6.6</td>
<td>22.9</td>
<td>18.3</td>
<td>80</td>
<td>13.52</td>
<td>3.54</td>
</tr>
<tr>
<td>7.2</td>
<td>20.8</td>
<td>16.6</td>
<td>80</td>
<td>12.46</td>
<td>3.30</td>
</tr>
<tr>
<td>7.8</td>
<td>14.7</td>
<td>7.4</td>
<td>50</td>
<td>8.51</td>
<td>0.06</td>
</tr>
<tr>
<td>8.2</td>
<td>9.4</td>
<td>2.8</td>
<td>30</td>
<td>5.43</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\( c = (b/a) \times 100; f = (e/d) \times 100; g = c - f \).

\( c \) represents nucleotide hydrolysis of all three types; \( f \), nucleotide hydrolysis of both types yielding inorganic phosphorous; \( g \), nucleotide hydrolysis of the type yielding phosphoric acid ester of ribose and free nitrogenous base.

See note below Table II for explanation of fractions.
quickly ((8) p. 167). Subject to that possibility and to two limitations of the analytical methods, the process of disintegration of yeast nucleic acid by Bacillus subtilis may be written

\[
\text{Nucleic acid} \rightarrow 4(\text{PRN}) \rightarrow 4(\text{PR}) + 4(\text{N}) \rightarrow 4P + 4R + 4N
\]

The limitations of the methods are the failure to discriminate between pyrimidine and purine compounds and the lack of sugar analyses.

SUMMARY

1. Methods are described for the study of the process of disintegration of yeast nucleic acid.
2. These methods were applied to cultures containing yeast nucleic acid and each of three members of the aerobic, spore-bearing group of bacilli.
3. Bacillus subtilis, Bacillus mesentericus vulgatus, and Bacillus megatherium were found to be agents for a rapid, intensive disintegration of yeast nucleic acid, particularly at pH 6.6.
4. The extent of substrate disintegration by these bacteria was not influenced by growth in an environment of low oxygen tension.
5. The bacilli retain their disintegrative power whether subcultured many times from agar or from solutions of yeast nucleic acid.
6. A tentative scheme for the process of disintegration of yeast nucleic acid by Bacillus subtilis was deduced from the results of analysis.

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