TRIPOLYPHOSPHATE AND TRIMETAPHOSPHATE
IN YEAST EXTRACTS*

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Appreciable amounts of inorganic polyphosphates have been observed
in bacteria, plants, and lower animals (1). Pyrophosphate has been char-
acterized and several biosynthetic sources established (1), but the re-
mainder of the polyphosphates, called "metaphosphate," is still unidenti-
ified. Ebel (2) found evidence for the presence of low molecular weight
polyphosphates in a yeast polyphosphate fraction and concluded that the
latter is a mixture of molecular species ranging from triphosphate to higher
polymers. We reported briefly (3) on the identification by ion exchange
chromatography of two polyphosphates in yeast extracts, the linear
tripolyphosphate and the cyclic trimetaphosphate. We have since isolated
specific phosphatases which served to confirm the identification of these
triphosphates.

Enzymes which split inorganic triphosphates have been reported in ex-
tracts of several biologic materials (e.g. (4-7)). Neuberg and Fischer (4)
detected PP\textsuperscript{1} during the hydrolysis of tripolyphosphate by extracts of
Taka-Diastase, but, in studies of trimetaphosphate hydrolysis (5-7), only
P\textsubscript{i} production was reported. By separating trimetaphosphatase from yeast
almost free of triplyphosphatase activity, we have shown that the cyclic
triphosphate is enzymatically converted to tripolyphosphate. The lat-
ter is completely degraded to P\textsubscript{i} by a yeast triplyphosphatase which
we prepared free of trimetaphosphatase, but not free of PP\textsuperscript{ase}. In the
present paper, we wish to give the details of the characterization of tri-
ployphosphate and trimetaphosphate and to present the confirmatory
results obtained with the use of tripolyphosphatase and trimetaphos-
phatase.

Materials and Methods

Bakers’ yeast (Fleischmann) was used within a day or two of receipt
from the manufacturer. Sodium tripolyphosphate was a product of the

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Health, United States Public Health Service, and the National Science Foundation.
\footnote{The abbreviations used are PP pyrophosphate, P\textsubscript{i} orthophosphate, PP\textsuperscript{ase} pyro-
phosphatase, Tris tris(hydroxymethyl)aminomethane, TCA trichloroacetic acid,
ATP adenosine triphosphate; ADP, adenosine diphosphate.}
Blockson Chemical Company. Sodium trimetaphosphate was synthesized and generously furnished us by Dr. David Lipkin. Crystalline PPase was kindly furnished by Dr. G. Perlmann and Dr. M. Kunitz. The resins used were purchased from The Dow Chemical Company. Protamine sulfate was a gift from Eli Lilly and Company. Radioactive phosphate was obtained from the Oak Ridge National Laboratory.

$\Delta$P$_i$ was determined by the Fiske-Subbarow method (8). Acid-labile phosphate was determined as P$_i$ after hydrolysis for 15 minutes at 100° in 1 N H$_2$SO$_4$. Total phosphate included the P$_i$ liberated after the material was ashed with an H$_2$SO$_4$-HNO$_3$ mixture. Radioactivity measurements were made with a Tracerlab end window counter on 1 ml. samples, and spectrophotometric measurements were made with a Beckman DU spectrophotometer. Protein was measured by the method of Lowry et al. (9).

**Results**

**Purification of Enzymes**

Tripolyphosphatase—Preparations were assayed for tripolyphosphatase as follows. The assay mixture contained, in 1 ml., 100 µmoles of succinate buffer, pH 6.1; MgCl$_2$, 2 µmoles; PPase, about 30 units; tripolyphosphate, 0.5 µmole, and about 1 unit of enzyme. After a 15 minute incubation at 37°, 1 ml. of 7 per cent perchloric acid was added, the mixture was centrifuged, and P$_i$ determined on a suitable aliquot of the supernatant fluid. A blank tube, to which enzyme was added after the incubation, was run with some of the crude fractions. A unit of enzyme is defined as the amount producing 1 µmole of P$_i$ per hour.

The isolation operations were carried out at 0–3°. 50 gm. of bakers' yeast and 50 ml. of 0.1 M Tris buffer, pH 8.0, were stirred to a smooth suspension. After sonic disintegration for 90 minutes in a Raytheon 10 kc. oscillator, the débris was removed by centrifugation and the supernatant suspension was filtered through glass wool to remove floating fat. To the crude extract (55 ml., 636 units per ml., 15 units per mg. of protein) were added, with stirring, 27.5 ml. of 1 per cent protamine solution. After 5 minutes, the precipitate was centrifuged off; the supernatant fluid contained 390 units per ml. (75 per cent recovery). This activity was unchanged after storage at -15° for 1 month. To 5 ml. of the solution (1950 units), 30 ml. of water and 5 ml. of aluminum hydroxide gel C$_\gamma$ (15 mg. of solids per ml.) (10) were added. After 5 minutes, the suspension was centrifuged, the supernatant fluid discarded, and the gel washed twice with 10 ml. of 0.2 M succinate buffer, pH 6.1. The washes were discarded. The enzyme was then eluted with two 5 ml. portions of 0.02 M tripolyphosphate, pH 9.4, and, after removing the gel by centrifugation, the supernatant fluid was neutralized and stored overnight at -15° (1300 units, 70 per cent recovery).
After thawing, the solution was freed of tripolyphosphate by passing it through a column of Dowex 1 (10 per cent cross-linked, formate form, 1 × 1 cm.). The neutralized enzyme preparation contained 890 units (34 per cent yield from the crude extract; 91 units per mg. of protein). Assayed with trimetaphosphate as substrate, this preparation had 1.5 units of activity, or only about 0.6 per cent of the activity present in the supernatant fluid of the protamine step. However, the PPase activity of the preparation was still high.

Trimetaphosphatase—Preparations were assayed for activity as described for the tripolyphosphatase assays, except that the substrate used was trimetaphosphate (7 μmoles) and 2 to 7 units of tripolyphosphatase were added. The unit of enzyme is defined as above. The isolation procedures were carried out at 0-3°. Yeast extract was prepared by the same procedure as for the tripolyphosphatase, except that sonic oscillation was carried out for 40 minutes. 25 ml. of the extract were diluted with 25 ml. of water and 40 ml. of a saturated ammonium sulfate solution were added with stirring; after 5 minutes the mixture was centrifuged and the precipitate discarded. To the solution were added 25 ml. of saturated ammonium sulfate, and after 5 minutes the precipitate was collected by centrifugation and dissolved in 10 ml. of Tris buffer (pH 8.0, 0.1 M). The solution (Fraction AS) was faintly turbid and had 30 per cent of the activity of the crude extract. To 8.0 ml. of Fraction AS and 2.0 ml. of water, cooled to −2° in a −15° bath, 1.5 ml. of absolute ethanol at −15° were added. After 2 minutes the suspension was centrifuged and the precipitate discarded. This procedure was repeated twice, the second time with 3.0 ml. of ethanol. The third precipitate was suspended and rubbed up in 4.0 ml. of 0.1 M Tris buffer (pH 8.0), and the insoluble residue discarded after centrifugation. In Table I is a summary of the three steps which increased the purity of the trimetaphosphatase with respect to tripolyphosphatase 30-fold.

In Table II are given the results of an experiment comparing the release of Pᵢ from trimetaphosphate by trimetaphosphatase in the presence or ab-
sence of added tripolyphosphatase. It can be seen that only 13 per cent of the total phosphate was found as \( P_1 \) after incubation with trimetaphosphatase for 2 hours, and that, when tripolyphosphatase and PPase were added, 69 per cent was converted to \( P_1 \), suggesting that trimetaphosphatase action is a conversion of trimeta- to tripolyphosphate. Further evidence for this conclusion derived from chromatographic studies will be presented in a later section.

**Extraction of Triphosphates from Yeast**

Tripoly- and trimetaphosphate were isolated from yeast that was cultivated under conditions shown by Wiame (11) to lead to accumulation of inorganic polyphosphate by the cells. Yeast cells which had been incu-

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**Table II**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Trimetaphosphatase</th>
<th>Trimetaphosphatase, PPase</th>
<th>Trimetaphosphatase, tripolyphosphatase, PPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( P_1 ) ( \mu \text{mole} )</td>
<td>( P_1 ) ( \mu \text{mole} )</td>
<td>( P_1 ) ( \mu \text{mole} )</td>
</tr>
<tr>
<td></td>
<td>Hydrolysis ( % )</td>
<td>Hydrolysis ( % )</td>
<td>Hydrolysis ( % )</td>
</tr>
<tr>
<td>30</td>
<td>0.07</td>
<td>0.06</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>60</td>
<td>0.11</td>
<td>0.08</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>54</td>
</tr>
<tr>
<td>120</td>
<td>0.33</td>
<td>13</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>69</td>
<td>69</td>
</tr>
</tbody>
</table>

The incubation mixtures contained, in 0.5 ml., 125 \( \mu \)moles of succinate buffer pH 6.1; \( \text{MgCl}_2 \), 1 \( \mu \)mole; trimetaphosphate, 2.5 \( \mu \)moles; trimetaphosphatase, 3 units; and tripolyphosphatase and PPase as indicated.

bated for 16 hours in phosphate-free medium were placed in a phosphate-containing medium to which \( P^{32} \)-phosphate was added. Uptake of phosphate by the yeast was determined by estimating the radioactivity in aliquots of the medium after centrifuging off the cells. Under these conditions, i.e. 1.5 gm. of yeast (wet weight) per 50 ml. of medium (containing 500 \( \mu \)moles of \( P_1 \)), phosphate uptake was usually complete within 60 minutes, corresponding to an uptake of 0.33 \( \mu \)mole of \( P_1 \) per mg. of yeast.

After the disappearance of \( P^{32} \) from the medium, the suspension was centrifuged and the supernatant fluid discarded. Extracts of the cells were made with cold TCA or with hot water. In the first procedure, essentially that of Wiame (11), 5 ml. of cold 10 per cent TCA were added to the washed cells; the cells were suspended and stirred for 1 hour at 2°. The extract was obtained by centrifugation and the residue was washed with 5 ml. of 5 per cent cold TCA. The combined supernatant fluid and wash was extracted twice with 10 ml. of ether. After the aqueous solution was neutralized with KOH, the ether was volatilized with air, and the insoluble residue removed.
The hot water extract was prepared as follows: The cells, suspended in 10 ml. of distilled water heated to 90°, were stirred for 10 minutes in a 90° water bath and then cooled in an ice bath and centrifuged.

Tripoly- and trimetaphosphate were obtained from both the acid and hot water extracts by chromatography on Dowex 1 columns (2 per cent cross-linked, chloride form). The chromatography was carried out at 2°. A typical example of this procedure follows.

A 4 ml. aliquot of a water extract, which contained 178 \( \mu \)moles of phosphate and \( 89 \times 10^6 \) c.p.m., was adsorbed on a 6 \( \times \) 1 cm. column. After washing the column with 20 ml. of water, a solution of 0.02 N HCl and 0.10 M KCl was passed through at a rate of 0.65 ml. per minute. The eluate was collected in 13 ml. fractions with an automatic fraction collector and the radioactivity in each fraction was determined.

### Table III

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Ash P</th>
<th>Acid-labile P</th>
<th>Radioactivity</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu )mole per ml.</td>
<td>( \mu )mole per ml.</td>
<td>c.p.m. per ml.</td>
<td>260 ( \mu )</td>
</tr>
<tr>
<td>11</td>
<td>0.175</td>
<td>0.172</td>
<td>( 1.2 \times 10^4 )</td>
<td>0.042</td>
</tr>
<tr>
<td>12</td>
<td>0.318</td>
<td>0.308</td>
<td>( 2.2 \times 10^4 )</td>
<td>0.037</td>
</tr>
<tr>
<td>13</td>
<td>0.230</td>
<td>0.228</td>
<td>( 1.6 \times 10^4 )</td>
<td>0.030</td>
</tr>
</tbody>
</table>

**Tripolyphosphate**—P\(_1\) and PP were completely eluted in 16 resin bed volumes and comprised 22 per cent of the radioactivity. This was followed by a symmetrical peak (which will be called the tripolyphosphate zone) from 25 to 40 volumes, with the maximum at 33 volumes. The component of this zone was identified as tripolyphosphate in the following manner.

All of the phosphate was found to be acid-labile; there was virtually no ultraviolet absorption (Table III), indicating slight if any contamination by nucleotides.

An aliquot (18,000 c.p.m.) of one of the fractions from the tripolyphosphate zone was mixed with commercial sodium tripolyphosphate (76 \( \mu \)moles of total phosphate) and chromatographed as described for the yeast extract. Total acid-labile phosphate, a measure of the known tripolyphosphate, and radioactivity, a measure of the yeast tripolyphosphate, were determined in each fraction eluted. The behavior of the two compounds was almost identical (Fig. 1).

When commercial tripolyphosphate and a sample of radioactive tripolyphosphate isolated from yeast were hydrolyzed in the same incubation mixture by Taka-Diastase tripolyphosphatase (12), the amount of known tripolyphosphate converted to P\(_1\) was the same as the amount of the radio-
active compound that appeared in the P$_1$ region of the chromatogram (Table IV). As in the preceding experiment, there was good correspondence be-

![Figure 1. Chromatography of tripolyphosphate](image)

**Table IV**

*Taka-Diastase Tripolyphosphatase Action on Authentic and Yeast Tripolyphosphate*

The incubation mixture (17 ml.) contained potassium acetate buffer, pH 5.5, 850 μmoles; MgCl$_2$, 17 μmoles; Taka-Diastase tripolyphosphatase solution, 3.4 ml.; commercial tripolyphosphate, 51.3 μmoles of total P; and 12 ml. (about 250,000 c.p.m.) of a tripolyphosphate zone eluate from a chromatographed TCA extract of yeast. After 15, 30, or 60 minutes at 37°, aliquots were removed, set in a boiling water bath for 2 minutes, analyzed for P$_1$, and chromatographed as described in the text. 2 ml. mixtures were made up for zero time analyses.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>P$_1$ per cent</th>
<th>P$_{32}^{32}$ in P$_1$ zone per cent</th>
<th>c.p.m. per μmole P</th>
<th>P$_{32}^{32}$ in tripolyphosphate zone per cent</th>
<th>c.p.m. per μmole total P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.7</td>
<td>4.4</td>
<td>85</td>
<td>5000</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>30</td>
<td>69</td>
<td>4400</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>45</td>
<td>35</td>
<td>55</td>
<td>5100</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>80</td>
<td>83</td>
<td>17</td>
<td>4900</td>
<td></td>
</tr>
</tbody>
</table>

tween total P and P$_{32}^{32}$ in the tripolyphosphate region and in the P$_1$ and PP regions as well.

Further evidence supporting the identification of tripolyphosphate was hydrolysis by the specific tripolyphosphatase of yeast. Under conditions in which tripolyphosphatase completely hydrolyzed known tripolyphos-
phate, yeast tripolyphosphate marked with $^{32}$P was also completely hydrolyzed. Similarly, when 88 per cent of the recovered radioactivity appeared in the $P_i$ region (Fraction 3, Table V) of the chromatogram, 86 per cent of the initial phosphate appeared with it. 3 to 6 per cent of the remaining phosphate appeared in the pyrophosphate (Fractions 4 to 8) and tripolyphosphate (Fractions 11 to 17) regions. In this experiment the radioactivity of the phosphate in the incubation mixture was 3150 c.p.m. per $\mu$ mole,

**Table V**

**Yeast Tripolyphosphatase Hydrolysis of Tripolyphosphate**

The incubation mixture (5.0 ml.) contained succinate buffer, pH 6.0, 500 $\mu$ moles; MgCl$_2$, 10 $\mu$ moles; commercial tripolyphosphate, 139 $\mu$ moles of total $P$; tripolyphosphate zone eluate from a chromatographed hot water extract of yeast, about 440,000 c.p.m.; enzyme, 50 units. The control tube contained the same mixture in a 2.0 ml. volume; enzyme was added at the end of the incubation period. During a 90 minute incubation at 37$^\circ$, aliquots were removed for $P_i$ analysis. At 90 minutes, when 86 per cent of the phosphate appeared as $P_i$, 1 ml. was added to 1 ml. of 7 per cent perchloric acid, the mixture was neutralized and chromatographed as described.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>c.p.m. per ml.</th>
<th>$\mu$ moles $P$ per ml.</th>
<th>$c$.p.m. per $\mu$ mole $P$</th>
<th>c.p.m. per ml.</th>
<th>$\mu$ mole $P$ per ml.</th>
<th>c.p.m. per $\mu$ mole $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5850</td>
<td>1.85</td>
<td>3160</td>
<td>112</td>
<td>0.161</td>
<td>3300</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>*</td>
<td></td>
<td>112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>*</td>
<td></td>
<td>534</td>
<td>0.481</td>
<td>2820</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>*</td>
<td></td>
<td>1350</td>
<td>0.704</td>
<td>2800</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>*</td>
<td></td>
<td>1970</td>
<td>0.595</td>
<td>3120</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>*</td>
<td></td>
<td>1700</td>
<td>0.322</td>
<td>3110</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td></td>
<td></td>
<td>1000</td>
<td>0.065</td>
<td>2800</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td></td>
<td></td>
<td>182</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These values were zero or too low for accurate measurement.

and that of the $P_i$ recovered after chromatography of the incubation mixture was 3160.

**Trimetaphosphate**—Continuing the elution described above, a broad zone with a peak at 110 resin bed volumes appeared. An aliquot (84,000 c.p.m.) from this peak (trimetaphosphate zone) was mixed with synthetic trimetaphosphate and chromatographed as before. Over 90 per cent of the labeled phosphate appeared in seven fractions and over 90 per cent of the acid-labile phosphate added as known trimetaphosphate was found in these fractions; the radioactivity of the phosphate per micromole remained essentially constant.

A similar experiment was carried out in which the mixture of known and isolated radioactive trimetaphosphate was adsorbed on 10 per cent cross-linked Dowex 1 resin, instead of the 2 per cent resin used before, and eluted...
with 0.25 M KCl-0.005 M potassium acetate, pH 5.0 (13). As before, there was no separation of isolated and authentic trimetaphosphate.

Enzymatic hydrolysis of known trimetaphosphate was compared with the hydrolysis of isolated trimetaphosphate. Table VI shows that when 40 and 60 per cent of the P$^{32}$-labeled yeast trimetaphosphate were removed, there was an equivalent disappearance of labile phosphate from the trimetaphosphate zone. This disappearance of trimetaphosphate and P$^{32}$ from the trimetaphosphate zone is accounted for by the appearance of both

**Table VI**

**Trimetaphosphatase Action on Yeast Trimetaphosphate**

Three vessels contained, in 2.5 ml., succinate buffer, pH 6.0, 250 μmoles; MgCl₂, 5 μmoles; trimetaphosphate, 24 μmoles of total P; trimetaphosphate from yeast, about 25,000 c.p.m.; enzyme, 18 units. After incubation at 37°, and the addition of 1 ml. of 7 per cent perchloric acid and KOH to neutralize, the solutions were chromatographed as described in the text (column, 4 cm. X 1 cm.).

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>P₁-PP zone</th>
<th>Triopolyphosphate zone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labile P</td>
<td>P$^{32}$</td>
</tr>
<tr>
<td>0 μmoles or c.p.m.</td>
<td>2.57</td>
<td>3000</td>
</tr>
<tr>
<td>45 % total recovery*</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>90 μmoles or c.p.m.</td>
<td>2.40</td>
<td>2550</td>
</tr>
<tr>
<td>90 % total recovery*</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

* Total recoveries were 88, 88, and 75 per cent of the original labile P at 0, 45, and 90 minutes, respectively; of the radioactivity, 88, 87, and 80 per cent, respectively, were recovered. Losses were due to eliminating fractions in which labile P was too low for accurate determination. The specific activity, counts per minute per micromole of P, of all fractions measured was in the range, 1060 to 1300.

P$^{32}$ and phosphate as triplyphosphate and as P₁ or PP or both. The ratios of P$^{32}$ to labile phosphate in the P₁-PP fractions and in the triopolyphosphate fractions were the same as in the trimetaphosphate fractions.

**DISCUSSION**

It is difficult to determine whether compounds extracted from biological materials exist as such in the living cell. Thus, it is possible that triplyphosphate and trimetaphosphate are fragments of larger molecules split during the course of extracting the yeast with cold acid or with hot water. For example, phospho triester linkages or bonds of comparable lability would be ruptured to a considerable extent under such conditions. Evidence for the natural occurrence of triplyphosphate is the finding (3) of the enzymatic
production of radioactive tripolyphosphate when ATP and radioactive PP are incubated with yeast autolysate. The detailed mechanism of the biosynthesis of tripolyphosphate is not yet known.

Tripoly- and trimetaphosphate do not show the properties of metachromasy and barium precipitability at acid pH which are characteristic of the fraction described by Wiame (11) as “metaphosphate.” In the water and acid extracts which we chromatographed, about 20 per cent of the phosphates was found in the P₁ zone, 10 to 20 per cent in the tripolyphosphate region, and 3 to 4 per cent in the trimetaphosphate region. About 50 per cent of the phosphates was held on the columns and probably contained metachromatic higher phosphate polymers. We observed little if any tripoly- or trimetaphosphate in the insoluble “metaphosphate” prepared according to Wiame.

**SUMMARY**

1. Tripolyphosphate (a linear triphosphate) and trimetaphosphate (a cyclic triphosphate) were identified in hot water and in trichloroacetic acid extracts of bakers’ yeast.

2. A tripolyphosphatase and a trimetaphosphatase were partially purified from bakers’ yeast, and were used in the identification of the respective polyphosphates. Tripolyphosphate was shown to be the product of the action of trimetaphosphatase on trimetaphosphate.

**BIBLIOGRAPHY**


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