THE STRUCTURE OF THE ADENOSINE TRIPHOSPHATE OF THIOBACILLUS THIOOXIDANS

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The adenosine triphosphate of *Thiobacillus thiooxidans* is of special interest because LePage and Umbreit (1943b) presented evidence that it differs from the adenosine triphosphate of other organisms by having the phosphate groups attached to the 3' instead of the 5' position of adenosine. All other bacteria and higher organisms so far examined contain adenosine-5'-triphosphate (5'-'ATP). Since diphosphopyridine nucleotide, triphosphopyridine nucleotide (Kornberg, 1950), flavin adenine dinucleotide (Schrec-ker and Kronberg, 1950), and coenzyme A (Novelli, 1953) are known to be formed from adenosine-5'-triphosphate in such a way as to preserve the original ribose-phosphate linkage, the existence of an unusual type of adenosine triphosphate in thiobacilli would imply also the presence of new types of these and probable other nucleotides.

Because of the theoretical importance of an unusual type of adenosine triphosphate, a re-investigation of the compound present in *T. thiooxidans* seemed desirable, particularly in view of the fact that substantial improvements in the techniques of isolation and characterization of nucleotides have been made in recent years. Our results show that *T. thiooxidans* contains small amounts of adenosine-5'-triphosphate and relatively large amounts of inorganic polyphosphate. No evidence for the occurrence of adenosine-2' or 3'-triphosphate was obtained.

MATERIALS AND METHODS

*Thiobacillus thiooxidans* was obtained from Dr. R. L. Starkey. The culture medium was that used by LePage and Umbreit (1943a) in their study of the structure of the bacterial ATP. The organism was grown at 30 C in the medium described by Starkey (1925) with sulfur as an energy source and under conditions similar to those used by Umbreit et al. (1942). Sixty liters of medium were distributed among 200 one liter Erlenmeyer flasks. To increase the rate of growth, the carbon dioxide partial pressure in the incubator room was maintained at 2 to 4 mm Hg. After 7 to 11 days of incubation the pH of the medium had decreased to 1.2 to 1.5. The medium was then cooled with ice to 10 C and filtered through cotton to remove residual sulfur. The cells were harvested with a Sharples centrifuge, washed twice with a cold solution containing 0.05 m KCl and 0.05 m NaCl, and finally resuspended in 15 ml of water. In a typical experiment approximately 1.7 g of dry weight of cells (176 mg total N) containing 775 μg of total phosphorus and 27 μg of orthophosphate were recovered from 60 liters of medium.

In most experiments, ATP and other soluble compounds were extracted by treating the cells with 3.5 per cent (w/v) perchloric acid for 60 minutes at 0 C. A quantity of 5'-ATP not exceeding 5 per cent of the total ATP was added as marker during the isolation. After centrifugation, the cells were reextracted twice with one-third the volume of the same reagent, the combined extracts were immediately neutralized with 5 N KOH, and after standing overnight at 2 C, the precipitated KClO₄ was removed by centrifugation. Approximately 96 per cent of
the 5'-ATP\textsuperscript{32}P was recovered in the extraction procedure. The nucleotides in the extract (35 ml) were concentrated by adsorption on an acid washed charcoal column (Nuchar 190, 5 cm\textsuperscript{2} by 2 cm) and elution with 50 per cent (v/v) acetone-water containing 0.2 per cent (v/v) of 14.8 M NH\textsubscript{4}OH. Eluate fractions containing 5'-ATP\textsuperscript{32}P were combined, most of the acetone was removed with a stream of helium, and the solution was concentrated to 5 ml by lyophilization. Dowex-1, 2 per cent cross linked, 200–400 mesh, in the chloride form, was used for column chromatography of ATP and related materials according to the method of Cohn and Carter (1950).

5'-ATP labeled with P\textsuperscript{32} in the two terminal positions was prepared from A-5'-P and P\textsuperscript{32} labeled orthophosphate using mouse liver mitochondria by the method of Kielley and Kielley (1951). The 5'-ATP\textsuperscript{32}P was freed of A-5'-P and 5'-ADP by chromatography on a Dowex-1 chloride column and was concentrated by adsorption and elution from charcoal as described above. The initial specific activity of the ATP\textsuperscript{32}P was of the order of 10 microcuries per micromole.

A commercial preparation of adenosine-5'-phosphate was used that gave a quantitative yield of orthophosphate by hydrolysis with purified bull semen 5'-nucleotidase kindly supplied by Dr. L. Heppel (Heppel and Hilme, 1951b). Adenylic acids "a" and "b" were also obtained from Dr. Heppel. P\textsuperscript{32} labeled A-5'-P was prepared from adenosine, and P\textsuperscript{32} labeled 5'-ATP by the use of a purified preparation of adenosine kinase (Kornberg and Pricer, 1951).

5'-ATP was estimated enzymatically as described by Kornberg (1950); adenine by ultraviolet absorption at 260 m\textmu; orthophosphate, total phosphorus, easily hydrolyzable phosphate (P\textsubscript{T}), and total nitrogen by the methods described by Umbreit et al. (1949). Inorganic pyrophosphate was estimated as orthophosphate after hydrolysis with a purified preparation of yeast inorganic pyrophosphatase supplied by Dr. Heppel (Heppel and Hilme, 1951a).

**EXPERIMENTAL RESULTS**

*Fractionation of phosphate compounds by barium precipitation.* The cells used for this fractionation contained 515 \mu\textmu; total P and 16 \mu\textmu; orthophosphate per gram dry weight compared with 650 \mu\textmu; total P and approximately 80 \mu\textmu; orthophosphate in the bacteria used by LePage and Umbrie (1943a). These investigators prepared a trichloroacetic acid extract of the bacteria, following a preliminary alkali treatment, and fractionated the extract to give (I) a barium-insoluble (pH 8.4) fraction, (II) a barium-soluble (pH 8.4) alcohol-insoluble fraction, and (III) a barium-soluble, alcohol-insoluble fraction. Only about 3 per cent of the acid soluble organic P was found in fraction III.

We used the same method of extraction and fractionation except that an additional fraction (IA) insoluble in the presence of barium ion at pH 4 was collected before the precipitation with barium ion at pH 8.2 (fraction IB). It should be noted that part of the barium salts precipitated at both pH 4 and pH 8.2 could not be redissolved in cold 0.2 n HCl even after long stirring. This accounts for the acid-soluble and acid-insoluble subfractions from each of these precipitations. In order to determine where 5'-ATP appears in this fractionation procedure, 1.5 \mu\textmu; of 5'-ATP\textsuperscript{32}P were added to the TCA extract. The presence of 5'-ATP\textsuperscript{32}P in the fractions was determined by P\textsuperscript{32} assay.

Analytical data on the initial TCA extract and the fractions derived therefrom are given in table 1. The data show that approximately 78 per cent of the TCA soluble P is present in compounds converted to orthophosphate by the usual 7 minute hydrolysis in 1 n HCl at 100 C. Comparison of the total P\textsubscript{T} with the total adenine in the fractions examined shows that the ratio is 9.3 \mu\textmu; P\textsubscript{T} per \mu\textmu; adenine. Since the corresponding ratio in ATP is 2, most of the P\textsubscript{T} in the extract must be in a form other than ATP.

In the fractionation of the phosphorus compounds, approximately 87 per cent of the P precipitated in the barium-insoluble fraction at pH 4 (fraction IA). This is indicative of the presence of inorganic poly- or metaphosphate (Jones, 1942; Mann, 1944). The relative insolubility of the barium precipitate in dilute acid is characteristic of some types of metaphosphate.

Fractions IA and IB were tested for pyrophosphate with yeast inorganic pyrophosphatase. Before applying the test to fraction IA, barium ion was removed by passing the solution through a Dowex-50 (acid form) column. Fraction IB was tested without removal of barium. No pyrophosphate (determined by the action of...
inorganic pyrophosphatase) could be detected in either fraction. Suitable control experiments demonstrated that the enzyme was active in the presence of both fractions.

The solubility characteristics of the phosphorus compound(s) in fraction IA, along with the absence of pyrophosphate, indicated that it consists of a polymetaphosphate. This conclusion was further supported by the use of the metachromasy test developed by Wiame (1947b). An aliquot of fraction IA was freed of salt, which interferes with the test, by precipitating

the phosphorus compound with lead acetate, decomposing the lead salt with hydrogen sulfide, and removing the lead sulfide by centrifugation. When an aliquot of the resulting clear solution containing 0.4 μM of P₇ was used in the metachromasy test, the ratio of the optical density at 530 με to that at 630 με was 0.235, compared to 0.125 in a control without phosphate, and 0.467 in a test solution to which 0.2 μM of synthetic metaphosphate (Jones, 1942) was added. The positive result indicates the presence of metaphosphate although on a molar basis the material derived from the bacteria is only about 20 per cent as active as synthetic metaphosphate in the metachromasy test.

The data of table 1 on the distribution of P₇ added as 5'-ATP added indicate that the barium fractionation procedure does not result in a satisfactory separation of the small amounts of 5'-ATP present in the TCA extract. 5'-ATP would be expected to appear in fraction IB. However, most of the 5'-ATP was found in fractions IA and II. Its presence in fraction IA can be attributed to co-precipitation on the bulky precipitate of barium metaphosphate. The presence of 5'-ATP in the barium-soluble alcohol-insoluble fraction II must be the result of incomplete precipitation of the small amount of ATP in the absence of alcohol (fraction IB). The total quantity of 5'-ATP in fraction II, estimated by the hexokinase-glucose-6-phosphate dehydrogenase test, was 1.56 μM, a large part of which can have been derived from the added 5'-ATP (1.49 μM).

LePage and Umbreit (1943a) reported that dried cells of T. thiooxidans contain approximately 32 μM of TCA-extractable ATP per gram dry weight of cells. Their value is based mainly upon determination of easily hydrolyzable P. For comparison we have calculated the maximum possible ATP content of our TCA extract from the P₇ and adenine data of table 1, on the assumption that all the light absorption at 260 mμ is due to adenine and all the adenine is associated with two equivalents of P₇ in the form of ATP. This gives a value of approximately 10 μM of ATP per gram of dry cells. Since not all of the 260 mμ absorption can be due to adenine and only part of the adenine can be in ATP, the actual ATP content of our cells must be considerably below the maximum value given above.

### TABLE 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>μM*</th>
<th>P/Per cent of Total in Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA soluble, total</td>
<td>397</td>
<td>51</td>
</tr>
<tr>
<td>TCA soluble, P₇</td>
<td>311</td>
<td>40</td>
</tr>
<tr>
<td>TCA soluble, Pᵢ</td>
<td>39</td>
<td>5</td>
</tr>
<tr>
<td>TCA insoluble, total</td>
<td>377</td>
<td>49</td>
</tr>
<tr>
<td>Total P in cells</td>
<td>774</td>
<td>100</td>
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<table>
<thead>
<tr>
<th>Fractions of TCA soluble P†</th>
<th>P₇</th>
<th>Pᵢ</th>
<th>P₇/&quot;Adenine&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA Barium insoluble, pH 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid soluble</td>
<td>101</td>
<td>13.1</td>
<td>1.80</td>
</tr>
<tr>
<td>Acid insoluble</td>
<td>105</td>
<td>3.20</td>
<td>0.59</td>
</tr>
<tr>
<td>IB Barium insoluble, pH 8.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid soluble</td>
<td>6.9</td>
<td>1.40</td>
<td>0.29</td>
</tr>
<tr>
<td>Acid insoluble</td>
<td>7.8</td>
<td>0.60</td>
<td>0.07</td>
</tr>
<tr>
<td>II Barium soluble-alcohol insoluble</td>
<td>15.5</td>
<td>7.44</td>
<td>4.49</td>
</tr>
<tr>
<td>III Barium soluble-alcohol insoluble</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>236.2</td>
<td>25.7</td>
<td>7.32</td>
</tr>
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</table>

* Quantities in 1.5 g of dry cells.
† Sample containing 328 μM total P.
‡ Calculated from the optical density at 260 με.

The fractionation methods are described in the text.
TABLE 2

Isolation of bacterial ATP

A washed suspension containing approximately 1.5 g dry weight of *Thiobacillus thiooxidans* and 480 μm total P was extracted twice for 90 minutes with cold 3.5 per cent PCA after the addition of 0.26 μm muscle ATP. The extract was neutralized with 5 N KOH, and after standing overnight at 0°C the precipitated KClO₄ was removed by centrifugation. The supernatant solution (fraction A) contained 85 per cent of the added ATP. The extract was acidified to pH 2 and passed through a 5.3 cm² by 1.2 cm column of acid washed charcoal which was washed with water (fraction B), and the nucleotides were eluted with 50 per cent acetone-0.2 per cent (v/v) NH₄OH. Most of the acetone was removed by aeration with helium, the remaining solution was adjusted to pH 8 by addition of acetic acid, concentrated by lyophilization (fraction C), and the residue was chromatographed on a 0.28 cm² by 6 cm Dowex-1 chloride, 2 per cent cross linked, 200-400 mesh, column. The eluting solvent was 0.01 N HCl plus 0.07 N KCl. Figure 1 gives the elution curve. Fractions 11 to 16 inclusive were combined and neutralized to pH 7 (fraction D). The figures in the table represent observed quantities uncorrected for losses, e.g., caused by removal of samples for analysis.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>ATP*</th>
<th>%</th>
<th>%</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA extract</td>
<td>32</td>
<td>1.9</td>
<td>100</td>
<td>100</td>
<td>0.9</td>
</tr>
<tr>
<td>Charcoal wash</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Charcoal eluate</td>
<td>4.3</td>
<td>0.98</td>
<td>52</td>
<td>58</td>
<td>3.8</td>
</tr>
<tr>
<td>Dowex-1 eluate</td>
<td>15.5</td>
<td>0.47</td>
<td>28</td>
<td>36</td>
<td>0.56</td>
</tr>
</tbody>
</table>

* Estimated by the hexokinase-myokinase-glucose-6-phosphate dehydrogenase test.
† Estimated from the OD at 260 μm without correction for other ultraviolet absorbing compounds.

Isolation of bacterial ATP. In order to avoid various difficulties encountered with the TCA extractions and barium fractionation procedures, we have used perchloric acid (PCA) extraction, charcoal adsorption, and elution of nucleotides, and column chromatography on Dowex-1 chloride for the isolation of bacterial ATP. PCA is preferable to TCA because it permits the estimation of adenine by the spectrophotometric method in the original extracts. Adsorption and elution from acid washed charcoal give a satisfactory recovery of nucleotides and result in removal of salts and other materials which interfere with subsequent procedures. Chromatography on Dowex-1 gives a large purification of ATP in a single step.

Data of Table 2 show that the PCA extract from 1.5 g cells contained at most 6.9 μM of adenine. This sets an upper limit for the amount of ATP. Actually, the true adenine and ATP content must have been considerably smaller since other compounds absorbing at 260 μM were undoubtedly present in the extracts. The amount of 5′-ATP in the perchloric acid extract, estimated by enzymatic assay, was 1.9 μM. Approximately 0.22 μM of this represented 5′-ATP added to the extract; the remainder was of bacterial origin. The lyophilized charcoal eluate contained 55 per cent of the 5′-ATP of the extract, by enzymatic assay, and almost the same percentage of the added 5′-ATP.

The fractionation of charcoal eluate on a Dowex-1 column is shown in figure 1. The optical densities of the eluate fractions indicate the presence of at least three major components. The initial high optical density which reaches a peak...
in fraction 3 undoubtedly represents a mixture of compounds, such as adenosine and adenylic acid, that contain not more than one phosphate group. Such compounds move rapidly down a Dowex-1 chloride column with the eluant used. A second, lower optical density peak appears in fraction 8. This is the region of the chromatogram in which diphasate esters such as ADP should be eluted. A P⁳₂ peak is also found in this region, indicating that a little of the added 5'-ATP³₂ was converted to 5'-ADP³₂.

The third optical density peak coincides with the main P⁳₂ peak in fraction 15. This obviously represents the elution of 5'-ATP³₂ and bacterial ATP. The zone from fractions 11 to 16 should include 3'-ATP, if present, as well as 5'-ATP since the rate of elution is determined primarily by the number of negative charges on the phosphate groups. From the P³₂ content of the ATP zone it can be estimated that 0.37 μM or 79 per cent of the 0.47 μM of ATP active in the hexokinase-glucose-6-phosphate dehydrogenase test must have been derived from the bacteria.

**Identification of adenosine-5'-triphosphate.** The identity of the bacterial ATP in the Dowex-1 eluate fractions 11 to 16 was determined in several ways. Comparison of the quantity of ATP, determined enzymatically, with the optical density at 260 μm indicates that at least 84 per cent of the adenosine is present in a compound that transfers two phosphate groups to glucose under the influence of yeast hexokinase and myokinase. This suggests that most of the ATP is 5'-ATP. However, since it is possible that 3'-ATP would also serve as a phosphoryl donor under the influence of yeast hexokinase, more definitive evidence concerning the structure of the ATP was sought. An aliquot of fractions 11 to 16 containing 0.43 μM of ATP was converted to AMP by the use of hexokinase, myokinase, and glucose-6-phosphate dehydrogenase; 0.87 μM of TPN was reduced in the reaction. The A-5'-P content of the reaction mixture was determined after heating for 2 minutes at 100°C to inactivate the enzymes by means of purified 5'-nucleotidase. The yield of orthophosphate, determined with an accuracy of ±10 per cent on a sample containing 0.0086 μM of AMP, was 112 per cent of the theoretical value to be expected from the observed TPN reduction in the enzymatic assay for 5'-ATP. Since neither the 2'- nor 3'-adenylic acid is hydrolyzed by 5'-nucleotidase, the results demonstrate that the main or possibly the only product of enzymatic dephosphorylation of bacterial ATP is A-5'-P. This means that the bacterial ATP, which phosphorylates glucose under the influence of yeast hexokinase, is 5'-ATP.

Further confirmation of the identity of the AMP resulting from enzymatic dephosphorylation of bacterial ATP was obtained by chromatographing it with adenosine-2'-phosphate (adenylic acid “a”), adenosine-3'-phosphate (adenylic acid “b”), and a tracer amount of P³₂-labeled A-5'-P on a Dowex-1 formate column, developed with 0.1 N formic acid plus 0.03 M ammonium formate. The bacterial AMP was found to move with the A-5'-P band which was well separated from the bands of the other two adenosine monophosphates.

**DISCUSSION**

*T. thiooxidans* is shown to contain relatively large amounts of an easily hydrolyzable inorganic phosphate compound that is probably a polyphosphate. Polypolynucleotides have not previously been recognized in this organism although the high levels of easily hydrolyzable phosphate observed by LePage and Umbreit (1943a) in TCA extracts of *T. thiooxidans* and the necessity of pretreatment of the bacteria with alkali to release acid soluble phosphorus suggest the presence of these compounds. Polynucleotides have been reported to be present in yeast (Wiam, 1947a; Juni et al., 1948), fungi (Malagreen, 1952; Houlahan and Mitchell, 1948), heterotrophic bacteria (Ebel, 1949), and green algae (Sommer and Booth, 1938); and they may occur in most microorganisms.

The ATP content of *T. thiooxidans* observed by us was 1.3 to 2.5 μM per gram of dry cells, determined by enzymatic assay. LePage and Umbreit (1943a) report a much higher value of 31.5 μM per gram, determined as easily hydrolyzable phosphate in the barium-insoluble fraction. Since both observations were made on the same strain of *T. thiooxidans* grown under similar conditions, it is unlikely that the striking difference in ATP levels can be attributed to the organism. A more probable explanation is that the calculated ATP values of LePage and Umbreit are too high because of the presence of polyphosphate in the samples used to determine easily hydrolyzable phosphate.

The bacterial ATP which we have partially purified by charcoal absorption and Dowex-1...
chromatography is undoubtedly adenosine-5'-triphosphate. This is proven by its enzymatic conversion to adenosine-5'-phosphate, identified by hydrolysis with a highly specific 5'-nucleotidase and by column chromatography on Dowex-1 formate.

Our data do not provide any evidence for the occurrence of adenosine-3'-triphasate in T. thiooxidans although they do not exclude the presence of a small amount of this compound. The charcoal adsorption and elution used for concentrating nucleotides present in the PCA extract are nonspecific and should not result in preferential accumulation of 5'-ATP unless 3'-ATP were so labile as to be extensively decomposed during this procedure. We endeavored to avoid decomposition by working rapidly and keeping the temperature at or below 3 C. The first and only intentional fractionation of the nucleotides was done on the Dowex-1 chloride column. Here again the possibility of preferential hydrolysis of an unusual type of ATP cannot be excluded although it appears unlikely in view of the fact that LeFage and Umbreit (1948a) exposed their product to 10 per cent TCA for 12 hours at 0 to 5 C in the course of isolation. Our material was exposed to 0.01 n HCl for 5 to 8 hours at 3 C during chromatography. If 3'-ATP is sufficiently stable to withstand these conditions, it should appear in or near the same region of the Dowex-1 chromatogram as 5'-ATP (figure 1, fractions 11 to 16). Actually 84 per cent of the adenine in this region was accounted for as 5'-ATP. After making a correction for the added 5'-ATP, it can be estimated that not more than 20 per cent of the adenine of bacterial origin in this region of the chromatogram can be present in a compound other than 5'-ATP.

It is possible, though unlikely, that an unusual type of ATP might move more rapidly than 5'-ATP on a Dowex-1 column and be eluted earlier in the ADP region (figure 1, fractions 6 to 10). Unfortunately, we did not examine the adenine nucleotides in this region to determine whether they were derivatives of A-5'-P or another type of adenylic acid. However, even if all the adenine in this region were present in an unusual type of ATP, figure 1 shows that the quantity would be considerably less than that found in 5'-ATP.

In conclusion, our data indicate that adenosine-5'-triphosphate is the main if not the only type of ATP present in T. thiooxidans.

**SUMMARY**

The adenosine triphosphate of *Thiobacillus thiooxidans* has been partially purified by charcoal adsorption and elution, and by column chromatography on Dowex-1 chloride. At least 80 per cent of the bacterial ATP, which moves with 5'-ATP on a Dowex-1 column, was identified as adenosine-5'-triphosphate by converting it with hexokinase and myokinase to adenosine-5'-phosphate and identifying the latter by dephosphorylation with a specific 5'-nucleotidase and by column chromatography on Dowex-1 formate. No evidence was obtained for any other type of ATP. Most of the acid soluble phosphorus was present in an easily hydrolysable compound tentatively identified as inorganic polyphosphate.

**REFERENCES**


ADENOSINE TRIPHOSPHATE OF T. THIOOXIDANS 661


