URIDINE-5' PYROPHOSPHATE DERIVATIVES
I. ISOLATION FROM STAPHYLOCOCCUS AUREUS

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The first evidence for the existence of uridine diphosphate compounds was limited to the observation that Staphylococcus aureus cells, when grown in the presence of penicillin, accumulated a new form of acid-labile phosphate in their internal environment (1). The labile phosphate appeared to be part of a complex compound which also contained uracil. In a brief note (2), it has since been reported that this material contains at least three compounds, all of which contain uracil, two phosphate groups, pentose, and an unknown sugar. Two of these compounds were shown to contain amino acids. The purpose of this paper is to describe the methods used for the production and purification of these compounds, with particular attention to the use of phenol in partition chromatography. Papers II and III deal with chemical characterization of the compounds (3).

EXPERIMENTAL

Production of Compounds—The labile phosphate compounds accumulate rapidly in S. aureus after the addition of penicillin (1). Apparently the growing cells are able to synthesize the compounds only for the short period they remain viable after the addition of penicillin (4). This period is 30 to 45 minutes in length. Glucose must be used in order to obtain rapid synthesis.

In order to obtain maximum yield per liter of culture, growth was allowed to proceed until about one-half the maximum population was reached before addition of penicillin. The amount of penicillin required to demonstrate labile phosphate accumulation is approximately that concentration needed to prevent multiplication of this organism; that is, about 0.1 unit of penicillin G per ml. However, 0.5 unit or more of penicillin per ml. was used routinely because a larger accumulation of labile phosphates results. Thus, quantities of S. aureus were produced and treated with penicillin in the following manner, which is based on a consideration of the above factors. A medium composed of 1 per cent Basamin-Busch yeast preparation (Anheuser-Busch) and 0.1 per cent K$_2$HPO$_4$ was adjusted to pH 7.1 to 7.2 and sterilized. After the medium had cooled to the incubation temperature of 37°, sterile glucose solution was added to make the
final concentration of glucose 0.2 per cent, and an inoculum of 1 per cent by volume of a 10 to 12 hour culture of *S. aureus* H was introduced. The inoculated culture was aerated to obtain rapid growth, but the rate of aeration was not critical. Lard oil was used as an antifoam agent when required.

Growth was observed indirectly by measurement of pH. When the pH dropped to 6.1 or 6.2, the population was approximately half maximum. The time required to reach this point was usually between 2.5 and 3.5 hours. When the point of half maximum growth was reached, 0.5 unit of penicillin was added per ml. of culture. 30 minutes after the addition of penicillin, harvesting of the cells with a Sharples centrifuge was begun.

The yield was about 2 gm. of packed cells, roughly 30 per cent dry matter, per liter of culture. Approximately 8 to 9 μM of labile phosphate were present per gm. of packed cells. Of this, about 60 per cent was represented by the uridine-5'-pyrophosphate compounds which had accumulated after the addition of penicillin. Thus, about 10 μM of uridine-5'-pyrophosphate compounds were present in the penicillin-treated cells from 1 liter of culture.

Five criteria were used in the following purification.

1. **Micromoles of Labile Phosphate Per Mg. of Dry Weight**—Labile phosphate was the principal basis for comparison, since for some time this was the only known characteristic of the compounds which accumulated in penicillin-treated *S. aureus*. In crude preparations, the labile phosphate content was measured by determination of the amount of orthophosphate released when the sample, contained in 1 ml. of 1 N HCl, was heated in a boiling water bath for 10 minutes. The short hydrolysis period roughly compensated for the phosphate split from the more stable forms of organic phosphate present in crude preparations. With purified preparations, in which less allowance was necessary to correct for liberation of orthophosphate from the "stable" forms of phosphate present, hydrolysis for 12 to 14 minutes was used in the estimation of labile phosphate. In this time, 98 to 99 per cent of the labile phosphate is split and about 1 per cent of the stable phosphate is hydrolyzed. The inorganic phosphate was determined colorimetrically with the Fiske and Subbarow reagents (5).

2. **Stable Phosphate Per Labile Phosphate**—Stable phosphate is defined as total phosphate less inorganic phosphate and labile phosphate. Samples for total phosphate were digested by the method of Johnson (6), cooled, mixed with 1 ml. of water, and then heated in a boiling water bath for 15 minutes to hydrolyze the inorganic pyrophosphate before analysis. The compounds of interest contain one stable phosphate per mole.

3. **Nitrogen Per Labile Phosphate**—Total nitrogen was determined by nesslerization according to the method of Johnson (6).
4. Uridine Per Labile Phosphate—Uridine derivatives were determined with the model DU Beckman spectrophotometer by measuring the optical density at 262 mµ. The amount of compound was calculated from the molecular absorption coefficient for uridine of 9900 (7). In purified preparations, uridine derivatives were the only ultraviolet-absorbing materials detectable (3).

5. Potential Reducing Power Per Labile Phosphate—Though the intact molecules are non-reducing, a reducing substance is liberated by 0.1 N HCl in 3 minutes at 100°. The reducing power is measured by the reduction of ferricyanide ions (8), and the results are expressed as glucose equivalents. The unknown reducing substance has only about 0.8 of the reducing power of glucose.

Purification of Compounds—The purification procedure used consisted of four steps: (1) extraction with trichloroacetic acid (TCA), (2) fractionation of the barium salts with ethanol, (3) removal of basic impurities with cation exchange resin, and (4) separation of the three components by partition chromatography.

Extraction—The packed cells were suspended in 1 volume of ice-cold water, and then 1 volume of ice-cold 10 per cent TCA was added. The cell suspension was centrifuged and the residue washed with 5 per cent TCA. The combined supernatant solutions were extracted twice with ether to remove TCA.

To the extract from 100 gm. of packed cells, 2.5 gm. of barium hydroxide and sufficient NaOH were added to adjust the pH to 9. The precipitate was removed by centrifugation and washed with 5 per cent TCA. The combined supernatant solutions were extracted twice with ether to remove TCA.

The uridine-5'-pyrophosphates were then precipitated from the concentrated solution, which contained 25 to 30 μM per ml., by the addition of 4 volumes of absolute ethanol. This precipitate, when washed with ethanol and ether and dried in vacuo at room temperature, usually contained from 0.25 to 0.4 μM of mixed uridine-5'-pyrophosphate derivatives per mg.

Ethanol Fractionation—At this stage the material contains about 18 atoms of nitrogen and 3 to 4 moles of stable phosphate per mole of labile phosphate present. The material can be purified considerably by fractional precipitation with ethanol. From a solution containing about 8 μM of labile phosphate per ml., roughly 80 per cent of the labile phosphate is precipitated as the ethanol concentration is raised from 50 to 80 per cent by volume. Table I lists data on several preparations at this stage of purification, illustrating the reproducibility of the yield and purity of
the preparations. It is seen that the preparations still contain considerably more than one stable phosphate per mole of labile phosphate and that the potential reducing power per labile phosphate is high compared with the 0.8 found in the purest preparations.

Use of Cation Exchange Resin—By passage of a solution of the material through cation exchange resin, some impurities were removed. The

| TABLE I |
|-----------------|-----------------|-----------------|-----------------|
| Culture volume* | Weight of wet packed cells | Dry weight of preparation | Labile P | Stabile P per labile P | Potential reducing power per labile P |
| liters | gm. | gm. | μm per mg. | |
| 30 | 55 | 1.01 | 0.29 | 2.3 | 1.07 |
| 30 | 50 | 0.92 | 0.38 | 1.9 | 0.98 |
| 30 | 43 | 0.80 | 0.38 | 1.8 | 0.91 |
| 60 | 100 | 1.50 | 0.41 | 1.6 | 0.91 |
| 60 | 87 | 1.58 | 0.42 | 1.7 | 0.95 |
| 90 | 125 | 1.68 | 0.42 | 1.8 | 0.97 |

* The medium contained 0.5 per cent peptone, 0.5 per cent yeast extract, 0.1 per cent glucose, and 0.03 per cent K₂HPO₄.

| TABLE II |
|-----------------|-----------------|-----------------|-----------------|
| Use of Cation Exchange Resin for Purification of Labile Phosphate Preparations |
| Sample | Stable P per labile P | Nitrogen per labile P | "Uridine" per labile P | Labile P recovered per cent |
| Untreated* | 1.40 | 10.1 | 1.23 |
| Treated (Experiment 1) | 1.12 | 6.35 | 1.09 | 91 |
| " ( Experiment 2) | 1.15 | 6.36 | 1.09 | 92 |

11 to 13 mg. of the sample in 1 ml. of water were washed through a 50 mm. × 5 mm. column of cation exchange resin, IR-100, with 2 ml. of water.

* The preparation used in this experiment had been fractionally precipitated twice with ethyl alcohol.

resins used were Amberlite IR-100 or IR-105 (Rohm and Haas). As shown in Table II, over 60 per cent of the stable phosphate and ultraviolet-absorbing substances, and much of the nitrogenous materials which were present as contaminants, were removed.

Our experience during the preparation of a quantity of the material will illustrate the results obtained from these procedures. The combined extracts from about 5 kilos of wet packed cells contained 45 mM of labile phosphate. After removal of compounds whose barium salts were insoluble at pH 8.2, 31 mM of labile phosphate remained in solution. This solution was concentrated under reduced pressure to 1 liter, and then 4
liters of cold ethanol were added to precipitate the material. The material was dissolved in 500 ml. of water, from which it was subsequently reprecipitated. Since the precipitate was very gummy, it was dissolved in 300 ml. of water and dried by lyophilization. The dry material contained 29 mM of labile phosphate, 0.25 μM of labile phosphate per mg., and 3.5 moles of stable phosphate and 18.3 atoms of nitrogen per mole of labile phosphate. 28 mM of the preparation were treated with sulfuric acid to remove barium. The liter of solution was then passed through a 14 cm. bed of cation exchange resin (500 gm. of acid-washed IR-105) at the rate of 40 ml. per minute. The sample was washed through with 2 liters of water. The effluent was adjusted to pH 7.8 by the addition of 100 mM of barium hydroxide. 1 volume of alcohol was added; the precipitate which formed was dissolved in 1100 ml. of water and reprecipitated with 1100 ml. of ethanol. This precipitate, which contained 3.4 mM of labile phosphate, was discarded. The supernatant solutions from the two precipitations were combined and concentrated under reduced pressure to 300 ml., and the alcohol-insoluble barium salts were recovered by the addition of 1200 ml. of alcohol. The precipitate contained 22.5 mM of labile phosphate. The dry material contained 0.56 μM of labile phosphate per mg. and assayed 1.45 moles of stable phosphate, 1.15 moles of uridine, and 7.8 atoms of nitrogen per mole of labile phosphate.

Separation of Components by Partition Chromatography—The results of a Craig distribution experiment, in which a sample of purified material was partitioned between aqueous phenol and sulfate buffer, are shown in Fig. 1. The distribution curves clearly demonstrate that at least two different labile phosphate compounds are present in the purified preparations and that the stable phosphate, uridine, and much nitrogen are closely associated with the labile phosphate compounds. The components are seen to differ markedly in nitrogen content only.

In order to obtain more effective separation and on a larger scale, partition chromatography with aqueous phenol as the mobile solvent was used. The system adopted was prepared as described below. 0.1 M sulfuric acid, adjusted to pH 2 with NaOH, was shaken with 5 volumes of 75 per cent phenol. Since the system was quite sensitive to temperature changes, final equilibration usually was carried out after the phases had reached the desired temperature and the chromatogram was then run in a constant temperature room. For the column pack, Celite 545 (Johns-Manville) was used as the inert support. The Celite was washed several times with normal sulfuric acid and then with water and dried before use. The pack was prepared by mixing phenol-saturated sulfate buffer with Celite in the proportion 0.7 ml. per gm. A slurry of this mixture in phenol saturated with sulfate buffer was poured into a chromato-
graphic tube and packed under about 30 cm. (Hg) of pressure (with proper precautions). The pack was tamped firmly.

The sample was dissolved in water, adjusted to pH 2 with sulfuric acid, and centrifuged. The residue (barium sulfate) was washed, the wash

water was added to the solution of sample, and the whole was saturated with phenol by the addition of phenol crystals. The sample was then placed on the column pack and eluted with sulfate buffer-saturated phenol. A flow rate of approximately 0.2 ml. per sq. cm. cross-section was used. Aliquots of effluent were collected, extracted with ether, and analyzed for labile phosphate in order to locate the position of compounds in the effluent. Fig. 2 illustrates the type of separation of labile phosphate compounds obtained. The position of the compounds is expressed in terms of hold-

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**Fig. 1.** Craig distribution pattern of a crude preparation (phenol-0.1 M sulfate buffer system, pH 1.5).

**Fig. 2.** Separation of the components by partition chromatography (phenol-0.1 M sulfate buffer system, pH 1.5 to 2.0).
back volume, which is the volume of mobile phase present in the pack. In practice, the amount of mobile phase present in the pack was found to be approximately 3 times that of non-mobile phase used.

About 95 per cent of the labile phosphate was ordinarily recovered from such chromatograms. However, the maximum solubility of the third component in the phenol phase is only about 1 $\mu$M per ml. The size of the sample should be such that the third component can be recovered in less than 2 holdback volumes. With larger samples, the additional effluent required to recover the third component contains stable phosphate compounds. This occurred on some preparative runs in which 12 gm. samples were used (2000 $\mu$M of Compound 1) and the holdback volume was about 525 ml. Analyses of the dry barium salts of the pooled samples of the three compounds are given in Table III.

### Table III

**Analytical Data on Labile Phosphate Compounds Separated by Partition Chromatography**

<table>
<thead>
<tr>
<th></th>
<th>Original material</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labile P, $\mu$M per mg.</td>
<td>0.56</td>
<td>1.00</td>
<td>0.78</td>
<td>0.68</td>
</tr>
<tr>
<td>Stable &quot; per labile P.</td>
<td>1.45</td>
<td>1.33</td>
<td>1.40</td>
<td>1.12</td>
</tr>
<tr>
<td>Uridine &quot; &quot; &quot;</td>
<td>1.15</td>
<td>1.05</td>
<td>1.15</td>
<td>1.02</td>
</tr>
<tr>
<td>Potential reducing power per labile P.</td>
<td>0.76</td>
<td>0.8</td>
<td>0.91</td>
<td>0.81</td>
</tr>
<tr>
<td>Nitrogen per labile P.</td>
<td>7.8</td>
<td>3.1</td>
<td>4.9</td>
<td>9.9</td>
</tr>
</tbody>
</table>

* Compound most soluble in phenol.

It can be seen that the preparations of the separated components are considerably purer than the starting material. The preparations are similar in the molecular ratio of labile phosphate to stable phosphate to uridine to reducing power, although two of the preparations are still quite contaminated with stable phosphorus compounds. It should be further noted that the principal difference between the preparations lies in their nitrogen content, and, as has been indicated (2), this difference is accounted for by the presence of amino acids in Compounds 2 and 3.

By repetition of some of the purification steps, preparations of each compound were obtained in which the molar ratio of labile phosphate to stable phosphate to uridine to potential reducing power was 1:1:1:0.8, and the nitrogen content of the preparations approached 3.0, 4.0, and 9.0 atoms per mole for Compounds 1, 2, and 3, respectively.

### DISCUSSION

On the basis of what is known of the compounds (3), the preparations of Compounds 1, 2, and 3 are about 75, 65, and 80 per cent pure, respec-
tively. In the best preparations obtained, all of the nitrogen, phosphorus, and ultraviolet absorption have been accounted for and found to be bound to the compounds; therefore, most of the impurities in the preparations may be inorganic.

**SUMMARY**

A procedure for the isolation of uridine-5'-pyrophosphate compounds from penicillin-treated *Staphylococcus aureus* is described. The use of aqueous phenol in partition chromatography for the separation of three uridine-5'-pyrophosphate derivatives is reported.

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URIDINE-5'-PYROPHOSPHATE
DERIVATIVES: I. ISOLATION FROM
STAPHYLOCOCCUS AUREUS
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