Microbial Growth on C\textsubscript{1} Compounds

INCORPORATION OF C\textsubscript{1} UNITS INTO ALLULOSE PHOSPHATE BY EXTRACTS OF PSEUDOMONAS METHANICA

BY M. B. KEMP* AND J. R. QUAYLE*

Department of Biochemistry, University of Sheffield

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1. Incubation of cell-free extracts of methane- or methanol-grown Pseudomonas methanica with [14C]formaldehyde and d-ribose 5-phosphate leads to incorporation of radioactivity into a non-volatile product, which has the chromatographic properties of a phosphorylated compound. 2. Treatment of this reaction product with a phosphatase, followed by chromatography, shows the presence of two compounds whose chromatographic properties are consistent with their being free sugars. 3. The minor component of the dephosphorylated products has been identified as fructose. The major component has been identified as allulose (psicose) on the basis of co-chromatography, co-crystallization of the derived phenyllosazone and dinitrophenyllosazone with authentic derivatives of allulose and behaviour towards oxidation with bromine water. 4. It is suggested that the bacterial extracts catalyse the condensation of a C\textsubscript{1} unit identical with, or derived from, formaldehyde with ribose 5-phosphate to give allulose 6-phosphate. 5. Testing of hexose phosphates and pentose phosphates as substrates has so far shown the reaction to be specific for ribose 5-phosphate. 6. The condensation reaction is not catalysed by extracts of methanol-grown Pseudomonas AM1. 7. A variant of the pentose phosphate cycle, involving this condensation reaction, is suggested as an explanation for the net synthesis of C\textsubscript{3} compounds from C\textsubscript{1} units by P. methanica.

The pathway by which carbon is incorporated into cell constituents during growth of Pseudomonas methanica on methane or methanol has previously been investigated by chromatographic analysis of the metabolites labelled during incubation of the organism with 14C-labelled substrates (Johnson & Quayle, 1965). The results of this study showed that over 90\% of the radioactivity fixed from [14C]methane or [14C]methanol at the earliest times of sampling appeared in phosphorylated compounds, of which glucose and fructose phosphates constituted the largest part (70–90\%); [14C]bicarbonate was incorporated mainly into malate and aspartate.

These results, together with the fact that no 3-phospho-D-glycerate carboxy-lyase (dimerizing) (EC 4.1.1.39) (ribulose diphosphate carboxylase) could be detected in cell-free extracts of the organism, show that the net incorporation of carbon is not effected by the ribulose diphosphate cycle of carbon dioxide fixation. Instead, some biosynthetic pathway operates which results in the direct incorporation of C\textsubscript{1} units into sugar phosphates.

This paper presents evidence that cell-free extracts of methane-grown P. methanica can catalyse the condensation of formaldehyde with ribose 5-phosphate to form allulose (psicose) phosphate. Such a reaction enables a modified pentose phosphate cycle to be constructed which may explain the net incorporation of C\textsubscript{1} units into cell constituents by this organism.

A preliminary account of part of this work has been published (Kemp & Quayle, 1965).

MATERIALS AND METHODS

Growth of the organism. P. methanica was grown in the medium described by Johnson & Quayle (1965). The sterile medium (600ml.), contained in a 21. Buchner flask, was inoculated with a 10ml. sample of starter culture. The flask was then gassed with a methane + air (50:50) mixture through a sterile cotton-wool filter, sealed and agitated by shaking on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 30°. After 2 days the cells were harvested by centrifuging, freeze-dried and stored at −15°.

Special chemicals. We are indebted to Dr F. J. Simpson and Dr B. E. Stacey for gifts of allulose, to Dr L. Szabo for deoxy sugar phosphates and to Dr R. J. Stoodley, Dr D. A. L. Davies and Dr W. G. Overend for other sugars.
Cylinders of methane were obtained from the Middlesex County Council, Main Drainage Dept., Mogden Works, Isleworth.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. [14C]Methanol was purified by vacuum-distillation. [14C]Formaldehyde was obtained by heating [14C]para-formaldehyde (1% in an end-window Geiger-Müller tube (type EHM28). At least 1000 counts were recorded and corrections for background were made.

Preparation and recrystallization of osazones. The unknown radioactive compound U was (see the Results section) was mixed with authentic allulose and derivatives were prepared from the resulting solution. (1) Phenyllosazone: allulose (25 mg.) plus radioactive unknown, sodium acetate (75 mg.) and phenylhydrazine hydrochloride (75 mg.) in water (2 ml.) were heated on a boiling-water bath for 2 hr. The product was collected by filtration, washed with water and recrystallized from acq. 40% (v/v) ethanol (Hough & Stacey, 1963). (2) Dinitrophenyllosazone: allulose (25 mg.) plus radioactive unknown and 2,4-dinitrophenylhydrazine (75 mg.) in 2 N-HCl (5 ml.) were heated in a stoppered tube in an oven at 100°C overnight. The precipitate was collected by filtration, washed with water and recrystallized from 2-methoxyethanol (Neuberg & Strauss, 1946). The precipitate at each stage was dried and dissolved in the solvent used for recrystallization to give a concentration of 10 mg./ml. A 0.1 ml. sample was plated on an aluminium disk (1 in. diam.) and the radioactivity assayed.

Oxidation of sugars with bromine water. The sugar (200 mg.) was dissolved in 0.5 ml. of 0.1N-sodium acetate buffer, pH 5-5, and treated with 0.5 ml. of bromine water (0.4%, v/v) at 37°C for 20 min. (Horecker, Smyrniotis & Seegmiller, 1951). Excess of bromine was removed by aeration and the solution was evaporated down and chromatographed one-dimensionally in phenol-formic acid-water. Sugars and radioactivity were located as described above.

Large-scale incubation of ribose 5-phosphate with formaldehyde. In the complete system 50 μmoles of ribose 5-phosphate, 50 μmoles of [14C]formaldehyde (containing 10 μC of 14C) and 100 μmoles of sodium phosphate buffer, pH 7.0, in a total volume of 5 ml., were incubated with 0.1 ml. of crude extract (containing 1 mg. of protein) for 30 min. at 30°C. The reaction was stopped by heating the tubes in boiling water for 5 min. A 25 μl. sample of the mixture was taken for pentose phosphate determination (Horecker et al. 1953) and a 0.2 ml. sample for hexulose phosphate determination (Dische & Devi, 1960). Incorporation of radioactivity was determined by chromatographing 0.5 ml. of the mixture one-dimensionally in phenol-formic acid-water and assaying the radioactivity in the phosphate area of the chromatogram. These phosphate areas were eluted, dephosphorylated with phosphaete from Polidase and rechromatographed two-dimensionally. The sugar phosphates present in the original incubation mixtures were also analysed by a method similar to that used by Gibbs & Simpson (1964). In this method, the pH of 1 ml. of the mixture was adjusted to 5-5 with 2N-acetic acid. MgCl₂ was added to give a final concentration of 2 mM, and the tubes were incubated with 200 μg. of the phosphaete from Polidase for 36 hr. at 37°C. Then HClO₄ was added to give a final concentration of 10% (w/v) and the precipitated protein removed by centrifuging. Cold 4 N-KOH was added to the supernatant cooled to 2°C until the solution was neutral and the KClO₄ removed by centrifuging. The supernatant was concentrated to 0.5 ml. and passed through a small (10 cm. x 1 cm.) column of mixed-bed ion-exchange resin (Amberlite MB-3). The deionized effluent was evaporated and chromatographed one-dimensionally in phenol-formic acid-water, and the sugars present were revealed by spraying.
Routine procedure for incubation of [14C]methanol or [14C]formaldehyde with cell-free extracts. The complete system contained 10 μmoles of sodium phosphate buffer, pH 7-0, 1 μmole of ribose 5-phosphate, 1 μmole of [14C]-methanol or [14C]formaldehyde, containing 1 or 0.2 μc of 14C respectively, and 0-0^{-}0^{-}15 ml. of extract (0-0^{-}25 mg. of protein), in a total volume of 0-2 ml. Reaction was started by the addition of extract, proceeded for 15 min. at 30° and was stopped by the addition of 8 ml. of methanol. Blank incubations were performed in which ribose 5-phosphate was omitted from the reaction mixture. Incorporation of radioactivity was followed in the case of [14C]methanol by plating 0-2 ml. samples on aluminium disks and washing twice with methanol-formic acid-water (16:1:3, by vol.) to remove volatile radioactivity. [14C]Formaldehyde was not completely volatile under these conditions, so the mixtures were centrifuged, evaporated to dryness and the residue taken up in 80% (v/v) methanol and chromatographed one-dimensionally in phenol-formic acid-water. The areas expected to include sugar phosphates were then assayed for radioactivity with an end-window Geiger-Müller tube.

RESULTS

Incorporation of [14C]methanol and [14C]formaldehyde. The crude extract of P. methanica was incubated with ribose 5-phosphate and [14C]-methanol or [14C]formaldehyde. Radioactivity was fixed in non-volatile compounds; these products were analysed by chromatography and radioautography (Table 1). The complete system, with either tracer compound, fixed much more radioactivity into the area expected for sugar phosphate than did the controls. This area included the chromatogram origin. When these areas were eluted from the chromatograms prepared from the complete systems, treated with an acid phosphatase and rechromatographed, much of the radioactivity appeared as two spots in the region characteristic of free sugars. These two unknown compounds are designated U_A and U_B. Considerable radioactivity remained near the origin. When these origin areas were eluted, again treated with phosphatase and rechromatographed, more of the radioactivity appeared in U_A and U_B. This indicates that much of the radioactivity remaining in the origin area of the first chromatogram was probably contained in sugar phosphate that had not been hydrolysed by the Polidase preparation. The incompleteness of action of Polidase S may have been due to the use of substrate amounts of sugar phosphate, instead of the tracer amounts that are usually involved in the use of this technique. The use of more Polidase, however, impaired the subsequent chromatography.

Identification of the reaction products. The nature of the unknown compounds U_A and U_B obtained from the incubation with [14C]formaldehyde was investigated by co-chromatography with authentic sugars. The minor component, U_B, was found to co-chromatograph with fructose, but U_A did not correspond to any common sugar. A condensation between formaldehyde and ribose 5-phosphate might be expected to yield the 6-phosphate of one of the hexoses, allose, altrose or allulose. When these sugars were tested, U_A was found to co-chromatograph with allulose (Fig. 1).

This identification has been confirmed as follows: (1) One-dimensional chromatography of U_A with authentic allulose in two further solvent systems, ethyl acetate-acetic acid-water (9:2:2, by vol.) and butan-1-ol-pyridine-water (10:3:3, by vol.). (2) Bromine water, in a buffered solution, oxidizes aldooses to aldonic acids, but leaves ketoses essentially unchanged (Horecker et al. 1951); when U_A and 200 μg. of authentic allulose were treated with bromine water as described in the Materials and Methods section and then chromatographed, the

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction system</th>
<th>Radioactivity in phosphate area of chromatogram (counts/min.)</th>
<th>Radioactivity in dephosphorylated compounds (counts/min.)</th>
<th>Origin area of chromatogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]Methanol</td>
<td>Complete</td>
<td>4 630</td>
<td>Unknown A 1 826</td>
<td>1 806</td>
</tr>
<tr>
<td></td>
<td></td>
<td>931</td>
<td>Unknown B 379</td>
<td>427</td>
</tr>
<tr>
<td></td>
<td>Complete, ribose omitted</td>
<td>407</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 848</td>
<td></td>
<td>4 816</td>
</tr>
<tr>
<td></td>
<td>Complete, extract boiled</td>
<td>4 647</td>
<td></td>
<td>183</td>
</tr>
<tr>
<td>[14C]Formaldehyde</td>
<td>Complete</td>
<td>68 088</td>
<td>Unknown A 34 729</td>
<td>24 953</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 420</td>
<td>Unknown B 3431</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Complete, ribose omitted</td>
<td>10 848</td>
<td></td>
<td>4 816</td>
</tr>
<tr>
<td></td>
<td>Complete, extract boiled</td>
<td>4 647</td>
<td></td>
<td>183</td>
</tr>
</tbody>
</table>
R<sub>p</sub> of the radioactivity and the sugar appeared unchanged. This shows that U<sub>A</sub> is a ketose rather than an aldose. (3) The phenyllosazone and dinitrophenyllosazone of a mixture of U<sub>A</sub> and authentic allulose (25 mg.) were prepared and recrystallized (see the Materials and Methods section). The specific radioactivities were determined by plating a known weight on aluminium disks and assaying the radioactivities with an end-window Geiger–Müller tube. The results given in Table 2 show that for the phenyllosazone the specific activity remains constant on successive recrystallizations. The specific activity of the dinitrophenyllosazone was not changed by the first recrystallization but fell by 18% during the second crystallization. It is considered that this fall is due to inaccuracies of the method, which necessarily involved small amounts of carrier. Occasional variations of this order of magnitude were encountered when authentic [14C]glucose was tested on the same scale under exactly the same conditions.

The possibility was also considered that formaldehyde might condense at C-2 of ribose 5-phosphate, yielding hamamelose phosphate. When U<sub>A</sub> was co-chromatographed with authentic hamamelose, U<sub>A</sub> ran slightly but significantly faster in phenol–formic acid–water and in ethyl acetate–acetic acid–water (Fig. 1). As a further check, U<sub>A</sub> was separately mixed with hamamelose and with allulose and each mixture oxidized with bromine water as described in the Materials and Methods section. On chromatography in ethyl acetate–acetic acid–water, U<sub>A</sub> and allulose appeared unchanged, whereas hamamelose had disappeared.

Chemical detection of hexulose formation. To detect the reaction product U<sub>A</sub> chemically, a larger-scale experiment was carried out with 50 μmoles each of ribose 5-phosphate and formaldehyde, as described in the Materials and Methods section. The results are shown in Table 3. The amount of pentose phosphate remaining after the incubation was determined by the method of Horecker et al. (1953). More disappeared in the presence of formaldehyde than in its absence, and this difference was paralleled by the appearance of hexulose phosphate, measured by the method of Dische & Devi (1960) (in both methods sugar phosphates react as free sugars).

Samples of the incubation mixtures were chromatographed one-dimensionally in phenol–formic acid–water, and the area of each chromatogram characteristic of sugar phosphates was assayed for radioactivity, eluted, dephosphorylated and re-chromatographed two-dimensionally. Ketose was detected in the same place on these chromatograms by spraying with naphtharesorcinol as radioactive U<sub>A</sub> was revealed by radioautography.

When samples of the incubation mixture were directly dephosphorylated without prior chromatography, one-dimensional chromatographic analysis of the resulting deionized solution also revealed, by spraying with naphtharesorcinol, orcinol, ketose in the area of the chromatogram characteristic of fructose and allulose, and most of the radioactivity was shown by radioautography to be

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**Table 2. Preparation of the phenyllosazone and dinitrophenyllosazone of unknown compound U<sub>A</sub> and their co-crystallization with the corresponding derivatives of allulose**

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Phenyllosazone</th>
<th>Dinitrophenyllosazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>573</td>
<td>642</td>
</tr>
<tr>
<td>First recrystallization</td>
<td>595</td>
<td>651</td>
</tr>
<tr>
<td>Second recrystallization</td>
<td>570</td>
<td>537</td>
</tr>
</tbody>
</table>
in this area. However, the presence of [14C]-formaldehyde in the original incubation mixtures caused streaks of radioactivity on the chromatograms which interfered with the precise correlation between chemically revealed ketose and radioactive product. (Prior chromatography of the incubation mixtures as described above avoids this difficulty.) Sedoheptulose, identified by Rf and colour with the orcinol spray, was present in samples of the complete system and that lacking formaldehyde, indicating the presence of enzymes of the pentose phosphate cycle such as D-ribose 5-phosphate ketol isomerase (EC 5.3.1.6) (ribose phosphate isomerase) and D-sedoheptulose 7-phosphate-D-glyceraldehyde 3-phosphate glycolaldehydetransferase (EC 2.2.1.1) (transketolase).

**Specificity of the reaction.** The specificity of the reaction was investigated with a number of sugar phosphates. The standard assay procedures were used as described in the Materials and Methods section and the results are shown in Table 4.

When [14C]-methanol was used as the C1 source, the only incorporation into non-volatile compounds significantly greater than that shown by the control (no sugar phosphate present) was given by D-ribose 5-phosphate. When [14C]-formaldehyde was used the presence of ribose 5-phosphate caused a tenfold stimulation in the radioactivity incorporated in the absence of acceptor. Smaller stimulations (twofold or less) were shown in tubes to which D-2-deoxyribose 5-phosphate and ribulose 1,5-diphosphate had been added. However, when these radioactive areas were eluted, treated with phosphate and rechromatographed one-dimensionally in phenol–formic acid–water, no significant amount of radioactivity was found in the area of the chromatogram characteristic of free sugars. Radioactive sugars were observed only when ribose

### Table 3. Formation of hexulose phosphate by incubation of formaldehyde and ribose 5-phosphate

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>Ribose 5-phosphate utilized (µmoles/5ml.)</th>
<th>Hexulose phosphate formed (µmoles/5ml.)</th>
<th>Radioactivity in phosphate area of chromatogram (counts/min./5ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>32-2</td>
<td>8-1</td>
<td>66273</td>
</tr>
<tr>
<td>Ribose 5-phosphate omitted</td>
<td>—</td>
<td>0</td>
<td>8334</td>
</tr>
<tr>
<td>[14C]Formaldehyde omitted</td>
<td>22-0</td>
<td>1-6</td>
<td>—</td>
</tr>
<tr>
<td>Bacterial extract omitted</td>
<td>12-0</td>
<td>0</td>
<td>14214</td>
</tr>
<tr>
<td>Extract and ribose 5-phosphate omitted</td>
<td>—</td>
<td>0</td>
<td>9876</td>
</tr>
<tr>
<td>Complete, but extract boiled</td>
<td>2-2</td>
<td>0</td>
<td>10950</td>
</tr>
</tbody>
</table>

### Table 4. Specificity of sugar phosphate acceptor for the incorporation of [14C]methanol and [14C]formaldehyde by extracts of P. methanica

<table>
<thead>
<tr>
<th>Sugar phosphate acceptor</th>
<th>From [14C]-methanol</th>
<th>From [14C]-formaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose 6-phosphate</td>
<td>53</td>
<td>136</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>42</td>
<td>116</td>
</tr>
<tr>
<td>Mannose 6-phosphate</td>
<td>37</td>
<td>136</td>
</tr>
<tr>
<td>2-Deoxyribose 5-phosphate</td>
<td>26</td>
<td>226</td>
</tr>
<tr>
<td>3-Deoxyribose 5-phosphate</td>
<td>38</td>
<td>106</td>
</tr>
<tr>
<td>2-Deoxyxylose 5-phosphate</td>
<td>28</td>
<td>136</td>
</tr>
<tr>
<td>Ribulose 1,5-diphosphate</td>
<td>46</td>
<td>186</td>
</tr>
<tr>
<td>Ribose 5-phosphate</td>
<td>102</td>
<td>1074</td>
</tr>
<tr>
<td>None</td>
<td>41</td>
<td>115</td>
</tr>
</tbody>
</table>

5-phosphate had been used as acceptor compound in the initial incubation mixture. Glyceraldehyde 3-phosphate and free D-ribose were also tested with [14C]-methanol, with negative results.

**Cofactor requirements for the reaction.** So far, no stimulation of the reaction has been observed on the addition of common cofactors or metal ions to the crude extract.

**Occurrence of reaction in extracts of methanol-grown P. methanica.** Extracts prepared in the same way from P. methanica grown on methanol (0-5%, v/v) showed similar incorporation of [14C]-
methanol or $^{[14]C}$formaldehyde and formation of hexulose phosphate as determined colorimetrically.

Non-occurrence of reaction in extracts of Pseudomonas $AM$. When cell-free extracts of methanol-grown Pseudomonas AM1 were substituted for those of $P$. methanica in the standard assay procedure, no incorporation of radioactivity greater than that in the controls was observed.

DISCUSSION

The quantitative difference in incorporation between $^{[14]C}$formaldehyde and $^{[14]C}$methanol (Table 1) suggests that the C1 unit which undergoes condensation is more closely related to formaldehyde than it is to methanol. Condensation of a formaldehyde equivalent with ribose 5-phosphate might be expected most simply to give a 6-phosphate of allose, altrose, allulose or hamamelose.

\[
\begin{align*}
\text{CHO} & \quad \text{CHO} \\
\text{HC-OH} & \quad \text{HO-CH} \\
\text{HC-OH} & \quad \text{CH}_2\text{-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{CH}_2\text{-OH} & \quad \text{CHO} \\
\text{CHO} & \quad \text{CHO} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH}
\end{align*}
\]

All the available evidence reported in this paper indicates that this condensation catalysed by extracts of $P$. methanica leads mainly to the formation of allulose phosphate (presumably the 6-phosphate). Hamamelose has chromatographic properties so similar to those of allulose that it would be difficult to rule its formation out by chromatography alone. However, the evidence that the product is a ketose rather than an aldose eliminates this possibility. If the true substrate for the reaction were formed in the crude extract by prior epimerization of ribose 5-phosphate at positions 2, 3 or 4, the products expected would be hexoses whose chromatographic co-ordinates, insofar as they are known, are not consistent with those of $U_1$. Not all of the theoretical possibilities of the nature of the reaction product, such as those resulting from the migration of the carbonyl group to positions 3 or 4 of the pentose skeleton before condensation, or rearrangement of the pentose skeleton, have been eliminated in this study. They are less likely to occur than that leading to allulose and would lead to rather recondite intermediary metabolism, no evidence for which has been obtained from whole-cell work with tracers.

Chemical study of substrate amounts of the product, here identified as allulose phosphate, is needed to resolve any remaining uncertainties.

$\delta$-Allulose has been isolated previously from natural sources. It occurs in the antibiotic $6$-$\alpha$-amino-$9$-$\beta$-psicofuranosylpurine (Schroeder & Hoeksma, 1959) and as a component of plants of Itea species (Hough & Stacey, 1963). Epimerization of allulose 6-phosphate to fructose 6-phosphate has been implicated in the conversion of $\delta$-allose into fructose 6-phosphate by allulose-grown Aerobacter aerogenes (Gibbins & Simpson, 1964).

The overall condensation reaction (1) represents an acyloin condensation:

\[
\begin{align*}
\text{CHO} & \quad \text{CHO} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{HC-OH} \\
\text{HC-OH} & \quad \text{CH}_2\text{-OH}
\end{align*}
\]

Formaldehyde Ribose Allulose 5-phosphate 6-phosphate

This is a familiar type of reaction catalysed by enzymes involving TPP* as coenzyme (Holzer, 1961). In such reactions one molecule of aldehyde is combined with TPP to form a complex in which the carbonyl carbon atom of the aldehyde is rendered strongly nucleophilic. This then condenses with the acceptor aldehyde to form the acyloin (reaction 2).

\[
\begin{align*}
\text{CHO} & \quad \text{OH} \\
\text{HC-OH} & \quad \text{O} \\
\text{HC-OH} & \quad \text{C} \\
\text{HC-OH} & \quad \text{C}
\end{align*}
\]

It has been shown that a corresponding formaldehyde derivative of TPP is formed during decarboxylation of glyoxylate by pyruvate oxidase obtained from pig heart muscle (Kohlhaw, Deus & Holzer, 1965). There is evidence that the formaldehyde–TPP can undergo an acyloin condensation with glyoxylate to form tartronic acid semialdehyde (reaction 3) in the presence of

* Abbreviation: TPP, thiamine pyrophosphate.
carboligase (Kohlhaw et al. 1965; Jaenicke & Koch, 1962).

\[ \text{[OHC\textcdot TPP]} + \text{OHC\textcdot CO}_2\text{H} \rightarrow \text{OHC\textcdot CH(OH)\textcdot CO}_2\text{H} + \text{TPP} \]  

(3)

In all such acyloin condensations the aldehyde which is activated by the TPP forms the carbonyl group of the acyloin. Hence, if the condensation reaction observed in extracts of \textit{P. methanica} involved formaldehyde–TPP as a reactant, it would be expected that an aldohexose, rather than a hexulose, would be formed as the product. From the known stereochemistry of the addition of glycolaldehyde–TPP to aldoses, catalysed by transketolase, it may be predicted that the aldohexose formed by the condensation of formaldehyde–TPP with ribose 5-phosphate would be altrose 6-phosphate. The finding that the major product is allulose phosphate is thus not in accord with this suggested mechanism.

It is unlikely that the equilibrium of an isomerization reaction between altrose phosphate and allulose phosphate would be in favour of the ketose, as the equilibrium of the similar isomerization between glucose 6-phosphate and fructose 6-phosphate lies towards the aldose. Thus, even if the first product of the condensation were altrose phosphate, the presence of an altrose phosphate isomerase enzyme in the crude extract would not be expected to lead to the predominant formation of allulose phosphate.

If indeed the acyloin condensation does proceed through TPP as a coenzyme, then it would seem more likely that the nucleophilic component would be a novel ribose 5-phosphate–TPP complex, which might condense with a formaldehyde acceptor to give allulose phosphate (reaction 4).

\[ \text{OH} \]

\[ C\text{[(CH(OH))]_3\cdot CH_2\cdot O\cdot PO}_3\text{H}_2 + \text{H\textcdot CHO} \]

\[ \rightarrow \text{TPP + allulose phosphate} \]  

(4)

Fractionation of the enzyme system is clearly needed for investigation of the mechanism of the reaction. To date, no stimulation of the reaction has been observed on addition of common cofactors, including TPP, to the incubation mixture.

The overall reaction has obvious implications in the problem of biosynthesis of cell constituents from methane or methanol by \textit{P. methanica} (Kemp & Quayle, 1965). There is evidence from several

![Scheme 1. Possible route for the net incorporation of reduced C\textsubscript{1} units by \textit{P. methanica}.](image)
groups of workers (Dworkin & Foster, 1956; Harrington & Kallio, 1960; Brown, Strawinski & McCleskey, 1964; Johnson & Quayle, 1964) that microbial oxidation of methane or methanol proceeds via formaldehyde. Condensation of formaldehyde with ribose 5-phosphate to give allulose 6-phosphate, followed by epimerization at C-3 to give fructose 6-phosphate, opens the possibility of constructing a modified pentose phosphate cycle, as in Scheme 1. This cycle would result in synthesis of triose phosphate from 3mol. of formaldehyde and 1mol. of ATP. The regeneration of 3mol. of acceptor ribose 5-phosphate from 2mol. of fructose 6-phosphate and 1mol. of triose phosphate could follow essentially the reactions established for the similar rearrangement in the ribulose diphosphate cycle of carbon dioxide fixation. The fact that sedoheptulose phosphate formation occurs during incubation of ribose 5-phosphate in crude extracts of *P. methanica* suggests that the enzymes necessary for the rearrangement are present in this organism. The main difference between the proposed new cycle and the ribulose diphosphate cycle is the by-passing, in formaldehyde fixation, of the reductive step, namely phosphoglycerate to glyceraldehyde phosphate, which is necessary to reduce the entering carbon dioxide to the level of formaldehyde. The stepwise condensation of formaldehyde to carbohydrate was proposed by Baeyer (1870) as an explanation of incorporation of carbon in photosynthesis; studies over the last 20 years have disproved this idea. It is noteworthy, nevertheless, that the scheme proposed for growth of *P. methanica* would accomplish, in a cyclical rather than a stepwise form, condensation of formaldehyde to carbohydrate.

The failure to find any allulose phosphate synthesis in extracts of methanol-grown *Pseudomonas* AM1 is consistent with the completely different pattern of 14C-labelled substrate incorporation observed in whole cells of the latter organism, and confirms the view (Johnson & Quayle, 1965) that entirely different mechanisms of synthesis of cell constituents from C1 units operate in the methane-utilizing organism *P. methanica* as compared with other organisms such as *Pseudomonas* AM1, *Hyphomicrobiunm vulgare* and *Pseudomonas* PRL-W4 (Kaneda & Roxburgh, 1959).

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