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Microbial Growth on C_1 Compounds

INCORPORATION OF C_1 UNITS INTO ALLULOSE PHOSPHATE BY EXTRACTS OF *PSEUDOMONAS METHANICA*

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1. Incubation of cell-free extracts of methane- or methanol-grown *Pseudomonas methanica* with [^{14}C]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 phenylosazone and dinitrophenylosazone 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_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* AM 1. 7. A variant of the pentose phosphate cycle, involving this condensation reaction, is suggested as an explanation for the net synthesis of C_3 compounds from C_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 ^{14}C-labelled substrates (Johnson & Quayle, 1965). The results of this study showed that over 90% of the radioactivity fixed from [^{14}C]methane or [^{14}C]methanol at the earliest times of sampling appeared in phosphorylated compounds, of which glucose and fructose phosphates constituted the largest part (70–90%); [^{14}C]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_1 units into sugar phosphates.

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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_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 2l. 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. [^{14}C]Methanol was purified by vacuum-distillation. [^{14}C]Formaldehyde was obtained by heating [^{14}C]paraformaldehyde (1mc) with 5ml. of water in a stoppered tube at 100° for 1hr.

D-*Ribose 5-phosphate* (*sodium salt*). The barium salt of D-ribose 5-phosphate (0·365g.) was dissolved in water (1·5ml.), the Ba^{2+} precipitated with 2N-H_2SO_4, and the supernatant neutralized with 2N-NaOH and diluted to 5ml. to give a 0·2M solution of the sodium salt in Na_2SO_4 soln.

Preparation of cell-free extracts. Freeze-dried cells (0·3–0·5g. dry wt.) were suspended in 3ml. of 20mM-sodium phosphate buffer, pH7·0, crushed in a Hughes (1951) press at −25° and centrifuged at 100000*g* for 30min. at 2°.

Chemical determinations. Protein was determined by the Folin–Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951), pentose phosphate by the orcinol method (Horecker, Smyrniotis & Klenow, 1953) and hexulose phosphate by the cysteine–H_2SO_4 method of Dische & Devi (1960), with fructose as the chromogenic standard.

Chromatography and radioautography. The methods of chromatography, radioautography, identification of labelled compounds and radioassay were as described by Large, Peel & Quayle (1961). Some chromatograms were developed one-dimensionally with the solvent system of Kornberg (1958): phenol–90% (w/v) A.R. formic acid–water (500:13:167, w/v/v). Phosphates were dephosphorylated with an acid phosphatase that had been purified from Polidase S (Schwartz Laboratories Inc., Mount Vernon, N.Y., U.S.A.) by precipitation with $(NH_4)_2SO_4$ according to the method of Cohen (1953).

Identification of radioactive sugars eluted from the chromatograms was achieved by co-chromatography with authentic samples in the same solvent systems as were used in the initial chromatography and also in ethyl acetate–acetic acid–water (9:2:2, by vol.) (Gibbins & Simpson, 1964) and in butan-1-ol–pyridine–water (10:3:3, by vol.) (Hough & Stacey, 1963). The radioactivity was revealed by radioautography and the sugars were located by spraying, using the following reagents: reducing sugars by spraying first with $AgNO_3$ in aq. acetone, followed by 0·5N-NaOH in aq. ethanol (Trevelyan, Procter & Harrison, 1950); aldose sugars by spraying with a solution of aniline (0·9ml.) and phthalic acid (1·7g.) in 100ml. of water-saturated butan-1-ol, followed by heating at 105° (Partridge, 1949); ketose sugars by spraying with a solution of orcinol (0·5g.) and trichloroacetic acid (15g.) in 100ml. of water-saturated butan-1-ol, followed by heating at 100° (Klevstrand & Nordal, 1950); hexuloses by spraying with 1% (w/v) naphtharesorcinol in ethanol–2N-HCl (1:9, v/v) (Forsyth, 1948), followed by heating at 90° for 10min.

Evaporation of samples to small volumes was carried out at 40° and approx. 30mm. Hg pressure in a Rotary Evapo-Mix (Buchler Instruments, New York, N.Y., U.S.A.).

Measurement of radioactivity. The radioactivity on paper chromatograms was assayed with a mica end-window Geiger–Müller tube (type 2B2; General Electric Co.). Radioactive compounds plated on aluminium disks (1in. diam.) were assayed in a Panax lead 'castle' assembly, with an end-window Geiger–Müller tube (type EHM2S). At least 1000 counts were recorded and corrections for background were made.

Preparation and recrystallization of osazones. The unknown radioactive compound U_A (see the Results section) was mixed with authentic allulose and derivatives were prepared from the resulting solution. (1) Phenylosazone: allulose (25mg.) plus radioactive unknown, sodium acetate (75mg.) and phenylhydrazine hydrochloride (75mg.) in water (2ml.) were heated on a boiling-water bath for 2hr. The product was collected by filtration, washed with water and recrystallized from aq. 40% (v/v) ethanol (Hough & Stacey, 1963). (2) Dinitrophenylosazone: allulose (25mg.) plus radioactive unknown and 2,4-dinitrophenylhydrazine (75mg.) in 2N-HCl (5ml.) were heated in a stoppered tube in an oven at 100° 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 10mg./ml. A 0·1ml. sample was plated on an aluminium disk (1in. diam.) and the radioactivity assayed.

Oxidation of sugars with bromine water. The sugar (200μg.) was dissolved in 0·5ml. of 0·1M-sodium acetate buffer, pH5·5, and treated with 0·2ml. of bromine water (0·4%, v/v) at 37° for 20min. (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 50μmoles of ribose 5-phosphate, 50μmoles of [^{14}C]formaldehyde (containing 10μc of ^{14}C) and 100μmoles of sodium phosphate buffer, pH7·0, in a total volume of 5ml., were incubated with 0·1ml. of crude extract (containing 1mg. of protein) for 30min. at 30°. The reaction was stopped by heating the tubes in boiling water for 5min. A 25μl. sample of the mixture was taken for pentose phosphate determination (Horecker *et al.* 1953) and a 0·2ml. sample for hexulose phosphate determination (Dische & Devi, 1960). Incorporation of radioactivity was determined by chromatographing 0·5ml. 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 phosphatase 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 Gibbins & Simpson (1964). In this method, the pH of 1ml. of the mixture was adjusted to 5·5 with 2N-acetic acid, $MgCl_2$ was added to give a final concentration of 20mM, and the tubes were incubated with 200μg. of the phosphatase from Polidase for 36hr. at 37°. Then $HClO_4$ was added to give a final concentration of 10% (w/v) and the precipitated protein removed by centrifuging. Cold 4N-KOH was added to the supernatant cooled to 2° until the solution was neutral and the $KClO_4$ removed by centrifuging. The supernatant was concentrated to 0·5ml. and passed through a small (10cm. × 1cm.) 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 [^{14}C]*methanol or* [^{14}C]*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 [^{14}C]-methanol or [^{14}C]formaldehyde, containing 1 or 0·2 μc of ^{14}C respectively, and 0–0·05 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 0·8 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 [^{14}C]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. [^{14}C]Formaldehyde was not completely volatile under these conditions, so the mixtures were centrifuged, evaporated to dryness and the residue taken up in aq. 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 [^{14}C]*methanol and* [^{14}C]*formaldehyde.* The crude extract of *P. methanica* was incubated with ribose 5-phosphate and [^{14}C]-methanol or [^{14}C]formaldehyde. Radioactivity was fixed into 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 [^{14}C]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 aldoses 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 1. *Incorporation of* [^{14}C]*methanol and* [^{14}C]*formaldehyde by cell-free extracts of* P. methanica

The complete reaction mixture contained: 0·1 ml. of extract (0·5 mg. of protein), 4 μmoles of ribose 5-phosphate, 50 μmoles of sodium phosphate buffer, pH 7·0, and either [^{14}C]methanol (2·5 μmoles: 10 μc of ^{14}C) or [^{14}C]-formaldehyde (1·5 μmoles; 4 μc of ^{14}C), in a total volume of 0·5 ml. After 15 min. at 30° the reaction was stopped with 2 ml. of methanol, the mixture centrifuged and the supernatant evaporated and analysed by chromatography and radioautography.

Substrate	Reaction system	Radioactivity in phosphate area of chromatogram (counts/min.)	Radioactivity in dephosphorylated compounds (counts/min.)		
			Unknown A (U_A)	Unknown B (U_B)	Origin area of chromatogram
[^{14}C]Methanol	Complete	4630	1826	379	1806
	Ribose 5-phosphate omitted	931	0	0	427
	Complete, but extract boiled	407	0	0	86
[^{14}C]Formaldehyde	Complete	68088	34729	3431	24953
	Ribose 5-phosphate omitted	10848	3420	190	4516
	Complete, but extract boiled	4647	339	53	183

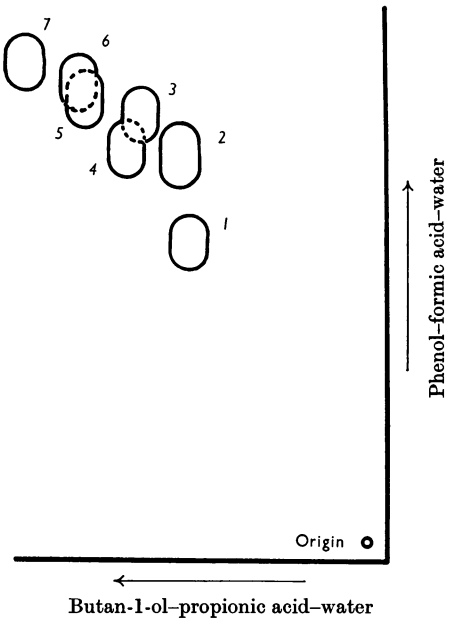


Fig. 1. Diagram of two-dimensional chromatogram of some sugars: *1*, Glucose; *2*, allose; *3*, fructose; *4*, altrose; *5*, hamamelose; *6*, allulose; *7*, ribose.

R_F of the radioactivity and the sugar appeared unchanged. This shows that U_A is a ketose rather than an aldose. (3) The phenylosazone and dinitrophenylosazone of a mixture of U_A and authentic allulose (25mg.) 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 phenylosazone the specific activity remains constant on successive recrystallizations. The specific activity of the dinitrophenylosazone 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 [^{14}C]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_A was co-chromatographed with authentic hamamelose, U_A ran slightly but significantly faster in phenol–formic acid–water and in ethyl acetate–acetic acid–water (Fig. 1). As a further check, U_A 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_A and allulose appeared unchanged, whereas hamamelose had disappeared.

Table 2. *Preparation of the phenylosazone and dinitrophenylosazone of unknown compound U_A and their co-crystallization with the corresponding derivatives of allulose*

The phenyl- and dinitrophenyl-osazones of a mixture of U_A and authentic allulose were prepared as described in the Materials and Methods section and recrystallized twice. At each stage 1mg. was plated in duplicate on aluminium disks and the radioactivity assayed with an end-window Geiger–Müller tube.

Purification stage	Specific radioactivity (counts/min./mg.) Phenylosazone	Dinitrophenylosazone
Crude	573	642
First recrystallization	595	651
Second recrystallization	570	537

Chemical detection of hexulose formation. To detect the reaction product U_A chemically, a larger-scale experiment was carried out with 50μmoles 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 rechromatographed two-dimensionally. Ketose was detected in the same place on these chromatograms by spraying with naphtharesorcinol as radioactive U_A 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, or orcinol, ketose in the area of the chromatogram characteristic of fructose and allulose, and most of the radioactivity was shown by radioautography to be

Table 3. *Formation of hexulose phosphate by incubation of formaldehyde and ribose 5-phosphate with cell-free extracts of* P. methanica

The complete system contained 50 μmoles of ribose 5-phosphate, 50 μmoles of [^{14}C]formaldehyde (containing 10 μc of ^{14}C), 100 μmoles of sodium phosphate buffer, pH 7·0, and 0·1 ml. of bacterial extract (containing 1 mg. of protein), in a total volume of 5 ml. The system was incubated at 30° for 30 min. and the reaction stopped by placing the tubes in boiling water for 5 min. Samples were withdrawn for colorimetric estimation of pentose phosphate and hexulose phosphate, and for chromatographic analysis followed by radioassay of the area of the chromatogram characteristic of sugar phosphates (see the Materials and Methods section).

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

in this area. However, the presence of [^{14}C]-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 R_F 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 [^{14}C]methanol was used as the C_1 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 [^{14}C]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 phosphatase 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 5-phosphate had been used as acceptor compound in the initial incubation mixture. Glyceraldehyde 3-phosphate and free D-ribose were also tested with [^{14}C]methanol, with negative results.

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

The routine assay procedure given in the Materials and Methods section was used. The radioactivity incorporated from [^{14}C]methanol was assayed by plating on aluminium disks, that from [^{14}C]formaldehyde by chromatographing the reaction mixture and assaying the radioactivity in the area characteristic of phosphorylated compounds.

Sugar phosphate acceptor	Radioactivity incorporated (counts/min.) From [^{14}C]-methanol	From [^{14}C]-formaldehyde
Fructose 6-phosphate	53	136
Glucose 6-phosphate	42	116
Mannose 6-phosphate	37	136
2-Deoxyribose 5-phosphate	26	226
3-Deoxyribose 5-phosphate	38	106
2-Deoxyxylose 5-phosphate	28	136
Ribulose 1,5-diphosphate	46	186
Ribose 5-phosphate	102	1074
None	41	115

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.

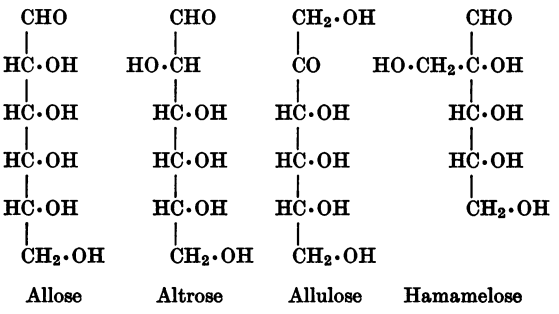
Occurrence of reaction in extracts of methanol-grown P. methanica. Extracts prepared in the same way from *P. methanica* grown on methanol (0·5%, v/v) showed similar incorporation of [^{14}C]-

methanol or [^{14}C]formaldehyde and formation of hexulose phosphate as determined colorimetrically.

Non-occurrence of reaction in extracts of Pseudomonas *AM* 1. When cell-free extracts of methanol-grown *Pseudomonas* AM 1 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

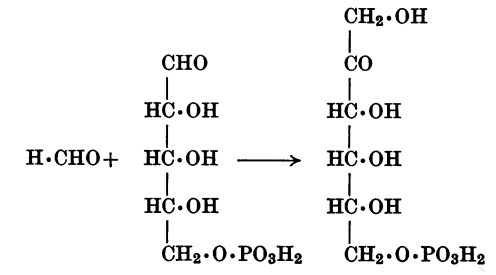
The quantitative difference in incorporation between [^{14}C]formaldehyde and [^{14}C]methanol (Table 1) suggests that the C_1 unit which undergoes condensation is more closely related to formaldehyde than it is to methanol. Condensation of a formaldehyde equivalent with ribose 5-phosphate might be expected most simply to give a 6-phosphate of allose, altrose, allulose or hamamelose.



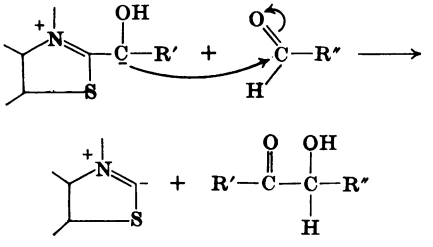
All the available evidence reported in this paper indicates that this condensation catalysed by extracts of *P. methanica* leads mainly to the formation of allulose phosphate (presumably the 6-phosphate). Hamamelose has chromatographic properties so similar to those of allulose that it would be difficult 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_A. 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.

D-Allulose has been isolated previously from natural sources. It occurs in the antibiotic 6-amino-9-D-psicofuranosylpurine (Schroeder & Hoeksema, 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 D-allose into fructose 6-phosphate by allose-grown *Aerobacter aerogenes* (Gibbins & Simpson, 1964).

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



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).



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.

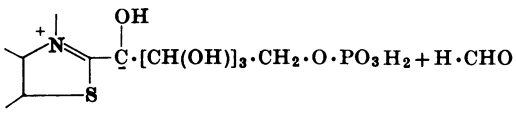
carboligase (Kohlhaw *et al.* 1965; Jaenicke & Koch, 1962).

$$[OHC\cdot TPP]+OHC\cdot CO_2H \longrightarrow OHC\cdot CH(OH)\cdot CO_2H+TPP \quad (3)$$

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 *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.

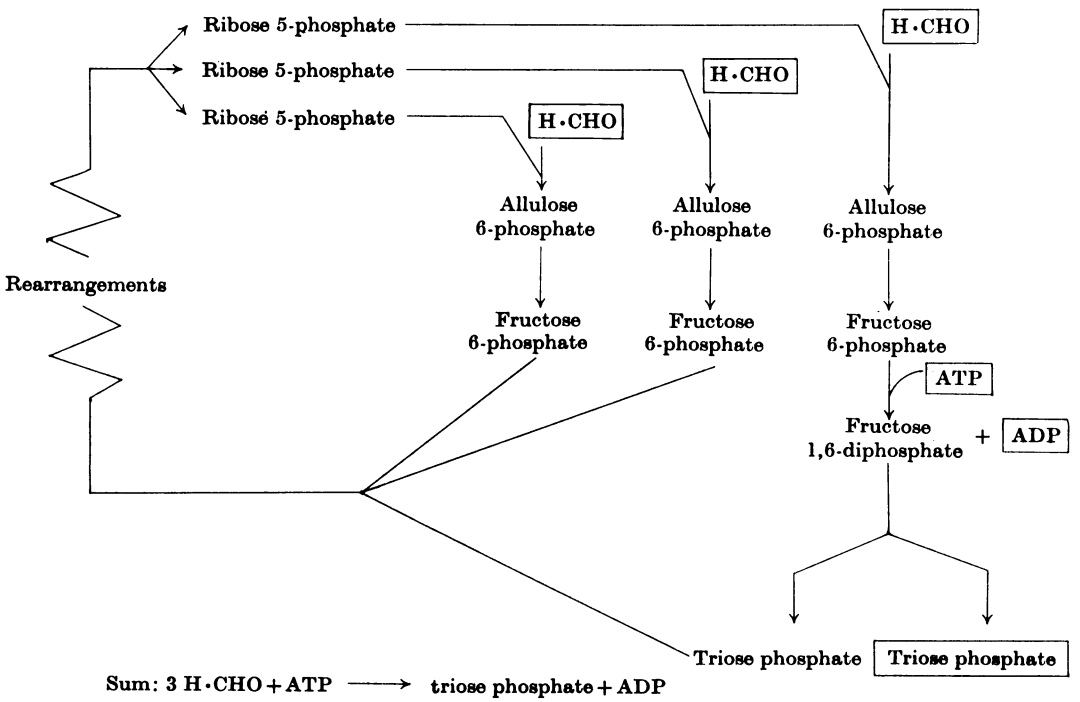
If indeed the acyloin condensation does proceed through TPP as a coenzyme, then it would seem more likely that the nucleophilic component would be a novel ribose 5-phosphate–TPP complex, which might condense with a formaldehyde acceptor to give allulose phosphate (reaction 4).



$$\longrightarrow \text{TPP + allulose phosphate} \quad (4)$$

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 *P. methanica* (Kemp & Quayle, 1965). There is evidence from several



Scheme 1. Possible route for the net incorporation of reduced C_1 units by *P. methanica*.

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 ^{14}C-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 C_1 units operate in the methane-utilizing organism *P. methanica* as compared with other organisms such as *Pseudomonas* AM1, *Hyphomicrobium vulgare* and *Pseudomonas* PRL–W4 (Kaneda & Roxburgh, 1959).

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