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Microbial Growth on C_1 Compounds

UPTAKE OF [^{14}C]FORMALDEHYDE AND [^{14}C]FORMATE BY METHANE-GROWN *PSEUDOMONAS METHANICA* AND DETERMINATION OF THE HEXOSE LABELLING PATTERN AFTER BRIEF INCUBATION WITH [^{14}C]METHANOL

BY M. B. KEMP AND J. R. QUAYLE
Department of Microbiology, University of Sheffield

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1. A study has been made of the incorporation of carbon from [^{14}C]formaldehyde and [^{14}C]formate by cultures of *Pseudomonas methanica* growing on methane. 2. The distribution of radioactivity within the non-volatile constituents of the ethanol-soluble fractions of the cells, after incubation with labelled compounds for periods of up to 1 min., has been analysed by chromatography and radioautography. 3. Radioactivity was fixed from [^{14}C]formaldehyde mainly into the phosphates of the sugars, glucose, fructose, sedoheptulose and allulose. 4. Very little radioactivity was fixed from [^{14}C]formate; after 1 min. the only products identified were serine and malate. 5. The distribution of radioactivity within the carbon skeleton of glucose, obtained from short-term incubations with [^{14}C]methanol of *Pseudomonas methanica* growing on methane, has been investigated. At the earliest time of sampling over 70% of the radioactivity was located in C-1; as the time increased the radioactivity spread throughout the molecule. 6. The results have been interpreted in terms of a variant of the pentose phosphate cycle, involving the condensation of formaldehyde with C-1 of ribose 5-phosphate to give allulose phosphate.

Previous work has shown that, on incubation of methane-grown *Pseudomonas methanica* with [^{14}C]-methane or [^{14}C]methanol, the radioactivity was incorporated initially into sugar phosphates, mainly those of fructose and glucose (Johnson & Quayle, 1965). Cell-free extracts of the same organism have been shown to catalyse the following reaction (Kemp & Quayle, 1965, 1966):

D-Ribose 5-phosphate + formaldehyde → allulose phosphate

On the basis of these findings it was suggested that the net incorporation of C_1 units by this organism might follow a modified pentose phosphate cycle.

In this paper the oxidation levels at which C_1 units are incorporated by whole cells have been further localized and the pattern of labelling in the radioactive hexose phosphate obtained from whole-cell experiments has been correlated with that predicted for operation of the proposed cycle.

MATERIALS AND METHODS

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 with water in a stoppered tube at 100° for 1 hr. Sodium [^{14}C]formate was purified by chromatography and vacuum distillation as described by Large, Peel & Quayle (1961).

Growth of the organism. P. methanica was grown as described previously (Johnson & Quayle, 1965; Kemp & Quayle, 1966).

Brief incorporation of ^{14}C*-labelled compounds.* These experiments were performed as described by Johnson & Quayle (1965). Very brief exposure at 30° of methane-grown cells to [^{14}C]methanol was accomplished by adding [^{14}C]methanol (200 μc, 30 μmoles) to 10 ml. of bacterial suspension (6 mg. dry wt. of bacteria/ml.) which was being gassed with methane–air (1:1, v/v) and immediately pouring the whole mixture into 4 vol. of absolute ethanol. The time of exposure to isotope was estimated to be less than 3 sec. In one experiment, the temperature of the culture was reduced from 30° to 16° by surrounding the culture vessel with a bath at 16°; the gas mixture was passed through a copper coil placed in the same bath before being blown through the suspension. The resulting suspensions of killed cells were prepared for chromatography as described by Large *et al.* (1961). The methods of chromatography, radioautography and identification by co-chromatography with authentic compounds were also as described by Large *et al.* (1961).

Fructose phosphate was partly converted into glucose phosphate by treatment of the eluted sugar phosphate area of the chromatogram with 10 μg. of glucose phosphate

isomerase (D-glucose 6-phosphate ketol-isomerase; EC 5.3.1.9) (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) in 0·5ml. of 0·4M-triethanolamine–HCl buffer, pH 7·6, for 30min. at 30°. The reaction was stopped by heating the tube in boiling water for 5min. and the sugar phosphates were isolated again by two-dimensional chromatography.

Degradation of labelled compounds

The degradation methods described below are summarized diagrammatically in Fig. 1. All carbon atoms were converted into carbon dioxide and assayed as such.

Preparation of samples. The purified radioactive sugars were eluted from the chromatograms. A portion was mixed with 100μg. of authentic sugar and the identification and purity checked by co-chromatography. The remainder was diluted with 1–3m-moles of carrier sugar and stored as an aqueous solution (approx. 0·5M) at −15°.

Combustion. The specific activity of labelled compounds was determined by total oxidation to carbon dioxide with the Van Slyke–Folch reagent as described by Sakami (1955).

Fermentation of glucose. Glucose was degraded as described by Sakami (1955) by bacterial fermentation with *Leuconostoc mesenteroides* (strain obtained from Department of Microbiology, University of Sheffield) giving 1 mole each of carbon dioxide, ethanol and lactate. The oxidation of the resulting ethanol to acetic acid, the oxidative decarboxylation of the lactic acid and the oxidation of the resulting acetaldehyde to acetic acid were performed as described by Sakami (1955), except that the acetic acid was steam-distilled directly from chromic acid without the

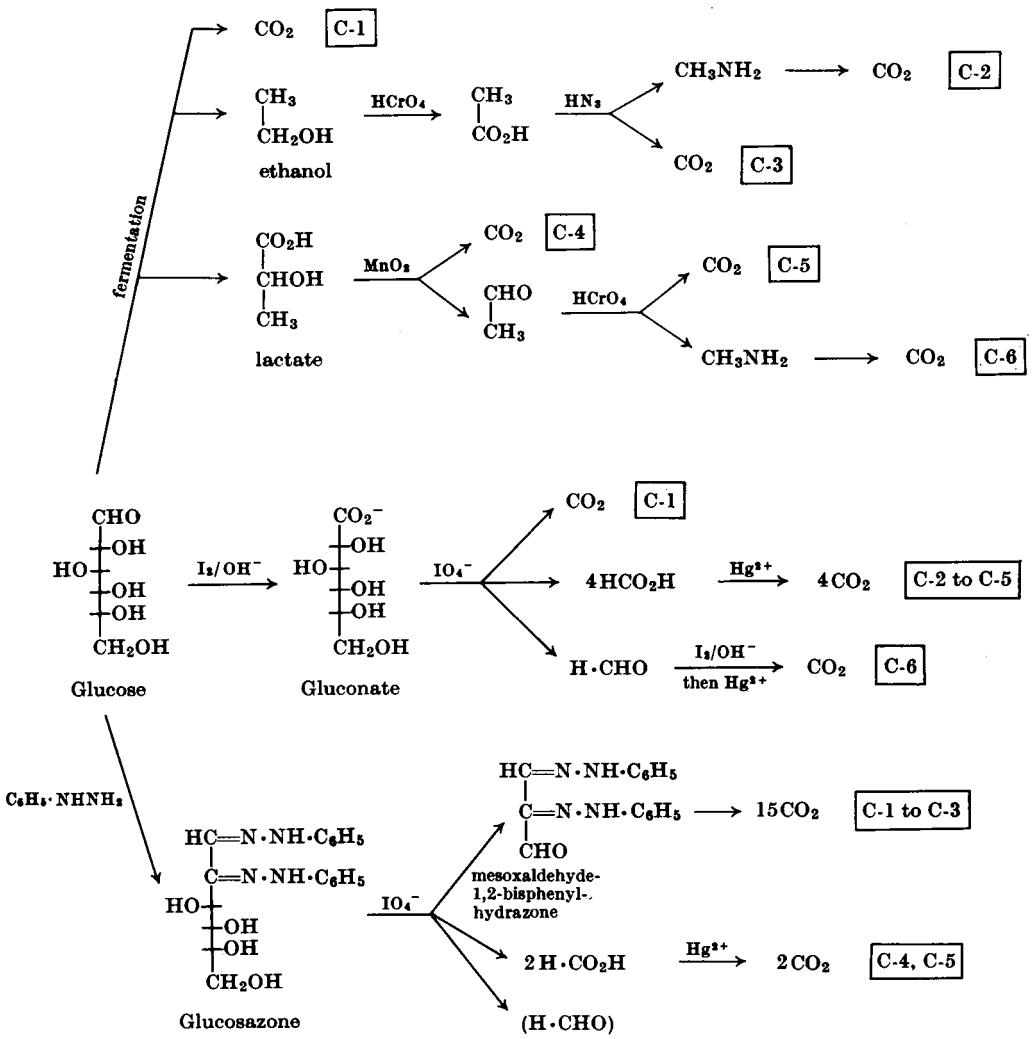

Fig. 1. Chemical degradations of glucose.

addition of stannous sulphate. Acetic acid was degraded by the Schmidt reaction (Phares, 1951) and the resulting methylamine sulphate produced was oxidized to carbon dioxide by combustion with the Van Slyke–Folch reagent.

Preparation and degradation of glucosazone. Glucose was converted into the osazone by heating 0·5m-mole of sugar with 250mg. of hydrated sodium acetate and 250mg. of phenylhydrazine hydrochloride in 4ml. of water at 100° for 2hr. The product was collected by filtration, washed with water and recrystallized from aq. 50% (v/v) ethanol. Glucosazone was oxidized by periodate by using the procedure described by Bassham *et al.* (1954) for the osazones of ribulose and sedoheptulose. Glucosazone (0·15m-mole) and 0·2ml. of sodium bicarbonate were dissolved by warming in 20ml. of aq. 66% (v/v) ethanol; after cooling the solution to 30°, 0·5m-mole of 1N-periodic acid was added. After 15min. the precipitate of mesoxaldehyde-1,2-bisphenylhydrazone was filtered off and recrystallized from aq. ethanol. The filtrate was treated with 2N-H_2SO_4, 20% (w/v) potassium iodide and 2M-sodium arsenite to remove the remaining periodic acid. The formic acid and formaldehyde produced were steam-distilled from 15ml., collecting 250ml. of distillate. They were separately oxidized to carbon dioxide as described by Sakami (1955) except that 0·3M-mercuric acetate in 0·5N-acetic acid was used to oxidize the formic acid.

Preparation and degradation of potassium gluconate. Glucose was oxidized to potassium gluconate with hypoiodite as described by Sakami (1955) and the product was recrystallized by the addition of methanol to the aqueous solution. It was degraded with sodium metaperiodate as described by Sakami (1955). The formate and formaldehyde were isolated and converted into carbon dioxide as described above under glucosazone.

Assay of [^{14}C]*carbon dioxide.* [^{14}C]Carbon dioxide, which was trapped in 2N-NaOH, was converted into barium [^{14}C]carbonate by precipitation with 5% (w/v) barium chloride solution, collected on filter-paper disks and dried with an infrared lamp. The samples were assayed for radioactivity by gel-scintillation counting as follows. The barium [^{14}C]carbonate was lifted from the filter-paper disk, ground in a small agate mortar (5cm. diam.), weighed, normally in triplicate, into empty counting vials and 5ml. of gel scintillator was added. The gel scintillator consisted of a 4% (w/v) suspension of Cab-O-Sil gelling agent in NE213 liquid scintillator (both from Nuclear Enterprises Ltd., Sighthill, Edinburgh). The products of degradation of the shortest-time samples (see the Results section) were counted in a Nuclear–Chicago coincidence liquid-scintillation counter (model 6801; Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.) and the remainder in a Panax assembly (type SC-LP) connected to a scaler type D657 (Panax Equipment Ltd., Redhill, Surrey). No self-absorption occurred in the range 0–75mg. of $BaCO_3$; at least 4000 counts were recorded and background counts were subtracted.

RESULTS

Bacterial incorporation of ^{14}C*-labelled substrates*

Incorporation of [^{14}C]*formaldehyde by methane-grown* Pseudomonas methanica. This experiment was performed as described by Johnson & Quayle (1965) for the incorporation of [^{14}C]methanol. Owing to the chemical reactivity of formaldehyde, analysis of the extract obtained from the boiled-cell control gave a two-dimensional radioautogram with a number of radioactive spots. Nevertheless a clear difference was evident between the test samples and the control; the former showed an intense radioactive spot on the chromatograms in the position characteristic of sugar phosphates. No radioactivity was present in the corresponding region of the control. These suspected radioactive sugar phosphates were eluted, treated with an acid phosphatase from Polidase S, and rechromatographed. The results are shown in Table 1. They are closely analogous to those obtained by Johnson & Quayle (1965) with [^{14}C]methanol. The percentage of the total activity found in fructose decreased with time, whereas that in glucose increased. However, in the present experiment, another radioactive spot was found, the percentage of which also decreased with time. On the basis of two-dimensional co-chromatography this compound is the same as that formed by cell-free extracts of *P. methanica*, the evidence for whose identification as allulose is discussed by Kemp & Quayle (1965, 1966). As was found by Johnson & Quayle (1965), the total radioactivity recovered in non-volatile compounds was substantially less than that present in the original phosphate area of the parent chromatograms. It is not known at present how much of these losses is due to the presence of phosphates of volatile compounds.

Incorporation of [^{14}C]*formate by methane-grown* Pseudomonas methanica. When [^{14}C]formate was added to a suspension of *P. methanica* growing on methane–air, very little fixation of radioactivity occurred compared with that occurring from [^{14}C]-methane, [^{14}C]methanol or [^{14}C]formaldehyde under similar conditions. Thus a suspension of *P. methanica* (5·1mg. dry wt./ml.) in 10mM-sodium phosphate buffer (pH7·0) containing 10mM-ammonium chloride was divided into two 5ml. portions, each of which was placed in a vessel with a sintered-glass bottom. Both vessels were aerated with methane–air (1:1, v/v) at 30°; to one vessel was added 60μc of sodium [^{14}C]formate (3·4μmoles) and to the other 60μc of [^{14}C]methanol (9μmoles) was added. They were incubated for 1min. and the contents of each poured into separate batches of 4vol. of ethanol. The amount of radioactivity fixed was estimated by chromatographic and radioautographic analysis, as previously described, of one-tenth of the resulting solutions. It was found that 22487 counts/min. of ^{14}C had been incorporated into the phosphate area of the chromatogram in the [^{14}C]methanol experiment, compared with 484 counts/min. on the whole of the chromatogram from the [^{14}C]formate experiment. Furthermore, of this radioactivity fixed from [^{14}C]formate, 199

Table 1. *Distribution of radioactivity among the labelled phosphates obtained from the ethanol-soluble fractions of methane-grown* Pseudomonas methanica *incubated with* [^{14}C]*formaldehyde*

[^{14}C]Formaldehyde (80μmoles; 200μc) was added at zero time to 10ml. of a bacterial suspension (7·5mg. dry wt./ml.) in 10mM-sodium phosphate buffer (pH7·0) containing 10mM-ammonium chloride, vigorously aerated with methane–air (1:1 v/v). Samples (approx. 1ml.) were withdrawn by hypodermic syringe at known times into 4ml. of absolute ethanol, centrifuged and the precipitates extracted with 0·5ml. of aq. 20% (v/v) ethanol. The combined extracts were evaporated and resuspended in 0·2ml. of aq. 20% (v/v) ethanol; 0·1ml. of each sample was analysed by chromatography and radioautography. The resulting phosphate area was eluted and incubated overnight at 30° with the phosphatase from Polidase S. The resulting dephosphorylated non-volatile compounds were analysed by two-dimensional chromatography. Values in parentheses represent the percentages of the total radioactivity on the chromatogram present in that particular compound.

Time (sec.)	Original phosphate area	Radioactivity (counts/min.) Dephosphorylated compounds						
		Glucose	Sedoheptulose	Fructose	Allulose	Ribose	Glycerate	Total
8	4213	574	711	1241	478	72	23	3099
		(18·5)	(22·9)	(40·1)	(15·5)	(2·3)	(0·7)	
20	7740	2115	1145	2003	827	157	78	6325
		(33·4)	(18·1)	(31·7)	(13·1)	(2·5)	(1·2)	
30	9672	3303	1786	2154	870	221	153	8487
		(38·9)	(21·1)	(25·3)	(10·3)	(2·6)	(1·8)	
39	7407	2645	1133	1930	529	79	39	6355
		(41·6)	(17·8)	(30·4)	(8·3)	(1·3)	(0·6)	
54	9034	3386	1396	1954	673	160	71	7640
		(42·7)	(17·7)	(28·2)	(8·5)	(2·0)	(0·9)	

Table 2. *Degradation of samples of authentically labelled* [^{14}C]*glucose with* Leuconostoc mesenteroides

The [^{14}C]glucose was mixed with carrier glucose and 1m-mole samples were degraded by fermentation with *L. mesenteroides*. The individual carbon atoms were obtained as $Ba^{14}CO_3$, which was assayed as a gel in a liquid-scintillation counter (see the Materials and Methods section). Limits of error are expressed as standard deviations.

[^{14}C]Glucose	Percentage of total radioactivity recovered in degradation present						Recovery of ^{14}C in degradation (expressed as percentage of ^{14}C obtained by total combustion)
	In C-1	In C-2	In C-3	In C-4	In C-5	In C-6	
[U-^{14}C]*	22·1±0·59	19·1±0·69	15·2±0·73	18·4±1·06	10·9±0·35	14·3±0·95	87±3·7
[1-^{14}C]	97·5	1·6	<0·2	0·3	<0·2	0·6	97
[2-^{14}C]	0·3	95·5	1·6	0·3	0·7	1·6	84
[6-^{14}C]	0·4	2·8	0·2	0·7	0·5	95·4	84
[2,6-$^{14}C_2$]	<0·2	50·2	0·8	0·4	0·3	48·2	80

* Results are the means of five separate degradations.

counts/min. were present in serine and 56 counts/min. in malate.

Degradations of labelled hexoses

Accuracy of fermentation degradation of [^{14}C]*-glucose.* Before degrading the samples of radioactive glucose obtained from the experiments, the accuracy of the degradation method involving *L. mesenteroides*, as carried out by us, was checked. This was done by degrading samples of uniformly labelled glucose obtained from The Radiochemical Centre. The procedure was repeated five times with samples of varying specific activity and gave reproducible results, the mean of which is given in Table 2. It will be noted that a departure from uniformity was obtained. The most striking features were that C-1 and C-5 apparently contained

respectively more and less radioactivity than uniformity would demand. Significant cross-contamination during the degradation was ruled out by the results of degrading samples of authentically labelled glucose (Table 2). The uniformity of the labelling of the [U-^{14}C]glucose was kindly checked by Dr J. R. Catch and Dr J. C. Turner, of The Radiochemical Centre, who showed by chemical degradation that the radioactivity in C-1 was exactly one-sixth of the radioactivity contained in the entire molecule. It is thus clear that, in our hands, the degradation gave results which erroneously indicated a departure from uniform labelling of $\pm 6\%$. The most likely cause of the error is that of unlabelled material coming from the strain of *L. mesenteroides* cells used. In support of this is the fact that the mean of the sum of the specific activities of the individual carbon atoms as obtained by degradation was 13% lower than that obtained by total combustion. Our experience might be borne in mind in other investigations using this method where small departures from uniformity of labelling are important. It would seem advisable to check the method first against uniformly labelled glucose.

The results of these checks made it important that incubations of *P. methanica* with isotope were done for such short time-intervals that the pattern of labelling in hexose was sufficiently far removed from uniformity to make the experimental error of the degradation relatively insignificant. Furthermore, an independent check on the results was made by partial chemical degradation of the same [^{14}C]glucose.

Degradation of [^{14}C]*glucose obtained from* Pseudomonas methanica. Samples of radioactive glucose obtained from incubation of methane-grown *Pseudomonas methanica* with [^{14}C]methanol were degraded by fermentation with *L. mesenteroides* and the results are shown in Table 3. As the time of incorporation decreased the percentage of the total radioactivity which was located in C-1 increased, but even at the shortest time (3 sec.) the radioactivity was distributed throughout the carbon skeleton.

To obtain a more distinctive labelling pattern, a further short-term incubation was performed at a lower temperature, 16° instead of 30° (see the Materials and Methods section). A portion of the radioactive sugar phosphate area of the resulting chromatogram was analysed after treatment with phosphatase in the usual way (Johnson & Quayle, 1965) and was found to contain 50% of fructose and only 22% of glucose. The radioactivity contained in this amount of [^{14}C]glucose was too small for accurate degradation. To obtain more radioactive glucose, another portion of the eluted phosphate area (before dephosphorylation) was treated with glucose phosphate isomerase as described in the Materials and Methods section. The products were then dephosphorylated with the phosphatase from Polidase S, and chromatographed. After this treatment, 59% of the radioactivity was then found in glucose and 13% in fructose. This amount of radioactivity then permitted a degradation of glucose to be carried out by fermentation with *L. mesenteroides* (Table 4).

The pattern of radioactivity which was obtained by the fermentation method of degradation was then checked by partial chemical degradation of the same [^{14}C]glucose. The results obtained are recorded in Table 5 and compared with the com-

Table 3. *Distribution of radioactive carbon in glucose derived from methane-grown* Pseudomonas methanica *incubated with* [^{14}C]*methanol at* 30°

The first sample of labelled glucose, relating to 3 sec. incubation of cells with [^{14}C]methanol, was obtained from an experiment performed as described in the Materials and Methods section. The two samples relating to 9 sec. and 75 sec. incubation with [^{14}C]methanol were obtained from a similar experiment performed by Johnson & Quayle (1965) and described in that reference under Tables 3 and 4. Each sample of radioactive glucose was purified by rechromatography, diluted with 2·5 m-moles of carrier glucose, and 1 m-mole of each was degraded with *L. mesenteroides* (see the Materials and Methods section). The values in parentheses represent the percentages of the total radioactivity in the glucose which is located in the particular carbon atom.

Time (sec.)	Specific radioactivity of $Ba^{14}CO_3$ (counts/min./mg. of $BaCO_3$)						Sum of specific activities of individual carbon atoms	6 × Specific activity of $Ba^{14}CO_3$ obtained from combustion
	From C-1	From C-2	From C-3	From C-4	From C-5	From C-6		
3	18·3 (43·9)	10·2 (24·4)	4·1 (10·0)	1·8 (4·2)	1·2 (2·8)	6·1 (14·7)	41·7	54·4
9	11·3 (27·8)	8·5 (20·9)	4·0 (9·7)	2·1 (5·0)	3·2 (7·8)	11·7 (28·8)	40·8	40·9
75	41·5 (21·4)	34·8 (17·9)	30·7 (15·7)	30·7 (15·7)	27·4 (14·1)	29·3 (15·1)	194·4	254·4

Table 4. *Distribution of radioactive carbon in glucose derived from methane-grown* Pseudomonas methanica *incubated with* [^{14}C]*methanol at* 16° (L. mesenteroides *method of degradation*)

The radioactive glucose was obtained from a bacterial culture (10 ml.) which was incubated with [^{14}C]methanol for approx. 3 sec. at 16° as described in the Materials and Methods section. The purified [^{14}C]glucose was mixed with carrier glucose and degraded with *L. mesenteroides*.

Carbon atom no.	Specific radioactivity of $Ba^{14}CO_3$ derived from each carbon atom (counts/min./mg. of $BaCO_3$)	Sum of specific activities of individual carbon atoms	6 × Specific activity of $Ba^{14}CO_3$ obtained from combustion	Percentage of total radioactivity in molecule contributed by each carbon atom
1	34·2	46·9	44·4	72·9
2	7·4			15·8
3	1·9			4·1
4	0·2			0·5
5	0·6			1·2
6	2·6			5·5

Table 5. *Partial chemical degradation of* [^{14}C]*glucose derived from methane-grown* Pseudomonas methanica *incubated with* [^{14}C]*methanol at* 16°

Degradation	Carbon atoms obtained as $Ba^{14}CO_3$	Specific activity of $Ba^{14}CO_3$ obtained (counts/min./mg.)	Specific activity of $Ba^{14}CO_3$ × no. of carbon atoms (counts/min./mg. of $Ba^{14}CO_3$)	Comparable results obtained from Table 4 (counts/min./mg. of $Ba^{14}CO_3$)
Periodate oxidation of gluconate	1	32·8	32·8	34·2
	2–5	2·3	9·2	10·1†
	6	2·4	2·4	2·6
Periodate oxidation of glucosazone	1–3	14·5*	43·5	43·5†
	4, 5	1·2	2·4	0·8†
Glucosazone combustion	1–6	8·4*	50·4	46·9†
Glucose combustion		7·4	44·4	
Gluconate combustion		8·1	48·6	
Gluconate total			44·4†	

* Values corrected for presence of ^{12}C in phenylhydrazine residues.
† Values obtained by addition.

parable results obtained by the fermentation degradation. It is clear that both the fermentation method and the chemical method give essentially the same labelling pattern for the [^{14}C]glucose, in which the specific activities of the individual carbon atoms are C-1 ≫ C-2 > C-3 > C-4 < C-5 < C-6.

DISCUSSION

The oxidation of methane by *P. methanica* is considered to occur in a stepwise manner via methanol, formaldehyde and formate to carbon dioxide (Dworkin & Foster, 1956; Brown, Strawinski & McCleskey, 1964; Johnson & Quayle, 1964). The studies of Johnson & Quayle (1965) showed that [^{14}C]methane and [^{14}C]methanol are rapidly assimilated by growing cell suspensions mainly into sugar phosphates, whereas [^{14}C]carbon dioxide is incorporated mainly into C_4 dicarboxylic acids. It was concluded that C_1 units are assimilated at reduction level(s) between methanol and formate by one pathway, and as carbon dioxide by another. The results described in the present work allow this localization to be further narrowed; [^{14}C]formaldehyde is incorporated mainly into phosphorylated compounds, whereas [^{14}C]formate is assimilated mainly into serine and malate. There is thus a qualitative difference in the path of carbon from C_1 units derived from methane, methanol and formaldehyde on one hand, and formate and carbon dioxide on the other. Furthermore, there appears to be a quantitative difference in the amount of ^{14}C fixation which occurs from the different tracers; high fixation occurs between the oxidation levels CH_4–H·CHO, and low fixation between H·CO_2H–CO_2. The results are represented diagrammatically in Fig. 2. If the assumption is made that the conversions involved in the oxidation

of methane to carbon dioxide are essentially irreversible, and this seems likely for an aerobic organism such as *P. methanica*, then the results point to formaldehyde as the oxidation level at which most of the carbon is assimilated into cell constituents. This is consistent with the operation of the postulated modified pentose phosphate cycle (Fig. 3), which involves hydroxymethylation of ribose 5-phosphate with formaldehyde to give allulose phosphate (Kemp & Quayle, 1965, 1966).

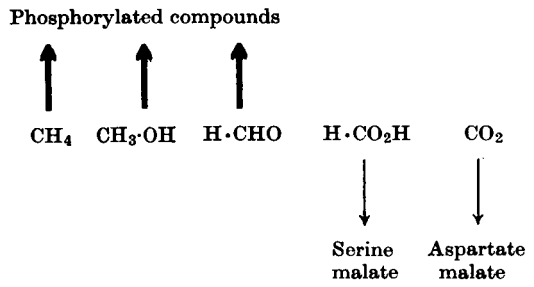

Fig. 2. Incorporation of carbon from C_1 units at different oxidation levels.

In previous isotopic experiments by Johnson & Quayle (1965), no [^{14}C]allulose phosphate had been detected amongst the early-labelled sugar phosphates after incubation of cells with [^{14}C]methane or [^{14}C]methanol. In the present work, when [^{14}C]formaldehyde was administered to cells growing on methane, 15% of the radioactivity fixed after 8sec. incubation was found in allulose phosphate. The percentage dropped thereafter. This result is taken as further evidence in favour of the proposed cycle of reactions. The failure to detect allulose phosphate amongst the early labelled sugar phosphates when [^{14}C]methane or [^{14}C]methanol is used may be due to a rate-limiting step in the conversion of methanol into formaldehyde. Under these conditions allulose phosphate might be metabolized almost as quickly as it is formed, whereas addition of formaldehyde to the system might cause a temporary rise in the pool of allulose phosphate.

The key reaction of the modified pentose phosphate cycle is the formation of allulose phosphate by hydroxymethylation of ribose 5-phosphate; subsequent epimerization and isomerization then leads to formation of fructose phosphate and glucose phosphate. It follows therefore that radio-

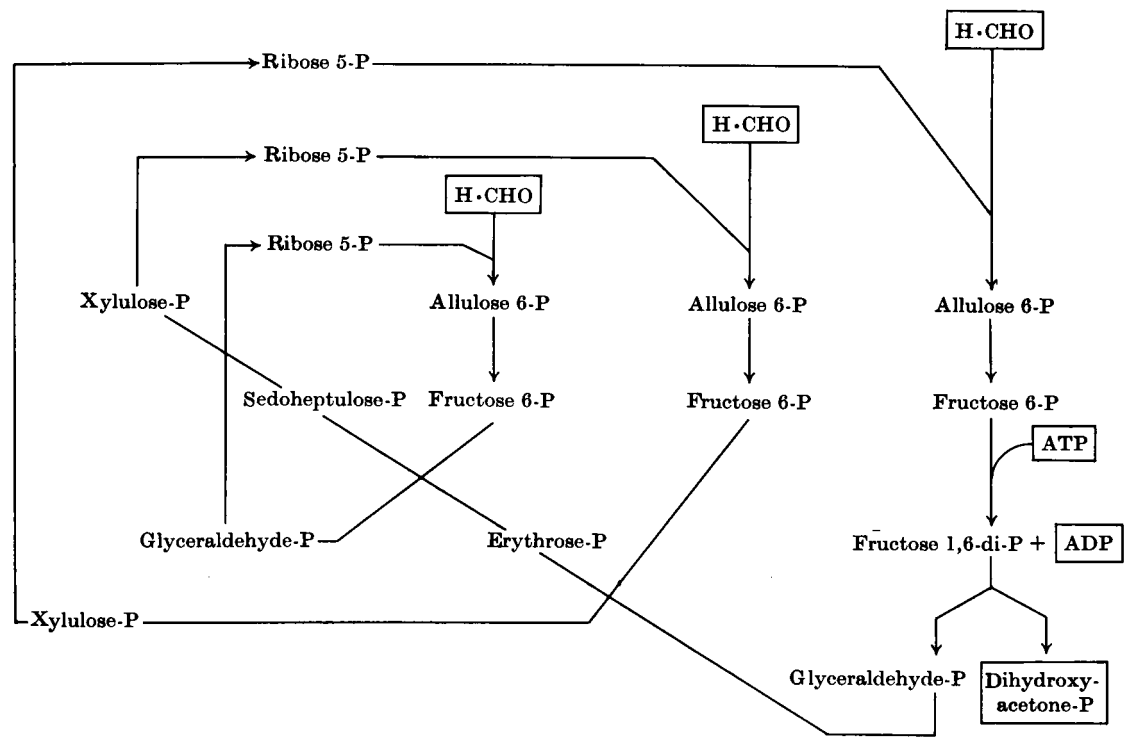

Fig. 3. Ribose phosphate cycle of formaldehyde fixation.

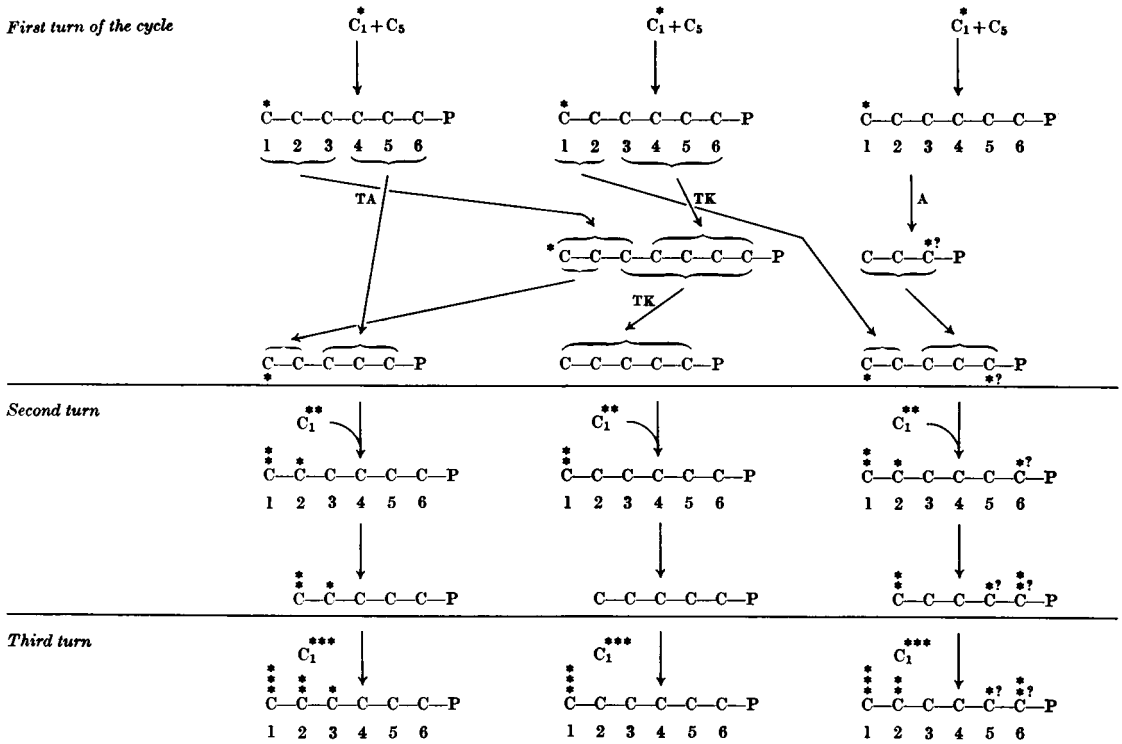

Fig. 4. Predicted labelling pattern of hexose phosphate. TA, transaldolase; TK, transketolase; A, fructose diphosphate aldolase.

active hexose phosphate obtained after brief incubation of the cells with ^{14}C-labelled growth substrate should be predominantly labelled in C-1. The labelling pattern found after 3 sec. incubation of cells with [^{14}C]methanol at 16° indeed shows this feature, 73% of the radioactivity being found in C-1. Furthermore, the effect of recycling of the carbon skeleton on the labelling pattern may be predicted. If three turns of the cycle are followed (Fig. 4), in which each turn involves the incorporation of a new ^{14}C unit, here represented as C*, C** and C*** respectively, then the pattern of the spread of radioactivity through the hexose skeleton becomes apparent. It will be noted that at each turn, one of the hexose molecules is cleaved by fructose diphosphate aldolase (D-fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13) to give 1 molecule each of dihydroxyacetone phosphate and glyceraldehyde phosphate. The latter is used as acceptor for the glycolaldehyde fragment transferred from another fructose phosphate molecule by transketolase (sedoheptulose 7-phosphate–D-glyceraldehyde 3-phosphate glycolaldehydetransferase, EC 2.2.1.1). However, any interconversion of the two triose phosphates catalysed by triose phosphate isomerase (D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1) would lead to some appearance of the labelling pattern of dihydroxyacetone phosphate in that of glyceraldehyde 3-phosphate. Hence the labelling pattern of C-1 to C-3 of hexose phosphate would appear in C-5 to C-3 of pentose phosphate and finally in C-6 to C-4 of hexose phosphate at the next turn of the cycle. The extent of this randomization cannot be predicted, and hence the label in the affected carbon atoms is indicated in Fig. 4 with a question mark. Nevertheless, the qualitative pattern in the hexose molecule may be clearly forecast. Operation of the cycle would demand the radioactivity to be spread such that C-1 > C-2 > C-3; this pattern would then be mirrored in C-4 < C-5 < C-6, the extent of this mirroring of the pattern depending on the relative rates of reaction of glyceraldehyde 3-phosphate with transketolase and triose phosphate isomerase. The pattern observed in the early-labelled glucose (which is derived from

an equilibrium mixture of glucose phosphate and fructose phosphate) precisely bears out this prediction. After longer times of assimilation of ^{14}C-labelled substrate, isotope should spread throughout the hexose skeleton whose labelling pattern should approach that of uniformity. This prediction is also borne out by the present work. The combined isotopic data here described are thus consistent with the operation *in vivo* of the cycle previously proposed (Kemp & Quayle, 1965, 1966).

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