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The Dissimilation of Glucose and Gluconate by *Acetobacter xylinum*

1. THE ORIGIN AND THE FATE OF TRIOSE PHOSPHATE

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Carbohydrate metabolism by the acetic acid bacteria has been studied extensively for many years (Rainbow, 1961). Most of these studies (Ameyama & Kondô, 1958; Kondô & Ameyama, 1958) have been directed at the ability of various species to oxidize a wide range of substrates and, particularly, to produce gluconate or oxogluconates. De Ley (1961) and his group have investigated the enzymes associated with oxogluconate metabolism in certain *Acetobacter* spp.

Only in a few cases has any attempt been made to determine which metabolic pathways are active in the conversion of glucose into pyruvate and acetate. The pentose phosphate cycle has been demonstrated in several *Acetobacter* spp. (De Ley, 1961). Katznelson (1958) obtained enzymic evidence for this pathway in *A. melanogenum*, as did Gromet, Schramm & Hestrin (1957) with *A. xylinum*. *A. suboxydans* is believed to dissimilate glucose predominantly via the pentose phosphate cycle (Kitos, Wang, Mohler, King & Cheldelin, 1958). The Entner & Doudoroff (1952) pathway, which involves the cleavage of 6-phosphogluconate to pyruvate and glyceraldehyde 3-phosphate, has been detected in *A. melanogenum* (Katznelson, 1958) and *A. suboxydans* (R. M. Hochster, personal communication). Pyruvate and acetate may also originate from 5-oxogluconate in *A. suboxydans* (Murooka, Kobayashi & Asai, 1960). Glycolytic activity is either weak or absent in *Acetobacter* spp. (De Ley, 1961; Rainbow, 1961).

The present work is concerned with an elucidation of the routes of glucose and gluconate dissimilation in *A. xylinum*, a cellulose-synthesizing bacterium. Previous radiorespirometric experiments by Wang & Bjerre (1961) on the utilization of specifically ^{14}C-labelled glucose by whole cells indicated that C-6 of glucose appeared very rapidly as carbon dioxide, followed by C-3 and C-4. Such an observation was surprising since C-6, by virtue of its incorporation into the methyl group of pyruvate or acetate by most known metabolic schemes, is less readily oxidizable to carbon dioxide than either C-3 or C-4. Moreover, the finding cannot be accounted for by the en-

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zymic data of Gromet *et al.* (1957) which suggested that only the pentose phosphate cycle was operative in *A. xylinum*.

EXPERIMENTAL

Biochemicals and radioactive substrates. D-[1-^{14}C]Glucose was purchased from Volk Radiochemical Co., Chicago, Ill., U.S.A. D-[2-^{14}C]Glucose and D-[6-^{14}C]glucose were supplied by the U.S. National Bureau of Standards, Washington, D.C. The sample of D-[3-^{14}C]glucose was generously provided by Dr H. Isbell of the National Bureau of Standards. Uniformly labelled [^{14}C]glucose ([U-^{14}C]glucose) was obtained from Tracerlab Inc., Richmond, Calif., U.S.A. D-[3,4-$^{14}C_2$]Glucose was prepared by the procedure of Wood, Lifson & Lorber (1945). D-[6-^{14}C]Glucuronate was supplied by the U.S. National Bureau of Standards, Washington, D.C.

Specifically ^{14}C-labelled gluconates were synthesized from the correspondingly ^{14}C-labelled D-glucose by the method of Moore & Link (1940). Potassium 2-oxogluconate and potassium 5-oxogluconate were provided by the Takeda Chemical Co., Osaka, Japan.

Sodium pyruvate (chemically pure), fructose 1,6-diphosphate (calcium salt) and fructose 6-phosphate (barium salt) were supplied by Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. The fructose 1,6-diphosphate (calcium salt) was dissolved in water and the Ca^{2+} ions were removed by treatment with Dowex 50 (X4; H^+ form) resin. The fructose 1,6-diphosphate solution was adjusted to pH 7·5 and analysed by the method of Roe, Epstein & Goldstein (1949). The same method was used for determining fructose 6-phosphate.

Sigma Chemical Co., St Louis, Mo., U.S.A., supplied 6-phosphogluconate (barium salt), β-NAD, DL-glyceraldehyde 3-phosphate (barium diethylacetal derivative), D-3-phosphoglycerate (barium salt), ADP (muscle), ATP, ribose 5-phosphate (sodium salt) and glucose 6-phosphate. The barium salts were converted into the sodium salts before use. 6-Phosphogluconate was prepared immediately before each experiment since it is unstable at neutral pH (Hochster & Katznelson, 1958). 3-Phosphoglycerate was analysed as acid-resistant phosphate by using procedures given by LePage (1957). Dihydroxyacetone phosphate was prepared from DL-glyceraldehyde 3-phosphate with crystalline triose isomerase (Sigma Chemical Co.). Crystalline (2 ×) aldolase was provided by Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Sodium arsenite solutions were prepared and neutralized immediately before use.

Cell culture and fractionation. Slant cultures of *A. xylinum*, ATCC 10821, were maintained on an agar medium consisting of 5·0 g. of peptone, 5·0 g. of yeast extract, 2·0 g. of KH_2PO_4, 0·80 g. of $CaCO_3$, 20·0 g. of glucose and 25·0 g. of agar/l. of water. Cells were grown in a liquid culture medium consisting of 20 g. of glucose, 2·5 g. of peptone, 2·5 g. of yeast extract, 2 g. of KH_2PO_4, 2 g. of K_2HPO_4 and 0·25 g. of $MgSO_4$/l. of water. This medium was adjusted to pH 6·8 and sterilized. As general procedure, a stock culture of cells was grown in 80 ml. of medium on a rotary shaker (60 cycles/min.) for 24 hr. at 30°. Portions of cell suspension so obtained were used to inoculate 80 ml. lots of sterile medium for the production of cells.

The bacteria were harvested in their exponential phase of growth by centrifuging for 3 min. at 3000*g*. The acidic culture supernatant was discarded. The wet packed cells, including some cellulose fibrils, were subsequently washed with buffer, being treated differently depending on the type of experiment. Cells to be used for the radiorespirometric studies were washed once in cold 0·01M-phosphate buffer, pH 6·0, recentrifuged and finally resuspended in buffer and diluted to a density giving a known dry weight of cells/ml.

For enzyme experiments, the cells were washed twice with cold 0·01M-tris buffer, pH 7·5, centrifuged and slurried into cold tris buffer. Cells were disrupted for 15 min. at 5° by using a Raytheon 250w 10 kcyc./sec. ultrasonic oscillator set at 1·1A. The preparation was centrifuged at 4000*g* at 1° for 15 min., giving an opalescent supernatant containing the enzymes to be assayed. Different extracts were standardized on the basis of substance (dried over P_2O_5) precipitated by 10% (w/v) trichloroacetic acid.

Radiorespirometric experiments. The rate of $^{14}CO_2$ production from cells utilizing specific carbon atoms of glucose and gluconate was followed by the method of Wang *et al.* (1958).

Assay of enzymes. Enzyme activities were measured in air. Aldolase was determined by the chromogenic procedure of Sibley & Lehninger (1949). Phosphofructokinase was determined by measuring the amount of triose phosphate trapped with hydrazine when fructose 6-phosphate was incubated with an excess of ATP. Fructose diphosphatase was assayed by following the release of inorganic phosphorus from fructose 1,6-diphosphate. The conversion of 3-phosphoglycerate into pyruvate was determined by analysis of deproteinized reaction mixtures for pyruvate as the 2,4-dinitrophenylhydrazone (Murooka *et al.* 1960). The mixture was assayed also for acid-resistant phosphate and acid-labile phosphate (phosphoenolpyruvate). The Entner & Doudoroff (1952) enzymes, 6-phosphogluconate dehydrase and 3-deoxy-2-oxo-6-phosphogluconate aldolase, were assayed by measuring the amounts of pyruvate and triose phosphate formed when enzyme extract was incubated with 6-phosphogluconate. Methods given by LePage (1957) were used to determine alkali-labile, acid-labile, inorganic and total phosphate. Acid-resistant phosphate was calculated by subtracting the acid-labile phosphate from the total phosphate and correcting the result for unhydrolysed ADP and NAD.

Radioactivity measurements. All ^{14}C-labelled material other than $Ba^{14}CO_3$ was counted in a Packard Tri-Carb liquid-scintillation spectrometer. Radioactive sugars and amino acids were standardized by counting an aqueous solution in a scintillation mixture consisting of 4·9 ml. of ethanolic hyamine hydroxide [(*p*-di-isobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide] and 0·3 mg. of 2,2′-*p*-phenylenebis-5-phenyloxazole plus 30 mg. of terphenyl dissolved in 10 ml. of toluene. Counter efficiency was determined by the use of internal standards. Pyruvate 2,4-dinitrophenylhydrazone was counted in toluene containing phosphor in the absence of hyamine hydroxide. Appropriate corrections were made for colour quenching. Calculations of specific activity were made on at least triplicate samples. Radioactivity in CO_2, cells and medium was measured as described in detail by Wang & Krackov (1962). Barium carbonate was centrifuged on to aluminium planchets and counted in a Geiger–Müller gas-flow counter

(low-background type with thin Mylar window; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). Samples were counted to a standard deviation of not more than 2%.

Incorporation of ^{14}C *from* [^{14}C]*glucose and* [^{14}C]*gluconate and separation of labelled amino acids.* Cells were allowed to metabolize ^{14}C-labelled substrate in air-swept radiorespirometric flasks (Wang *et al.* 1958). The $^{14}CO_2$ evolved was trapped in ethanolic 0·25N-hyamine hydroxide and counted at half-hour intervals. At the point where the rate of $^{14}CO_2$ output began to decline, the cell suspension was poured into centrifuge cups chilled in ice. The reaction flask was rinsed several times with water. Cells were separated from the medium by centrifuging, washed with water, and then recentrifuged and dried over P_2O_5 *in vacuo.*

Isolation of cellular amino acids. Dried cells were hydrolysed in 20% HCl in sealed test tubes for 6·5–16 hr. at 15 lb./in.^{2} Partial separation of amino acids in concentrated hydrolysate was obtained on Whatman no. 4 chromatography paper developed with descending phenol–water (4:1, v/v). The solvent was allowed to run to the end of the paper, at which time the sheet was dried in warm air and washed with peroxide-free ether. Glutamate and aspartate were separated on the phenol–water chromatogram. Alanine was eluted with 4% (v/v) propan-2-ol and resolved from contaminating amino acids by chromatography on Whatman no. 4 paper developed with butan-2-ol–aq. 3% NH_3 (Roland & Gross, 1954). The alanine was eluted, concentrated to dryness and dissolved in water. Samples were taken for colorimetric analysis by the procedure of Yemm & Cocking (1955) as well as for radioactivity determinations. Glutamate and aspartate fractions were rechromatographed and treated in a manner similar to that used for the alanine fraction.

Degradation of labelled amino acids. Carrier-diluted alanine was degraded sequentially first by decarboxylation with ninhydrin to yield C-1 as CO_2 and C-2 plus C-3 as acetaldehyde. The latter was trapped in 2% (w/v) bisulphite solution. Acetaldehyde was released from the bisulphite complex, separated by steam-distillation and allowed to react with hypoiodite to yield C-2 as formate and C-3 as iodoform. The iodoform was recrystallized from methanol–water solution and dried over P_2O_5. Formic acid was isolated by steam-distillation and oxidized to CO_2 with HgO (Osburn, Wood & Werkman, 1933). The specific activity of iodoform and alanine was determined by combustion to CO_2 (Van Slyke & Folch, 1940). The CO_2 from each degradation step was trapped in CO_2-free 0·2N-NaOH. Overall recoveries of carbon from C-1, C-2 and C-3 averaged 80, 25 and 30% respectively.

Glutamate was degraded, in part, by ninhydrin decarboxylation to yield C-1 and by the Schmidt reaction to yield C-5 of the molecule. Total specific activity was found by combustion with potassium persulphate (Chen & Lauer, 1957). Aspartate was allowed to react with ninhydrin to yield C-1 plus C-4. The activity of C-2 plus C-3 was obtained by difference after persulphate combustion of the whole molecule.

Incorporation of ^{14}C *from* [^{14}C]*glucose and separation of labelled pyruvate.* Cells were suspended in buffer in radiorespirometric flasks at 30°. Labelled glucose was tipped into the cell suspension and the evolved CO_2 trapped in CO_2-free N-NaOH. A CO_2 blank having the same amount of buffer but no cells was run simultaneously. At the end of the incorporation interval, the cell suspension and flask washings were placed in cold centrifuge tubes. After centrifuging for 5 min., the supernatant fluid was decanted off and the cells were washed and recentrifuged. Supernatants were combined and diluted with water to 50 ml. A 2 ml. sample was stored frozen for paper chromatography, sugar analysis and radioactivity determination.

The NaOH in the CO_2 trap was diluted to 50 ml. with CO_2-free water. For activity determination, 1 ml. and 0·5 ml. samples were added to separate flasks having non-radioactive Na_2CO_3 solution (53·7 mg. of Na_2CO_3/20·0 ml.) and precipitated as $Ba^{14}CO_3$. The $^{14}CO_2$ left in the diluted trap solution was recovered as $Ba^{14}CO_3$, dried and weighed. Results so found were used to calculate the specific activity of the metabolic $^{14}CO_2$.

A portion (2 ml.) of 0·4% solution of 2,4-dinitrophenylhydrazine in 2N-HCl was added to the diluted radioactive supernatant and permitted to remain for 4 hr. at room temperature. Keto acid phenylhydrazones were extracted into ethyl acetate and then into 10% (w/v) Na_2CO_3 solution. This was acidified at 0° and re-extracted with ethyl acetate. The acidic phenylhydrazones were resolved on Whatman no. 3 paper, and developed, descendingly, with butan-1-ol–ethanol–water (7:1:2, by vol.). Standard 2,4-dinitrophenylhydrazone of pyruvate was co-chromatographed with the radioactive phenylhydrazones. The pyruvate derivative was sectioned out and eluted by pulping for 2 hr. in 10% (w/v) Na_2CO_3. The slurry was filtered, washed with Na_2CO_3 solution and acidified in the cold before quantitative extraction with ethyl acetate. The ethyl acetate solution was evaporated to dryness in a stream of dry air. The residue of the 2,4-dinitrophenylhydrazone of pyruvate was dissolved in 15 ml. of ethanol. Samples were taken for counting and quantitative analysis. The remainder was diluted with unlabelled 2,4-dinitrophenylhydrazone of pyruvate and recrystallized twice from hot ethanol–water solution.

Degradation of 2,4-dinitrophenylhydrazone of pyruvate. The pyruvate derivative was decarboxylated with ceric sulphate, releasing C-1 of the pyruvate moiety as CO_2 (Krebs, 1938). C-2 and C-3 of the pyruvate moiety were converted into acetate by oxidation of the dinitrophenylhydrazone derivative with 5% (w/v) $KMnO_4$ in N-H_2SO_4. The reaction solution was made alkaline to phenolphthalein and concentrated to 30 ml. after the excess of $KMnO_4$ had been removed with thiosulphate and the precipitate filtered off. The solution was acidified with 10N-H_2SO_4 (to Congo Red paper), and a few drops of 2% (w/v) $KMnO_4$ in N-H_2SO_4 were added. Acetate was recovered as the sodium salt after steam-distillation, titration of the steam-distillate with CO_2-free 0·2N-NaOH and evaporation of the solution to dryness over P_2O_5 *in vacuo.* Acetate samples were diluted with 1 m-mole of unlabelled sodium acetate and degraded by the method of Katz, Abraham & Chaikoff (1955). The specific activity of the 2,4-dinitrophenylhydrazone of pyruvate was determined by Van Slyke–Folch combustion.

Analysis of the incorporation medium. The radioactive medium from the glucose experiment was deionized with Dowex 50 (X4; H^+ form) resin and chromatographed with standard 2-oxogluconate, 5-oxogluconate and gluconate on Whatman no. 1 paper. Descending butan-1-ol–formic acid–water (40:7:10, by vol.) was used to develop the chromatogram. Acids were located with basic 0·05% bromophenol blue in ethanol.

Total reducing power in a sample of non-deionized medium was estimated by the procedure of Somogyi (1952). The amount of 2-oxogluconate was determined with *o*-phenylenediamine (Lanning & Cohen, 1951). The difference between the total reducing power and 2-oxogluconate represented glucose.

Glucose and glycerol were chromatographed on tris-washed Whatman no. 1 paper (Gordon, Thornburg & Werum, 1956). Sugar phosphates were separated by chromatography on Whatman no. 1 paper with ascending ethyl acetate–acetic acid–water (3:3:1, by vol.). The hexose phosphate spots were detected by the method of Bandurski & Axelrod (1952).

RESULTS

Time-course of production of [^{14}C]*carbon dioxide from intact cells metabolizing* [^{14}C]*glucose*. The radiorespirometric patterns, i.e. the relative rates of $^{14}CO_2$ production, for 27 hr. cells utilizing specifically ^{14}C-labelled glucose are presented in Table 1. Given also is an inventory of the radioactivity remaining in the cells and medium and the total percentage of ^{14}C recovery at the period when the output of $^{14}CO_2$ approached zero. The relative rates of $^{14}CO_2$ recovery from individual C atoms of glucose were in the order:

$$\text{C-1} > \text{C-2} > \text{C-6} = \text{C-3} > \text{C-4}.$$

The cells utilizing [1-^{14}C]glucose retained only a small percentage of the substrate activity, with the bulk of it being converted into $^{14}CO_2$. The amount of activity remaining in the cells and medium was highest from glucose labelled at C-3, C-4 and C-6, showing that these C atoms were preferentially incorporated into cell constituents, including cellulose and oxogluconates in the medium. The results indicated clearly that the pathway of glucose dissimilation in *A. xylinum* was unique, particularly in view of the high yield of $^{14}CO_2$ from [6-^{14}C]-glucose.

The organism is known to be capable of converting glucose into various sugar acids, predominantly gluconate with smaller amounts of 2-oxogluconate and 5-oxogluconate. This fact prompted a study of the relative rates of $^{14}CO_2$ production from cells utilizing specifically ^{14}C-labelled gluconate. The results, qualitatively similar to those obtained with glucose, are given in Table 2. The order of $^{14}CO_2$ output was

$$\text{C-1} = \text{C-6} > \text{C-2} = \text{C-3} > \text{C-4}.$$

Total recoveries of $^{14}CO_2$ from gluconate were lower than those from glucose and due, it appeared, to a greater amount of keto sugar acids accumulat-

Table 1. *Time-course of production of* [^{14}C]*carbon dioxide from* Acetobacter xylinum *cells metabolizing specifically* ^{14}C*-labelled glucose*

The reaction mixture (volume, 16·0 ml.) contained: initial glucose, 33·3 μmoles; cells (age, 27 hr.), 13·5 mg.; phosphate buffer, 304 μmoles. The initial pH was 5·8, and the temperature 28°. The values for the distribution of radioactivity are given for 6 hr. after the introduction of glucose. The values for [4-^{14}C]glucose were calculated from the results obtained for [3-^{14}C]glucose and [3,4-$^{14}C_2$]glucose.

		$^{14}CO_2$ recovery (%)			Distribution of radioactivity (%)			
Substrate	Radioactivity (μc)	At 1 hr.	At 2 hr.	At 3 hr.	CO_2	Cells	Medium	Total
[1-^{14}C]Glucose	0·25	65	21	5	93	1	5	99
[2-^{14}C]Glucose	0·24	56	17	8	85	7	10	102
[6-^{14}C]Glucose	0·24	46	16	5	69	14	15	98
[3-^{14}C]Glucose	0·02	46	18	5	72	11	12	95
[3,4-$^{14}C_2$]Glucose	0·03	41	16	5	66	15	16	97
[4-^{14}C]Glucose	—	36	15	5	58	18	20	96

Table 2. *Time-course of production of* [^{14}C]*carbon dioxide from* Acetobacter xylinum *cells metabolizing specifically* ^{14}C*-labelled gluconate*

The reaction mixture (volume, 16·0 ml.) contained: initial gluconate (potassium salt), 46·9 μmoles; cells (age, 27 hr.), 9·0 mg.; phosphate buffer, 304 μmoles. The initial pH was 5·8 and the temperature 29°. The values for the distribution of radioactivity are given for 6 hr. after the introduction of gluconate. The values for [4-^{14}C]gluconate were calculated from the results obtained for [3-^{14}C]gluconate and [3,4-$^{14}C_2$]gluconate.

		$^{14}CO_2$ recovery (%)			Distribution of radioactivity (%)			
Substrate	Radioactivity (μc)	At 1 hr.	At 2 hr.	At 3 hr.	CO_2	Cells	Medium	Total
[1-^{14}C]Gluconate	0·21	37	22	5	66	1	32	99
[2-^{14}C]Gluconate	0·23	34	25	3	66	8	21	95
[6-^{14}C]Gluconate	0·23	38	22	1	63	13	24	99
[3-^{14}C]Gluconate	0·06	35	24	2	64	12	24	100
[3,4-$^{14}C_2$]Gluconate	0·08	29	21	2	54	14	26	94
[4-^{14}C]Gluconate	—	23	17	2	45	15	27	87

ing in the medium. As with glucose, little of the C-1 of gluconate entered synthetic processes in the cell.

The relative rates of $^{14}CO_2$ output observed in the gluconate experiments again suggested a novel route of carbohydrate utilization in *A. xylinum*. The pathway is one which allows a preferred conversion of C-6 into carbon dioxide as compared with C-3 and C-4. However, the yields of $^{14}CO_2$ provide rather limited clues as to the exact nature of the pathways involved in hexose breakdown in *A. xylinum*. The pentose phosphate cycle is presumably operative in this organism in view of the rapid output of carbon dioxide from C-1 and C-2 of glucose and gluconate. To gain further insight into this problem it seemed advisable to study the mode of incorporation of glucose carbon into metabolic intermediates and cellular constituents which are closely associated with the main pathways (Katz & Wood, 1960; Dawes & Holms, 1958).

Incorporation of ^{14}C *from* [^{14}C]*glucose into alanine.* In the first series of experiments study was focused on cellular alanine which is known to be closely related to pyruvate, a key metabolic intermediate of glucose metabolism.

The ^{14}C distribution patterns in alanine samples, isolated from cells utilizing specifically ^{14}C-labelled glucose, are given in Table 3. The incorporation experiments were conducted over a period of 6 hr. until the $^{14}CO_2$ production had nearly ceased. At this time, 33% of the initial activity from [U-^{14}C]-glucose remained in the medium as cellulose and oxogluconates (results not shown). There was a preferential incorporation of C-1 and C-2 of glucose into C-1 and C-2 respectively of alanine. Most intriguing was the finding that 52% of the total label in alanine derived from [6-^{14}C]glucose resided at C-1, with 8% at C-2 and 40% at C-3.

Table 4 presents the results obtained in another experiment on the incorporation of glucose carbon

Table 3. *Experiment I: the distribution of* ^{14}C *in alanine from* Acetobacter xylinum *metabolizing specifically* ^{14}C*-labelled glucose*

The reaction mixture (volume, 40·0 ml.) contained: initial glucose, 27·8 μmoles (11·1 × 10^6 disintegrations/min.); cells (age, 20 hr.), as indicated. The initial pH was 5·5 and the final pH 5·1; the temperature was 28°. The rate of aeration was 41·0 ml./min. The reaction was stopped at 6 hr.

Substrate	[1-^{14}C]Glucose	[2-^{14}C]Glucose	[6-^{14}C]Glucose
Sp. activity of C-1 of alanine (counts/min. per mg.atom of C)	1920 (91%)	820 (27%)	883 (52%)
Sp. activity of C-2 of alanine (counts/min. per mg.atom of C)	42 (2%)	1852 (61%)	144 (8%)
Sp. activity of C-3 of alanine (counts/min. per mg.atom of C)	154 (7%)	373 (12%)	682 (40%)
Total sp. activity of alanine (counts/min. per m-mole)	2116	3045	1709
Sp. activity of undiluted alanine (disintegrations/min. per m-mole)	1·29 × 10^6	4·23 × 10^6	3·57 × 10^6
Dry wt. of cells (mg.)	18·7	17·9	18·3
Radioactivity of CO_2 (disintegrations/min.)	8·1 × 10^6	5·7 × 10^6	5·8 × 10^6
Radioactivity of cells (disintegrations/min.)	0·2 × 10^6	1·4 × 10^6	1·8 × 10^6
Radioactivity of medium (disintegrations/min.)	2·2 × 10^6	3·6 × 10^6	3·4 × 10^6
^{14}C recovery (%)	94·0	95·8	99·0

Table 4. *Experiment II: the distribution of* ^{14}C *in alanine isolated from* Acetobacter xylinum *metabolizing specifically* ^{14}C*-labelled glucose*

The reaction mixture (volume, 21·3 ml.) contained: initial glucose, 66·6 μmoles (13·3 × 10^6 disintegrations/min.); cells (age, 27 hr.), as indicated; phosphate buffer, 304 μmoles. The initial pH was 5·8 and the final pH 5·6; the temperature was 28°. The reaction was stopped at 2½ hr.

Substrate	[U-^{14}C]Glucose	[1-^{14}C]Glucose	[2-^{14}C]Glucose	[6-^{14}C]Glucose
Sp. activity of C-1 of alanine (counts/min. per mg.atom of C)	264 (39%)	650 (97%)	73 (12%)	406 (64%)
Sp. activity of C-2 of alanine (counts/min. per mg.atom of C)	233 (35%)	5 (1%)	506 (85%)	46 (7%)
Sp. activity of C-3 of alanine (counts/min. per mg.atom of C)	173 (26%)	13 (2%)	18 (3%)	185 (29%)
Total sp. activity of alanine (counts/min. per m-mole)	670	668	597	637
Sp. activity of undiluted alanine* (disintegrations/min. per m-mole)	2·02 × 10^6	0·43 × 10^6	1·16 × 10^6	1·6 × 10^6
Dry wt. of cells (mg.)	67·3	49·2	50·9	65·5
Radioactivity of CO_2 (disintegrations/min.)	8·67 × 10^6	11·4 × 10^6	8·8 × 10^6	8·7 × 10^6
Radioactivity of cells† (disintegrations/min.)	1·8 × 10^6	0·0 × 10^6	1·5 × 10^6	1·7 × 10^6
Radioactivity of medium (disintegrations/min.)	2·8 × 10^6	2·3 × 10^6	2·9 × 10^6	2·9 × 10^6

* Corrected for difference in cell weight. ‡ Calculated by difference.

into alanine of older (27 hr.) cells. The cells were permitted to metabolize labelled substrate, in this case, for only 2·5 hr., i.e. until the time when the rate of $^{14}CO_2$ production began to decline. It was visualized that a shorter duration of incubation would minimize the extent of randomization of labelling in alanine or its precursor, pyruvate. An examination of the results given in Table 4 shows that the results are qualitatively the same as those observed in the first experiment. The unusual distribution of ^{14}C seen in the alanine synthesized from [6-^{14}C]glucose (Expt. I and Table 3) was strikingly apparent in the second experiment. The almost equal activities of $^{14}CO_2$ arising from C-2 and C-6 of glucose and the slightly lower (but possibly not significant) specific activity of alanine from [6-^{14}C]glucose hints at a reaction scheme whereby C-6 appears in an intermediate which is quickly decarboxylated to yield this carbon atom.

Sufficient clues are provided by the results of the glucose-incorporation experiments to account for the rapid rate of carbon dioxide evolution from C-6 of glucose. It appeared that C-6 of glucose was converted extensively into C-1 of pyruvate which, in turn, is readily decarboxylated by intact cells of *A. xylinum* (Gromet *et al.* 1957).

Incorporation of ^{14}C *from* [^{14}C]*gluconate into alanine.* When specifically ^{14}C-labelled gluconate was supplied to the cells, the distribution of ^{14}C in alanine was as shown in Table 5. The incorporation period was terminated 2 hr. after the introduction of substrate. The most significant finding is the very large percentage (84 %) of ^{14}C at C-1 of alanine derived from [6-^{14}C]gluconate as compared to 12 % at C-3 and 4 % at C-2. The alanine produced from [1-^{14}C]gluconate had all the ^{14}C at C-1. The relative specific activities of the alanine derived from the gluconate labelled in C-1, C-2 and C-6 were 0·63, 1·0 and 0·86 respectively, indicating a smaller incorporation of C-6 than C-2 into pyruvate. The similarity in the distribution of ^{14}C in alanine from ^{14}C-labelled glucose and gluconate shows a close relationship in the dissimilation pathways of these two compounds.

Accumulation of pyruvate in arsenite-inhibited cells. Gromet *et al.* (1957) reported that pyruvate did not accumulate in the medium of *A. xylinum* cells (freeze-dried) exposed to glucose and 5 mM-arsenite. This point was reinvestigated with freshly grown cells where arsenite was found to cause the accumulation of pyruvate analysed as the 2,4-dinitrophenylhydrazone. However, total pyruvate yields were small (see Table 6) and decreased gradually with time of incubation. Concentrations of arsenite from 1·5 mM to 6·0 mM were equally effective. Measurable amounts of pyruvate did not appear in a system containing arsenite but minus glucose. Arsenite thus has little influence on glucose metabolism and pyruvate decarboxylation in *A. xylinum*, but does lead to the accumulation of small amounts of pyruvate, presumably by affecting the tricarboxylic acid cycle.

Incorporation of ^{14}C *from* [^{14}C]*glucose into pyruvate.* Table 6 shows the distribution of ^{14}C in pyruvate biosynthesized from specifically ^{14}C-labelled glucose. The incubation medium was analysed for total pyruvate, glucose and 2-oxogluconate. The pyruvate derived from [U-^{14}C]-glucose had 34 % of the activity at C-1. Of the ^{14}C in the pyruvate arising from [1-^{14}C]glucose 97 % was located at C-1. Pyruvate arising from [2-^{14}C]-glucose had most of the ^{14}C at C-2. C-6 of glucose was incorporated to the extent of 52 % into C-1 of the keto acid. The relative specific activities of the pyruvate derived from glucose labelled at C-1, C-2 and C-6 were 0·46, 1·0 and 0·74 respectively. With arsenite present, the specific activity of the $^{14}CO_2$ from cells utilizing [2-^{14}C]glucose was much lower

Table 5. *Experiment III: the distribution of* ^{14}C *in alanine isolated from* Acetobacter xylinum *metabolizing specifically* ^{14}C*-labelled gluconic acid*

The reaction mixture (volume, 21·3 ml.) contained: initial gluconate, 114 μmoles; cells (age, 27 hr.), as indicated; phosphate buffer, 304 μmoles. The initial pH was 5·8 and the final pH 5·6; the temperature was 28°. The reaction was stopped at 2 hr.

Substrate ...	[1-^{14}C]Gluconate	[2-^{14}C]Gluconate	[6-^{14}C]Gluconate
Sp. activity of C-1 of alanine (counts/min. per mg.atom of C)	695 (100 %)	45 (6 %)	460 (84 %)
Sp. activity of C-2 of alanine (counts/min. per mg.atom of C)	0 (0 %)	711 (93 %)	20 (4 %)
Sp. activity of C-3 of alanine (counts/min. per mg.atom of C)	0 (0 %)	5 (1 %)	67 (12 %)
Total sp. activity of alanine (counts/min. per m-mole)	695	761	547
Sp. activity of undiluted alanine* (disintegrations/min. per m-mole)	0.884×10^6	1.41×10^6	1.24×10^6
Dry wt. of cells (mg.)	50·6	51·4	65·7
Initial radioactivity of gluconate (disintegrations/min.)	15.6×10^6	13.3×10^6	13.3×10^6
Radioactivity of CO_2 (disintegrations/min.)	9.5×10^6	8.7×10^6	7.7×10^6
Radioactivity of medium (disintegrations/min.)	3.3×10^6	2.9×10^6	3.2×10^6
Radioactivity of cells† (disintegrations/min.)	2.8×10^6	1.7×10^6	2.4×10^6

* Corrected for initial sp. activity of gluconate and cell weight.
† Calculated by difference.

than that from cells using [6-^{14}C]glucose. The C-1 and C-6 groups of glucose were being rapidly oxidized to carbon dioxide as evidenced by the high specific activity of the $^{14}CO_2$. Only a small amount (0·14–0·17 mg.) of pyruvate accumulated in 70 min. During this time, it was estimated that about 177 μmoles of the initial glucose were used with the concomitant formation of carbon dioxide, 2-oxogluconate, cellulose and, mostly, gluconate. About 50 μmoles of the initial glucose were oxidized beyond the gluconate level and presumably to carbon dioxide since the weight of carbon trapped (as barium carbonate) was equivalent to approx. 50 μmoles of hexose.

Paper chromatography of deionized medium showed distinct spots corresponding to gluconate and 2-oxogluconate. An extremely faint spot co-chromatographing with authentic 5-oxogluconate was present. Separation of the acidic 2,4-dinitrophenylhydrazone fraction and subsequent chromatography revealed two yellow bands other than that of pyruvate 2,4-dinitrophenylhydrazone. One of these, which was barely visible, was identified as α-oxoglutarate, 2,4-dinitrophenylhydrazone. The other (not positively identified) was considered to be 2-oxogluconate 2,4-dinitrophenylhydrazone.

Enzymes in cell-free extracts. From the radioactivity results it was obvious that the pathways of oxidative glucose and gluconate metabolism in *A. xylinum* are somewhat different from those described by Gromet *et al.* (1957). These investigators showed that cell-free extracts of *A. xylinum* could: oxidize glucose to 2-oxogluconate and 5-oxogluconate via gluconate; phosphorylate (with ATP) glucose, gluconate and fructose; catalyse the oxidation of glucose 6-phosphate and 6-phosphogluconate by a 'complete' pentose phosphate cycle; convert triose phosphate into 3-phosphoglycerate and pyruvate; and rapidly oxidize acetate and pyruvate to carbon dioxide and water. Of the glycolytic enzymes, phosphohexose isomerase and aldolase were active, whereas phosphofructokinase was apparently not. The Entner–Doudoroff pathway was excluded on the sole criterion that dried-cell extracts cleaved 6-phosphogluconate very slowly under nitrogen.

The small fraction of activity at C-3 of alanine and pyruvate derived from [1-^{14}C]glucose suggested weak glycolysis. The extensive incorporation of C-2 of glucose and gluconate into C-2 of alanine and pyruvate hinted at a functional Entner–Doudoroff system. Supporting the latter thesis was the fact that almost 100 % of the ^{14}C in pyruvate and alanine arising from [1-^{14}C]glucose and [1-^{14}C]gluconate was located at C-1. The preferred conversion of C-6 of glucose and glucon-

Table 6. *Experiment IV: the distribution of* ^{14}C *in pyruvate isolated from the medium of* Acetobacter xylinum *metabolizing specifically* ^{14}C*-labelled glucose*

The reaction mixture (volume, 36·8 ml.) contained: initial glucose, 194 μmoles; cells (age, 24 hr.), 61·6 mg. (initial); arsenite, 250 μmoles. CO_2 was trapped in N-NaOH. The temperature was 30°. The reaction was stopped at 70 min. N.D., Not determined.

Substrate	[U-^{14}C]Glucose	[1-^{14}C]Glucose	[2-^{14}C]Glucose	[6-^{14}C]Glucose
Sp. activity of C-1 of pyruvate (counts/min. per mg.atom of C)	269 (34 %)	347 (97 %)	174 (26 %)	281 (52 %)
Sp. activity of C-2 of pyruvate (counts/min. per mg.atom of C)	N.D. (66 %) (C-2 + C-3)	N.D. (3 %) (C-2 + C-3)	N.D. (63 %)*	N.D. (9 %)*
Sp. activity of C-3 of pyruvate (counts/min. per mg.atom of C)			N.D. (11 %)*	N.D. (39 %)*
Total sp. activity of diluted pyruvate (counts/min. per m-mole)	787	359	659	541
Sp. activity of undiluted pyruvate† (disintegrations/min. per m-mole)	17·8 × 10^6	9·6 × 10^6	21 × 10^6	15·5 × 10^6
Initial radioactivity of glucose (disintegrations/min.)	8·88 × 10^6	8·88 × 10^6	7·36 × 10^6	8·88 × 10^6
Sp. activity of CO_2† (disintegrations/min. per mg.atom of C)	7·71 × 10^6	13·82 × 10^6	8·78 × 10^6	12·02 × 10^6
Wt. of $BaCO_3$ (mg.)	52·5	67·6	58·6	60·7
Radioactivity of CO_2 (disintegrations/min.)	2·05 × 10^6	4·73 × 10^6	2·16 × 10^6	3·7 × 10^6
Radioactivity of cells (disintegrations/min.)	0·125 × 10^6	0·016 × 10^6	0·077 × 10^6	0·225 × 10^6
Radioactivity of medium (disintegrations/min.)	6·69 × 10^6	4·18 × 10^6	5·07 × 10^6	5·6 × 10^6
^{14}C recovery (%)	99·8	99·5	99·2	107
Total pyruvate (μg.)	138	174	159	164
Total reducing power remaining‡ (mg.)	8·75	7·84	7·53	7·90
Glucose remaining (mg.)	N.D.	2·17	2·78	N.D.
2-Oxogluconate formed (mg.)	N.D.	5·67	4·75	N.D.
Gluconate + cellulose formed§ (mg.)	19·4	N.D.	N.D.	N.D.

* Activity in C-2 and C-3 determined by degradation of carrier-diluted acetate.
† Corrected to initial sp. activity of 8·88 × 10^6 disintegrations/min.
‡ Glucose + 2-oxogluconate.
§ Calculated value.

ate into a carboxyl group and thence to carbon dioxide via pyruvate suggested a process that would introduce C-6 of glucose into C-1 of hexose phosphate and thence into C-1 of pyruvate via the Entner–Doudoroff pathway. A series of experiments were designed with the purpose of demonstrating the presence of enzymes involved in these pathways.

Formation of fructose 6-phosphate from glyceraldehyde 3-phosphate by cell-free extract. Since the main argument of the present investigation centres around triose recombination in *A. xylinum*, it was of importance to see if hexose phosphate could indeed be formed from glyceraldehyde 3-phosphate *in vitro*. The results of an experiment are shown in Table 7. Glyceraldehyde 3-phosphate, incubated with cell extract, disappeared rapidly and was accompanied by the appearance of inorganic phosphate and fructose 6-phosphate. After 40 min., 0·2 μmole of glyceraldehyde 3-phosphate remained, and 16·5 μmoles of inorganic phosphate and 3·3 μmoles of fructose 6-phosphate had been formed. A small amount (1·3 μmoles) of inorganic phosphate but no fructose 6-phosphate was produced by boiled enzyme plus substrate.

At 40 min., 5·3 μmoles of inorganic phosphate could not be accounted for as alkali-labile phosphate or as inorganic phosphate. Of these 5·3 μ-moles, 3·3 are found in fructose 6-phosphate, the remainder probably being in glucose 6-phosphate.

Phosphohexose isomerase is present in extracts of the bacterium (Gromet *et al.* 1957). Spots corresponding to authentic fructose 6-phosphate and glucose 6-phosphate were detected on chromatograms of trichloroacetic acid-treated reaction media. Fructose 1,6-diphosphate was not detected, nor was oxogluconate. Another reaction product, located near orthophosphate on the chromatogram, appeared in the system initially, then became undetectable at 40 min. The transitory intermediate co-chromatographed with dihydroxyacetone phosphate. The existence is thus demonstrated of an active triose phosphate isomerase in *A. xylinum* extracts.

Table 7. *Direct evidence for the cell-free synthesis of hexose phosphate from* D-*glyceraldehyde* 3-*phosphate*

The reaction mixture (volume, 5·0 ml.) contained: DL-glyceraldehyde 3-phosphate, 22·0 μmoles; $MgCl_2$, 24 μ-moles; tris buffer, pH 7·5, 205 μmoles; enzyme extract, 0·5 ml. (18 mg. of trichloroacetic acid ppt.). The temperature was 37°. At 0, 10, 20 or 40 min. 4·0 ml. of 10% (w/v) trichloroacetic acid was added.

Time (min.)	Triose phosphate present (μmoles)	Inorganic phosphate formed (net) (μmoles)	Fructose 6-phosphate formed (net) (μmoles)
0	19·5	1·7	0·5
10	9·8	7·6	1·2
20	5·3	11·4	2·4
40	0·2	16·5	3·3

The action of a phosphatase is indicated since the theoretical yield of inorganic phosphate is 5·5 μmoles (11·0 μmoles of D-glyceraldehyde 3-phosphate should produce 5·5 μmoles of both fructose 6-phosphate and inorganic phosphate, if glyceraldehyde 3-phosphate, via triose recombination and fructose 1,6-diphosphate, forms hexose monophosphate). The results in Table 7 can best be explained if it is assumed that most of the D-isomer is converted into hexose phosphate, whereas the L-isomer is dephosphorylated. Such might be the case if, in a competition between a phosphatase and enzymes synthesizing hexose phosphate from D-glyceraldehyde 3-phosphate, the latter prevail.

Formation of pyruvate and triose phosphate from 6-phosphogluconate. Fresh cell extracts contained an enzyme system which cleaved 6-phosphogluconate to triose phosphate and pyruvate. The findings of a typical experiment are given in Table 8. Triose phosphate is expressed as alkali-labile phosphate. Pyruvate, triose phosphate and inorganic phosphate were detected in the presence of arsenite (system 2). At 40 min., a small increase in pyruvate, a twofold increase in inorganic phosphate and a slight loss of triose phosphate occurred. Pyruvate formed in the absence of arsenite (system 1) disappeared slowly and, therefore, was not readily decarboxylated by cell-free enzymes as it is by intact cells (Gromet *et al.* 1957). The non-equivalence of pyruvate and triose phosphate (on the basis of a mole-to-mole theoretical ratio) may be due to incomplete trapping by hydrazine. The liberation of inorganic phosphate is considered to be due partly to the condensation of triose phosphate to form fructose 1,6-diphosphate followed by hydrolysis of one phosphate group and partly to the action of a phosphatase on glyceraldehyde 3-phosphate.

The addition of NAD (system 3) caused a slight decrease in the concentration of keto acid, probably by accelerating the activity of 6-phosphogluconate dehydrogenase and making less 6-phosphogluconate available to the Entner–Doudoroff system. A lower inorganic phosphate value suggests that some triose phosphate reacted with glyceraldehyde 3-phosphate dehydrogenase instead of reacting with phosphatase or condensing to form fructose 1,6-diphosphate and thence fructose 6-phosphate plus inorganic phosphate. When 6-phosphogluconate was incubated with extract minus hydrazine (system 4), triose phosphate vanished at 40 min. Concurrently, the amount of pyruvate decreased and the amount of inorganic phosphate increased. Thus the loss of triose phosphate resulted in an increase in inorganic

phosphate rather than pyruvate, again suggesting that some of the triose phosphate actually recombined via aldolase to yield fructose 1,6-diphosphate which was subsequently dephosphorylated. Gromet *et al.* (1957) found little or no release of inorganic phosphate from glucose 6-phosphate and fructose 6-phosphate by cell-free extracts of *A. xylinum*. The results of Table 8 tend to support the contention that only a small portion of the triose phosphate formed via the Entner–Doudoroff and pentose phosphate-cycle pathways is converted into pyruvate, most of it being recycled into hexose phosphate via aldolase.

Ribose 5-phosphate incubated with cell extract and hydrazine under the same conditions gave rise to small amounts of pyruvate and triose phosphate. The anaerobic conversion of ribose 5-phosphate into hexose phosphate and heptose phosphate, consistent with the presence of pentose phosphate-cycle enzymes, has been demonstrated by Gromet *et al.* (1957).

Demonstration of phosphofructokinase and aldolase activities. On the basis of their inability to detect fructose 1,6-diphosphate on incubation with fructose 6-phosphate and ATP, Gromet *et al.* (1957) reported that phosphofructokinase is absent from extracts of *A. xylinum*. It is possible that the strong fructose diphosphatase present in the extract may have dephosphorylated any fructose 1,6-diphosphate synthesized, thus masking the kinase reaction. A reinvestigation showed that phosphofructokinase could indeed be detected in cell-free extracts, although the activity was extremely weak. Triose phosphate was found when hydrazine was added to a system containing fructose 6-phosphate and ATP. The fructose 1,6-diphosphate formed was split by endogenous aldolase and the triose moieties were trapped by hydrazine. Table 9 presents the findings. Assays were done at two different pH values (7·5 and 8·6) since both aldolase and phosphofructokinase are known to have different pH optima depending on their source. The phosphofructokinase was most active at pH 7·5. System 1 showed that less fructose 6-phosphate was converted into triose phosphate in the presence of sodium fluoride than in its absence. Fluoride was added to inhibit fructose-diphosphatase activity, but it appeared to inhibit the kinase. The diphosphatase was undoubtedly competing with aldolase for the kinase-synthesized fructose 1,6-diphosphate. Approx. 56% of the initial fructose 1,6-diphosphate (system 8) does not appear as triose phosphate in the absence of fluoride. As a result, the values for phosphofructokinase activity might be higher by a factor of 2. Even so, the activity is not striking (2 μmoles of triose phosphate/hr./150 mg. of fresh cells).

By contrast, aldolase was much more active. The results are shown in systems 5–11 in Table 9. The enzyme was about 50% more active at pH 7·5 than at pH 8·6. With sodium fluoride, 61·8% of the fructose 1,6-diphosphate was converted into triose phosphate. With no sodium fluoride, the amount fell to 44% (system 8). A system lacking sodium fluoride and hydrazine (system 9) yielded only 7% of the theoretical triose phosphate. The addition of NAD (system 11) decreased this value to 3%. The introduction of sodium fluoride with NAD (system 10) increased the amount of triose phosphate. The results from systems 7–11 once more suggest that both aldolase and fructose diphosphatase compete for fructose 1,6-diphosphate. Fluoride partially inhibits the diphosphatase, allowing more fructose 1,6-diphosphate to be split (the amount of triose phosphate rises). With no

Table 8. *Metabolism of 6-phosphogluconate by cell-free enzymes*

The reaction mixture (volume, 5·0 ml.) contained: 6-phosphogluconate, 10 μmoles; NAD (where indicated), 0·62 μmole; arsenite (where indicated), 25 μmoles; $MgCl_2$, 24 μmoles; hydrazine sulphate, 252 μmoles; tris buffer, pH 7·5, 205 μmoles; enzyme extract, 0·5 ml. (19·4 mg. of trichloroacetic acid ppt.). The temperature was 35°. At 20 or 40 min. 4·0 ml. of 10% (w/v) trichloroacetic acid was added. N.D., Not determined.

		Pyruvate formed (net) (μmoles)		Alkali-labile phosphate formed (net) (μmoles)		Inorganic phosphate formed (net) (μmoles)	
	System	At 20 min.	At 40 min.	At 20 min.	At 40 min.	At 20 min.	At 40 min.
1	6-Phosphogluconate + hydrazine sulphate	5·7	4·0	4·0	3·3	2·0	3·3
2	6-Phosphogluconate + hydrazine sulphate + arsenite	6·2	6·4	5·0	4·6	1·2	2·3
3	6-Phosphogluconate + hydrazine sulphate + NAD	4·9	4·9	3·8	3·8	0·9	2·3
4	6-Phosphogluconate; hydrazine sulphate added after 10% trichloroacetic acid	4·2	3·0	0·8	0·0	4·0	5·8
5	6-Phosphogluconate and hydrazine sulphate added after 10% trichloroacetic acid	N.D.	0·0	N.D.	0·0	0·0	0·0

Table 9. *Estimation of aldolase and phosphofructokinase activities*

The reaction mixture (volume, 5·0 ml.) contained: fructose 6-phosphate (where indicated), 20 μmoles; fructose 1,6-diphosphate (where indicated), 10 μmoles; ATP (where indicated), 10 μmoles; NAD (where indicated), 0·5 μmole; $MgCl_2$, 24 μmoles; NaF (where indicated), 100 μmoles; hydrazine sulphate, 252 μmoles; tris buffer, 154 μmoles; enzyme extract, 0·5 ml. (17·1 mg. of trichloroacetic acid ppt.). The temperature was 35° for systems 1–6 and 37° for systems 7–11. The enzymes for systems 7–11 were stored in the cold overnight (no activity loss). At 30 min. (for systems 1–6) and at 40 min. (for systems 7–11) 4·0 ml. of 10% (w/v) trichloroacetic acid was added. The percentage conversion of hexose phosphate into triose phosphate was given by the percentage of total chromogen as determined with crystalline aldolase where the total colour units equalled 6120 Klett units. The values are corrected for boiled enzyme controls.

	System	pH	Percentage conversion of hexose phosphate into triose phosphate	Calculated triose phosphate formed (μmoles)
1	Fructose 6-phosphate + hydrazine sulphate + ATP + NaF	7·5	1·5	0·3
2	Fructose 6-phosphate + hydrazine sulphate + ATP	7·5	2·5	0·5
3	Fructose 6-phosphate + hydrazine sulphate + ATP + NaF	8·6	0·14	0·03
4	Fructose 6-phosphate + hydrazine sulphate + ATP	8·6	0·73	0·15
5	Fructose 1,6-diphosphate + hydrazine sulphate + NaF	8·6	31·0	6·2
6	Fructose 1,6-diphosphate + hydrazine sulphate	8·6	18·2	3·6
7	Fructose 1,6-diphosphate + hydrazine sulphate + NaF	7·5	61·8	12·4
8	Fructose 1,6-diphosphate + hydrazine sulphate	7·5	43·7	8·8
9	Fructose 1,6-diphosphate; hydrazine sulphate added after 10% trichloroacetic acid	7·5	7·4	1·5
10	Fructose 1,6-diphosphate + NAD + NaF; hydrazine sulphate added after 10% trichloroacetic acid	7·5	18·4	3·7
11	Fructose 1,6-diphosphate + NAD; hydrazine sulphate added after 10% trichloroacetic acid	7·5	2·9	0·6

hydrazine available, the equilibrium is shifted towards the dephosphorylation of fructose 1,6-diphosphate. Actually, the temperature-dependent equilibrium of aldolase sides towards fructose 1,6-diphosphate synthesis (Meyerhof & Junowicz-Kocholaty, 1943). If NAD is added without hydrazine, some of the glyceraldehyde 3-phosphate is dehydrogenated by glyceraldehyde 3-phosphate dehydrogenase which is active in extracts of this organism (Gromet *et al.* 1957). When sodium fluoride is added to the latter system, the diphosphatase is inhibited, permitting more triose phosphate accumulation (system 10).

Metabolism of fructose 1,6-*diphosphate and* 3-*phosphoglycerate*. The information in Table 9 suggested that fructose diphosphatase was extremely active and that fructose 1,6-diphosphate occupied a 'pivot' position in the metabolism of hexose by *A. xylinum*. That is, fructose 1,6-diphosphate could be cleaved or resynthesized by means of aldolase, dephosphorylated by fructose diphosphatase but formed only slowly from fructose 6-phosphate by a weak phosphofructokinase. If triose phosphate, as postulated, recombined extensively to form hexose monophosphates (fructose 6-phosphate and glucose 6-phosphate), the mechanism would necessitate an active aldolase (already shown) and a fructose diphosphatase. Further, an extensive resynthesis of fructose 1,6-diphosphate would presuppose the existence of a 'bottleneck' in the multi-enzyme system forming pyruvate from triose phosphate. If these assumptions were correct, the *A. xylinum* extract should possess high fructose-diphosphatase activity and lack a vigorous route of pyruvate synthesis from glyceraldehyde 3-phosphate. Some indirect clues for these processes exist in the studies of Gromet *et al.* (1957).

Table 10 presents the evidence for fructose-diphosphatase activity. In the extracts studied, 90% of the theoretical amount of inorganic phosphate was generated from fructose 1,6-diphosphate at 60 min. at pH 7·6 (20·1 μmoles based on hydrolysis of 1-phosphate). The small fraction of triose phosphate formed during the initial reaction time gradually fell to zero at 50 min., perhaps as a result of both phosphatase action and recondensation via aldolase to hexose phosphate. Pyruvate was not detected in system 1. The enzyme was inactive at pH 8·9, in contrast with fructose diphosphatases from other organisms (Hochster & Katznelson, 1958).

Pyruvate was formed from fructose 1,6-diphosphate when NAD was added as a cofactor for glyceraldehyde 3-phosphate dehydrogenase, and excess of ADP as a phosphate acceptor for 3-phosphoglycerate kinase and pyruvate kinase (system 2). The concentrations of pyruvate and inorganic phosphate increased with time while, simultaneously, the concentration of triose phosphate dropped to zero. In 60 min., only 5% of the fructose 1,6-diphosphate was accounted for as pyruvate, on the basis that 1 mole of fructose 1,6-

diphosphate gives 2 moles of the keto acid. One has to visualize the latter system as complex because of possible competition for glyceraldehyde 3-phosphate among triose isomerase, aldolase, glyceraldehyde 3-phosphate dehydrogenase and phosphatase. The action of the phosphatase may be minor since pyruvate was produced from triose phosphate.

Paper chromatography of the reaction contents of system 1 (without ADP or NAD) at 10 min. showed sugar phosphate spots with R_F values of 0·075, 0·15, 0·21, 0·26, 0·32 and 0·42. These were identified respectively as 6-phosphogluconate (faint), glucose 6-phosphate, fructose 6-phosphate, ribose 5-phosphate (faint), 3-phosphoglycerate and inorganic phosphate (strong). A similar study of the sugar phosphates in system 2 revealed the same components plus fructose 1,6-diphosphate, ADP and NAD, and the spots corresponding to 3-phosphoglycerate and ribose 5-phosphate were more intense than in system 1. Hence fructose 1,6-diphosphate can be metabolized bidirectionally depending on cofactor availability.

Production of pyruvate from 3-phosphoglycerate. Evidence for a sluggish conversion of triose phosphate into pyruvate is provided in Table 11. Cell extract incubated with 3-phosphoglycerate minus cofactors contained small quantities of pyruvate and acid-labile phosphate (assumed to be phosphoenolpyruvate) at 60 min. Arsenite (system 2) elicited a minor increase in pyruvate. If ADP was added (system 3), the concentration of pyruvate rose but no acid-labile phosphate was found. This would be expected if pyruvate kinase is activated. The possibility that the observed amounts of pyruvate were too low owing to an arsenite-insensitive decarboxylation of the keto acid was excluded on the grounds that at least 98% of the carbon (systems 2 and 3) could be recovered as pyruvate, acid-labile phosphate and 3-phosphoglycerate (acid-resistant phosphate). Inexplicably, the inclusion of NAD (systems 5 and 6) approximately doubled the yield of pyruvate. This pyruvate was not synthesized from endogenous substrates since the amount of 3-phosphoglycerate decreased in

Table 10. *Estimation of fructose-diphosphatase activity in cell-free extract*

The reaction mixture (volume, 5·0 ml.) contained: fructose 1,6-diphosphate, 20·1 μmoles; ADP (where indicated), 31·8 μmoles; NAD (where indicated), 1·88 μmoles; $MgCl_2$, 24 μmoles; tris buffer, 205 μmoles; enzyme extract, 0·5 ml. (21·3 mg. of trichloroacetic acid ppt.). The final pH was 7·6 for system 1 and 7·65 for system 2; the temperature was 36°. At 10, 20, 30, 40, 50 or 60 min. 4·0 ml. of 10% (w/v) trichloroacetic acid was added. The results are corrected for a control where fructose 1,6-diphosphate was added after the trichloroacetic acid.

	System	Product measured	Product formed (net) (μmoles) At 10 min.	At 20 min.	At 30 min.	At 40 min.	At 50 min.	At 60 min.
1	Fructose 1,6-diphosphate	Inorganic phosphate	13·4	15·3	15·6	16·6	17·7	18·2
		Pyruvate	0·0	0·0	0·0	0·0	0·0	0·0
		Triose phosphate	2·3	1·4	1·1	0·5	0·0	0·0
2	Fructose 1,6-diphosphate + ADP + NAD	Inorganic phosphate	5·3	8·7	10·2	11·0	14·4	14·9
		Pyruvate	0·5	1·2	1·2	1·6	2·2	2·1
		Triose phosphate	5·1	3·7	2·6	0·8	0·1	0·0

Table 11. *Cell-free formation of pyruvate from 3-phosphoglycerate*

The reaction mixture (volume, 5·0 ml.) contained: 3-phosphoglycerate (by analysis), 51·0 μmoles; ADP (where indicated), 31·7 μmoles; NAD (where indicated), 1·24 μmoles; $MgCl_2$, 24 μmoles; NaF (where indicated), 150 μmoles; arsenite (where indicated), 25 μmoles; tris buffer, 145 μmoles; enzyme, 0·5 ml. (18·8 mg. of trichloroacetic acid ppt.). The pH was 7·7 and the temperature 34°. At 60 min. 4·0 ml. of 10% (w/v) trichloroacetic acid was added. Values for acid-resistant phosphate are corrected for unhydrolysed ADP and NAD plus 2% hydrolysis of 3-phosphoglycerate/3 hr. Values for pyruvate are corrected for controls where 3-phosphoglycerate was added after the trichloroacetic acid. N.D., Not determined.

	System	Acid-resistant phosphate remaining (μmoles)	Pyruvate formed (net) (μmoles)	Acid-labile phosphate formed (net) (μmoles)	Total phosphate + pyruvate (net) (μmoles)
1	3-Phosphoglycerate	41·5	2·1	6·1	49·7
2	3-Phosphoglycerate + arsenite	39·4	2·3	7·0	48·7
3	3-Phosphoglycerate + ADP	43·3	5·5	N.D.	48·8
4	3-Phosphoglycerate + ADP + arsenite	42·5	4·5	N.D.	47·0
5	3-Phosphoglycerate + ADP + NAD	38·2	9·4	N.D.	47·6
6	3-Phosphoglycerate + ADP + NAD + arsenite	40·3	7·8	N.D.	48·1
7	3-Phosphoglycerate + ADP + NAD + NaF	51·5	0·2	N.D.	51·7

accordance with the rise in pyruvate. Fluoride characteristically prevented the formation of pyruvate. There was no apparent action of a phosphatase on 3-phosphoglycerate since the fraction not converted into pyruvate was recovered only as acid-resistant phosphate.

DISCUSSION

The evidence at hand suggests that both the pentose phosphate-cycle and the Entner–Doudoroff systems are involved in hexose breakdown in *A. xylinum*. The small extent of incorporation of C-1 of glucose into C-3 of pyruvate or alanine from [1^{14}-C]glucose (Tables 3, 4 and 6) is indicative of weak glycolysis, as is the fact that cells incubated under nitrogen evolve $^{14}CO_2$ from [U-^{14}C]glucose and cell extracts slowly convert fructose 6-phosphate into triose phosphate.

Support for a pentose phosphate cycle is that cell-free extracts contain each enzyme of the cycle and can transform ribose 5-phosphate into a mixture of hexose phosphate and heptose phosphate (Gromet *et al.* 1957). Triose phosphate and pyruvate are additional products. Also, the randomization of ^{14}C from C-2 of glucose and gluconate into C-1 and C-3 of pyruvate and alanine is typical of the pentose phosphate cycle in conjunction with glycolysis or the Entner–Doudoroff pathway (Wang, Doyle & Ramsey, 1962; Wang & Ikeda, 1961). Minor, Greathouse & Shirk (1955) noticed a transfer of ^{14}C from C-2 of substrate glucose to C-1 and C-3 of cellulosic glucose synthesized by *A. xylinum*, in accordance with randomization via the pentose phosphate cycle. The participation of the Entner–Doudoroff pathway is obvious from the results of the present experiments. Schramm & Racker (1957) described an enzyme, phosphofructoketolase, that cleaves fructose 6-phosphate to acetyl phosphate and erythrose 4-phosphate in extracts from a cellulose-less mutant of *A. xylinum*. If the enzyme were active in glucose metabolism, ^{14}C from [1-^{14}C]glucose (isomerized to [1-^{14}C]fructose via phosphohexose isomerase) should enter the methyl group of acetate as well as label various derivatives of the tricarboxylic acid cycle. Aspartate and glutamate isolated from cells given [2-^{14}C]acetate were strongly radioactive (G. A. White & C. H. Wang, unpublished work). The fact that cells using [1-^{14}C]glucose retained little activity, most of it in alanine and almost none in aspartate and glutamate, tends to eliminate phosphofructoketolase as an important pathway in our strain of cells. Reactions of the reverse transketolase–transaldolase type (Wood & Katz, 1958) could account for the small amount of ^{14}C in C-2 of alanine and pyruvate derived from [6-^{14}C]hexose.

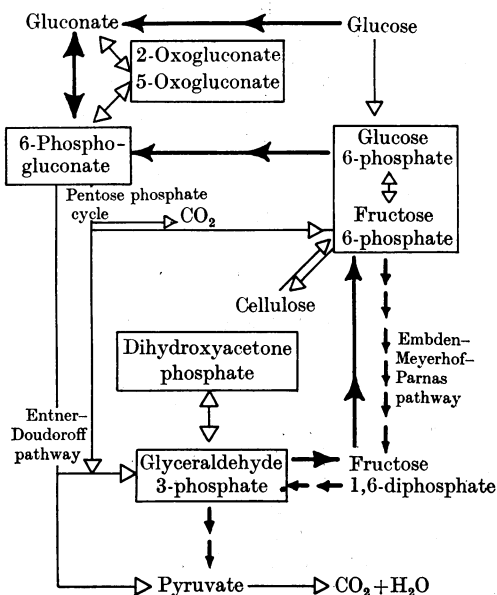
The main thesis of the present investigation concerns the fate of triose phosphate formed by the pentose phosphate-cycle and Entner–Doudoroff systems. It appears that the triose fragment, derived from substrate glucose, undergoes extensive recombination via triose phosphate isomerase and aldolase to yield fructose 1,6-diphosphate and hexose monophosphate. The evidence to support this is: (*a*) C-1 of pyruvate becomes extensively labelled from [6-^{14}C]glucose; (*b*) the high yield of respiratory carbon dioxide from C-6 of glucose and gluconate is consistent with the decarboxylation of 6-phosphogluconate and pyruvate labelled in the carboxyl group; (*c*) cell-free extracts have strong aldolase and fructose-diphosphatase activities but convert 3-phosphoglycerate slowly into pyruvate; and, most important, (*d*) cell-free extracts can synthesize fructose 6-phosphate and glucose 6-phosphate from D-glyceraldehyde 3-phosphate.

The corrected relative specific activity of the pyruvate derived from glucose labelled at C-1, C-2 and C-6 was 54 %, 118 % and 87 % respectively, as calculated by the method of Blumenthal, Lewis & Weinhouse (1954). Thus C-6 is not used preferentially for pyruvate synthesis since the relative specific activity would be 100 % for exclusive Entner–Doudoroff reactions and 200 % for exclusive pentose phosphate-cycle reactions. A loss of activity from C-6 as $^{14}CO_2$ via C-1 of 6-phosphogluconate would explain the low relative specific activity. Similarly, the relative specific activity of pyruvate arising from [2-^{14}C]glucose would be 0 % if there were 100 % participation of the pentose phosphate cycle and 100 % if there were 100 % participation of the Entner–Doudoroff pathway. A value of 118 % implies that C-4, C-5 and C-6 of glucose are not routed as extensively to pyruvate as are C-1, C-2 and C-3. The low specific activity of pyruvate derived from [1-^{14}C]glucose could result from two processes: a loss of $^{14}CO_2$ via decarboxylation of 6-phosphogluconate and dilution of [1-^{14}C]-glucose by unlabelled hexose formed by the pentose phosphate cycle and triose recombination.

It appears that the 'hexose cycle' functions in *A. xylinum*. This system was originally proposed by Hochster & Katznelson (1958) to explain the dissimilation of glucose in cell-free extracts of *Xanthomonas phaseoli* XP 8 and, later, by Vardanis & Hochster (1961) studying *Agrobacterium tumefaciens*. The hexose cycle entails a system where an equilibrium condition favouring triose recombination over the conversion of glyceraldehyde 3-phosphate into pyruvate exists. The basis of the cycle is that triose phosphate formed through the simultaneous action of the pentose phosphate-cycle and Entner–Doudoroff pathways can enter a common pool and then form fructose 1,6-diphosphate by aldolase activity. The fructose 1,6-

diphosphate is attacked by an active fructose diphosphatase hydrolysing the 1-phosphate group (Hochster, 1962) to yield fructose 6-phosphate plus inorganic phosphate. The fructose 6-phosphate is isomerized to glucose 6-phosphate which joins the pool of hexose phosphate produced from the pentose phosphate cycle as well as from the phosphorylation of substrate glucose. This glucose 6-phosphate, which cannot be readily reconverted into fructose 1,6-diphosphate owing to the weak phosphofructokinase activity, again enters the pentose phosphate-cycle or Entner–Doudoroff systems via 6-phosphogluconate, and so on. A proposed scheme for glucose metabolism and the hexose cycle in *A. xylinum* is presented in Scheme 1.

A ready formation of pyruvate from the Entner–Doudoroff pathway could satisfy the main energy requirements of the organism. *A. xylinum* grown on glucose rapidly metabolizes acetate and pyruvate (G. A. White & C. H. Wang, unpublished work) which are not utilized in hexose resynthesis. Whereas pyruvate is not converted into cellulose in glucose-cultured cells (Schramm, Gromet & Hestrin, 1957*a*), it is in cells grown on succinate (Benziman & Burger-Rachamimov, 1962). The hexose cycle is not an inefficient process in energy production since the oxidation of resynthesized hexose phosphate could be linked to nicotinamide nucleotide reduction and possibly oxidative phosphorylation.



Scheme 1. Scheme for glucose metabolism and the 'hexose cycle' in *Acetobacter xylinum*. Heavy arrows, strong pathways; medium-heavy arrows, weak pathways; open-head arrows, main pathways.

Schramm *et al.* (1957*a*) found glycerol and dihydroxyacetone to be excellent precursors of cellulosic glucose in glucose-grown *A. xylinum*. Presumably such synthesis would necessitate triose recombination. Other studies by Schramm, Gromet & Hestrin (1957*b*) indicated that no condensation of triose occurred since C-6 of [6-^{14}C]glucose failed to appear in C-1 of polymerized glucose. However, a labelling ratio of 3:2 has been found in C-1:C-6 of cellulosic glucose synthesized from [6-^{14}C]fructose (Hestrin, 1961). Radiotracer experiments by Greathouse (1957), using stationary-phase cultures of *A. xylinum*, have given evidence for hexose resynthesis from triose phosphate. Whereas such differences in results are not easily reconciled, it is possible that they are related to variations in the age and metabolic state of the cells. Schramm *et al.* (1957*a*) noted that sodium fluoride had little effect on oxygen–carbon dioxide exchange in glucose-metabolizing cells. Also, cellulose synthesis was relatively insensitive to iodoacetate. One explanation of these findings is that triose recombination, coupled with the Entner–Doudoroff system, would effectively by-pass the regions of glycolysis sensitive to these inhibitors. Thus ATP generated from the oxidation of pyruvate formed by the Entner–Doudoroff pathway could 'drive' the synthesis of cellulose from recombined hexose and in the presence of compounds preventing pyruvate synthesis from glyceraldehyde 3-phosphate.

The increased rate of $^{14}CO_2$ output from C-6 of glucose over that from C-2 (Table 6) may result from arsenite inhibition of the tricarboxylic acid cycle and inhibition of acetaldehyde dehydrogenation (De Ley & Schell, 1962). The net result would be a lowering of the rate of $^{14}CO_2$ output from [1-^{14}C]acetate (corresponding to C-2 of the original glucose) without markedly changing the rate of $^{14}CO_2$ output from [1-^{14}C]pyruvate (corresponding to C-6 of the original glucose). Again, this is in line with the view that C-6 becomes partly transposed to the C-1 position of recombined hexose phosphate and can form carbon dioxide either through the 6-phosphogluconate-dehydrogenase reaction or by decarboxylation of pyruvate formed by the Entner–Doudoroff pathway. The lack of pronounced accumulation of pyruvate in the medium from arsenite-treated cells can be explained by the results of De Ley & Schell (1962) which show that the conversion of pyruvate into acetate in *Acetobacter* spp. involves two enzyme systems, pyruvate decarboxylase and acetaldehyde dehydrogenase, the former being arsenite-insensitive.

Various other metabolic pathways could also give the observed distribution of ^{14}C in pyruvate and alanine and the relative rates of $^{14}CO_2$ output from cells using [6-^{14}C]glucose. The most likely prospect would be C-1–C-6 interchange resulting

from glycolytic cleavage and resynthesis of hexose. This possibility seems remote since glycolytic activity is limited in this organism. Another possible route would be the glucuronate pathway where C-6 is released as carbon dioxide after inversion of the molecule to L-gulonate (Eisenberg, Dayton & Burns, 1959). Fresh cells failed to produce $^{14}CO_2$ from [6-^{14}C]glucuronate. In addition, it is difficult to rationalize the labelling of C-1 of pyruvate by [6-^{14}C]glucose if the glucuronate pathway is involved. Fixation of carbon dioxide could also place ^{14}C in C-1 of pyruvate or alanine. Three lines of evidence are available which tend to discount this as a mechanism. First, amino acids in the radioactive hydrolysate from cells given D-[1-^{14}C]glucose were separated on Whatman no. 1 paper and scanned with a 4 pi chromatogram counter. The only radioactive area was identified as alanine. Little activity was incorporated into aspartate and glutamate which should normally be labelled via any of the usual C_1-plus-C_3 fixation systems. Secondly, the fact that C-1 of pyruvate and alanine derived from [2-^{14}C]glucose was labelled to a minor extent compared with C-1 of the intermediates arising from [6-^{14}C]glucose suggested that $^{14}CO_2$ fixation was not the mechanism. Lastly, the specific activity of C-1 of pyruvate or alanine should be lowered as a result of fixation of unlabelled carbon dioxide (Dawes & Holms, 1958). As seen in Table 6, C-1 of pyruvate derived from [U-^{14}C]glucose contained nearly the expected theoretical amount (33·3 %) of activity. The sample of [U-^{14}C]glucose used in Expt. II (Table 4) was not uniformly labelled considering the percentage distribution of ^{14}C in alanine. Even so, there was no apparent dilution of label at C-1 by fixation of unlabelled carbon dioxide.

Two alternative mechanisms that might label C-1 of pyruvate derived from [6-^{14}C]glucose could be a condensation of acetate with [^{14}C]formate or a direct exchange of $^{14}CO_2$ with unlabelled C-1 of pyruvate. The last two arguments against appreciable fixation of carbon dioxide apply here.

Considering the relative rates of $^{14}CO_2$ production, it was plausible that the hexose was partly converted into oxoglutarate via a pentonic acid derivative like 4,5-dihydroxy-2-oxopentanoate (Weimberg, 1961) or via 2,5-dioxogluconate as in *A. melanogenum* (Datta & Katznelson, 1957). If such pathways were present in *A. xylinum*, oxoglutarate derived from [2-^{14}C]glucose would be predominantly labelled at C-1 and at C-5 if derived from [6-^{14}C]glucose. Both carboxyl groups should appear readily as carbon dioxide when oxoglutarate enters the tricarboxylic acid cycle. The observed distribution of ^{14}C in glutamate (representing oxoglutarate) was as follows: with [2-^{14}C]-glucose: C-1 (34 %), C-2, C-3 and C-4 (22 %), C-5 (44 %); with [6-^{14}C]glucose: C-1 (22 %), C-2, C-3 and C-4 (74 %), C-5 (4 %). These results are indicative of acetate metabolism via the tricarboxylic acid cycle (Reed, Cheldelin & Wang, 1958) rather than one of the above mechanisms. Degradation of aspartate samples showed a distribution of ^{14}C corroborating this view.

The question of randomization of ^{14}C in a symmetrical intermediate like glycerol or dihydroxyacetone must be considered since it could introduce C-6 of glucose into C-1 of pyruvate. It seems unlikely that *A. xylinum* forms glycerol or dihydroxyacetone under conditions where these C_3 units are readily oxidized (Schramm *et al.* 1957*a*). Neither triose could be detected in the radioactive medium.

Operation of the hexose cycle is the result of the equilibria of a series of enzyme reactions. The foremost point is that the fructose-diphosphatase reaction is essentially irreversible. If it were not, no blockage of glycolysis would occur even if phosphofructokinase were missing. The equilibrium of the phosphohexose-isomerase reaction favours glucose 6-phosphate formation (Lohmann, 1933). Since glucose 6-phosphate is drained off by its dehydrogenase, reverse aldolase action occurs whereby the formation of glucose 6-phosphate from glyceraldehyde 3-phosphate is promoted. Therefore the overall equilibrium between glucose 6-phosphate and glyceraldehyde 3-phosphate is directed towards hexose phosphate synthesis. The situation is considerably accentuated if phosphofructokinase is weak or inactive. A further consideration is that the total equilibrium of the Entner–Doudoroff pathway strongly encourages removal of 6-phosphogluconate and, therefore, glucose 6-phosphate (Kovachevich & Wood, 1955*a*, *b*). Vardanis & Hochster (1961) have discussed the possible regulation of the hexose cycle through inhibition of phosphohexose isomerase by 6-phosphogluconate. An interplay among enzymes for 'key' substrates like glyceraldehyde 3-phosphate as well as substrate inhibition could have a directive role in the hexose cycle by regulating the proportion of carbohydrate metabolized via a given pathway.

The hexose cycle was previously believed to be confined to relatively few organisms (Gibbs, 1959). Including the bacteria already mentioned, the cycle is present to a lesser degree in *Azotobacter vinelandii* (G. G. Still & C. H. Wang, unpublished work) and in *Pseudomonas fluorescens* (Lewis, Blumenthal, Weinrach & Weinhouse, 1955). As in *A. xylinum*, C-1 of alanine or pyruvate is labelled when these organisms incorporate [6-^{14}C]glucose. Mortensen, Hamilton & Wilson (1955) have shown hexose phosphate synthesis from triose phosphate in cell-free extracts of *Azotobacter vinelandii*. Triose phosphate recombines to form fructose 1,6-diphos-

phate in *A. suboxydans*, which lacks glyceraldehyde 3-phosphate dehydrogenase (Hauge, King & Cheldelin, 1955). The hexose cycle may thus occur in this species which has a strong pentose phosphate-cycle (Kitos *et al.* 1958) and a weak Entner–Doudoroff system (M. Isono & C. H. Wang, unpublished work). A limited interchange of C-6 and C-1 of glucose has been noted during polysaccharide synthesis in *Aerobacter aerogenes* (Segal & Topper, 1957) and in *Pseudomonas aeruginosa* (Hauser & Karnovsky, 1958). In these cases it appears to result from triose recombination during glycolysis rather than by the hexose cycle. Triose recombination is common in green plants (Shibko & Edelman, 1958) and seemingly rare in animal tissues, which direct triose phosphate to glycerol, amino acids, fatty acids and carbon dioxide (Wood & Katz, 1958; Katz & Wood, 1960). Taylor & Juni (1961*a*) have described a peculiar capsular coccus that has a highly modified hexose cycle. Rhamnose from cells given [6-^{14}C]glucose showed ^{14}C in C-6:C-1 in the ratio of 50:43 (Taylor & Juni, 1961*b*).

The hexose cycle, thus far, seems largely confined to Gram-negative bacteria that: have an active Entner–Doudoroff system; have a pentose phosphate cycle; show weak or no glycolysis; rapidly oxidize glucose to sugar acids, chiefly gluconate or 6-phosphogluconate; and, finally, synthesize some type of hexose-containing polysaccharide (Davies, 1960).

The question as to why the hexose cycle is present or needed in these organisms is intriguing. For *A. xylinum*, at least, the main pool of glucose for cellulose synthesis seems to be not the original substrate hexose but hexose phosphate formed via the pentose phosphate cycle (Gromet *et al.* 1957) and, as the present study shows, by triose recombination. Khan & Colvin (1961) believe that the glucose unit becomes attached to a lipid-containing precursor engaged in extracellular cellulose synthesis. Since glucose is readily oxidized to gluconate and since the pentose phosphate-cycle activity is relatively weak (see White & Wang, 1964), the immediate source of hexose phosphate for cellulose synthesis is likely from recombination of triose fragments arising from the Entner–Doudoroff pathway. This may be generally true for bacteria having a hexose cycle and synthesizing polysaccharide. That glucose was not formed by a reversal of the glucose 6-phosphate-dehydrogenase reaction is supported by the labelling pattern in pyruvate derived from [1-^{14}C]gluconate, on the assumption that, via glycolysis, a small proportion of the ^{14}C at C-1 would appear in C-3 of pyruvate.

Calculations based on the distribution of ^{14}C in alanine and pyruvate have shown that 80–90 % of the triose phosphate recombines to produce hexose phosphate. The amounts of glucose and gluconate being metabolized via the pentose phosphate-cycle and Entner–Doudoroff systems have likewise been estimated. White & Wang (1964) present these results and a mathematical model of hexose metabolism in *A. xylinum*.

SUMMARY

1. The dissimilation of glucose and gluconate in *Acetobacter xylinum* has been investigated by giving specifically ^{14}C-labelled substrates to actively metabolizing cells, and then isolating biosynthesized alanine and pyruvate for degradation and determination of ^{14}C distribution.

2. Alanine and pyruvate derived from [1-^{14}C]-glucose contained over 90 % of the total activity at C-1 and 2–7 % at C-3. Alanine derived from D-[1-^{14}C]gluconate had 100 % of the label at C-1.

3. C-2 of glucose appeared largely in C-2 of pyruvate and alanine. The remaining ^{14}C was distributed between C-1 and C-3. Alanine derived from [2-^{14}C]gluconate was similarly labelled.

4. In three separate experiments, alanine and pyruvate arising from [6-^{14}C]glucose had 52–64 % of the total activity at C-1 and 29–40 % at C-3. Alanine from [6-^{14}C]gluconate had 84 % of the total activity at C-1 and 12 % at C-3.

5. Cell-free extracts contained aldolase, fructose diphosphatase and enzymes of the Entner–Doudoroff system. Phosphofructokinase activity and therefore glycolysis is weak. Extracts convert 3-phosphoglycerate into pyruvate at a very slow rate. Direct evidence shows that hexose phosphate is synthesized from glyceraldehyde 3-phosphate.

6. The Entner–Doudoroff pathway appears to be more active than the pentose phosphate cycle in hexose phosphate utilization. Gluconate and 2-oxogluconate are formed rapidly in the medium of glucose-metabolizing cells.

7. Glyceraldehyde 3-phosphate generated via the pentose phosphate-cycle and Entner–Doudoroff pathways appears to undergo extensive recombination at the triose phosphate-isomerase and aldolase levels to yield fructose 1,6-diphosphate. A situation of 'reverse' glycolysis is present whereby hexose diphosphate is converted into 6-phosphogluconate via glucose 6-phosphate. The whole system is termed the 'hexose cycle'.

8. The operation of the hexose cycle randomizes C-6 of glucose or gluconate into C-1 of hexose phosphate by way of triose recombination. The pyruvate ensuing from the [1-^{14}C]-6-phosphogluconate via the Entner–Doudoroff pathway is thus labelled at C-1. Pyruvate is decarboxylated to acetate which is oxidized by the tricarboxylic acid cycle.

9. Consistent with the hexose cycle was a rapid output of $^{14}CO_2$ from [6-^{14}C]glucose and [6-^{14}C]-gluconate. The rate of carbon dioxide output was highest from C-1 and lowest from C-4 followed by C-3.

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