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Synthesis of Cellulose by *Acetobacter xylinum*

4. ENZYME SYSTEMS PRESENT IN A CRUDE EXTRACT OF GLUCOSE-GROWN CELLS

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The synthesis of cellulose from glucose by washed cells of *Acetobacter xylinum* has been shown to be conditional upon a concurrent oxidative process (Hestrin, Aschner & Mager, 1947; Schramm, Gromet & Hestrin, 1957*a*, *b*). To understand the synthesis, a knowledge of the nature of the oxidative process seemed desirable. Some of the reactions of glucose oxidation that occur in an extract of *A. xylinum* cells are demonstrated in this paper.

METHODS AND MATERIALS

A. xylinum *extracts*. All operations were carried out in the cold room. Cell-free extracts were prepared from washed *A. xylinum* cells harvested from glucose medium (Schramm *et al.* 1957*b*) as follows.

(*a*) '*Alumina*' *extract*: Freeze-dried cells (100 mg.) were triturated in a hand mortar with 300 mg. of alumina (Alumina A-301, Alcoa Ltd., Pittsburgh, Pa., U.S.A.) for 10 min. Then 3 ml. of 0·04 M-sodium citrate buffer, pH 6·4, was added to the mixture. After 10 min. the suspension was centrifuged (3000 *g* for 15 min.) and the packed sediment was re-extracted in 2 ml. of buffer. The supernatant fluids (about 4 ml.) were pooled and partially cleared by centrifuging (3000 *g* for 15 min.). The turbid, slightly pink supernatant fluid was stored at −20°.

(*b*) '*Mickle*' *extract*: Cells (100 mg. dry wt.) were washed in water (10 ml.) and then suspended in 3 ml. of 0·1 M-2-amino-hydroxymethylpropane-1:3-diol (tris) buffer (Cl^-; pH 7·5). Treatment in a Mickle tissue disintegrator (30 min. with about 1 g. of Ballotini glass beads) served to disrupt the cells. This 'homogenate' was centrifuged (3000 *g* for 15 min.), the sediment re-extracted in 2 ml. of buffer and the supernatant fluids (about 4 ml.) pooled and partially cleared (3000 *g* for 15 min.). The product was an opalescent fluid. It formed CO_2 oxidatively from glucose 6-phosphate (G 6-P) even after storage for 1 month at −20°. Such extracts have been obtained from fresh cells, freeze-dried cells and from frozen cells.

Test systems. The temperature was 30±0·2°. Anaerobic experiments were conducted in Thunberg vessels under N_2 at reduced pressure. Conventional Warburg techniques were used in all the manometric determinations, CO_2 being absorbed by alkali in the central cup. Systems with alumina extract were buffered by 0·02 M-sodium citrate, pH 6·4, and the systems with Mickle extract by 0·05 M-tris (Cl^-), pH 7·5. In a Mickle extract acting aerobically on G 6-P, the formation of CO_2 was found to be accelerated by phenazine methochloride (Fig. 5). This compound (final concn. 50–100 μg./ml.) was included in the composition of all the aerobic reaction systems except as otherwise stated specifically in the text.

Reagents. The origin of reagents were as follows: phenazine methochloride (Bios, New York, N.Y., U.S.A.); diphosphopyridine nucleotide (DPN) as a sodium salt (Pabst Laboratories, Milwaukee, Wis., U.S.A.); triphosphopyridine nucleotide (TPN) of 80% purity and a crystalline preparation of adenosine monophosphate (AMP) of muscle (Sigma Chemical Co., St Louis, Mo., U.S.A.); adenosine triphosphate (ATP) and diphosphate (ADP) as sodium salts (Pabst Laboratories); 3-phosphoglycerate (PGA), G 6-P, fructose 6-phosphate (F 6-P) and fructose 1:6-diphosphate (F 1:6-P) as barium salts (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.). 6-Phosphogluconate (6-PG) was a barium salt kindly given by Dr B. L. Horecker. Glucose 4-phosphate (G 4-P) was used in the form of a crude preparation kindly given by Dr R. J. Reithel. β-Glucose 1-phosphate (β-G 1-P) and ribose 5-phosphate (R 5-P) were prepared in the laboratory (Schramm *et al.* 1957*b*). Uridine diphosphate glucose (UDPG) was obtained from Dr H. Kalckar. To generate triose phosphate (triose-P) from F 1:6-P a thrice-recrystallized preparation of rabbit-muscle aldolase, kindly given by Dr J. Mager, was used. The barium salts were all converted before use into their soluble sodium forms.

Analytical methods. Samples were clarified by the following methods: acidification with HCl to pH 1 in the estimations of total reducing power by copper reduction and in the enzymic assay of G 6-P; treatment with barium–zinc mixture (Somogyi, 1945) in the assays of non-phosphorylated reducing sugars; 12% (w/v) $HClO_4$ in the estimations of oxogluconates (OGn) by colorimetric procedures; treatment with 5% (w/v) trichloroacetic acid in other cases.

Hexose (as glucose) and heptose (as sedoheptulose) were estimated by reaction with cysteine (Dische, 1955), total ketose (as fructose) with resorcinol (Roe & Papadopoulos, 1954) and pentose (as xylose) with orcinol (LePage, 1949*b*). Heating time with the orcinol reagent was 40 min. F 6-P was estimated by reaction with the resorcinol reagent as described by Roe, Epstein & Goldstein (1949). The colour formed by F 6-P with this reagent was only half as intense as that formed by fructose. Phosphorylated carbohydrate was calculated in all the colorimetric assays as the fraction removed from the test portions by the treatment with barium–zinc mixture. G 6-P was assayed enzymically by reduction of TPN as catalysed by yeast G 6-P dehydrogenase. The latter was prepared from a brewery yeast (Nesher Brewery, Rishon LeZion, Israel) according to Kornberg (1950) and stored as a lyophilized powder. To enhance its specificity, this reagent was always aged in solution (48 hr. at 4°) before use. After ageing, it contained some hexokinase in addition to G 6-P dehydrogenase, but was free from 6-PG dehydrogenase, phosphohexoisomerase and glucose dehydrogenase. Other methods of estimation of carbohydrate have been noted in the preceding communication (Schramm *et al.* 1957*b*).

Orthophosphate (P_i) was determined by the method of Fiske & Subbarow (1925), acid-labile P as ΔP_i in samples held at 97° in N-HCl for 10 min., and alkali-labile P as ΔP_i in samples held at room temperature in N-KOH for 20 min. Adenylic acid was precipitated as its barium salt with ethanol and assayed spectrophotometrically (260 mμ at pH 7). Reduction of DPN and TPN was followed spectrophotometrically at 340 mμ. Protein nitrogen was estimated with a colorimetric reagent (Lowry, Rosebrough, Farr & Randall, 1951). A dried-plasma preparation of known nitrogen content served as the protein standard.

Endogenous rates represented ⩽10% of total readings. Values are reported after having been corrected for endogenous rates, except as otherwise is indicated.

RESULTS

Formation of gluconate from glucose. A cell-free extract of *A. xylinum* oxidized glucose in the presence of phenazine methochloride without attendant evolution of CO_2. The O_2 uptake was 0·5 mol.prop. (Fig. 1). Gluconate was not oxidized.

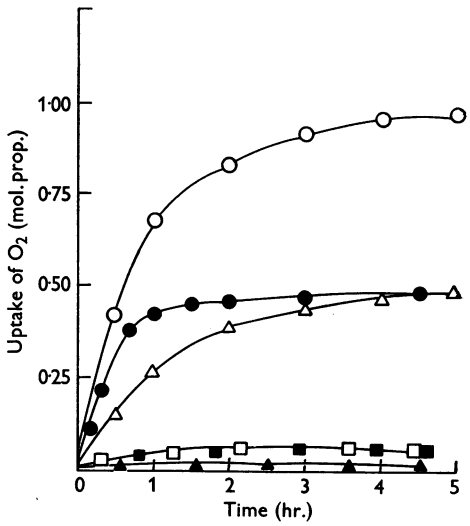

Fig. 1. Conversion of glucose via gluconate into oxogluconate by *A. xylinum* extract. Reaction mixtures (4 ml.) contained 5 μmoles of substrate (glucose or gluconate), Mickle extract (0·9 mg. of protein N), and phenazine methochloride with and without DPN (100 μg./ml.). The gas phase was air. None of the tested mixtures formed CO_2. Endogenous O_2 uptake (with and without DPN) amounted to 0·7 μmole in 5 hr. Values are plotted after correction for the endogenous values. Copper-reducing power (relative units) in the system oxidizing glucose without added DPN was found to be 100, 4 and <1 respectively at 0, 2 and 3 hr. ▲, ●, Gluconate and glucose without DPN; △, ○, gluconate and glucose with DPN; □, ■, gluconate with and without DPN in the absence of phenazine methochloride.

Renewal of glucose supply in the system after O_2 uptake had ceased resulted in renewal of oxidation. The product of reaction was non-reducing to copper. These observations suggest that the product of glucose oxidation was gluconolactone or gluconate.

Formation of oxogluconates from glucose and gluconate. When the extract with added phenazine methochloride was further supplemented with DPN, CO_2 was again not formed from glucose, but the total O_2 uptake was now twice as high (1·0 mol.-prop.). Moreover, this system oxidized gluconate, taking up 0·5 mol.prop. of O_2 (Fig. 1). It was found that glucose and gluconate had been converted almost quantitatively into oxogluconate (OGn), the yield of 2- and 5-OGn from 5 μmoles of substrate being 4 and 0·6 μmoles respectively. The extract without phenazine methochloride failed to catalyse an anaerobic reduction of DPN by gluconate. Aerobic oxidation of gluconate required the presence of both phenazine methochloride and DPN in the reaction mixture. Thus the observed requirement for DPN need not mean that this substance necessarily intervened in the reaction in the role of an electron carrier.

Formation of carbon dioxide, from glucose in the presence of adenosine triphosphate. The extract with added DPN and phenazine methochloride oxidized glucose at substantially the same rate in the presence and absence of ATP. In the presence of ATP, however, the oxidation proceeded with evolution of CO_2. The initial R.Q. was approx. 0·7, the rate of O_2 uptake being 10 μmoles/hr./mg. of protein N, i.e. of the same order of magnitude as observed in a suspension of whole cells (Fig. 2). The yield of CO_2 attained >4 glucose equivalents/mole of added ATP, i.e. significantly >2. These results suggest that a reaction induced by ATP was one which generates ATP.

Formation of phosphate esters from glucose and gluconate. In the presence of extracts of the cells, glucose and gluconate were phosphorylated by ATP. With extract prepared from cells grown in fructose, fructose was also phosphorylated. Satisfactory equivalence between the decrease in free hexose and that of acid-labile P was observed in experiments in which alumina extract acted anaerobically in the presence of fluoride (Table 1). With Mickle extract in the same conditions, a large discrepancy between the decreases in acid-labile P and free hexose was experienced, the loss of the latter being the greater. An explanation of the discrepancy might perhaps be suggested by the finding of Schramm & Racker (1957) that the *A. xylinum* extract comprises an enzyme system or systems which split F 6-P and xylulose 5-phosphate anaerobically into acetyl phosphate (acetyl-P) and carbohydrate phosphate (erythrose 4-phosphate and triose-P respectively) with attendant uptake of P_i.

The experiment with glucose reported in Table 1 was repeated on a larger scale in an attempt to obtain unequivocal evidence for the presence of glucokinase. The reaction mixture contained 42 μmoles each of ATP and glucose. The decreases in acid-labile P and free hexose in this reaction mixture were equal, amounting each to 26 μmoles. The products of reaction were resolved by barium (LePage, 1949*a*) into a water-insoluble fraction (I) and a water-soluble ethanol-insoluble fraction (II).

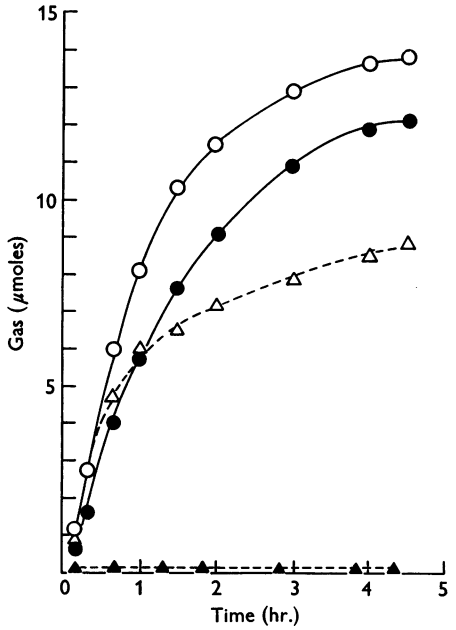

Fig. 2. Oxidation of glucose via phosphorylated intermediates. Reaction mixtures (2 ml.) with or without ATP (300 μg.) contained 0·5 ml. of Mickle extract (0·9 mg. of protein N) of fresh *A. xylinum* cells, 10 μmoles of glucose, DPN (160 μg.), phenazine methochloride and 12 μmoles of $MgCl_2$. The gas phase was air. ○, ●, ΔO_2 and ΔCO_2 with ATP; △, ▲, ΔO_2 and ΔCO_2 without ATP.

Table 1. *Phosphorylation of glucose and gluconate by adenosine triphosphate*

Reaction mixture (2 ml.) with and without substrate (5 μmoles) or ATP (5 μmoles) or both contained 1 ml. of alumina extract (0·7 mg. of protein N), 5 mM-$MgCl_2$ and 10 mM-NaF. Gas phase was N_2. ΔP-ester was calculated from the values of Δ acid-labile P.

System		Changes during 120 min. (μmoles)		
Substrate	ATP	Δ Glucose	Δ Acid-labile P	ΔP-ester
None	+	—	+0·6	—
Glucose	−	−0·2	+0·7	—
Gluconate	−	—	+0·5	—
Gluconate	+	—	−1·7	+2·2
Glucose	+	−2·7	−2·0	+2·7

The amount of G 6-P in II was 11 μmoles (assayed with G 6-P dehydrogenase), and that of F 6-P, 6 μmoles. The total reducing power of (II) proved to be exactly equal to the calculated sum of the reducing powers of these amounts of G 6-P and F 6-P. The sum of recovered hexose-P represents about 60 % of the yield expected on the basis of the observed values of Δ acid-labile P and Δ free glucose. The quotient G 6-P/F 6-P observed is that of a system at equilibrium. Thus the findings suggest that this extract contained phosphohexoisomerase, glucokinase and gluconokinase.

AMP was estimated on the basis of adenine and pentose in fraction (II). These were equal, amounting each to 12·5 μmoles. Assuming myokinase to be present, the observed disappearance of 26 μmoles of acid-labile P would be expected to yield 13 μmoles of AMP. The good correspondence between the observed value and that calculated suggests that myokinase may indeed be present in the extract. α-G 1-P was rapidly equilibrated at pH 7·4 with G 6-P (rate of conversion of G 6-P: $\geqslant$ 100 μmoles/hr./mg. of protein N). Thus this system also contained phosphoglucomutase.

As the decreases of free glucose and acid-labile P in the total reaction mixture had been found to be equal, it seemed likely that formed F 6-P had not been further phosphorylated to F 1:6-P. This is also in accord with the finding that fraction (I), which would have contained any formed F 1:6-P, gave a negative test for ketose.

Reduction of pyridine nucleotides by glucose 6-phosphate and 6-phosphogluconate. Both DPN and TPN could be reduced by G 6-P and 6-PG in the cell-free extract. In one set of conditions, G6-P reduced DPN and TPN at roughly equal rates,

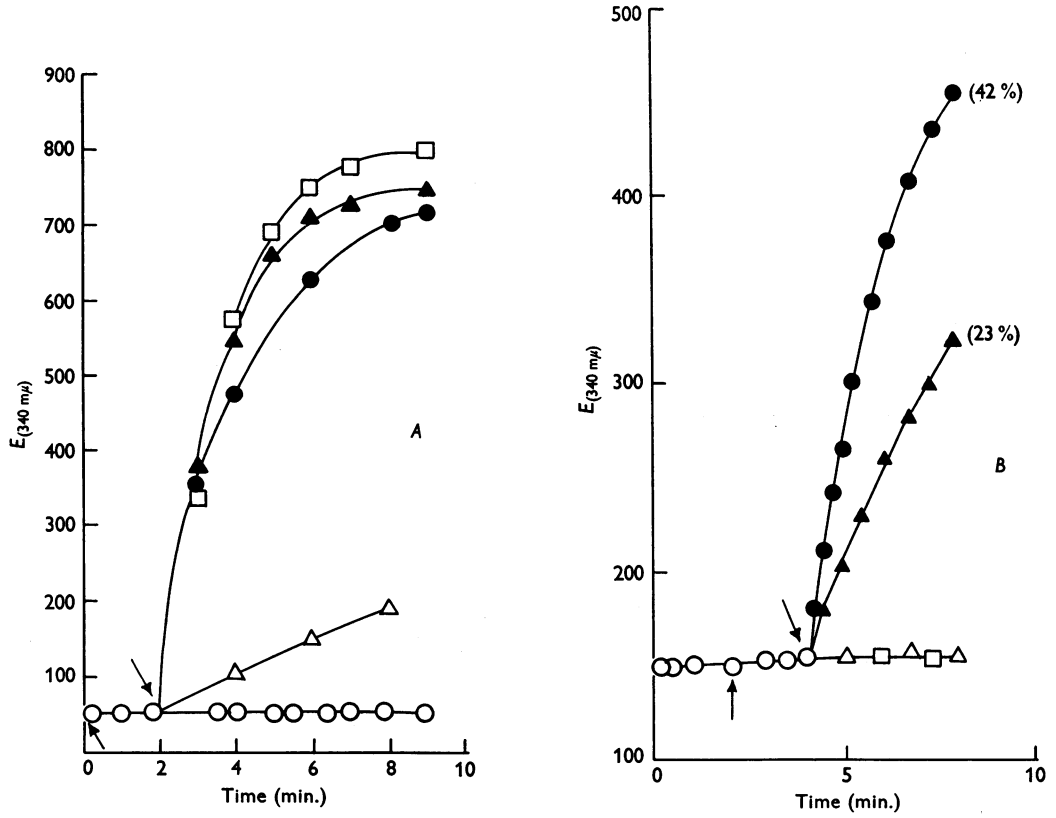

Fig. 3*A*, *B*. G 6-P and 6-PG dehydrogenase activities of *A. xylinum* extract. Reaction mixtures (3 ml.) in systems of Fig. 3*A* contained Mickle extract (0·1 mg. of protein N) of fresh cells, 3 μmoles of substrate (G 6-P or 6-PG), 0·5 μmole of DPN, 0·45 μmole of TPN, 20 μmoles of $MgCl_2$ and 25 mM-cacodylate buffer; pH 6·4. Systems of Fig. 3*B* contained Mickle extract (0·1 mg. of protein N) of frozen cells, 2·5 μmoles of substrate (G 6-P or 6-PG), 0·36 μmole of nucleotide (DPN or TPN), 4 mM-cysteine, 20 mM-NaF and 50 mM-tris buffer; pH 8·2. Light path (quartz cuvette) 1 cm. Values in parentheses beside curves represent the percentage of added nucleotide reduced. ↑, Time of addition of nucleotide; ↓, time of addition of substrate. ○, Systems before substrate addition; ●, 6-PG with DPN; △, 6-PG with TPN; ▲, G 6-P with TPN; □, G 6-P with DPN.

Table 2. *Anaerobic transformation of ribose 5-phosphate into hexose phosphate and heptose phosphate*

Reaction mixtures (3 ml.) with and without R 5-P (12·5 μmoles) contained 1 ml. of Mickle extract (made from 40 mg. dry wt. of fresh cells) with 0·01 M-$MgCl_2$ and 0·01 M-NaF. Gas phase was N_2.

	Composition of reaction mixture after 15 min. (μmoles)				
	Hexose-P	Pentose-P	Heptose-P	P_i	Total
Without added R 5-P	0·8	0·9	0·0	0·8	—
With added R 5-P	6·5	3·7	0·6	1·3*	—
Analytical recovery of added C of R 5-P at 15 min. (%)	55†	26	7	—	88‡

* Despite this observed release of P_i, the amount of non-phosphorylated reducing sugar formed was negligible.
† This value, which corresponds to 34 μatoms of C, was obtained by a direct colorimetric analysis. G 6-P and F 6-P, each determined separately, amounted together to a somewhat larger value, namely 27 + 14 = 41 μatoms of C.
‡ At 90 min. the analytical recovery of added C had fallen to 75%. In a similar system, but without added F^-, the recovery of added C was even lower (50% at 90 min.).

Table 3. *Anaerobic transformation of fructose 1:6-diphosphate into triose phosphate*

Reaction mixtures (2 ml.) with and without F 1:6-P (6·3 μmoles) contained 1 ml. of Mickle extract (made from 20 mg. of dried cells) and neutral 0·06 M-hydrazine sulphate. Gas phase was N_2. Δ Triose-P was calculated from the alkali-labile phosphate.

	Changes during 35 min. (μmoles)		
	Δ Total ketose	P_i	Δ Triose-P
With added F 1:6-P	−4·4	+1·9	+7·5
Without added F 1:6-P	0·0	+0·5	0·0

whereas 6-PG reduced DPN significantly more rapidly than it did TPN (Fig. 3*A*). Under other conditions and with another preparation of extract, TPN but not DPN was reduced by G 6-P whereas DPN but not TPN was reduced by 6-PG (Fig. 3*B*). [Dehydrogenases of G 6-P and 6-PG that are activated both by DPN and TPN have been encountered by other investigators in several bacterial systems. In some of these, the crude mixture of enzymes showed no marked specificity as between DPN and TPN but such specificity became apparent after the enzymes were purified (see, for example, De Moss & Gibbs, 1955).] The reductions of TPN by G 6-P and of DPN by 6-PG were not inhibited by 20 mM-fluoride. The reduction of G 6-P by TPN was not noticeably retarded by 10 mM-monoiodoacetate.

Anaerobic cleavages of carbon chains of carbohydrate phosphates and 6-phosphogluconate. When the Mickle extract of dried cells was incubated in the absence of added nucleotide anaerobically with 6-PG and hydrazine a sluggish production of alkali-labile P plus pyruvate took place (about 0·4 μmole/hr./mg. of protein N). Therefore it seems probable that cleavage of 6-PG to triose-P and pyruvate (Entner & Doudoroff, 1952) did not play a significant role in the metabolism of glucose by this extract. The formation of pyruvate was not augmented by 5 mM-arsenite.

R 5-P was rapidly converted anaerobically into hexose-P (equilibrium mixture of G 6-P and F 6-P) and heptose-P (Table 2). This transformation is consistent with the presence of the following enzymes in the extract: phosphopentoisomerase, phosphopentoepimerase, transketolase, transaldolase and phosphohexoisomerase. Carbon balances drawn up on this basis were found to be incomplete, the deficit increasing as the reaction time increased (see footnote ‡ in Table 2). The anaerobic cleavages described by Schramm & Racker (1957), to which reference has already been made, may have accounted for this disappearance of carbon.

Under anaerobic conditions the extract acting in the presence of hydrazine as added trapping agent formed alkali-labile P (presumably triose-P) from F 1:6-P (Table 3). Whether cleavage of F 1:6-P by aldolase or the anaerobic pathways described by Schramm & Racker (1957) or both accounted for this transformation remains undetermined.

Formation of P_i from carbohydrate phosphates. F 1:6-P was split anaerobically with rapid formation of P_i accompanied by release of a relatively small amount of reducing free sugar. For example, it was found that 12 μmoles of F 1:6-P incubated with Mickle extract (equivalent of 20 mg. dry wt. of frozen cells) in 2 ml. of 20 mM-glycylglycine buffer, pH 7·2, gave 8·1 μmoles of P_i and only 0·5 μmole of reducing free sugar in a reaction time of 120 min. The results suggest that F 1:6-P was hydrolysed largely into a hydrolysis-resistant hexose-P (presumably F 6-P in equilibrium with G 6-P). In the same conditions, α-G 1-P was rapidly converted into reducing acid-stable hexose-P (presumably G 6-P in equilibrium with F 6-P) with little or no release of P_i. When β-G 1-P and UDPG were tested as substrates, the values of acid-labile P and of reducing power released by mild acid hydrolysis remained

practically constant. Presumably, these last substrates were not attacked.

In contrast to the behaviour of the extract, the homogenate of the cells exhibited versatile phosphatase activity. P_i was released rapidly by the homogenate from G 6-P, α-G 1-P, R 5-P and ATP in an anaerobic system in tris buffer at pH 7·5.

Oxidation of triose-P to pyruvate via 3-phosphoglycerate. DPN was rapidly reduced by the cell-free extract acting on F 1:6-P and yeast aldolase (Fig. 4). The velocity of this reaction was increased very markedly by the addition of arsenate to the reaction mixture. The reaction was suppressed by mM-monoiodoacetate. TPN was reduced by this system to some extent, but as such reduction soon ceased it conceivably may have been due to presence of a contaminant (e.g. F 6-P) in the F 1:6-P preparation. In this system TPN but not DPN was reduced by G 6-P (Fig. 3*B*). It could be concluded on the basis of these findings that the extract contained triose-P dehydrogenase.

PGA was converted by the extract into pyruvate and P_i (Table 4). Addition of ADP to the system did not alter the yield of pyruvate though it increased that of P_i. In the system containing PGA plus ADP, fluoride suppressed pyruvate formation completely and lowered the value of P_i released to a level equal to that found in the system containing ADP without PGA. Fluoride did not alter the rate of formation of P_i from ADP. To rationalize these findings the concurrence of the following enzymes in this extract could be assumed: phosphoglyceromutase, a fluoride-sensitive enolase, pyruvate kinase and a fluoride-resistant enzyme system forming P_i from ADP. The inability of added ADP to increase the rate of pyruvate formation might be explained by assuming that a catalytic amount of ADP or its equivalent was present in the enzyme system and could be utilized as a P-acceptor in a cyclic manner, the ADP being converted by reaction with phosphoenolpyruvate into ATP and the latter then back again into ADP. Though pyruvate has been found to be readily oxidized by the whole cells and to be rapidly decarboxylated by them under anaerobic conditions (Schramm *et al.* 1957*b*), it was not oxidized by their extract under the conditions in which PGA was converted into pyruvate.

Evidence has been presented above that when the extract acts in conjunction with ATP to oxidize glucose, ATP is generated. Oxidation of triose-P via PGA into pyruvate and the attendant conversion of P_i into high-energy P could have accounted in part for such ATP synthesis. Additional mechanisms for substrate-level phosphorylation in

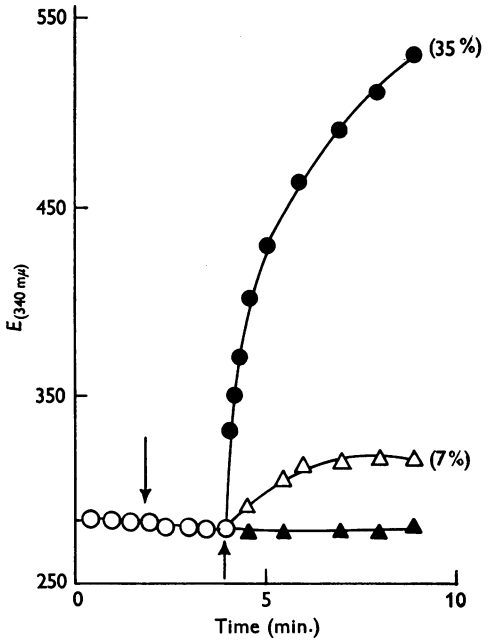

Fig. 4. Oxidation of triose-P by *A. xylinum* extract. Reaction mixtures (2·8 ml.) contained Mickle extract (0·2 mg. of protein N) of frozen cells, crystalline muscle aldolase (50 μg. of protein N), sugar phosphate (2·5 μ-moles), pyridine nucleotide (0·36 μmole), 20 mM-NaF, 17 mM-arsenate (Na^+), 4 mM-cysteine and 50 mM-tris buffer; pH 8·2. Light path (quartz cuvette) 1 cm. Values in parentheses beside curves represent the percentage of added nucleotide found to have been reduced. ↓, Time of addition of pyridine nucleotide; ↑, time of addition of sugar phosphate. ○, Systems before substrate addition; ●, F 1:6-P with DPN; △, F 1:6-P with TPN; ▲, G 6-P with DPN.

Table 4. *Formation of pyruvate from 3-phosphoglycerate*

Reaction mixtures (2 ml.) contained 1 ml. of Mickle extract (15 mg. dry wt. of frozen cells) and 5 mM-$MgCl_2$ in 50 mM-tris buffer, pH 7·8, under N_2. The reaction time was 120 min.

Composition of test system			Products (μmoles)	
PGA (20 μmoles)	ADP (10 μmoles)	Fluoride (12 mM)	Δ Pyruvate	ΔP_i
+	−	−	2·5	3·0
+	+	−	2·5	8·6
+	+	+	0·0	5·2
−	+	−	0·0	5·1

this system have been revealed by the work of Schramm & Racker (1957).

Aerobic formation of carbon dioxide from carbohydrate phosphates. G 6-P was oxidized to CO_2 by a cell-free extract, supplemented only by DPN + TPN, at a slowly falling rate and with an R.Q. slightly less than 1·0. The effect of phenazine methochloride on this system is illustrated in Fig. 5. It is evident that phenazine methochloride increased the velocity of reaction by about fivefold but did not affect the value of R.Q. Conceivably, the phenazine methochloride could have intervened in the oxidation chain as an efficient electron carrier either between reduced pyridine nucleotide and O_2 or between the substrate and pyridine nucleotide, and thus produced the observed increase in reaction rate.

Single addition of either DPN and TPN markedly accelerated the formation of CO_2 from G 6-P (Fig. 6; compare also Fig. 3). However, in these conditions, a mixture of DPN and TPN produced an effect which was significantly greater than the sum of the single effects of the nucleotides.

Oxidations of P-esters to CO_2 were found to be retarded by added adenine nucleotides. For example, 2·5 mM-AMP in an extract supplemented by DPN + TPN + phenazine methochloride lowered the rate of formation of CO_2 from G 6-P by $\geqslant 80\%$. ADP was tested in another experiment in which F 1:6-P was added as substrate and was found to lower the rate of formation of CO_2 by about 50%. The mechanism by which these marked inhibitory effects was accomplished is not apparent to us.

It is noteworthy that in the presence of added Mg^{2+} ions the oxidation of G 6-P attained a high CO_2 yield (83% of theory) (Fig. 7). Reaction in the absence of added Mg^{2+} ions was bimodal in course and showed a steep fall in rate at a relatively low CO_2 yield ($\leqslant 41\%$ of theory) (cf. Fig. 6; see also Fig. 5). In this system, renewal of the G 6-P supply after CO_2 evolution had become slow led promptly to a restoration of a high reaction rate. The findings indicate that the oxidative process occurring in the

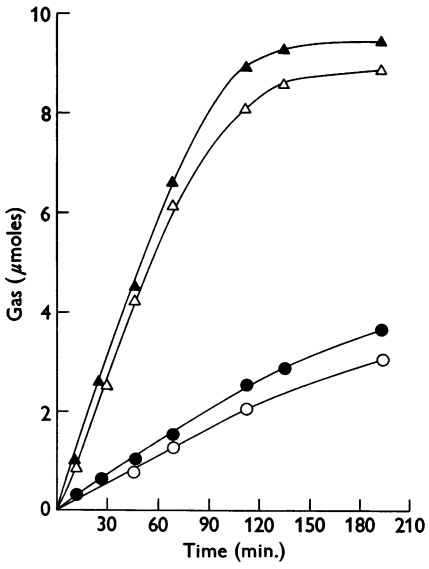

Fig. 5. Effect of phenazine methochloride on the aerobic oxidation of G 6-P by *A. xylinum* extract. Reaction mixtures (2 ml.) contained 5 μmoles of G 6-P, Mickle extract made from 20 mg. of freeze-dried cells, 200 μg. of DPN and 100 μg. of TPN with and without phenazine methochloride. The gas phase was air. The pH was initially about 7 but decreased during the reaction. Values for CO_2 were calculated from the pressure readings on the basis of the assumption that the pH was 6. ○, ●, ΔCO_2 and ΔO_2 without phenazine methochloride; △, ▲, ΔCO_2 and ΔO_2 with phenazine methochloride.

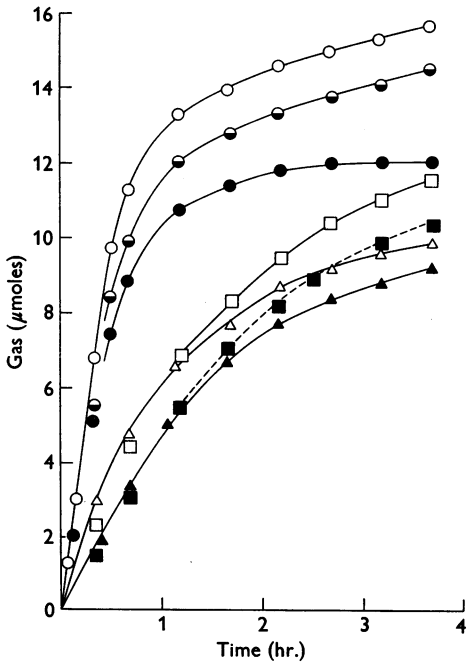

Fig. 6. Effects of pyridine nucleotide on aerobic oxidation of G 6-P by *A. xylinum* extract. Reaction mixtures (2 ml.) with 200 μg. of DPN or 200 μg. of TPN or 200 μg. of their mixture (1:1) and with or without 10 mM-fluoride contained Mickle extract (1 mg. of protein N) of fresh cells, 50 μg. of phenazine methochloride/ml. and 5 μmoles of G 6-P. The gas phase was air. In the system with DPN + TPN, ΔF 1:6-P (μmoles) at 240 min. was found to be 0·5 and 1·0, without and with fluoride respectively. Corresponding values for ΔP_i were 2·0 and 0·5 μmoles. For the method of calculation of CO_2 see the legend to Fig. 5. □, ■, ΔO_2 and ΔCO_2 with DPN; △, ▲, ΔO_2 and ΔCO_2 with TPN; ○, ΔO_2 with DPN + TPN (with and without fluoride); ◒, ●, ΔCO_2 with DPN + TPN without and with fluoride respectively.

absence of Mg^{2+} ions had resulted in an intermediary piling up of a slowly oxidizable product or products. The nature of the latter is still unknown.

The rates of formation of CO_2 from G 6-P, 6-PG and R 5-P in a system consisting of cell-free extract supplemented by DPN + phenazine methochloride were found to be all practically equal. In accord with the assumption that the formation of CO_2 had involved the pentose cycle, 10 mM-fluoride did not decrease the initial rate of these oxidations. Moreover, an extract with added phenazine methochloride + DPN + TPN + Mg^{2+} ions oxidized F 1:6-P to CO_2 at the same rate as it oxidized G 6-P. In both cases the R.Q. was 1·0 (Fig. 7).

Rates of measured part reactions and those of CO_2 formation are collected in Table 5. It can be seen that the part reactions proceeded at rates greater than or in the same rough order of magnitude as the rates of the production of CO_2 from carbohydrate phosphates. Thus the interpretation that the production of CO_2 occurred via observed part reactions does not present difficulty on kinetic grounds.

Homogenates prepared either by grinding the cells with alumina or exposing them to vibration with glass beads as described in the Methods section, sediments prepared from such homogenates

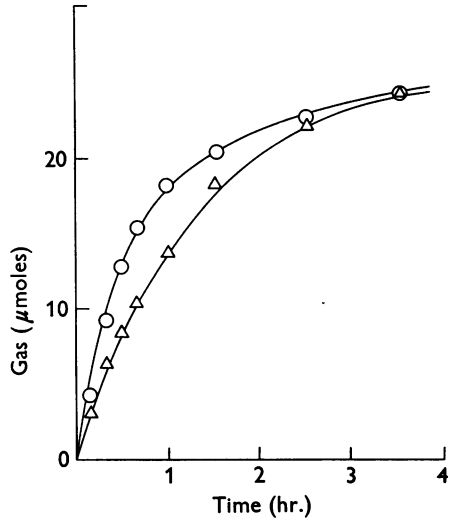

Fig. 7. Aerobic oxidation of F 1:6-P and 6-P by *A. xylinum* extract. Reaction mixtures (2 ml.) contained 1 ml. of Mickle extract (made from 20 mg. dry wt. of frozen cells), 5 μmoles of substrate (G 6-P or F 1:6-P), 200 μg. of TPN, 200 μg. of DPN, 100 μg. of phenazine methochloride and 4 mM-$MgCl_2$. The gas phase was air. The R.Q. of all the oxidations was 1·0 throughout, both with G 6-P and F 1:6-P. ○, G 6-P; △, F 1:6-P.

Table 5. *Reactions catalysed by a cell-free extract of* A. xylinum

Cell-free Mickle extracts of glucose-grown cells were used. Substrate concentration varied from 2 to 5 μmoles/ml. Systems were buffered with tris at pH 7·4 and 8·2, and with citrate (Na^+) at pH 6·4. The temperature was 30°. Aerobic systems were supplemented with phenazine methochloride (100 μg./ml.) as added electron carrier. To anaerobic systems 10 mM-fluoride was added in order to inhibit side reactions catalysed by phosphatases. Mixtures tested at pH 8·2 contained 4 mM-cysteine. Gas phase was air or N_2. Triose-P was generated from F 1:6-P by crystalline aldolase.

Substrate	O_2	pH	Measured products and their rates of formation (μmoles/hr./mg. of protein N)	Cofactors*
Glucose	+	6·4	CO_2, 0·0; Gluconate, 4	—
	+	7·5	CO_2, 4	ATP, DPN, Mg^{2+} ions
	−†	6·4	G 6-P, 3	ATP, Mg^{2+} ions
Gluconate	+	6·4	CO_2, 0·0; 2-OGn, 2	DPN
	−†	6·4	6-PG, 3	ATP, Mg^{2+} ions
F 6-P	−†	6·4	F 1:6-P, 0·0	ATP, Mg^{2+} ions
G 6-P	+	7·4	CO_2, 12	DPN, TPN
	+	7·4	CO_2, 5	DPN
	−	7·4	F 6-P, ⩾50	—
	−	8·2	TPNH, 15	TPN‡
α-G 1-P	−	7·4	G 6-P, ⩾100	—
F 1:6-P§	−	7·4	Triose-P, 8	—
R 5-P	+	7·4	CO_2, 5	DPN
	−	7·4	F 6-P, 10	—
6-PG	+	7·4	CO_2, 5	DPN
	−	8·2	DPNH, 30	DPN‡
Triose-P	−	8·2	DPNH, 20	DPN,‖ AsO_4^{3-} ions
PGA	−	7·4	Pyruvate, 3	—

* Components listed are those which markedly accelerated reaction.
† Extract was prepared by grinding cells with alumina.
‡ Under another set of experimental conditions, both DPN and TPN were able to accelerate this reaction.
§ Hydrazine was added as trapping agent. Fluoride was not added.
‖ TPN was not reduced.

by centrifuging and supernatant fluids separated by the same means failed to form cellulose from glucose, G 6-P, UDPG, α-G 1-P, β-G 1-P and a crude specimen of G 4-P in the presence of a range of tested cofactors. As the outcome of these experiments was negative they are not described in detail. G 6-P incubated aerobically in a suspension of whole cells within their Mickle extract supplemented by a mixture of cofactors gave no cellulose. This result suggests that during the oxidation of G 6-P by the cell extract no cellulose precursor which could be polymerized by the cells on their outer surface had been formed.

DISCUSSION

A scheme of alternate pathways for glucose metabolism

In constructing a scheme for glucose metabolism in this organism, it is necessary to make provision for the following observations (Schramm *et al.* 1957*a*, *b*). (1) The isotope configuration of cellulose formed by the cells from specifically-labelled [^{14}C]hexoses corresponds to that of hexose-P in a pentose cycle. (2) The cells form cellulose not only from glucose but also from gluconates (gluconate, 2-OGn, 5-OGn) and certain three-carbon substrates (glycerol and dihydroxyacetone), and, after adaptation, from fructose. (3) Cells acting on glucose rapidly form gluconate, demonstrable in the medium. Cells injured selectively by freeze-drying or by 2:4-dinitrophenol convert glucose into 2- and 5-OGn, which pile up in the medium. (4) Depending on experimental conditions and the history of the cells, 2- and 5-OGn may be converted into carbon dioxide with and without attendant formation of cellulose. (5) Pyruvate, acetate and citrate-cycle intermediates, though oxidized by the cells to carbon dioxide, do not yield cellulose. (6) The concentration of fluoroacetate which stops acetate oxidation does not block cellulose formation from glucose. (7) A concentration of fluoride which

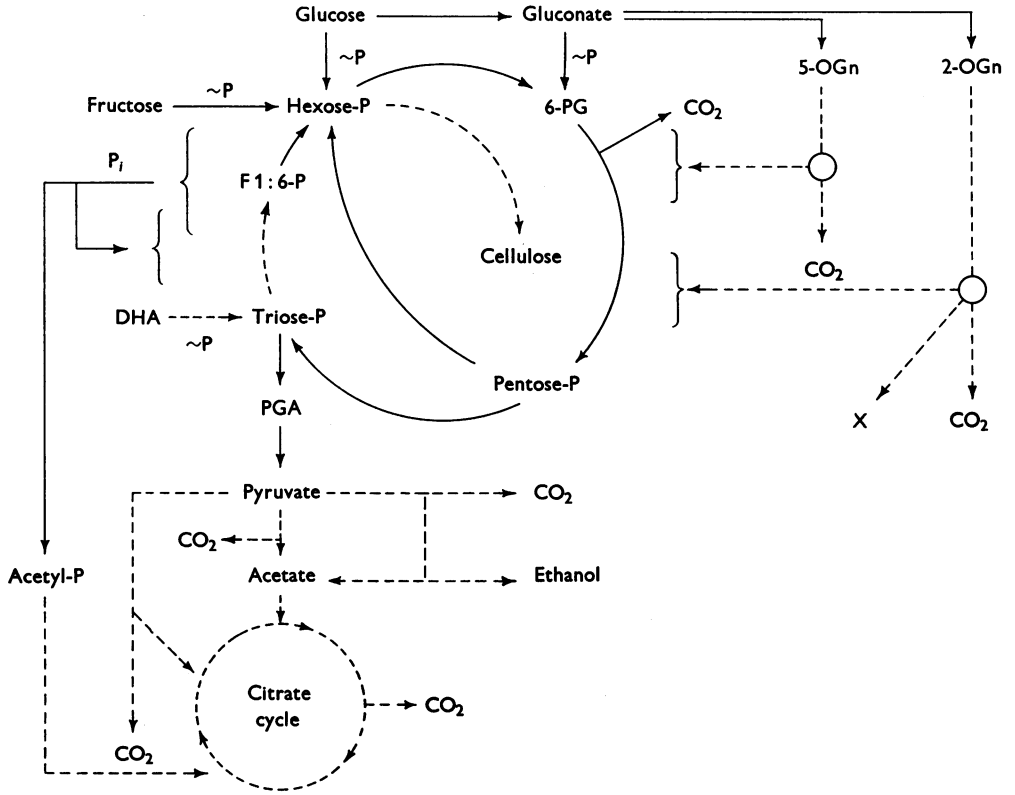

Fig. 8. Scheme of alternate pathways of oxidative carbohydrate metabolism in *A. xylinum*. Reactions drawn with a continuous line have been demonstrated in the cell-free extract. Reactions not so demonstrated but which probably occur in the intact cell are drawn with a broken line. ~P indicates an uptake of ATP. Anaerobic cleavages by which pentose-cycle intermediates are converted into acetyl-P with uptake of P_i are represented by an arrow on the left-hand side of the scheme. Pathways of 2- and 5-OGn metabolism whose mode of entry into the pentose cycle and ways of by-passing it are not known are represented by arrows on the right-hand side of the scheme. An unknown product of 2-OGn metabolism has been designated *X*.

blocks *A. xylinum* enolase in an extract does not interfere with the production of carbon dioxide from glucose by a suspension of cells.

In addition to the above observations made on whole cells, the scheme of metabolism to be constructed should also take account of those reactions pertinent to carbohydrate oxidation that have been demonstrated by us in cell-free preparations of the organism, namely the following: (1) oxidation of glucose via gluconate to OGn (largely 2-OGn); (2) phosphorylation of glucose, gluconate and fructose; (3) oxidation of phosphorylated carbohydrates and 6-PG via a pentose cycle; (4) oxidation of triose-P via PGA to pyruvate; (5) oxidation of tri- and di-carboxylic acids to carbon dioxide (unpublished experiments); and (6) anaerobic cleavages of F 6-P and xylulose 5-phosphate leading to formation of acetyl-P with uptake of P_i (Schramm & Racker, 1957) in a manner resembling a cleavage of pentose-P which was first described by Heath, Hurwitz & Horecker (1956).

It is evident that no single known pathway of glucose metabolism can account by itself for the versatile activities of *A. xylinum* cells and their extracts. A scheme which presents alternate pathways for glucose metabolism is therefore proposed (Fig. 8). It is predicated upon the notion that these cells operate a pentose cycle and that the intermediate of the cycle which is closest to cellulose is a hexose-P. Thus substrates which give cellulose are all shown as being capable of entering the pentose cycle, whereas substrates which give carbon dioxide without attendant formation of cellulose are shown by-passing the pentose cycle. With 2- and 5-OGn, pathways which enter the cycle and others which by-pass it are indicated [in this connexion, cf. Wood (1955) and De Ley (1954)].

The pentose cycle shown is one entered in different ways: the way mediated by glucokinase and several other ways in which primary conversion of glucose into gluconate plays a part. It is indicated that gluconate can be introduced into the cycle directly by the action of gluconokinase and indirectly via 2- and 5-OGn respectively.

Several ways by which carbon can be withdrawn from the pentose cycle and fed into a citrate cycle are shown. One of these is an oxidative shunt operated at the level of triose-P, similar to that which has been found in conjunction with the pentose cycle in a number of other micro-organisms (see, for example, Vandemark & Fukui, 1956). Additional means by which carbon can be moved out of the pentose cycle and fed into the citrate cycle involve anaerobic transformations of pentose-cycle intermediates leading to formation of acetyl-P.

The pentose cycle is thought to be a major pathway of nucleotide syntheses and as such might be essential for cell proliferation. The failure of some strains of *A. xylinum* to grow on acetate (or acetate-forming ethanol) as a sole carbon source in a medium containing ammonium as a nitrogen source (Shimwell, 1957*a*), whereas they grow on glucose in this medium and can utilize acetate in the presence of glucose, is consistent with the proposal that the pentose cycle can be entered in such strains by glucose but not by acetate and that the pentose cycle plays an essential role in growth.

Among the enzymes that are concerned with glycolysis via the classical Embden–Meyerhof pathway, phosphofructokinase was conspicuous by its apparent absence from an extract of *A. xylinum*. The finding that these cells did not metabolize glucose anaerobically was consistent with this result. The fact that anaerobic oxidations of glucose by dye systems and ferricyanide did not sustain either cellulose synthesis or formation of carbon dioxide by the cell suspension (Schramm *et al.* 1957*b*) can probably be traced to the reason that such oxidations do not generate ATP. It is not as easy, however, to rationalize the finding that these cells possess anaerobic mechanisms for the cleavage of carbohydrate phosphate esters with attendant uptake of P_i and generation of high-energy P yet fail to form cellulose anaerobically from glucose.

Note on biochemical evolution in Acetobacter

It is evident that a series of successive intermediary steps of carbohydrate oxidation in *A. suboxydans* (cf. Hauge, King & Cheldelin, 1955; Kitos, King & Cheldelin, 1956; Fewster, 1956) and in *A. xylinum* are identical. It should also be noted, however, that there are at least two enzyme functions in *A. xylinum* which appear to be missing from *A. suboxydans*, namely, (1) an ability to convert a P-ester participating in the pentose cycle into cellulose, and (2) an ability to oxidize acetate to carbon dioxide. Both functions are positioned terminally in the metabolic scheme; neither of them need be regarded as indispensable to the metabolic utilization of carbohydrates by *A. xylinum*, either for energy or carbon. It has indeed been demonstrated experimentally that in *A. xylinum* the mutative deletion of the property of cellulose production is compatible with survival of the cell and with vigorous proliferation (Schramm & Hestrin, 1954). This loss mutation, moreover, is a reversible one (Shimwell, 1956) and consonant with further mutations by which strains indistinguishable from established type species of *Acetobacter* arise under laboratory conditions from an *A. xylinum* parent (Shimwell, 1957*b*). As yet, mutative loss or acquisition of the ability to oxidize acetate does not appear to have been encountered experimentally in this bacterial genus. Still, the notion that this ability is likewise a mutable property seems permissible. Biochemically, the

hypothesis that *A. xylinum* is a phylogenetic ancestor of *A. suboxydans* thus might imply only that *A. xylinum* has been able to undergo consecutive loss mutations of two non-essential enzyme systems. An opposing view, namely that evolution proceeded from a simple state to a more complex one, may be more attractive on philosophical grounds. In that case, the interesting possibility has to be considered that the ancestor of *Acetobacter* was equipped with a mutative potential so broad as to comprise within its range two enzyme systems (respectively for cellulose synthesis and acetate oxidation) which are facultative in *Acetobacter* but have elsewhere assumed cardinal roles in cellular survival.

SUMMARY

1. A cell-free extract of *Acetobacter xylinum* failed to form cellulose from a range of tested substrates.

2. The extract (with added phenazine methochloride) oxidized glucose aerobically to gluconate. When this system was further supplemented with diphosphopyridine nucleotide, it oxidized gluconate to oxogluconate (largely 2-oxogluconate).

3. The extract with adenosine triphosphate oxidized glucose aerobically to carbon dioxide. The extract contained kinases (glucokinase and gluconokinase), phosphoglucomutase, a complete pentose-cycle set of enzymes, and a set of enzymes by which triose phosphate could be converted into pyruvate.

4. A scheme of alternate pathways for glucose metabolism that can be operated in an *A. xylinum* cell is proposed. The scheme shows a pentose cycle which has several ports of entry and exit. The latter lead from the pentose cycle into a citrate cycle.

5. Biochemical evolution in the genus *Acetobacter* is briefly discussed.

Note added in proof. See Colvin, J. R. (1957). *Arch. Biochem. Biophys.* **70**, 294. Glaser, L. (1957). *Biochim. biophys. Acta*, **75**, 436. Greathouse, G. A. (1957). *J. Amer. chem. Soc.* **79**, 4503.

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The Excretion of *allo*Tetrahydrocortisol in Human Urine

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It is now agreed that cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione; hydrocortisone) is the principal glucocorticoid hormone secreted by the normal human adrenal cortex (Mason & Sprague, 1948; Bush & Sandberg, 1953; Romanoff, Hudson & Pincus, 1953; Sweat, Abbott, Jeffries & Bliss, 1953; Roberts & Szego, 1955). As with other steroids having the Δ^4-3-keto group in ring A, the principal known metabolites of cortisol are steroids in which this group has been reduced to a saturated 3-hydroxyl group (Fig. 1) (Lieberman & Teich, 1953; Fukushima *et al.* 1955). Although four possible stereoisomeric 3-alcohols exist, only one has been found so far among the C_{21} metabolites of cortisol

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