DISSIMILATION OF 6-PHOSPHOGLUCONATE BY *AZOTOBACTER VINELANDII**

by

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It has been established[1,2] that the product of glucose-6-phosphate dehydrogenase in *Azotobacter* is 6-phosphogluconate (6-PG) and that 6-phosphogluconolactone precedes the formation of 6-PG in dried yeast[3]. Furthermore, in yeast and *Escherichia coli* it has been found that 6-PG is oxidized to ribulose-5-phosphate by an enzyme, 6-phosphogluconate dehydrogenase, specific for TPN[4,5]. However, all attempts to demonstrate the presence of this enzyme in the *Azotobacter* have failed.

Using isotopic labeled glucose, ENTNER AND DOUDOROFF in 1952 established that 6-PG is split into two three carbon fragments, pyruvate and glyceraldehyde-3-phosphate (G-3-P), by *Pseudomonas saccharophila*[6]. These authors, as well as KOVACHEVISCH AND WOOD[7], have proved that the intermediate in this reaction is the dehydration product of 6-PG, 2-keto-3-deoxy-6-phosphogluconate. One enzyme was required for this dehydration; another required for the splitting of this product into pyruvate and G-3-P by these organisms.

This paper is concerned with the demonstration of the splitting of 6-PG into pyruvate and G-3-P by extracts from *Azotobacter vinelandii*. The results support the conclusion that most of the oxidation of glucose by this organism passes through these steps.

The further metabolism of pyruvate, one of the products of 6-PG splitting, has been discussed previously[8], but the status of the remaining product, G-3-P, has not been studied. STONE AND WERKMAN[9] reported that small amounts of phosphoglyceric acid accumulate in the *Azotobacter* during oxidation of glucose in the presence of fluoride, indicating that triose phosphate dehydrogenase is present. However, the direct demonstration of this enzyme by the reduction of DPN (diphosphopyridine nucleotide) is not possible since *Azotobacter* extracts apparently contain alpha-glycerophosphate dehydrogenase and isomerase and no net DPN reduction is observed[2]. *Azotobacter* preparations, however, do convert 3-phosphoglyceric acid to pyruvate as will be shown in this paper.

Since these preparations also contain aldolase and hexosediphosphatase, as will be demonstrated, it is possible that part of the G-3-P is converted to hexose phosphate and recycles through glucose-6-phosphate dehydrogenase and the splitting enzyme.

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METHODS AND MATERIALS

The 6-PG used in these experiments was obtained from W. A. WOOD, University of Illinois; the F-1,6-P (fructose-1,6-diphosphate) and 3-PGA (3-phosphoglyceric acid) were obtained from Nutritional Biochemicals Corp.

To demonstrate the presence of the splitting and dehydrating enzymes, the following system was used[10]: 0.5 ml extract (23.4 mg protein) from *Azotobacter vinelandii* O (*Azotobacter agile* var. *vinelandii*), 1.0 ml glycylglycine buffer at pH 7.6, 1.5 ml 6-PG solution (31 μmoles), 1.0 ml of 0.56 *M* hydrazine at pH 7.4, and 1.0 ml of 10^{-2} *M* arsenite. The hydrazine was added to trap the G-3-P formed and the arsenite to prevent further metabolism of pyruvate. In one experiment the hydrazine was omitted to determine if more pyruvate would be formed from the G-3-P through G-3-P dehydrogenase, enolase, and phosphoenolkinase, or whether instead F-1,6-P or alpha-glycerophosphate might be formed.

The reaction was followed by appearance of pyruvate which was determined by a method already described[2]. G-3-P was estimated by alkali lability[11]; by reaction with orcinol and measurement of the optical density at 440 mμ[12]; and by reaction with anthrone as will be described in the results. In addition any pentose formed was measured by reaction with orcinol. Standards of each compound were used for comparison.

To establish the identity of the keto acid formed, the mixture after deproteinization was treated with 1.0 ml of 0.1 % 2,4-dinitrophenylhydrazine in 1 *N* HCl, and the derivative formed was extracted with ether. This procedure was also carried out with known pyruvate. The derivatives were then chromatographed on paper using three different solvent systems: (a) water saturated phenol; (b) water saturated butanol; and (c) 50% butanol, 10% ethanol, 40% water. All spots on the chromatograms were identified by the yellow color of the derivatives, by the formation of a red color upon spraying with an alkali solution, and by the appearance under ultraviolet light. Although G-3-P formed a hydrazone, it appeared to remain water soluble and non-extractable by ether.

Aldolase was determined using a system similar to that of SIBLY AND LEHNINGER[13]. The triose phosphate formed was determined by alkali labile phosphate, and by reaction with anthrone. The concentration of the F-1,6-P remaining and triose phosphate formed, at varying times during the incubation, was estimated by reacting an aliquot with anthrone reagent, measuring the optical density of the color formed at 490 and 600 mμ and determining the concentrations by solution of simultaneous equations.

Fructose diphosphatase was assayed by measurement of the phosphate released from F-1,6-P when the latter substrate was incubated with an *Azotobacter* preparation and Mg^{++}. After completion of the reaction the products were separated on Dowex-1 resin[14].

The system from 3-PGA to pyruvate (phosphoglyceromutase, enolase, and phosphoenolkinase) was measured by the appearance of pyruvate when 3-PGA, ADP (adenosine diphosphate), Mg^{++} and arsenite were incubated with an azotobacter extract. Arsenite was added to prevent the further metabolism of pyruvate.

RESULTS

The stoichiometry of the action of *Azotobacter* extracts on 6-PG is presented in Table I. From 31 μmoles of 6-PG, 30.5 μmoles of pyruvate and 28 μmoles of G-3-P were formed. In addition, a small amount of pentose was produced as was reported previously[2]. The *RF* values of the 2,4-dinitrophenylhydrazone derivatives of the product of 6-PG splitting, as well as those of known pyruvate, are recorded in Table II. The spectra of the color produced when the dephosphorylated product was reacted with orcinol and anthrone reagent together with the spectra of known glyceraldehyde and alpha-glycerophosphate are presented in Figs. 1 and 2, and agree closely with known glyceraldehyde, thus confirming the product as this compound. Pyruvate did not interfere with these reactions. Also it was found that if the hydrazine was omitted from the system used for measuring the splitting of 6-PG and if DPN was added, approximately 1/3 of the G-3-P was found as would be expected on the basis of splitting.

The spectra obtained after reacting G-6-P (glucose-6-phosphate), glyceraldehyde, R-5-P (ribose-5-phosphate), and a mixture of the three with anthrone is presented in Fig. 3. Although R-5-P gave little or no color, glyceraldehyde and G-6-P (any hexose)

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TABLE I

STOICHIOMETRY OF THE SPLITTING OF 6-PG (31 μmoles) BY *Azotobacter vinelandii* O EXTRACTS*

Time	Pyruvate** μmoles		G-3-P μmoles		
			Method of analysis		
	PO_4*	No PO_4	Anthrone	Alkali labile PO_4***	Orcinol§
0	0.0	0.0	0.1	0.1	0.0
30 min	18.0	18.1	—	—	—
60 min	30.4	30.6	28.5	28.3	27.5

* Incubation mixture as in Fig. 1; one mixture contained 100 μmoles of PO_4, the other glycylglycine buffer.
** Determined by method of KING[18], confirmed by chromatography (Table II).
*** Determined by the method of LOHMANN AND MEYERHOF[11].
§ Determined by orcinol reaction against standard glyceraldehyde as in Fig. 1.

TABLE II

CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONE OF REACTION PRODUCTS OF 6-PG SPLITTING*

Solvent system	RF		
	Hydrazone of pyruvate	Hydrazone of reaction products	Hydrazone of mixture
Water saturated phenol	0.86	0.86	0.87
Water saturated butanol	0.30	0.30	0.29
50% butanol 10% ethanol 40% water	0.53	0.53	0.52

* Derivatives were prepared by adding 1.0 ml of 0.1% 2,4-dinitrophenylhydrazone in 1 N HCl to the deproteinized mixture, allowing the hydrazones to form, and extracting with ether. The derivative in ether was spotted, and the separation made by the respective solvent systems.

gave pronounced peaks at 490 and 620 mμ respectively. The fact that the extinction of hexose is at a minimum when glyceraldehyde is at a maximum and vice versa, readily lends itself to estimation of mixtures of these compounds by solution of simultaneous equation[15]. This is further substantiated since the absorbancies at these wavelengths, 490 and 620 mμ, when added yield as sum the absorbancy of the mixture (see Fig. 3). Good recovery was obtained using this method for determining concentrations of triose and hexose in mixtures. For example, when a mixture of 0.51 μmoles of G-6-P and 1.2 μmoles of glyceraldehyde was analyzed by solution of simultaneous equations, the results indicated that the solution contained 0.52 μmoles of G-6-P and 1.14 μmoles of glyceraldehyde.

The stoichiometry of the action of aldolase on F-1,6-P is given in Table III. Here it is seen that aldolase splits F-1,6-P into triose phosphate and dihydroxyacetone phosphate at the rate of approximately 13 μmoles split/h/46 mg extract protein. Fructose diphosphatase from *Azotobacter* hydrolyzes the # 1 phoshate from F-1,6-P at the rate of 11 μmoles/h/46 mg extract protein, and the products of the hydrolysis of 19.4 μmoles of F-1,6-P were 3.0 μmoles free hexose, 14.3 μmoles of a mixture of G-6-P and F-6-P

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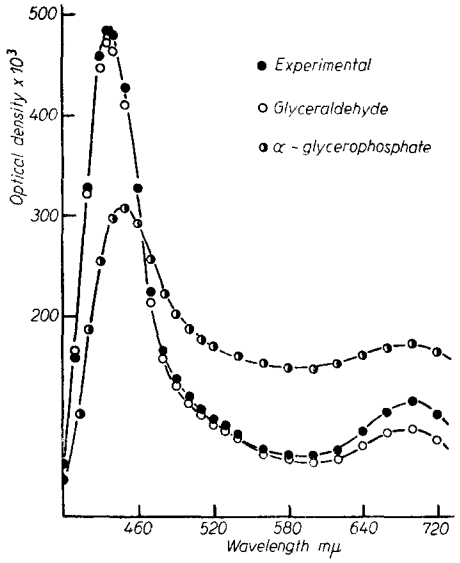

Fig. 1. Orcinol spectrum of the products of 6-PG splitting. The reaction mixture contained: 0.5 ml extract protein (23 mg), 1.0 ml of 0.56 *M* hydrazine at pH 7.4, 1.0 ml 10^{-2} *M* arsenite, and 1.5 ml containing 31 μmoles 6-PG. Duplicate mixtures were made, one containing in addition to the above, 100 μmoles of phosphate. Trichloro acetic acid was added after 60 minutes incubation at 30° C and the ppt. protein removed. One tenth ml of the mixture after treatment with phosphatase was reacted with orcinol and a spectroanalysis of the color developed was recorded against a blank without substrate treated in the same manner. α-glycerophosphate and glyceraldehyde were included for comparison.

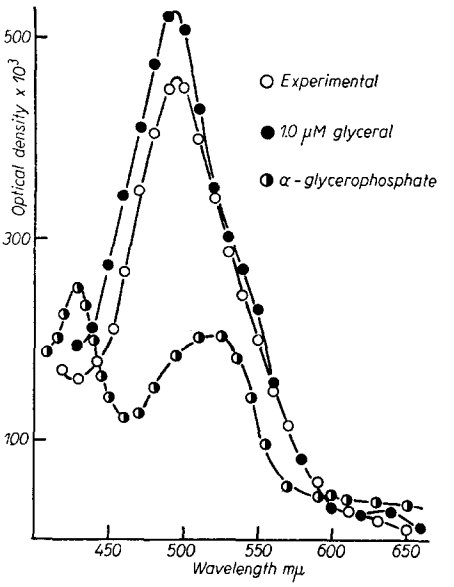

Fig. 2. Anthrone spectrum of the products of 6-PG splitting by extracts of *Azotobacter vinelandii* O. The reaction mixture and treatment were as in Fig. 1; 0.2 ml of the mixture after treatment with phosphatase and removal of the free sugars by treatment with Dowex-1 resin was reacted with anthrone reagent and a spectroanalysis of the color was made. Known glyceraldehyde and α-glycerophosphate were included for comparison.

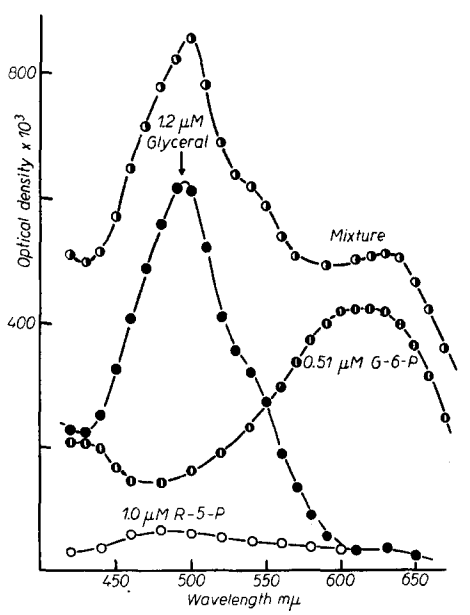

Fig. 3. Comparison of the spectra of glyceraldehyde, G-6-P, and R-5-P alone and mixed after reaction with anthrone. The mixture contained 1.2 μmoles of glyceraldehyde, 0.51 μmoles of G-6-P and 1.0 μmoles of R-5-P. The quantities indicated were diluted to 1.5 ml and 4.5 ml of anthrone reagent was added. The mixture was heated in a boiling water bath for 3 minutes, cooled by immersing in cold water and the spectra recorded in the Beckman spectrophotometer against a reagent blank.

(65% to 35% respectively), and 23 μmoles of phosphate (corrected for phosphate in preparation). Fluoride (10^{-2} *M*) completely inhibited hexose diphosphatase from this organism.

That 3-PGA in the presence of arsenite is readily converted into pyruvate by extracts of *Azotobacter* is shown in Table IV. The concentration of pyruvate accumulating never reached that of the initial 3-PGA and, in fact, decreased after reaching a level of 9.5 μmoles. Fluoride (10^{-2} *M*) completely inhibited the production of pyruvate from 3-PGA. The rate of conversion of 3-PGA to pyruvate was approximately 5 μmoles/h/46 mg extract protein.

TABLE III

STOICHIOMETRY OF THE SPLITTING OF F-1,6-P BY *Azotobacter* ALDOLASE

Time	*Triose phosphate*		*F-1,6-P*
	Alkali labile PO_4 μmoles	*By anthrone reagent μmoles*	*By anthrone reagent μmoles*
0 min	0.0	0.3	19.4
30 min	—	12.8	12.9
60 min	21.8	19.5	8.5

Conditions: Incubation mixture contained: 19.5 μmoles of F-1,6-P, 2 ml glycylglycine buffer at pH 7.4, 0.56 *M* hydrazine, and 1.0 ml of enzyme protein (46 mg) made to a total volume of 5 ml.

TABLE IV

CONVERSION OF 3-PGA TO PYRUVATE BY EXTRACTS OF *Azotobacter vinelandii* O

Conditions	*Pyruvate μmoles Time in minutes*		
	30	*120*	*210*
Fluoride + PO_4	0.0	0.0	0.0
No fluoride + PO_4	0.5	8.5	8.7
No fluoride No PO_4	2.4	9.5	7.2

System contained: 10 μmoles ADP, 10^{-2} *M* Mg^{++}, 10^{-2} *M* fluoride when designated, 22.3 μmoles of PO_4 when designated, AsO_2^- 10^{-2} *M*, 3-PGA 20 μmoles, and 1 ml enzyme protein (46 mg) diluted to 5 ml with 0.05 *M* glycylglycine buffer at pH 7.4.

DISCUSSION

The conversion of 31 μmoles of 6-PG into 30 μmoles of pyruvate and 28 μmoles of G-3-P by *Azotobacter* extracts indicates the presence of the 6-PG splitting system. The dehydration of 6-PG into 2-keto-3-deoxy-6-phosphogluconate before splitting demonstrated by MACGEE AND DOUDOROFF[16] and KOVACHEVIC AND WOOD[7] probably also occurs in this organism. Since G-6-P and 6-PG are oxidized rapidly by these *Azotobacter* extracts with the final difference in oxygen consumption consistent with the one step oxidation of G-6-P to 6-PG (17), since these extracts contain high levels of *zwischenferment* (2) and the splitting system, and since phosphohexokinase and 6-PG dehydrogenase are not demonstrable[2], it appears that all G-6-P and 6-PG are metabolized through pyruvate and G-3-P. The rate of conversion of G-6-P to 6-PG, 600

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μmoles/h/46 mg extract protein[2], and of the splitting of 6-PG, about 72 μmoles split/h/46 mg extract protein, is consistent with this view.

Of the further metabolism of the products of 6-PG splitting, only pyruvate dissimilation has been investigated[8]. Two possible pathways for further metabolism of G-3-P are: (a) conversion by triose phosphate dehydrogenase, phosphoglycericmutase, enolase, and phosphoenolkinase to pyruvate; and (b) conversion of part of the G-3-P to dihydroxyacetone phosphate, condensation of the latter compound with G-3-P by aldolase to yield F-1,6-P, and hydrolysis of the # 1 phosphate of F-1,6-P by fructose diphosphatase to yield F-6-P. The F-6-P could be converted into G-6-P[2], and whole system recycled through *zwischenferment* and the splitting system.

The respective rates of conversion of G-3-P to pyruvate by system *a* and to F-6-P by system *b* are consistent with the operation of both these pathways in *Azotobacter* extracts. For example, although in the presence of hydrazine 72 μmoles of 6-PG is split into 72 μmoles of G-3-P in one hour, only 55 μmoles of 6-PG disappear in its absence. From these 55 μmoles of 6-PG, 24 μmoles appear as G-3-P, leaving 31 μmoles of G-3-P to be accounted. Since the rate of conversion of G-3-P to hexose phosphate (G-6-P and F-6-P) by isomerase, aldolase, and fructose diphosphatase is approximately 22 μmoles/h/46 mg extract protein (2 glyceraldehyde-3-phosphate $\rightarrow$ 1 hexose phosphate + 1 phosphate) and the rate of conversion to pyruvate *via* enolase is approximately 5 μmoles/h/46 mg extract protein, these systems combined could account for the disappearance of 27 μmoles of the G-3-P formed from the splitting of 55 μmoles of 6-PG. Therefore, of the 55 μmoles of G-3-P 51 μmoles (24 + 27) could be accounted for by the remaining G-3-P and that converted to pyruvate and hexose phosphate by systems *a* and *b*. It is likely that part of the G-3-P formed recycles through G-6-P to yield again G-3-P and pyruvate, the overall result being the oxidation of one molecule of G-3-P to pyruvate.

SUMMARY

A system for splitting 6-phosphogluconate to glyceraldehyde-3-phosphate and pyruvate at the rate of approximately 72 μmoles/h/46 mg enzyme protein has been demonstrated in extracts of *Azotobacter vinelandii* O. Part of the glyceraldehyde-3-phosphate formed appears to recycle through aldolase, fructose diphosphatase, and phosphohexoisomerase to G-6-P; the rest is converted into pyruvate through triose phosphate dehydrogenase and enolase. The ratio of conversion by these two pathways is limited by the level of triose phosphate dehydrogenase which is unknown in this organism.

The use of simultaneous equations to determine the concentrations of trioses and hexoses in mixtures after reaction with anthrone reagent is proposed. Good confirmation of this method was obtained when it was checked with mixtures of these compounds in known concentrations.

RÉSUMÉ

Un système transformant le 6-phosphogluconate en glycéraldéhyde-3-phosphate et pyruvate, à une vitesse d'environ 72 μmoles/h/46 mg de protéine enzymatique, a été mis en évidence dans des extraits *d'Azotobacter vinelandii* O. Une partie du glycéraldéhyde-3-phosphate formé rentre dans le cycle après transformation en G-6-P en présence d'aldolase, de fructose diphosphatase et de phosphohexoisomérase; le reste est transformé en pyruvate sous l'action d'une triose phosphate déshydrogénase et d'une énolase. L'importance relative de ces deux voies métaboliques est déterminée par la teneur en triose phosphate déshydrogénase, teneur inconnue chez cet organisme.

L'emploi d'équations simultanées pour le dosage des trioses et des hexoses en mélanges, après réaction avec le réactif à l'anthrone, est proposé. La validité de la méthode a été contrôlée à l'aide de mélanges de compositions connues.

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ZUSAMMENFASSUNG

Ein System, das 6-Phosphoglukonat in Glycerinaldehyd-3-Phosphat und Pyruvat mit einer Spaltungsrate von 72 μMol/Std. und 46 mg Enzymprotein umsetzt, wird demonstriert in Extrakten von *Azotobacter vinelandii O*. Ein Teil des gebildeten Glycerinaldehyd-3-Phosphats scheint sich zurückzubilden zu 6-P-G durch Aldolase, Fruktosediphosphatase, und Phosphohexoisomerase. Der Rest wird in Brenztraubensäure verwandelt durch Triosephosphatdehydrogenase und Enolase. Das Verhältnis der Umwandlung auf diesen beiden Wegen ist begrenzt durch den Triosephosphatdehydrogenase-Gehalt, der bei diesem Organismus unbekannt ist.

Es wird die Benutzung von Simultan-Gleichungen vorgeschlagen, um die Konzentrationen von Triose und Hexose in Gemischen nach Reaktion mit Anthron-Reagenz zu bestimmen. Eine gute Bestätigung dieser Methode wurde erhalten, bei ihrer Prüfung durch Mischungen der beiden Verbindungen in bekannten Konzentrationen.

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