PHOSPHORYLATION OF GLUCONATE IN YEAST EXTRACTS*

BY HENRY Z. SABLE AND ARMAND J. GUARINO†

(From the Department of Biochemistry and Nutrition, Tufts College Medical School, Boston, Massachusetts)

(Received for publication, December 26, 1951)

The formation of gluconate by direct oxidation of glucose in extracts of molds (1) and liver (2) has been known for many years, and more recently Stetten and Stetten have demonstrated that gluconate may be utilized for glycogen synthesis in vivo (3). Enzymes which catalyze oxidation of phosphogluconate are present in many animal tissues and microorganisms (4–6). The present study was undertaken to investigate the possibility that gluconate might be phosphorylated as the first step in its utilization.

An enzyme has now been found in extracts of yeast which catalyzes the reaction

\[ \text{Gluconate} + \text{adenosinetriphosphate} \xrightarrow{M^{2+}} 6\text{-phosphogluconate} \]

Some of the properties of this enzyme will be discussed.

While this work was in progress, a similar enzyme was described in extracts of gluconate-adapted Escherichia coli (7), and the name gluconokinase proposed. This name has been adopted in the present work.

EXPERIMENTAL

Materials—Potassium gluconate was purchased from Eimer and Amend Division. Adenosinetriphosphate (ATP), the disodium salt as purchased from the Pabst Brewing Company, was adjusted to pH 6.8 with KOH and stored in the frozen state. Tris(hydroxymethyl)aminomethane (TRIS) was purified as described elsewhere (8). Triphosphopyridine nucleotide (TPN) was prepared from beef liver by the method of Warburg et al. (9). Myokinase1 was obtained from rabbit muscle by the procedure of Colowick and Kalckar (10); hexokinase,1 free of ATPase and myokinase, was prepared from bakers’ yeast by the method of Berger et al. (11). Phosphogluconic dehydrogenase2 was prepared according to

* This report is abstracted from a dissertation to be presented by Armand J. Guarino to the Graduate School of Tufts College in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† Fellow of the Baxter Laboratories and of the Charlton Fund.

1 Kindly supplied by Dr. W. W. Kielley.

2 We are grateful to Dr. Horecker for supplying the details of these methods a year in advance of publication.
Horecker and Smyrniotis (12) and barium-6-phosphogluconate6,8 by oxidation of barium glucose-6-phosphate (13). Special active dried brewers’ yeast, purchased from Anheuser-Busch, Inc., was used in all but one of the experiments reported.

Analytical Methods—Inorganic orthophosphate was determined by King’s modification (14) of the Fiske-Subbarow method (15). “Easily hydrolyzable phosphate” was determined by measuring the increase in inorganic orthophosphate after 10 minutes hydrolysis in n H2SO4 at 100°. Reducing sugar was estimated by the method of Nelson (16) and protein according to Robinson and Hogden (17). Phosphogluconate was determined by the enzymatic procedure of Horecker and Smyrniotis (12). Hexokinase was determined by incubation with glucose, ATP, and Mg++ and measuring the disappearance of reducing substance not precipitated by the Ba(OH)2-ZnSO4 reagent of Nelson (16). Myokinase was determined by adding the material to be tested to the hexokinase test system and measuring the increase in the amount of glucose phosphorylated in the presence of limiting amounts of ATP. One-dimensional paper chromatograms were prepared according to Bandurski and Axelrod (18), and phosphate compounds located by treatment with the perchloric acid-molybdate reagent of Hanes and Isherwood (19). Maceration juice was prepared by incubating the dried yeast with 3 volumes of 0.067 m Na2HPO4 for 4 hours at 38°.

Determination of Enzyme Activity—Evidence for phosphorylation of gluconate was first obtained by measuring the decrease of easily hydrolyzable organic phosphate when dialyzed yeast maceration juice was incubated with ATP, Mg++, and gluconate (Fig. 1, A). Confirmatory evidence was obtained by the following type of experiment. 5 μm of gluconate and 6 μm of ATP were incubated at 30° with 1 ml. of dialyzed maceration juice (44 mg. of protein), 0.01 m TRIS buffer, pH 7.6, and 0.003 m Mg++ in a final volume of 3.5 ml. After 1 hour the reaction mixture was fixed in 0.2 n HCl and neutralized with NaOH, upon which treatment the protein precipitated. The protein-free filtrate was analyzed with phosphogluconic dehydrogenase and 3.42 μm of 6-phosphogluconate were found. In a parallel experiment the conversion of acid-labile to acid-stable phosphate was measured. In 1 hour 44 mg. of protein catalyzed the formation of 4.0 μm of acid-stable phosphate.

Neither of the foregoing methods of assay was completely satisfactory for routine work in following the purification of the enzyme. It was found convenient to assay gluconokinase by measuring the change in pH which accompanies the formation of a new acid group as a result of phos-

---

6 We thank Miss Helen F. Elliott for assistance in this preparation.

8 All incubations were carried out at 30°.
phate transfer (10). This was done with external electrodes of a Beckman pH meter. A typical experiment is shown in Fig. 1, B. Although not an absolute measure of the reaction, pH measurement can be used to give an accurate estimate of the relative activity of different enzyme preparations.

**Purification**—Because of the presence of interfering enzymes, particularly ATPases, in the crude maceration juice it was necessary to purify the enzyme before studying its properties. This was accomplished as follows: All operations were carried out in a cold room and protein solutions kept chilled in an ice bath. To the maceration juice (150 ml.) were added 183.5 ml. of a solution of (NH₄)₂SO₄, pH 7.6, saturated at 25°, to give a saturation of 0.55. After chilling for 20 to 30 minutes more the mixture was centrifuged at high speed on a Servall SS-1 centrifuge. The precipitate was dissolved in 60 ml. of cold water and dialyzed for 18 hours at 2° against large volumes of distilled water. The resulting solution (ammonium sulfate fraction) was adjusted to pH 4.7 by addition of 0.1 N acetic acid, and, after standing for 20 minutes, the precipitate was removed by centrifugation. The clear solution (acid supernatant fraction, volume 86 ml.) was adjusted to 23 per cent ethanol concentration by addition of 27.5 ml. of cold 95 per cent ethanol and centrifuged immediately. The precipitate was discarded and the solution (108 ml.) adjusted to 55 per

---

**Fig. 1.** A, utilization of ATP in the presence and absence of gluconate. The incubation mixtures contained, in a final volume of 2 ml., 9.8 mg. of protein (dialyzed maceration juice), 0.01 M Mg⁺⁺, 0.013 M TRIS buffer, pH 7.6, 6 μM of ATP, and 4 μM of gluconate. Phosphorus was determined in aliquots of the protein-free filtrate. Upper curve, gluconate omitted. B, change in pH during the phosphorylation of gluconate. The incubation mixtures contained, in a final volume of 6 ml., 50 mg. of protein (acid supernatant fraction), 0.02 M Mg⁺⁺, 0.007 M TRIS buffer, pH 7.0, 15 μM of gluconate, and 20 μM of ATP. Upper curve, gluconate omitted.
cent ethanol concentration by addition of 86 ml. of 95 per cent ethanol. The mixture was centrifuged and the precipitate suspended in 30 ml. of water and adjusted to pH 6.8 with 0.1 N NaOH. A considerable amount of undissolved material remained. The mixture was dialyzed with rotation for 4 hours against 2 liters of distilled water at 2° and then frozen overnight. After thawing, the mixture was centrifuged and the precipitate discarded. The clear solution (23 to 55 per cent ethanol fraction), when stored in the frozen state, lost activity slowly over a period of several weeks. In several preparations we achieved 6- to 8-fold purification by this procedure. The final products were free of ATPase, as shown by constancy of pH in the absence of gluconate, but contained a trace of hexokinase and large quantities of myokinase. The rate of the reaction was found to be proportional to the amount of protein added (Fig. 2). The possibility that phosphorylation of gluconate was due to the action of hexokinase was ruled out by the experiment shown in Fig. 3.

**Enzyme Affinity Constants**—When experiments similar to those shown in Fig. 1, B were carried out in the absence of Mg++, there was no change in pH over a period of 1 hour. Upon the addition of the usual amount of Mg++, acid production began immediately in the mixture which contained gluconate. Mn++ at the same concentration was about 50 per cent as effective as Mg++. The optimum magnesium concentration lay between $5 \times 10^{-3}$ and $7 \times 10^{-3}$ M. Higher concentrations were inhibitory.
To determine the affinity of the enzyme for Mg\(^{++}\) and gluconate the rate of acid production at different concentrations of these substances was measured. Calculations were made according to Lineweaver and Burk (20). The Michaelis constants were determined from the data shown in Fig. 4. These were found to be \(K_m = 1.68 \times 10^{-3} \, \text{M}\) for magnesium and \(1.74 \times 10^{-3} \, \text{M}\) for gluconate.

The affinity of the enzyme for ATP could not be determined in the same way because the buffer capacity of the solution varied with different concentrations of ATP. To overcome this difficulty the rate of the reaction was measured by titrating to maintain the pH at 7.2 with 0.01 \(\text{N}\) NaOH. The rate of alkali consumption in such an experiment was an absolute measure of the reaction velocity. The reaction vessels were covered with a rubber membrane pierced by the electrodes, the tip of the burette, and a nitrogen inlet tube. Stirring was accomplished with a stream of nitrogen directed at an angle across the surface of the liquid to keep it in rotary motion.\(^6\) The data obtained by this method are recorded in Fig. 5, and the \(K_m\) for ATP was found to be \(2.0 \times 10^{-4} \, \text{M}\).

The pH optimum was determined by measuring the rate of alkali addition required to maintain constant pH in the assembly described above.

\(^6\) We are grateful to Dr. Harry H. Powers for suggesting this assembly.
A sharp optimum was found at pH 7.2 (Fig. 5). This was confirmed by experiments in which the formation of acid-stable phosphate was measured.

**Product of Reaction**—As has already been indicated, a substance was formed which reacted in the phosphogluconic dehydrogenase system and therefore probably is 6-phosphogluconate. Confirmatory evidence was obtained by paper chromatography. In two chromatograms, in which the formic acid-methanol-water solvent of Bandurski and Axelrod was used, authentic 6-phosphogluconate had an $R_F$ value of 0.32, while the incubated mixtures contained a component with an $R_F$ of 0.30.

**Fig. 5.** Effect of varying ATP concentration and pH on enzyme activity. Velocity is expressed as ml. of 0.01 N NaOH required to maintain a constant pH for 30 minutes. A, in 6 ml. there were 15 $\mu$M of gluconate, 0.005 M Mg++, 4.3 mg. of protein (23 to 55 per cent ethanol fraction, Preparation 7), and pH maintained at 7.20. B, in 6 ml. there were 15 $\mu$M of gluconate, 20 $\mu$M of ATP, 0.007 M Mg++, 35 mg. of protein (23 to 55 per cent ethanol fraction, Preparation 5).

**Enzyme Specificity**—In tests with the crude maceration juice carried out as indicated in Fig. 1, B, we obtained no evidence for the phosphorylation of glycerol, glycolaldehyde, D- or L-arabinose, or D-xylose.

Bakers' yeast was also tested as a source of the enzyme. Anheuser-Busch starch-free bakers' yeast was crumbled and dried at room temperature. Maceration juice was prepared in the usual way and dialyzed for 18 hours at 2$^\circ$ against large volumes of distilled water. A large precipitate which formed on dialysis was removed by centrifugation, and the supernatant fluid tested in an experiment arranged similarly to that shown in Fig. 1, B. The rate of acid production in this experiment was approximately the same as that observed when dialyzed maceration juice prepared from the brewers' yeast was used.
DISCUSSION

The presence of gluconokinase as a constitutive enzyme of yeast may indicate that some free gluconate is formed during the metabolism of hexoses. In any case it is certain that free gluconate can be utilized by the organism. Since the enzyme is present in both bakers' and brewers' yeasts, the possibility of contamination with another microorganism is minimized.

The equivalence, within the limits of experimental error, of the Michaelis constants for gluconate and Mg$$^{++}$$ is evidence in favor of Najjar’s theory (21) of metallosubstrates; i.e., “in determination of $$K_m$$ for either the substrate or the divalent cation, one is actually measuring $$K_m$$ of the metallosubstrate complex.”

As in the case of hexokinase and fructokinase (22) the affinity of the enzyme for ATP was an order of magnitude greater than that for the substrate. This may be an indication of the efficiency with which cells utilize available ATP.

SUMMARY

Extracts of bakers’ and brewers’ yeast contain an enzyme, gluconokinase, which catalyzes a reaction between gluconate and ATP. The product of the reaction is 6-phosphogluconate.

The enzyme has been partially purified and has been found to be activated by Mg$$^{++}$$.

The affinity of the enzyme for Mg$$^{++}$$, gluconate, and ATP has been determined, and the significance of these affinities is discussed.

BIBLIOGRAPHY


---

6 Personal communication from Dr. V. A. Najjar.
PHOSPHORYLATION OF GLUCONATE IN YEAST EXTRACTS
Henry Z. Sable and Armand J. Guarino


Access the most updated version of this article at http://www.jbc.org/content/196/1/395.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/196/1/395.citation.full.html#ref-list-1