Purification and Properties of Galactose 1-Phosphate Uridyl Transferase from *Escherichia coli*

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Galactose 1-phosphate uridyl transferase found in galactose-adapted yeast by Kalckar et al. (1), catalyzes the following reaction: UDP-glucose + galactose-1-P = UDP-galactose + glucose-1-P. A method for partial purification of galactose 1-phosphate uridyl transferase from calf liver and application of this enzyme to the determination of galactose 1-phosphate have been described (2). In the latter work it was indicated that this method can be applied for the determination of galactokinase activity in a two-step analysis involving an incubation for the formation of galactose 1-phosphate from adenosine triphosphate and galactose followed by its determination (24). As the partial purification of galactose 1-phosphate uridyl transferase from adenosine triphosphate and galactose followed by its determination (24).

This paper deals with the partial purification of galactose 1-phosphate uridyl transferase from *Escherichia coli* K12 galactokinase-less mutant and its application in the measurement of galactokinase activity. Some of the properties of the partially purified galactose 1-phosphate uridyl transferase are also presented.

**METHODS AND MATERIALS**

**Chemicals**—Glucose-1-P, glucose-6-P, galactose-1-P, and UDP-glucose were the products of the Sigma Chemical Company. ATP was a product of the Abbott Laboratories. Activated Carbon, Nuchar, Grade XXX was purchased from the Industrial Chemical Sales. UDP-galactose was kindly prepared by Dr. E. Maxwell of this Institute (5). Galactose was a product of Mann Research Laboratories and was recrystallized twice from 70% ethanol. DEAE-cellulose was purchased from the Eastman Kodak Company. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (6). The dry weight of the gel was 21.9 mg per ml. Aluminum Cj gel was prepared according to the method of Wittstätter and Kraut (6). The dry weight of gel was 12.2 mg per ml. Glucose-1,6-diphosphate was kindly supplied by Dr. L. Leloir of the Instituto de Investigaciones Bioquimicas, Buenos Aires through Dr. V. Ginsburg of this institute.

**Enzymes**—UDP-galactose-4-epimerase was kindly prepared by Dr. E. Maxwell of this Institute (5). UDP-glucose dehydrogenase was kindly prepared by Dr. N. Kirkman of this Institute according to the method of Strominger et al. (7). Phosphoglucomutase was prepared according to the method of Najjar (8).

Glucose 6-phosphate dehydrogenase was prepared from brewers' yeast according to the method of Kornberg and Horecker (9). The gel adsorption and acid precipitation steps in this method are repeated twice, in order to remove 6-phosphogluconate dehydrogenase and galactose-1-P uridyl transferase.2

*E. coli* strain—*E. coli* K12 strain W3092 which was isolated by Morse et al. (10) and identified as galactokinase-less (3) was kindly supplied by Drs. E. and J. Lederberg of Stanford University.

Culture of *E. coli* mutant—A loopful of bacteria from a 24-hour culture of *E. coli* K12 W3092 at 37° on a nutrient agar slant (Difco) is used to inoculate 50 ml of medium which contains 10 g of casamino acid (Difco), 5 g of yeast extract (Difco), 3 g of KH2PO4, 1 g of KH2PO4, 5 g of D-glucose, and 1 g of D-glucose per liter (11). After incubation at 37° for 8 to 10 hours with aeration by rotatory shaking, the entire culture is transferred into 1 liter of fresh culture medium of the same composition as described above and incubation is continued for 10 hours at 37° with rotatory shaking.

Preparation of Cell-Free Extract—The cells are harvested by centrifuging in a Lourdes model AT centrifuge with volume rotor at 6,000 x g for 10 minutes and the cells were washed with 200 ml of cold water. Ordinarily 1 liter of culture yields about 4 to 5 g of packed cells. For each gram of packed cells 4 ml of potassium phosphate buffer (pH 7.4, 0.05 M) are added, and the cells are disintegrated in a sonic disintegrator (Raytheon 10 kc) for 10 minutes.

The broken cell suspension is centrifuged in a Servall model SS-1 centrifuge at 20,000 x g for 10 minutes. The supernatant solution is used as starting material for purification. The protein concentration of this crude extract, determined by the biuret method (12), ranges between 23 and 28 mg per ml, unless the disintegration of cells is unusually inefficient.

**RESULTS AND DISCUSSION**

**Purification of Galactose 1-P Uridyl Transferase**—The crude extract is adjusted to contain 20 mg per ml of protein and 100 nmoles of potassium phosphate per ml by addition of enough water and 0.5 M potassium phosphate buffer (pH 7.4 when diluted to 0.05 M). Ordinarily 60 ml of the above solution were worked up at one time.

3 Dr. N. Kirkman of this Institute has developed a method of purification of glucose 6-phosphate dehydrogenase from human red blood cells, which is free of 6-phosphogluconate dehydrogenase and galactose-1-P uridyl transferase (personal communication).
The diluted solution is incubated at 37° for 2 hours to enhance the breakdown of nucleic acids (this fraction can be stored at -10° for several days without much loss of activity). After cooling it is placed in a hot water bath at 65 to 70°, and the temperature is brought to 53° rapidly (1.5 to 2 minutes). The solution is maintained at 53° for 11 minutes and then chilled in an ice bath. After this step, all the procedures are carried out at 2°.

The heat-denatured solution is fractionated by the addition of (NH₄)₂SO₄ solution (pH 7.0) saturated at 2° which contains 0.006 M Versene. An active fraction is obtained between 0.45 and 0.67 saturation of (NH₄)₂SO₄. After each addition of (NH₄)₂SO₄ solution, the mixture is stirred for 10 minutes in an ice bath before centrifugation in a Servall model SS-1 centrifuge at 20,000 × g for 5 minutes. After collecting the precipitate between 0.45 and 0.67 saturation with (NH₄)₂SO₄, the supernatant solution is drained off completely by inverting the centrifuge tubes for 5 minutes, because excessive (NH₄)₂SO₄ interferes with the effectiveness of Ca₃(PO₄)₂ gel in the following step. The precipitate is dissolved in 10 ml of water, and the protein concentration is determined by the biuret method (12).

The solution is adjusted to contain 7.5 mg of protein and 50 μmoles of potassium phosphate per ml by the addition of enough water and potassium phosphate buffer (0.5 M, pH 6.5 when diluted to 0.05 M). Then calcium phosphate gel (1 mg dry weight per mg protein) is added. After stirring for 10 minutes in an ice bath, the gel is removed by centrifugation at 20,000 × g for 5 minutes. The enzymic activity remains in the supernatant solution.

After determining the protein concentration spectrophotometrically (13), the supernatant solution is treated with Nuchar, 1 mg per 1 mg of protein, and stirred for 4 minutes with a stirring rod. Then another equivalent amount of Nuchar is added and stirred for another 4 minutes. The charcoal is removed by centrifugation at 20,000 × g for 5 minutes. This treatment removes the residual nucleic acid and the ratio of the absorption of the enzyme solution at 280 μm to the absorption at 260 μm reaches 1.75. Aluminum C₇ gel is then added to the supernatant solution, 0.3 mg dry weight per mg protein. After stirring for 10 minutes the gel is removed by centrifugation at 20,000 × g for 5 minutes.

The supernatant solution is refractionated with (NH₄)₂SO₄ and the precipitate between 0.5 and 0.6 saturation is collected. The solution is adjusted to contain 7.5 mg of protein and 50 μmoles of potassium phosphate per ml by the addition of enough water and potassium phosphate buffer (pH 7.4) which contains 0.05 M NaCl. Then the active enzyme fractions are eluted from the column by 0.005 M potassium phosphate buffer (pH 7.4) which contains 0.12 M NaCl. Two milliliter fractions are collected. Ordinarily the bulk of the enzymic activity is recovered in fractions 3 to 7. The most active fractions (the 3rd and 4th) are combined and dialyzed for 1 hour against 0.005 M potassium phosphate buffer (pH 7.4) at 2°. The dialyzed fraction is then lyophilized after addition of 1/2 volume of reduced glutathione (15 mg per ml) and 4 ml of 0.25 M glycylglycine buffer (pH 7.4). The amount of enzyme protein in this lyophilized powder was determined by the method of Lowry et al. (16) after precipitating the enzyme protein by trichloro-acetic acid. The lyophilized powder can be stored at -10° in a desiccator for several months. An enzyme solution is prepared by dissolving the powder in 1% bovine albumin solution. This solution can be stored at -10° for 1 week with gradual loss of activity. The over-all purification achieved is 60- to 70-fold above the crude extract with an over-all yield of 15 to 20%.

Table I summarizes the results of the purification of galactose-1-P uridyl transferase achieved by the above described method. All of the following studies of properties of the enzyme were carried out with this partially purified preparation.

The assay of galactose-1-P uridyl transferase was carried out by the method described in the following section. One unit of activity is defined as that amount of enzyme which causes the incorporation of 1 μmole of galactose-1-P into the uridine nucleotide per minute.

The partially purified galactose-1-P uridyl transferase is free of phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, and UDP-galactose-4-epimerase. Galactokinase is missing in this strain of E. coli.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Purification of galactose-1-P uridyl transferase</th>
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<tbody>
<tr>
<td>Fractions</td>
<td>Total volume</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>54.3</td>
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<tr>
<td>2. Incubation at 37°</td>
<td>60.0</td>
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<tr>
<td>3. (NH₄)₂SO₄, 0.45 to 0.67 saturation</td>
<td>10.6</td>
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<tr>
<td>4. Ca₃(PO₄)₂ gel supernatant solution</td>
<td>37</td>
</tr>
<tr>
<td>5. Nuchar supernatant solution</td>
<td>33</td>
</tr>
<tr>
<td>6. Aluminum C₇ gel supernatant solution</td>
<td>33</td>
</tr>
<tr>
<td>7. (NH₄)₂SO₄, 0.5 to 0.6 saturation</td>
<td>4.4</td>
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<tr>
<td>8. DEAE chromatography</td>
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<tr>
<td>Fraction 3</td>
<td>2.0</td>
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<tr>
<td>Fraction 4</td>
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<td>Fraction 5</td>
<td>2.0</td>
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<tr>
<td>Fraction 6</td>
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</table>

* See the text.

# Notes

1. Personal communication from Dr. F. Borg at Stanford University, California.

2. Which contains 0.05 M NaCl. Then the active enzyme fractions are eluted from the column by 0.005 M potassium phosphate buffer (pH 7.4) which contains 0.12 M NaCl. Two milliliter fractions are collected. Ordinarily the bulk of the enzymic activity is recovered in fractions 3 to 7. The most active fractions (the 3rd and 4th) are combined and dialyzed for 1 hour against 0.005 M potassium phosphate buffer (pH 7.4) at 2°. The dialyzed fraction is then lyophilized after addition of ½ volume of reduced glutathione (15 mg per ml) and 4 ml of 0.25 M glycylglycine buffer (pH 7.4). The amount of enzyme protein in this lyophilized powder was determined by the method of Lowry et al. (16) after precipitating the enzyme protein by trichloro-acetic acid. The lyophilized powder can be stored at -10° in a desiccator for several months. An enzyme solution is prepared by dissolving the powder in 1% bovine albumin solution. This solution can be stored at -10° for 1 week with gradual loss of activity. The over-all purification achieved is 60- to 70-fold above the crude extract with an over-all yield of 15 to 20%.

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The partially purified galactose-1-P uridyl transferase is free of phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, and UDP-galactose-4-epimerase. Galactokinase is missing in this strain of E. coli.

Assay of Galactose-1-P Uridyl Transferase—Galactose-1-P uridyl transferase activity is measured by 14NH₂ formation in a coupled reaction with phosphoglucomutase and glucose-6-P dehydrogenase. The reaction mixture consists of 60 μmoles of glycine buffer (pH 8.75), 4.8 μmoles of cysteine hydrochloride (pH adjusted to 8.5 before use), 5 μmoles of MgCl₂, 0.4 μmole of TPN,
Fig. 1. Dependence of the reaction upon enzyme, galactose-1-P, and UDP-glucose. Assay conditions are described in the text except the additions as follows: At Arrow 1, 0.35 µmole of galactose-1-P, to Experiments A and B, and 0.15 µg of enzyme protein to Experiment C. At Arrow 2, 0.15 µg of enzyme protein, 0.25 pmole of UDP-glucose and 0.35 pmole of galactose-1-P to Experiments A, B, and C, respectively.

Fig. 2. Relation of reaction rate to enzyme concentration. The assay conditions are the same as those described in the text except for the amount of enzymes, as indicated.

0.25 µmole of UDP-glucose, 0.35 µmole of galactose-1-P, 0.07 unit4 of glucose-6-P dehydrogenase, 0.7 unit4 of phosphoglucomutase together with 2 × 10^-5 µmole of glucose-1,6-diphosphate

4 One unit of glucose-6-P dehydrogenase is defined as that amount of enzyme which causes the reduction of 1 µmole of TPN per minute under the assay conditions used for galactose-1-P uridyl transferase except that 0.4 µmole of glucose-6-P is used as the substrate. One unit of phosphoglucomutase is similarly defined as the amount of enzyme which causes the reduction of 1 µmole of TPN per minute when 0.4 µmole of glucose-1-P (free of glucose 1,6-diphosphate) is used as the substrate.

4 Some of the phosphoglucomutase preparations contain enough glucose 1,6 diphosphate, especially when freshly prepared. However, since galactose-1-P was found to be inhibitory for the phosphoglucomutase reaction (17, 18), glucose 1,6-diphosphate and phosphoglucomutase are used routinely in excess. It is also necessary to avoid prolonged contact of phosphoglucomutase with galactose-1-P before starting the galactose-1-P uridyl transferase assay.

and an appropriate amount of galactose-1-P uridyl transferase. The total volume is made up to 0.62 ml. The galactose-1-P uridyl transferase fraction for assay is diluted with 1% bovine albumin to give an optical density change at 340 mµ between 0.03 and 0.05 per minute under the assay condition. The optical density change at 340 mµ due to the reduction of TPN is measured in a Beckman DU spectrophotometer with a pinhole attachment.

Fig. 1 shows the dependence of the reaction upon galactose-1-P, UDP-glucose, and galactose-1-P uridyl transferase. At the beginning of the reaction there is always a short lag period, and then the reaction proceeds linearly with time when the reaction is started by the addition of enzyme or UDP-glucose. Therefore, the linear part of the curve is utilized for the calculation of the rate of reaction. The small initial rise of optical density after addition of galactose-1-P may be due to the contamination of galactose-1-P by small amounts of glucose-1-P.

Fig. 2 shows the relation between the reaction rate and enzyme protein concentration. The linear relation persists up to 0.48 µg of galactose-1-P uridyl transferase protein, then it levels off possibly due to the limiting activity of the phosphoglucomutase.

Fig. 3 shows the relation between the enzyme activity and the concentration of UDP-glucose and galactose-1-P. The Kₐ values for UDP-glucose and galactose-1-P under the assay conditions are estimated to be 1.5 × 10^-4, and 4.1 × 10^-4, respectively, from the Lineweaver and Burk (19) plot on the right half of the figure. The assay conditions are the same as described above except for the galactose-1-P and UDP-glucose concentrations indicated. For the determination of the Kₐ values for UDP-glucose and for galactose-1-P, 0.4 µmole of galactose-1-P and 0.4 µmole of UDP-glucose are present in 0.62 ml of reaction mixture, respectively.

Alternative Method of Enzyme Assay—For the purpose of studying some of the properties of this enzyme it is necessary to modify the assay method described in the previous section. One of the methods utilized involves incubation of galactose-1-P uridyl transferase with UDP-glucose and galactose-1-P and analysis for glucose-1-P formed during the incubation period by a coupled reaction with phosphoglucomutase and glucose-6-P dehydrogenase.
The incubation mixture consists of 20 μmoles of glycine buffer (pH 8.7), 2 μmoles of MgCl₂, 1.6 μmoles of cysteine HCl (pH adjusted to 8.5 before use), 0.13 μmole of UDP-glucose, 0.21 μmole of galactose-1-P, and an appropriate amount of galactose-1-P uridyl transferase (0.004 to 0.006 unit under the standard assay condition described in the previous section). The total volume is made up to 0.20 ml. After incubation at 30°, the reaction is stopped by immersing the tube in a boiling water bath for 1.5 minutes. After centrifugation, 100 μl aliquots are analyzed for glucose-1-P by phosphoglucomutase and glucose-6-P dehydrogenase in a reaction mixture which is essentially similar to the one described in the previous section except for the omission of UDP-glucose, galactose-1-P, and galactose-1-P uridyl transferase. The amount of glucose-1-P formed during the incubation is corrected by the amount in the blank, found in a reaction which was stopped at zero time.

Fig. 4 shows the amount of glucose-1-P formed plotted against the time of preincubation. The reaction proceeds linearly up to 8 minutes. The results of a study of the stoichiometry of the reaction in an experiment similar to that of Fig. 4 are presented in Table II. The amount of UDP-glucose remaining at the end of the reaction is determined by UDP-glucose dehydrogenase (7). The amount of galactose-1-P remaining is determined by the standard method, using galactose-1-P uridyl transferase in the presence of excess UDP-glucose and TPN. The amount of UDP-galactose formed is determined by a coupled reaction of UDP-galactose-4-epimerase and UDP-glucose dehydrogenase (5). The equilibrium constant

$K = \frac{[UDP-galactose][Glucose-1-P]}{[UDP-glucose][Galactose-1-P]}$

was calculated from the data in Table II to be 1.1.

By the use of this modified method studies of some of the properties of galactose-1-P uridyl transferase were made. Table III summarizes the effect of magnesium ions, cysteine, and sodium fluoride. It is observed that the enzyme shows an absolute requirement of cysteine. Mg²⁺ ions are rather inhibitory. NaF shows a slight inhibition.

Fig. 5 shows the relation between pH and the rate of the reaction. The pH values shown are the final pH values of the reaction mixture determined by a Beckman pH meter with a one-drop electrode. The pH of the reaction mixture was adjusted with 20 μmoles of glycine buffer between pH 7 and 10. Galactose-1-P uridyl transferase has a pH optimum between 8.5 and 8.9.

$K_n$ of Galactose-1-P Uridyl Transferase for UDP-Galactose and Glucose-1-P—The $K_n$ values for UDP-galactose and glucose-1-P are estimated by determining the rate of formation of UDP-glucose in an incubation mixture of UDP-galactose, glucose-1-P, and galactose-1-P uridyl transferase by UDP-glucose dehydrogenase in the presence of DPN. This method is only applicable for the determination of galactose-1-P uridyl transferase activity when it is completely free of UDP-galactose-4-epimerase.

The reaction mixture consists of 10 μmoles of glycine buffer (pH 8.7), 0.044 μmole of UDP-galactose, 0.1 μmole of glucose-1-P, 1 μmole of MgCl₂, 0.8 μmole of cysteine hydrochloride (pH adjusted to 8.5), and 0.003 to 0.006 unit of galactose-1-P uridyl transferase. The total volume is made up to 0.10 ml. The reaction is started by the addition of galactose-1-P uridyl transferase. After 4 minutes incubation at 30°, the reaction is stopped.
Application of Galactose-1-P Uridyl Transferase for Determination of Galactokinase Activity—Since galactose-1-P uridyl transferase purified from E. coli K12 strain W3092 is free of galactokinase, it can be applied for the determination of galactokinase activity in a coupled reaction with phosphoglucomutase and glucose-6-P dehydrogenase. The reaction mixture consists of 30 μmoles of potassium phosphate buffer (pH 7.4), 4.8 μmoles of cysteine hydrochloride (pH adjusted to 7.4), 10 μmoles of MgCl₂, 0.4 μmole of TPN, 0.3 μmole of UDP-glucose, 1 μmole of galactose, 1 μmole of ATP, 0.17 unit of glucose-6-P dehydrogenase, 0.2 unit of phosphoglucomutase together with 1 × 10⁻³ μmole of glucose 1,6-diphosphate, 0.1 unit of galactose-1-P uridyl transferase, and an appropriate amount of a dialyzed galactokinase fraction. The total volume is made up to 0.62 ml with water. The reaction is started by the addition of the galactokinase fraction. The optical density change at 340 μm due to the reduction of TPN, is followed in the Beckman DU spectrophotometer. The galactokinase fraction used in this study is a dialyzed crude extract of E. coli K12 strain W3092 (10) (galactose-1-P uridyl transferase-less (3)) which was kindly supplied by Drs. E. and J. Lederberg of Stanford University.

The cells are cultured under conditions similar to those described earlier for E. coli W3092 for the preparation of galactose-1-P uridyl transferase. Two grams of harvested cells are disintegrated at 0⁰ in a mechanical disintegrator described by Nossal (20) with 8 ml of potassium phosphate buffer (pH 7.4, 0.04 M) and 8 g of glass beads (Minnesota Mining Company Superbrite glass beads type No. 150, 75 μ in diameter). The disintegrated cells are centrifuged at 17,000 × g for 10 minutes. The cell-free extract is dialyzed against 6 liters of potassium phosphate buffer (pH 7.4, 0.005 M) for 3 hours. The protein concentration of this extract is 28 mg per ml.

Fig. 7 shows the dependence of the reaction on galactose, ATP, galactokinase, and UDP-glucose. It is observed there is a lag period at the beginning of the reaction. The rate of the galactokinase reaction is measured at the linear portion of the curve. The rate of reaction in Experiment C in which 5 μl of E. coli extract was used is greater than the rate in Experiment D in which 7 μl of E. coli extract was used because of the accumulation of galactose-1-P in the former before the addition of UDP-glucose. The rate of reaction in Experiment C is 28 mg per ml.

A method of purification of galactose 1-phosphate uridyl transferase from an Escherichia coli galactokinase-less mutant was described. An overall purification of 60- to 80-fold above the crude extract was achieved. With the use of the partially purified enzyme preparation, some of its properties were studied. Kₐ values for uridine diposphogluconate, galactose 1-phosphate, uridine diphosphoglactose, and glucose 1-phosphate were estimated to be 1.5 × 10⁻⁴, 4.1 × 10⁻⁴, 2.5 × 10⁻⁴, and 2.4 × 10⁻⁴, respectively. In the direction of uridine diposphoglactose formation the equilibria were determined.

SUMMARY

A method of purification of galactose 1-phosphate uridyl transferase from an Escherichia coli galactokinase-less mutant was described. An overall purification of 60- to 80-fold above the crude extract was achieved. With the use of the partially purified enzyme preparation, some of its properties were studied. Kₐ values for uridine diposphogluconate, galactose 1-phosphate, uridine diphosphoglactose, and glucose 1-phosphate were estimated to be 1.5 × 10⁻⁴, 4.1 × 10⁻⁴, 2.5 × 10⁻⁴, and 2.4 × 10⁻⁴, respectively. In the direction of uridine diposphoglactose formation the equilibria were determined.

One unit of enzyme activity here is defined as that amount of enzyme which oxidizes 1 μmole of UDP-glucose per minute under the assay conditions.
K. Kurahashi and A. Sugimura

1.5
1.0
0.5
0

TIME (MINUTES)

FIG. 7. Dependence of galactokinase assay on galactose, ATP, galactokinase, and UDP-glucose. The reaction mixture of Experiment A consisted of 30 µmoles of potassium phosphate buffer (pH 7.4), 10 µmoles of MgCl₂, 0.4 µmole of TPN, 0.3 µmole of UDP-glucose, 1 µmole of galactose, 1 µmole of ATP, 4.8 µmoles of cysteine HCl (pH adjusted to 7.4), 0.07 unit of glucose-6-P dehydrogenase, 0.2 unit of phosphoglucomutase together with 1 X 10⁻² µmole of glucose 1,6-diphosphate, 0.1 unit of galactose-1-P uridyl transferase, and water to make up the total volume to 0.60 ml. At Arrow 1, 10 µl of E. coli extract (galactokinase) were added. The reaction mixture of Experiment B consisted of the same components as Experiment A except for the omission of galactose. At Arrow 2, 10 µl of E. coli extract were added. At Arrow 3, 1 µmole of galactose was added. The reaction mixture of Experiment C consisted of the same components as Experiment A except for the omission of UDP-glucose. At Arrow 4, 5 µl of E. coli extract were added. At Arrow 6, 0.5 µmole of UDP-glucose was added. The reaction mixture of Experiment D consisted of the same components as Experiment A except for the omission of ATP. At Arrow 1, 7 µl of E. coli extract were added. At Arrow 8, 1 µmole of ATP was added.

REFERENCES


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