Ureidoglycolate Synthetase of *Streptococcus allantoicus*

I. Measurement of Glyoxylate and Enzyme Purification

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Received for publication 26 June 1965

ABSTRACT

GAUDY, ELIZABETH T. (University of Illinois, Urbana), R. BOJANOWSKI, R. C. VALENTINE, AND R. S. WOLFE. Ureidoglycolate synthetase of *Streptococcus allantoicus*. I. Measurement of glyoxylate and enzyme purification. *J. Bacteriol.* **90**:1525-1530. 1965.—A new spectrophotometric method for the determination of glyoxylate is described. The technique is based on measurement of the initial rate of formation of glyoxylic acid phenylhydrazone in neutral solution. Its advantages include rapidity and convenience, suitability for use with mixtures containing acid-labile substrates, and elimination of possibly inhibitory reagents from the enzyme incubation mixture. Ureidoglycolate synthetase, which cleaves ureidoglycolate to glyoxylate and urea, was purified from crude extracts of *Streptococcus allantoicus* grown on allantoin-containing medium. The purification procedures include treatment with $MnCl_2$, fractionation on calcium phosphate gel, fractional precipitation with ammonium sulfate, and column chromatography on diethylaminoethyl cellulose. The final enzyme preparation was purified 77-fold and contained 35% of the total activity of the extract.

In previous communications, we have proposed a scheme for the fermentation of allantoin by *Streptococcus allantoicus* (Valentine et al., 1962; Valentine and Wolfe, 1961; Gaudy et al., 1962). The conversion of allantoic acid to glyoxylate and urea in other organisms has been attributed to the action of a single enzyme, allantoicase (Laskowski, 1951; DiCarlo, Schultz, and Kent, 1953; Campbell, 1954). Present evidence indicates that in *S. allantoicus* at least two enzymes are involved in this cleavage; allantoic acid is hydrolyzed to urea and ureidoglycolate, the latter being cleaved to glyoxylate and urea by the enzyme ureidoglycolate synthetase. [In this communication the name, ureidoglycolate, is used in place of the former name, glyoxylurea, and ureidoglycolate synthetase is used in place of glyoxylurease (see Discussion).]

The method used previously (Valentine et al., 1962; Valentine and Wolfe, 1961) for deter-

mination of the amount of glyoxylate formed in the reaction mixture involved the exposure of the sample containing excess, acid-labile ureidoglycolate to acid conditions. This communication presents a new, rapid spectrophotometric method for measurement of glyoxylate in neutral solution and describes the purification of ureidoglycolate synthetase from allantoin-grown cells of *S. allantoicus*. The preparation of sodium ureidoglycolate has been described (Valentine and Wolfe, 1961). The compound used as substrate in the present studies was prepared by an improved synthetic procedure, details of which will be reported elsewhere.

MATERIALS AND METHODS

Preparation of calcium phosphate and diethylaminoethyl (DEAE) cellulose. Calcium phosphate gel, for batch adsorption of protein, was prepared by the method of Swingle and Tiselius (1951). The dry weight of the gel preparation was 51 mg/ml. DEAE cellulose was prepared as described by Peterson and Sober (1962) and was packed under pressure to form a column (9 by 61 mm), which was washed with 1 liter of distilled water at 4 C before use. The exchange capacity of the DEAE cellulose used was 0.87 meq per g.

Protein The biuret-phenol method of Sutherland et al. (1949) was used for determination of the protein content of crude and purified enzyme

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preparations, with bovine serum albumin (Armour and Co., Chicago, Ill.) as standard. For fractions in which high phosphate buffer content interfered with the biuret-phenol method, the protein content was determined by the method of Warburg and Christian (1942). Results of the two methods agreed closely when applied to purified fractions of ureidoglycolate synthetase.

Preparation of cell-free extract. Cells of *S. al-lantoicus* were grown as described by Bojanowski et al. (1964) and were harvested after 18 hr by use of a cooled Sharples centrifuge. The cell paste was frozen at -20°C , and the cells were disrupted in a Hughes press. The crushed cells from 20 liters of medium were suspended in 85 ml of 0.05% sodium mercaptoacetate. Cells and cell debris were removed by centrifugation for 30 min at $30,000 \times g$ in a Servall RC-2 centrifuge at 5°C .

Enzyme assay. One unit of ureidoglycolate synthetase activity is defined as the amount of enzyme required for the formation of 1 μmole of glyoxylate per min. The standard incubation mixture contained 2 to 5 mg (12.8 to 32 μmoles) of sodium ureidoglycolate, 40 μmoles of sodium bar-bital buffer at pH 8.4, a sufficient amount of enzyme to catalyze the formation of 0.5 to 1.0 μmole of glyoxylate in 10 min, and distilled water to a total volume of 1.0 ml. Tubes containing no enzyme or heated enzyme were used as controls. The reaction was initiated by the addition of substrate. After exactly 10 min of incubation in a water bath at 30°C , a 0.1-ml sample was removed for determination of the amount of glyoxylate formed.

Measurement of glyoxylate. A solution of phenylhydrazine-HCl (certified reagent; Fisher Scientific Co., Pittsburgh, Pa.), 10 mg per ml, was freshly prepared and stored in an ice bath. A 1.0 M solution of K_2HPO_4 was prepared and its concentration was adjusted, if necessary, so that 0.4 ml exactly neutralized 2.0 ml of the phenylhydrazine solution. The enzyme-substrate mixture was prepared as described above and was incubated for 10 min at 30°C . Immediately before the end of the incubation period, 2.0 ml of the phenylhydrazine solution were pipetted into a test tube containing 0.4 ml of 1.0 M K_2HPO_4 and 1.5 ml of distilled water. At the end of the incubation period, 0.1 ml of the incubation mixture was added rapidly to the prepared tube containing neutralized phenylhydrazine. The contents of the tube were mixed and poured immediately into a cuvette, and the change in optical density at $322.5 \text{ m}\mu$ was recorded as a function of time, by use of a Cary 14 recording spectrophotometer. The recording was continued until the tracing became obviously nonlinear (usually 1 to 3 min).

For measurements with standard sodium glyoxylate solutions or when both enzymatic and nonenzymatic cleavage rates of ureidoglycolate were to be determined, a blank cuvette containing 2.0 ml of phenylhydrazine solution, 0.4 ml of 1.0 M K_2HPO_4 , and 1.6 ml of water was used. By use of this blank, the enzymatic cleavage of ureido-

glycolate was calculated from the difference in rates of phenylhydrazone formation obtained in a cuvette which received a 0.1-ml sample from a reaction mixture without enzyme and one which received a 0.1-ml sample from the complete reaction mixture. Alternatively, the control and experimental tubes could be incubated and sampled simultaneously, with 0.1 ml from the control tube added to the phenylhydrazine mixture for the reference cuvette. By this procedure, the enzymatically formed glyoxylate was determined directly, with the measured reaction rate being proportional to the difference in glyoxylate concentrations in the two samples.

RESULTS

Measurement of glyoxylate. Figure 1 presents the difference spectrum for the glyoxylic acid phenylhydrazone formed by the described procedure and a solution of neutral phenylhydrazine (reagent blank). The maximal absorption is at $322.5 \text{ m}\mu$.

The rate of phenylhydrazone formation was constant for approximately 1.5 min after mixing and was proportional to the amount of glyoxylate present. The linear relationship between the initial rate of formation of glyoxylic acid phenylhydrazone and amounts of glyoxylate over a wide range of concentrations is shown in Fig. 2. The range of variation from the mean (rate of

FIG. 1. Spectrum of glyoxylic acid phenylhydrazone. The reaction mixture contained 0.2 μmole of sodium glyoxylate (Sigma Chemical Co., St. Louis, Mo.), 10 mg of phenylhydrazine-HCl, 200 μmoles of K_2HPO_4 , and water to a total volume of 4.0 ml. The pH of the reaction mixture was 7.0. The reference cuvette contained all components except glyoxylate. Values are plotted from a Cary spectrophotometer recording.

FIG. 2. Initial rate of phenylhydrazone formation as a function of glyoxylate concentration. A 2.0-ml amount of a solution of phenylhydrazine-HCl, 10 mg/ml, was added to a tube containing 0.4 ml of 1.0 M K_2HPO_4 and sufficient water to bring the final volume after addition of the sample to 4.0 ml. Glyoxylate solution was added, the tube contents were mixed rapidly and poured into a cuvette, and the change in optical density at 322.5 m μ was recorded as a function of time by use of a Cary spectrophotometer. The reference cuvette received a freshly prepared blank containing all reagents except glyoxylate.

change of optical density per minute per micromole) in one series of measurements was -3 to $+2\%$ and in another, -5 to $+6\%$. For the latter series, the mean rate per micromole was 2.47 and the standard deviation was 0.07.

Recovery of glyoxylate in the presence of a large excess of ureidoglycolate is shown in Table 1.

Purification of ureidoglycolate synthetase. Crude extract (fraction A), prepared as described above, was kept at 0 C in an ice bath, and 0.05 volume of 1.0 M $MnCl_2$ was added at a rate of one drop per minute with stirring. After continuous, slow stirring for an additional 15 min, the precipitate was removed by centrifugation at 5 C. The supernatant solution was stored at -20 C for 8 to 10 hr and was then gradually warmed to 10 C. The precipitate formed during the freezing and thawing procedure was removed by centrifugation at 5 C, and the supernatant solution was dialyzed against 3 liters of 0.05% mercaptoacetate for 8 to 10 hr at 4 C. The con-

tents of the flask were replaced with fresh solution after 4 hr.

Calcium phosphate gel treatment. The dialyzed extract (fraction B) was stirred slowly in an ice bath, and 0.02 volume of calcium phosphate gel was added dropwise. Stirring was continued for 15 min, and the gel was removed by centrifugation and discarded. A second treatment with 0.08 volume of gel was performed in the same manner, except that the second portion of gel was resuspended with slow stirring in 10 volumes of ice-cold 0.01 M potassium phosphate buffer at pH 7.25, in an ice bath. Stirring was continued for 1 hr, and the gel was removed by centrifugation and discarded.

Ammonium sulfate fractionation. The gel eluate (fraction C) was stirred slowly in an ice bath, and 0.312 g of solid ammonium sulfate was added per ml of extract (50% saturation). Stirring was continued for 15 min, and the precipitate was collected by centrifugation and dissolved in ice-cold 0.01 M potassium phosphate buffer at pH 7.25 (fraction D-1). The supernatant solution was brought to 55% saturation by addition of 0.032 g of solid ammonium sulfate per ml, and the precipitate was collected in a similar manner (fraction D-2). A third fraction (D-3) was obtained by adding 0.033 g of solid ammonium sulfate per ml (55 to 60% saturation). The three fractions were dialyzed against 3 liters of 0.05%

TABLE 1. Recovery of glyoxylate measured in the presence of sodium ureidoglycolate*

Glyoxylate	Ureidoglycolate	Change in optical density per min	Per cent recovery
μ moles	μ moles		
0.06	—	0.168	
0.06	6.4	0.166	99
0.06	12.8	0.167	99
0.10	—	0.274	
0.10	12.8	0.277	101
0.16	—	0.439	
0.16	12.8	0.476	108

* For measurement of glyoxylate alone, a tube containing the indicated amount of glyoxylate, 10 μ moles of potassium phosphate buffer at pH 7.0, and water to a total volume of 1.0 ml was incubated for 10 min at 30 C. A 0.1-ml sample was used to determine rate of phenylhydrazone formation. For measurement of glyoxylate in the presence of ureidoglycolate, a control tube containing ureidoglycolate and an experimental tube containing both glyoxylate and ureidoglycolate were incubated simultaneously. Samples of 0.1 ml were used in the reference and sample cuvettes, respectively, to determine net rate of phenylhydrazone formation.

TABLE 2. Purification of ureidoglycolate synthetase*

Fraction	Vol	Units/ml	Total units	Per cent recovery	Specific activity	Protein	Total protein	Per cent recovery
	ml					mg/ml	mg	
A. Crude extract.....	640	10.3	6,600	100	0.64	16.1	10,300	100
B. Mn-treated.....	595	19.3	10,480	174	4.8	4.0	2,380	23
C. Calcium phosphate...	475	19.4	9,210	139	14.9	1.3	617	6
D. Ammonium sulfate...	57	121	6,890	104	36.7	3.3	188	2
E. DEAE.....	428	8.6	3,680	56	86	0.1	43	0.4

* One unit of ureidoglycolate synthetase activity is the amount of enzyme required for the formation of 1 μ mole of glyoxylate per minute. Specific activity is expressed as units per milligram of protein. The purification procedures are explained in detail in the text. Each enzyme fraction was assayed at three or more concentrations.

mercaptoacetate at 4 C for 6 hr. Fraction D-1 contained 27% of the total protein of fraction C and only 7% of the ureidoglycolate synthetase activity, and was discarded. Fractions D-2 and D-3 contained approximately equal amounts of ureidoglycolate synthetase activity and were combined as fraction D.

Chromatography on DEAE cellulose. A 5-ml amount of fraction D, containing 605 units of ureidoglycolate synthetase activity and 16.5 mg of protein, was applied to a column of DEAE cellulose. The protein was eluted from the column as indicated in Fig. 3, with a linear gradient of potassium phosphate buffer (total volume, 200 ml). Tubes 23 to 28 were combined as fraction E. This fraction contained 23% of the protein applied to the column and 54% of the ureidoglycolate synthetase activity.

A summary of the complete purification procedure is given in Table 2. The final enzyme preparation contained 100 μ g of protein per ml and had a specific activity 135 times that measured in the crude extract. It contained 0.4% of the total protein of the crude extract; it possessed no carboligase and no activity for allantoin acid.

DISCUSSION

The exact degree of purification obtained in these studies is difficult to assess. The procedures employed for purification of ureidoglycolate synthetase resulted in a final enzyme preparation with a specific activity 135 times that measured in the crude extract. However, the measurement of ureidoglycolate synthetase activity in crude preparations is complicated by the presence of other enzymes which affect the apparent rate of glyoxylate formation or compete for substrate with ureidoglycolate synthetase. According to the present knowledge of allantoin fermentation in *S. allantoinicus*, ureidoglycolate may be acted upon by two enzymes, a dehydrogenase, which carries out the initial step in an energy-yielding reaction sequence, and ureidoglycolate synthetase, which recently has been shown to be the catalyst for the initial reaction in a pathway leading to biosynthesis through tartronic semialdehyde (Valentine, Drucker, and Wolfe, 1964). The pertinent reactions from the proposed pathway (Valentine et al., 1962) are shown below.

FIG. 3. Elution of ureidoglycolate synthetase from DEAE cellulose. Protein was eluted with a linear gradient of 0.0 to 0.2 *M* potassium phosphate buffer (pH 7.0) at a flow rate of 40 ml per hr. Each tube contained 6.25 ml.

The apparent activity of ureidoglycolate synthetase, as measured by rate of formation of glyoxylate, is thus dependent upon the rate of removal of glyoxylate by the action of the glyoxylic acid carboligase, and it also may be affected by high levels of the dehydrogenase. In preliminary studies, it was found that storage for 2 months at -20°C in 0.05% mercaptoacetate inactivated the glyoxylic acid carboligase almost completely. Carboligase activity at pH 8.4 in fraction A after storage (measured as rate of glyoxylate disappearance by the spectrophotometric assay for glyoxylate) was $0.012\ \mu\text{mole}$ of glyoxylate per min per mg of protein. The activity of the crude extract (fraction A) given in Table 2, therefore, was measured by use of a sample which had been stored for 2 months. It was not possible to determine whether ureidoglycolate synthetase activity had been lost upon storage, since the apparent activity increased due to carboligase inactivation. However, purified fractions were stable for several months under the same conditions of storage.

In view of these considerations, it was not possible to obtain an accurate measurement of the initial level of ureidoglycolate synthetase activity in the crude extract. Based upon the measurement of total enzyme activity in fraction B, the specific activity of the crude extract may be estimated as 1.74 times that actually measured. The purification achieved, calculated on this basis, was 77-fold, and the recovery of enzyme activity was 35%.

None of the methods available for measurement of glyoxylate was suitable for use in assays of ureidoglycolate synthetase activity. Methods involving quantitative conversion of glyoxylate

to a derivative, such as the phenylhydrazone or semicarbazone (Young and Conway, 1942; Christman, Foster, and Esterer, 1944; Friedemann and Haugen, 1943; Neish, 1957), require exposure of the reaction mixture containing excess acid-labile substrate to acid conditions. The methods of Olson (1959*a, b*) and Dixon and Kornberg (1959), in which derivative formation is measured in the enzyme reaction mixture, cannot be used with an enzyme which has maximal activity at an alkaline pH. In addition, it was found that phenylhydrazine inhibited ureidoglycolate synthetase activity approximately 50% when added to the reaction mixture in quantities sufficient to react completely with the glyoxylate formed. In the procedure developed in these studies, glyoxylate is measured at pH 7.0 and the enzyme is not exposed to possibly inhibitory reagents. Whereas these advantages are particularly applicable in the ureidoglycolate synthetase assay, they also may be of value in assays of other enzymatic reactions in which glyoxylate is involved or in the determination of glyoxylate in biological samples.

The sample size used in these studies was chosen for convenience in measuring the slope of the recorded optical-density change, but larger amounts of glyoxylate may be measured with equal precision (Fig. 2). In all cases, only the initial, linear portion of the recorded optical-density curve was used for measurement of the slope. Since the actual slope obtained (but not its numerical value) is dependent upon both glyoxylate concentration and the chart speed of the recorder, the limiting amount of glyoxylate measurable by this technique would depend upon the instrumentation available. By preparing in advance the required number of tubes containing water and K_2HPO_4 and overlapping enzyme incubation periods, assays could be completed at 3-min intervals. Since phenylhydrazine is unstable at pH 7, it was necessary to delay its neutralization until immediately before addition of the sample.

Recently, Vogels (1963) confirmed our preliminary studies on glyoxylurease and suggested that the trivial name, glyoxylurea, be changed to the more descriptive term, ureidoglycolate, and that the enzyme be named ureidoglycolate ureohydrolase. We have adopted his suggestion for renaming the substrate; however, there is no evidence that the reaction catalyzed by this enzyme is hydrolytic. Therefore, the name ureidoglycolate synthetase appears preferable.

ACKNOWLEDGMENTS

This investigation was supported by grant G-19393 from the National Science Foundation and

by Public Health Service grant AI-05401 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

- BOJANOWSKI, R., E. GAUDY, R. C. VALENTINE, AND R. S. WOLFE. 1964. Oxamic transcarbamylase of *Streptococcus allantoicus*. *J. Bacteriol.* **87**:75-80.
- CAMPBELL, L. L., JR. 1954. The mechanism of allantoin degradation by a *Pseudomonas*. *J. Bacteriol.* **68**:598-603.
- CHRISTMAN, A. A., P. W. FOSTER, AND M. B. ESTERER. 1944. The allantoin content of blood. *J. Biol. Chem.* **155**:161-171.
- DICARLO, F. J., A. S. SCHULTZ, AND A. M. KENT. 1953. The mechanism of allantoin catabolism by yeast. *Arch. Biochem. Biophys.* **44**:468-474.
- DIXON, G. H., AND H. L. KORNBERG. 1959. Assay methods for key enzymes of the glyoxylate cycle. *Biochem. J.* **72**:3P.
- FRIEDEMANN, T. E., AND G. E. HAUGEN. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. *J. Biol. Chem.* **147**:415-442.
- GAUDY, E., R. C. VALENTINE, R. BOJANOWSKI, AND R. S. WOLFE. 1962. Glyoxylurease, a new enzyme in allantoin degradation. *Bacteriol. Proc.*, p. 104.
- LASKOWSKI, M. 1951. Allantoinase and allantoinase, p. 946-950. In J. B. Sumner and K. Myrback [ed.], *The enzymes*, vol. 1, part 2. Academic Press, Inc., New York.
- NEISH, W. J. P. 1957. α -Keto acid determinations. *Methods Biochem. Anal.* **5**:109-179.
- OLSON, J. A. 1959a. Spectrophotometric measurement of α -keto acid semicarbazones. *Arch. Biochem. Biophys.* **85**:225-233.
- OLSON, J. A. 1959b. Purification and properties of yeast isocitric lyase. *J. Biol. Chem.* **234**:5-10.
- PETERSON, E. A., AND H. A. SOBER. 1962. Column chromatography of proteins: substituted celluloses, p. 3-27. In S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 5. Academic Press, Inc., New York.
- SUTHERLAND, E. W., C. F. CORI, R. HAYNES, AND N. S. OLSEN. 1949. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. *J. Biol. Chem.* **180**:825-837.
- SWINGLE, S. M., AND A. TISELIUS. 1951. Tricalcium phosphate as an adsorbent in the chromatography of proteins. *Biochem. J.* **48**:171-174.
- VALENTINE, R. C., R. BOJANOWSKI, E. T. GAUDY, AND R. S. WOLFE. 1962. Mechanism of the allantoin fermentation. *J. Biol. Chem.* **237**:2271-2277.
- VALENTINE, R. C., H. DRUCKER, AND R. S. WOLFE. 1964. Glyoxylate fermentation by *Streptococcus allantoicus*. *J. Bacteriol.* **87**:241-246.
- VALENTINE, R. C., AND R. S. WOLFE. 1961. Glyoxylurea. *Biochem. Biophys. Res. Commun.* **5**:305-308.
- VOGELS, G. D. 1963. On the microbial metabolism of allantoin. Dissertation, Technische Hogeschool, Delft, Netherlands.
- WARBURG, O., AND W. CHRISTIAN. 1942. Isolierung und Kristallisation des Garungsferments Enolase. *Biochem. Z.* **310**:384-421.
- YOUNG, E. C., AND C. F. CONWAY. 1942. On the estimation of allantoin by the Rimini-Schryver reaction. *J. Biol. Chem.* **142**:839-853.