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OXAMIC TRANSCARBAMYLASE OF *STREPTOCOCCUS ALLANTOICUS*

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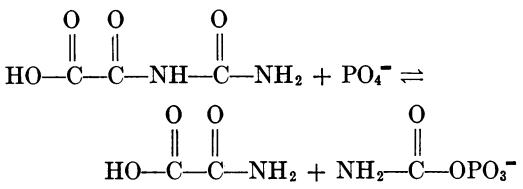
ABSTRACT

BOJANOWSKI, R. (University of Illinois, Urbana), ELIZABETH GAUDY, R. C. VALENTINE, AND R. S. WOLFE. Oxamic transcarbamylase of *Streptococcus allantoicus*. J. Bacteriol. 87:75-80. 1964. —An improved colorimetric assay for carbamyl oxamate, which allows the precise measurement of the activity of oxamic transcarbamylase, has been developed. Activity is maximum over the pH range from 8.3 to 8.7. A cation requirement is satisfied by 2.5×10^{-3} M Mg^{++} or Mn^{++}. The equilibrium constant for the phosphorolysis of carbamyl oxamic acid is 1.6, corresponding to a negative free energy change of −285 cal per mole.

The conversion of carbamyl oxamate to oxamate by *Streptococcus allantoicus* is a unique phosphorolytic reaction catalyzed by oxamic transcarbamylase (Valentine and Wolfe, 1960*a*; Bojanowski et al., 1962):



The carbamyl phosphate formed is converted to CO_2 and NH_3 with the formation of adenosine triphosphate (ATP; Valentine and Wolfe, 1960*b*). In the complete pathway of allantoin fermentation which has been proposed (Valentine et al., 1962), the decomposition of the ureido moiety of carbamyl oxamate in this manner provides the organism with its primary source of energy.

This report contains the results of a study of the role of carbamyl oxamate in the pathway of allantoin degradation by *S. allantoicus*.

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MATERIALS AND METHODS

Organism and growth conditions. S. allantoicus was kindly supplied by H. A. Barker. The culture medium for *S. allantoicus* was prepared as follows: component A contained (per 700 ml): yeast extract (Difco), 1.0 g; K_2HPO_4, 1.0 g; sodium mercaptoacetate, 0.5 g; *p*-aminobenzoic acid, 1.0 mg; and biotin, 10 μg. This mixture was sterilized and cooled to room temperature. Component B contained (per 300 ml): allantoin (Nutritional Biochemicals Corp., Cleveland, Ohio), 4.0 g; $MgSO_4$, 160 mg; $MnSO_4$, 20 mg; $CaCl_2$, 10 mg; and $FeSO_4$, 2.0 mg. This mixture was heated to 60 C to dissolve the allantoin, and was then cooled to 40 C; the pH was adjusted with KOH to 7.4. Higher temperatures destroyed allantoin.

Component B was filter-sterilized while still warm, and was added aseptically to component A to form 1 liter of complete medium. Stationary cultures were incubated at 30 C in containers filled almost to capacity with culture medium. For certain experiments, *S. allantoicus* was grown on the glucose medium described by Barker (1943). In all cases, incubation was at 30 C for 18 hr.

Preparation of crude extracts. Harvested cells were frozen at −20 C and crushed in a Hughes press. Crushed cells from 20 liters of medium were suspended in 85 ml of distilled water at 4 C, and were centrifuged to remove cell debris. The protein content of crude and purified enzyme preparations was determined by the biuret-phenol method of Sutherland et al. (1949). An Evelyn colorimeter (Rubicon Co., Philadelphia, Pa.) was used for all colorimetric assays.

Assay of oxamic transcarbamylase. One unit of oxamic transcarbamylase activity is defined as the amount of enzyme required for the formation of 1 μmole of carbamyl oxamic acid per min. The standard incubation mixture contained 15 μmoles of oxamate (K & K Laboratories, Jamaica, N.Y.), 15 μmoles of carbamyl phosphate (Sigma Chemi-

cal Co., St. Louis, Mo.), 5 μmoles of $MgSO_4$, 40 μmoles of tris(hydroxymethyl)aminomethane (tris) buffer (pH 8.3), an amount of enzyme sufficient to catalyze the formation of 0.2 to 2.0 μmoles of carbamyl oaxmic acid in 10 min, and distilled water to a total volume of 1.0 ml. Oxamate was omitted from control mixtures. The reaction was initiated by the addition of enzyme and, after 10 min at 30 C, was stopped by the addition of the H_2SO_4-H_3PO_4 acid mixture used in the assay for carbamyl oxamate. A new colorimetric method for the determination of carbamyl oxamate was developed and is presented below.

Assay of ornithine transcarbamylase. For the determination of ornithine transcarbamylase activity, the reaction mixture consisted of 20 μmoles of ornithine-HCl (Nutritional Biochemicals Corp.), 15 μmoles of carbamyl phosphate, 40 μmoles of tris buffer (pH 8.3), a suitable amount of enzyme, and distilled water to a total volume of 1.0 ml. Control mixtures contained either no enzyme or no ornithine. The reaction was initiated by addition of the enzyme and allowed to continue at 30 C for 10 min; it was stopped by the addition of the acid mixture used in the assay for citrulline (Archibald, 1944).

Colorimetric determination of carbamyl oxamate. Carbamyl oxamate responds to both the Archibald method for determination of citrulline and the method of Koritz and Cohen (1954) for the estimation of carbamylamino acids and related compounds. The structural requirement for response to these methods is the ureido grouping: $R—NH—\overset{\overset{O}{\|}}{C}—NH_2$. Neither of these procedures afforded a precise method for colorimetric analysis of carbamyl oxamate. A colorimetric procedure which utilized certain aspects of both methods was performed as follows. To 3 ml of sample were added 6 ml of a 1:1 (v/v) mixture of concentrated H_2SO_4 and 85% H_3PO_4, 0.1 ml of a 1% aqueous solution of *p*-diphenylamine sulfonic acid, and 0.25 ml of a 3% aqueous solution of 2,3-butanedione monoxime. Each tube was mixed thoroughly, capped with a marble, and placed in a boiling-water bath for 10 min. While still hot, the contents of each tube were transferred to a colorimeter tube containing 0.25 ml of 1% potassium persulfate. The violet color which formed immediately was allowed to develop for 5 min as the tube equilibrated at room temperature; absorbancy was read at 540 mμ. With the exception of the acid mixture, all reagents were prepared on the day they were to be used and kept cold while not in use.

RESULTS

Assay for carbamyl oxamate. A typical standard curve is shown in Fig. 1. The reproducibility of values for quantities below 0.5 μmole was in the range of ± 4% of the mean, and, for quantities above 0.5 μmole, ± 7%. The color formed, which had a broad absorption with a maximum at 540 mμ, was stable for at least 0.5 hr and was not affected by light. Chloride ion in a low concentration increases the sensitivity of the color reaction (Crokaert and Schram, 1958), and one must be careful to employ chloride-free reagents. Urea and other ureido compounds also reacted in this procedure.

This colorimetric assay provided a precise quantitative procedure which could be used for the reliable measurement of oxamic transcarbamylase activity. The complete assay procedure usually took 30 min. Interference due to urea contained in the enzyme preparation or to urea that formed nonenzymatically from carbamyl phosphate (or from its decomposition product, cyanate) in the reaction mixture was corrected for by using appropriate controls.

Fractionation of extracts. An extensive purification of the enzyme was not achieved, because conditions for storing the fractions without loss of activity were not realized. The enzyme preparation used in these studies was prepared in the following manner. Crude extract (fraction A)

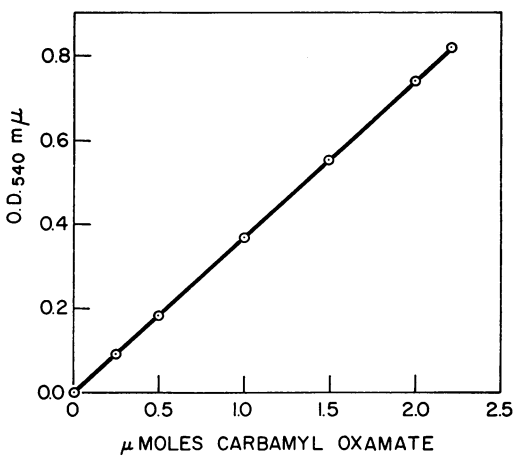


FIG. 1. *Colorimetric determination of carbamyl oxamate.*

contained (per ml) 16.5 mg of protein and 4.2 units of activity. To 70 ml of this fraction in an ice bath were added (dropwise) 3.5 ml of 1 M $MnCl_2$: the precipitate was discarded. To remove excess Mn^{++} from the extract, 3.5 ml of 1 M K_2HPO_4 at pH 8.0 were added dropwise with stirring; the precipitate was discarded. To remove chloride ions, the extract was then dialyzed against 3 liters of 0.02 M K_2HPO_4 at pH 8.0 for 4 hr; the dialyzing medium was changed after 2 hr. The precipitate formed during dialysis was discarded. This fraction (B) contained (per ml) 7.9 mg of protein and 4.8 units of activity. To 63 ml of fraction B were added 25 g of solid ammonium sulfate. The resulting precipitate was suspended in 20 ml of cold distilled water and dialyzed against 2 liters of 0.02 M K_2HPO_4 at pH 8.0 for 2 hr. This fraction was designated fraction C, and contained (per ml) 15.8 mg of protein and 10.5 units of activity, representing a 2.6-fold purification of the crude extract with 81% recovery.

Effect of protein concentration. The velocity of the oxamic transcarbamylase reaction was proportional to the amount of protein in the incubation mixture. These data are shown in Fig. 2 for a greater than tenfold range of enzyme concentrations.

Incubation time. The rate of carbamyl oxamate formation from oxamate and carbamyl phosphate was linear over a period of approximately 15 min (Fig. 3). A 10-min incubation time was chosen for the standard assay. Because only a slight purification of the enzyme was obtained, a detailed study of substrate-concentration kinetics was not attempted.

Effect of pH. The effect of pH on the velocity of the reaction catalyzed by oxamic transcarbamylase is shown in Fig. 4. Tris buffers adjusted to the desired pH with dilute H_2SO_4 were used to control the pH in the range indicated. At pH values below 7.0 and above 9.0, the activity at constant pH was difficult to determine owing to the decomposition of carbamyl phosphate (Jones and Lipmann, 1960).

The optimal pH of 8.3 was chosen for the standard assay, to minimize the decomposition of carbamyl phosphate. The maximum in the pH curve at pH 8.5 was approximately the same as that for the glyoxylurease of *S. allantoicus* (Gaudy, 1962), and for the ornithine transcarbamylase from *S. faecalis* (Caravaca and Grisolia, 1960).

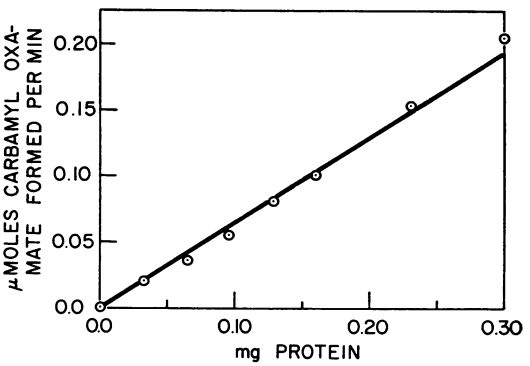


FIG. 2. *Activity of oxamic transcarbamylase as a function of protein concentration. Each reaction mixture contained: 15 μmoles of oxamate, 15 μmoles of carbamyl phosphate, 5 μmoles of $MgSO_4$, 40 μmoles of tris buffer (pH 8.3), a volume of fraction C containing the amount of protein indicated, and water to a total volume of 1.0 ml. Incubation: 30 C for 10 min.*

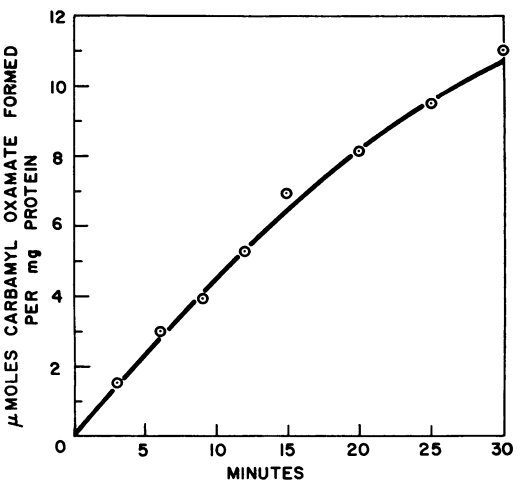


FIG. 3. *Activity of oxamic transcarbamylase as a function of time. Each reaction mixture contained: 100 μmoles of oxamate, 100 μmoles of carbamyl phosphate, 50 μmoles of $MgSO_4$, 200 μmoles of tris buffer (pH 8.3), 1.0 ml of a 1:10 dilution of fraction C, and water to a volume of 10 ml. Incubation was at 30 C. At the time intervals indicated, 0.5-ml samples were removed and the carbamyl oxamate content was determined.*

Cation requirement. Oxamic transcarbamylase requires Mg^{++} for activity (Valentine and Wolfe, 1960*a*). The cation requirement was satisfied by 2.5×10^{-3} M $MgSO_4$ (Fig. 5). The effects of two other metal cations on the activity of oxamic transcarbamylase were determined.

In a typical experiment, the following specific activities were observed when 5 μmoles of each of the following salts were added: 0.60 with added $MgSO_4$, 0.59 with added $MnSO_4$, and 0.11 with added $CaCl_2$.

Comparison of oxamic and ornithine transcarbamylases in crude extracts. The enzymatic system responsible for the metabolism of allantoin by *S. allantoicus* is inducible, and exists in the organism only when grown on allantoin (Barker, 1961). Table 1 presents a comparison of the crude extracts of glucose-grown and allantoin-grown cells with respect to the activity of oxamic transcarbamylase. The absence of oxamic transcarbamylase in glucose-grown cells supports the conclusion that the enzyme is involved specifically in the metabolism of allantoin in *S. allantoicus*. The data obtained for ornithine transcarbamylase in the same extracts illustrate that the two enzymes are in fact different, and are probably not related in function. The response to added Mg^{++} points out another difference between the two enzymes: the activity of oxamic transcarbamylase in crude extracts is stimulated by addition of the cation, whereas ornithine transcarbamylase activity is not affected. The ornithine transcarbamylase of *S. faecalis* has been reported to require no Mg^{++} (Jones, Spector, and Lipmann, 1956).

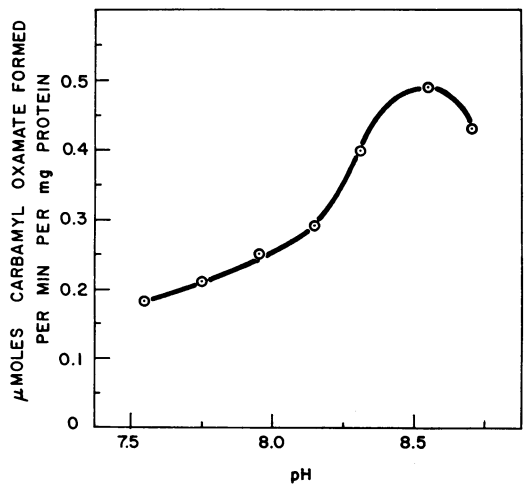


FIG. 4. *Effect of pH on the activity of oxamic transcarbamylase. Each reaction mixture contained: 15 μmoles of oxamate, 15 μmoles of carbamyl phosphate, 5 μmoles of $MgSO_4$, 40 μmoles of tris buffer at the indicated pH, 0.1 ml of a 1:25 dilution of fraction C, and water to a 1.0-ml volume. Incubation time: 10 min at 30 C. Each point is the average of three separate measurements.*

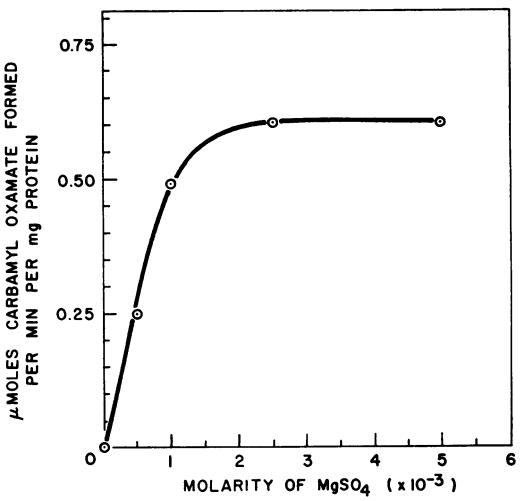


FIG. 5. *Dependence of oxamic transcarbamylase activity on Mg^{++}. Each reaction mixture contained: 15 μmoles of oxamate, 15 μmoles of carbamyl phosphate, the concentration of $MgSO_4$ indicated, 40 μmoles of tris buffer (pH 8.3), 0.1 ml of a 1:20 dilution of fraction C, and glass-distilled water to a 1.0-ml volume. Incubation: 30 C for 10 min.*

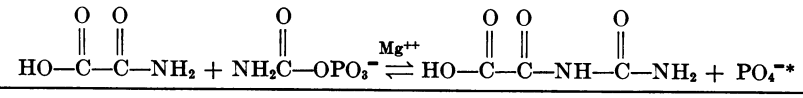
Oxamic transcarbamylase equilibrium. Since the stoichiometry of the oxamic transcarbamylase reaction has been established (Valentine and Wolfe, 1960*a*), an apparent equilibrium constant for the reaction could be calculated from the equilibrium concentration of carbamyl oxamate and the initial concentrations of reactants (Table 2). The calculations were based on determinations of carbamyl oxamate at equilibrium; the good agreement of values obtained indicated that the error involved was not large. The average

TABLE 1. *Comparison of oxamic and ornithine transcarbamylases in crude extracts of Streptococcus allantoicus**

Description of extract	Specific activity	
	Oxamic transcarbamylase	Ornithine transcarbamylase
Glucose-grown cells.....	0.00	0.80
Allantoin-grown cells....	0.11	1.23
Allantoin-grown cells (Mg^{++} added).....	0.21	1.23

* Incubation mixtures contained: 20 μmoles of ornithine or 15 μmoles of oxamate, 15 μmoles of carbamyl phosphate, 40 μmoles of tris buffer (pH 8.3), 5 μmoles of $MgSO_4$ where indicated, enzyme, and water to a 1.0-ml volume. Incubation was for 10 min at 30 C.

TABLE 2. *Apparent equilibrium constant for the reaction:*



Expt no.	Initial concn†		Equilibrium concn†			Kapp = $\frac{[COx][Pi]}{[Ox][CAP]}$
	[Ox]	[CAP]	[COx] = [Pi]	[Ox]	[CAP]	
1	75	75	31.71	43.29	43.29	0.536
2	75	75	33.88	41.12	41.12	0.680
3	90	90	39.21	50.79	50.79	0.596
4	90	75	36.89	53.02	38.11	0.674
5	75	90	36.25	38.75	53.75	0.631
Avg						0.623

* Incubation mixtures contained: concentrations of oxamate and carbamyl phosphate indicated in the table, 25 μmoles of $MgSO_4$, 200 μmoles of tris buffer (pH 8.3), enzyme, and water to a 5.0-ml volume. In experiment 1, 3.2 mg of protein were included in the reaction mixture; 4.8 mg of protein were added in experiments 2 through 5; 0.2-ml samples were removed from the reaction mixture at 10-min intervals until an equilibrium condition was established.

† Concentrations (in μmoles) present in the complete reaction mixture (5 ml). Abbreviations: Ox = oxamate; CAP = carbamyl phosphate; COx = carbamyl oxamate; Pi = inorganic phosphate.

equilibrium constant calculated, 0.623, represented a readily reversible reaction, and corresponded to a free energy change of 285 cal per mole in the direction of carbamyl oxamate synthesis.

DISCUSSION

In *S. faecalis*, the phosphorolytic cleavage of citrulline may be adaptively developed into a pathway of energy derivation (reactions B and C):

citrulline + phosphate
$\leftrightarrows$ carbamyl phosphate + ornithine (B)

carbamyl phosphate + adenosine diphosphate
$\xrightarrow{Mg^{++}}$ ATP + CO_2 + NH_3 (C)

Reaction B is dependent upon the removal of carbamyl phosphate in the ATP-yielding reaction (C), the equilibrium of the latter reaction being far in the direction of ATP formation (Jones et al., 1956). As suggested by Krebs and Kornberg (1957), these reactions are probably an adaptation evolved from the reverse process, which is an important synthetic process in growing bacteria. The use of the ureido group of citrulline in this instance is analogous to the oxamic transcarbamylase reaction found in *S. allantoicus*. For the phosphorolysis of carbamyl oxamate, however, there is no apparent requirement for removal of carbamyl phosphate by the ATP-yielding reaction.

ACKNOWLEDGMENT

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