JOURNAL OF BACTERIOLOGY, June 1975, p. 943-948

Vol. 122, No. 3
Printed in U.S.A.

Anacystis nidulans Mutants Resistant to Aromatic Amino Acid Analogues

W. PHARES AND L. F. CHAPMAN*

Microbiology Section, Division of Biological Sciences, University of Missouri, Columbia, Missouri 65201

Received for publication 20 January 1975

Three classes of mutants of *Anacystis nidulans* were selected on the basis of resistance to fluorophenylalanine and 2-amino-3-phenylbutanoic acid. The most frequent type exhibited DAHP synthetase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate-D-erythrose-4-phosphate-lyase [pyruvate phosphorylating], EC 4.1.2.15) activity identical to that of the parental strain. The second type was characterized by extremely low levels of the activity. The third type had a DAHP synthetase showing decreased sensitivity to inhibition by L-tyrosine. The enzyme was purified 140-fold from wild-type and feedback-insensitive strains, and the kinetics of the reaction was examined. The activity of the wild-type enzyme was inhibited 75% in the presence of 2.0×10^{-3} M tyrosine, and the altered enzyme was inhibited 10%. The following apparent constants were obtained from kinetic studies with partially purified wild-type enzyme: $S_{0.5}$ for D-erythrose-4-phosphate = 7.1×10^{-4} M; $S_{0.5}$ for phosphoenolpyruvate = 1.4×10^{-4} M. Inhibition by tyrosine was mixed with respect to binding of both D-erythrose-4-phosphate and phosphoenolpyruvate. In addition, tyrosine promoted cooperative interactions in the binding of phosphoenolpyruvate. For the altered enzyme the following apparent constants were obtained: $S_{0.5}$ for D-erythrose-4-phosphate = 7.1×10^{-4} M; $S_{0.5}$ for phosphoenolpyruvate = 2.9×10^{-4} M. Inhibition by tyrosine was mixed with respect to D-erythrose-4-phosphate and competitive with respect to phosphoenolpyruvate. Tyrosine did not promote cooperative effects in the binding of phosphoenolpyruvate to the altered enzyme.

The first reaction unique to the synthesis of phenylalanine, tyrosine, and tryptophan is the condensation of phosphoenolpyruvate and D-erythrose-4-phosphate to give 3-deoxy-D-*arabino*-heptonate-7-phosphate (DAHP) synthetase. In the blue-green bacterium *Anacystis nidulans* this key regulatory enzyme exists as a single activity under the feedback control of a single end product, tyrosine (9). Inhibition of enzyme activity can often be mediated by synthetic analogues of naturally occurring amino acids. Since the analogue may substitute for the natural product in a variety of reactions, analogue resistance is often a useful tool in the selection of variants in which a control mechanism has been modified (15). This method has proved to be of much practical value for work on regulation of the metabolism in several species of bacteria (6, 11-13). Work in this laboratory demonstrates that mutants of *A. nidulans* may be selected on the basis of resistance to fluorophenylalanine and 2-amino-3-phenylbutanoic acid. One of these mutants is a feedback-insensitive strain, the isolation of which is of interest for two reasons. First, the characterization of such a strain expands the possibilities for study of the regulation of aromatic amino acid biosynthesis in *A. nidulans*. Second, examination of catalytic and feedback properties of the altered enzyme provides further description of the interaction between substrate, inhibitor, and enzyme molecules; it was previously reported that tyrosine exerted a differential effect on the binding of the two substrates, which suggested a relationship to the mechanism of the enzymatic reaction (9). Kinetic studies of the DAHP synthetase from a feedback-insensitive strain and a preliminary analysis of the activity in crude extracts of other mutants are described in the present report.

MATERIALS AND METHODS

Growth of cells, preparation of crude extracts, method of protein determinations, procedure for assay of DAHP synthetase, and calculation of units of activity were described previously (9). The feedback-resistant mutant *A. nidulans* AP10 is a spontaneous derivative of strain 625 (14), resistant to 2.0×10^{-3} M 2-amino-3-phenylbutanoic acid.

Isolation of analogue-resistant mutants. Strain 625 was grown to early stationary phase (9) and

harvested by centrifugation, and the pellet was washed with 0.02 M potassium phosphate (pH 7.0) plus 10^{-3} M $MgCl_2$. The cells were resuspended in one-tenth the volume of the same buffer, and 0.3 ml of cell suspension was spread on minimal agar plates containing 200 μg of analogue per ml. Colonies appearing after 5 days of incubation at 37 C under constant illumination were picked and purified three times by successive streaking on analogue-supplemented medium. Strains resistant to fluorophenylalanine and 2-amino-3-phenylbutanoic acid are designated FP and AP, respectively.

Assay of substrates. Both phosphoenolpyruvate and D-erythrose-4-phosphate used for the assay of DAHP synthetase were hydrolyzed in 1 N HCl at 100 C. The kinetics of this reaction was examined, and for quantitative purposes it was determined that an incubation period of 1 h was sufficient for maximum hydrolysis. The inorganic phosphate liberated was determined by the method of Ames et al. (1). A 0.6-ml amount of hydrolyzed sample was incubated with 1.4 ml of Chen reagent at 45 C for 20 min. The absorbance at 820 nm was read against a similarly treated water blank. The molar extinction coefficient was 2.1×10^4 M^{-1} under these assay conditions. Concentrations of both substrates were corrected for inorganic phosphate present initially.

D-Erythrose-4-phosphate was concomitantly determined colorimetrically (8); phosphoenolpyruvate was determined by its absorbance at 240 nm under specified conditions (5). The results from both of these assays substantiate values obtained from inorganic phosphate determinations.

Purification of DAHP synthetase. All of the following operations were performed at 0 to 5 C.

(i) Precipitation of nucleic acids. Crude extracts of the wild-type strain were treated with protamine sulfate (9). Homogenates prepared from analogue-resistant cells were treated with streptomycin sulfate instead. Streptomycin sulfate was dissolved in 0.05 M potassium phosphate (pH 6.8) plus 0.5 mM ethylenediaminetetraacetate and added to a final concentration of 2% (wt/vol) with continuous, slow stirring. The resulting suspension was stirred for an additional 15 min. The precipitate was removed by centrifugation at 12,100 $\times$ *g* for 15 min and discarded.

(ii) $(NH_4)_2SO_4$ fractionation. Fractions of 0 to 30, 30 to 50, and 50 to 65% saturation were prepared by the dropwise addition of an appropriate volume of a saturated solution of the salt (7) with continuous, slow stirring. The pH was monitored and adjusted to 6.8 with KOH when necessary. Stirring was continued for 20 min, and then the suspension was centrifuged at 12,100 $\times$ *g* for 15 min. The precipitate was dissolved in 0.05 M potassium phosphate (pH 6.8) plus 1.0 mM $MgCl_2$ and dialyzed against 1,000 volumes of the same buffer.

(iii) Sephadex G-100 fractionation. A Sephadex G-100 column (2.0 by 70.5 cm) was prepared according to the method of Andrews (2) and equilibrated with 0.05 M potassium phosphate (pH 6.8) plus 1.0 mM $MgCl_2$ plus 0.1 mM phosphoenolpyruvate. The 30 to 50% $(NH_4)_2SO_4$ fraction was layered on the gel and washed into the column with a few milliliters of buffer. Elution was then begun at a flow rate of 20 to 30 ml/h. Fractions of 3.0 ml were collected. Protein concentrations in column fractions were estimated by measuring the absorbance at 280 nm in a Coleman 124 spectrophotometer.

(iv) $(NH_4)_2SO_4$ concentration. The Sephadex G-100 eluate was brought to a final concentration of 5% glycerol by the addition of one-tenth the volume of 55% glycerol (vol/vol) in 0.05 M potassium phosphate (pH 6.8) plus 1.0 mM $MgCl_2$ plus 0.2 mM phosphoenolpyruvate. The resulting solution was then brought to 80% saturation with $(NH_4)_2SO_4$ by addition of the solid salt (7). The precipitate was removed by centrifugation, resuspended in 0.05 M potassium phosphate (pH 6.8) plus 1.0 mM $MgCl_2$ plus 0.2 mM phosphoenolpyruvate plus 5% glycerol, and then dialyzed against 1,000 volumes of the same buffer without glycerol.

(v) Hydroxyapatite fractionation. Hydroxyapatite was prepared and a column (1.7 by 23.0 cm) was poured (4). The column was equilibrated with 0.01 M potassium phosphate (pH 6.8) plus 1.0 mM $MgCl_2$ plus 0.2 mM phosphoenolpyruvate. Unadsorbed protein was eluted with 80 ml of buffer at a flow rate of 40 to 45 ml/h. A 200-ml potassium phosphate (pH 6.8) linear gradient, 0.01 to 0.30 M, containing 1.0 M $MgCl_2$ and 0.2 mM phosphoenolpyruvate, was used to elute the enzyme. Fractions of 2.8 ml were collected.

Chemicals. Phosphoenolpyruvate trisodium salt, L-tyrosine, and protamine sulfate were obtained from Sigma Chemical Co. D-Erythrose-4-phosphate-dimethylacetal-1,1-cyclohexylammonium salt was obtained from Calbiochem and was converted to D-erythrose-4-phosphate (3). Sephadex was obtained from Pharmacia Fine Chemicals, Inc. Streptomycin sulfate, fluorophenylalanine, and 2-amino-3-phenylbutanoic acid were obtained from Nutritional Biochemicals Co. Other reagents were obtained from Fisher Scientific Co., Sigma Chemical Co., or Mallinckrodt Chemical Works. Weights of substrates and reagents used were corrected according to the purity stated by the supplier.

RESULTS

DAHP synthetase activity of mutants. DAHP synthetase activity in 23 mutants was compared to that of the parental strain with regard to specific activity and feedback inhibition by tyrosine. Strains isolated thus far fit one of three classes (Table 1). The most frequent type exhibits DAHP synthetase activity identical to that of the parental strain. The second type is characterized by extremely low levels of the activity under standard assay conditions. Mutants of these two classes are currently being investigated. Mutants of the third class have a tyrosine-insensitive DAHP synthetase. The enzyme from a feedback-insensitive strain was purified and examined further; the wild-type activity was also purified in the same manner.

Purification of DAHP synthetase. A representative purification from the analogue-resist-

ant strain is presented in Table 2. The use of streptomycin sulfate to precipitate nucleic acids from cell-free extracts of the analogue-resistant strain was chosen as an alternative to the use of protamine sulfate, since the latter resulted in almost complete loss of activity. The same observation applies to the use of streptomycin sulfate with the extract from the wild-type strain. Otherwise, the two enzymes behaved similarly during each purification step. During the $(NH_4)_2SO_4$ fractionation procedure, approximately 85% of the activity recovered was precipitated in the 30 to 50% fraction. The activity in the remaining two fractions exhibited feedback properties identical to that of the 30 to 50% fraction. Both enzymes eluted from Sephadex G-100 as single symmetrical peaks, which appeared at the decline of the major peak of eluted protein. Both enzymes adsorbed to hydroxyapatite at a phosphate concentration of 0.01 M and were eluted as single peaks, skewed slightly to the right, about midpoint in the linear gradient.

Kinetic studies with the purified enzyme. Kinetic studies were performed using fraction VI preparations of DAHP synthetase from strains 625 and AP10. These studies consist of measuring initial reaction velocities as a function of varying substrate concentration; the concentration of one substrate is varied at saturating levels of the second substrate. Measurements were then conducted in the presence of two concentrations of L-tyrosine.

TABLE 1. *Representative classification of mutants*

Strain	Sp act[a]	% Inhibition by 10^{-4} M L-tyrosine
625	1.3	75
AP5	1.4	71
AP7	1.6	71
FP2	1.5	76
AP11	<0.1	
AP10	4.5	9

[a] Nanomoles of DAHP formed per minute per milligram of protein.

Kinetic studies of the wild-type enzyme present double-reciprocal plots similar to those previously published (9). The $S_{0.5}$ for D-erythrose-4-phosphate is 7.1×10^{-4} M. L-Tyrosine, at concentrations of 10^{-5} and 2.5×10^{-5} M, affects both the $S_{0.5}$ for D-erythrose-4-phosphate and the V_{max} of the reaction. The $S_{0.5}$ for phosphoenolpyruvate is 1.4×10^{-4} M. The effect of tyrosine is to alter both the $S_{0.5}$ and V_{max}. The reaction is inhibited 75% in the presence of 2.0×10^{-3} tyrosine, although inhibition by tyrosine at this concentration varied somewhat for different enzyme preparations.

For the purified enzyme from the feedback-insensitive strain, a plot of the velocity as a function of D-erythrose-4-phosphate concentration gives a nonlinear, double-reciprocal curve (Fig. 1a). An $S_{0.5}$ for D-erythrose-4-phosphate of 7.1×10^{-4} M is obtained. The effect of tyrosine, at concentrations of 10^{-4} and 10^{-3} M, is to alter both the $S_{0.5}$ and V_{max} and to increase the degree of concavity of the plot (Fig. 1b). The double-reciprocal plot of velocity as a function of phosphoenolpyruvate concentration is nonlinear, and an $S_{0.5}$ for phosphoenolpyruvate of 2.9×10^{-4} M is obtained (Fig. 2a). Tyrosine, at concentrations of 10^{-4} and 10^{-3} M, appears to affect the $S_{0.5}$ for phosphoenolpyruvate but not the V_{max} for the reaction (Fig. 2b). Inhibition of the reaction by tyrosine, at saturating concentrations of both substrates (2.0×10^{-3} M), is not proportional to the amount of inhibitor at the concentrations tested, indicating a partial inhibition system. The inhibition obtained at tyrosine concentrations as high as 2.0×10^{-3} M is 10%.

The effect of tyrosine on reaction velocity as a function of varying substrate concentration was determined by Hill plots (not shown) for both enzymes. The Hill coefficient, n, for phosphoenolpyruvate is increased from 1.4 in the absence of tyrosine to 2.5 in the presence of 2.5×10^{-5} M tyrosine for the wild-type enzyme, whereas no such effect is observed for the altered enzyme. Cooperativity indexes (10), de-

TABLE 2. *Purification of mutant DAHP synthetase*

Fraction	Vol (ml)	Total protein (mg)	Total act (U)[a]	Sp act (U/mg)	Purification (fold)	Yield act (%)
I. Crude extract	12.5	250.0	1,030	4.1	1.0	100
II. Streptomycin-SO_4 supernatant	10.0	260.0	984	3.8	0.9	96
III. $(NH_4)_2SO_4$ fraction (30–50%)	3.4	47.0	388	8.2	2.0	38
IV. Sephadex G-100 eluate	8.4	5.3	156	29.0	7.1	15
V. $(NH_4)_2SO_4$ concentrate	2.8	3.1	130	42.0	10.0	13
VI. Hydroxyapatite eluate	18.0	0.14	80.5	575.0	140.0	8

[a] Nanomoles of DAHP formed per minute.

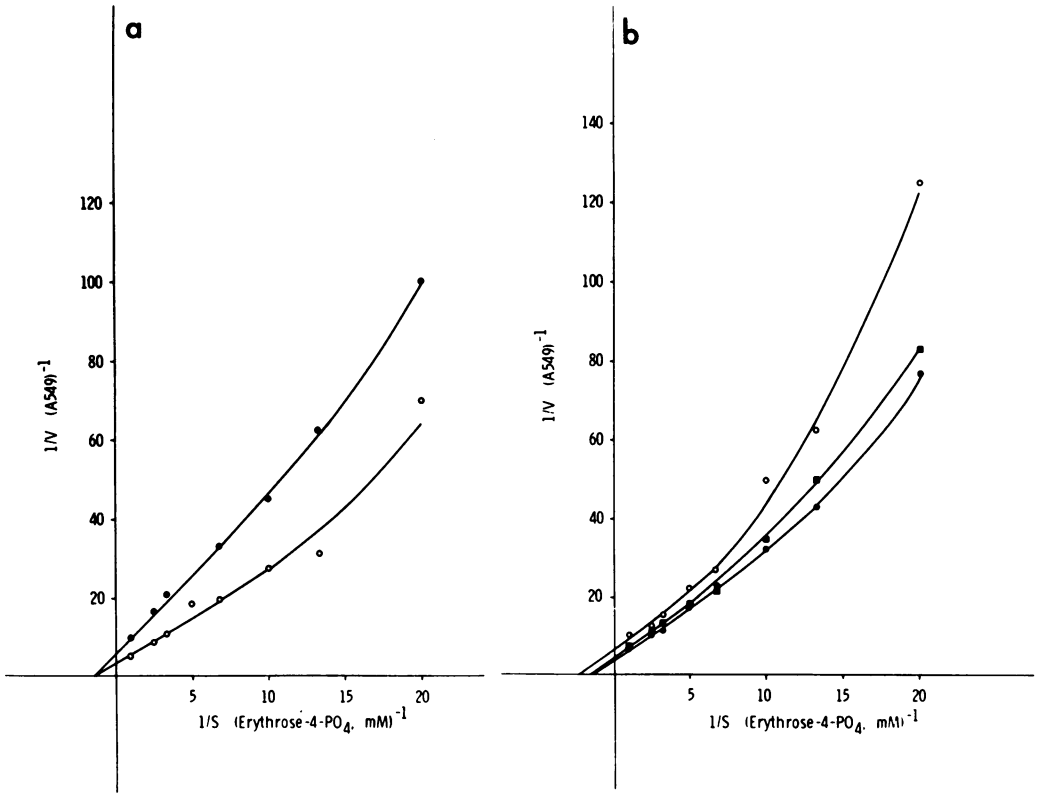

FIG. 1. *(a) Double-reciprocal plot of reaction velocity as a function of* D*-erythrose-4-phosphate concentration, strain API0. Symbols:* ●, *10 μg of protein;* ○, *20 μg of protein. (b) API0: Double-reciprocal plot of reaction velocity as a function of* D*-erythrose-4-phosphate concentration, effect of* L*-tyrosine. Symbols:* ●, *no tyrosine;* ■, *10^{-3} M tyrosine;* ○, *10^{-4} M tyrosine.*

rived from double-reciprocal plots (Fig. 2b), were calculated. For the wild-type enzyme, R_s = 125 in the absence of inhibitor and R_s = 28 in the presence of 2.5×10^{-5} M tyrosine; for the altered enzyme, R_s = 83 in the absence of inhibitor and R_s = 100 in the presence of 10^{-3} M tyrosine. These data may be related to the Hill data by the equation

$$R_s = \frac{S_{0.9}}{S_{0.1}} = \sqrt[n]{81}$$

and from this relationship it is seen that the double-reciprocal data indicate a 1.4-fold increase in n for the wild-type enzyme in the presence of 2.5×10^{-5} M tyrosine. These two sets of data are derived from different graphs, and analysis of R_s is more rigorously confined to binding studies. However, to a first approximation the results indicate that tyrosine promotes positive cooperative effects between phosphoenolpyruvate molecules with the wild-type enzyme. No such effect is observed with the feedback-insensitive enzyme.

DISCUSSION

The present study demonstrates that it is possible to obtain mutants of *A. nidulans* resistant to aromatic amino acid analogues. One type of variant isolated in this manner has a DAHP synthetase insensitive to inhibition by tyrosine. It is quite plausible that the feedback-insensitive system is primed for higher levels of pathway metabolites than the parental strain; thus, toxic effects of the analogue during growth are diminished by an effective dilution with naturally occurring end products.

The examination of catalytic and feedback properties of the altered enzyme is also of interest. It was previously observed (with a less purified preparation from wild-type cells) that in the absence of tyrosine the response of reaction velocity to substrate concentration approaches Michaelis kinetics. The presence of tyrosine appeared to facilitate cooperative interactions between molecules of phosphoenolpyruvate but had little effect on the binding of D-erythrose-4-phosphate. These observations

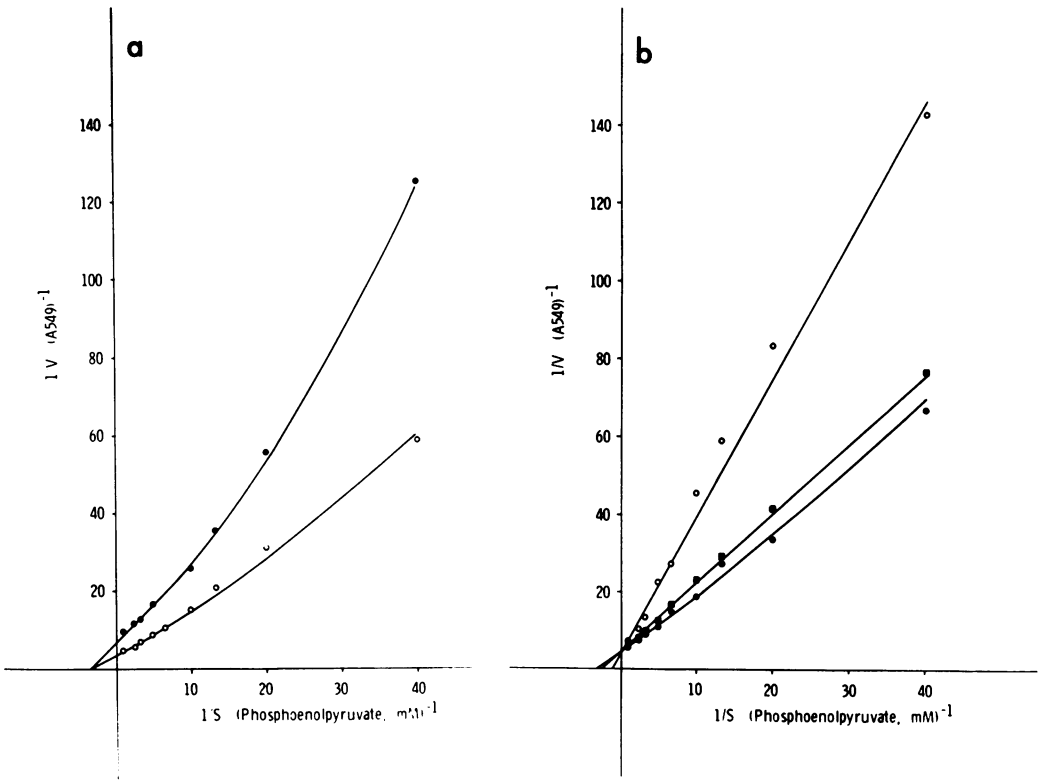

FIG. 2. *(a) Double-reciprocal plot of reaction velocity as a function of phosphoenolpyruvate concentration, strain APIO. Symbols:* ●, *10 μg of protein;* ○, *20 μg of protein. (b) AP10: Double-reciprocal plot of reaction velocity as a function of phosphoenolpyruvate concentration, effect of* L*-tyrosine. Symbols:* ●, *no tyrosine;* ○, 10^{-3} *M tyrosine;* ■, 10^{-4} *M tyrosine.*

suggested that the reaction proceeds without a definite combination between D-erythrose-4-phosphate and the enzyme-phosphoenolpyruvate complex (9). The results of the present study substantiate this view. Both wild-type and altered enzymes exhibit the same apparent affinity for D-erythrose-4-phosphate. The altered enzyme shows decreased sensitivity to tyrosine inhibition and decreased affinity for phosphoenolpyruvate; enhanced cooperative binding of phosphoenolpyruvate was not observed. An alteration in the affinity of the enzyme for tyrosine is accompanied by changes in the binding of phosphoenolpyruvate but not D-erythrose-4-phosphate.

ACKNOWLEDGMENTS

This investigation was supported by grant GB-36424 from the National Science Foundation.

LITERATURE CITED

1. Ames, B. N., B. Garry, and L. A. Herzenberg. 1960. The genetic control of the enzymes of histidine biosynthesis in *Salmonella typhimurium*. J. Gen. Microbiol. **22**:369-378.
2. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. **91**:222-233.
3. Ballou, C. E., H. O. L. Fischer, and D. L. MacDonald. 1955. The synthesis and properties of D-erythrose-4-phosphate. J. Am. Chem. Soc. **77**:5967-5970.
4. Bernardi, G. 1971. Chromatography of proteins on hydroxyapatite, p. 325. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 22. Academic Press Inc., New York.
5. Bücher, T. 1955. Enolase from brewer's yeast, p. 427. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
6. Cohen, G. N., and J.-C. Patte. 1963. Some aspects of the regulation of amino acid biosynthesis in a branched pathway, p. 513. *In* Synthesis and structure of macromolecules, vol. 28. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. di Jeso, F. 1968. Ammonium sulfate concentration conversion nomograph for 0°. J. Biol. Chem. **243**:2022-2023.
8. Dische, Z., and M. R. Dische. 1958. Methods for the qualitative and quantitative determinations of tetroses by two new specific color reactions. Biochim. Biophys. Acta **27**:184-188.
9. Holtzclaw, W. D., L. F. Chapman, and C. S. Gowans. 1971. Some regulatory properties of phospho-2-keto-3-deoxyhepatonate aldolase from the blue-green alga *Anacystis nidulans*. Biochim. Biophys. Acta **268**:562-572.

10. Koshland, D. E., Jr. 1970. The molecular basis for enzyme regulation, p. 341. *In* P. D. Boyer (ed.), The enzymes, vol. 1. Academic Press Inc., New York.
11. Moyed, H. S. 1960. False feedback inhibition: inhibition of tryptophan biosynthesis by 5-methyltryptophan. J. Biol. Chem. **235**:1098–1102.
12. Moyed, H. S. 1961. Interference with the feed-back control of histidine biosynthesis. J. Biol. Chem. **236**:2261–2267.
13. Sheppard, D. E. 1964. Mutants of *Salmonella typhimurium* resistant to feedback inhibition by L-histidine. Genetics **50**:611–623.
14. Starr, R. C. 1960. The culture collection of algae at Indiana University. Am. J. Bot. **47**:67–86.
15. Umbarger, H. E. 1971. p. 447–462. *In* H. J. Vogel (ed.), Metabolic regulation. Academic Press Inc., New York.