# ACETYLORNITHINASE OF ESCHERICHIA COLI: PARTIAL PURIFICATION AND SOME PROPERTIES*

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In *Escherichia coli*, ornithine synthesis has been shown to proceed via glutamate, *N*-acetylglutamate, *N*-acetylglutamic γ-semialdehyde, and $N^{\alpha}$-acetylornithine (1–3). The last step in the formation of ornithine

$$\begin{array}{l} \mathrm{NH{\cdot}COCH_3} \\ | \\ \mathrm{H_2N{\cdot}(CH_2)_3{\cdot}CH{\cdot}COOH} \end{array} + \mathrm{H_2O} = \begin{array}{l} \mathrm{NH_2} \\ | \\ \mathrm{H_2N{\cdot}(CH_2)_3{\cdot}CH{\cdot}COOH} \end{array} + \mathrm{CH_3{\cdot}COOH}$$

is catalyzed by the enzyme acetylornithinase (1, 4). The present report is concerned with a characterization of this enzyme.

## *Materials and Methods*

*Compounds Used*—$N^{\alpha}$-Acetyl-L-ornithine was synthesized as previously described (5, 3).[1] L-Ornithine monohydrochloride was obtained from the Mann Research Laboratories. Several of the acetylamino acids used, including acetyl-DL-methionine, were supplied by the California Foundation for Biochemical Research.[2]

*Organisms*—The organisms used are *E. coli* (ATCC 9637) and ornithine-requiring mutant 160-37 of *E. coli* (5, 1).

*Cultivation*—The growth medium used is prepared at 50 × strength as follows. In distilled water (670 ml.) are dissolved, successively, $MgSO_4 \cdot 7H_2O$ (10.0 gm.), citric acid·$H_2O$ (100.0 gm.), $K_2HPO_4$·anhydrous (500.0 gm.), and $NaNH_4HPO_4 \cdot 4H_2O$ (175.0 gm.), the final volume being about 1.00 liter.[3] On 50-fold dilution with distilled water, the resulting single strength medium, designated E, has a pH of about 7.0; it is sterilized by autoclaving. Medium E is supplemented with dextrose (autoclaved sepa-

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[1] Certain laboratory facilities used in this synthesis were generously made available by Dr. J. S. Fruton.

[2] These acetylamino acids were received as a gift. The generosity of the California Foundation for Biochemical Research is gratefully acknowledged.

[3] Mallinckrodt "analytical reagents" were found satisfactory. The 50 × strength medium is stored at room temperature; chloroform (about 1 ml.) may be added as a preservative.

rately) at a concentration of 5 gm. per liter. For strain 160-37 the medium is further supplemented with L-arginine (50 $\gamma$ per ml.).

The organisms are grown with shaking at 37° in Fernbach flasks containing suitably supplemented medium.

### *Assay of Enzyme Activity*

For acetylornithinase assays, the enzyme is permitted to act under a set of desired conditions, for example "standard conditions" (see below), and the ornithine produced is determined by a new modification of the colorimetric ninhydrin method. The development of this modification was necessitated by the properties of the substrate; at pH values near 5 (*cf.* (6)), and to a lesser extent in strongly acid solution (*cf.* (7)), $N^{\alpha}$-acetylornithine gives a ninhydrin reaction. In the present method, the ninhydrin is permitted to act at a pH of about 2.5 (measured in the absence of the organic solvent used). Under these conditions $N^{\alpha}$-acetylornithine gives but a very slight ninhydrin reaction.

*Ninhydrin Reagent*—The reagent used is prepared by mixing 2 volumes of a 1 per cent solution of ninhydrin in Methyl Cellosolve with 1 volume of 0.4 M aqueous citric acid.[4]

*Determination of Ornithine*—The ornithine-containing samples are prepared in 0.1 M aqueous phosphate (pH 7),[5] 1 mM with respect to glutathione. To 0.5 ml. portions of each of the samples are added 1.5 ml. of the ninhydrin reagent. Appropriate standards and blanks (see below) are run along with the samples. The resulting reaction mixtures are heated in a boiling water bath for 10 minutes and then cooled. If ornithine is present, a blue color is produced.[6] To each reaction mixture are added 3 ml. of 0.7 N aqueous sodium hydroxide with immediate stirring.[7] A transient intense yellow color appears even in the absence of ornithine. 20 minutes after addition of the alkali, the final reaction mixtures are read against reference blanks (see below) in a Klett-Summerson colorimeter with the No. 42 filter. Ornithine gives rise to an orange-yellow color which is stable for hours.

[4] The ninhydrin reagent is stored at 3°; it remains usable for at least 1 week.

[5] The phosphate and the citric acid (in the ninhydrin reagent) provide the desired pH.

[6] If the glutathione is omitted from the reaction mixtures, the color yield is lowered. Certain reducing agents other than glutathione also enhance the color yield.

[7] The reasons for adding the alkali are 2-fold: (*a*) the spectrum of the initially produced blue color is a rather sensitive function of the hydrogen ion concentration, whereas the orange-yellow color obtained on addition of alkali is not, and (*b*) the alkali tends to dissolve any added protein that may have precipitated under acid conditions. The assay can be carried out even in the presence of coarse débris or whole cells.

A linear color response to ornithine is obtained up to at least 0.3 μmole of ornithine per sample. Absorption maxima occur at 350 and 470 mμ. A Klett-Summerson reading of 160 corresponds to optical densities of 0.495 and 0.320 at 350 and 470 mμ, respectively, as determined in the Beckman spectrophotometer (light path, 1 cm.).

The present modification of the ninhydrin method may be applied to the determination of proline. Certain other amino acids, including lysine, cysteine, and α,ε-diaminopimelic acid,[8] also produce colors.

*Standard Conditions for Enzyme Assays*—Assays are performed in 4 inch test-tubes by incubating potassium phosphate at pH 7 (50 μmoles), $N^{\alpha}$-acetylornithine (3 μmoles), glutathione (0.5 μmole), cobaltous chloride (0.1 μmole), and enzyme (usually added last)[9] in aqueous solution (total volume, 0.5 ml.) at 37° for 10 minutes.

*Colorimetry*—At the end of the incubation period the enzymatic reaction is stopped by the addition of 1.5 ml. of the ninhydrin reagent to each tube, and ornithine is determined as described above. In addition to the samples to be assayed, reference blanks (substrate and enzyme omitted), enzyme blanks (substrate omitted), and substrate blanks (enzyme omitted) are run. Assay results are computed by subtracting the values obtained for the respective enzyme and substrate blanks from the values for the assay samples. Typical blanks as well as the response to ornithine are shown in Table I.[10]

*Unit of Enzyme Activity*—1 unit of acetylornithinase activity is defined as that amount of enzyme which will catalyze the formation of 0.1 μmole of ornithine under standard conditions.

*Protein Determination*—Protein was determined by the method of Lowry *et al.* (8).

## EXPERIMENTAL

### *Partial Purification of Enzyme*[11]

*Crude Acetylornithinase*—For the preparation of acetylornithinase, *E. coli* (ATCC 9637) cells, grown on 3.5 liters of Medium E with 0.5 per cent dextrose, were harvested by centrifugation, suspended in 0.1 M phosphate buffer at pH 7, collected again, and resuspended in phosphate buffer to give a final volume of about 35 ml. The resulting suspension was sub-

[8] Kindly furnished by Dr. C. Gilvarg.

[9] When necessary, enzyme preparations are diluted in 0.1 M phosphate buffer (pH 7) made 1 mM in glutathione.

[10] The absolute magnitude of the color response was found to vary somewhat from batch to batch of the reagents used.

[11] Carried out at 0–5°.

jected to sonic vibration in a 9 kc. Raytheon oscillator for 30 minutes to yield a crude extract of acetylornithinase.

*Protamine Treatment and Precipitation with Ammonium Sulfate*—The crude extract obtained was treated with protamine sulfate (210 mg.), dissolved in 0.1 M phosphate at pH 7 (7 ml.). The resulting precipitate was removed by centrifugation. The supernatant solution was treated similarly with half the amount of protamine sulfate previously used, and the precipitate formed was again removed. In the supernatant liquid (37 ml.), ammonium sulfate (16 gm., 60 per cent of saturation) was dissolved with stirring. The resulting precipitate was collected, dissolved in 20 ml. of 0.1 M phosphate at pH 7, and reprecipitated by addition of ammonium sulfate (8.6 gm., 60 per cent of saturation). The precipitate was then collected and dissolved by addition of 0.1 M phosphate buffer at

TABLE I

*Color Response Obtained with Ninhydrin Reagent*

| Sample | Colorimeter reading* | Sample | Colorimeter reading* |
|---|---|---|---|
| Reference blank | 0 | Ornithine, 0.1 μmole | 160 |
| Substrate " | 7 | " 0.2 " | 318 |
| Enzyme blank | 2 | " 0.3 " | 482 |

* A Klett-Summerson instrument with No. 42 filter was used. The reference blank was set at zero; it gives a reading of about 15 against distilled water set at zero.

pH 7, made 1 mM in glutathione. The final volume of the solution obtained (PS + $AS_{0-60}$) was 17.5 ml.

*Fractionation with Ammonium Sulfate*—In the solution obtained (PS + $AS_{0-60}$), ammonium sulfate (3.8 gm., 30 per cent of saturation) was dissolved with stirring, and the resulting precipitate was collected and dissolved by addition of phosphate buffer made 1 mM in glutathione (14.5 ml.). The solution obtained ($AS_{0-30}$) had a volume of 15 ml. Solution $AS_{0-30}$ was treated with ammonium sulfate (3.0 gm., 28 per cent of saturation) and the precipitate formed was removed by centrifugation and discarded. The supernatant solution was then treated with ammonium sulfate (0.75 gm., 35 per cent of saturation), and the resulting precipitate was dissolved in phosphate buffer, 1 mM in glutathione, to give a volume of 5 ml. ($AS_{28-35}$).

*Fractionation with Acetone*—To solution $AS_{28-35}$, acetone (3.5 ml., 41 per cent by volume), previously chilled to 0°, was added with stirring. The precipitate obtained was removed by centrifugation and cold acetone

(4.0 ml., 60 per cent by volume) was added to the supernatant solution. A precipitate formed which was dissolved in phosphate buffer at pH 7 made 1 mM in glutathione to give a volume of 5 ml. The resulting solution was finally dialyzed against the same glutathione-containing buffer. The final dialyzed preparation $Ac_{41-60}$ had a volume of 5 ml. and was stored at $-15°$.

The procedure described results in a 30- to 40-fold purified preparation with an over-all yield of about 25 per cent. The progress of a typical purification is summarized in Table II. Unless otherwise stated, the experiments reported were performed with at least 30-fold purified preparations.

TABLE II

*Summary of Enzyme Purification*

| Fraction* | Total volume | Protein | Enzyme activity | Specific activity | Over-all yield |
|---|---|---|---|---|---|
| | *ml.* | *mg. per ml.* | *units per ml.* | *units per mg.* | *per cent* |
| Crude extract | 35.0 | 37.4 | 950 | 25 | 100 |
| PS + $AS_{0-60}$ | 17.5 | 21.7 | 1420 | 65 | 75 |
| $AS_{0-30}$ | 15.0 | 9.0 | 1340 | 149 | 60 |
| $AS_{28-35}$ | 5.0 | 8.9 | 2750 | 309 | 41 |
| $Ac_{41-60}$ | 5.0 | 2.1 | 1680 | 800 | 25 |

* PS, protamine sulfate; AS, ammonium sulfate; Ac, acetone. The subscripts used with AS refer to per cent of saturation; that used with Ac refers to per cent of volume. See the text for details.

## *Properties of Enzyme and of Reaction*

*Reaction Products*—L-Ornithine has previously been recognized as one of the products of the enzymatic reaction (1). In the present experiments, the formation of 0.93 mole of acetate could be demonstrated per mole of ornithine produced. The acetate was determined by passing the reaction mixture through a bed of the acid form of a sulfonic type ion exchanger, distilling the eluate, and titrating the distillate with alkali. The value obtained is in agreement, within the accuracy of the method used, with the expected equimolar ratio of acetate to ornithine formed.

*Extent of Reaction*—To ascertain the extent of the reaction, 40 units of enzyme were permitted to act on the substrate. The conditions were standard, except that an incubation period of 3 hours was used. The reaction mixture was then appropriately diluted and assayed for ornithine. The results showed that the reaction had gone to completion within the sensitivity of the method employed.

*Time-Course and Dependence on Enzyme Concentration*—Under standard

conditions with an amount of enzyme of the order of magnitude of 1 unit, the quantity of substrate cleaved was found to be proportional to time for at least 20 minutes. Over a range of enzyme concentrations, including 0.5 to 3 units per 0.5 ml., the initial rate of cleavage was proportional to enzyme concentration.

*Effect of Cobalt*—It has been briefly reported that acetylornithinase is stimulated by the cobaltous ion (1). This stimulatory effect is illustrated in Table III. Preincubation of the enzyme in the presence of 0.2 mM cobaltous chloride failed to increase the reaction rate over that obtained under standard conditions. Of a number of other metal ions tested, none proved stimulatory and some were found to be inhibitory (see below).[12]

*Effect of Glutathione*—The stimulatory effect of added glutathione is also presented in Table III. If enzyme preparations are stored in phos-

TABLE III

*Stimulation of Acetylornithinase Activity by Cobaltous Ion and Glutathione*

| $CoCl_2$ | Glutathione | Relative activity* | $CoCl_2$ | Glutathione | Relative activity* |
|---|---|---|---|---|---|
| *mM* | *mM* | | *mM* | *mM* | |
| 0 | 1.0 | 50 | 0.20 | 0.2 | 40 |
| 0.02 | 1.0 | 70 | 0.20 | 1.0 | 100 |
| 0.20 | 1.0 | 100 | 0.20 | 2.0 | 100 |
| 0.40 | 1.0 | 100 | | | |

* Each value was obtained with 1 unit of enzyme. The conditions used were standard, except for the variations shown.

phate buffer without glutathione, their activity decreases markedly; such preparations can be largely reactivated by addition of glutathione.[13] A number of reducing agents tested, including thioglycolate, ascorbate, and hydrosulfite, failed to produce the same stimulating and preserving effect as glutathione.

*Effect of pH and Buffers*—The optimal pH for the action of acetylornithinase was found to be about 7.0, as shown in Table IV. The reaction rate was unchanged when a sodium phosphate buffer at pH 7 was substituted for potassium phosphate. A number of other buffers, in-

[12] Upon addition of alkali in the determination of ornithine, certain heavy metal ions produced extraneous colors which in general faded within several hours; the extraneous color produced by the cobaltous ion was found to fade within 20 minutes.

[13] 30- to 40-fold purified preparations, when stored at −15° in the presence of glutathione, were found to retain their activity for at least several months; however, on repeated thawing and freezing the activity tended to diminish. 12-fold purified preparations, such as $AS_{28-35}$ (see Table II), were more resistant to thawing and freezing.

cluding tris(hydroxymethyl)aminomethane, cacodylate, succinate, bicarbonate, Veronal-acetate, and pyrophosphate, were tested as substitutes for phosphate. The conditions were standard, except for the buffers, and the pH range used was 6.5 to 7.5. None of the buffers examined permitted as high a reaction rate as phosphate at corresponding pH values.

TABLE IV

*Effect of pH on Acetylornithinase Activity*

| pH | Relative activity* |
|---|---|
| 6.0 | 33 |
| 6.5 | 75 |
| 7.0 | 100 |
| 7.5 | 87 |
| 8.0 | 54 |

* Each value was obtained with 1 unit of enzyme. The conditions employed were standard, except that the pH was varied by using suitable mixtures of $KH_2PO_4$ and $K_2HPO_4$ at constant total phosphate concentration.

TABLE V

*Effect of Various Substances on Acetylornithinase Activity*

| Substance | Relative activity* | Substance | Relative activity* |
|---|---|---|---|
| None | 100† | $Cu^{++}$ | 60 |
| $Mg^{++}$ | 95 | $Zn^{++}$ | 16 |
| $Ca^{++}$ | 95 | $Ni^{++}$ | 27 |
| $Mn^{++}$ | 96 | Ethylenediaminetetraacetate | 57 |
| $Fe^{++}$ | 99 | *p*-Chloromercuribenzoate | 43 |

* Each value was obtained with 2 units of enzyme. *p*-Chloromercuribenzoate was tested at a concentration of 1 mM under standard conditions. All other substances listed were tested at a concentration of 0.2 mM under standard conditions modified by the omission of cobaltous chloride.

† This value was obtained under standard conditions modified by the omission of cobaltous chloride.

*Dependence on Substrate Concentration*—The effect of substrate concentration on the reaction velocity was examined over a range of initial concentrations from 1 to 8 mM under otherwise standard conditions. The substrate concentration giving half maximal velocity was calculated (9) to be $K_s = 2.8$ mM.

*Metals and Inhibitors*—The effect of certain divalent metal ions was studied by substituting them singly for the cobaltous ion.[12] Stimulation of acetylornithinase activity was obtained with the cobaltous ion only.

Salts of magnesium, calcium, manganese, and iron, at the concentrations tested, affected the reaction but slightly; however, salts of copper, zinc, and nickel proved inhibitory (see Table V).

Acetylornithinase was also found to be inhibited by the metal binder, ethylenediaminetetraacetate, and by the sulfhydryl reagent, *p*-chloromercuribenzoate, as shown in Table V.[14]

*Specificity*—A number of acylamino acids, including acetyl-DL-alanine, acetyl-DL-valine, acetyl-DL-leucine, acetyl-DL-methionine, acetyl-DL-proline, acetyl-DL-glutamate, chloroacetyl-L-tyrosine, and benzoyl-L-arginine, were tested (6) as possible substrates for acetylornithinase preparations. Under the conditions employed, only acetyl-DL-methionine was found to be deacetylated at a rate approximating that of acetylornithine. The cleavage of acetylmethionine, like that of acetylornithine, was found to be stimulated by the cobaltous ion and by glutathione. The ratio acetylornithinase to acetylmethioninase activity was found to be approximately constant in crude, 12-fold, and 30-fold purified preparations. Extracts of the ornithine-requiring mutant 160-37 of *E. coli*, which had previously been shown to contain no detectable acetylornithinase (5, 1), have now been shown to be devoid also of detectable acetylmethioninase activity. It therefore appears that the cleavage of acetylmethionine is largely, if not exclusively, mediated by acetylornithinase.

## DISCUSSION

The present study has revealed that acetylornithinase resembles other acylases (10, 11) and certain peptidases (12) in a number of features. These features include the observed stimulation by the cobaltous ion, which in the present case appears to be specific. The stimulating and preserving action of glutathione and the inhibiting action of *p*-chloromercuribenzoate are also shared with functionally related enzymes. For example, prolidase (13) from animal (14) and microbial (15) sources is similar to acetylornithinase in being both a "metal" and "sulfhydryl" enzyme. Furthermore, the enhanced reaction rates with phosphate compared to those obtained with other buffers are not without precedent. In some cases, this enhancement has been ascribed to the selective binding of toxic metal ions by the phosphate (*cf.* (12)).

In view of the voluminous earlier work on acetylamino acids and enzymes that participate in their metabolism, it seems of special interest that acetylornithinase was found to have a definite biosynthetic function, namely the catalysis of a step in the biosynthesis of ornithine (1). More-

[14] Although glutathione was found to antagonize the inhibitory effect of *p*-chloromercuribenzoate, the latter was tested under standard conditions (in the presence of glutathione) because of the instability of the enzyme in the absence of glutathione.

over, the function of this acylase appears to be an essential one, at least under the conditions employed, since it could be shown (5, 1) by means of an *E. coli* mutant that absence of detectable acetylornithinase is associated with a growth requirement for ornithine (or an equivalent metabolite).

Acetylornithinase activity has been demonstrated in all of several Enterobacteriaceae tested, including strains K-12 and B of *E. coli* and strains of *Aerobacter*, *Klebsiella*, *Erwinia*, *Serratia*, *Proteus*, *Salmonella*, and *Shigella* (16).[15] In contrast, no appreciable acetylornithinase activity could be detected in *Neurospora crassa* (17), several other fungi, or certain Bacillaceae (16).[15] The presence of acetylornithinase activity in the Enterobacteriaceae suggests that they, like *E. coli*, produce ornithine via acetylated intermediates. On the other hand, the absence of detectable acetylornithinase activity in the Bacillaceae and fungi provides evidence that these organisms do not use an acetylation mechanism in ornithine synthesis. It is not unlikely that the Bacillaceae as well as the fungi synthesize ornithine from glutamic $\gamma$-semialdehyde, as has been reported for *N. crassa* (17) and *Torulopsis utilis* (18).

## SUMMARY

Acetylornithinase has been extracted from *Escherichia coli* and partially purified by a procedure including treatment with protamine and fractionation with ammonium sulfate and acetone.

Acetylornithinase appears to be specifically stimulated by the cobaltous ion and by glutathione; it is inhibited by the divalent ions of copper, zinc, and nickel as well as by ethylenediaminetetraacetate and *p*-chloromercuribenzoate.

Under the conditions used, the substrate concentration giving half maximal velocity is 2.8 mM, and the pH optimum of the enzymatic reaction is about 7.0.

Acetylornithinase appears capable of deacetylating *N*-acetylmethionine at a rate approximating that obtained with $N^{\alpha}$-acetylornithine.

Enzyme assays have been carried out by means of a convenient modification of the ninhydrin method of determining ornithine.

A simple growth medium for the organisms used has also been described.

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