DEGRADATION OF ERGOTHIONEINE BY CELL-FREE EXTRACTS OF *ALCALIGENES FAECALIS*[1]

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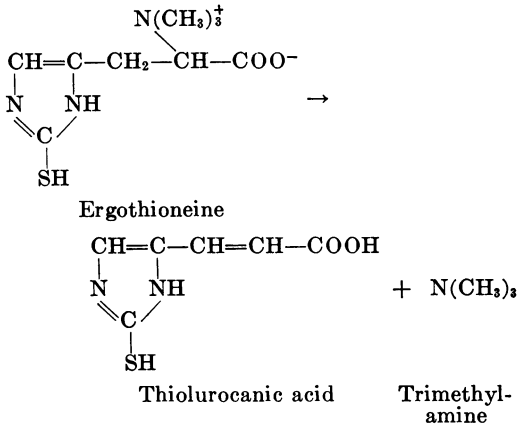
Yanasugondha and Appleman (1957) reported that *Alcaligenes faecalis* is capable of adaptively degrading ergothioneine, with the production of thiolurocanic acid and trimethylamine, followed by the disappearance of the thiolurocanic acid, presumably due to its degradation to unknown products:



Cell-free enzyme preparations have now been shown to be effective in degrading ergothioneine, the production of thiolurocanic acid has been separated from the rest of the degradative pathway, and additional data in support of the identification of the first isolated product as thiolurocanic acid have been offered. A more convenient method has been developed for studying reactions in which thiolurocanic acid is concerned.

MATERIALS AND METHODS

Ergothioneine·HCl·H_2O was obtained from the California Corporation for Biochemical Research.

Enzyme preparations and cell suspensions were usually prepared from cells which had been grown in nutrient broth (Difco) plus yeast extract and then adapted by incubating overnight in phosphate buffer containing 1.0 μmole ergothioneine per ml. In a few experiments cells were grown in a defined medium and then adapted by the same procedure.

The phosphate buffer used was a mixture of 0.067 M KH_2PO_4 and 0.067 M Na_2HPO_4. In the earlier experiments buffer at pH 7.0 was used. Later, buffer at pH 8.0 was substituted.

The defined medium had the following composition: lactic acid, 2.5 ml; NaOH, 1.12 g; asparagine, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1.5 g; NaCl, 8.0 g; and distilled water, 1,000 ml; adjusted to pH 8.0.

Enzyme preparations. 1) Glass-ground cells: Washed cells of *A. faecalis* were placed in a cold mortar with double the amount of powdered glass, and ground to a paste, the entire procedure taking approximately 5 min. Cold phosphate buffer, of the same pH as that used for the adapting procedure, was added. Usually 20 ml of buffer were used per batch of cells, approximately 3.5 g wet weight. The preparation was extracted for 20 to 40 min at 4 C, and glass and debris were removed by high speed centrifugation.

2) Sonic preparations: Washed cells of *A. faecalis* were suspended in phosphate buffer, 20 ml per 3.5 g wet weight of cells, and treated for 8 min at 0.76 amp in a Raytheon sonic oscillator model DF 101. After extracting for 20 to 40 min at 4 C, the debris was removed by high speed centrifugation.

The protein concentration of the glass-ground preparations, as estimated by the trichloroacetic method, using egg albumin as a standard, was 7 to 9 mg per ml, whereas that of the sonic preparations was 13 to 15 mg per ml.

In some experiments the enzyme preparations were fractionated by precipitation with $(NH_4)_2SO_4$, the calculated amount of crystalline salt being dissolved in the preparation. The mixture was allowed to stand 20 to 30 min at 4 C before

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centrifuging and suspending in the original quantity of phosphate buffer.

Chromatography. Chromatograms were developed on Whatman no. 1 paper by the ascending technique in butanol-acetic acid-water (4:1:5) using the upper phase to which 10% excess butanol was added.

Developed chromatograms were examined under ultraviolet light (Mineralight model SL 2537) under which ergothioneine appears as a dark spot, whereas thiolurocanic acid produces a bright fluorescent spot.

Chromatograms were also sprayed with diazo reagent (Ames and Mitchell, 1952) with which both ergothioneine and thiolurocanic acid give orange-red spots, and with 0.2% 2,6-dichloroquinonechloroimide in ethyl alcohol, with which ergothioneine appears brick red and thiolurocanic acid purple (Heath and Wildy, 1956).

In some experiments ninhydrin spray reagent was used: 0.25% solution in 95% butanol in water. The paper was heated 10 min at 80 C before reading.

Reaction mixtures for chromatography. Aerobic reaction mixtures for chromatography consisted of 0.2 ml enzyme preparation or cells in phosphate buffer, 0.1 ml substrate (final concentration 5 μmoles per ml), and 0.1 ml phosphate buffer. At intervals, 0.01-ml amounts were removed and spotted for chromatography without removing proteins.

Anaerobic experiments for chromatographic analysis were set up in a series of Warburg flasks containing 0.5 ml enzyme preparation or cells suspended in phosphate buffer, 0.25 ml ergothioneine (final concentration 5 μmoles per ml), and 0.25 ml phosphate buffer. The flasks were flushed with nitrogen for 20 min and then sealed before the substrate was tipped into the reaction mixture.

Hydrogen sulfide production. In some chromatographic experiments, H_2S production was read qualitatively by using lead acetate paper strips. In aerobic experiments the strips were suspended in the necks of reaction tubes. In anaerobic experiments they were placed in the center well of Warburg flasks.

Spectrophotometry. The disappearance of ergothioneine in reaction mixtures was followed using a spectrophotometer at a wavelength of 258 mμ. It was found that as ergothioneine disappeared a new peak at 314 mμ appeared. Since subsequent testing of crystalline thiolurocanic acid showed this maximum to be a property of thiolurocanic acid, in later experiments the appearance and disappearance of thiolurocanic acid were followed spectrophotometrically at 314 mμ. Aerobic reactions were followed directly in cuvettes containing 2.85 ml phosphate buffer, 0.15 ml substrate, and 0.02 ml enzyme preparation. Anaerobic reactions were followed by setting up a series of Thunberg tubes, each containing the above components. The tubes were flushed with N_2 and gassed for 20 min before the substrate was tipped in. At intervals one tube was opened, and the contents were transferred to a cuvette and read immediately.

RESULTS AND DISCUSSION

A crude glass-ground or sonic preparation derived from ergothioneine-adapted cells is capable of degrading ergothioneine with the production of thiolurocanic acid, and of further degrading thiolurocanic acid.

By the use of an enzyme dialyzed against 0.033 M phosphate buffer, pH 8.0, for 24 to 72 hr, or simply aged in the refrigerator for 72 hr, it was found possible to degrade ergothioneine to thiolurocanic acid while substantially eliminating subsequent degradative steps.

In Fig. 1*A* the production of thiolurocanic acid from ergothioneine, as determined by the increase in optical density at 314 mμ, is shown with crude enzyme, dialyzed enzyme, and aged, refrigerated enzyme. In Fig. 1*B* the degradation of thiolurocanic acid, as judged by decrease in optical density at 314 mμ, is shown with the same enzyme preparations.

In some preparations of enzyme, $MnCl_2$ (0.05 M final concentration) was added to remove nucleic acids. No loss of activity against thiolurocanic acid occurred on aging in the refrigerator up to 6 days, although this activity was lost upon dialyzing against 0.033 M phosphate buffer. Addition of $MnCl_2$ to a dialyzed enzyme did not restore the ability to degrade thiolurocanic acid.

In enzymes fractionated with $(NH_4)_2SO_4$ the bulk of the activity was distributed between the 20 to 40 and the 40 to 60 fractions. No significant separation of activity against ergothioneine and activity against thiolurocanic acid was achieved by this means.

A sample of thiolurocanic acid was prepared enzymatically from ergothioneine, using $MnCl_2$

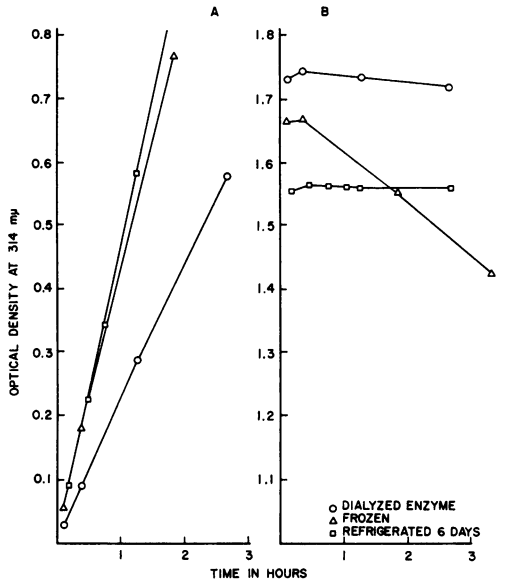

Fig. 1 Accumulation and degradation of thiolurocanic acid with dialyzed, refrigerated, and frozen enzyme preparations. *A*, Production of thiolurocanic acid from ergothioneine. *B*, Degradation of thiolurocanic acid. Contents of cuvettes: 0.02 ml enzyme preparation, 2.85 ml phosphate buffer, (pH 8.0), 0.15 ml substrate in phosphate buffer (pH 8.0). (*A*) Ergothioneine, 0.10 μmole per ml final concentration. (*B*) Thiolurocanic acid approximately 0.075 μmole per ml final concentration.

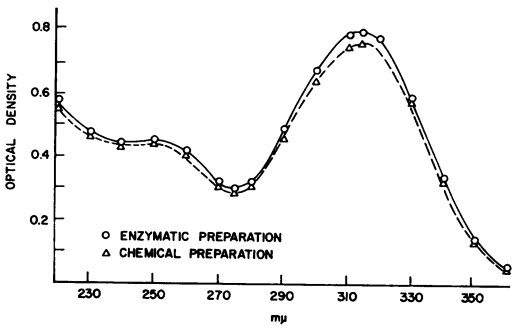

Fig. 2. Comparison of enzymatically and chemically prepared thiolurocanic acid. Contents of cuvettes: 0.05 μmole per ml solutions in 7.5×10^{-5} N NaOH.

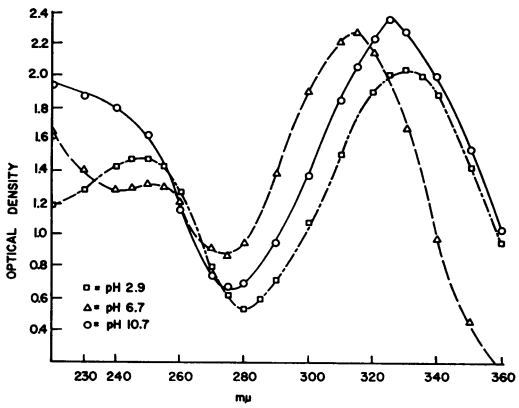

Fig. 3. Ultraviolet spectrum of thiolurocanic acid at different pH values. A few crystals of thiolurocanic acid were dissolved in 0.01 N NaOH and adjusted to the indicated pH by addition of a few drops of 0.1 N HCl.

treated, dialyzed enzyme, and purifying by repeated crystallization from HCl. From 113.4 mg of ergothioneine, 33.1 mg (49%) of bright yellow crystals of thiolurocanic acid were obtained. For comparison, a sample was prepared chemically from ergothioneine by the method of Barger and Ewins (1911), a yield of 19.3 mg of thiolurocanic acid being obtained from 200 mg ergothioneine (16.4%).

The enzymatic and chemical preparations were compared in several ways. On a Fisher-Johns melting point apparatus, the chemical preparation began to char at 265 C, the enzymatic preparations at 275 C. Both were dark brown but not melted at 295 C.

Ultraviolet absorption spectra were compared, as shown in Fig. 2, and proved to be almost identical, with maxima at 314 and 250 mμ, a shallow dip at approximately 240 mμ, and a sharp minimum at 275 mμ. The ratios of extinctions at 314 mμ and at 275 mμ are 2.63 for the enzymatic preparation, and 2.65 for the chemical preparation.

Over a pH range from 6.0 to 9.0, the spectrum of thiolurocanic acid remains essentially the same. Outside this range a reversible shift takes place, as shown in Fig. 3. At 314 mμ the optical density is proportional to the concentration of thiolurocanic acid.

The strong absorption at 314 mμ at physiological pH values provides a convenient method for following enzymatic reactions, eliminating the need for a laborious, and possibly less specific, method such as that previously used in this laboratory. Neither ergothioneine nor urocanic acid gives significant absorption at 314 mμ. The possibility that unknown products with similar absorption characteristics may occur in the reaction

mixtures cannot be eliminated until the complete series of degradative products is known.

The enzymatic and chemical preparations of thiolurocanic acid were also compared by chromatographing individual and mixed solutions. Development in butanol-acetic acid-water, and in ether-formic acid-water (5:2:1) did not separate the mixed solutions. Examination with ultraviolet light showed fluorescent spots with R_F 0.7 on the ether-formic acid-water papers, and with R_F 0.74 on the butanol-acetic acid papers. When sprayed with diazo reagent an orange red color appeared in areas corresponding to the fluorescent spots. An additional faint spot (R_F 0.18), showing a yellow color with diazo reagent, appeared on both the individual and mixed samples developed in butanol-acetic acid-water. This spot could be eliminated by developing in the dark and possibly represented a photochemical degradation product of thiolurocanic acid.

Thiolurocanic acid in solution proved to be relatively unstable. Chromatography of a solution (either in the dark or in the light, after storage for several days at refrigerator temperature) showed a series of spots, including one corresponding (in position and in reaction to spray reagents) to urocanic acid, i.e., R_F approximately 0.5, dark under ultraviolet light, red with diazo reagent, no reaction with 2,6-dichloroquinonechloroimide. Thiolurocanic acid was therefore used as a substrate for enzyme activity only in short term experiments using freshly prepared solutions.

In later work it was found that under certain ill-defined conditions, double fluorescent spots were occasionally produced from freshly dissolved crystalline thiolurocanic acid, whether enzymatically or chemically produced. Although attempts to define the conditions under which these double spots appeared were hampered by the small amount of material available for experimentation, the phenomenon appeared to be independent of pH and redox potential.

The production of thiolurocanic acid from ergothioneine by a crude enzyme preparation, and degradation of thiolurocanic acid by the same crude preparation were compared at pH 6, 7, and 8 by comparing the reactions spectrophotometrically at 314 mμ (Fig. 4). Since both reactions proceeded most rapidly at pH 8.0, phosphate buffer at this pH was used routinely in subsequent experiments. In previous work (Yanasugondha and Appleman, 1957) the degradation of thiolurocanic acid was found to be most rapid at pH 7.0. But in these experiments whole cells were incubated with ergothioneine, and degradation of thiolurocanic acid was determined indirectly.

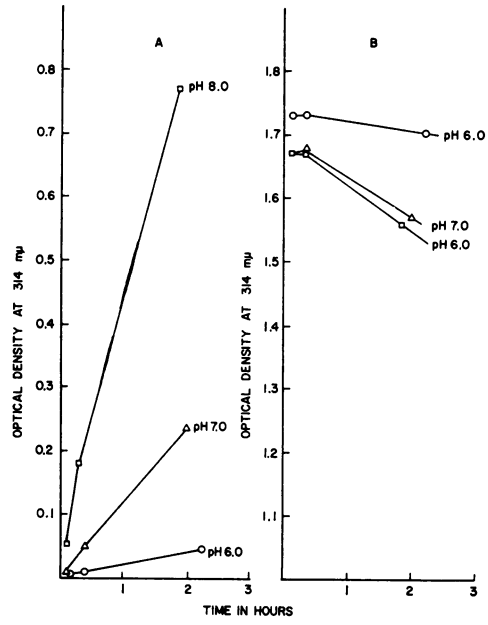

Fig. 4. Accumulation and degradation of thiolurocanic acid at pH 6.0, 7.0, and 8.0. *A*, Production of thiolurocanic acid from ergothioneine. *B*, Degradation of thiolurocanic acid. Contents of cuvettes: 0.02 ml crude, undialyzed enzyme preparation, 2.85 ml phosphate buffer of the indicated pH, 0.15 ml substrate in phosphate buffer (pH 8.0). (*A*) Ergothioneine, 0.10 μmole per ml final concentration. (*B*) Thiolurocanic acid, approximately 0.075 μmole per ml final concentration.

The crude enzyme produces H_2S from ergothioneine and from thiolurocanic acid. The dialyzed enzyme, which does not degrade thiolurocanic acid, does not produce H_2S. Experiments are continuing in an effort to determine whether the H_2S is produced directly from thiolurocanic acid or if there are intermediate products involved. Preliminary attempts to separate the degradation of thiolurocanic acid from the production of H_2S by fractionation of the enzyme with $(NH_4)_2SO_4$, by heating, and by treatment with metabolic poisons have been unsuccessful. Two possible sulfur containing intermediates, thiourea and thiocyanate, were tested with crude enzyme preparations capable of rapid production

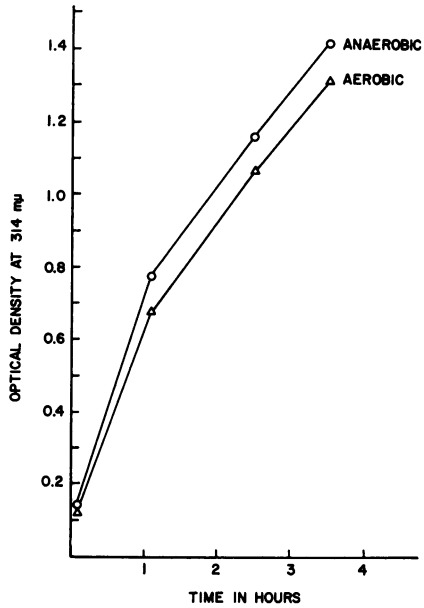

Fig. 5. Aerobic and anaerobic production of thiolurocanic acid from ergothioneine. Three milliliters phosphate buffer (pH 8.0) and 0.03 ml dialyzed enzyme preparation were placed in a series of Thunberg tubes. After alternately evacuating and filling with N_2, tubes in the anaerobic series were gassed for 20 min. Ergothioneine, 0.15 ml of a 2 μmoles per ml solution, was tipped in from the side arm and the tubes were incubated at room temperature. At intervals as shown, one tube of each series was opened and read immediately on the spectrophotometer.

of H_2S from thiolurocanic acid, but showed no activity. The possibility was considered that pyridoxal phosphate, which is necessary for the production of H_2S from cystine, might be a cofactor in this system. But attempts to restore the ability to degrade thiolurocanic acid, and to produce H_2S to dialyzed enzymes by the addition of pyridoxal phosphate, with and without Mg and Mn, have been unsuccessful.

Anaerobically, with crude undialyzed enzyme and with whole cells, the degradation of ergothioneine proceeds beyond thiolurocanic acid, as determined chromatographically and by the production of H_2S. The rate of production of thiolurocanic acid by a dialyzed enzyme preparation is essentially the same anaerobically and aerobically, as determined spectrophotometrically (Fig. 5) on reactions carried out in a series of Thunberg tubes.

The possibility that thiolurocanic acid might be degraded to urocanic acid was considered. However, no accumulation of urocanic acid was observed as a result of enzymatic activity, either chromatographically or spectrophotometrically at 277 mμ, with any of the various enzyme preparations used.

The enzyme as usually prepared (from cells adapted to ergothioneine after previous growth in nutrient broth plus yeast extract) was able to degrade urocanic acid, as determined both chromatographically and spectrophotometrically. However, preparations obtained from cells similarly adapted after being grown in a defined medium, although able to degrade ergothioneine and thiolurocanic acid, were unable to attack urocanic acid. It seems probable that the ability to attack urocanic acid found in some of the enzyme preparations is a result of adaptation to the histidine present in the complex growth medium, rather than a result of adaptation to ergothioneine, since urocanic acid is a known degradation product of histidine (Magasanik and Bowser, 1955; Tabor et al., 1952).

SUMMARY

Degradation of ergothioneine by cell-free enzyme preparations from *Alcaligenes faecalis*, with production of thiolurocanic acid as an intermediate product, has been shown. The production and degradation of thiolurocanic acid can be followed spectrophotometrically at 314 mμ.

Dialysis of a crude enzyme preparation against 0.033 M phosphate buffer, or refrigeration for several days, destroys the ability to degrade thiolurocanic acid, but the ability to produce thiolurocanic acid from ergothioneine is retained.

Crude enzyme preparations and whole cells further degrade thiolurocanic acid with the production of H_2S and unknown products. No evidence for the occurrence of urocanic acid in the reaction sequence was obtained.

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