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Acetoacetate Decarboxylase. Preparation of the Enzyme*

Burt Zerner,† Stephen M. Coutts,‡ Florence Lederer,§ Horace H. Waters, and F. H. Westheimer

ABSTRACT: Crystalline acetoacetate decarboxylase can be prepared from *Clostridium acetobutylicum* by the following steps: Preparation of an acetone powder from the bacteria, extraction of this powder at pH 5.9, precipitation of the enzyme at pH 3.8, a single fractionation with ammonium sulfate, and chromatography over diethylaminoethylcellulose, followed by crystallization. The yield of crystalline enzyme is about 180 mg from 120 l. of bacterial broth.

This is the first of a series of four papers describing the preparation, properties, and mechanism of action of acetoacetate decarboxylase. Earlier work was reviewed in 1963 (Westheimer). Crystalline acetoacetate decarboxylase from *Clostridium acetobutylicum* (American Type Culture Collection 862) was announced in an earlier communication (Hamilton and Westheimer, 1959) and a scheme for the preparation of purified but noncrystalline enzyme has been published from this laboratory (Fridovich, 1963). This paper describes the production, in about 70% yield from the initial extracts, of crystalline enzyme with several times higher specific activity than that obtained previously. Particular emphasis is placed on the changes in procedure which allow five times as much enzyme to be prepared per week. The bacterial strain (ATCC 862) with which the work began has been lost, but the work was continued with a presumably identical strain, which was kindly donated by the Northern Utilization Research and Development Division of the Department of Agriculture in Peoria.[1]

A slight modification of the spectrophotometric assay of Fridovich was used throughout. One (arbitrary) activity unit is defined as the enzyme required to produce a change in optical density at 270 mμ of one unit per 100 sec in a solution of 0.03 M acetoacetate at 30° at pH 5.9. Calibration experiments with a Warburg apparatus show that one arbitrary unit of enzyme activity corresponds to the formation of 14.5 μmoles of carbon dioxide per mg of enzyme per minute. The growth of the bacteria is described in the Experimental Section. Here it should be noted (Thimann, 1955) that the decarboxylase is not a constitutive enzyme of the *Clostridia*, but is produced late in their growth cycle, presumably as part of a defense mechanism against the accumulation of acetoacetic acid in the medium. Furthermore, after repeated transfers the bacteria, although viable, lose their ability to make the enzyme. In our laboratory, growth was carried out in six 20-l. carboys at a time, and when the bacteria were harvested, half of one carboy was used for transfer to six carboys containing fresh growth medium. These transfers could be repeated successfully about half a dozen times, but then growth had to be begun again in test tube cultures from spores.

The preparation of the crystalline enzyme depends on

* From the James Bryant Conant Laboratory of the Department of Chemistry, Harvard University, Cambridge, Massachusetts. *Received October 18, 1965.* This work was supported by Grant GM 04712 from the Institute of General Medical Studies of the National Institutes of Health. Preliminary communication: Hamilton and Westheimer (1959).

† Present address: Biochemistry Department, Queensland University, Brisbane, Australia.

‡ National Science Foundation Predoctoral Fellow 1962–1965.

§ Centre National de la Recherche Scientifique; NATO Fellow 1963-1965.

[1] Letters to F. H. Westheimer from R. W. Jackson and C. W. Hesseltine. Because of a copying error, the bacterial strain was incorrectly referred to as B-527 in an earlier paper (Fridovich, 1963).

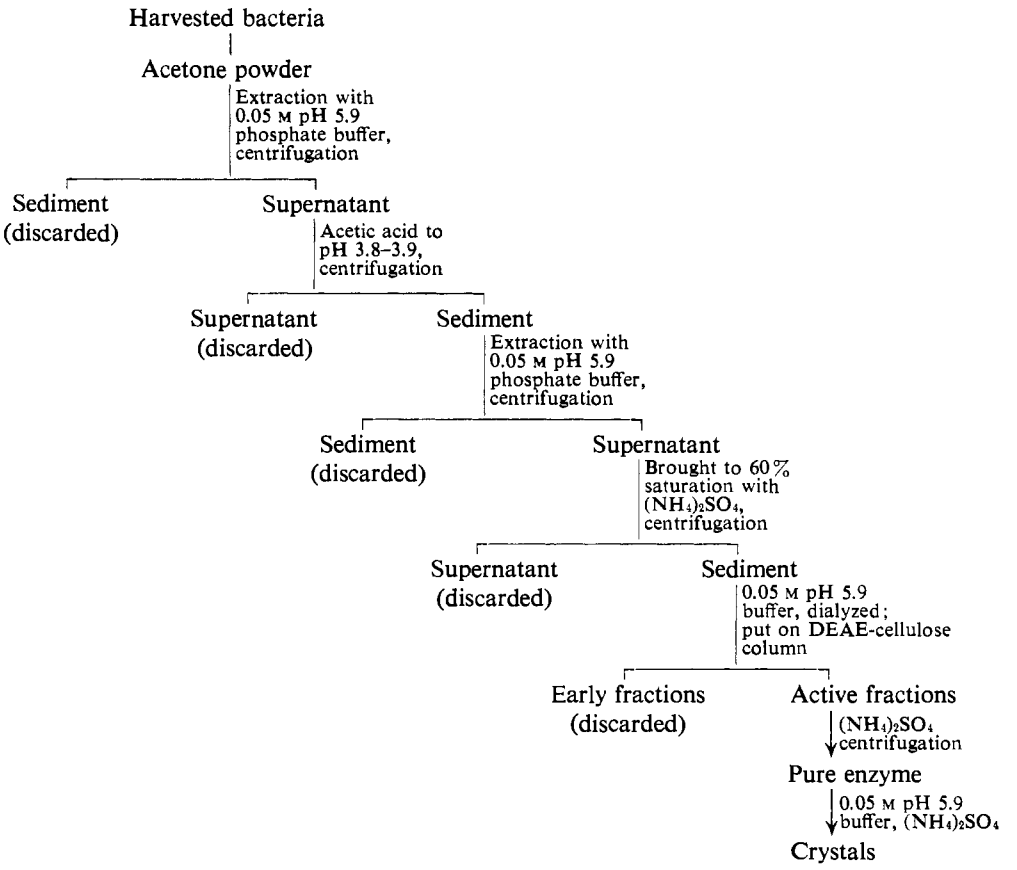

FIGURE 1: Enzyme purification scheme.

the strain of bacteria used. A similar method of preparation from *Clostridium madisonii* results in equally active enzyme (Colman, 1962), but the material is not crystalline or even homogeneous (Lee, 1965). The scheme for the purification of the enzyme is shown in Figure 1.

Experimental Section

Bacterial Growth. Spores of *Cl. acetobutylicum*, strain NRRL B-528 from the U. S. Department of Agriculture at Peoria, are grown in deep liver medium (Haynes *et al.*, 1955). The resulting culture can be used to prepare further samples of spores on sterile earth or sand (Colman, 1962). The bacteria are grown in test tubes from spores on sand in the following medium, autoclaved at 121°.

Growth Medium. SOLUTION A. Trypticase (Baltimore Biological Laboratory, Baltimore, Md.) 20 g, Bacto yeast extract (Difco Certified, Difco Laboratories, Detroit, Mich.) 1 g, KH_2PO_4 1 g, L-asparagine 600 mg, ferrous sulfate heptahydrate 20 mg, ascorbic acid 20 mg, salt solution 10 ml, distilled water 900 ml. The salt solution had the following composition: magnesium sulfate heptahydrate, 40 g/l., manganous sulfate monohydrate, 1.24 g/l., and sodium chloride, 2 g/l.

Growth Medium. SOLUTION B. L(+)Arabinose (20 g) in 100 ml of distilled water. Solutions A and B are separately autoclaved and mixed; about 15 ml of the mixed medium and about a half-teaspoonful of spores on sand are introduced into each test tube, and the tubes closed with sterile cotton. Growth is generally evident (gas evolution) after 24 hr. After 4–7 days, the contents of two test tubes per liter flask are used to initiate growth; each liter flask is filled with about 700 ml of growth medium of the same composition as that used for test tube growth. Finally, the contents of one of the one liter flasks is used to inoculate the medium in each carboy. All the transfers, at the test tube, liter, and carboy stages, are carried out with sterile techniques, using autoclaved medium and glassware, flaming of the mouths of opened flasks, using sterile cotton for plugs, face-masks for laboratory personnel, etc.

Carboy-Stage Culture. The growth medium for each 20-l. carboy consists of the following: (solution A) 720 g of trypticase, 36 g of yeast extract, 21.6 g of L-asparagine, and 2.5 l. of demineralized water; (solution B) 36 g of potassium hydrogen phosphate, 360 ml of salt solution, 720 mg of ferrous sulfate heptahydrate, 720 mg of ascorbic acid, about 1 ml (30 drops) of Dow Corning Antifoam B, and 12 l. of distilled water; (solution C) 720 g of technical grade sucrose and 2.5 l. of distilled water. Solutions A and C are autoclaved for 45 min at 121°; solution B is autoclaved for 90 min.

The solutions, after they are autoclaved, may be allowed to stand overnight before use. Solutions A and C are added to solution B in 20-l. carboys and inoculated; the carboys are plugged with sterile cotton and transferred to a 37° room, where they are placed under a hood to vent the hydrogen evolved during fermentation.

Preparation of Acetone Powder. After 3-days' growth, the bacteria are harvested with a Sharples Supercentrifuge. The acetone powder is prepared from the bacteria as described by Fridovich (1963) except that it is not dried with ether. The acetone is removed from the moist powder by placing it in a vacuum desiccator connected to a pump through a Dry Ice trap and pumping until no more acetone condenses in the trap. The powder is then stored in a deep-freeze until needed. About 250 g of acetone powder, with up to 10,000 activity units, is obtained from 120 l. of broth.

Purification of the Enzyme. Each 100 g of acetone powder is extracted for 2 hr at 37° on a rotary shaker with 1500 ml of 0.05 M phosphate buffer, pH 5.9. The most important step in the purification procedure consists in lowering the pH of the clear extract to 3.8–3.9 with 2 M acetic acid. In most cases the enzyme precipitates at this point with little loss of activity, with purification factors ranging, with different preparations, from 20- to 67-fold. (The pH is lowered to 3.8 when effective precipitation is not attained at 3.9.) Occasionally the activity remains in the supernatant, even at pH 3.8. Although the reason for the variation is unknown, purification can, nevertheless, be accomplished by a modification of the procedure. The suspension at pH 3.8 is centrifuged to remove inactive protein, and the enzyme is next precipitated, along with much other protein, by addition of ammonium sulfate to 30–50% saturation; as additions of ammonium sulfate are made, each precipitate and supernatant must be assayed. The protein is collected by centrifugation at 3900 rpm for 2 hr at 4°, and is resuspended in about 100 ml of 0.05 M buffer, pH 5.9, for each 100 g of acetone powder. Much of the proteins fails to dissolve, and the extraction may have to be repeated several times, sometimes with 0.1 M buffer, before most of the activity is separated from the precipitate and brought into solution. The suspension is clarified by centrifugation at 10,000 rpm and the enzyme precipitated by bringing the solution to 60% saturation with solid ammonium sulfate at 25°. When (as almost always occurs) the enzyme is precipitated at pH 3.9 or 3.8 with acetic acid, the extraction with pH 5.9 buffer is carried out with that precipitate, and the extract treated with ammonium sulfate as outlined above. Of course the procedure is then much shorter and easier, but the final result is in any case the same. The enzyme after precipitation with ammonium sulfate is again collected by centrifugation, dissolved in a minimum (10–15 ml) amount of 0.05 M phosphate buffer, and desalted by dialysis against pH 5.9 buffer or by passing the solution through a short column of Sephadex G-25.

Chromatography. The enzyme is then purified by chromatography, using a column set up with an automatic fraction collector and an ultraviolet absorption device (Gilson Medical Electronics) coupled to a recorder. A column of Type 40 DEAE cellulose (Brown Co.; 0.94 mequiv/g) 4 cm in diameter and only 11 cm long will serve to purify as much as 200 mg of protein at a time. The column is first equilibrated with pH 5.9 phosphate buffer, and then the protein is applied to the column in this buffer. The column is washed with buffer until no more material absorbing at 280 mμ appears, and then subjected to gradient elution where 0.1 M ammonium sulfate in 0.05 M phosphate buffer, pH 5.9, is introduced into 0.05 M phosphate buffer, pH 5.9. The enzyme is eluted as a well-defined peak with a sharp leading edge usually after about 300 ml of the gradient eluent have passed through the column. Although the enzyme is not completely separated from the protein that follows it, it is sufficiently pure for crystallization.

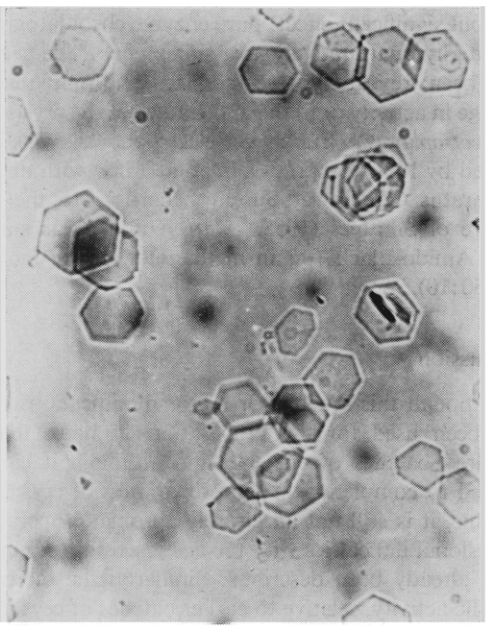

FIGURE 2: Photomicrograph of crystals of acetoacetate decarboxylase.

Crystallization. Samples where the 280 mμ/260 mμ ratio is 1.9 or greater are brought to 75% saturation with ammonium sulfate, and the precipitated protein collected by centrifugation at 10,000 rpm. It is again dissolved in a minimum amount (5–10 ml) of 0.05 M phosphate buffer, and crystallized at room temperature by the successive addition of small amounts of solid ammonium sulfate on a stirring rod. When such an addition produces turbidity, a few drops of buffer suffice to dissolve amorphous material; the resulting suspension of seeds promotes the gradual growth of fine crystals, which settle to the bottom of the tube after about 12 hr at 4°. They consist of thin hexagonal plates (see Figure 2). The activity of the enzyme is usually about 38 units/mg, although on one occasion significantly higher activity (about 50 units/mg) was noted. The activity is higher than that of the earlier samples of crystalline enzyme. The chromatography over diethylaminoethylcellulose has been repeated on crystallized enzyme

without significant effect; pure enzyme chromatographs essentially quantitatively as a single peak. The enzyme has been repeatedly recrystallized without marked change in activity or in the appearance of the crystals.

Electrophoresis. Starch gel electrophoresis was conducted by Mrs. Roberta Colman at 155 v with an E-C Apparatus Co. Power Supply according to the procedure of Smithies (1955, 1959). The gel was stained with Amido-Black 10B in methanol–water–acetic acid (50:50:10).

Discussion

Although this scheme, or minor modifications of it, has been used for the preparation of more than a hundred batches of enzyme, and although it can be carried to completion in the preparation of crystalline enzyme, it is still not an entirely automatic operation. Occasional difficulties with the acetic acid precipitation have already been described. Similarly, the increased specific activity, relative to earlier batches of crystalline enzyme, has been noted. Despite these variations, the evidence at hand indicates that the protein is essentially pure. This evidence includes the following: (1) the enzyme is pure with respect to column chromatography on diethylaminoethylcellulose; (2) the activity is unaffected by prolonged dialysis or repeated crystallizations; (3) the enzyme migrates as a single species on starch gel electrophoresis at pH 5.4; (4) the enzyme is monodisperse with respect to ultracentrifugation; (5) chemical evidence suggests a pure material; for example, only methionine has been found as an N-terminal group, and the stoichiometry on analysis is consistent with purity; (6) the spectral properties have not changed from one batch to the next. Ultracentrifugal and chemical evidence is presented in an accompanying paper (Lederer *et al.*, 1966). The enzyme from any particular batch appears to be unaffected by repeated attempts to further purify it. One possible explanation for the variability in activity is bacterial mutation. An alternative possibility is based on the finding that the decarboxylase is an induced enzyme. Perhaps in some batches of bacteria an inhibitor molecule is incompletely removed; if it is a small molecule, its presence might go undetected by the methods of purification used. The preparation of the enzyme continues under active investigation.

References

Colman, R. F. (1962), Ph.D. Thesis, Radcliffe College.
Fridovich, I. (1963), *J. Biol. Chem. 238*, 592.
Hamilton, G., and Westheimer, F. H. (1959), *J. Am. Chem. Soc. 81*, 2277.
Haynes, W. C., Wickerham, L. J., and Hesseltine, C. W. (1955), *Appl. Microbiol. 3*, 361.
Lederer, F., Coutts, S. M., Laursen, R. A., and Westheimer, F. H. (1966), *Biochemistry 5*, 823 (this issue).
Lee, P. (1965), Ph.D. Thesis, Harvard University.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Smithies, O. (1959), *Advan. Protein Chem. 14*, 65.
Thimann, K. (1955), The Life of Bacteria, New York, Macmillan, Chapter XV.
Westheimer, F. H. (1963), *Proc. Chem. Soc.*, 253.