SPECTROPHOTOMETRIC MEASUREMENTS OF THE ENZYMATIC FORMATION OF FUMARIC AND *CIS*-ACONITIC ACIDS

by

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Fumaric and *cis*-aconitic acids are intermediates in the main pathway of substances oxidized through the tricarboxylic acid cycle. With the exception of the keto-acid oxidases, the enzymes participating in the cycle have been obtained in solution and after purification can be studied in isolated and defined systems. Compounds such as fumaric and *cis*-aconitic acid with an unsaturated C = C linkage have a marked absorption in the ultraviolet. This property can be utilized in a spectrophotometric test, measuring appearance and disappearance of these substances in the course of enzymatic reactions.

A rapid and convenient test for the enzymes catalysing the formation of fumaric acid from malic acid or aspartic acid and the formation of *cis*-aconitic acid from citric acid or isocitric acid will be described in this paper.

EXPERIMENTAL

Ultraviolet Absorption Spectrum of Fumaric Acid and Cis-Aconitic Acid

The ultraviolet absorption spectrum of the sodium salts of these two acids is recorded in Fig. 1. The fumaric acid used in this experiment was a recrystallized commercial preparation; the *cis*-aconitic acid was kindly supplied by Dr S. OCHOA. As can be seen from Fig. 1, the absorption of these compounds shows a steady rise toward the short wave lengths. Because proteins and nucleic acids absorb considerable amounts of ultraviolet light in this region, enzymes used for spectrophotometric studies must have a fairly high turnover number so that activity measurements can be carried out at high enzyme dilutions. The activity of enzymes with a low turnover number can be tested spectrophotometrically only after considerable purification, with

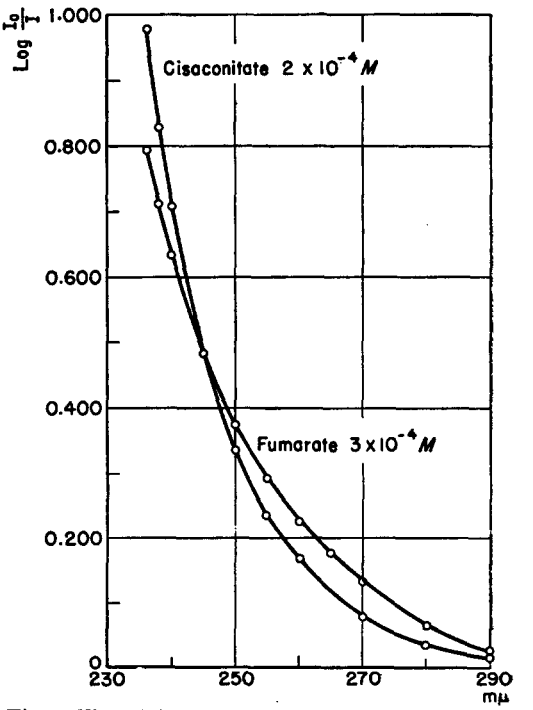

Fig. 1. Ultraviolet absorption spectrum of sodium fumarate and sodium *cis*-aconitate.

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removal of interfering absorbing substances, particularly proteins and nucleic acid.

Of the enzymes catalyzing the formation of unsaturated intermediates of metabolism, fumarase, aconitase and aspartase were selected for study.

PREPARATION OF ENZYMES

a) Fumarase. Fumarase was prepared according to the method of LAKI AND LAKI[1] and fumarase activity was measured at each stage of the purification[2]. It was found that the preparation at the final stage still contained contaminating proteins. The crystalline precipitate obtained was found to have lost most of the fumarase activity after four subsequent recrystallizations while the supernatant retained the fumarase activity[2]. These findings confirm the report by SCOTT[3] who observed that the crystalline fraction lost fumarase activity on recrystallization while the amorphous fraction had a specific activity equal to that ascribed to the crystals by LAKI AND LAKI[1]. Furthermore, the purified fumarase preparations of LAKI AND LAKI still contain considerable quantities of contaminating proteins. Appreciable aconitase activity has been found in these preparations as will be described below, as well as very active lactic acid dehydrogenase which represents about 20% of the protein present[2].

b) Aconitase. Fumarase prepared by the method of LAKI AND LAKI[1], and kindly supplied by Dr J. B. V. SALLES, was found to contain an active aconitase as noted above. This preparation of fumarase had been kept at 0° for several weeks and retained considerable aconitase activity. Because of the known lability of purified aconitase, it was decided to investigate this preparation further.

Fumarase was prepared, therefore, according to the method of LAKI AND LAKI[1] and fumarase and aconitase activity were measured in all fractions[2]. A large proportion of the aconitase activity was retained by the heart muscle pulp after thorough washing with water; the pulp was then extracted by the phosphate buffer treatment used for obtaining the fumarase activity [1]. Both aconitase and fumarase were purified. Aconitase showed a somewhat greater sensitivity to the acid p_H used in the course of the purification. On fractionation with ammonium sulphate, the fumarase precipitated at lower salt concentrations, so that partial separation of the two enzymes was accomplished.

An aconitase preparation was also made from FLEISCHMANN's baker's yeast. Maceration juice was obtained by extracting dried yeast with M/15 disodium phosphate for 3 hours at 37°. The maceration juice was fractionated at −5° with acetone. An active fraction was obtained which precipitated between 30 and 50% acetone concentration. This was dissolved in cold water and dialysed for two hours against running tap water. Following centrifugation, the supernatant was further fractionated by the addition of solid ammonium sulphate. The precipitate obtained at 50% saturation was collected. Solid ammonium sulphate was added to the supernatant and the fractions precipitated up to 80% saturation were also collected. The aconitase activity of these fractions will be described later in this paper.

c) Aspartase. This enzyme was prepared from *E. coli* (strain B). The bacteria were grown in neopeptone broth for 18 hours at 37° with vigorous aeration, then centrifuged and washed once distilled water. They were then suspended in a small volume of distilled water and disintegrated by sonic vibration[1] for five minutes. After centrifugation for 20 minutes at 18000 rpm in a refrigerated centrifuge, the supernatant was fractionated by means of ammonium sulphate. The precipitate obtained at 50% saturation was dissolved and dialysed against distilled water at 0° for 24 hours. This preparation of aspartase was used for the studies described in this paper and was found to be quite stable if kept at 0°.

SPECTROPHOTOMETRIC MEASUREMENTS

a) Fumarase. The enzymatic activity of fumarase was determined in a Beckman DU quartz spectrophotometer. The final volume was 3 ml including 0.05 M potassium-phosphate buffer at p_H 7.4 and 0.05 M sodium L-malate. After addition of the enzyme, the changes in absorption at 240 mμ were recorded at intervals of 15 seconds. The control cell contained all the solutions except the substrate. The enzymatic reaction follows a zero order course for several minutes and is measured during this period. One unit is defined as a change of $\log \frac{I_0}{I}$ of 0.001 per minute. The increments in optical density at 240 mμ are proportional to the amount of enzyme added (Fig. 2).

* Sonic oscillator manufactured by Ratheon Corp., Waltham, Massachusetts, U. S. A.

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Under these experimental conditions the MICHAELIS constant for fumarase as determined by the method of LINEWEAVER AND BURK[4] was $4.1 \cdot 10^{-3}$ (moles $\times$ liter^{-1}) with sodium L-malate as substrate.

The enzymatic activity of fumarase can also be followed with sodium fumarate as the substrate. Due to the high specific absorption of fumaric acid, only limited amounts of this substrate, which are not sufficient to saturate the enzyme, can be used in the spectrophotometric test. The rates, therefore, are slower and fall off more rapidly than with L-malic acid as the substrate. However, with an active enzyme preparation the equilibrium is quite rapidly established from either direction.

b) *Aconitase.* For the measurement of aconitase activity the test system was the same as that for fumarase except that the substrate used was either 0.03 M sodium citrate or 0.01 M sodium D,L-isocitrate (kindly supplied by Dr S. OCHOA). Since the enzyme is unstable in dilute solutions, all estimations were carried out immediately following dilution in 0.1 M phosphate buffer. The enzymatic activity followed a zero order course for several minutes and was proportional to the amount of enzyme added (Fig. 2).

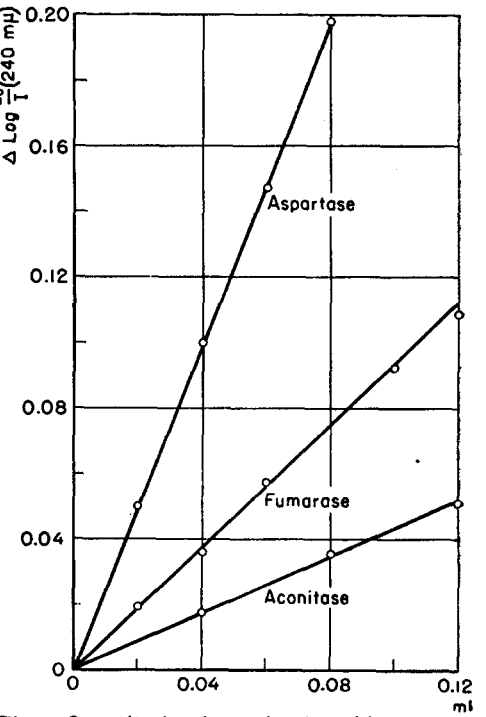

Fig. 2. Quantitative determination of fumarase, aconitase and aspartase. Relation of enzyme concentration to activity per minute.

The specific activity (units/mg protein) of aconitase preparations when tested with isocitrate was always found to be greater than with citrate. Considerable variation in the relative activities was found in different fractions during purification. Although no evidence was obtained of a separation of the enzyme activity for the two substrates, the respective activities, for the sake of convenience, are referred to as citrase and isocitrase. Thus, in a crude heart extract, a ratio isocitrase/citrase activity of 2.1 was found, while the purified preparation[2] had a ratio of 7.5. Similarly, the fractions obtained from yeast by acetone and ammonium sulphate precipitation, showed considerable variation in the relative citrase and isocitrase activities. The ammonium sulphate precipitate obtained at 50% saturation showed an isocitrase/citrase ratio of 2.0, while the fractions obtained between 60 and 80% saturation showed a ratio of about 7.0.

The MICHAELIS constant of aconitase measured with sodium citrate as substrate was found to be $1.1 \cdot 10^{-3}$ and for D-isocitrate $4 \cdot 10^{-4}$ M.

c) *Aspartase.* This enzyme was measured in the same manner as the other hydrases with 0.15 M sodium aspartase as the substrate. A high concentration of substrate is required for maximal activity of this enzyme. With substrate concentration sufficient for enzyme saturation, proportionality between enzyme concentration and increments in optical density was found (Fig. 2).

The MICHAELIS constant of aspartase was found to be in the neighbourhood of

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$3 \cdot 10^{-2}$ M. Some variation around this value was found with different preparations. This variation might be explained by the presence of two different aspartases reported by GALE[5].

DISCUSSION

Rapid and convenient spectrophotometric methods for the determination of glycolytic enzymes of the MEYERHOF-EMBDEN scheme have been developed by WARBURG and his school. These methods have been valuable in following purification and also for kinetic studies of these enzymes. The high absorption coefficients in the ultraviolet of unsaturated compounds such as fumaric and aconitic acid have been made the basis for a method of measuring their enzymatic formation. Other compounds such as crotonic and vinyl-acetic acid were also found to show a high absorption in the ultraviolet. These latter compounds are known to be metabolized by animal tissues and by bacteria and may be intermediates of fatty acid metabolism. In view of their high specific light absorption, their enzymatic formation and breakdown could be followed by spectrophotometric tests similar to those described in this paper.

The occurrence of unsaturated compounds as intermediates of metabolism of amino acids such as serine and threonine has been postulated[6]. The probably high absorption in the ultraviolet of such intermediates may help in the elucidation of the pathway of the metabolic breakdown of these amino acids. Advantage has been taken of the high absorption coefficients of reduced coenzymes I and II, keto acids, dehydropeptides, and amino acids, such as tyrosine for enzymatic studies with these compounds. The present study shows that the metabolism of unsaturated organic substances may be followed by a similar technique.

SUMMARY

A spectrophotometric method of measuring the enzymatic formation and disappearance of umaric and *cis*-aconitic acids is reported.

RÉSUMÉ

Nous décrivons une methode spectrophotométrique qui permet de mesurer la formation et la disparation enzymatique de l'acide fumarique et de l'acide *cis*-aconitique.

ZUSAMMENFASSUNG

Eine spektrophotometrische Methode zur Messung der enzymatischen Bildung und Zerstörung von Fumarsäure und *cis*-Akonitsäure wird beschrieben.

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Received April 28th, 1949