Glutamic Acid Decarboxylase of Lemons and Oranges

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The flavedo of lemon and orange fruit is among the richest plant sources of glutamic acid decarboxylase hitherto reported. Even a most rudimentary fractionation of the enzyme from orange flavedo yields a preparation with an activity of about 40,000 cu. mm. of carbon dioxide per hour per milligram of protein nitrogen. The enzyme can be conveniently assayed by an electrometric titration. The requirement of this enzyme for additional pyridoxal phosphate to prevent loss of activity when it is in contact with its substrate at 37° C. and pH 5.6 is shown to disappear when either the temperature is lowered to 15° C. or the pH changed to 6.5.

ORANGE FLAVEDO for some years (*2*) has been known to contain relatively high glutamic acid decarboxylase activity. Recently, Rockland (*6*) found that there is a positive correlation between maturity of oranges and γ-aminobutyric acid content in the juice. Since the decarboxylation of glutamic acid yields γ-aminobutyric acid as end product, it became of interest to study this enzyme in more detail. Okunuki (*5*) was first to discover this enzyme in higher plants. Schales, Mims, and Schales (*7*) have made an extensive investigation of this enzyme in a number of different plant species and developed evidence that pyridoxal phosphate acts as the coenzyme. This report reveals that both lemon and orange flavedo are rich in this enzyme. Enzyme activity was easily determined by an electrometric titration. This enzyme was found to have a fairly broad pH optimum between 5.3 and 5.6 and to be specific, acting only on L-glutamic acid. A zero-order reaction can be obtained at 37° C. and pH 5.6 only in the presence of pyridoxal phosphate; however, at 15° C. or pH 6.5 the reaction is one of zero order without the addition of pyridoxal phosphate.

Materials and Methods

The fruit used in these experiments, Navel and Valencia oranges and Eureka lemons, were grown in southern California. The pyridoxal phosphate used in the early phases of this work was synthesized by the method of Umbreit, Bellamy, and Gunsalus (*10*), and that used later was a preparation purchased from the California Foundation for Biochemical Research. The γ-aminobutyric acid was obtained from 2-pyrrolidone by the method of Tafel and Stern (*9*). The ornithine was purified from a commercial preparation in the manner described by Adamson (*1*). Nitrogen was determined by the micro-Kjeldahl procedure.

Preparation of Enzyme. Flavedo was obtained by grating the fruit with a metal kitchen grater. The flavedo was suspended in three times its weight of ice-cold distilled water and blended in a chilled electrical blender for 2 to 3 minutes at minimum speed. Enzymatic assays were determined with homogeneous aliquots of this suspension.

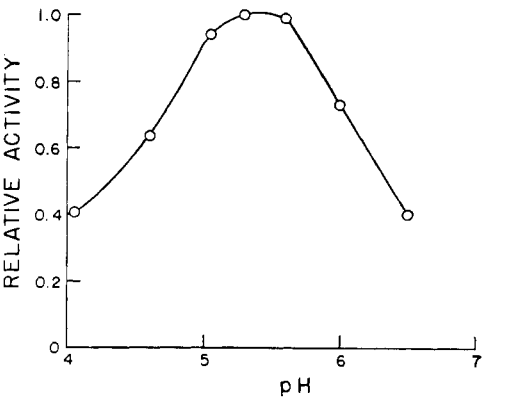
Electrometric Enzyme Assay. In this procedure the enzyme activity is assayed by determining the rate at which standardized acid must be added to maintain the pH of the reaction mixture at 5.6. The pH is measured with a glass-calomel electrode pair connected to a Model G Beckman pH-meter, locked for continuous reading, and adjusted to give a null reading on the galvanometer at pH 5.6. The assay is conducted in a 30-ml. reaction vessel, in a 37° C. water bath. Carbon dioxide–free air is bubbled through the reaction mixture, via a capillary tube connected to the bottom of the reaction vessel. The reaction mixture contains 5.0 ml. of 2% L-glutamic acid (optimum substrate concentration) at pH 5.6, 0.5 mg. of ammonium pyridoxal phosphate, enzyme, and sufficient water to give a final volume of 15 ml. The pH is maintained at 5.6 ± 0.1 by the addition of standardized 0.0200*N* hydrochloric acid. Since the citrus enzyme has a fairly broad optimum, pH variations of this magnitude do not introduce any appreciable errors. Activity determinations can be made as frequently as convenient, by noting the volume of hydrochloric acid consumed and the time elapsed between null point readings of the galvanometer. However, in routine assays it is convenient and satisfactory to make just one reading after 7 to 8 minutes. Results are expressed in milliequivalents of hydrochloric acid per minute. The potentiometric method is to be preferred for routine work, and it may be scaled down satisfactorily to use one third of the above volume.

Manometric Method. The procedure is essentially as described by Schales, Mims, and Schales (*7*). Since substitution of nitrogen for air did not reduce the output of carbon dioxide, air was used as the gas phase. Coenzyme pyridoxal phosphate was supplied in excess by either 0.1 ml. of the Umbreit, Bellamy, and Gunsalus preparation (*10*) (in the stage after the removal of the inorganic phosphate) or 0.1 mg. of the commercial preparation. This procedure was used for some of the specificity studies, especially in the early phases of the work and was largely abandoned in favor of the electrometric method, except when limited amounts of substrate were available.

pH Optimum. The pH optimum for this enzyme is shown in Figure 1.

Figure 1. pH activity curve

Conditions same as in potentiometric assay method except for pH



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At pH 4.05, the activity dropped rapidly because of acid instability. The curve was constructed from the average activities of the first 10-minute reaction period.

Purification of Enzyme from Orange Flavedo. First, a flavedo suspension was prepared as described above. The suspension was then strained through cheesecloth and centrifuged at about 10,000 × gravity for 15 to 20 minutes at 5° C. Attempts to filter instead of centrifuging were unsuccessful. Filtration through a Celite pad was rapid, but virtually all of the activity was retained on the pad and was not easily eluted. The supernatant from centrifugation was subjected to ammonium sulfate fractionation at 0 to 0.25, 0.25 to 0.40, and 0.40 to 0.50 saturation at 25° C. The 0.25 to 0.40 fraction was further fractionated at 0.30 to 0.33, 0.33 to 0.36, and 0.36 to 0.39 saturation. From a preliminary study of this type, the results showed that the specific activity of the original suspension, 0.003 meq. per minute per mg. of protein nitrogen, was improved to 0.034 meq. per minute per mg. of protein nitrogen in the 0.36 to 0.39 fraction.

Effect of Pyridoxal Phosphate. Schales, Mims, and Schales (*7*) initially observed that the rate of decarboxylation of glutamic acid by a carrot root enzyme preparation decreased more rapidly than predicted on the basis of first-order kinetics unless pyridoxal phosphate was added. A similar anomalous decline was noted with the flavedo preparations; here, under the experimental conditions employed, pyridoxal phosphate was required to maintain zero-order reaction. The observation by these workers that this apparent inactivation of the carrot enzyme occurred only in the presence of glutamic acid was shown to hold equally for the flavedo preparation. It was also established that if pyridoxal phosphate was added after the reaction had begun, the lower rate, existing at the time of the addition, was now maintained, but the original rate was not restored. If, however, the reaction was carried out at 15° C., the addition of pyridoxal phosphate was not necessary to preserve the initial rate (Figure 2). At 37° C. the addition of pyridoxal phosphate was not required to maintain the initial rate if the pH were 6.5.

Specificity. Of a number of substances tested, only L-glutamic acid was attacked by the crude Naval orange flavedo enzyme, essentially confirming the results of Okunuki (*5*). However, he did report that pyrrolidone carboxylic acid was attacked by a preparation made from the pollen of *Lilium auratum*. This substrate was not attacked by the flavedo enzyme. The pyrrolidone carboxylic acid used was chromatographically pure, melted at 184° C., and was free of ninhydrin reactive material. The following substances were tested with negative results: D,L-α-aminoadipic acid, L-arginine, D,L-aspartic acid, L-glutamine, glutaric acid, glutathione, α-ketoglutaric acid, D,L-ornithine, D,L-methionine, D,L-methionine sulfoxide, pyrrolidone carboxylic acid, D,L-serine, and L-tyrosine. The ornithine was of commercial origin and showed considerable activity, giving rise to an amino acid chromatographically indistinguishable from γ-aminobutyric acid. Examination revealed the ornithine to be contaminated with glutamic acid. After purification (*7*), it was completely inert.

Table I. Inhibitors of Glutamic Acid Decarboxylase[a]

	Concn., M	Inhibition, %
α-Ketoglutaric acid	0.0045	15
	0.0091	26
	0.018	37
Versene	0.027	32
Aspartic acid	0.045	7
Glutaric acid	0.045	50
$MnCl_2$	0.0067	0
	0.067	42
Ethyl alcohol	0.142	11
	0.94	32
NaCl	0.167	46

[a] Test performed as in standard electrometric assay.

Inhibitors. A variety of compounds were tested for their inhibitory qualities (Table I). The usual carbonyl reagents whose effect on pyridoxal phosphate enzymes is well-known were not tried. Since, in general, pyridoxal phosphate enzymes have not been shown to have a metal requirement (*4*) except for the indication of Yanofsky (*13*), the inhibition by ethylenediamine tetraacetic acid [(ethylenedinitrilo)tetraacetic acid, Versene] may be of interest.

Figure 2. Effect of pyridoxal phosphate on glutamic acid decarboxylase activity at 15 and 37

Conditions same as in titrimetric assay method except for absence of pyridoxal phosphate and lower temperature

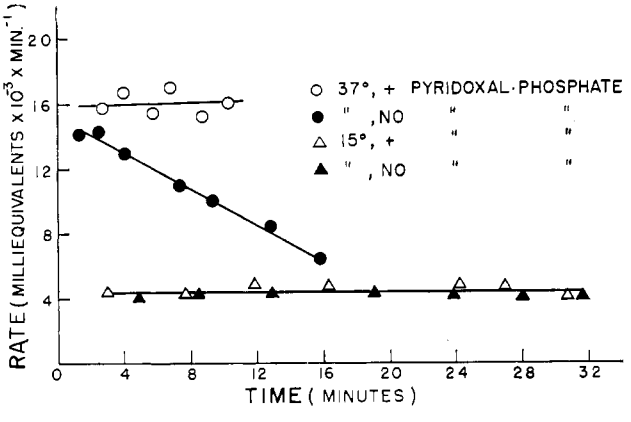

Table II. Specific Activities of Flavedo Glutamic Acid Decarboxylase from Various Citrus Species

	Meq. × 10^{-3}/ G./Min. (Fresh wt.)	Meq. × 10^{-3}/ Mg. PN/Min.
Naval oranges, fresh	5.1	3.7
Valencia oranges, fresh	8.4	4.2
Valencia oranges, stored	15.3	3.0
Lemons, green	10.0	10.0
Lemons, ripe	5.5	5.5

Occurrence of Enzyme in Various Citrus Species. Results in Table II show that there is very little difference in specific activity on a protein nitrogen (PN) basis between Naval and Valencia oranges. It is of interest that there is a negative correlation between maturity and enzyme activity in the lemon—i.e., the ripe fruit in which γ-aminobutyric acid has accumulated contained considerably less enzyme than the green fruit.

Discussion

The anomalous decline in activity, which carrot glutamic acid decarboxylase undergoes when extra pyridoxal phosphate is absent from the reaction medium as observed by Schales, Mims, and Schales (*7*) and considered in more detail by Schales and Schales (*8*), was also observed for citrus glutamic acid decarboxylase. Like these authors the present authors also found that this decrease was not the result of inhibition by the γ-aminobutyric acid formed. Schales, Mims, and Schales (*7*) have interpreted the effect as due to the inactivation of the carrot enzyme per se owing to the decomposition of its prosthetic group. Both the citrus and carrot preparations suffered such inactivation only while acting upon the substrate and not just merely on incubation. In the case of the orange enzyme this inactivation was irreversible, because the addition of pyridoxal phosphate during the course of the reaction halted the inactivation, but did not restore the enzyme to its original activity. The observation that inactivation did not occur even in the absence of added coenzyme if the reaction temperature was lowered to 15° C. suggests a possible explanation. It may be that by some mechanism not yet understood the small amount of endogenous phosphate present

is decomposed in the presence of the substrate and that the enzyme, now devoid of coenzyme, is susceptible to thermal inactivation. A case in point is rabbit muscle glyceraldehyde phosphate dehydrogenase, which Velick, Hayes, and Harting (*11*) have demonstrated to be very unstable at room temperature when deprived of the coenzyme, diphosphopyridine nucleotide, with which it is normally associated. Beevers (*3*) reported barley glutamic acid decarboxylase to be subject to thermal inactivation. The failure to observe the anomalous inactivation at pH 6.5 as opposed to lower pH in the absence of pyridoxal phosphate may also be explicable on the basis of the increased sensitivity of the enzyme to inactivation at lower pH values.

Citrus flavedo tissue is a relatively good source of the enzyme, being 10 times richer on a fresh-weight basis than the most active of the plant materials listed by Schales, Mims, and Schales (*7*). The activity of the 0.36 to 0.39 ammonium sulfate fraction from the purification study when expressed in terms of cubic millimeters of carbon dioxide per milligram protein nitrogen per hour is approximately 40,000 at 37° C. Assuming the reaction velocity to be halved at 30° C., this value becomes 20,000 as compared to 12,500 obtained by Weinberger and Clendenning (*12*) with a highly purified wheat leaf preparation. In view of the fact that the flavedo purification was of the most preliminary sort, it would seem that orange flavedo is the starting material of choice.

Acknowledgment

The following gifts are gratefully acknowledged: D,L,α-aminoadipic acid from Peter Lowy, California Institute of Technology; L-pyrrolidone carboxylic acid from J. B. Stark of the Western Utilization Research Branch, U. S. Department of Agriculture; and 2-pyrrolidone from the General Aniline & Film Corp.

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Received for review August 1, 1955. Accepted September 6, 1955.

PECTIC PRODUCTS IN FRUIT

Determination of Galacturonic Acid in Tomatoes and Its Changes on Storage

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The degradation of pectic substances to D-galacturonic acid is a possible explanation of the fate of these compounds in fruits and vegetables. Using the tomato as the test fruit, the object of this investigation was to devise an analytical procedure for D-galacturonic acid in tomatoes and to determine the changes in galacturonic acid content of tomatoes during storage.

THE FATE OF PECTIC SUBSTANCES in fruits and vegetables was thought to be due to degradation of these compounds to D-galacturonic acid. Therefore an investigation was undertaken, using the tomato as the test fruit, with the objective of devising an analytical procedure for determining D-galacturonic acid in tomatoes and determining the changes in galacturonic content of tomatoes during storage. An extraction procedure and a method for separating interfering substances were required.

Preliminary Investigations

Two procedures using naphthoresorcinol—those of Kertesz (*7*) and of Winkler (*9*)—were studied. The Winkler procedure used 50 mg. of naphthoresorcinol per sample of galacturonic acid, so that a recrystallized lot of reagent sufficed to analyze only 7 to 15 samples, when adequate checks on the reliability of the reagent were made. The Kertesz method was investigated because only 4 mg. of naphthoresorcinol was used per analysis, and this permitted adequate checks and duplicate determinations; at least 100 samples could be analyzed with one sample of recrystallized naphthoresorcinol. Modifications in acid concentration and reaction temperature of the Kertesz procedure were developed which permitted the analysis of galacturonic acid solutions containing between 0.0050 and 0.0550 mg. per ml. with a precision within 0.0004 mg. per ml.

In the modified procedure the hydrogen ion concentration was reduced from 3.90*N* to 3.56*N* and the reaction temperature was lowered from 100° to 91° C. The reduction of the hydrogen ion concentration increased the range of galacturonic acid that could be determined.

Experiments indicated that neither color nor melting point was satisfactory as an index of purity of naphthoresorcinol. Therefore each lot of recrystallized naphthoresorcinol was checked with standard solutions of galacturonic acid before use.

In order to separate the tomato carbohydrates that react with naphthoresorcinol from the galacturonic acid, the ion exchange technique was used.