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Determination of L-Glutamic Acid by Use of L-Glutamic Acid Decarboxylase from *Mycobacterium phlei*. (22266)

YEHESKEL S. HALPERN AND NATHAN GROSSOWICZ.

Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Amino-acid decarboxylases of bacterial origin are now widely used for the quantitative estimation of certain amino-acids(1-7,9). Gale(1) suggested the use of *Clostridium welchii* SR-12 for the determination of glutamic acid. This organism releases CO_2 not only from L-glutamic acid, but from L-glutamine(3), and L-aspartic acid(5,6) as well. Additional tests and special conditions are necessary(3,5,6) to obtain separate values for each of these compounds. Glutamic acid decarboxylases obtained from different strains of *E. coli* were used by several investigators(2,7-9) for the assay of L-glutamic acid. In general, the *E. coli* preparations possessed in addition to L-glutamic acid decarboxylase, a glutaminase(7), or a L-lysine and L-arginine decarboxylases(2,8,9). Recently, Najjar and Fisher(9) reported that they were able to destroy the arginine and lysine decarboxylases without seriously affecting the L-glutamic acid decarboxylase.

In the present paper a description of the use of cell-free extracts from *Mycobacterium phlei* for the determination of L-glutamic acid will be given. Extracts from this organism decarboxylate quantitatively L-glutamic acid only and do not react with 18 other amino-acids tested. Furthermore, no glutaminase or glutamo-racemase activity could be detected under conditions optimal for the decarboxylase activity.

Methods. A strain of *Mycobacterium phlei* from the stock of this laboratory is

grown in Roux bottles on nutrient agar medium (peptone—1%, meat extract "Bovril" —0.3% • NaCl—0.50%, Na_2HPO_4 • $12H_2O$ —0.25%), to which 0.1% Tween 80 was added, and incubated for 3-4 days at 35°C. The total crop from 6 bottles is washed 3 times with saline, resuspended in 20 ml distilled water and then disintegrated in a 9 KC Raytheon sonic vibrator for 25 minutes. An alternative method for preparing the extracts by grinding the bacterial paste with alumina A-301, according to McIlwain(10) was also used and proved quite satisfactory. After disruption of the cells the debris are removed by a Servall centrifuge at 10,000 r.p.m. for 20 minutes and the clear and somewhat opalescent supernatant is dialyzed against running tap water in the cold-room for 13-15 hours. The protein content of the dialyzed solution is determined by Mehl's Biuret method(11).

Results. Measurements of glutamic acid decarboxylation. a). The Warburg manometric technic is used and from the amount of CO_2 evolved the quantity of glutamic acid that underwent decarboxylation can be calculated. Suitable controls containing no substrate and other controls containing substrate with KOH in the central well are run simultaneously with the complete system. All measurements are made at 37°C in citrate-phosphate buffer, pH 5.2, final concentration as indicated by McIlvaine(12), with air as the gas phase. The main compartment contains the enzyme and the buffer; the substrate is tipped in from the side-arm after equilibration for 20 minutes. For estimation of the reaction rate measurements are taken every 5-10 minutes. For quantitative estimation of glutamic acid readings are made until no changes in gas pressure are seen. The recovery of glutamic acid is 95-98% and owing to the low pH of the system there is hardly any retention of CO_2 by the liquid phase. b) Paper chromatography was used as an alternative method for the quantitative determination of the other product of decarboxylation of glutamic acid—γ-amino-butyric acid. Circular chromatography after Giri *et al.*(13) was employed; the solvent used was water-saturated phenol. The effect of pH on the activity of the enzyme is presented graphically in Fig. 1. The optimum activity is at pH 5.2-5.4, falling off rapidly at both sides of the pH curve, the slope being even steeper on the alkaline side.

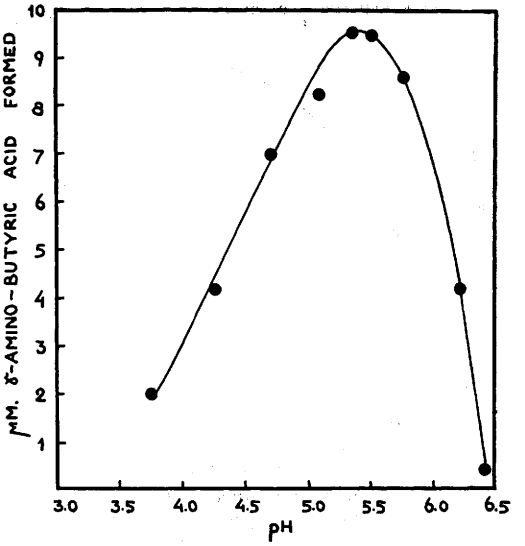

FIG. 1. The system contained: 10 μM L-glutamic acid, cell-free extract equivalent to 6.8 mg protein, McIlvaine's buffer, pH values as indicated, total volume—1 ml; time of incubation—2 hr at 37°C. The γ-amino butyric acid was determined by quantitative paper chromatography(13).

The influence of enzyme concentration on the reaction rate was studied and as is appar-

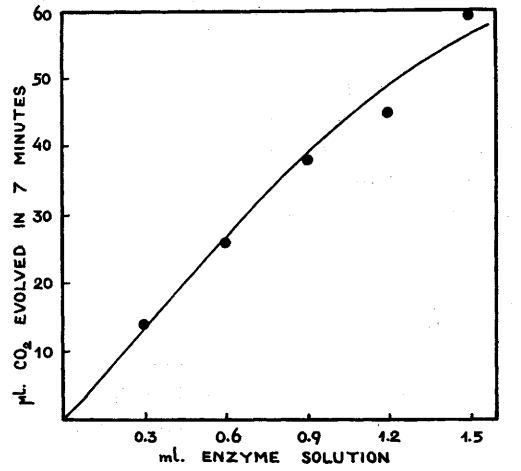

FIG. 2. The system contained: 10 μM L-glutamic acid, varying quantities of a cell-free extract (7.5 mg protein/ml), McIlvaine's buffer—pH 5.2; total volume—2.5 ml. Incubation at 37°C.

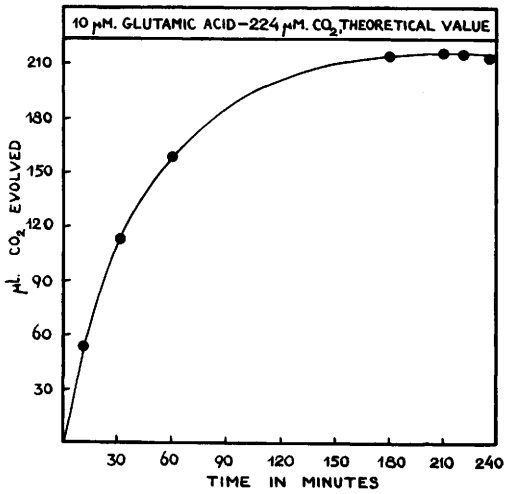

FIG. 3. The system contained: 10 μM L-glutamic acid, cell-free extract equivalent to 15 mg protein, McIlvaine's buffer—pH 5.2; total volume—2.5 ml. Incubated at 37°C.

ent from Fig. 2, activity is proportional to enzyme concentration as long as the enzyme is saturated with substrate. For the assay the enzyme is always taken in excess (15-20 mg protein per vessel). A typical experiment is illustrated in Fig. 3. Dialysis of the enzyme solution is not essential. However, when undialyzed extracts were used, the blanks showed considerably higher values. Only slight differences in activity (about 10%) were noticed when dialyzed and undialyzed extracts were compared. The enzyme preparation is quite stable; about ⅔ of activity were preserved after storage at 0°C for 4 months.

Specificity for L-glutamic acid. In repeated experiments with a series of amino-acids the cell-free extracts from *Mycobacterium phlei* did not decarboxylate any amino-acid other than L-glutamic acid. The amino-acids tested were: L-alanine, L-arginine, L-aspartic acid, D-glutamic acid, L-glutamine, glycine, DL-histidine, DL-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-ornithine, DL-proline, DL-serine, DL-threonine, DL-tyrosine, L-valine. Furthermore, there is no appreciable change in decarboxylation rate of L-glutamic acid when D-glutamate, L-aspartate, L-glutamine, or L-asparagine are present. (Table I).

Partial purification of enzyme. A cell-free extract was prepared as already described. Ammonium sulphate was added gradually to obtain 33% saturation. The precipitate (fraction I) was centrifuged off and to the clear supernatant a further amount of ammonium sulphate was added until 50% saturation was obtained; centrifugation was then repeated and after removing the precipitate (fraction II) more ammonium sulphate was added to the supernatant until 70% saturation was reached. The precipitate (fraction III) was removed by centrifugation. All the manipulations were carried out in the cold-room. Table II shows the activities of the various fractions (after dialysis to remove the $(NH_4)_2SO_4$) as tested for glutamic acid decarboxylase. Fraction II (precipitate from 33-50% saturated $(NH_4)_2SO_4$ solution) contained over 50% of the original activity with a 3-4-fold purification.

Discussion. The enzyme preparation described in this paper has the advantage of being highly specific towards L-glutamic acid; none of the other 18 amino-acids tested is attacked by the enzyme. Closely related amino acids and amides (D-glutamic acid, L-glutamine, L-aspartic acid and L-asparagine) did not interfere with the activity of the glutamic decarboxylase. The lack of any measurable glutaminase activity enables direct estimation of L-glutamic acid in natural

TABLE I. Influence of Related Substances on Decarboxylation of L-Glutamic Acid.

Substrates (μM)	CO_2 evolved* (μM)	γ-amino butyric acid formed* (μM)
L-glutamic acid, 10	.60	.60
D-glutamic acid, 10		.0
L-glutamine, 10	.0	.0
L-aspartic acid, 10	.0	.0
L-glutamic acid, 10 + D-glutamic acid, 10		.58
L-glutamic acid, 10 + L glutamine, 10	.60	.58
L-glutamic acid, 10 + L-aspartic acid, 10		.62
L-glutamic acid, 10 + L-asparagine, 10		.62

* Values calculated/mg enzyme protein/hr.

The reaction mixture was set up in McIlvaine's buffer, pH 5.2, in a total volume of 1 ml, and incubated at 37°C; the γ-amino butyric acid formed was determined by quantitative paper chromatography(13).

TABLE II. Partial Purification of L-Glutamic Acid Decarboxylase with Ammonium Sulfate.

	Total activity (units)*	Total protein (mg)	Specific activity (units per mg protein)
Crude extract	216	1200	.18
F I .33 s†	46	760	.06
F II .50 s	113	180	.63
F III .70 s	5	40	.12
Supernatant at .70 s	0	213	.0

* 1 unit is defined as that amount of enzyme which will decarboxylate 1 μM of glutamic acid in 10 min.

† F = fraction; s = saturation.

The tests were performed with dialyzed enzyme samples; the γ-amino butyric acid formed was determined by quantitative paper chromatography.

products containing considerable amounts of glutamine. The cultivation of the bacteria and the preparation of the extract do not present any difficulties. It is possible to prepare a larger amount of extract and preserve it for a long period by cold-storage.

Summary. 1. A preparation from *Mycobacterium phlei* with decarboxylase activity towards L-glutamic acid only, was described. The preparation has no detectable decarboxylase activity towards other 18 amino-acids tested, and no glutaminase or glutamo-racemase activities. This enables direct estimation of L-glutamic acid in natural products. 2. The optimal pH range is between pH 5.2-5.4, with a sharp drop in activity at pH values of 4 and 6 respectively. 3. Some purification of the enzyme was achieved by fractionation with ammonium sulphate.

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Interrelationships of Glucose and Inorganic Phosphorus in Blood and Urine of Patients with Diabetes Mellitus.* (22267)

JOSEPH L. IZZO. (Introduced by W. S. McCann.)
(With the technical assistance of Angela M. Roncone and Ann Eilers.)
Department of Medicine, University of Rochester, and Medical Clinic of Rochester Medical Center, Rochester, N. Y.

Studies in relationships of inorganic phosphorus and glucose in blood, both in experimental animals and in man, have contributed to the development of present-day concepts concerning the role of phosphorus in metabolic processes(1). It has been established that marked lowering of blood glucose following administration of insulin is associated with simultaneous fall in phosphorus in blood and urine(2,3). A similar decline in inorganic phosphate also occurs following administration of glucose(4-6). The fall in serum phosphate has been shown to be related to active entry of glucose into the glycolytic cycle in muscle, rather than into the glycogeneic cycle in the liver(7). These observations have been extended and related to the action of insulin, epinephrine, and certain hypophyseal hormones(8). On the basis of these findings, the fall of serum inorganic phosphorus following intravenous administration of glucose has become an accepted criterion for peripheral utilization of glucose.

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