LXVIII. MICRODETERMINATION OF GLUTAMIC ACID

By PHILIP P. COHEN¹

From the Department of Biochemistry, University of Sheffield

(Received 1 March 1939)

In the course of experiments on the intermediary metabolism of amino-acids it was found necessary to determine small quantities of glutamic acid. Methods used by previous workers either require large quantities or are unspecific [Foreman, 1914; Jones & Moeller, 1928; Braunstein & Kritzmman, 1937]; on the suggestion of Dr H. A. Krebs a new method has therefore been worked out which is more specific than the older methods and permits the accurate determination of glutamic acid in amounts from 0.1 mg. upwards.

Principle of the method

Glutamic acid is converted into succinic acid by treatment with an excess of chloramine T and subsequent acid hydrolysis [Dakin, 1917]:

The succinic acid is determined manometrically [Szent-Györgyi & Gösz, 1935; Krebs, 1937].

Optimum conditions for reaction (2)

β -Cyanopropionic acid was prepared from glutamic acid according to Dakin [1917], and the optimum conditions for its hydrolysis determined by studying the rate of succinic acid formation.

Reaction (2) occurs readily in the presence of strong HCl at 100°. The influence of HCl concentration on the reaction is given in Table I. Hydrolysis is complete within 15 min. when the concentration is at least 12.5%. The effect of the time of heating on the rate of hydrolysis is given in Table II. Since prolonged heating is to be avoided (see discussion of specificity) heating for 15 min. with 12.5% HCl was adopted as the standard procedure.

¹ National Research Council Fellow in Medicine.

Table I. *Influence of HCl concentration on hydrolysis of β -cyanopropionic acid*

Heating time 15 min., 100°. After hydrolysis succinic acid was extracted and determined manometrically.

	mg. β -Cyano- propionic acid	% HCl (final conc.)	Succinic acid found (μ l. O ₂)	Succinic acid calc. (μ l. O ₂)	Hydrolysis %
1	3.22	2.6	153	366	42
2	3.22	6.5	249	366	68
3	3.22	10.4	344	366	94
4	3.22	12.5	363	366	99
5	3.64	15.0	414	414	100

Table II. *Effect of time of heating on rate of hydrolysis of β -cyanopropionic acid*

12.5% HCl. After heating succinic acid was extracted and determined manometrically.

	mg. β -Cyano- propionic acid	Time of heating min.	Succinic acid found (μ l. O ₂)	Succinic acid calc. (μ l. O ₂)	Hydrolysis %
1	3.69	0	12.3	418.5	3
2	3.69	2.5	105.3	418.5	25
3	3.69	5	276.3	418.5	66
4	3.69	7.5	345.3	418.5	83
5	3.64	10	393.6	414	95
6	3.64	15	413.1	414	100
7	3.64	20	414.6	414	100

Optimum conditions for reaction (1)

Reaction (1) was studied by determining the formation of β -cyanopropionic acid by measuring the succinic acid formed from it on acid hydrolysis.

Dakin [1917] found that reaction (1) took place only when the molar concentration of chloramine T was at least twice that of glutamic acid. We find that an excess of chloramine T does not interfere. The investigation of the influence of time, temperature and pH on reaction (1) showed that quantitative yields of β -cyanopropionic acid were obtained when the reaction was carried out at 40° for 10 min. at pH 4.7. At pH 2.7 the average recovery was 83%, while at pH 7 the recoveries were as low as 38%. Thus it is important to keep the pH in the range of 4-5. At high temperatures (100°) the recoveries average 20%.

The rate of reaction (1) may be followed manometrically by measuring the CO₂ produced. From Table III it can be seen that the theoretical value for CO₂ is obtained within 5 min. It should be noted however that the final CO₂ value is slightly higher than the theoretical. This extra CO₂, which amounts to 1-2.5%,

Table III. *Rate of CO₂ production from glutamic and β -cyanopropionic acids measured manometrically*

Each cup contains 2 ml. 10% chloramine T, 1.1 ml. citrate buffer, pH 4.7, 2 ml. substrate solution. Temp. 40°. 1.51 mg. glutamic acid is expected to yield 230 μ l. CO₂ according to (1).

Substrate	Time min.	μ l. CO ₂ formed	Yield of CO ₂ % of theory
Glutamic acid (1.51 mg.)	5	231.4	100.5
	10	233.1	101.2
	15	235.1	102
	20	235	102
β -Cyanopropionic acid (1.62 mg.)	5	1.9	—
	10	1.7	—
	15	1.7	—
	20	2.4	—

implies that glutamic acid is oxidized by chloramine T at some other point in the molecule besides the amino group. Since this "side reaction" occurs simultaneously with reaction (1) it was not found possible to prevent it. As a result of this the corresponding yields of β -cyanopropionic acid are low by 1–2.5%. The extra CO_2 is not due to further oxidation of β -cyanopropionic acid (Table III).

Reagents

(1) Citrate buffer (pH 4.7): 17.65 g. $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $2\text{H}_2\text{O}$ and 8.40 g. $\text{C}_6\text{H}_8\text{O}_7$, $1\text{H}_2\text{O}$ are dissolved in water and diluted to 50 ml.

(2) 0.1 M phosphate buffer (pH 7.4): 17.8 g. Na_2HPO_4 , $2\text{H}_2\text{O}$ are dissolved in about 500 ml. water and 20 ml. N HCl added. The solution is then diluted to 1 l.

(3) 10% chloramine T solution (to be freshly prepared before use).

(4) Conc. HCl.

(5) 5% NH_4Cl solution.

(6) Sat. NaOH solution (50–60%).

(7) Succinoxidase preparation.

The preparation of this enzyme system has been previously described by Krebs [1937]. As noted by Krebs, the enzyme preparation acquires an increasing blank O_2 uptake on storage. To avoid this, the following procedure may be adopted.

The finely minced pigeon muscle (about 50 g.) is washed with 500 ml. distilled water and filtered by suction through muslin. This procedure is twice repeated. The muscle pulp is sucked as dry as possible after the third washing. For use, a portion of the pulp is suspended in four to five times its weight of 0.1 M phosphate buffer, pH 7.4. For storage, it is moistened with distilled water and kept in the refrigerator in a tightly glass-stoppered bottle. Such a preparation remains active without increasing its blank O_2 uptake for as long as 7–10 days.

The fresh enzyme, prepared by suspension in 0.1 M phosphate buffer, usually has a small blank of 1–2 μl . O_2 per ml. suspension. If such a suspension is allowed to stand in the refrigerator for 4–5 days the blank may increase to as much as 10–15 μl . O_2 per ml. However, when the enzyme is stored as described above and freshly suspended in 0.1 M phosphate buffer as needed the blank is rarely greater than 2–3 μl . O_2 per ml. after as long as 1 week.

Procedure

(1) *Deproteinization*. When tissue slices are employed, it is not necessary to deproteinize, since the small amount of protein present does not interfere. Suspensions of minced tissue are deproteinized by the addition of $\frac{2}{3}$ vol. of $\frac{3}{4}N$ H_2SO_4 plus $\frac{1}{3}$ vol. of 10% Na_2WO_4 . An aliquot of the filtrate is used for the determination.

(2) *Oxidation by chloramine T*. The solution to be analysed is brought to pH 4.7 by the addition of 1–1.5 ml. citrate buffer. 2 ml. 10% chloramine T are added and the solutions well mixed by shaking. They are then placed in a rack and shaken at 40° for 10 min. The reaction is carried out in small Erlenmeyer flasks, or, where tissue slices are employed, the reaction may be carried out in the manometric flask after the removal of the slices and the alkali in the centre well. After 10 min. shaking the containers are removed and placed in an ice bath for 15–20 min. to precipitate most of the p -toluenesulphonamide formed as a reaction product, and most of the unused chloramine T. The solutions are filtered, the precipitate is washed with several small volumes of water and the combined filtrate and washings are collected in large test tubes (25×200 mm.).

(2A). *Extraction of β -cyanopropionic acid.* In the determination of glutamic acid in blood, tissue extracts and other fluids containing glutathione, it is necessary to separate the β -cyanopropionic acid from another compound derived from glutathione which forms succinic acid on hydrolysis (see discussion on specificity). This is accomplished by extraction with ethyl ether in which the β -cyanopropionic acid is readily soluble, while the glutathione derivative remains in the aqueous layer.

The filtrate described above is acidified with 4 ml. 10 % H_2SO_4 and extracted in a continuous extractor of the Kutscher-Steudel [1903] type. With the extractors and conditions used in this study extraction for 1–2 hr. was usually sufficient. 2.5 ml. phosphate buffer are added to the extraction flask and the ether is slowly distilled off. The remaining aqueous solution is then chilled to precipitate the *p*-toluenesulphonamide, which is filtered off, and the filtrate containing the β -cyanopropionic acid is collected in test tubes.

(3) *Hydrolysis of β -cyanopropionic acid.* Conc. HCl is added to the filtrate to make a final concentration of not less than 12.5 %. The tubes are placed in a boiling water bath for 15 min. At the end of this time the tubes are removed and allowed to cool. Conc. NaOH (50–60 %) is added dropwise until the solution becomes hot. At this point 0.5 ml. 5 % NH_4Cl is added and the contents are well mixed. The NH_4Cl decomposes traces of chloramine T, which if present will decolorize the indicator. A few drops of phenol red are now added and the solution is made alkaline to a purple colour. A large excess of alkali should be avoided as the *p*-toluenesulphonamide forms a salt in strongly alkaline solution. The solution is then cooled and transferred to a Kutscher-Steudel extractor.

(4) *Extraction with ether.* The ether must be free from peroxides, otherwise the manometric readings will be inaccurate owing to the decomposition of the peroxides by the catalase in the enzyme preparation [Krebs, 1937]. The alkaline solution is extracted with freshly distilled ether in a Kutscher-Steudel extractor for a time sufficient to remove the remaining traces of *p*-toluenesulphonamide (usually 1–2 hr.). While *p*-toluenesulphonamide in a concentration as high as 0.10 *M* has no inhibiting effect on succinic acid oxidation, it will separate during concentration of the extract if it is not removed and thus make the quantitative transfer of the concentrated solution difficult.

After the period of alkaline extraction, the extraction flask is removed and the contents of the extractor are acidified strongly to phenol red (light yellow-pink colour) with 3 ml. 10 % H_2SO_4 . A clean extraction flask with fresh ether is attached and the extraction continued for 2 or more hours, during which the phenol red is also extracted from the aqueous solution.

(5) *Preparation of solution for succinic acid determination.* When the extraction of the succinic acid is complete, 2–3 ml. *M*/10 phosphate buffer are added to the ether solution followed by dropwise addition of 2 *N* NaOH until the aqueous solution assumes the colour of neutral or slightly alkaline phenol red. The ether is distilled off and after the remaining neutral or slightly alkaline aqueous solution has been concentrated further on a steam bath to about 1 ml. it is then transferred to a small graduated cylinder by means of a small pipette. The extraction flask is rinsed with 0.5–2 ml. *M*/10 phosphate and the washings are combined with the solution.

For further details of the succinic acid determination see Krebs [1937].

Calculation

The $\mu\text{l. O}_2$ uptake observed is converted into mg. glutamic acid as follows:

$$\frac{\mu\text{l. O}_2}{112} \times 1.47 = \text{mg. glutamic acid.}$$

Recovery of known amounts of glutamic acid

The recoveries of known amounts of glutamic acid from pure solutions are recorded in Table IV. The average recovery is 96 %. The deficit of 4 % is partly due to an over-oxidation of the glutamic acid (as evidenced by the excess CO₂ production) and partly to unavoidable loss during the various manipulations.

Table IV. *Recovery of glutamic acid*

mg. glutamic acid	% recovery	mg. glutamic acid	% recovery
0.1	99	3.02	91.5
0.36	99	3.60	97.0
0.65	100	5.27	94.4
1.05	95	6.03	98.2
1.32	96.6	8.04	95.2
2.63	92.8	10.05	91.5

The recovery values for glutamic acid added to protein solutions are of the same order as those from pure solutions. Thus, of 8.80 mg. glutamic acid added to a muscle suspension, 8.20 mg. or 94 % were recovered.

The smallest amount of glutamic acid which can be determined is limited by the accuracy of the manometric succinic acid determination. Since 1 mg. glutamic acid is equivalent to 76.2 μ l. O₂, as little as 0.1 mg. can be determined by this method. Since the error in the manometric determination is greater with small readings, the method is more accurate with amounts above 1 mg.

Specificity of the method and interfering substances

Succinic acid and substances other than glutamic acid which form succinic acid during the course of the procedure will interfere with the determination unless taken into account. Among the substances tested which are likely to occur in biological material, glutamine and glutathione are the only compounds which have been found to yield significant amounts of succinic acid (see Table V).

Table V. *Succinic acid formation from compounds other than glutamic acid*

Compound	Amount (mg.)	Yield of succinic acid %
<i>DL</i> -Proline	29.3	0
α -Ketoglutaric acid	30.9	0
Ornithine-HCl	6.9	0
<i>DL</i> -Alanine	3.3	0
<i>DL</i> -Valine	4.4	0
<i>DL</i> -Leucine	2.5	0
Arginine-HCl	12.5	0
<i>l</i> (-)-Methionine	20.0	0
<i>l</i> (-)-Tyrosine	20.0	0
Aspartic acid	10.0	0
Asparagine	11.4	0
Oxidized glutathione	8.5	17.5
Reduced glutathione	8.5	17.5
Glutamine	1-3.6	70-80

As Weil-Malherbe [1937] has reported that α -ketoglutaric acid yields succinic acid when autoclaved with H₂SO₄, it should be noted that no succinic acid is formed from relatively large amounts of α -ketoglutaric acid under the conditions employed in this method.

Glutathione. When glutathione is treated with chloramine T under the conditions used for the determination of glutamic acid, it forms a compound or compounds, probably β -cyanopropionylcysteylglycine and the disulphide, which give rise to succinic acid on hydrolysis. In experiments where only negligible amounts of glutathione are present, the glutamic acid can be determined without separating the β -cyanopropionic acid before hydrolysis (step 2A). However, if significant amounts of glutathione are present, as in blood or in certain tissue extracts, it is necessary to remove the β -cyanopropionic acid from the interfering compounds, as described in the procedure (2 A). The recovery of glutamic acid in the presence of glutathione using step 2A is listed in Table VI.

Table VI. *Recovery of glutamic acid in presence of glutathione*

β -Cyanopropionic acid extracted before hydrolysis.

	μ l. O ₂ found	μ l. O ₂ calc.	% recovery
Glutathione (7.2 mg.)	4.9	284*	1.7
Glutamic acid (3.3 mg.)	234	251	94
Glutathione (7.2 mg.) + glutamic acid (3.3 mg.)	242	251	96

* Calc. for the equiv. quantity of glutamic acid.

Glutamine. In quantities of 1 to 3.6 mg. glutamine yields 70–80 % of the theoretical amounts of succinic acid (Table V). The yields were not improved by hydrolysis with acid or alkali under a variety of conditions before the determination. Attempts to convert glutamine into pyrrolidone-carboxylic acid under conditions described by Vickery *et al.* [1935] and subsequent conversion of the pyrrolidone-carboxylic acid into glutamic acid in acid or alkali according to Wilson & Cannan [1937] failed to improve the yields. Since no satisfactory method has been found for converting glutamine quantitatively into glutamic acid, the problem of determining glutamic acid in the presence of glutamine remains unsolved. However, since glutamine occurs only under special conditions [Krebs, 1935], this difficulty will be met with in a few instances only.

Succinic acid. When present in appreciable quantities succinic acid can be determined either separately on an aliquot [Krebs, 1937] or it can be removed from the glutamic acid solution by extraction with ethyl ether. Glutamic acid in strongly acid solution is not extracted with ether. Thus a mixture containing 2.62 mg. succinic acid plus 3.96 mg. glutamic acid was acidified with 3 ml. 10 % H₂SO₄ and extracted with ether for 30 min. The ether extract was found to contain 2.62 mg. succinic acid, or 100 % recovery, while the aqueous solution when analysed was found to contain 3.78 mg. glutamic acid or 95.5 % recovery.

Inhibitors of succinoxidase. Compounds such as malonic, fumaric, oxaloacetic and α -ketoglutaric [Weil-Malherbe, 1937] acids inhibit the oxidation of succinic acid. The last three compounds only slow up the oxidation, but do not affect the final values. For example, we found that in the presence of 0.125 *M* α -ketoglutaric acid theoretical results for the added succinic acid were obtained in 60 min.

Neither aspartic acid nor asparagine yields succinic when treated with chloramine T and subsequently hydrolysed (Table VI). However, by analogy with reactions (1) and (2) it might be expected that they would yield malonic acid. To test this, solutions of aspartic acid and asparagine were treated according to the outlined procedure. These were then added to an enzyme preparation and the O₂ uptake of a known amount of added succinic acid was determined. The theoretical malonic acid concentration in the case of aspartic acid was 0.012 *M*, of asparagine 0.014 *M*. However, the theoretical O₂ uptake of the added

succinic acid (0.0025 *M*) was observed in the same period of time as in the controls. Thus neither aspartic acid nor asparagine interferes with the determination of glutamic acid.

Application

An example showing the application of the method to studies in intermediary amino-acid metabolism is shown in Table VII in which are listed data from experiments on transamination ("Umaminierung" of Braunstein & Kritzmann [1937]). It is to be seen that the formation of glutamic acid from

Table VII. *Rate of anaerobic glutamic acid formation and disappearance in pigeon breast muscle*

1 part pigeon breast muscle suspended in 5 parts *M*/10 phosphate buffer (pH 7.4); final substrate concentration = 0.016 *M*.

Glutamic acid formation from α -keto-glutaric and <i>l</i> (+) alanine		Glutamic acid disappearance	
min.	mg. glutamic acid	min.	mg. glutamic acid
0	0.61	0	8.82
10	6.67	60	4.57
20	5.40	60	(pyruvic acid added) 8.20
40	5.00		(no pyruvic acid added)

α -ketoglutaric acid and *l*(+)alanine proceeds very rapidly in minced muscle and reaches a peak within the first 10 min.; thereafter the glutamic acid formed slowly disappears. When glutamic acid alone is added to muscle it also disappears slowly, but when pyruvate is added 50% disappears in 60 min. The findings, which are being investigated further, in general confirm those reported by Braunstein & Kritzmann [1937].

The glutamic acid contents of different tissues are listed in Table VIII. The tissues were minced shortly after the death of the animal and extracted with

Table VIII. *Glutamic acid content of tissues*

	mg. glutamic per 100 g. wet wt.
Pigeon breast muscle	34
Pigeon gizzard	94
Guinea-pig kidney	94
Guinea-pig liver	47
Sheep brain (hemispheres)	80
Sheep heart muscle	171
Blood plasma (human)	2.8

2 vol. of boiling water. The glutamic acid contents of these tissues are remarkably high. In contrast, blood plasma has a very low glutamic acid content. Determinations of the glutamic acid content of the erythrocytes from the same specimen of blood show a value of the order of 20 mg. 100 ml.

A sample of human urine was found to contain 4.3 mg. 100 ml. of glutamic acid.

SUMMARY

A micro method for the determination of glutamic acid in biological material is described. Glutamic acid is oxidized by chloramine T to β -cyanopropionic acid; the latter is hydrolysed to succinic acid; succinic acid is determined

manometrically by means of a succinoxidase preparation. The method is highly specific; no other compound except glutamine occurring in biological material has been found to react.

The glutamic acid content of different tissues has been found to be remarkably high, the highest value observed being 171 mg. per 100 g. heart muscle.

I wish to express my thanks to Dr H. A. Krebs for suggestions and help throughout this study.

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