A MANOMETRIC METHOD FOR DETERMINING THE KINETICS OF AN ENZYMATIC HYDROLYSIS OF PEPTIDES*

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(Received for publication, March 29, 1947)

Although a number of standard methods are available for determining the rate of hydrolysis of peptide bonds (1–6), no one of these procedures provides the flexibility of operation which characterizes the manometric techniques evolved in the study of the respiratory enzymes. The present procedure was worked out in the hope of providing a manometric method suitable for use in studying the behavior of catheptic enzymes in crude tissue extracts. It is in dealing with such preparations that the greatest difficulties are encountered with titration procedures. The present method depends on the ability of a bacterial decarboxylase to split off carbon dioxide from free L-tyrosine, but not from tyrosine as bound in peptide linkage in the substrate carbobenzoxy-L-glutamyl-L-tyrosine.1 Thus, a second or “indicator” enzyme is used to record the reaction kinetics of the catheptic enzyme under investigation.

Stimulation for this approach came from recent estimation (7) of the amino acid composition of protein hydrolysates by means of bacterial decarboxylases, and the observation (8) that L-tyrosine decarboxylase will not attack glycyl-L-tyrosine. An analogous method has been used by Zeller (9, 10) in following the distribution of peptidase in the brain. Whereas Zeller’s procedure, in which a snake venom amino acid oxidase is employed, is most suitable for dealing with peptidases at pH 7, the method to be described below operates optimally at pH 5.0 to 5.5.

The principle of the decarboxylase-catheptic enzyme method is to be distinguished from that of the urease-arginase method of Weil and Russell (11). In the former the entire course of the enzymatic hydrolysis is followed dynamically, whereas in the latter method, at the termination of the arginase reaction, a single determination of the total amount of urea liberated during that period is made.

* This work was supported by a grant from the Donner Foundation, Inc. This is Reprint No. 633 of the Cancer Commission of Harvard University.

1 The substrate carbobenzoxy-L-glutamyl-L-tyrosine was obtained through the courtesy of Dr. G. W. Irving, Jr., and the late Dr. Max Bergmann.
ENZYMATIC HYDROLYSIS OF PEPTIDES

Materials and Methods

Preparation of Decarboxylase—The tyrosine decarboxylase was prepared from *Streptococcus lactis* R, obtained from the American Type Culture Collection. The culture medium of Bellamy and Gunsalus (12) was used. The decarboxylase was purified according to the procedure of Epps (8), through stage three, with citrate buffer. At this point, the coenzyme is still attached to the apoenzyme.

For assay purposes, a 20 mg. per cc. concentration of dry powder was suspended in 0.4 M citrate buffer at pH 5.5, incubated 16 hours at 37°, and centrifuged 15 minutes at 2400 r.p.m. The supernatant was either employed immediately or was frozen in a dry-ice box and used a few days later. Addition of synthetic pyridoxal phosphate (13) nearly doubled the activity of the decarboxylase, but the activity was sufficient for present purposes without the addition of this coenzyme.

Preparation of Cathepsin—A cathepsin preparation was made from swine kidneys by ammonium sulfate fractionation, according to the procedure of Fruton and Bergmann (14). The active fraction was dissolved in 1 per cent sodium chloride, dialyzed against 1 per cent sodium chloride for 48 hours at 3°, and frozen in a dry-ice box. Enzyme stored under these conditions has maintained a high order of activity for over 3 years.

Manometric Details—L-Tyrosine decarboxylase activity was measured in Warburg manometers by a modification of the procedure of Epps (8). Unless otherwise stated, all reactions were carried out at 25°, in the presence of nitrogen, buffered by 0.20 M citrate buffer at pH 5.4 to 5.5. In some cases the nitrogen was purified by passage over hot copper. Substrate was usually tipped in from the side arm after equilibration. The main compartment contained the cathepsin, decarboxylase, and buffer. The carbon dioxide evolved has been expressed as micromoles per cc. of test solution. All flask readings have been corrected for untipped controls. Since the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by a catheptic "pepsinase" (15) does not require the presence of an activator such as cysteine (16), none was added.

In some cases, the course of the enzymatic hydrolysis was followed in parallel with the manometric method by determination of amino nitrogen, according to the method of Van Slyke (4), or by the titration procedure of Grassmann and Heyde (17). As previously recorded (16), the end-point in the latter titration procedure is difficult with tyrosine-containing substrates.

Results

The relation of the reaction rate to the concentration of cathepsin is shown in Fig. 1. There is a good proportionality between the cathepsin concentration and the measured activity.
The relation of the reaction rate to the concentration of decarboxylase is shown in Fig. 2. In the presence of a large excess of decarboxylase (upper two curves), the reaction rate is relatively independent of the decarboxylase concentration and strictly dependent on the cathepsin concentration. It is therefore desirable to employ decarboxylase concentrations in this range.

The rate of reaction of the decarboxylase on L-tyrosine alone is proportional to the decarboxylase concentration, as is shown in Fig. 3.

The relation of the reaction rate to the concentration of carbobenzoxy-L-glutamyl-L-tyrosine is illustrated in Fig. 4. When the initial substrate concentration is above 0.017 M, the reaction rate is relatively independent of the substrate concentration during the first portion of the hydrolysis. $K_r = 3.0 \times 10^{-3}$, as calculated according to Lineweaver and Burk (18). At concentrations above 0.05 M, an inhibiting effect due to substrate ex-
cess begins to appear. The reaction kinetics are discussed in greater detail by Frantz and Stephenson (19).

![Graph showing the effect of varying the decarboxylase concentration on the rate of splitting of L-tyrosine.](image)

**Fig. 3.** Effect of varying the decarboxylase concentration on the rate of splitting of L-tyrosine. The decarboxylase concentration is expressed as mg. of nitrogen per cc. of test solution. CO₂ evolved during a 75 minute reaction time. The test solution contained the following: 0.2 cc. of 0.033 M L-tyrosine and 0.6 cc. of decarboxylase-citrate (1.02 mg. of nitrogen per cc.); dilutions made with buffer.

![Graph showing the effect of varying the concentration of carbobenzoxy-L-glutamyl-L-tyrosine.](image)

**Fig. 4.** Effect of varying the concentration of carbobenzoxy-L-glutamyl-L-tyrosine, the concentrations of cathepsin and decarboxylase being kept constant. The figures on the curves indicate the molar concentration of carbobenzoxy-L-glutamyl-L-tyrosine in the test solution. The test solution contained the following: 0.7 cc. of decarboxylase-citrate, 0.1 cc. of cathepsin, 0.3 cc. of carbobenzoxy-L-glutamyl-L-tyrosine at five different concentrations. The experiment was carried out in 95 per cent nitrogen-5 per cent carbon dioxide.

It was previously shown (7) that at pH 5.5 carbon dioxide retention in a tyrosine decarboxylase system was very slight, amounting to less than 5 per
Our results are in agreement with this finding, both on a basis of the theoretical 100 per cent carbon dioxide evolution and as a result of tipping in acid.

Comparison of the manometric method with Van Slyke amino nitrogen (4) determinations, run in parallel on the same type of test solution (but with the omission of decarboxylase), were made. The satisfactory agreement of the two methods is illustrated in Fig. 5. Fig. 5 further indicates that removal of one of the split-products (tyrosine) has no noticeable effect on the rate of the hydrolytic reaction. In order to check particularly on this point, the colorimetric amino nitrogen method of Frame, Russell, and Wilhelmi (20, 21) was used as a further standard. Comparisons were made of the rate of production of tyrosine in this enzymatic reaction as determined manometrically and colorimetrically. Good agreement was obtained between the two methods, and there was no evidence of acceleration of the enzymatic reaction as a consequence of removal of one of the split-products.
The effect of adding the other split-product of peptide hydrolysis, carbo- 
benzoxoeyglutamic acid, has been studied by Frantz and Stephenson (19). 
Their results show that carbobenzoxoeyglutamic acid has an inhibitory effect 
on the enzymatic splitting of carbobenzoxoeyglutamytyrosine. Thus from 
the hydrolysis of this peptide, one split-product (carbobenxoeyglutamic 
acid) inhibits the peptidase reaction, whereas the other split-product 
(l-tyrosine) does not.

Zeller and Maritz (9) used snake venom amino acid oxidase to measure 
the rate of hydrolysis of peptides by brain peptidases in the vicinity of pH 7. 
They reported an acceleration of peptidase activity as a consequence of the 
removal of amino acid from the reaction mixture by means of the amino 
acid oxidase. The amino acid oxidase method thus differs strikingly in 
this respect from the decarboxylase method.

### Table 1

**Reproducibility of Decarboxylase Method**

Manometric details are given in Fig. 5. The figures represent micromoles of car-
bon dioxide evolved. Theoretical 100 per cent hydrolysis is 15.03 micromoles.

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* \( S = \sqrt{\Sigma(x^2)/(N - 1)} \) (Arkin and Colton (22)).
As a test of the reproducibility of the method, six Warburg vessels containing cathepsin, carbobenzoxy-L-glutamyl-L-tyrosine, decarboxylase, and buffer were run in replicate. The results are recorded in Table I.

According to Umbreit et al. (23), the over-all accuracy of the Warburg apparatus, as used under conditions similar to the present ones, is within 5 per cent. It may be concluded that the reproducibility of the decarboxylase method is within the limit of accuracy of the apparatus.

As is evident from Table I, the hydrolysis of quantities of substrate of the order of 1 micromole may be followed with satisfactory accuracy.

![Graph showing the effect of varying the concentration of liver extract on the rate of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine.](http://www.jbc.org/)

**Fig. 6.** Effect of varying the concentration of liver extract on the rate of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine. Micromoles of CO₂ evolved between 6 and 20 minutes elapsed time after tipping. The test solution contained the following: 0.3 cc. of 0.055 M carbobenzoxy-L-glutamyl-L-tyrosine, 0.6 cc. of decarboxylase-citrate, and 0.4 cc. of enzyme diluted with 0.9 per cent sodium chloride. The enzyme solution was prepared by grinding up 450 mg. of lyophilized liver with 5 cc. of 0.9 per cent sodium chloride, centrifuging, and filtering through Pyrex glass wool. In the controls, water instead of substrate was tipped into the reaction mixture from the side arm.

As a test of the method in dealing with a catheptic enzyme as extracted from crude tissue powder, the type of experiment recorded in Fig. 6 was performed. A fairly linear relationship between concentration of tissue extract and enzymatic activity is present during the early phase of the reaction. The limit of sensitivity of the method under present working conditions is in the region of 0.1 micromole (cf. Fig. 6). The substrate carbobenzoxyglycyl-L-tyrosine is hydrolyzed by swine kidney carboxypeptidase (15), and the course of this reaction has been followed manometrically. Cysteine, which is an activator for this enzyme, has been found, however, to have an inconstant mild inhibitory effect on the tyrosine decarboxylase system. 0.01 M glutathione also activates this catheptic carboxypeptidase, but does not appreciably inhibit the tyrosine decarboxylase system.
DISCUSSION

The good agreement of results in which decarboxylase is omitted with those in which decarboxylase is present (cf. Fig. 5) is evidence that the decarboxylase does not contain substances which activate or inhibit the cathepsin appreciably. Likewise, a concentrate of the supernatant portion of a boiled decarboxylase preparation had no effect on the catheptic reaction.

When the activity of crude cathepsin preparations, as contained in aqueous extracts of tissue powders, is followed by titration procedures, the autolysis blank is found to comprise a disturbingly large fraction of the total titration. The autolysis blank in such a case measures the splitting of numerous types of peptide bonds by a number of enzymes. This difficulty is minimized by the use of the present method, by which the rate of scission of very few types of peptide bonds is measured.

Specific decarboxylases have been found for the natural forms of the amino acids lysine, ornithine, arginine, histidine, and glutamic acid (24). It is therefore theoretically possible to follow the hydrolysis of peptide substrates containing these amino acids by an adaptation of the present method. In agreement with Gale (24), we have been unable to find decarboxylases for glycine or for leucine, using cultures of soil bacteria and of Proteus vulgaris.

It should be mentioned that, although some experiments have been carried out over a period of hours, the possibility of an artifact due to bacterial multiplication makes it advisable to rely on short reaction periods or to add a bacteriostatic agent.

While the decarboxylase is used in the present experiments simply as a convenient indicator of the activity of a second proteolytic enzyme, it is worth recalling that both of these enzymes are present in the kidney (25–27). It is thus possible that the present "in vitro" coupled reaction sequence may serve as a model for a process which occurs naturally within the cell under certain circumstances, resulting in liberation of the pressor amine, tyramine.

From the finding of Zeller and Maritz (9) on the acceleration of peptidase activity by removal of split-products, and the one above on carbobenzoxyglutamic acid, the interesting question arises of whether the rate of peptide hydrolysis in the cell may be influenced by the fate of certain amino acid split-products. By remaining in the equilibrium mixture they may slow down the degradative reaction. Their removal by participation in further reactions such as decarboxylation, deamination, or transamination may facilitate continuation of peptide hydrolysis.

SUMMARY

A manometric method has been described for following the kinetics of the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by a preparation con-
taining catheptic enzymes. The method depends on the ability of a bacterial decarboxylase to split off carbon dioxide from L-tyrosine, but not from carbobenzyoxo-L-glutamyl-L-tyrosine.

The authors are indebted to Professor Joseph C. Aub for encouragement and interest and to Dr. Ivan D. Frantz, Jr., for performing the Van Slyke amino nitrogen determinations. Dr. Joseph S. Fruton and Dr. Irwin W. Sizer offered helpful criticisms.

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