MANOMETRIC ESTIMATION OF PEPTIDASE ACTIVITY

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In the course of investigations involving measurement of the hydrolysis of triglycine by bacterial suspensions or extracts, a manometric method has been developed for estimating with fair accuracy the extent of hydrolysis of this peptide. The method, which is based on the well known reaction between formol and amino groups, may prove to be useful in investigations involving breakdown and synthesis of peptides other than those with which we have worked, and it has, therefore, been thought worthwhile to publish the details. The method is simple, requiring use only of the Warburg manometric apparatus, and has given good duplication of results.

It is well known that the reaction between formol and an amino acid in aqueous solution results in a lowering of the pH of the solution. If the reaction is allowed to take place in a solution of bicarbonate, saturated with CO_2, an evolution of CO_2 will result. The amount of gas evolution is proportional to the extent of reaction between formol and the amino group involved, and to the number of such amino groups present. Thus, a quantity of dipeptide, after complete hydrolysis, may be expected to release twice as much carbon dioxide, after formol treatment in a bicarbonate solution, as is produced by this quantity of dipeptide, before hydrolysis.

This method has been used successfully by us for the estimation of di- and triglycine hydrolysis by suspensions and extracts of *Clostridium sporogenes*.

PROCEDURE

Preparation of formol

Two drops of phenolphthalein were added to 90 ml of pure formalin and sufficient alkali was then added to change the colour of the indicator to a faint pink.

After dilution with water to 100 ml a few grams of charcoal were added and the solution was filtered. The clear filtrate was gassed with a mixture of 93% nitrogen and 7% carbon dioxide and stored in a well stoppered bottle in a dark cupboard. A fresh solution was prepared weekly.

Method of analysis

The solution of amino acid or peptide to be analysed was added to the main compartment of a Warburg manometric vessel, with 0.2 ml of 0.28 *M* sodium bicarbonate solution. 0.7 ml of the formol solution was placed in the side arm of the vessel and the final volume in the flask was made up to 3.2 ml.

After gassing with the mixture of 93% nitrogen and 7% carbon dioxide, and thermal equilibration at 37°, the formol solution was tipped into the main compartment of the vessel and the gas evolution measured. The time required for complete

evolution of the gas was usually 20–25 minutes for a solution containing, for example, 0.0066 *M* triglycine. Readings were taken until the volume of gas was constant.

RESULTS

Initial results made it evident that gas evolutions, expected on the basis of a complete reaction between formol and the amino group, are rarely obtained. However, the reaction attains a well defined equilibrium, and an equilibrium constant may be obtained for any particular substance. The equilibrium point varies with the chemical nature of the compound, and, therefore, equilibrium constants have to be determined for all the substances under investigation. This constant is given by the ratio of the theoretical gas evolution (*i.e.* one NH_2 group producing one mole CO_2) to the observed amount of gas evolution. Typical values of such constants for a variety of substances are given in Table I.

TABLE I

FORMOL EQUILIBRIUM CONSTANTS OF PEPTIDES AND AMINO ACIDS

$$\text{Formol equilibrium constant} = \frac{\text{Gas evolution expected for conversion of one free } NH_2 \text{ group to one mole } CO_2}{\text{Observed gas evolution}}$$

Substance	*Formol equilibrium constants*	*Substance*	*Formol equilibrium constants*
Triglycine	1.74, 1.80, 1.88	L-Aspartate	3.60, 3.78
Glycyltyrosine	1.79, 1.81, 1.91	L-Asparagine	1.19, 1.28
Diglycine	1.54, 1.54, 1.57	DL-Alanine	2.21, 1.95
Glycyltryptophan	1.84, 1.86, 1.92	L-Threonine	1.30, 1.35
Glycine	1.17, 1.21	L-Tryptophan	1.92, 2.03
L-Glutamate	3.10, 2.90	Ammonium chloride	1.30, 1.20
L-Glutamine	1.14, 1.12		

It can be seen from these results that glycine and triglycine have different formol equilibrium constants. These constants have to be taken into account in calculating the extent of hydrolysis.

When triglycine and glycine are mixed, the CO_2 evolution of the mixture, after formol treatment, is the sum of the CO_2 evolutions of the two compounds. Theoretical and observed values for CO_2 evolution given by triglycine and glycine are given in Table II. The results indicate good agreement between duplicates. Moreover, a mixture of triglycine and glycine gives the expected additive values. For example, a mixture of triglycine ($\equiv$ 440 μl CO_2) and glycine ($\equiv$ 120 μl CO_2), giving a theoretical total CO_2 evolution of 560 μl, would result in a release of $\frac{440}{1.80} = 244.4$ μl CO_2 from the peptide alone, and $\frac{120}{1.20} = 100$ from glycine alone. The sum is 344.4, in good agreement with the observed figures of 344 and 338 (see Table II).

Calculation of extent of hydrolysis

Let $K_{f'}$ = formol equilibrium constant for tripeptide
Let $K_{f''}$ = formol equilibrium constant for glycine
Let V_0 = initial amount of tripeptide = A micromoles $\equiv 22.4\,A$ μl CO_2
$\equiv \frac{22.4\,A}{K_{f'}}$ μl $\equiv$ observed gas evolution of CO_2.

TABLE II

ESTIMATION OF FORMOL EQUILIBRIUM CONSTANTS OF GLYCINE AND TRIGLYCINE

Addition	*Theoretical value (µl)* (*1 mole $-NH_2 \equiv 1$ mole CO_2*)	*µl CO_2 evolved*	*µl CO_2 evolved (corrected)*	*Formol equilibrium constant* $= \frac{\text{theoretical value}}{\text{observed value}}$
Nil	0	30.0	0	
Triglycine	660	402.0	372.0	1.77 } average = 1.80
	660	390.0	360.0	1.83 }
Glycine	150	155.0	125.0	1.20 } average = 1.20
	150	154.5	124.5	1.20 }
Mixture of triglycine and glycine				µl CO_2 evolved (calculated)
Triglycine + glycine	440 + 120 = 560	374.0	344.0	$\frac{440}{1.8} + \frac{120}{1.2} = 344.4$
Triglycine + glycine	440 + 120 = 560	368.0	338.0	$\frac{440}{1.8} + \frac{120}{1.2} = 344.4$
Triglycine + glycine	660 + 90 = 750	472.0	442.0	$\frac{660}{1.8} + \frac{90}{1.2} = 441.6$

At time 't' let x micromoles of tripeptide be hydrolysed.

Therefore, tripeptide at time 't' $= (A - x)$ micromoles $\equiv 22.4\ (A - x)$ µl CO_2

$$\equiv \frac{22.4\ (A-x)}{K_{f'}} = \text{observed gas evolution of } CO_2 \text{ due to tripeptide.}$$

Glycine formed $= 3x$ micromoles $= 67.2x$ µl CO_2

$$\equiv \frac{67.2\ x\ \mu l}{K_{f''}} = \text{observed gas evolution of } CO_2 \text{ due to glycine formed.}$$

V_t = total volume of gas evolution observed at time 't' after adding formol

$$= \frac{22.4\ (A-x)}{K_{f'}} + \frac{67.2x}{K_{f''}}\ \mu l.$$

Difference between observed volumes of gas evolution at time 't' and zero time $= V_t - V_0$

$$= \frac{22.4\ (A-x)}{K_{f'}} + \frac{67.2\ x}{K_{f''}} - \frac{22.4A}{K_{f'}}\ \mu l$$

$$= x\left(\frac{67.2}{K_{f''}} - \frac{22.4}{K_{f'}}\right)\mu l.$$

Therefore, $x = \dfrac{V_t - V_0}{\dfrac{67.2}{K_{f''}} - \dfrac{22.4}{K_{f'}}} \equiv$ micromoles of tripeptide hydrolysed at time 't'.

This equation is based on the assumption that the triglycine is fully hydrolysed to glycine. This assumption is justified in our experiments, as chromatographic data, and data obtained with the manometric formol technique, indicate that diglycine is hydrolysed much more rapidly than triglycine with suspensions or extracts of *Clostridium sporogenes*.

The above equation applies to the calculation of results obtained with peptides of one amino acid. Changes in the equation obviously have to be made with peptides containing two or more different amino acids. We have limited ourselves to the utilisation of di- and triglycine in these experiments.

A typical experiment, using the manometric formol technique, to estimate peptide hydrolysis, is as follows:

Enzymic hydrolysis of triglycine is carried out in 0.028 *M* $NaHCO_3$, in an atmosphere of 93% nitrogen and 7% carbon dioxide, with a suspension, or a cell-free extract, of *Clostridium sporogenes* as source of enzyme. After a given time, usually 60 minutes, the cells are removed by centrifugation at 20,000 *g*. If a cell-free preparation is used, the enzyme is heated for 10 minutes in a boiling water bath and the precipitated protein is removed by centrifugation before the analyses are carried out. A 2 ml sample of the supernatant is used for analysis. Since this aliquot already contains the required bicarbonate concentration, further bicarbonate is not added. The side arm contains 0.7 ml of neutral formol solution and the volume is made up to 3.2 ml. Control experiments on the (organism) suspension or extract, incubated without tripeptide, are always carried out. It is necessary to determine the "blank" of the (organism) suspension or extract, as the organism may have previously been in contact with amino acids or ammonium chloride, and traces of these adhering to the organism will affect the CO_2 evolution. Standard values of gas evolution given by known quantities of triglycine and glycine are determined with each experiment.

Table III gives the results of a typical experiment, showing the gas evolutions and the calculation of the amount of tripeptide hydrolysed.

TABLE III

PROTOCOL OF A TYPICAL EXPERIMENT ON TRIGLYCINE HYDROLYSIS BY A BACTERIAL SUSPENSION

Contents	*µl CO_2 observed*	*Corrected µl CO_2*	*Theoretical CO_2 (µl)*	*Formol equilibrium constant*
Nil	44.5	—	—	
Glycine standard	147.0	103.5	120	$\frac{120}{103.5} = 1.18 = K_{f''}$
Triglycine standard	198.0	153.5 (V_0)	275	$\frac{275}{153.5} = 1.79 = K_{f'}$
Bacteria "blank" A	73.5	—	—	—
Bacteria "blank" B	84.5	—	—	—
After time "t"				
Bacteria A + triglycine	330.0	256.5 (V_t)	—	—
Bacteria B + triglycine	430.0	345.5 (V_t)	—	—

$$\mu M \text{ Peptide hydrolysed in aliquot (Bacteria A)} = \frac{V_t - V_0}{\frac{67.2}{K_{f''}} - \frac{22.4}{K_{f'}}} = \frac{256.5 - 153.5}{44.5} = 2.31\ \mu M.$$

$$\mu M \text{ Peptide hydrolysed in aliquot (Bacteria B)} = \frac{345.5 - 153.5}{44.5} = 4.31\ \mu M.$$

Bacteria A = *Clostridium sporogenes* suspension previously incubated only in bicarbonate buffer solution.

Bacteria B = *Clostridium sporogenes* suspension previously incubated in a mixture of 0.05 *M* glucose and 0.1 *M* L-proline.

SUMMARY

A manometric method has been devised for following peptide hydrolysis. The method depends on the reaction of amino acids with formol, in bicarbonate solution, whereby carbon dioxide is evolved. Peptides and certain amino acids give equilibrium values of gas evolution. Such values enable the extent of hydrolysis of peptides to be calculated.

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