A PROCEDURE FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY*

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The difficulties introduced by the desire to maintain a constant pH during an enzyme-catalyzed hydrolysis of peptide-like substrates and at the same time to determine the extent of hydrolysis by an acid-base titration have been pointed out (1), but to date no completely satisfactory solution of the problem has been given. With those enzymes whose pH optima lie in the region between pH 7.5 to 8.5, *e.g.* trypsin and chymotrypsin, the poor buffering capacity of phosphate in this region prompted us, as it has others (2–5), to consider the use of organic amines whose pK'_a values were near to or identical with the pH optimum of the enzyme being used. In the course of such studies it soon became evident that coincidental use of a suitable primary or secondary amine buffer system and a formol titration (1) would insure adequate buffering capacity with low buffer concentration during the hydrolysis and at the same time permit the final acid-base titration to be conducted under nearly ideal conditions. In this communication we shall limit the discussion to results obtained with chymotrypsin and specific acylated-α-amino acid amide substrates, since the application of the general method to other proteolytic enzymes and other types of substrates will be obvious.

Initially the system $\overset{+}{N}H_3CH_2CH_2\overset{+}{N}H_3\text{-}\overset{+}{N}H_3CH_2CH_2NH_2\text{-}NH_2CH_2CH_2\text{-}NH_2$ (pK'_{a_1} = 10.0; pK'_{a_2} = 7.0) (6) was employed as a buffer for chymotrypsin studies at pH 7.8[1] and, while superior to phosphate or veronal, was subsequently discarded in favor of the system $(CH_2OH)_3C\overset{+}{N}H_3\text{-}(CH_2OH)_3CNH_2$ (pK'_a = 8.1) which is not only an excellent buffer at pH 7.8 (7) but is also monovalent. While formaldehyde would be expected to react with either a primary or secondary amine, the titration curves given in Figs. 1 and 2 will serve to emphasize the point that the reaction is not necessarily quantitative or irreversible (1). Thus for any given case in which maximum accuracy is desired it is clear that, as for other formol titrations (1), the end-point of the titration must be determined experimentally and in the system containing buffer, substrate,

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[1] Unpublished experiments of R. V. MacAllister.

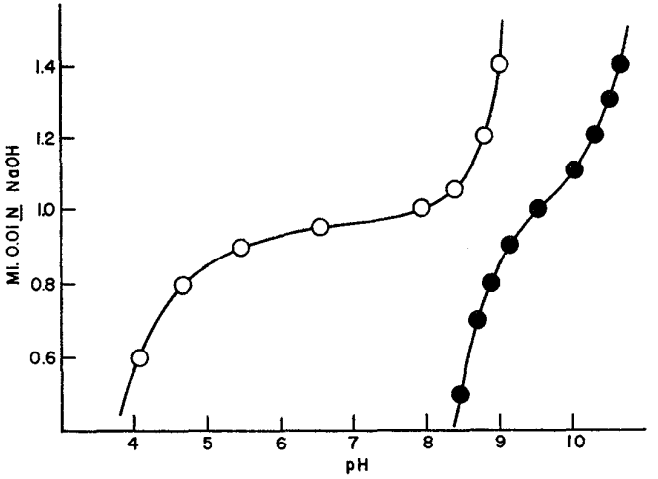

FIG. 1. Titration of tris(hydroxymethyl)aminomethane-hydrochloric acid buffer. ●, 2.0 ml. of 0.01 M buffer; ○, 1.0 ml. of 0.02 M buffer and 1.0 ml. of 36 per cent aqueous formaldehyde adjusted to pH 7.0.

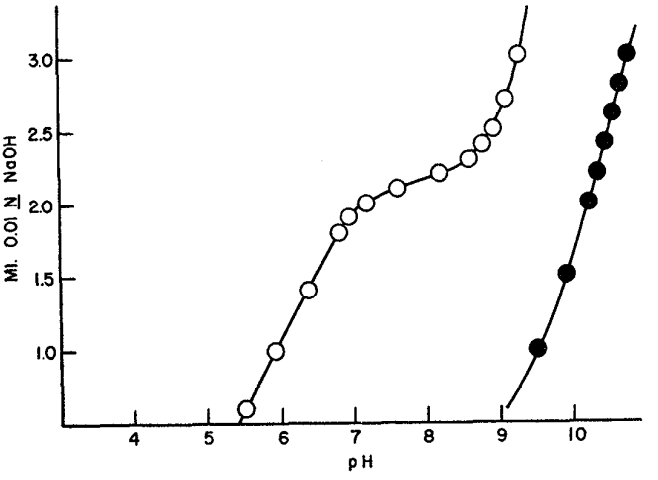

FIG. 2. Titration of ethylenediamine-hydrochloric acid buffer. ●, 2.0 ml. of 0.01 M buffer; ○, 1.0 ml. of 0.02 M buffer and 1.0 ml. of 36 per cent aqueous formaldehyde adjusted to pH 7.0.

enzyme, and hydrolysis products. The effect of the hydrolysis products, either singly or in combination, upon the end-point of the titration is illustrated by the titration curves given in Fig. 3. The data of Fig. 4 clearly

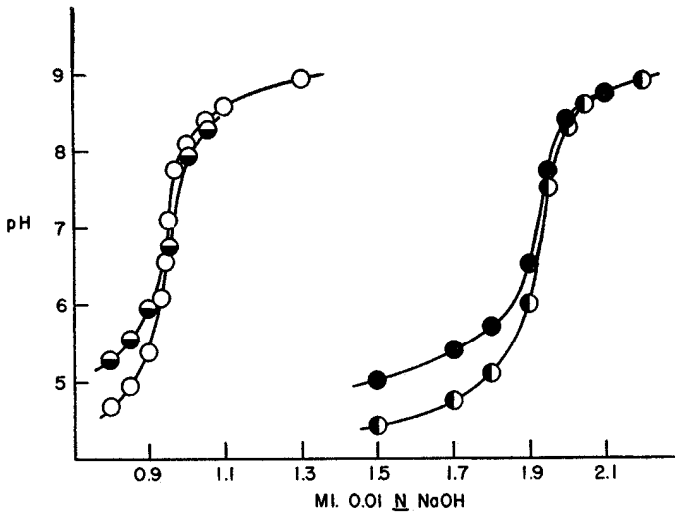

FIG. 3. Formol titration of tris(hydroxymethyl)aminomethane-hydrochloric acid buffer and of buffer and hydrolysis products. ○, 1.0 ml. of 0.02 M buffer and 1.0 ml. of 36 per cent aqueous formaldehyde adjusted to pH 7.0; ◒, 1.0 ml. of solution 0.02 M in buffer and 0.01 M in ammonia and 1.0 ml. of formaldehyde solution; ◐, 1.0 ml. of solution 0.02 M in buffer and 0.01 M in acetyl-DL-phenylalanine and 1.0 ml. of formaldehyde solution; ●, 1.0 ml. of solution 0.02 M in buffer, 0.01 M in ammonia, 0.01 M in acetyl-DL-phenylalanine, and 1.0 ml. of formaldehyde solution.

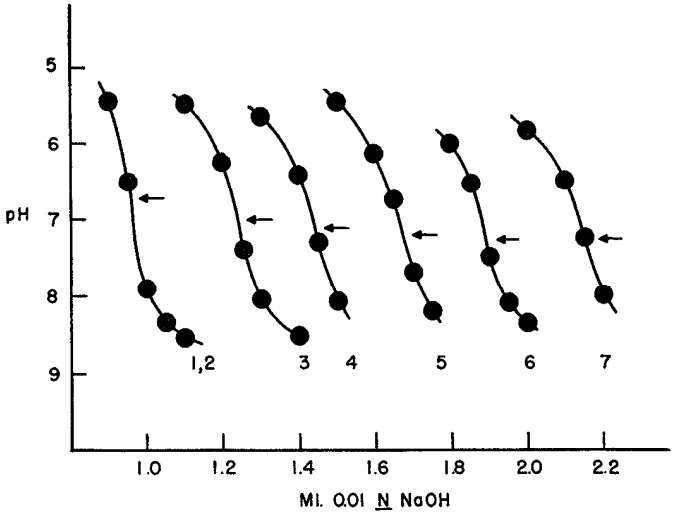

FIG. 4. Formol titration curves obtained during hydrolysis of nicotinyl-L-tryptophanamide by chymotrypsin at pH 7.8 and 25°. Curve 1, buffer; Curve 2, buffer plus substrate; Curve 3, buffer plus substrate plus enzyme, t = 1 minute; Curve 4, same as Curve 3 except t = 10 minutes; Curve 5, same as 3 except t = 20 minutes; Curve 6, same as Curve 3 except t = 30 minutes; Curve 7, same as Curve 3 except t = 60 minutes. End-point indicated by arrow.

show that every component in an enzymatic digest, with the exception of neutral substrates which do not react with formaldehyde, can influence the end-point of the formol titration. Curves 1 and 2, which are superimposable, are formol titration curves of a 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer of pH 7.8 and of this buffer plus 10 μM of nicotinyl-L-tryptophanamide per ml. of solution respectively. Curve 3 is the formol titration curve of a solution 0.02 M in buffer containing 10 μM of substrate and an amount of chymotrypsin equivalent to 0.15 mg. of protein nitrogen per ml. of solution determined 1 minute after the addition of the enzyme. Curves 4 to 7 are similar titration curves determined 10, 20,

TABLE I

*Hydrolysis of Nicotinyl-L-tryptophanamide by Chymotrypsin in Presence of Various Buffers**

Time	Per cent hydrolysis						
	$NH_2CH_2CH_2NH_2$·HCl			$(CH_2OH)_3CNH_2$·HCl			Phosphate
	0.005 M	0.02 M	0.05 M	0.005 M	0.02 M	0.1 M	0.02 M
min.							
10	25	25	24	24	23	21	27
20	45	45	51	46	46	50	52
30	64	67	67	68	68	69	70
40	75	77	81	77	79	83	82
50	83	85	85	86	87	87	87
60	89	92	88	93	94	94	94

* At 25°, pH 7.8, initial substrate concentration s_0 = 10 μM per ml. of reaction mixture, initial enzyme concentration E_0 = 0.15 mg. of protein nitrogen per ml. of reaction mixture.

30, and 60 minutes after the addition of the enzyme. With the end-point of the titration varying with the extent of hydrolysis, it may be concluded that in order to obtain maximum accuracy in the determination of proteolytic activity by the above method it is imperative to use a potentiometric titration (8) so that the end-point of the titration may be determined coincidentally with the extent of hydrolysis at any given time.

In any enzyme-catalyzed reaction it is necessary to demonstrate that the buffer used does not participate in the reaction other than to control the pH of the system if unambiguous results are to be obtained. Accordingly the chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide was studied at pH 7.8 and 25° in three different buffer systems and at three different concentrations of two of the three buffers. These data are given in Table I. In a second series of experiments a similar study was made in respect to the hydrolysis of nicotinyl-L-phenylalaninamide, though in this

latter case only two buffers were compared at a single buffer concentration (Table II). The data given in Tables I and II clearly show that within the limits of experimental error the rates of hydrolysis of the two substrates above at pH 7.8 and 25° are essentially independent of the nature of the buffer, and it may be concluded that none of the three buffers participates in the hydrolytic reaction other than to control the pH of the system. In view of the fact that similar results have been obtained with a number of other specific chymotrypsin substrates[2] it would appear that either ethylenediamine or tris(hydroxymethyl)aminomethane buffers may be used without fear of complications in any chymotrypsin-catalyzed hydrolysis provided the buffer concentration is not allowed to exceed that required to insure adequate buffering of the system.

TABLE II

*Hydrolysis of Nicotinyl-L-phenylalaninamide by Chymotrypsin in Presence of Various Buffers**

Time	Per cent hydrolysis	
	$NH_2CH_2CH_2NH_2$-HCl, 0.02 M	Phosphate, 0.02 M
min.		
15	21	20
30	40	38
60	63	62
90	79	78
120	92	90

* At 25°, pH 7.8, initial substrate concentration $s_0 = 10$ μM per ml. of reaction mixture, initial enzyme concentration $E_0 = 0.15$ mg. of protein nitrogen per ml. of reaction mixture.

In Figs. 1 to 4 it will be noted that there is a large potential change in the region of the end-point when tris(hydroxymethyl)aminomethane is used as a buffer. While there is no doubt that coincidental determination of the end-point of the titration and the extent of hydrolysis by a potentiometric titration will give the most accurate results, it is obvious that in some cases an indicator titration could be used, though with some sacrifice in accuracy. The data given in Table III illustrate the point that, if the pH of the end-point is known and is invariant with respect to time, the precision of the indicator titration is of the same order of magnitude as that of the potentiometric titration. However, in the determination of proteolytic activity there is no guarantee that the end-point of the titration will be the same for all substrates, for all proteolytic enzyme preparations, and for all concentrations of these components. This fact and the

[2] Unpublished experiments of R. V. MacAllister, H. T. Huang, and B. M. Iselin.

fact that for any given initial substrate and enzyme concentration the pH of the end-point of the titration does vary with time clearly establish the limitations of the above indicator titration even though it is decidedly superior to those in which the titration is conducted in a highly buffered system (9–11).

TABLE III

*Titration of Simulated Reaction Mixture**

0.10 N NaOH consumed per 2.0 ml. mixture		
Potentiometric titration; end-point, pH 7.25	Phenol red titration; end-point, orange color	Brom thymol blue titration; end-point, blue color
ml.	*ml.*	*ml.*
1.94	1.97	1.94
1.96	1.95	1.96
1.96	1.96	1.93
1.95	1.93	1.95
1.95	1.95	1.96
1.95	1.96	1.97
1.95 ± 0.01	1.95 ± 0.02	1.95 ± 0.02

* 1.0 ml. of a solution 0.02 M in tris(hydroxymethyl)aminomethane-hydrochloric acid buffer, 0.01 M in acetyl-DL-phenylalanine, and 0.01 M in ammonia and 1.0 ml. of 36 per cent aqueous formaldehyde, pH 7.0.

EXPERIMENTAL

Reagents—A 0.20 M stock solution of the tris(hydroxymethyl)aminomethane-hydrochloric acid buffer was prepared by dissolving 12.11 gm. of the amine (obtained from the Commercial Solvents Corporation), m.p. 168–169° after two recrystallizations from ethanol, in the minimum quantity of water, adding 50 ml. of 1 N hydrochloric acid, and making the solution up to 500 ml. The pH of this solution was 8.05 and that of a 0.02 M solution 7.85. A 0.20 M stock solution of the ethylenediamine-hydrochloric acid buffer was prepared from 6.01 gm. of freshly distilled ethylenediamine and 107 ml. of 1.0 N hydrochloric acid made up to 500 ml. The pH of this solution was 8.0 and that of a 0.02 M solution 7.80. The concentration of all buffers is given in respect to the amine component. The two substrates used in this study, *i.e.* nicotinyl-L-tryptophanamide and nicotinyl-L-phenylalaninamide, were prepared by the condensation of nicotinyl azide with the amino acid ester and subsequent ammonolysis (12). The chymotrypsin used was an Armour preparation.

Procedure—The desired quantity of substrate was weighed into a 10.0 ml. calibrated glass-stoppered volumetric flask, dissolved in 5 to 7 ml. of

hot water, 1.0 ml. of 0.20 M buffer solution added, the solution brought to thermal equilibrium, the desired amount of enzyme preparation added,[3] the solution made up to volume, and thoroughly mixed. 1 ml. aliquots, withdrawn from the solution immediately after mixing and at subsequent selected time intervals, were added to 1.0 ml. of 35 per cent aqueous formaldehyde, previously adjusted to pH 7.0 by the addition of 0.1 N sodium hydroxide, and contained in a 20 mm. × 50 mm. shell vial, and the mixture was immediately titrated potentiometrically with standard 0.01 N sodium hydroxide by use of a Beckman model G pH meter equipped with No. 270-6 calomel electrode and a No. 290-11 glass electrode. The semi-automatic burette, graduated in 0.01 ml., was equipped with a capillary tip of sufficient length to permit introduction of the reagent beneath the surface of the solution being titrated which was stirred by rotation of the shell vial (13). The end-point of the titration and extent of hydrolysis were determined by examination of the curve constructed for each titration. It should be pointed out that this procedure consumes no more time than that taken in trying to decide whether or not the end-point has been reached in an indicator type of titration and is far more objective. In all cases blank experiments in which enzyme or substrate was omitted were performed coincidentally.

Results

The data given in Figs. 1 to 3 are self-explanatory. The potentiometric formol titration curves given in Fig. 4 were obtained by the titration of 1.0 ml. aliquots of a system 0.02 M in tris(hydroxymethyl)aminomethane-hydrochloric acid buffer with an initial substrate concentration, s_0, of 10 μM per ml. of solution and an initial enzyme concentration, E_0, of 0.15 mg. of protein nitrogen per ml. of solution after the addition of 1.0 ml. of 36 per cent formaldehyde previously adjusted to pH 7.0. The data given in Tables I and II were obtained as described above and those in Table III are self-explanatory.

SUMMARY

A procedure for the determination of proteolytic activity, based upon coincidental use of a primary or secondary amine buffer system and a potentiometric formol titration, has been described and it has been shown that in order to obtain accurate results the end-point of the titration must be determined coincidentally with the extent of hydrolysis. For studies with chymotrypsin, a tris(hydroxymethyl)aminomethane-hydrochloric acid buffer is recommended.

[3] It is recommended that whenever possible the solid enzyme preparation be weighed out for each individual hydrolysis experiment and dissolved in about 1 ml. of water just prior to the addition to the system.

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