THE EFFECT OF pH AND CATIONS ON THE THERMAL DENATURATION OF TRYPSIN

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Summary

Dialysed or very dilute solutions of crystalline trypsin are rapidly and irreversibly inactivated at 40°C. At this temperature and in dilute solution the enzyme is most stable at pH values of about 2.7, 6.6, and 10.7, with intermediate regions of instability.

Between pH 3.0 and 6.5 all cations stabilize trypsin, salts of Cr, Al, Th, La, Fe+++, and Be affording considerable protection at 1×10^{-4}M, salts of Ca, Ba, Mg, and Mn at 1×10^{-2}M, and salts of K, Na, and NH_4 at 2M. Salts of Zn, Co, Ni, Cd, and Hg increase the stability of trypsin at 5×10^{-4}M but tend to inactivate the enzyme at higher concentrations. Between pH 7.0 and 9.5 where the most rapid inactivation takes place, cations soluble at the experimental pH stabilize the enzyme. The affinity of trypsin for cations increases with increasing pH.

Complexes of ferric salts with tartrate and pyruvate stabilize the enzyme at pH 8.0. Anionic ferritartrate is ineffective, stabilization being obtained only with the non-ionic complex which is formed in solutions of sufficiently low tartrate concentration.

On changing the conditions of inactivation to favour autodigestion of the enzyme, either by increasing the enzyme concentration or by heating for an extended period at 30°C, Mg was found to have little stabilizing effect whereas Ca was more effective than in dilute solutions of the enzyme at 40°C.

Evidence is presented that in dilute solutions of trypsin irreversible denaturation takes place at 40°C by a process which does not involve autodigestion or the formation of an intermediate reversibly denatured form of the enzyme.

I. Introduction

The earliest report of inactivation of trypsin by dilution was made by Rona and Kleinmann (1928) who showed that dilute solutions of crude trypsin lost up to 50 per cent. of their activity unless suitable concentrations of electrolytes were present. At pH values between 5.0 and 6.0 Al salts showed a considerable stabilizing effect at 0.003M, Mg salts at 0.01-0.05M, and Na salts at 2M.

Bier and Nord (1951) and Gorini (1951) showed crystalline trypsin to be unstable in solution at pH 8.0 and claimed that Ca and Mn salts were the only electrolytes providing significant protection against thermal inactivation.

There are also reports of "activation" of trypsin by metal ions. Michaelis and Stern (1931) found trypsin to be "activated" by certain coordinated Fe

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compounds such as trisdipyridyl iron (II) sulphate and "inhibited" by others such as ferritartrate. Kikuo Sugai (1944) reported that salts of Na, K, Mg, and Ca did not affect tryptic activity whereas $AlCl_3$ caused some increase in activity and salts of Mn, Fe, Co, and Ni caused marked increases in activity. Bresler and Rosetzveig (1951) found dialysed preparations of crystalline trypsin to be "activated" by salts of Mg and Cr and "inhibited" by metal-chelating agents. The "inhibition" of commercial preparations of the enzyme by such reagents was ascribed to their reaction with Cr.

The data in many of these papers are not sufficient to distinguish between activation and stabilization by metal ions during the preliminary warming up and subsequent incubation. Changes in pH of the enzyme solution on addition of salts before incubation of the enzyme with the substrate were not checked, and the course of breakdown of the substrate in the presence and in the absence of metal ions was not investigated. This paper describes the influence of pH, temperature, cations in the presence and absence of buffers, and coordinated metal complexes on the activity and stability of trypsin, and reports some preliminary kinetic experiments on the thermal inactivation of trypsin.

II. Materials and Methods

Crystalline trypsin (Armour) was used in most experiments and some crucial experiments were repeated with a crystalline preparation kindly supplied by Dr. Elizabeth Work.

Apart from $Th(NO_3)_4$ and $BeCl_2$, which were of "Laboratory Reagent" quality, all chemicals were of "Analar" quality. Except for Th, which was added to the enzyme as the nitrate, chlorides were used in all experiments.

The activity of the trypsin solutions was measured by the gravimetric method for determining gelatin hydrolysis (Crewther 1952) and in some experiments by the haemoglobin digestion method of Anson (1938). The gelatin used for preparing buffer substrate solution for the gravimetric method contained considerable amounts of Ca salts, which could be readily demonstrated by precipitation with oxalate. Unless otherwise specified, digestions were carried out at 40°C and pH 7.0, the optimum pH at that temperature as estimated by both methods. In experiments using the gravimetric method, thermal inactivation of the enzyme was carried out by pipetting 0.1 ml of a well-dialysed solution containing approx. 1 mg crystalline trypsin per ml into 20 ml of a solution of the electrolyte standing in a water-bath at the required temperature. The solution was immediately shaken and returned to the water-bath for the experimental period before estimating residual activity. When the haemoglobin digestion method was used to estimate residual activity the concentration of the enzyme was increased tenfold and in some experiments only 10 ml of the electrolyte solution was used. Dialysis of a solution containing 10 mg/ml of the enzyme was carried out through Visking cellulose tubing in distilled water without agitation for 2-3 days at 0-3°C. The solution was then diluted with distilled water if necessary. A glass electrode assembly was used to measure pH.

III. Experimental

(*a*) *Effects of Dilution and Dialysis of Trypsin Solution*

Figure 1 shows the activities of a series of dilutions of a trypsin solution using distilled water as diluent, and of similar solutions diluted with the boiled diffusate from a concentrated solution of crude trypsin. At low enzyme concentrations there was considerable inactivation in the solution diluted with water. Dialysis of solutions of crystalline trypsin against running tap water* for several hours resulted in almost complete inactivation, and activity was not restored by addition of salts of K, Na, Mg, Ca, Mn, Fe, Co, or Cu, or by the addition of diffusate from crude trypsin solutions (Table 1). However, dialysis against salts of any of the above metals or dialysis in the cold caused considerably less diminution in activity (Table 1). Similarly the use of various electrolyte solutions for dilution of trypsin solutions reduced the inactivation at high dilution.

Table 1

EFFECTS OF DIALYSIS AND METAL IONS ON THE STABILITY OF TRYPSIN

Trypsin Solution	Additions	Activity (gravimetric units)
Undialysed	Nil	12·6
Dialysed against distilled water, 24 hr at room temperature	Nil	0·5
	$MgSO_4$ 0·01M	0·6
	$CaCl_2$ 0·01M	0·7
	$CoCl_2$ 0·01M	0·4
	$MnCl_2$ 0·01M	0·5
Dialysed against boiled diffusate from crude trypsin, 24 hr	Nil	9·7
Dialysed against 0·001M $MgSO_4$	Nil	9·7
Dialysed against 0·1M NaCl	Nil	8·5
Dialysed against distilled water, 48 hr at 2°C	Nil	11·8
Dialysed at 2°C, held at 40°C 1 hr, pH 7·8	Nil	0·3
pH 8·4	$MgSO_4$* 0·001M	9·2
pH 8·1	$CaCl_2$* 0·003M	10·2
pH 7·6	$MnCl_2$* 0·003M	9·3
pH 9·4	KCl* 1M	8·0
pH 7·5	$Al_2(SO_4)_3$* <0·0001M	3·4

* Added before heating the enzyme to 40°C.

(*b*) *Relation between pH and Stability of Trypsin Solutions*

A dialysed solution of trypsin was diluted 1/200 with dilute HCl or NaOH at 40°C to give a suitable range of final pH values, and held at that temperature in stoppered tubes. Residual activity was measured after 60 min and the

* The tap water in Melbourne contains approx. 5 p.p.m. Ca and may reach a temperature of 21°C in summer.

final pH values determined immediately. There were three peaks of maximum stability at pH approx. 2.9, 6.6, and 10.7 (Fig. 2) and inflexions in the curve at pH 3.5 and 6.0. The activity values at the extreme acid end of the curve

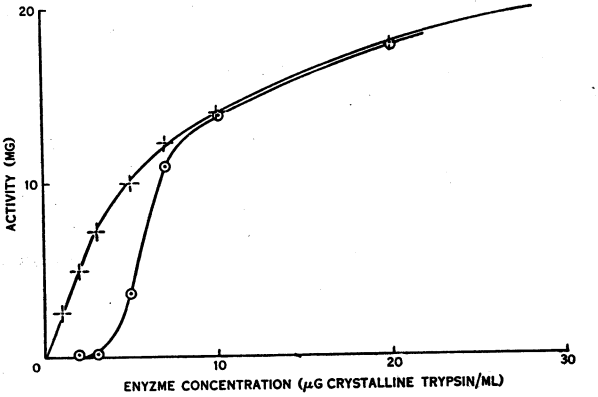

Fig. 1.—Inactivation of trypsin by dilution. ⊙, Trypsin diluted with distilled water. +, Trypsin diluted with diffusate from crude trypsin solution.

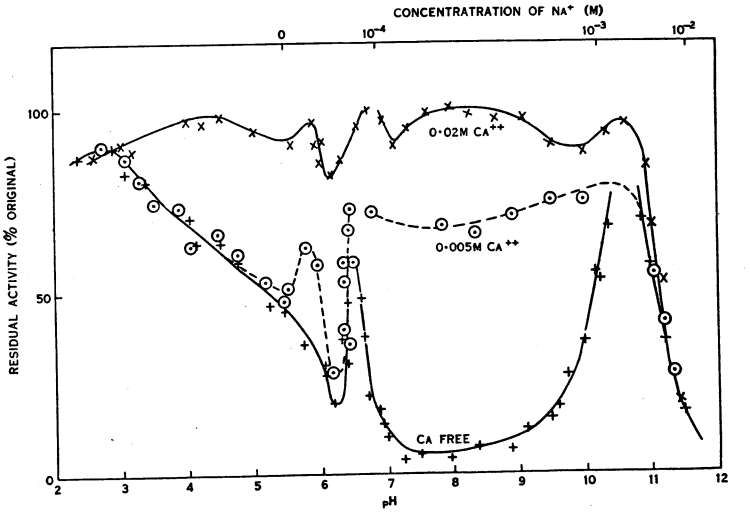

Fig. 2.—Effect of pH on the stability of trypsin in the presence and absence of calcium salts. +, pH-stability curve in the absence of added cations. ⊙, pH-stability curve in 0.005M $CaCl_2$. ×, pH-stability curve in 0.02M $CaCl_2$. The ordinates are the residual activities after holding 30 min at 40°C.

were slightly low, owing to a small reduction in pH of the buffer substrate on adding the enzyme solution. The accurate estimation of pH in the final unbuffered enzyme solutions was made difficult by solution of CO_2 and by the

slow rate at which the glass electrode came to equilibrium with the solution in the absence of buffer. Errors of ± 0.1 pH unit probably occurred. A similar relationship between trypsin stability and pH was obtained using the haemoglobin digestion method for estimating residual activity.

(*c*) *pH-Activity Curves*

Under the conditions usually employed for estimation of tryptic activity (26°C for 10 min (Anson 1938)), a pH optimum of 7.5 is obtained. With a digestion temperature of 40°C the gravimetric technique provided the curves shown in Figure 3, the optimum activity being at pH *c.* 7.0 with a subsidiary peak at *c.* 9.0. To ensure that a single enzyme was being estimated, the pH-activity curve of a solution of trypsin was compared with that of the same solution after partial heat inactivation at pH 8.0. The ratio of activities over the whole pH range was constant within experimental error (Fig. 3). This

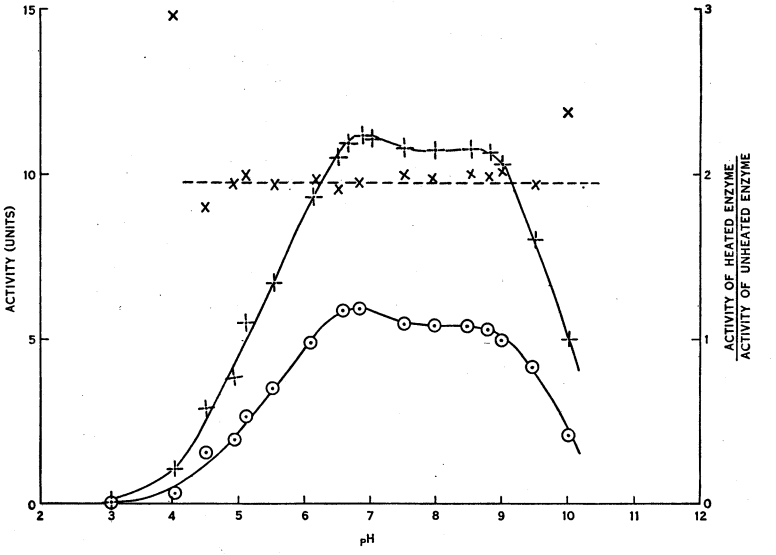

Fig. 3.—pH-activity curves of trypsin and partially inactivated trypsin. +, Native trypsin. ⊙, Trypsin partially inactivated at 40°C. ×, (Activity native trypsin)/(Activity partially inactivated trypsin).

suggests that the dual peaks were due to inactivation of the enzyme during incubation with the substrate at 40°C between pH 7.0 and 9.0, and not to the presence of more than one enzyme. The incorporation of 0.005M $CaCl_2$ in the buffer substrate solution or reduction of the temperature of incubation to 25°C provided curves with a single peak at a pH of approx. 7.6. When Anson's haemoglobin method was used at 40°C to determine the pH-activity curve, a single activity peak was obtained at pH 7.0. The differences in the pH-activity curves obtained by the gravimetric method and the haemoglobin digestion method are attributable to the greater amount of inactivation of the enzyme during the longer incubation period of the gravimetric method.

(*d*) *Stabilization of Trypsin by Cations*

It was difficult to ensure that a predetermined pH would be obtained on mixing dialysed enzyme solution with unbuffered salt solutions, particularly between pH 7.0 and 9.0. By pre-testing mixtures of enzyme and the salt solutions the range of final pH values for a series of dilutions of a particular salt could be limited sufficiently for practical purposes, but considerable variations in pH occurred from one salt to another.

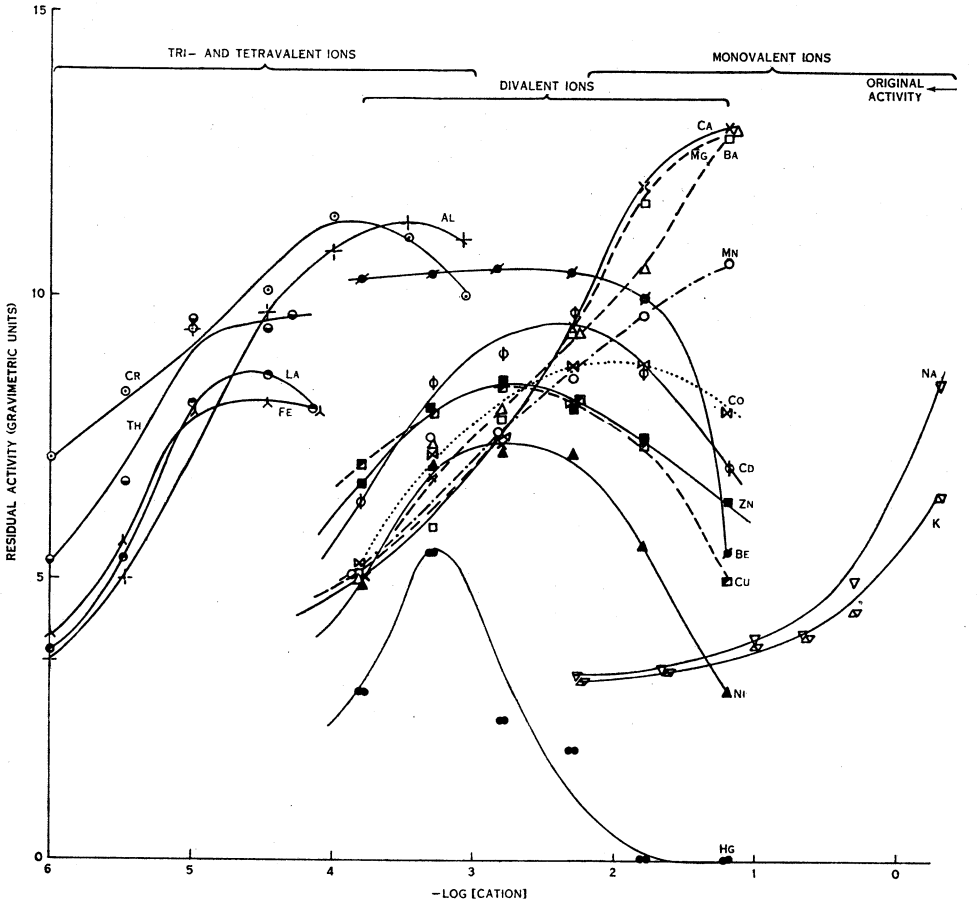

Fig. 4.—Stabilization of trypsin against treatment for 1 hr at 40°C in the presence of metal chlorides. The pH ranges for the various cations were:

Th (as the nitrate) 5.05 ± 0.10	Ca 6.10 ± 0.15	Zn 5.80 ± 0.15
Cr 6.0 ± 0.25	Mg 6.05 ± 0.15	Cu 6.62 ± 0.10
Al 6.20 ± 0.25	Ba 5.95 ± 0.15	Ni 6.10 ± 0.20
La 4.91 ± 0.06	Mn 5.9 ± 0.30	Hg 5.33 ± 0.25
Fe 4.95 ± 0.08	Co 6.0 ± 0.40	Na 6.05 ± 0.10
Be 6.00 ± 0.15	Cd 6.90 ± 0.20	K 6.12 ± 0.15

Figure 4 shows the residual activity after 1 hr at 40°C of trypsin solutions containing various concentrations of salts at pH values between 4.9 and 6.2.

Salts of Th, Cr, Al, La, Fe^{+++}, and Be stabilized the enzyme to a considerable degree at concentrations of 0.0001M whereas most divalent cations gave maximum stabilizing effects at 0.0005M to greater than 0.05M. It will be seen that, in general, the divalent cations providing the greatest degree of stability at concentrations of 0.005M caused the greatest inactivation or inhibition of the enzyme at 0.05M. The monovalent cations showed little effect at concentrations below 0.5M, and even at 2M preserved only 45-60 per cent. of the original activity. In the pH range 7.0-9.0 Mg, Co, and NH_4 stabilize trypsin (Fig. 5); Table 1 provides data for other cations in this pH range.

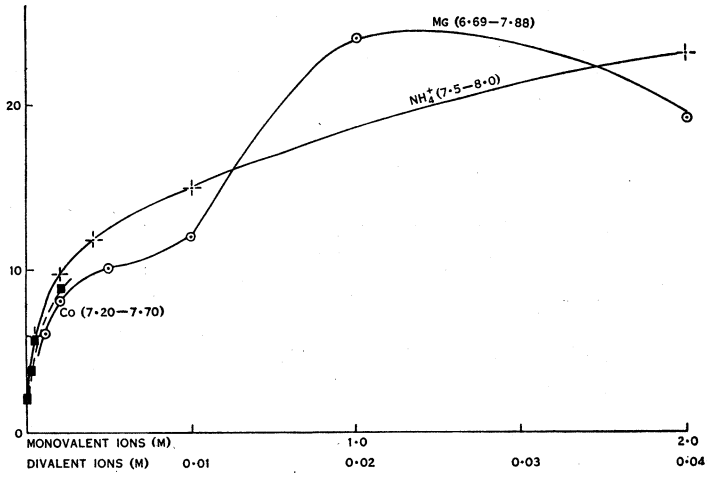

Fig. 5.—Comparison of the stabilizing effects of cations (40°C for 1 hr) in the pH range 7.0-9.0. pH ranges of the final solutions are indicated in brackets.

In view of the results in Figure 4 and the findings of Gorini (1951) and Bier and Nord (1951) that Ca^{++} and Mn^{++} are the only cations which stabilize trypsin at pH 8.0, a further comparison of the stabilizing effects of Ca^{++} and Mg^{++} was made in unbuffered solutions, with particular attention to pH control. Figure 6 shows $CaCl_2$ and $MgCl_2$ to have equal stabilizing effects at pH 6.9 ± 0.05.

Ca^{++} and Mg^{++} were also tested for stabilization at pH 7.9 in solutions containing 0.1M borate buffer and in 0.1M NaCl solution (this being the approximate Na^+ concentration of the buffer). Control solutions containing no cations other than those added in pH adjustment, and buffer solution and NaCl solution containing no added cations were also tested for stabilization of the enzyme. With each of the unbuffered solutions a series of tests was conducted to give final pH values on either side of 7.9, and the residual activity at pH 7.9 was estimated graphically. In solutions containing NaCl, Ca^{++} was slightly more effective than Mg^{++}, whereas in borate the order was reversed (Table 2), possibly owing to the greater tendency for Ca^{++} to form a complex with borate. The stabilizing effects of the Na^+ of the borate and NaCl solutions

were also apparent. A similar experiment using higher concentrations of the enzyme, and using the haemoglobin digestion method for estimating trypsin activity, is summarized in Table 3. In this experiment the undoubted stabilizing effects of Mg^{++} were masked by the comparable effects of the buffer. However, under the conditions of this experiment Mg^{++} was inferior to Ca^{++} whether in borate, in NaCl solution, or in the absence of other electrolytes.

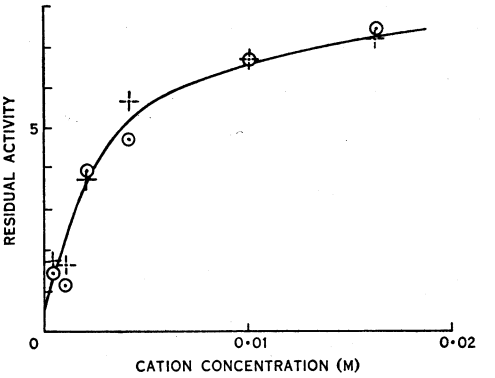

Fig. 6.—Comparison of the stabilizing effects of calcium and magnesium ions at pH 6.9 ± 0.05. Trypsin solutions held 1 hr at 40°C. ⊙, Ca^{++}; +, Mg^{++}.

TABLE 2

STABILIZATION OF TRYPSIN SOLUTIONS BY 0·001M $CaCl_2$ AND $MgCl_2$ IN THE PRESENCE OF 0·1M BORATE BUFFER OR 0·1M NaCl AT pH 7·9

Trypsin solutions held at 40°C for 30 min

Buffer (0·1M)	Cation Added (0·001M)	Residual Gravimetric Activity (% original activity)
Borate	Nil	36
Borate	Mg	61
Borate	Ca	60
NaCl	Nil	49
NaCl	Mg	63
NaCl	Ca	71
Nil	Na	7

That these effects are due to a real stabilization of the enzyme and not to a secondary effect of the cation on the method of estimating residual activity was demonstrated as follows: An enzyme solution of twice the normal concentration was pipetted into salt solutions and water controls at 40°C. After 1 hr the salt solutions were diluted with an equal volume of ice-water and the control solutions with an equal volume of chilled salt solution; the activity of each solution was then determined. Table 4 shows that trypsin solutions which

contained 0.005M $MgCl_2$ at pH 5.9, or 2M KCl at pH 6.7 during heating at 40°, retained a much greater proportion of the original activity than enzyme solutions at the same pH to which the salts were added after heating.

TABLE 3

STABILIZATION OF TRYPSIN BY CALCIUM AND MAGNESIUM IN ABSENCE AND PRESENCE OF BORATE BUFFER, AS DETERMINED BY THE DIGESTION OF HAEMOGLOBIN

Dialysed solution (0·1 ml) of crystalline trypsin containing 10 mg/ml pipetted into 10 ml of electrolyte solution at 40°C and held 1 hr at pH 8·3

Buffer	Metal Ion	Residual Acitivty (trypsin units)
Nil	Nil	0·6*
Nil	Ca 0·005M	5·8*
Nil	Ca 0·0005M	5·7*
Nil	Ca 0·00005M	1·0*
Nil	Mg 0·005M	5·2*
Nil	Mg 0·0005M	3·1*
Nil	Mg 0·00005M	0·9*
Borate 0·1M	Nil	6·4
Borate 0·1M	Ca 0·005M	11·0
Borate 0·1M	Ca 0·0005M	8·1
Borate 0·1M	Ca 0·00005M	6·2
Borate 0·1M	Mg 0·005M	7·9
Borate 0·1M	Mg 0·0005M	6·0
Borate 0·1M	Mg 0·00005M	6·2
NaCl 0·1M	Nil	6·3*
NaCl 0·1M	Ca 0·005M	10·5*
NaCl 0·1M	Ca 0·0005M	7·3*
NaCl 0·1M	Ca 0·00005M	6·1*
NaCl 0·1M	Mg 0·005M	6·8*
NaCl 0·1M	Mg 0·0005M	5·6*
NaCl 0·1M	Mg 0·00005M	5·5*

* Activities at pH 8·3 determined graphically from a series of determinations.

(*e*) *Effect of pH on Stabilization by Cations*

Curves relating pH and the residual activity after heating 1 hr at 40°C were obtained for trypsin solutions containing various concentrations of $CaCl_2$. Figure 2 shows that, in the pH range 7.0-10.5, Ca stabilized the enzyme at a lower concentration than in the pH range 3.0-6.5. Similarly 0.1M Na stabilized trypsin to a greater extent at pH 7.9 than at 6.0 (cf. Fig. 4 and Table 2).

(*f*) *Reaction of Cations with Trypsin and their Role in the Digestion of Proteins*

A 2 per cent. gelatin substrate solution containing the usual amounts of glacial acetic acid and NaOH to give a pH of 8.0 was prepared from gelatin which had been freed of cations by passage through a column of "Amberlite

IR120." No alkaline earth cations could be detected by precipitation as oxalates. Four solutions containing 20-ml aliquots of the buffer-substrate with 1 ml of a dialysed solution containing 0.1 mg/ml crystalline trypsin and 4 ml of water

TABLE 4

STABILIZATION OF TRYPSIN BY 2M KCl AT pH 6·5 AND BY 0·005M $MgCl_2$ AT 5·9 WHEN HEATED AT 40°C FOR 1 HR

Final trypsin solutions of both control and test solutions contained either 1M KCl or 0·0025M $MgCl_2$

Electrolyte Present During Heating	Final pH	Residual Activity (units)
KCl 2M	6·7	7·9
NaOH approx. 10^{-4}M	6·7	1·0
	6·8	0·9
$MgCl_2$ 0·005M	5·9	5·8
NaOH approx. 10^{-4}M	6·0	2·6
Enzyme not heated		9·5

or of a 0.02M solution of $CaCl_2$, $MgCl_2$, or $MnCl_2$ at pH 8.0 were incubated at 40°C. The progress of digestion in each solution was followed at intervals by the gravimetric technique using 2.5-ml samples. Figure 7 shows that the initial

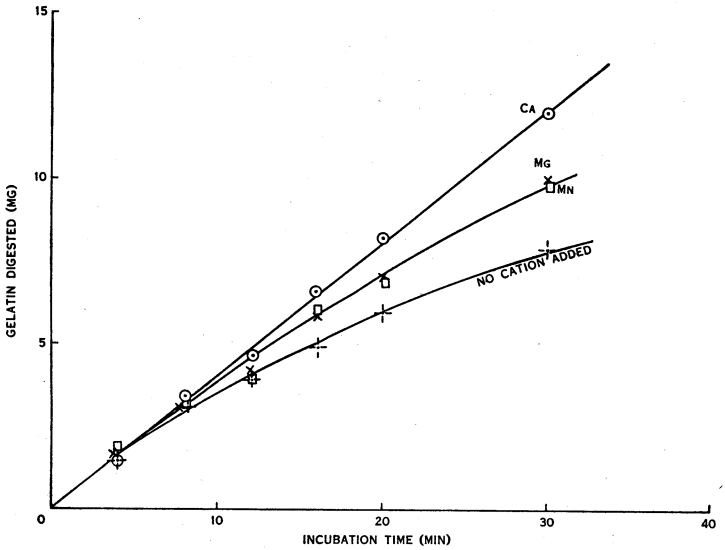

Fig. 7.—Relationship between digestion of gelatin by trypsin at 40°C and time, in the presence and absence of cations. ⊙, Ca++; ×, Mg++; □, Mn++; +, no cation.

rate of digestion was independent of the presence of cations and demonstrates a considerable progressive inactivation of the protease during incubation with a substrate solution containing no cations but Na^+.

The reaction between enzyme and cations was very rapid. Thus addition of $CaCl_2$ to the enzyme solution before mixing with a solution of $CaCl_2$ of the same concentration at 40°C and pH 8.0 did not affect the rate of inactivation.

TABLE 5

EFFECT OF TRYPSIN CONCENTRATION ON ITS RATE OF INACTIVATION AT 40 AND 30°C

Enzyme heated in 0·02M borate buffer at pH 8·0 with and without addition of calcium or magnesium salts, 40°C for 1 hr or 30°C for 24 hr

Cation Added	Concentration of Trypsin (mg/ml)	Heat Treatment	Dilution Before Estimation	Residual Activity (gravimetric units)
Nil	0·6	1 Hr at 40°	1/100	0·6
Ca 0·002M	0·6	1 Hr at 40°	1/100	4·9
Ca 0·005M	0·6	1 Hr at 40°	1/100	5·1
Mg 0·002M	0·6	1 Hr at 40°	1/100	0·6
Mg 0·005M	0·6	1 Hr at 40°	1/100	0·8
Nil	0·06	1 Hr at 40°	1/10	1·0
Ca 0·002M	0·06	1 Hr at 40°	1/10	3·3
Ca 0·005M	0·06	1 Hr at 40°	1/10	4·0
Mg 0·002M	0·06	1 Hr at 40°	1/10	1·6
Mg 0·005M	0·06	1 Hr at 40°	1/10	3·5
Nil	0·006	1 Hr at 40°	1/1	0·5
Ca 0·002M	0·006	1 Hr at 40°	1/1	2·3
Ca 0·005M	0·006	1 Hr at 40°	1/1	2·9
Mg 0·002M	0·006	1 Hr at 40°	1/1	1·8
Mg 0·005M	0·006	1 Hr at 40°	1/1	3·7
Ca 0·001M+Mg 0·001M	0·006	1 Hr at 40°	1/1	2·0
Ca 0·0025M+ Mg 0·0025M	0·006	1 Hr at 40°	1/1	3·6
Nil	0·6	24 Hr at 30°	1/100	0·4
Ca 0·005M	0·6	24 Hr at 30°	1/100	6·3
Mg 0·005M	0·6	24 Hr at 30°	1/100	1·7
Nil	0·006	24 Hr at 30°	1/1	0·7
Ca 0·005M	0·006	24 Hr at 30°	1/1	5·6
Mg 0·005M	0·006	24 Hr at 30°	1/1	2·0

(g) *Effect of Trypsin Concentration on Stabilization by Cations*

Three different concentrations of trypsin in 0.1M borate buffer at pH 8.0 were heat-treated in the presence and in the absence of $CaCl_2$ or $MgCl_2$, diluted to the concentration of the most dilute solution with ice-cold 0.001N HCl, and residual activities measured. Table 5 shows that at high trypsin concentrations Ca^{++} was a more effective stabilizing agent than at low enzyme concentrations, whereas Mg^{++} had relatively little effect at high enzyme concentrations but was as effective as Ca^{++} at low enzyme concentrations. With lower temperatures of inactivation and greater time of heating, Ca^{++} was more effective than Mg^{++} at each trypsin concentration tested.

(*h*) *Stabilization of Trypsin with Coordinated Ferric Complexes*

Solutions of 0.0017M $FeCl_3$ containing various concentrations of tartaric or pyruvic acids were compared for stabilizing effect at pH 8.0 with similar solutions of the same acids containing 0.005M NaCl (approximating the concentration of NaOH required to neutralize the $FeCl_3$) in place of the $FeCl_3$ salt. The iron-containing solutions with the lower concentrations of the organic acids con-

TABLE 6

STABILIZATION OF TRYPSIN BY COORDINATION COMPLEXES OF FERRIC IRON WITH TARTRATE AND PYRUVATE AT pH APPROX. 8·0

Enzyme-complex mixture heated at 40°C for 30 min

Stabilizing Solution	Final pH	Residual Activity	
		Gravimetric Method (units)	Anson's Method* (units × 10^4)
$FeCl_3$ 0·003M +			
tartrate 0·1M (yellow)	8·3		1·9
0·05M (yellow)	7·8		1·9
0·025M (yellow)	7·3		2·0
0·012M (orange)	6·8		6·1
0·003M (orange)	7·1		5·0
pyruvate 0·1M	8·1		1·5
0·05M	7·8		1·8
0·025M	7·4		3·6
0·012M	7·8	7·3 (pH 8·9)	4·5
0·003M	8·1		2·8
NaCl (0·01M) +			
tartrate 0·1M	7·0		1·6
0·05M	7·0		1·7
0·025M	7·1		2·0
0·012M	6·8		2·7
0·003M	7·1		2·0
pyruvate 0·1M	7·3		2·0
0·05M	7·0		1·1
0·025M	7·3		—
0·012M	7·8	1·3 (pH 8·7)	0·8
0·003M	7·6		0·7
Fe^{+++} saturated at pH 6·8	6·5	2·0	—
HCl (0·001N) (enzyme not heated)	2·8	9·4	7·3

* As the experimental conditions were not standard these units are not identical with standard trypsin units.

tained precipitates of basic salts which were filtered off. Tartrate interfered with the gravimetric protease method by co-precipitating with the gelatin; the haemoglobin digestion method was therefore used. Table 6 demonstrates the considerable stabilizing effects of the Fe coordination complexes as compared

with Fe salts at the same pH, or with Na^+ ions at the concentration present in the complex solutions. Stabilization occurred chiefly in the solutions containing a low concentration of the ligand. With higher concentrations of the ligand the stabilization could be attributed to the Na^+ ions in the solution introduced during pH adjustment.

Filter-paper ionophoresis of the complex solutions containing 0.1M tartrate and 0.003M tartrate was carried out in tartrate of the same concentration as the test solution at pH 8.0 with a potential gradient of 2.5 V/cm for 1 hr, the paper then being sprayed with thiocyanate. In the lemon-yellow solution containing 0.1M tartrate the iron was anionic and in the solution containing 0.003M tartrate it was non-ionic.

(*i*) *Kinetic Experiments*

The progress of trypsin inactivation with time was followed at various pH values. There were slight differences in the form of the curves obtained with buffered and unbuffered solutions which may be due to changes in the pH of the solutions during denaturation. The results of the experiments in which buffers were used are shown in Figures 8 and 9.

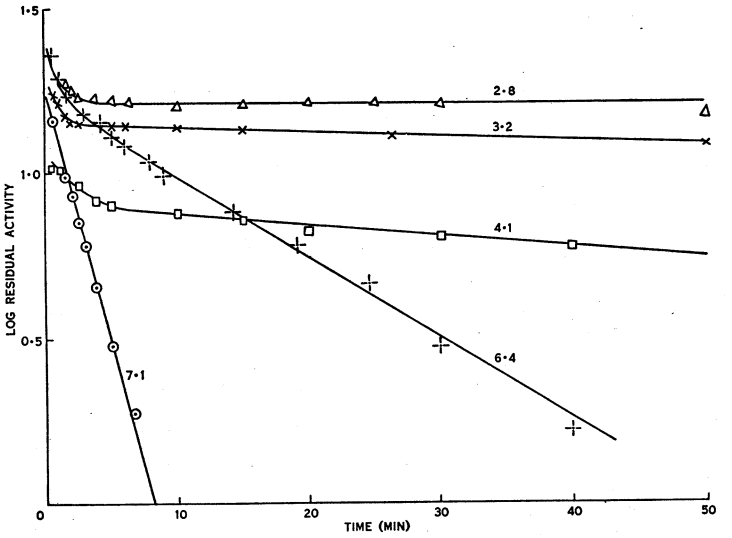

Fig. 8.—Kinetics of inactivation of trypsin at 40°C. pH values are indicated on the curves. Buffers were 0.01M formate (pH < 4.0) and acetate (pH 4.0-6.5).

IV. Discussion

The pH-stability curve obtained by Kunitz and Northrop (1934) for crystalline trypsin, after holding at 30°C for 24 hr, shows optimum stability at pH 2.3, the residual activity decreasing with increasing pH to give almost complete inactivation at *c.* pH 10.0. Pace (1930), using a partly purified preparation of trypsin, reports optimum stability at 50°C at pH 6.5. The difference in the

results obtained by Kunitz and Northrop and those in Figure 2 may be due to the wide spacing of the experimental points on the curve presented by these authors or to the different conditions of inactivation. The results of Pace indicate that a protecting substance was present in the enzyme solutions since the greatest value obtained by Pace for the first-order reaction constant was of the order of 0.01 min^{-1} as compared with 0.7 min^{-1} in the present experiments (Fig. 8).

The inflexions in the curves of Figure 2 at pH 3.5, 6.0, 6.6, 6.8, 10.0, and 11.2 suggest that at least six different ionizable groups play a part in determining the thermal stability of trypsin. The peak of stability at pH 10.7 corresponds with the value obtained by Bier and Nord (1951) for the isoelectric point of the enzyme.

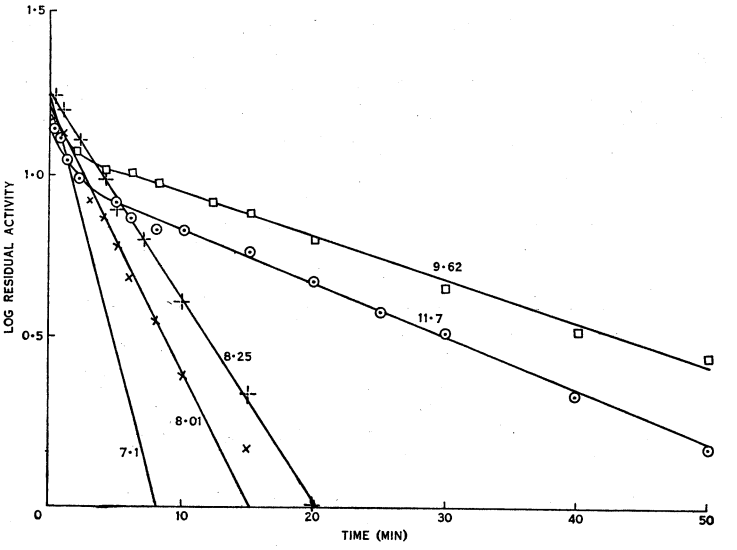

Fig. 9.—Kinetics of inactivation of trypsin at 40°C. pH values are indicated on the curves. Buffers were 0.1M borate (pH 7.0-9.0) and carbonate (pH > 9.0).

The apparent activation of trypsin by certain coordination complexes of Fe and its inactivation by others (Michaelis and Stern 1931), and the apparent activation of the enzyme by various cations observed by Kikuo Sugai (1944) and Bresler and Rosetzveig (1951), may be due in part to stabilization of the enzyme during the preliminary warming-up period and incubation with the substrate and in part to pH changes. The conclusions of the above authors and the assumption by Gorini (1951) and by Bier and Nord (1951) that divalent cations are necessary for the activity of trypsin are shown to be incorrect; the initial rate of hydrolysis of gelatin by the enzyme is unaffected by the presence or absence of cations such as Ca, Mg, or Mn in a substrate solution containing Na^+ as the only cation (Fig. 7). The stabilizing effects of a wide range of cations explain the greater yield of trypsin from trypsinogen in the presence of various cations (McDonald and Kunitz 1941).

In general the present results bear out those of Rona and Kleinmann (1928) except that the trivalent and tetravalent ions are effective stabilizing agents at even lower concentrations than these authors suggest. The greater efficacy of the cations of high valence may be due in part to the concentration of polyvalent ions at the protein-water interface as predicted by the Gibbs-Donnan theory (Danielli 1949). However, since the tetra- and trivalent cations all have pK_1 values considerably below the experimental pH values the ions would be present largely in the form $M(OH)^{++}$, $M(OH)^{+}{}_2$, and $M(OH)_3$ and the considerable difference between the concentrations of tri- and divalent cations required to provide a certain degree of stability cannot be adequately explained on this basis.

It seems more probable that the cations coordinate with certain groups on the protein. At the lowest concentration of divalent cations used in these experiments (1.5×10^{-4}M) the order of their stabilizing effects was Be > Cu > Zn > Cd > Co > Mg > Mn > Ca > Ba > Ni > Hg (Fig. 4). Except for Ni and Hg, which probably inhibit to some extent even at 1.5×10^{-4}M, this series resembles that for ease of formation of complexes with 8-hydroxyquinoline, acetylacetone, and salicylaldehyde (Maley and Mellor 1949*a*) and with amino acids (Maley and Mellor 1949*b*; Albert 1950; Monk 1951). On arranging these cations in order of their inactivating effects at 0.05M, namely, Hg > Ni > Cu > Be > Zn > Cd > Co > Mn > Mg > Ba > Ca, a comparable series is obtained. The two series can only approximate the true order of stabilization or inactivation owing to overlapping of the two effects, variations in the pH ranges at which the cations were tested, and precipitation of some of the cations as hydroxides in the more concentrated solutions.

Pauling (1948), on the basis of his neutrality theory, suggests that the ability of metals such as Fe, Cr, and Al to form trivalent ions is a result of their ability to form with the solvent coordinated complexes in which the bond between the metal and oxygen of the water is partly covalent. He considers that the non-coordinated form of the ions does not exist as such in solution. The formation of a coordinate complex between trypsin and a cation would therefore involve displacement of water from the complex. In complexes of ferric ions with other ligands such as tartrate or pyruvate the ligand would not be displaced as readily as water, and the ability of such a complex to stabilize the enzyme would depend on the relative affinities of the cation for the ligand and enzyme, and on their concentrations. The increase in stability of trypsin with decreasing concentrations of tartrate and pyruvate in solutions containing ferric ions held in solution by these complexing agents (Table 6) is in accord with this view.

Uncharged amino, guanidino, indole, or imidazole groups of the enzyme proteins would be expected to displace water from the cation complex more readily than phenolic, carboxyl, or alcohol groups (Fyfe 1952), and the increase in affinity of trypsin for cations with increasing pH conforms with this view. Thus the increase in affinity for cations over the pH range 6.0-7.5 (Fig. 2) could be due to the formation of metal complexes as protons are removed from

the histidine side chains. Groups coordinated in this way could still form hydrogen bonds (Fyfe 1952) which may in fact be strengthened by the increased charge on the hydrogen atoms of the ligand (Pauling 1945). The reduction in the overall positive charge of the enzyme molecules with increasing pH would also facilitate the approach of the cation to the protein.

The manner in which stability of the enzyme is increased on formation of such a complex may be explained by two hypotheses. The first postulates that the cation, in forming a complex, increases the strength of certain labile hydrogen bonds either, as suggested above, by increasing the positive charge on amino or imino groups, or by providing additional cross linkages in the protein. The second hypothesis, based on the views of Levy and Benaglia (1950) concerning denaturation of ricin, suggests that each ionized form of the protein denatures at a characteristic rate and not necessarily via the same path. If the most stable arrangement of the molecule chelates preferentially with cations, this stable form will predominate at the expense of the unstable forms according to the Law of Mass Action, and the apparent pK values of the protein will be changed. In this way, when the pH of such a protein solution is raised, it may be possible for the molecule to pass from one pH zone of stability to another without assuming the intermediate unstable configuration to an appreciable degree. Stabilization according to both hypotheses could operate concurrently. The fall in pH of trypsin solutions in the pH range 7.0-9.5 on adding to the enzyme a solution of a salt at the same pH suggests that the second hypothesis is operative. It could be ascribed to the formation of a complex between the metal ion and the stable form of the protein having the primary amino groups or lysine amino groups in the uncharged condition, with a consequent dissociation of the ionized form of the protein during complex formation to yield protons. In the pH range 3.0-6.5, the presence of optimal concentrations of cations limits the formation of reversibly inactivated enzyme (Fig. 2) and this may also be a simple mass action effect.

Figures 8 and 9 show that between pH 7.0 and 8.0 the inactivation of trypsin is first-order with respect to trypsin, the maximum rate of inactivation being at *c.* 7.1. At pH values outside this range the plot of log (residual activity)-time is initially curvilinear but approaches a rectilinear relationship as inactivation proceeds. More than one type of equation can be fitted satisfactorily to such a curve but of these the most probable is that derived from a reversible inactivation, such as that demonstrated by Kunitz and Northrop (1934), together with an irreversible denaturation of the native protein thus:

$$\begin{array}{l} \mathrm{P} \rightleftharpoons \mathrm{P}', \\ \downarrow \\ \mathrm{P}'' \end{array}$$

where P is the native enzyme, P′ the reversibly inactivated form, and P″ the irreversibly inactivated form. If k_1, k_2, and k_3 are the rate constants for the reactions

$$\begin{array}{l} \mathrm{P} \rightharpoonup \mathrm{P}', \\ \mathrm{P}' \rightharpoonup \mathrm{P}, \end{array}$$

and

$$P \rightarrow P'',$$

and $[P_0]$ is the initial concentration of the enzyme

$$\begin{aligned}\frac{d[P]}{dt} &= -(k_1+k_3)[P]+k_2[P'] \\ &= -(k_1+k_3)[P]+k_2([P_0]-[P]-[P'']) \\ &= -(k_1+k_2+k_3)[P]+k_2[P_0]-k_2k_3\int[P].dt.\end{aligned}$$

Integrating,

$$[P] = Ae^{-mt}+Be^{-nt}, \quad .. \quad .. \quad .. \quad (1)$$

where A and B are constants such that $A + B = [P_0]$, and m and n have the form

$$\frac{k_1+k_2+k_3 \pm \sqrt{[(k_1+k_2+k_3)^2-4k_2k_3]}}{2}.$$

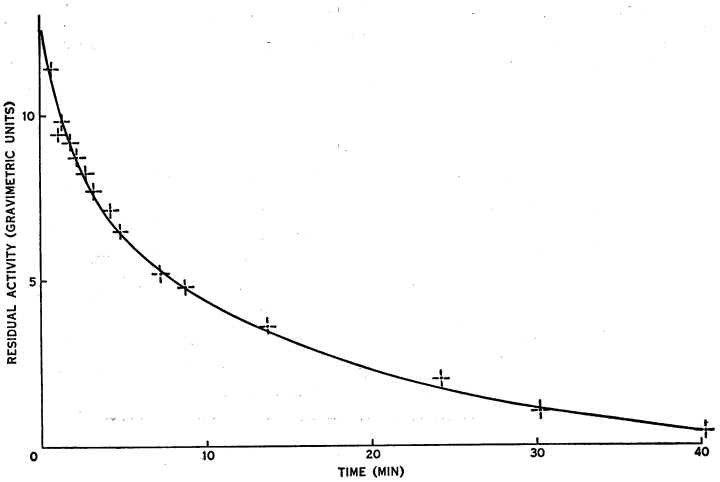

Fig. 10.—Comparison of inactivation of trypsin at pH 6.4 with the equation $[P] = 3.5e^{-0.711t} + 8.9e^{-0.068t}$, where [P] is the residual tryptic activity and t the time (min).

If the reversibly inactivated form of the enzyme were to change back to the native form on addition to the substrate the activity measured would be that of $([P] + [P'])$.

Since

$$\begin{aligned}\frac{d([P]+[P'])}{dt} &= -\frac{d[P'']}{dt} = -k_3[P], \\ [P]+[P'] &= -k_3\int(Ae^{-mt}+Be^{-nt}) \,.\, dt \\ &= k_3(Am\, e^{-mt}+Bn\, e^{-nt}). \quad .. \quad .. \quad (2)\end{aligned}$$

An equation having the form of (1) and (2) may be fitted satisfactorily to the kinetic data shown in Figures 8 and 9. Thus Figure 10 compares experimental values obtained at pH 6.4 and 40°C with an equation of this form.

In view of the first-order kinetics of the inactivation at pH 7.1, and the low concentration of trypsin used (10^{-6}M), of which less than 10 per cent. would be in the reversibly inactivated form at pH 7.1 (Kunitz and Northrop 1934) it is unlikely that irreversible inactivation of the enzyme is due to digestion of the reversibly inactivated enzyme by the active form. In the digestion of other protein substrates considerably higher concentrations of the substrate are required for the rate of hydrolysis to remain constant over a period of 10 min at 40°C. Furthermore, the maximum rate of irreversible denaturation takes place at a pH of *c.* 7.1 where the present experiments (Figs. 8, 9) and those of Kunitz and Northrop (1934) indicate the formation of reversibly inactive trypsin to be a minimum. Since the optimum pH for the hydrolytic action of trypsin is certainly higher than 7.1 maximum inactivation would be expected to occur at a higher pH. Furthermore, the initial rate of irreversible inactivation of trypsin at pH 7.1 is greater than the greatest initial rate of reversible inactivation under the conditions investigated in these experiments (Figs. 8, 9). This also suggests that the reversibly inactivated enzyme is not an intermediate in the formation of the irreversibly inactivated form.

TABLE 7

EFFECTS OF CATIONS ON THE FORMOL TITRATION

Titration of 5 ml enzyme-substrate mixture (50 ml 5 per cent. casein in collidine buffer at pH 7·6+2 ml trypsin solution) + 2 ml 0·05M cation solution + 3ml 36 per cent. formaldehyde, with 0·02N NaOH. The values given are differences between titres for an enzyme-substrate mixture held at 100°C during mixing and for a similar mixture incubated 15 min at 20°C before adding cations

Cation	Titration Difference (ml)
Nil	2·23
Ca	2·04
Mg	1·61
Ba	1·78

The differences in results of the present investigation and those obtained by Gorini (1951) and Bier and Nord (1951) can be explained by several differences in experimental conditions. Gorini's results are very similar to those obtained in the presence of sodium borate and sodium chloride (Table 3) and it is probable that his use of 0.1M sodium borate buffer masked the stabilizing effects of Mg^{++} and other cations. His use of trypsin at four times the concentration used in the present experiments would also tend to enhance the stabilizing effect of Ca^{++} and decrease that of Mg^{++} (Table 5). The differences in experimental conditions used by Bier and Nord and the manner in which they would affect the results are:

(1) A lower temperature and a longer time of heating were used by Bier and Nord and, although the concentration of trypsin used was not stated, experiment showed that they probably employed about 200 times the concentration of trypsin used in the present experiments. Table 6 shows that each of these factors would tend to increase the stabilizing effect of Ca^{++} and decrease that of Mg^{++}.

(2) The formol titration method was used to estimate residual protease activity. Investigation showed (Table 7) that the titration differences obtained with a trypsin-casein mixture before and after incubation differ according to the cations present. This effect would also tend to lessen the apparent stabilizing effects of Mg^{++} and Ba^{++} as compared with that of Ca^{++}.

(3) The concentration of cations used by Bier and Nord was higher than in the present experiments. At the concentration used (0.1M) many cations would be inhibitory (Fig. 4). Moreover, 0.05M borate or glycine buffers were used in most of the experiments by Bier and Nord, while in experiments with unbuffered solutions it was not stated whether the pH values quoted were those of the initial solutions or of the final mixture of enzyme and electrolyte.

Of these differences in technique the first appears to be the most important. The conditions used by Bier and Nord favoured autodigestion of the enzyme whether the substrate was the native enzyme, a reversibly inactivated form of the enzyme, or an irreversibly inactivated form. Possibly all three forms of the enzyme are digested to some extent. Cunningham *et al.* (1953) demonstrated the existence of a complex variation of the sedimentation rate of trypsin with enzyme concentration and pH. They suggested that in the pH range in which trypsin was active the protein existed in a concentration-dependent monomer-polymer equilibrium tending toward polymerization as protein concentration increased. In very dilute solutions, such as were used in the present study, the enzyme would be present largely as the monomer. The results suggest therefore that the monomer is thermally unstable, and its inactivation prevented or retarded by suitable concentrations of any cation. The dimer, on the other hand, is either readily hydrolysed by active molecules of the monomer, or constitutes an enzyme-substrate complex as suggested by Cunningham *et al.* (1953). Ca^{++} and Mn^{++} may decrease the formation of this complex.

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