THE EFFECT OF CALCIUM CHLORIDE ON SEVERAL α-CHYMOTRYPSIN-CATALYZED HYDROLYSES

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In 1950 JANDORF[1] observed that the addition of magnesium sulfate caused a marked increase in the rate of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine ethyl ester and in 1952 SHINE AND NIEMANN[2] extended the above observation to include an amide type of specific substrate, *i.e.*, chloroacetyl-L-tyrosinamide. At approximately the same time NEURATH *et al.*[3], using acetyl-L-tyrosine ethyl ester and unspecified amide-type specific substrates, reported that only Ca^{++} produced significant activation. It was also stated[3] that with the above ester, in systems 0.005 *M* in a THAM**–HCl buffer, a maximum activation of about 50% was attained at 10^{-2} *M* Ca^{++}, and about one-half of this amount at $2 \cdot 10^{-4}$ *M*. More recently WU AND LASKOWSKI[4] examined the effect of calcium chloride upon reactions catalyzed by both α- and β-chymotrypsin and, with L-phenylalanine ethyl ester as the specific substrate, obtained an extent of activation comparable to that reported by NEURATH *et al.*[3].

In a study completed several years ago[5] it was observed that the addition of calcium chloride caused a significant increase in the rate of the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydroxamide, in a system 0.1 *M* in the cacodylic acid component of a cacodylic acid–sodium cacodylate buffer, that did not appear to arise from an increase in the ionic strength of the reaction system (Table I) and in this sense was distinguishable from the increase in rate associated with the addition of sodium or potassium chloride to a system containing α-chymotrypsin and chloroacetyl-L-tyrosinamide[6]. The lesser increase in activity observed with L-tyrosinhydroxamide (Table I), relative to that reported for acetyl-L-tyrosine ethyl ester[3] and L-phenylalanine ethyl ester[4] could have arisen either from a dependency of the extent of activation by calcium chloride upon the nature of the specific substrate or upon the nature and/or concentration of the buffer components of the reaction systems. There was no indication that the extent of activation by calcium chloride was pH-dependent between pH 6.0 and 7.0. Furthermore, in view of the earlier experience with magnesium sulfate[1,2] the data given in Table I indicated that the nature of the anionic component of the added salt may be of some consequence.

From a second set of experiments, conducted with acetyl-L-tyrosinhydroxamide in aqueous solutions at 25° and pH 7.6 and 0.3 *M* in the THAM component of a THAM–HCl buffer and in the absence and presence of 0.04 *M* calcium chloride, *of*

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** Tris-(hydroxymethyl)-aminomethane.

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TABLE I

EFFECT OF SODIUM, MAGNESIUM AND CALCIUM CHLORIDE ON THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF L-TYROSINHYDROXAMIDE

L-Tyrosinhydroxamide in aqueous solutions at 25° with $[E]$ = 0.208 mg protein-nitrogen/ml of Armour preparation No. 90,402 and $[S]_0 = 10 \cdot 10^{-3}$ M; pH to within ± 0.1 of a pH unit; buffer: 0.1 M in the cacodylic acid component of a cacodylic acid–sodium cacodylate buffer; $[S]_0/[S]_t$ in units of 10^{-3} min^{-1}.

pH	*Added component*	*Ionic strength*			$1/60 \times ln([S]_0/[S]_t)$	*Relative activity*
		Added comp.	*Buffer*	*Total*		
7.0	None	—	0.09	0.09	15.5	1.00
7.0	0.02 M $MgCl_2$	0.06	0.09	0.15	16.4	1.06
7.0	0.02 M $CaCl_2$	0.06	0.09	0.15	18.3	1.18
6.0	None	—	0.05	0.05	5.39	1.00
6.0	0.06 M $NaCl_2$	0.06	0.05	0.10	5.50	1.02
6.0	0.02 M $MgCl_2$	0.06	0.05	0.10	5.41	1.00
6.0	0.02 M $CaCl_2$	0.06	0.05	0.10	6.33	1.17

TABLE II

EFFECT OF CALCIUM CHLORIDE ON THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDROXAMIDE

L-Tyrosinhydroxamide in aqueous solutions at 25° and pH 7.6 ± 0.1 and 0.3 M in the THAM component of a THAM–HCl buffer with $[E]$ = 0.040 mg protein-nitrogen/ml of Armour preparation No. 10,705; $[S]_0$ in units of 10^{-3} M; v_0 in units of 10^{-5} M/min; $[S]_0/v_0$ in minutes.

$[S]_0$	v_0	$[S]_0/v_0$	$[S]_0$	v_0	$[S]_0/v_0$
				0.04 M $CaCl_2$	
3.00	8.77	34.2			
10.0	25.2	39.6			
10.0	24.3	41.1	5.21	18.0	29.0
30.0	55.5	54.0	16.79	47.5	35.2
50.0	72.0	69.5	39.84	82.0	48.5
70.0	86.3	81.0	70.44	101.0	69.6

Table II, a subjective estimate of the slopes and intercepts of the two v_0 *vs.* $[S]_0/v_0$ plots led to values of K_S* of 45 and 40 and k_3* of 35 and 40. The values of K_S and k_3 observed in the absence of added calcium chloride are in reasonable agreement with those obtained previously[7], *i.e.*, 43 ± 4 and 33 ± 3. Those obtained in the presence of calcium chloride suggest that K_S is relatively insensitive to the presence of calcium chloride and that the value of k_3 may be greater than in the absence of this salt. However, from the ratios of k_3/K_S it follows that 0.04 M calcium chloride led to an expected increase in rate of 28% when both reactions are examined under conditions where they are apparent first order with respect to specific substrate.

In order to obtain additional information of the dependence of the extent of activation associated with the presence of calcium chloride upon the concentration of the buffer components of the reaction system a series of experiments were conducted with α-N-nicotinyl-L-tyrosinhydrazide under conditions where the buffer concentration was maintained at 0.02 and 1.0 M in the THAM component of a THAM–HCl buffer and the calcium chloride concentration was varied over a 10^4 to 10^5 fold range,

* All values of K_S are in units of 10^{-3} M and those of k_3 in units of 10^{-3} M/min/mg protein-nitrogen/ml.

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cf. Table III. The initial velocities were evaluated by the method of JENNINGS AND NIEMANN[8] and by the empirical orthogonal polynomial procedure of BOOMAN AND NIEMANN[9].

TABLE III

EFFECT OF CALCIUM CHLORIDE AND OF VERSENE ON THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-NICOTINYL-L-TYROSINHYDRAZIDE

Versene: Disodium hydrogen salt of ethylenetetraacetic acid; α-N-nicotyl-L-tyrosinhydrazide in aqueous solution at 25° and pH 7.9 ± 0.1 with $[E]$ = 0.218 mg protein-nitrogen/ml of Armour preparation No. 00592 and $[S]_0 = 1.003 \cdot 10^{-3}$ *M*; buffer: *M* of the THAM component of a THAM–HCl buffer; v_0 in units of 10^{-5} *M*/min evaluated by the method of JENNINGS AND NIEMANN[8]; v'_0 in units of 10^{-5} *M*/min, evaluated by the procedure of BOOMAN AND NIEMANN[9]; P_m: order of polynomial used for fitting of nine points observed for maximum time of reaction of 9 or 10 min corresponding to extents of reaction of from 22.0 to 44.6%.

(*Buffer*) *M*	($CaCl_2$) *M*	v_o	v'_o	P_m
0.02	0	2.62	2.95 ± 0.22	3
	0	2.67	2.45 ± 0.27	3
	10^{-6}	2.53	2.42 ± 0.27	3
	10^{-5}	2.68	2.49 ± 0.28	3
	10^{-4}	3.15	3.32 ± 0.18	2
	10^{-3}	4.24	4.30 ± 0.15	3
	10^{-2}	4.35	4.52 ± 0.25	3
	10^{-1}	4.63	4.45 ± 0.56	2
1.00	0	3.80	3.63 ± 0.18	3
	10^{-5}	4.20	4.18 ± 0.26	3
	10^{-4}	4.42	4.49 ± 0.34	3
	10^{-3}	5.10	4.97 ± 0.41	2
	10^{-2}	5.32	5.32 ± 0.52	2
	(Versene) *M*			
0.02	0	2.70	2.51 ± 0.39	3
	$5.3 \cdot 10^{-6}$	2.58	2.43 ± 0.08	2
	$8.9 \cdot 10^{-6}$	2.66	2.55 ± 0.20	2
	$15.1 \cdot 10^{-6}$	2.72	2.67 ± 0.26	2

It will be seen from the data summarized in Table III that in 0.02 *M* THAM significant activation by calcium chloride begins to appear at concentrations between 10^{-5} and 10^{-4} *M*, reaches an apparent maximum, which in reality is a plateau, between 10^{-3} and 10^{-2} *M* and then continues to increase at concentrations above 10^{-2} *M*. The extent of activation at 10^{-3} to 10^{-2} *M* calcium chloride, *i.e.* about 60%, is comparable to that observed previously by NEURATH *et al.*[3] and by WU AND LAWKOWSKI[4] for systems of relatively low ionic strength. In 1.0 *M* THAM activation by calcium chloride is seen at 10^{-5} *M* and continues to increase, with no evidence for any marked discontinuities, with increasing concentration of the added salt. However, the extent of activation attained by the addition of 10^{-2} *M* calcium chloride in the latter case is but about two-thirds of that observed at the lower buffer concentration. It appears that the marked rise in activity that occurs between 10^{-4} and 10^{-3} *M* calcium chloride in the 0.02 *M* THAM buffer is lost at least in part in systems of higher buffer concentration. The fact that the rates in the absence and presence

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of Versene are identical with the limits of experimental error, *cf.* Table III, is consistent with the earlier conclusion of NEURATH *et al.*[3], based upon spectroscopic evidence, that α-chymotrypsin is substantially free of calcium salts.

It is clear that the dependence of the extent of activation associated with the presence of calcium chloride upon the nature and/or concentration of the buffer components of the reaction system, which has been demonstrated above, explains in part the discrepancies noted earlier in this communication. However, in view of this dependence and an additional possible dependence upon the nature of the specific substrate it follows that an interpretation of the nature of the apparent activation of α-chymotrypsin-catalyzed hydrolyses by calcium chloride must be deferred until further information is available with respect to the behavior of several specific substrates in systems of varying enzyme concentration under conditions where the systems are maintained at a constant pH but in the absence of conventional buffers.

EXPERIMENTAL

The experiments involving L-tyrosinhydroxamide and acetyl-L-tyrosinhydroxamide were conducted essentially as described by HOGNESS AND NIEMANN[10] and FOSTER, JENNINGS AND NIEMANN[11]. Those with α-N-nicotinyl-L-tyrosinhydrazide as described by LUTWACK, MOWER AND NIEMANN[12] and KERR AND NIEMANN[13].

SUMMARY

The extent to which the reactivity of systems containing α-chymotrypsin and specific substrates of this enzyme may be enhanced by the presence of calcium chloride has been shown to be dependent not only upon the concentration of this salt but also upon the nature and/or concentration of several buffer components. An additional dependence upon the nature of the specific substrate has not been excluded.

REFERENCES

1 B. J. JANDORF, *Federation Proc.*, 9 (1950) 186.
2 H. J. SHINE AND C. NIEMANN, *J. Am. Chem. Soc.*, 74 (1952) 97.
3 M. M. GREEN, J. A. GLADNER, L. W. CUNNINGHAM, JR. AND H. NEURATH, *J. Am. Chem. Soc.* 74 (1952) 2122.
4 F. C. WU AND M. LASKOWSKI, *Biochim. Biophys. Acta*, 19 (1956) 110.
5 R. R. JENNINGS, *Ph. D. Thesis*, Calif. Inst. Techn., Pasadena, Calif., 1955.
6 H. J. SHINE AND C. NIEMANN, *J. Am. Chem. Soc.*, 77 (1955) 4275.
7 H. J. FOSTER AND C. NIEMANN, *J. Am. Chem. Soc.*, 77 (1955) 1886.
8 R. R. JENNINGS AND C. NIEMANN, *J. Am. Chem. Soc.*, 75 (1953) 4687.
9 K. A. BOOMAN AND C. NIEMANN, *J. Am. Chem. Soc.*, 78 (1956) 3642.
10 D. S. HOGNESS AND C. NIEMANN, *J. Am. Chem. Soc.*, 75 (1953) 884.
11 R. J. FOSTER, R. R. JENNINGS AND C. NIEMANN, *J. Am. Chem. Soc.*, 76 (1954) 3142.
12 R. LUTWACK, H. F. MOWER AND C. NIEMANN, *J. Am. Chem. Soc.*, 79 (1957) 2179.
13 R. J. KERR AND C. NIEMANN, *J. Am. Chem. Soc.*, in the press.

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