Thus there exists an imposing body of kinetic evidence which requires that the mechanism of hydrolysis of "specific" substrates by α-chymotrypsin follow eq. 1.

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THE APPARENT MOLAR VOLUME OF SODIUM IN AMMONIA AT −45°[1]

Sir:

We have previously[2] reported measurements of the apparent molar volume, $\bar{V}$, of sodium in ammonia at 0°; $\bar{V}$, defined as (volume of solution − volume of solvent)/g. atoms Na, is nearly independent of concentration, being 56.3 ml. mole^{-1} at 0.35 *M* and about 2 ml. mole^{-1} greater at 0.009 *M*. The change is but little outside of experimental error. Evers and Filbert[3] have reported similar measurements at −45°; they found $\bar{V}$ to decrease slowly from 62 ml. mole^{-1} at 0.2 *M* to 60 at 0.050 *M* and steeply to 53 at 0.040 *M*, and then to rise steeply from 53 at 0.023 *M* to 59 at 0.018 *M*, 64 at 0.012 *M* and 65 at 0.0036 *M*. They have stated their accuracy to be better than 3%; however, it would be expected that the probable errors of such measurements in dilute solution would be roughly inversely proportional to the concentration.

The dissimilarity of these two reports has led us to perform measurements at −45° also; the procedure was essentially as previously described. A stirred methyl alcohol bath was used, cooled by a system described elsewhere,[4] controlled by a Hallikainen controller and electrical heater and monitored with a platinum thermometer, Mueller bridge, and recorder. The dilatometer bulbs were sealed together, rather than being assembled with waxed joints. Results are given in Table I; over-all uncertainties are estimated separately for each run. Within experimental accuracy, $\bar{V}$ is constant at 60.7. Agreement with the data of Evers and Filbert at the higher concentrations is satisfactory, but there is no evidence for a minimum or any considerable change at lower concentrations.

TABLE I

APPARENT MOLAR VOLUME OF Na IN NH_3 AT −45°

C (mole liter^{-1})	$\bar{V}$ (ml. mole^{-1})	*C* (mole liter^{-1})	$\bar{V}$ (ml. mole^{-1})
0.1640	60.7 ± 0.2	0.0276	$61.5^{+1.0}_{-1.5}$
.0940	60.7 ± 0.2		
.0493	59.8 ± 1.0	.0269	61.2 ± 0.8
.0428	$59.7^{+1.0}_{-0.5}$	.0234	59.9 ± 0.8
		.0161	60.4 ± 1.2
.0388	60.0 ± 0.7	.0114	59.4 ± 1.2
.0317	61.4 ± 1.2	.0058	59.9 ± 3.0

It may be noted that $\bar{V}$ decreases from −45 to 0°; for aqueous solutions of electrolytes at ordinary temperatures, $\bar{V}$ increases with increasing temperature.

It is also of interest to note that both the volume and absorption spectra[5] of sodium–ammonia solutions are essentially constant through a concentration range where the magnetic, electrochemical, and thermochemical properties change drastically. Becker, Lindquist, and Alder[6] proposed the equilibria $(1/2)M_2 = M = M^+ + e^-$ to explain the conductance and magnetic properties. Gold, Jolly and Pitzer[7] proposed that the intermediate non-conducting paramagnetic species M is an ordinary ion-pair and that M_2 is a quadrupolar ionic assembly. However, dissociation of salt ion-pairs in ammonia produces a decrease of 20 to 30 ml. mole^{-1} in $\bar{V}$[1].

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.

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SIGMOID AND BELL-SHAPED pH-RATE PROFILES IN α-CHYMOTRYPSIN-CATALYZED HYDROLYSES. A MECHANISTIC CORRELATION[1]

Sir:

The thesis enunciated earlier, that all chymotrypsin-catalyzed hydrolyses follow eq. 1, has been substantiated by spectrophotometric and kinetic evidence.[2,3] On experimental and theoretical grounds the two steps of acylation and deacylation (k_2 and k_3) were postulated

$$E + S \underset{}{\overset{K_m}{\rightleftarrows}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$+ P_1 \text{ (from } ES\text{')}$$

to be mechanistically equivalent, the enzymatic components of their transition states being identical.[3] On this basis, the same pH dependence might be expected in all chymotrypsin steps and reactions. However, some chymotrypsin-catalyzed reactions show bell shaped pH-rate profiles while others show sigmoid pH-rate profiles.[4]

The pH dependencies of the rate constants of the two *individual* steps (k_2 and k_3) (eq. 1 and Table I) indicate that pH-rate profiles of *deacylation* obey a *sigmoid* curve, while the pH-rate profile of the only *acylation* step observed over a sufficient pH range follows a *bell-shaped* curve.

The pH dependencies of the *catalytic rate constants* (turnover) (eq. 2 and Table II) of this research and literature data may be correlated (on the basis of Table

$$E + S \overset{K_m}{\rightleftarrows} ES \xrightarrow{k_{cat}} E + P \quad (2)$$

I) with the pH dependence of a rate-controlling acylation or deacylation step. In the hydrolyses of acetyl-L-tryptophanamide and acetyl-L-tryptophan ethyl ester, acylation and deacylation, respectively, were shown to be rate-controlling.[2] The respective bell-shaped and sigmoid pH dependencies of these reactions are in complete accord with such a designation. The steric and electronic similarity of acetyl-L-tryptophanamide to the other amides and hydroxamides in Table II suggests that they should exhibit acylation rate-controlling steps and therefore bell-shaped pH-rate profiles, as found experimentally (the other amide "bells" do not, however, reflect $k_{cat.}$). The similarity of the three ethyl ester substrates in Table II suggests that they should exhibit rate-controlling deacylation steps and sigmoid pH-rate profiles, as found experimentally. All these results are self-consistent and lead one to

(1) This research was supported by Grant H-5726 of the National Institutes of Health. Paper XIX in the series, The Mechanism of Action of Proteolytic Enzymes.

(2) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **85**, 356 (1963).

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(4) These observations apply to reactions in which K_m (or K_m(app)) has been separated from the appropriate rate constant.

TABLE I

THE pH DEPENDENCE OF THE ACYLATION AND DEACYLATION RATE CONSTANTS IN α-CHYMOTRYPSIN-CATALYZED REACTIONS

Substrate	pK_1	pK_2	pH Optimum	pH Range	Reference
		Acylation (k_2)			
p-Nitrophenyl acetate	6.7	8.4	7.9	5.91–9.21	[a]
2,4-Dinitrophenyl acetate	6.7	...	...	6.1–7.2	13
		Deacylation (k_3)			
Acetyl-α-chymotrypsin	6.96–7.3	...	...	5.6–8.8	12, 13, 14
trans-Cinnamoyl-α-chymotrypsin	7.15	...	...	6–13	15, 16

[a] This research. K_m varies by less than a factor of two over the entire pH range.

TABLE II

THE pH DEPENDENCE OF THE CATALYTIC RATE CONSTANT OF SOME α-CHYMOTRYPSIN-CATALYZED HYDROLYSES

Substrate	pK_1	pK_2	pH Optimum	pH Range	Reference
		Bell-shaped pH-Rate Profile			
Acetyl-L-tryptophanamide	7.16	8.66	7.91	5.73–9.70	[a]
	6.5	9?	7.8[g]	6.6–8.8[h,c]	6
Acetyl-L-tyrosinamide	6.7(6.85)	9?	7.8	6.7–8.5[h,b]	5, 13
Nicotinyl-L-tryptophanamide	6.5	9?	7.8[g]	6.6–8.8[h,d]	6
Acetyl-L-tyrosinhydroxamide	6.5	8.5	7.6	6.5–8.8[h,e]	7
Methyl hippurate	6.5	8.7	7.8[g]	6.6–8.7[h,f]	8
Methyl hydrocinnamate	7.0	8.4	7.8	5.0–9.5	9
		Sigmoid pH-Rate Profiles			
Acetyl-L-phenylalanine ethyl ester	6.8	...	...	6.5–9.0	10
Acetyl-L-tryptophan ethyl ester	6.7	...	...	5.5–9.0	11
Acetyl-L-tyrosine ethyl ester	6.74	...	...	5.5–8.3	11

[a] This research. K_m varies by less than a factor of two over the entire pH range. The product ammonia does not inhibit the reaction. [b] $S_0/K_m = 0.67$ at pH 7.9. [c] $S_0/K_m = 2.0$ at pH 7.9. [d] $S_0/K_m = 3.7$ at pH 7.9. [e] $S_0/K_m = 0.39$ at pH 7.9. [f] $S_0/K_m = 2.25$ at pH 7.9. [g] These pH optima are broad maxima extending over 1.5 pH units. [h] K_m and k_{cat} not separated.

predict that those substrates exhibiting a bell-shaped pH-rate profile of the catalytic rate constant have a rate-determining acylation step (either partially or fully),[17] while those substrates exhibiting a sigmoid pH-rate profile of the catalytic rate constant have a rate-determining deacylation step.[17]

If Eq. 1 is the pathway of all α-chymotrypsin-catalyzed hydrolyses, and if bell-shaped and sigmoid pH-rate profiles describe the acylation and deacylation steps, respectively, the meaning of the mechanistic symmetry of the acylation and deacylation steps may be questioned. Since deacylation is essentially the microscopic reverse of acylation, (with a change only in nucleophile), the acylation–deacylation system may be considered as an equilibrium in which the forward and reverse reactions have identical transition states but different pH dependencies, a common phenomenon in chemical reactions. The pK_1's of all reactions are reasonably similar and attributable to the same (basic) group which is operative in both acylation and deacylation. The pK_2's (corresponding to an acidic group) are reasonably similar but appear only in acylation. Therefore one must account for an equilibrium reaction, dependent in the forward direction on two ionizable groups, an acid of pK_a' 8.5 and a base of pK_a' 7.0, but in the reverse direction only on one ionizable group, a base of pK_a' 7.0.[18]

On this basis one may rule out general acid–general base catalysis in acylation, for microscopic reversibility would be violated in deacylation. These data can be explained in two general ways: (1) an ionizable group present in acylation is not present in deacylation; (2) one of the ionizable groups detected in acylation is not involved in the bond-changing process. Under category (1) the two ionizable groups could be identified as an imidazole group and a serine hydroxyl group which is acylated and whose pK_a' has been considerably perturbed[19]; alternatively the two groups could be identified as an imidazole group and a tyrosine hydroxyl group which is acylated.[20] Under category (2) one of the ionizable groups could be designated as a conformational stabilizer of the active site in acylation, unnecessary in deacylation because of the covalent link of the acyl-enzyme. Further experiments are needed to distinguish these possibilities.

The bell-shaped and sigmoid pH-rate profiles in acylation and deacylation, respectively, appear to be completely general for chymotrypsin reactions and point up another facet of the mechanism of the reaction.

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(17) The catalytic rate constant may be expressed by $k_{cat} = k_2k_3/(k_2 + k_3)$.[12] Thus when $k_2 \gg k_3$, $k_{cat} = k_3$, and when $k_3 \gg k_2$, $k_{cat} = k_2$. The pH dependence of the over-all reaction in these two extreme cases will be that of k_3 or k_2, respectively. When both k_2 and k_3 are rate limiting, the pH dependence of the over-all reaction will be a (non-additive) function of the pH dependencies of both steps. This may be the interpretation of some unpublished results on methyl hippurate kindly supplied by Professor C. Niemann.
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