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Surface Effects in α-Chymotrypsin-catalyzed Hydrolyses[1]

BY ROBERT L. BIXLER AND CARL NIEMANN[2]

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The adsorption of α-chymotrypsin on the surfaces of conventional glass volumetric equipment precludes the use of enzyme stock solutions more dilute than 10^{-5} M if losses of enzyme are to be avoided during subsequent transfer operations. In reaction systems with apparent enzyme concentrations of 10^{-6} M or less a fraction of the enzyme will be surface adsorbed.

In an earlier study conducted in these laboratories[3] it was shown that α-chymotrypsin may be adsorbed on glass surfaces and that the adsorbed enzyme is catalytically active.[4] Thus, wall or surface effects may become important in the examination of reactions catalyzed by this enzyme when the total amount being manipulated is relatively small.

In a practical sense surface effects may be of two kinds. With the first, one is concerned with the consequences of a situation where the total amount of enzyme present in the reaction system is known but where uncertainty arises with respect to the possibility of differing kinetic properties of molecularly dispersed and surface adsorbed enzyme[6] and the relative amounts of enzyme so distributed. With the second, one's attention is directed to the possibility that a significant proportion of the amount of enzyme presumed to be present may have been lost to the surfaces of volumetric apparatus used in establishing the reaction system. In this investigation our principal concern will be with surface effects of the second kind.

For kinetic studies involving reaction of α-chymotrypsin with acylated α-amino acid amides and hydrazides, it has been found that enzyme concentrations of *ca.* 1 to 6 × 10^{-5} M[7] give convenient rates of hydrolysis for those amides and hydrazides that can be considered to be useful specific substrates of this enzyme.[8-10] For the corresponding hydroxamides[8] the same situation prevails with enzyme concentrations as low as *ca.* 10^{-6} M.

While there are a number of acylated α-amino acid esters that can be studied at enzyme concentrations of the order of 10^{-5} M,[3,11] there are others, *i.e.*, those possessing an aromatic side chain, that are hydrolyzed so rapidly that lower enzyme concentrations are required if the rates of hydrolysis are to be held within the capabilities of available analytical devices.[3,11] Representative data with respect to the kinetics of the α-chymotrypsin-catalyzed hydrolysis of acylated α-amino acid esters[3,11-13] are summarized in Table I.

It will be seen from the data given in Table I that situations involving variation of values of k_3/K_S[14] of *ca.* 20-fold can be accommodated without significant alteration of the enzyme concentration. However, it is doubtful that this will be true for a variation in values of k_3/k_S of more than 100-fold, particularly if a *p*H-stat is used as the analytical device.[11] Since the variation in values of k_3/K_S noted for the extremes of the examples given in Table I is *ca.* 5 × 10^5, it may be estimated that the range of potentially determinable values of k_3/K_S characteristic of specific substrates of the acylated α-amino acid ester type may vary from *ca.* 10^{-4} to 10^5 min.$^{-1}$ (mg. protein-nitrogen/ml.)$^{-1}$, *i.e.*, *ca.* 10^9 fold.[15] Thus, it is evident that if specific substrates representative of the extremes of this type are to be studied it may be necessary to vary enzyme concentrations over a 10^5-fold range if optimal or near optimal observational conditions are to be preserved.

The results of previous experiments conducted in these laboratories[16,17] suggest that surface effects of the first or second kind are not observable with glass surfaces at enzyme concentrations greater than 10^{-6} M even though it may be inferred that

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) T. H. Applewhite, H. Waite and C. Niemann, THIS JOURNAL, **80**, 1465 (1958).

(4) In addition to the experiments described in Table I of ref. 3 it was observed[5] that when a glass reaction vessel used as a container for an α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine glycolamide ester in aqueous solutions at 25° and *p*H 7.9 and 0.01 M in sodium chloride was repeatedly rinsed with distilled water sufficient enzyme remained on the walls of the vessel to cause the hydrolysis of the above specific substrate in a system containing no additionally added enzyme. A reaction vessel so prepared was used in one instance to cause the asymmetric hydrolysis of acetyl-DL-phenylalanine glycolamide ester to the D-ester and the L-acid.

(5) Unpublished experiments of Dr. T. H. Applewhite.

(6) A. D. McLaren, *Science*, **125**, 697 (1957).

(7) Based upon an assumed molecular weight of 22,000 and a nitrogen content of 16.0% for monomeric α-chymotrypsin

(8) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 1886 (1955).

(9) R. Lutwack, H. F. Mower and C. Niemann, *ibid.*, **79**, 5690 (1957).

(10) It is axiomatic that usefulness is determined in part by the sensitivity of available analytical methods and by the requirement for a significant extent of reaction in a reasonable time, *i.e.*, not exceeding several hours for systems at 25°. Observations made over longer periods, or for the same period but at higher temperatures, are suspect because of the likelihood of a substantial change in the nature and/or concentration of the catalyticaly active species.

(11) T. H. Applewhite, R. B. Martin and C. Niemann, THIS JOURNAL, **80**, 1457 (1958).

(12) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

(13) B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).

(14) Values of k_3/K_S are useful indices of the reactivities of specific substrates when the latter are examined under conditions where the reaction rates are apparent first order with respect to the initial specific substrate and enzyme concentrations.

(15) It is probable that the upper limit of k_3/K_S for an ester type of specific substrate will not exceed 10^5 min.$^{-1}$ (mg. protein-nitrogen/ml.)$^{-1}$, *i.e.*, a value *ca.* 10 times greater than that observed for acetyl-L-tryptophan methyl ester, *cf.*, Table I, whereas it is doubtful that the enzyme catalyzed hydrolysis of a specific substrate with a k_3/K_S value of less than 10^{-4} min.$^{-1}$ (mg. protein-nitrogen/ml.)$^{-1}$, *i.e.*, *ca.* 100 times less reactive than acetyl-L-valine methyl ester, *cf.*, Table I, could be differentiated from the non-enzymatic base-catalyzed hydrolysis of the specific substrate.

(16) R. A. Bernhard and C. Niemann, THIS JOURNAL, **77**, 480 (1955).

(17) K. A. Booman and C. Niemann, *Biochem. Biophys. Acta*, **26**, 439 (1957).

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSES OF SEVERAL REPRESENTATIVE ACYLATED α-AMINO ACID ESTERS[a]

Ref.	Specific substrate	[E][b], M	K_S[c]	k_2[d]	k_2/K_S[e]
7	Acetyl-L-valine methyl ester[f]	4.16×10^{-5}	126	2.3	0.0185
7	Chloroacetyl-L-valine methyl ester[f]	4.16×10^{-5}	47	1.76	.0373
7	Benzoyl-L-valine methyl ester[f]	4.17×10^{-5}	4.9	0.7	.143
6	Methyl hippurate[g]	4.26×10^{-5}	7.6	2.9	.384
8	Acetyl-L-tyrosine ethyl ester[h]	10^{-7}	1.0	2460	2460
9	Acetyl-L-phenylalanine ethyl ester[i]	1.99×10^{-7}	1.0	2720	2720
8	Acetyl-L-tryptophan ethyl ester[j]	2.13×10^{-7}	0.093[k]	740[k]	7955

[a] In aqueous solutions at 25° and *p*H 7.9. [b] Apparent enzyme concentration based upon an assumed molecular weight of 22,000 and a nitrogen content of 16.0% for monomeric α-chymotrypsin. [c] In units of 10^{-3} M. [d] In units of 10^{-3} M/min./. mg. protein-nitrogen per ml. [e] In units of min.$^{-1}$ (mg. protein-nitrogen/ml.)$^{-1}$. [f] System 0.10 M in sodium chloride. [g] System 0.02 M in sodium chloride. [h] System 0.001 M in ethylenediaminetetraacetic acid and 0.1 M in sodium chloride. [i] System 0.005 to 0.05 M in ethylenediaminetetraacetic acid and 0.1 M in sodium chloride. [j] System 0.001 M in tris-(hydroxymethyl)-aminomethane and 0.1 M in calcium chloride. [k] Values interpolated from those given for *p*H 8.0.

such effects might be encountered with other types of surfaces at enzyme concentrations as high as 10^{-4} M.[18-20] Therefore, in order to span the anticipated requirement for enzyme concentrations of from 10^{-4} to 10^{-9} M, it was decided to examine the region from 10^{-6} to 10^{-9} M for possible surface effects of the second kind particularly in view of earlier evidence that such effects were observable at concentrations of 10^{-8} M.[3-5]

For the ideal case a study of the above type would involve the determination of values of v_0 for a single specific substrate at varying enzyme concentrations with all other reaction parameters being held constant. Under these conditions the ratio v_0/[E] should be invariant in the absence of surface effects provided the upper limit of [E] is less than that which is of significance with respect to association processes leading to the formation of dimeric or polymeric enzyme whose catalytic properties may differ from that of the monomer.[17,21-23] While this practice is applicable for a variation of enzyme concentration of 10, or in certain cases of *ca.* 100-fold,[17] it cannot be used for a range of 1000-fold because no known analytical procedure is useful over this latter range of enzyme concentrations. Thus, it became necessary to use a series of specific substrates which were selected with respect to their individual usefulness when employing a *p*H-stat[11] for following their hydrolysis over a limited range of enzyme concentrations but which collectively spanned the range of concentrations of interest. The specific substrates chosen were: α-N-acetyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester, used as the DL-mixture,[24] for the range 10^{-6} to 10^{-7} M [E]; α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester[24] for the range 10^{-7} to 10^{-8} M [E] and α-N-acetyl-L-tyrosine methyl ester for the range 10^{-8} to 10^{-9} M [E]. In general, the values of v_0 were obtained by the empirical procedure of Booman and Niemann[25] from data derived from *p*H-stat traces of extent of reaction *vs.* time.[11]

In practice the value of v_0 for the α-chymotrypsin-catalyzed hydrolysis of α-N-acetyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester, in the presence of an equivalent amount of the D-isomer, was determined, in aqueous solutions at 25.0 ± 0.1° and *p*H 7.90 ± 0.01 and 0.02 M in sodium chloride, for a value of $[S]_0 = 2.41 \times 10^{-3}$ M and for an apparent value of $[E] = 4.3 \times 10^{-6}$ M.[26] In this series of experiments, *cf.*, Table II, series 1, which involved the use of a 4.3×10^{-5} M enzyme stock solution and a single 1:10 dilution, it was assumed with considerable justification, *vide post*, that there was no significant loss of enzymatic activity for any cause and that the apparent and effective enzyme concentrations were identical. The values of v_0 for the hydrolysis of the same specific substrate were then determined for the same value of $[S]_0$ but for apparent values of $[E] = 5.8$ and 4.1×10^{-7} M. From the respective values of v_0/[E], *cf.*, Table II, series 1, 2 and 3, it is seen that in the latter two cases the effective enzyme concentration was 75 and 84% of the apparent enzyme concentration. Thus, in establishing a reaction system of apparent enzyme concentration of *ca.* 5×10^{-7} M, 20 ± 5% of the enzyme was lost, or rendered inactive, in the process of preparing the corresponding 10^{-6} M enzyme stock solution and performing a single 1:10 dilution.

A similar set of experiments were performed with α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester, *cf.*, Table II, series 5, 6, 7, 8 and 12. In this instance it was assumed, on the basis of the previous experiments, that the preparation of a reaction system with an apparent enzyme concentration of 4.1×10^{-7} M, by a 1:10 dilution of a 4.1×10^{-6} M enzyme stock solution, resulted in an effective enzyme concentration of but 80 ± 5% of the apparent concentration. Therefore, the values of v_0/[E] obtained for the experiments of series 5 and

(18) A. D. McLaren, *J. Phys. Chem.*, **58**, 129 (1954).

(19) A. D. McLaren, *Soil Sci. Soc. Amer. Proc.*, **18**, 170 (1954).

(20) A. D. McLaren and E. F. Estermann, *Arch. Biochem. Biophys.*, **61**, 158 (1956); **68**, 157 (1957).

(21) R. B. Martin and C. Niemann, THIS JOURNAL, **80**, 1473 (1958).

(22) I. Tinoco, Jr., *Arch. Biochem. Biophys.*, **68**, 367 (1957).

(23) R. J. Keri and C. Niemann, THIS JOURNAL, **80**, 1469 (1958).

(24) R. L. Bixler and C. Niemann, *J. Org. Chem.*, **23**, 575 (1958).

(25) K. A. Booman and C. Niemann, THIS JOURNAL, **78**, 3642 (1956).

(26) In this communication we shall use the term "apparent enzyme concentration" with reference to the amount of crystalline enzyme presumed to have been introduced into a given volume of reaction mixture and the term "effective enzyme concentration" with reference to the concentration inferred from the kinetic properties of the reaction system and the assumption that values of v_0 are linearly dependent upon the effective enzyme concentration when all other reaction parameters are held constant.

TABLE II

INITIAL SPECIFIC VELOCITIES *vs.* SPECIFIC SURFACE AREAS[a]

Exptl. series[b]	[E][c,d]	$v_0/[E]$[e]	$A_V/[E]$[f,g]	$A_R/[E]$[g,h]
α-N-Acetyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester, $[S]_0 = 2.41 \times 10^{-3}$ *M*[i]				
1[j]	4.3×10^{-6}	$2.82 \pm 0.03 \times 10^{-3}$	1.69	1.59
2[k]	5.8×10^{-7}	$2.11 \pm .01 \times 10^{-3}$	1.23×10	1.18×10
3[l]	4.1×10^{-7}	$2.37 \pm .03 \times 10^{-3}$	1.72×10	1.65×10
4[m]	4.5×10^{-7}	$2.74 \pm .04 \times 10^{-3}$	2.30×10^{4n}	2.30×10^{4n}
α-N-Benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester, $[S]_0 = 1.05 \times 10^{-3}$ *M*				
5[o]	4.1×10^{-7}	$1.98 \pm 0.01 \times 10^{-2}$	1.71×10	1.65×10
6[p]	4.1×10^{-7}	$2.23 \pm .09 \times 10^{-2}$	1.72×10	1.66×10
7[q]	5.9×10^{-8}	$0.91 \pm .08 \times 10^{-2}$	1.16×10^2	1.15×10^2
8[r]	6.2×10^{-8}	$0.87 \pm .09 \times 10^{-2}$	1.10×10^2	1.09×10^2
9[s]	4.5×10^{-7}	$3.39 \pm .07 \times 10^{-2}$	2.30×10^{4n}	2.30×10^{4n}
10[t]	4.8×10^{-7}	$3.30 \pm .10 \times 10^{-2}$	8.70×10^{3n}	8.70×10^{3n}
11[u]	4.5×10^{-7}	$2.90 \pm .0 \times 10^{-2}$	0.77	4.90×10^{4n}
12[v]	4.5×10^{-7}	$2.87 \pm .11 \times 10^{-2}$	0.77	1.53×10
13[w]	4.4×10^{-8}	2.5×10^{-2}	3.70×10^{4n}	3.70×10^{4n}
α-N-Acetyl-L-tyrosine methyl ester, $[S]_0 = 1.58 \times 10^{-3}$ *M*				
14[x]	6.2×10^{-8}	$9.4 \pm 0.8 \times 10^{-1y}$	1.10×10^2	1.09×10^2
15[z]	5.4×10^{-8}	$8.2 \pm 0.3 \times 10^{-1y}$	1.27×10^2	1.26×10^2
16[aa]	5.9×10^{-8}	$12.9 \pm 0.7 \times 10^{-1y}$	1.16×10^2	1.15×10^2
17[ab]	4.4×10^{-8}	$11.7 \pm 0.4 \times 10^{-1y}$	3.70×10^{4n}	3.70×10^{4n}
18[ac]	4.2×10^{-8}	13.3×10^{-1y}	3.90×10^{4n}	3.90×10^{4n}
19[ad]	6.2×10^{-8}	$14.1 \pm 0.4 \times 10^{-1y}$	1.70×10^{4n}	1.70×10^{4n}
20[ae]	4.1×10^{-9}	$1.7 \pm 1.0 \times 10^{-1y}$	1.70×10^4	1.66×10^3
21[af]	4.1×10^{-9}	$1.0 \pm 0.4 \times 10^{-1y}$	1.70×10^4	1.66×10^3
22[ag]	4.4×10^{-9}	4.0×10^{-1}	1.60×10^4	1.55×10^3
			3.70×10^{4n}	3.70×10^{4n}

[a] Based upon experiments conducted in aqueous solutions at 25.0 ± 0.1° and *p*H 7.90 ± 0.01 and 0.02 *M* in sodium chloride. [b] Each experimental series composed of triplicate experiments each involving nine observations of the extent of reaction at one minute intervals from zero to eight min. unless otherwise noted. [c] Apparent enzyme concentration in *M*. [d] To convert to mg. protein-nitrogen per ml. divide by 2.84×10^{-4} which is the factor for an assumed molecular weight of 22,000 and a nitrogen content of 16.0%. [e] In units of *M* ml./mg. protein-nitrogen min. with deviation of value being value of σ. [f] In units of 100 cm.²/mg. protein-nitrogen and pertaining to the surface in contact with the enzyme prior to final dilution. [g] To convert to cm.²/mole multiply by 3.52×10^6. [h] In units of 100 cm.²/mg. protein-nitrogen and pertaining to surface in contact with enzyme in reaction cell. [i] Concentration of L-component added as the DL-mixture. [j] Original enzyme solution made up at *p*H 4.0, mean extent of reaction 24%. [k] Original enzyme solution made up at *p*H 8.0, mean extent of reaction 2.8%, duplicate experiments. [l] Original enzyme solution made up at *p*H 4.0, mean extent of reaction 2.1%, quadruplicate experiments. [m] Crystalline enzyme dissolved in 5 ml. of "Ludox" at *p*H 8 and diluted to 100 ml., mean extent of reaction 2.8%, duplicate experiments. [n] Specific surface area of added "Ludox." [o] Original enzyme solution made up at *p*H 8.0, mean extent of reaction 20%. [p] Original enzyme solution made up at *p*H 5.0, mean extent of reaction 21%. [q] Same as *o* except mean extent of reaction 1.3%. [r] Original enzyme solution made up at *p*H 3.5, mean extent of reaction 1.2%. [s] Crystalline enzyme dissolved in 5 ml. of "Ludox" at *p*H 8.0 and diluted to 100 ml., mean extent of reaction 33%. [t] Crystalline enzyme dissolved in 2 ml. of "Ludox" at *p*H 8.0 and diluted to 100 ml., mean extent of reaction 33%, duplicate experiments. [u] 0.1 ml. syringe used for introduction of enzyme solution into reaction system which contained 0.0221 g./10 ml. of "highly porous" silica powder, mean extent of reaction 31%, duplicate experiments. [v] 0.1 ml. syringe used for introduction of enzyme solution into reaction mixture, mean extent of reaction 28%, duplicate experiments. [w] Crystalline enzyme dissolved in 8 ml. of "Ludox" at *p*H 8.0 and diluted to one liter, mean extent of reaction 2.8%, single experiment. [x] Original enzyme solution made up at *p*H 3.5, mean extent of reaction 45%. [y] Initial velocities estimated from slopes of tangents to curves at zero time, subjective graphical procedure used because extreme curvature of traces of extent of reaction *vs.* time could not be accommodated even with a fifth degree polynomial. [z] Same as *x* except mean extent of reaction 39%. [aa] Original enzyme solution made up at *p*H 8.0, mean extent of reaction 55%. [ab] Crystalline enzyme dissolved in 8 ml. of "Ludox" at *p*H 8.0 and diluted to one liter, mean extent of reaction 68%. [ac] Same as *ab* except mean extent of reaction 75%. [ad] Crystalline enzyme dissolved in 5 ml. of "Ludox" at *p*H 8.0 and diluted to one liter, mean extent of reaction 65%. [ae] System 0.10 *M* in sodium chloride, mean extent of reaction 0.86%. [af] Mean extent of reaction 0.72%, duplicate experiments. [ag] Enzyme solution prepared as in *ab* and then diluted 1:10, extent of reaction 2.1%, single experiment.

6 were divided by the factor 0.80 ± 0.05 to obtain a reference value of $v_0/[E] = 2.64 \pm 0.33 \times 10^{-2}$ [27] for an enzyme stock solution whose apparent enzyme concentration was *ca.* 4×10^{-7} *M*. In the experiments of series 12 a reaction system 4.5×10^{-7} *M* in apparent enzyme concentration was established by a 1:100 dilution of a 4.5×10^{-5} *M* enzyme stock solution where it may be assumed that the apparent and effective enzyme concentrations are identical. The agreement of the value of $v_0/[E] = 2.87 \pm 0.11 \times 10^{-2}$ so obtained with the reference value of $v_0/[E] = 2.64 \pm 0.33 \times 10^{-2}$ noted above suggests that the 20 ± 5% loss in enzymatic activity associated with the preparation of a reaction system *ca.* 5×10^{-7} *M* in apparent enzyme concentration by a 1:10 dilution of an apparent 5×10^{-6} *M* enzyme stock solution is due to losses incurred in the preparation and manipulation of the 5×10^{-6} *M* stock solution that can be circumvented by an alternative 1:100 dilution of a 5×10^{-5} *M* stock solution. The experiments of series 12 also are of value in provid-

(27) All values of $v_0/[E]$ given in this communication are in units of *M* ml./mg. protein-nitrogen min.

ing additional evidence that the loss in effective enzyme concentration observed in the experiments of series 2 and 3 is due to an actual loss of enzyme to the surface of the volumetric equipment rather than to an inactivation of the enzyme in solution by a process dependent upon the absolute enzyme concentration.

It will be seen from the data given in Table II for the experiments of series 7 and 8 that reaction systems with apparent enzyme concentrations of 5.9 and 6.2 × 10^{-8} *M*, and produced by a single 1:10 dilution of apparent 5.9 and 6.2 × 10^{-7} *M* enzyme stock solutions, possess effective enzyme concentrations that are but 34 ± 5% of the apparent concentrations if it is assumed that the reference value of $v_0/[E] = 2.64 \pm 0.33 \times 10^{-2}$, determined for the reaction systems with an apparent enzyme concentration of *ca.* 4 × 10^{-7} *M* can be applied to the stock solutions with an apparent enzyme concentration of *ca.* 6 × 10^{-7} *M*.

A third set of experiments were conducted with α-N-acetyl-L-tyrosine methyl ester, *cf.*, Table II, series 14, 15, 16, 20 and 21. The experiments of series 14, 15 and 16 were performed in order to establish a reference value of $v_0/[E]$ for reaction systems with an apparent enzyme concentration of *ca.* 6 × 10^{-8} *M* and established by a single 1:10 dilution of enzyme stock solutions with an apparent concentration of *ca.* 6 × 10^{-7} *M*. From these experiments and those of series 7 and 8 it follows that the effective enzyme concentrations of such reaction systems are but 34 ± 5% of the apparent concentrations and that the reference value of $v_0/[E] = 3.0 \pm 1.4$. If this reference value can be assumed to be applicable for enzyme stock solutions with apparent enzyme concentrations of *ca.* 4 × 10^{-8} *M* it follows, from the experiments of series 20 and 21, that for reaction systems with apparent enzyme concentrations of *ca.* 4 × 10^{-9} *M* the effective enzyme concentration is less than 10% of the apparent concentration.[28]

While the results summarized in Table III can be interpreted in terms of losses incurred because of surface effects of the second kind it is clear that additional supporting evidence for this interpretation is required. If reference is made to the specific surface areas given in Table II, and encountered in the experiments of series 1-3, 5-8, 14-16 and 20 and 21, it will be seen that a decrease in effective enzyme concentration can be associated with an increase in the total specific surface area, *i.e.*, $(A_V + A_R)/[E]$. However, it cannot be determined from these data whether the surface area of the reaction vessel or that of the volumetric apparatus is the most important in causing a decrease in effective enzyme concentration. In order to determine whether a greatly increased surface area associated with the reaction vessel would or would not lead to a decrease in the effective enzyme concentration, the experiments of series 11 were performed. In this instance, the conditions employed in the experiments of series 12 were duplicated except for the addition of a preparation of highly porous silica powder, with a surface area of 350 m.^{2}/g.[29] to the reaction vessel so as to increase its specific surface area by a factor of *ca.* 3 × 10^3. It will be seen from the data given in Table II that the values of v_0 for the experiments of series 11 and 12 are identical within the limits of experimental error and we may conclude that the increase in the surface area of the reaction vessel had little or no effect upon the effective enzyme concentration. Therefore, our attention was directed to the possibility that the decrease in effective enzyme concentration observed previously was largely due to a loss of enzyme to the surfaces of the volumetric apparatus employed in the preparation of the enzyme stock solutions and their transfer to the reaction vessels, particularly since the results of the experiments of series 5, 6 and 12, *vide ante*, afforded the same conclusion.

TABLE III

LOSS OF ENZYMATIC ACTIVITY THROUGH SURFACE EFFECTS OF THE SECOND KIND

Approx. apparent enzyme concn., *M*	Effective enzyme concn.,[a] %	
	Conventional procedure	With "Ludox"
5 × 10^{-6}	100[b]	100
4 × 10^{-7}	80 ± 5[c]	97; 125 ± 25
6 × 10^{-8}	34 ± 5	93 ± 9; *ca.* 50
4 × 10^{-9}	<10	*ca.* 10

[a] As per cent. of the apparent enzyme concentration and based upon a single 1:10 dilution of the enzyme stock solution. [b] Assumed value, see text. [c] A single 1:100 dilution of a 10^{-5} *M* enzyme stock solution appeared to provide a reaction system in which the apparent and effective enzyme concentrations were identical within the limits of experimental error.

Initially, an attempt was made to limit loss of enzyme to the volumetric apparatus by prior treatment of the apparatus with agents tending to form alternative hydrophobic surfaces. When this procedure failed to lead to significant improvement, it was decided to investigate the consequences of adding a mobile surface that could compete with the immobile surface of the volumetric apparatus for the enzyme and which could be transferred, along with the adsorbed enzyme, to the reaction vessel. It is obvious that the component providing the mobile surface must possess a large surface area relative to that of the volumetric apparatus and a sufficiently small particle size so that gravitational sedimentation would be of no consequence. Since stable silica sols with specific surface areas of *ca.* 400 m.^{2}/g. and a mean particle size of *ca.* 7 mμ are commercially available under the designation of "Ludox"[30] SM Colloidal Silica,[31] it was decided to determine whether this material would provide a satisfactory mobile surface.

A number of preliminary experiments indicated that the most suitable procedure involving the use of "Ludox" was to dissolve the crystalline enzyme in a limited volume of the commercial product adjusted to *p*H 7 to 8, then to perform the necessary dilution, recognizing the need for a possible final

(28) A more precise value cannot be given because of the extreme curvature of the traces of extent of reaction *vs.* time, *cf.*, Table II. This latter feature also suggests that in this instance enzyme may be being lost, at least in part, by a process other than one involving a surface effect of the second kind.

(29) The authors are indebted to Dr. Ralph Iler of the Grasselli Chemicals Department of E. I. du Pont de Nemours and Co. for this preparation.

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(31) A product of the Grasselli Chemicals Department, E. I. du Pont de Nemours and Co., Wilmington 98, Del.

adjustment of the *p*H of the solution and to use this enzyme stock solution as promptly as possible. It was found that it was not desirable to attempt solution of the enzyme in a "Ludox" solution of *p*H less than 7 since under these conditions polymerization of the colloidal silica, eventually leading to gel formation, resulted in a decrease in the effective enzyme concentration.

A second set of experiments were conducted under conditions similar to those described previously, *cf.* Table II. It will be seen from the results obtained in the experiments of series 1 and 4 that the presence of a mobile surface of specific surface area *ca.* 1.4×10^3 greater than that of the immobile surface accessible to the 4.5×10^{-6} *M* enzyme stock solution resulted, after a 1:10 dilution, in a reaction system with an apparent enzyme concentration of 4.5×10^{-7} *M* in which the effective enzyme concentration was 97% of the apparent concentration. This loss of 3% may be compared with the loss of 20 ± 5% encountered in the absence of a mobile surface. From the experiments of series 9 and 10 it will be seen that the effective enzyme concentration does not appear to be influenced by a 2.6-fold diminution in the specific surface area of the mobile surface when the latter is *ca.* 10^3 times greater than that of the immobile surface. However, in the experiments of series 9 and 10, which were conducted with a different specific substrate than those of series 4, the preparation of a reaction system with an apparent enzyme concentration of *ca.* 4.4×10^{-7} *M* by a 1:10 dilution of a "Ludox" containing stock solution resulted in a mean value of $v_0/[E]$, *i.e.*, $3.3 \pm 0.1 \times 10^{-2}$, that was greater than the reference value of $2.7 \pm 0.4 \times 10^{-2}$ based upon equality of apparent and effective enzyme concentration. These latter results, which cannot be explained solely on the basis of limitation of surface effects of the second kind, raises the question as to whether the increase in effective enzyme concentration observed in the presence of "Ludox" is due to an alternative activation phenomena that does not depend upon the presence of a mobile surface. The results of the experiments of series 11 suggest that this is not the case. However, it cannot be excluded that at least a fraction of the observed increase may be due to such an independent activation process which may or may not be a surface effect of the first kind.

It will be seen from the results of the experiments of series 13 that the extension of the "Ludox" technique to the preparation of reaction systems with an apparent enzyme concentration of 4.4×10^{-8} *M* led to a value of $v_0/[E] = 2.5 \times 10^{-2}$ which is 93 ± 9% of the reference value of $2.7 \pm 0.4 \times 10^{-2}$, or 76 ± 2% of the mean value of $3.3 \pm 0.1 \times 10^{-2}$ obtained for the "Ludox" system with apparent and effective enzyme concentrations of *ca.* 4.4×10^{-7} *M*. Since the mean value of $v_0/[E] = 0.9 \pm 0.1 \times 10^{-2}$ observed for a conventional reaction system with an apparent enzyme concentration of *ca.* 6×10^{-8} *M* in which the effective enzyme concentration was but 34 ± 5% of the apparent concentration, we may conclude that the presence of sufficient "Ludox" to provide a mobile surface area *ca.* 3.4×10^2 times greater than that of the immobile surface area of the volumetric equipment used in the preparation and transfer of the enzyme stock solution led to a 2 to 3-fold increase in the effective enzyme concentration of the reaction system. The observation that the "Ludox" was less effective in preventing the apparent loss of enzyme in the establishment of a reaction system with an apparent enzyme concentration of the order of 10^{-8} *M* than one of the order of 10^{-7} *M* may be due to the fact that in the latter system the ratio of mobile to immobile surface areas was of the order of 10^3 whereas in the former it was of the order of 10^2. This latter conclusion is reinforced by the observations made in the experiments of series 17, 18, 19 and 22 where a third specific substrate was used. The establishment of reaction systems with apparent enzyme concentrations of the order of 5×10^{-8} *M* in the presence of "Ludox" led to effective enzyme concentrations which were *ca.* 50% of the apparent concentration. While this latter value is lower than the value of 93 ± 8% obtained from the experiments of series 9, 10 and 11 conducted with the second specific substrate,[32] it may be used to demonstrate that when a reaction system with an apparent enzyme concentration of 4.4×10^{-9} *M* is established under conditions where the mobile surface area is of comparable magnitude to that of the immobile surface area of the volumetric equipment the effective enzyme concentration is not much greater than that observed in the absence of added "Ludox."

While it may be inferred from the preceding experiments that α-chymotrypsin is adsorbed on the "Ludox" particles, it was decided to demonstrate this directly. Two solutions were prepared, *i.e.*, one of *p*H 8.0 and containing 0.128 mg. of α-chymotrypsin protein-nitrogen per ml. and 0.08 ml. of "Ludox" solution per ml., to give an estimated ratio of silica particles to enzyme molecules of *ca.* 1.3:1, and a second of *p*H 8.0 containing 0.119 mg. of α-chymotrypsin protein-nitrogen per ml. Both solutions were centrifuged at 25° for 150 min. at 100,000 *g*. A comparison of the spectrophotometrically determined enzyme concentrations revealed that there was a diminution of 7.5% in the enzyme concentration of the top 40% of the solution containing no "Ludox" and of 84% in the same portion of the "Ludox" containing solution. The presence of silica particles at the bottom of this latter solution was visibly evident. Thus, it is clear that α-chymotrypsin is adsorbed on the "Ludox" particles present in a solution of *p*H 8.0.

A number of conclusions relative to the possible role of surface effects in α-chymotrypsin-catalyzed hydrolyses may be drawn from the results obtained in this study. First, with regard to surface effects of the second kind it is clear that the use of enzyme stock solutions more dilute than those of the order of 10^{-5} *M* can lead to significant losses of enzyme to the surface of the volumetric equipment used in the preparation and ma-

(32) It will be noted that in the presence of "Ludox" systems containing α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester appeared to be more reactive than equivalent ones containing α-N-acetyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester or α-N-acetyl-L-tyrosine methyl ester. The reason for this behavior is not known, but it suggests that if an independent activation process is operative, *vide ante*, it may be dependent upon the nature of the specific substrate.

nipulation of such solutions. Admitting the practicality of 1:10 to 1:100 dilutions of 10^{-5} M enzyme stock solutions, it follows that reaction systems with apparent enzyme concentrations of the order of 10^{-6} and 10^{-7} M are accessible without significant loss of enzyme through surface effects of the second kind. However, reaction systems with apparent enzyme concentrations of the order of 10^{-8} and 10^{-9} M are illusory with respect to the true enzyme concentration unless they can be prepared by direct 1:1000 or 1:10,000 dilutions of 10^{-5} M stock solutions.

While the device of providing a competitive mobile surface appeared to be instrumental in decreasing the amount of enzyme lost to the immobile surfaces of the volumetric equipment, it is clear that the "Ludox" so used was of limited effectiveness in the preparation of the more dilute enzyme solutions. Since it can be shown that whenever "Ludox" was used the number of "Ludox" particles was greater than the number of enzyme molecules present, one is left with the conclusion that per unit surface area the glass volumetric equipment was much more effective than "Ludox" as an adsorbent for the enzyme. Since this result was not anticipated when the experiments were performed, no attempt was made to keep a record of the source and past history of the various pieces of volumetric equipment. Therefore, it is impossible to determine at the present time whether flint or borosilicate glass is the more efficient adsorbent for α-chymotrypsin.

Although surface effects of the second kind can be eliminated as a significant factor in reaction systems with an apparent enzyme concentration greater than the order of 10^{-7} M, it follows from the results of the present study that surface effects of the first kind may be significant in systems with an apparent enzyme concentration of the order of 10^{-6} M or less. While it is true that the addition of a substantial amount of colloidal silica to a reaction system with apparent and effective enzyme concentrations of the order of 10^{-6} M was without demonstrable effect, it should be noted that when α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester was used as a specific substrate, the enhancement in rate upon the addition of "Ludox" was greater than that observed with the other two specific substrates. Furthermore, it was observed, particularly with the systems involving α-N-acetyl-L-tyrosine methyl ester, that the presence of "Ludox" not only led to an enhancement in rate but also resulted in the elimination of a large amount of curvature in the traces of extent of reaction *vs.* time seen in the absence of "Ludox." While all of the above phenomena are illy understood, they do suggest that surface effects of the first kind may be encountered in the presence of silicous surfaces and may become significant with glass apparatus at apparent enzyme concentrations of the order of 10^{-6} M or less, where surface effects of the second kind may or may not be significant.

Experimental

General Procedure.—All reactions were followed with a *p*H-stat essentially as described in an earlier communication.[11] All glassware was washed with an alkaline detergent, rinsed with distilled water and air-dried. To minimize the effect of residual enzyme on the electrodes in the reaction cell, the electrodes were washed by stirring for one min. with 1 N hydrochloric acid, to deactivate the residual enzyme,[3] and rinsed with distilled water. In one case where the electrodes previously had been exposed to an apparent 10^{-5} M enzyme solution and washed with distilled water the residual activity contained on the electrode was equivalent to that of an apparent 10^{-9} M enzyme solution.

Specific Substrates.—The preparation of α-N-acetyl-β-(4-pyridyl-1-oxide)-DL-alanine methyl ester and of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester has been described.[24] α-N-Acetyl-L-tyrosine methyl ester, m.p. 136–138°, $[\alpha]^{25}D$ +24.6°, was prepared by conventional procedures. It should be noted that the first two specific substrates appeared to contain *ca.* 1 to 3% of a more rapidly hydrolyzable component, probably the corresponding pyridyl derivative. Since these impurities were present to such a small extent and were hydrolyzed much more rapidly than the principal components, the initial portions of the *p*H-stat traces, *i.e.*, from 0 to t = 2 to 3 minutes, which possessed marked curvature were ignored and the more linear portion employed to extrapolate back to the initial situation.

Surface Areas of Volumetric Equipment.—The following values were computed on the basis of the stated capacity of the apparatus. Volumetric flasks: 10 ml., 3.5 cm.²/ml.; 25 ml., 2.0 cm.²/ml.; 50 ml., 1.4 cm.²/ml.; 100 ml., 1.5 cm.²/ml.; 1000 ml., 0.7 cm.²/ml.; pipets: 0.1 ml., 10 cm.²/ml.; 0.2 ml., 33 cm.²/ml.; 2.0 ml., 15 cm.²/ml.; 5.0 ml., 1.5 cm.²/ml.; syringe: 1.0 ml., 5.3 cm.²/ml.; beaker: 3.2 cm.²/ml.; reaction vessel: 2.4 cm.²/ml.

Enzyme Solutions.—In order to minimize losses of enzyme by the technique of serial dilution, the enzyme stock solutions were prepared by dissolving weighed amounts of the crystalline enzyme, *i.e.*, salt-free α-chymotrypsin Armour lot no. 3283, in the requisite amounts of water to produce stock solution containing ten times the amount of enzyme desired in the reaction mixtures. Immediately prior to use these stock solutions were adjusted to *p*H 7.90 ± 0.01 and a 1.0 ml. aliquot introduced into 9.0 ml. of a solution adjusted to *p*H 7.90 ± 0.01 and containing the specific substrate and 0.02 M sodium chloride. With this technique the number of transfers was reduced to an absolute minimum. When "Ludox" was employed the commercial product was usually adjusted to *p*H 8.0 with 1 N aqueous hydrochloric acid, the enzyme dissolved in this solution which was then made up to volume and used as promptly as was possible. Further details are given in the footnotes to Table II.

Sedimentation Studies.—A refrigerated Model L-Spinco Ultracentrifuge operated for 2.5 hr. at 40,000 r.p.m; was employed for the studies described in the text. Optical densities were determined in a Cary Model 11 Spectrophotometer.

PASADENA, CALIFORNIA