pies of activation for the resin-catalyzed hydrolysis are 13 to 18 cal. deg.$^{-1}$ mole^{-1} higher than for acid hydrolysis.[11] Thus the greater efficiency of the resin is explained by the increase in the entropy of activation as compared with the entropy of activation of the acid-catalyzed reaction. This increase in the entropy of activation is probably due to the fixation of the dipeptide molecule to the resin particle.

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[CONTRIBUTION NO. 1943 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Evaluation of the Enzyme–Inhibitor Dissociation Constants of α-Chymotrypsin and Several Pairs of Charged and Uncharged Competitive Inhibitors at *p*H 7.9 and 6.9[1,2]

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The enzyme–inhibitor dissociation constants of α-chymotrypsin and several pairs of charged and uncharged competitive inhibitors of this enzyme which were evaluated previously in aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM–HCl buffer now have been evaluated in aqueous solutions at 25° and *p*H 6.9 and 0.2 *M* in the THAM component of a THAM–HCl buffer. A comparison of these two sets of dissociation constants has led to the suggestion that the development of a negative charge in the environment of the catalytically active site of the enzyme particularly at *p*H 7.9 is responsible for the lesser affinity of α-chymotrypsin at *p*H 7.9 than at *p*H 6.9 for certain negatively charged competitive inhibitors of this enzyme. The effect of adding phosphate ion to the above systems has been discussed.

In 1937, Bergmann and Fruton[4] reported that α-chymotrypsin, acting in aqueous solutions *M*/15 in a phosphate buffer and at 40° and *p*H 7.6–7.8, hydrolyzed carbobenzoxy-L-tyrosylglycinamide, to give carbobenzoxy-L-tyrosine and glycinamide, but did not hydrolyze carbobenzoxy-L-tyrosylglycine. On the basis of the above evidence these authors concluded that the enzyme was incapable of causing the hydrolysis of specific substrates in which a carboxyl group was bonded to the non-carbonyl carbon atom immediately adjacent to the nitrogen atom involved in the susceptible peptide bond. This conclusion was substantiated further by the subsequent report[5] that α-chymotrypsin, acting in aqueous solutions *M*/15 in a phosphate buffer,[6] but at 25° and *p*H 7.1–7.5, hydrolyzed carbobenzoxy-L-phenylalanylglycinamide but did not hydrolyze carbobenzoxy-L-phenylalanylglycine, and by the claim[7] that carbobenzoxy-L-glutamyl-L-tyrosylglycinamide and L-glutamyl-L-tyrosylglycinamide were hydrolyzed in the presence of α-chymotrypsin but that carbobenzoxy-L-glutamyl-L-tyrosylglycine was not.[8] In 1950, Neurath and Schwert,[9] recognizing that the carboxyl group in question would be completely ionized in aqueous solutions in the region of *p*H 7–8, concluded, on the basis of the above evidence, that the presence of a negative charge near the susceptible bond caused a loss in substrate activity.

The first suggestion that a negative charge was present at or near the catalytically active site of the enzyme and that an electrostatic repulsion could arise from the interaction of this negative charge with that present in a competitive inhibitor containing a carboxylate group was offered by Neurath and Schwert[9] on the basis of experiments described by Kaufman and Neurath[10,11] which were conducted at 25° and *p*H 7.8 in the presence of a 0.1 *M* phosphate buffer when aqueous solutions were employed, or a 0.045 *M* phosphate buffer when the solvent system was 30% aqueous methanol. However, the experiments of Kaufman and Neurath[10,11] do not provide a direct and unambiguous demonstration that the affinity of α-chymotrypsin, when evaluated in aqueous solutions at *p*H 7.8 and in the presence of a phosphate buffer, for a competitive inhibitor containing a negatively charged carboxylate group is substantially less than for an uncharged competitive inhibitor which possesses the same structural features except for the replacement of the negatively charged carboxylate group by an uncharged group of approximately the same volume. The only uncharged competitive inhibitors which were studied by Kaufman and Neurath[10,11] were DL-1-phenyl-2-acetamidobutanone-3 and DL-1-*p*-hydroxyphenyl-2-acetamidobutanone-3 and the inhibition constants determined were for the DL-mixtures. It was a tenuous comparison of these composite inhibition constants with those of aceturic acid, hippuric acid, acetyl-DL-methionine, benzoyl-DL-methionine, benzoyl-D-, L- and DL-phenylalanine, and presumably that of O,N-diacetyl-L-tyrosine, that led Neurath and Schwert[9] to the conclusion that replacement of a carboxylate group by an acetyl group results in an increased affinity of the enzyme for the inhibitor, under the conditions previously specified, rather than a direct comparison of, for example, acetyl-L-phenylalanine with

(1) Supported in part by a grant from Eli Lilly and Co.

(2) *Cf.* R. J. Foster, Ph.D. Thesis, Calif. Inst. Tech., Pasadena, Calif., 1952.

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

(4) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **118**, 405 (1937).

(5) J. S. Fruton and M. Bergmann, *ibid.*, **145**, 253 (1942).

(6) Private communication from J. S. Fruton.

(7) M. Bergmann and J. S. Fruton, *Advances in Enzymol.*, **1**, 63 (1941).

(8) The authors have been informed by J. S. Fruton that these experiments were conducted in aqueous solutions at 38–39° and *p*H 7.6 and *M*/15 in a phosphate buffer and that in the experiment with carbobenzoxy-L-glutamyl-L-tyrosylglycinamide the specific substrate was initially present in suspension.

(9) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(10) S. Kaufman and H. Neurath, *Arch. Biochem.*, **21**, 245 (1949).

(11) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **181**, 623 (1949).

L-1-phenyl-2-acetamidobutanone-3 where the only structural factor involved is the replacement of a carboxylate group by an acetyl group.

In a subsequent study in which the enzyme–inhibitor dissociation constants of α-chymotrypsin and a relatively large number of carboxylic acids were evaluated in aqueous solutions at 25° and *p*H 7.8 in the presence of a 0.1 *M* phosphate buffer, Neurath and Gladner,[12] in an argument which was based upon the use of absolute values of the above dissociation constants, the binding energies reported for the interaction of certain dye anions and homologous alkyl sulfates with serum albumin,[13,14] and the behavior of an entirely different enzyme system,[15] again expressed the belief that an electrostatic repulsion arising from the interaction of a competitive inhibitor bearing a negatively charged carboxylate group with a negative charge at the catalytically active site was a likely possibility even though it was reported by these authors[12] that the K_I values of β-phenylpropionate and β-phenoxyethanol were almost identical.

In 1952, Huang and Niemann[16] called attention to the fact that when the enzyme–inhibitor dissociation constants of α-chymotrypsin and a series of competitive inhibitors of the type $R(CH_2)_nCO_2^-$ were evaluated at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* with respect to the THAM[17] component of a THAM–HCl buffer, one obtained K_I values which were approximately five times greater than those reported by Neurath and Gladner[12] for the same competitive inhibitors but based upon an evaluation at 25° and *p*H 7.8 in aqueous solutions 0.1 *M* with respect to a phosphate buffer. Recently we have re-evaluated the primary experimental data obtained by Huang and Niemann[16] with the aid of the procedure described by Jennings and Niemann[18] and the revised K_I values,[19] *i.e.*, the enzyme–inhibitor dissociation constants, for several of the carboxylic acids and amides studied by Huang and Niemann[16] are given in Table I along with the corresponding K_I values reported by Neurath and Gladner[12] for the same acids. As before,[16] it was found that the K_I values of four of the bifunctional competitive inhibitors of the carboxylate type were approximately five, actually four to six, times greater when evaluated in aqueous solutions at 25° and *p*H 7.9 in the presence of a 0.02 *M* THAM–HCl buffer than when evaluated in aqueous solutions at 25° and *p*H 7.8 and 0.1 *M* in a phosphate buffer.

From the data given in Table I it may be concluded that the affinity of the catalytically active site of the enzyme for a bifunctional competitive inhibitor bearing a negatively charged carboxylate group, when present in aqueous solutions at 25° and *p*H 7.9 ± 0.1, can be increased by an amount corresponding to a change in $-\Delta F^0$ at 25° of approximately 1 kcal. by simply transferring the system from a 0.02 *M* uni-univalent THAM–HCl buffer to a 0.1 *M* uni-polyvalent phosphate buffer. That this effect is due to an electrostatic repulsion of the negatively charged carboxylate group, present in the bifunctional competitive inhibitors, by a negative charge at or near the catalytically active site of the enzyme which is operative, in aqueous solutions at 25° and *p*H 7.9 ± 0.1 in a 0.02 *M* uni-univalent THAM–HCl buffer but which is not operative in a 0.1 *M* uni-polyvalent phosphate buffer is suggested by the fact that in the former system the K_I values of the bifunctional competitive inhibitors bearing a negatively charged carboxylate group are approximately five, actually 3.6–6.5, times greater than the K_I values of the corresponding uncharged amides, whereas the latter K_I values are identical, within the limits of experimental error, with the K_I values observed by Neurath and Gladner[12] for the corresponding carboxylate ions in the presence of a 0.1 *M* phosphate buffer.

Further evidence for the existence of an electrostatic repulsion arising from the interaction of a negatively charged species with a negative charge at or near the catalytically active site of the enzyme in systems containing a uni-univalent THAM–HCl buffer was obtained by Hogness and Niemann[20] in their study of the kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide wherein it was found that the effective concentration of the specific substrate at *p*H values greater than 7.6 was demonstrably diminished by an ionization process which resulted in the formation of increasing amounts of the negatively charged acetyl-L-tyrosinhydroxamate ion.

In order to gain additional information with respect to the possible interaction of negatively charged species with the catalytically active site of the enzyme, particularly in reaction systems containing a uni-univalent buffer, we have in this study evaluated the enzyme–inhibitor dissociation constants of α-chymotrypsin and a series of charged and uncharged competitive inhibitors of this enzyme when

TABLE I

ENZYME–INHIBITOR DISSOCIATION CONSTANTS

Inhibitor	H and N[a] K_I[c,d]	H and N[a] $-\Delta F^0$[f]	N and G[b] K_I[c]	N and G[b] $-\Delta F^0$[f]	δ $-\Delta F^0$
β-Phenylpropionate[e]	25 ± 5	2.2	5.5	3.1	0.9
β-Phenylpropionamide	7 ± 2	2.9	..	..	..
γ-Phenylbutyrate[e]	60 ± 10	1.7	14	2.5	0.8
γ-Phenylbutyramide	12 ± 3	2.6	..	..	..
β-(β-Indole)-propionate[e]	15 ± 3	2.5	2.5	3.6	1.1
β-(β-Indole)-propionamide	2.3 ± 0.4	3.6	..	..	..
γ-(β-Indole)-butyrate[e]	23 ± 5	2.2	3.6	3.4	1.2

[a] Revised values[19] of Huang and Niemann[16] for aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM–HCl buffer. [b] Values of Neurath and Gladner[12] for aqueous solutions at 25° and *p*H 7.8 and 0.1 *M* in a phosphate buffer. [c] In units of 10^{-3} *M*. [d] The values given in this column do not differ significantly from the previously reported values[16] except for a more realistic probable error. [e] Since this inhibitor was added to the reaction system in the form of its sodium salt the corresponding K_I value is subject to correction for an ionic strength effect[19] which in this instance is probably less than the estimated experimental error. [f] Values of $-\Delta F^0$ in kcal. per mole at 25° to the nearest 0.1 kcal.

(12) H. Neurath and J. A. Gladner, *J. Biol. Chem.*, **188**, 407 (1951).
(13) I. M. Klotz, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 97 (1950).
(14) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).
(15) E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **178**, 645 (1949).
(16) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 5903 (1952).
(17) Tris-(hydroxymethyl)-aminomethane.
(18) R. R. Jennings and C. Niemann, THIS JOURNAL, **75**, 4687 (1953).
(19) R. J. Foster and C. Niemann, *ibid.*, **77**, 3370 (1955).
(20) D. S. Hogness and C. Niemann, *ibid.*, **75**, 884 (1953).

the reactants were present in aqueous solutions at 25° and *p*H 6.9 and 0.2 *M* in the THAM component of a uni-univalent THAM–HCl buffer in order that they could be compared with the corresponding values which had been determined previously under the same conditions except at *p*H 7.9 and in solutions 0.02 *M* in the THAM component of a THAM–HCl buffer.[16,19,21–27]

While it would have been preferable, had it been experimentally feasible, to maintain the buffer concentration, in the experiments at *p*H 6.9, at 0.02 *M* in the THAM component of a THAM–HCl buffer, the fact that it was not so maintained is relatively unimportant for the case at hand since the anticipated increase in initial velocities expected from an increase in the ionic strength of the reaction system, and the concomitant underestimation of the respective values of K_I.[19,27] is probably well within the limits of experimental error encountered in the present study. The values of K_I so obtained are summarized in Table II. It should be noted that the values given in Table II are not the original values[2,16,21–26] but are the revised values[19,27] which have been obtained from the original primary data[2,16,21–26] through the use of evaluation procedures which are more objective than those used previously.[18,19,27,28]

TABLE II

*p*H DEPENDENCE OF SEVERAL ENZYME–INHIBITOR DISSOCIATION CONSTANTS[a,b]

Inhibitor	$K_I \times 10^3$ *M*[c] *p*H 7.9	*M*[c] *p*H 6.9	$-\Delta F^{0d}$ *p*H 7.9	*p*H 6.9	δ $-\Delta F^{0e}$
Acetyl-D-tryptophanamide	2.3 ± 0.4	1.7 ± 0.3	3.6	3.8	0.2
Acetyl-D-phenylalaninamide	12 ± 3	10 ± 2	2.6	2.7	0.1
Acetyl-D-tryptophanate[f]	7.5 ± 1.5	1.3 ± 0.2	2.9	3.9	1.0
Acetyl-L-tryptophanate[f]	10 ± 2	2.0 ± .3	2.7	3.7	1.0
β-(β-Indole)-propionate[f]	15 ± 3	1.5 ± .3	2.5	3.9	1.4
D-Tryptophanamide[g]	4.0 ± 1.0	5.0 ± 1.0	3.3	3.1	−0.2
Tryptamine[g]	2.3 ± 0.4	2.5 ± 0.5	3.6	3.5	−0.1

[a] In aqueous solutions at 25°. [b] Values at *p*H 7.9 determined with 0.02 *M* THAM–HCl buffer, those at *p*H 6.9 with 0.2 *M* THAM–HCl buffer. [c] Values of K_I are the revised values[19,27] based upon primary data presented either in previous publications[16,21–26] or in the Ph.D. thesis of R. J. Foster.[2] [d] In kcal. per mole at 25° to the nearest 0.1 kcal. [e] Positive values indicate a greater affinity at *p*H 6.9 than at *p*H 7.9. [f] Since this inhibitor was added to the reaction system in the form of its sodium salt, the corresponding K_I value is subject to additional correction for an ionic strength effect[19,27] which in this instance is probably less than the estimated experimental error. [g] See text.

It will be seen from the data presented in Table II that the K_I values of acetyl-D-tryptophanamide and acetyl-D-phenylalaninamide which were obtained at *p*H 7.9 in solutions 0.02 *M* in the THAM component of a THAM–HCl buffer are identical, within the limits of experimental error, with those obtained for these two uncharged competitive inhibitors at *p*H 6.9 in solutions 0.2 *M* in the THAM component of a THAM–HCl buffer. Thus, on the basis of the above evidence, it appears that not only is the increase in the buffer concentration from 0.02 to 0.2 *M* without significant effect upon the K_I values of the above two competitive inhibitors but that there is also no significant increase in the affinity of the catalytically active site of α-chymotrypsin for uncharged competitive inhibitors of this enzyme when the acidity of the reaction system is increased by a factor of ten, *i.e.*, from *p*H 7.9 to 6.9 for aqueous solutions at 25° and, respectively, 0.02 and 0.2 *M* in the THAM component of a THAM–HCl buffer.

In contrast to the situation noted immediately above, it will be seen, *cf.* Table II, that when negatively charged competitive inhibitors are considered, *e.g.*, acetyl-D-tryptophanate, acetyl-L-tryptophanate and β-(β-indole)-propionate, the K_I values at *p*H 7.9 are significantly greater than the corresponding values of K_I at *p*H 6.9 and that a tenfold increase in the acidity of the reaction system from *p*H 7.9 to 6.9 causes a five- to tenfold increase in the affinity of the catalytically active site of α-chymotrypsin for these anionic competitive inhibitors. Since there can be no doubt that each of the above three competitive inhibitors are present in aqueous solutions as the negatively charged anion in the region between *p*H 7.9 and 6.9 it follows that the increase in affinity that has been observed may be ascribed to a substantial protonation at *p*H 6.9 of a negatively charged group which is present at or near the catalytically active site of α-chymotrypsin and which at *p*H 7.9 appears to be protonated to a far lesser extent. It is interesting that this group which seems to be substantially ionized at *p*H 7.9, and thus contributes at this *p*H to a far greater extent than at *p*H 6.9 a negative charge to the environment of the catalytically active site also appears to be so disposed with respect to the other structural features of the active site of the enzyme as to be able to participate in a coulombic repulsion with both acetyl-D-tryptophanate and acetyl-L-tryptophanate to approximately the same degree when these latter two negatively charged species are present at the active site of the enzyme in their respective modes of combination.

The ammonium group of a protonated D-tryptophanamide is known to have a pK'_A value of 7.5 ± 0.1[23] and it follows from this information that at both *p*H 7.9 and 6.9 one is confronted with a mixture of the amino and ammonium forms of this competitive inhibitor. At *p*H 7.9 the amino form predominates, *i.e.*, in the ratio of *ca.* 7:3 and at *p*H 6.9 the ammonium form is the most abundant, *i.e.*, in the ratio of *ca.* 4:1. There is no *a priori* reason to expect that the group which appears to contribute a substantial negative charge to a region of the catalytically active site of the enzyme at *p*H 7.9, because of a far greater extent of ionization at *p*H 7.9 than at *p*H 6.9, must necessarily engage in a coulombic attraction with the positively charged ammonium group which is present in the protonated D-trytophanamide. Therefore, one could inter-

(21) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).
(22) *Idem., ibid.*, **73**, 1555 (1951).
(23) *Idem., ibid.*, **73**, 3223 (1951).
(24) *Idem., ibid.*, **74**, 101 (1952).
(25) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).
(26) H. T. Huang and C. Niemann, *ibid.*, **74**, 4634 (1952).
(27) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).
(28) R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).

pret the equivalence, within the limits of experimental error, of the two K_I values which are given in Table II for this competitive inhibitor, even though these constants are clearly composite in nature, to mean that in so far as combination in all modes is concerned it makes little or no difference whether the amino group of D-tryptophanamide is or is not protonated. However, it could be alternatively assumed that a coulombic attraction such as that postulated above, does take place and that the effect of increased protonation of the amino group of D-tryptophanamide in passing from *p*H 7.9 to 6.9 is almost exactly counterbalanced by a decreased ionization at *p*H 6.9 of the group which is capable of contributing a negative charge to the environment of the catalytically active site particularly at *p*H 7.9 where a pronounced decrease in the affinity of the active site for certain negatively charged competitive inhibitors of α-chymotrypsin is observed.

The case of tryptamine is in a sense less ambiguous than that of D-tryptophanamide since the former base with a pK'_A value of approximately ten[29] for the protonated species is, for all practical purposes, completely protonated at both *p*H 7.9 and 6.9. Therefore, the K_I values which are given in Table II for tryptamine at *p*H 7.9 and 6.9 can be taken as the enzyme–inhibitor dissociation constants of α-chymotrypsin and monoprotonated tryptamine at these respective *p*H values. The fact that the K_I values at *p*H 7.9 and 6.9 are identical, within the limits of experimental error, *cf.* Table II, affords no evidence of the existence of a coulombic attraction between the positively charged ammonium group of protonated tryptamine and the negative charge which is assumed to be present at or near the catalytically active site particularly at *p*H 7.9. Therefore, if it is assumed that at *p*H 7.9 only a single negative charge is present in the region of the catalytically active site, one also must assume that the modes of combination of protonated tryptamine with the catalytically active site of α-chymotrypsin are such as to minimize or avoid juxtaposition of the positively charged ammonium group which is present in the inhibitor with the group which appears to be present in a region of the active site of the enzyme and which seems to contribute a negative charge to this region to a far greater extent at *p*H 7.9 than at 6.9. However, it does appear strained to postulate two substantially different modes of combination for the positively charged protonated tryptamine and the negatively charged β-(β-indole)-propionate. Hence, it should be noted that all of the observations made in this study, with the possible exception of those relative to D-tryptophanamide where the K_I values observed at *p*H 7.9 and 6.9 are clearly composite in nature and consequently are difficult to interpret, *vide ante*, can be accommodated if it is assumed that in the environment of the catalytically active site there are two groups capable of ionization to give negative charges, one of which is completely ionized, or nearly so, both at *p*H 7.9 and 6.9 and the other of which is significantly ionized only at *p*H 7.9. If this situation prevails, then it would be expected that the K_I values of α-chymotrypsin and competitive inhibitors of this enzyme which are uncharged or bear a single positive charge, such as is present in monoprotonated tryptamine, would be substantially independent of the *p*H of the reaction system between the limits of *p*H 7.9 and 6.9 and that the K_I values of α-chymotrypsin and competitive inhibitors of the type of acetyl-D-tryptophanate, acetyl-L-tryptophanate or β-(β-indole)-propionate, in which a single negative charge is present, would be more dependent upon the *p*H of the reaction system within the above-mentioned limits with the K_I value of any one of these latter anionic competitive inhibitors being greater at *p*H 7.9 than at 6.9. While it cannot be said that the existence of one or more groups capable of ionization to give negative charges in the environment of the catalytically active site of α-chymotrypsin under a certain set of reaction conditions is well established the evidence that is presently available appears to afford no other interpretation of the data that has been obtained from the studies conducted in aqueous solutions at 25° in the presence of a uni-univalent THAM–HCl buffer.

If it is admitted that for a reaction system at *p*H 7.9 and containing a uni-univalent THAM–HCl buffer the active site of α-chymotrypsin contains a negative charge which is largely lost at *p*H 6.9, it is necessary to inquire as to why the replacement of a uni-univalent THAM–HCl buffer of *p*H 7.9 by a uni-polyvalent phosphate buffer of approximately the same *p*H should cause such a marked decrease in the K_I values of the anionic bifunctional competitive inhibitors which were considered in Table I.

The experiments of Bergmann and Fruton[4,5,7] with respect to the non-hydrolyzability of compounds of the type $RCONHCHR'CONHCH_2CO_2^-$, *vide ante*, throw little light on the problems associated with the presence of charged groups in the environment of the catalytically active site of α-chymotrypsin because it is not known whether the above compounds were not hydrolyzed because they were unable to combine with the catalytically active site of the enzyme or whether combination did take place but the susceptibility of the hydrolyzable bond to subsequent reaction was so decreased by the presence of the adjacent carboxylate group that no significant hydrolysis could be observed with the methods used by the above investigators. In contrast to this situation it is clear that the differing behavior, in phosphate and THAM–HCl buffers, of the series of so-called bifunctional competitive inhibitors of the type $R(CH_2)_nCO_2^-$ which were studied by Neurath and Gladner,[12] in a 0.1 *M* phosphate buffer at *p*H 7.8, and by Huang and Niemann,[16] in a 0.02 *M* THAM–HCl buffer at *p*H 7.9, may be interpreted in terms of an increased affinity of the active site of the enzyme for these bifunctional competitive inhibitors in the presence of phosphate over that which obtains in the THAM–HCl buffer. While it may seem odd that a negatively charged ion should enhance the affinity of a catalytically active site which possesses one or possibly two negative charges for a negatively charged bifunctional competitive inhibitor of the type $R(CH_2)_nCO_2^-$, we wish to suggest that such a situa-

(29) Unpublished observation of R. A. Bernhard.

tion could arise if through the interaction of phosphate ion with the enzyme in the neighborhood of the active site the conformation of the active site is so altered as to permit the combination of the above type of bifunctional anionic competitive inhibitor with the active site of the enzyme in modes which do not bring the negatively charged carboxylate group of the inhibitor into juxtaposition to the negative charge or charges which are present at the site and which are forbidden, because of steric reasons, in the conformation of the site which obtains in the absence of phosphate ion, *i.e.*, in the presence of the THAM–HCl buffer. While the above hypothesis provides a reasonable explanation of the differing results obtained by Neurath and Gladner[12] and by Huang and Niemann[16] we wish to point out that there is no evidence that is available at the present time with respect to the behavior of anionic trifunctional competitive inhibitors of the type $R'CONHCHRCO_2^-$ and until such studies, which are now in progress, are completed, it is necessary that we withhold judgment as to the general behavior of competitive inhibitors of the latter type.

Experimental

Specific Substrate.—All experiments were conducted with L-tyrosinhydroxamide as the specific substrate, which was prepared as described by Foster, Jennings and Niemann.[30] The values of K_S and k_3 for this specific substrate in aqueous solutions at 25° and *p*H 6.9 and 0.2 *M* in the THAM component of a THAM–HCl buffer are $41 \pm 2 \times 10^{-3}$ *M* and $3.6 \pm 0.2 \times 10^{-3}$ *M*/min./mg. protein-nitrogen/ml., respectively.[28,30]

Competitive Inhibitors.—All of the inhibitors employed in this study, with but one exception, have been described previously, *i.e.*, acetyl-D-tryptophanamide,[21,22,26] acetyl-D-phenylalaninamide,[25] acetyl-D-tryptophanate,[23] acetyl-L-tryptophanate,[21] β-(β-indole)-propionate,[16] D-tryptophanamide[23] and tryptamine.[24] D-Tryptophan, which had not been studied earlier, was prepared by the resolution of DL-tryptophan ethyl ester with α-chymotrypsin and the D-antipode was observed[29] to possess an $[\alpha]^{25}D$ 32.5° (*c* 0.5% in water). Berg[31] gives a value of $[\alpha]^{25}D$ 32.45°.

α-Chymotrypsin.—An Armour preparation No. 90402 was employed and in every instance the enzyme concentration was maintained at 0.104 mg. protein-nitrogen/ml.

TABLE III

SUMMARY OF INHIBITION EXPERIMENTS[a]

Competitive inhibitor[b]	$[S]_0 \times 10^3$ *M*	$[I] \times 10^3$ *M*	$E_I' \times 10^2$	I_1'	$K_I \times 10^3$ *M*
Acetyl-D-tryptophanamide	4–15[c,d]	1.5	1.7	0.88	1.7 ± 0.3
Acetyl-D-phenylalaninamide	4–15[c,d]	5.0	0.3	0.50	10 ± 2
Acetyl-D-tryptophanate	5–15[e,f]	1.0	2.3	0.77	1.3 ± 0.2
Acetyl-L-tryptophanate	5–15[g,f]	2.0	1.5	1.0	2.0 ± .3
β-(β-Indole)-propionate	5–15[g,f]	1.0	2.0	0.67	1.5 ± .3
D-Tryptophanamide[h]	5–15[e,f]	3.0	0.6	0.60	5.0 ± 1.0
Tryptamine[i]	5–15[e,f]	3.0	1.2	1.2	2.5 ± 0.5
D-Tryptophan	5–15[e,f]	2.4	0.5	0.40	6.0 ± 2.0

[a] In aqueous solutions at 25° and *p*H 6.9 and 0.2 *M* in the THAM component of a THAM–HCl buffer, enzyme concentration 0.104 mg. protein-nitrogen/ml., *i.e.*, [E] = 2.95×10^{-5} *M*. [b] *Vs.* L-tyrosinhydroxamide with $K_S = 41 \pm 2 \times 10^{-3}$ *M*, $k_3 = 3.6 \pm 0.2 \times 10^{-3}$ *M*/min./mg. protein-nitrogen/ml.,[28,30] and $E_S' = 0.07 \times 10^{-2}$. [c] Six experiments at six initial specific substrate concentrations within the limits indicated. [d] $S_S' = 0.10$–0.37. [e] Five experiments at five specific substrate concentrations within the limits indicated. [f] $S_S' = 0.12$–0.37. [g] Four experiments at four specific substrate concentrations within the limits indicated. [h] Partially protonated. [i] Fully protonated.

(30) R. J. Foster, R. R. Jennings and C. Niemann, THIS JOURNAL, **76**, 3142 (1954).

(31) C. P. Berg, *J. Biol. Chem.*, **100**, 79 (1933).

Enzyme Experiments.—All experiments were conducted in aqueous solutions at 25° and *p*H 6.9 and 0.2 *M* in the THAM component of a THAM–HCl buffer and the extent of reaction, at the various initial specific substrate and inhibitor concentrations was determined colorimetrically as described by Foster, Jennings and Niemann.[30] The initial velocities were determined by the method of Jennings and Niemann[18] from both zero and first-order plots of the primary data, and the respective values of K_I were evaluated from subsequent v_0 *versus* $v_0/[S]_0$ plots[19,27,28]; *cf.* Figs. 1 and 2. From the summary of the inhibition experiments which is given in Table III it will be seen that in every case [E] was of the order of 10^{-5} *M*, $E_S' < 0.1 \times 10^{-2}$, $E_I' < 2.5 \times 10^{-2}$, S_S' within the limits of 0.1–0.4 and $I_1' > 0.4$. Thus all experiments were conducted under conditions compatible with the evaluation procedures employed.[19,27,28] For purposes of calculation monomeric α-chymotrypsin was assumed to have a molecular weight of 22,000 and a nitrogen content of 16.0%.[19,27,28]

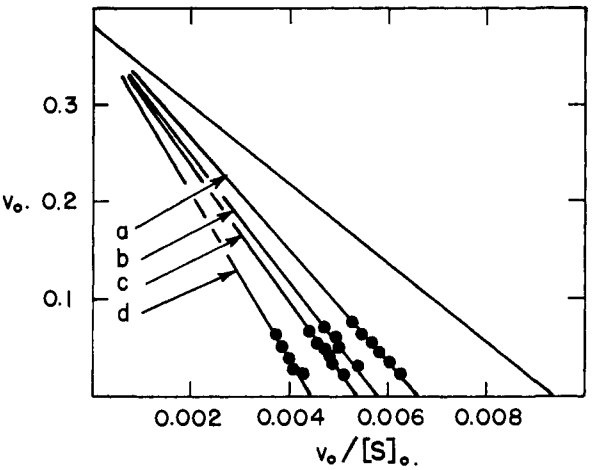

Fig. 1.—Competitive inhibition of the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide by: a, acetyl-D-phenylalaninamide, [I] = 5.0×10^{-3} *M*; b, β-(β-indole)-propionate, [I] = 1.0×10^{-3} *M*; c, acetyl-D-tryptophanamide [I] = 1.5×10^{-3} *M*; d, tryptamine, [I] = 3.0×10^{-3} *M*; v_0 in units of 10^{-3} *M*/min.; $[S]_0$ in units of 10^{-3} *M*.

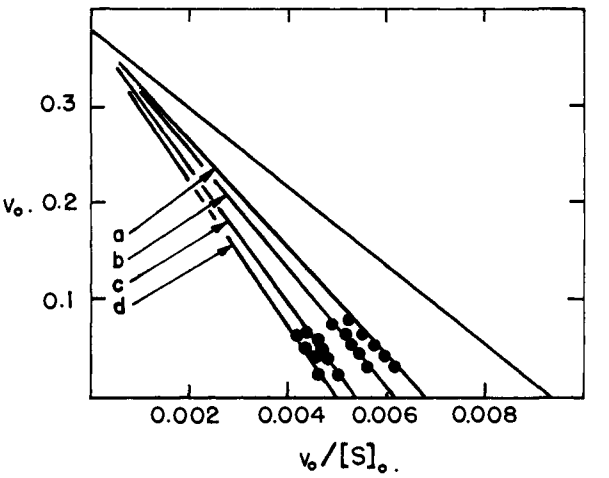

Fig. 2.—Competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydroxamide by: a, D-tryptophan, [I] = 2.4×10^{-3} *M*; b, D-tryptophanamide, [I] = 3.0×10^{-3} *M*; c, acetyl-D-tryptophanate, [I] = 1.0×10^{-3} *M*; d, acetyl-L-tryptophanate, [I] = 2.0×10^{-3} *M*; v_0 in units of 10^{-3} *M*/min.; $[S]_0$ in units of 10^{-3} *M*.

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