ml. aliquots were withdrawn at convenient time intervals, delivered into a series of volumetric flasks containing the 0.2 M citrate buffer of pH 4.8 ± 0.1, the solution immediately made up to volume with the citrate buffer and then analyzed as directed above.

Analysis of Data.—Data representative of a single kinetic experiment are given in Table VII. Sets of these data were graphically evaluated by the method of Foster and Niemann[13-15] as illustrated in Fig. 1.

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[CONTRIBUTION NO. 2172 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Dependence of the Enzyme-Inhibitor Dissociation Constants of Several Bifunctional Anionic Competitive Inhibitors of α-Chymotrypsin upon Added Potassium Phosphate[1]

BY RICHARD A. BERNHARD AND CARL NIEMANN[2]

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It has been observed that the enzyme-inhibitor dissociation constants of two representative bifunctional anionic competitive inhibitors of α-chymotrypsin, *i.e.*, β-(β-indole)-propionate and phenyl acetate, when evaluated in aqueous solutions at 25° and pH 7.9 ± 0.1 may be decreased by increasing the concentration of the uni-univalent buffer system, or by the addition of sodium chloride or potassium phosphate. A detailed analysis has indicated that the effect produced by added potassium phosphate is to be associated with a general increase in ionic strength and a specific ion effect.

It has been recognized that the enzyme-inhibitor dissociation constants of a number of bifunctional anionic competitive inhibitors of α-chymotrypsin, when evaluated in aqueous solutions at 25° and pH 7.9 and 0.02 M in the THAM[3] component of a THAM–HCl buffer,[4-6] are substantially greater than the constants obtained for the same inhibitors in aqueous solutions at 25° and pH 7.8 and 0.1 M in an unspecified phosphate buffer.[7,8] However, the lack of a suitable analytical procedure has prevented, until recently, a more direct comparison of the behavior of certain bifunctional anionic competitive inhibitors of α-chymotrypsin in the presence of THAM–HCl and phosphate containing buffers. With the development of a procedure which was suitable for following the rate of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.9 ± 0.1 in the presence of THAM–HCl or sodium or potassium phosphate buffers[9] it was thought desirable to re-examine several of the experiments reported earlier[4-8] and if possible to clarify and explain the behavior noted above.

In the first series of experiments the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.0 ± 0.1 and 0.02 M in the THAM component of a THAM–HCl buffer was examined in the presence of added potassium β-(β-indole)-propionate, β-(β-indole)-propionamide, potassium phenylacetate, phenylacetamide or tryptamine hydrochloride. As before[9] the reaction was followed by determining the rate of formation of ammonia and ammonium ion and the primary data so obtained were graphically evaluated by the method of Foster and Niemann.[10-12] The experimental conditions employed in this study are summarized in the footnotes to Table I. It will be seen from these data that the extent of reaction was sufficient to justify the use of the above evaluation procedure.[13] From the evaluated data given in Table I it is evident that all values of K_I were obtained under conditions that were compatible with the assumptions inherent in the procedures used for their evaluation.[6,10-12,14,15]

TABLE I

K_I VALUES OF FIVE COMPETITIVE INHIBITORS OF α-CHYMOTRYPSIN EVALUATED IN AQUEOUS SOLUTIONS AT 25° AND pH 7.9 ± 0.1 AND 0.02 M IN THE THAM COMPONENT OF A THAM–HCl BUFFER[a]

Competitive inhibitor	K_I[b]	E_I'[c]	I_I
Potassium β-(β-indole)-propionate	11 ± 1.5[e]	0.52	3.6
β-(β-Indole)-propionamide	1.7 ± 0.3[f]	3.4	0.88
Potassium phenylacetate	170 ± 15[g]	0.03	0.88
Phenylacetamide	10 ± 1[h]	0.57	1.0
Tryptamine hydrochloride	1.5 ± 0.3[i]	3.8	6.7

[a] Evaluated against acetyl-L-tyrosinamide with K_S = 32 × 10^{-3} M, [E] = 5.7 × 10^{-5} M, [d] E_S' = 0.17 × 10^{-2} and S_S' = 0.625 to 1.25. [b] In units of 10^{-3} M. [c] In units of 10^{-2}. [d] Based upon an assumed molecular weight of monomeric α-chymotrypsin of 22,000 and a nitrogen content of 16.0%. [e] Based upon 4 sets of experiments with $[S]_0$ = 20, 25, 30 and 40 × 10^{-3} M, [I] = 40 × 10^{-3} M, t = 150 minutes with a total of 7 observations of extent of reaction within this time interval, with a total extent of reaction of 36.5, 35.4, 32.2 and 30.0%, respectively, and with [E] = 0.200 mg. protein-nitrogen per ml. of Armour preparation no. 00592. [f] Same as *e* but with [I] = 1.5 × 10^{-3} M and total extent of reaction of 66.3, 64.0, 61.8 and 58.0% respectively. [g] Same as *e* but with [I] = 150 × 10^{-3} M and total extent of reaction of 61.3, 59.0, 56.8 and 52.8%, respectively. [h] Same as *e* but with [I] = 10 × 10^{-3} M and total extent of reaction of 58.5, 57.3, 55.2 and 53.4%, respectively. [i] Same as *h* but with total extent of reaction of 24.3, 23.8, 22.7 and 22.4%, respectively.

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

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

(3) Tris-(hydroxymethyl)-aminomethane.

(4) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 5963 (1952).

(5) R. J. Foster and C. Niemann, *ibid.*, **77**, 3365 (1955).

(6) R. J. Foster and C. Niemann, *ibid.*, **77**, 3370 (1955).

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

(8) H. Neurath and J. A. Gladner, *ibid.*, **188**, 407 (1951).

(9) R. A. Bernhard and C. Niemann, THIS JOURNAL, **79**, 4085 (1957).

(10) R. J. Foster and C. Niemann, *Proc. Natl. Acad. Sci.*, **39**, 999 (1953).

(11) T. H. Applewhite and C. Niemann, THIS JOURNAL, **77**, 4923 (1955).

(12) R. R. Jennings and C. Niemann, *ibid.*, **77**, 5432 (1955).

(13) K. A. Booman and C. Niemann, *ibid.*, **77**, 5783 (1955).

(14) R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).

(15) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

In a second series of experiments, *cf.* Table II, the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.1 *M* in the phosphate component of a potassium phosphate buffer was examined in the presence of the above five competitive inhibitors using the same procedures as before.

TABLE II

K_I VALUES OF FIVE COMPETITIVE INHIBITORS OF α-CHYMOTRYPSIN EVALUATED IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1 AND 0.1 *M* IN THE PHOSPHATE COMPONENT OF A POTASSIUM PHOSPHATE BUFFER[a]

Competitive inhibitor	K_I[b]	E_I'[c]	I_I'
Potassium β-(β-indole)-propionate	4.0 ± 0.4[e]	1.4	2.5
β-(β-Indole)-propionamide	1.8 ± 0.2[f]	3.2	0.83
Potassium phenylacetate	60 ± 5[g]	0.10	0.83
Phenylacetamide	11 ± 1[h]	0.52	0.90
Tryptamine hydrochloride	1.4 ± 0.3[i]	4.5	3.6

[a] Evaluated against acetyl-L-tyrosinamide with K_S = 32 × 10^{-3} *M*, [E] = 5.7 × 10^{-5} *M*[d], E_S' = 0.17 × 10^{-2} and S_S' = 0.156 to 1.25. [b] In units of 10^{-3} *M*. [c] In units of 10^{-2}. [d] Based upon an assumed molecular weight of monomeric α-chymotrypsin of 22,000 and a nitrogen content of 16.0%. [e] Based upon 4 sets of experiments with $[S]_0$ = 5, 10, 30 and 40 × 10^{-3} *M*, [I] = 10 × 10^{-3} *M*, *t* = 110 minutes for values of $[S]_0$ = 5 and 10 × 10^{-3} *M* and 150 minutes for values of $[S]_0$ = 30 and 40 × 10^{-3} *M* with a total of 7 observations of extent of reaction within these time intervals, with a total extent of reaction of 44.8, 43.7, 51.3 and 46.9%, respectively, and with [E] = 0.200 mg. protein-nitrogen per ml. of Armour preparation no. 00592. [f] Based upon 4 sets of experiments with $[S]_0$ = 20, 25, 30 and 40 × 10^{-3} *M*, [I] = 1.5 × 10^{-3} *M*, *t* = 150 minutes with a total of 7 observations of extent of reaction within this time interval, with a total extent of reaction 74.4, 72.8, 70.7 and 68.3%, respectively, and with [E] = 0.200 mg. of protein-nitrogen per ml. of Armour preparation no. 00592. [g] Same as *f* but with [I] = 50 × 10^{-3} *M* and total extent of reaction of 74.4, 72.8, 70.7 and 68.3%, respectively. [h] Same as *e* but with extent of reaction of 64.6, 61.3, 66.7 and 63.4%, respectively. [i] Same as *e* but with [*I*] = 5 × 10^{-3} *M* and extent of reaction of 40.4, 37.8, 47.3 and 44.6%, respectively.

The enzyme-inhibitor dissociation constants obtained from the two series of experiments are given in Table III where they are compared with the previously determined values.[4-6,8] It will be seen that in general the values of K_I obtained in this study are in reasonable agreement with those reported earlier. Where significant differences exist the more recent values are to be preferred.

TABLE III

SUMMARY OF ENZYME-INHIBITOR DISSOCIATION CONSTANTS

Competitive inhibitor	K_I × 10^3, *M*: In 0.02 *M* THAM H + N[a]	In 0.02 *M* THAM This study	In 0.1 *M* phosphate This study	In 0.1 *M* phosphate N + G[b]
β-(β-Indole)-propionate[c]	15 ± 3	11 ± 1.5	4.0 ± 0.4	2.5
β-(β-Indole)-propionamide	2.3 ± 0.4	1.7 ± 0.3	1.8 ± 0.2	..
Phenylacetate[c]	200 ± 50	170 ± 15	60 ± 5	42
Phenylacetamide	15 ± 3	10 ± 1	11 ± 1	..
Tryptamine[d]	2.3 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	..
Acetyl-L-tryptophanate[e]	8.0 ± 1.0[f]	.....	7.3 ± 0.3[f]	..
Acetyl-L-tyrosinate[e]	110 ± 30[f]	80 ± 20[f]	80 ± 20[f]	..

[a] Value of Huang and Niemann.[4-6] [b] Value of Neurath and Gladner[8]. [c] Present as the potassium salt. [d] Present as the hydrochloride. [e] Formed from the amide in the reaction mixture. [f] Actually K_P values, see ref. 9.

From the constants given in Table III we may conclude as before[4-6] that the K_I values of the two bifunctional uncharged competitive inhibitors, *i.e.*, β-(β-indole)-propionamide and phenylacetamide, and the single bifunctional cationic competitive inhibitor, *i.e.*, tryptammonium ion, for aqueous systems at 25° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM component of a THAM–HCl buffer or 0.1 *M* in the phosphate component of a potassium phosphate buffer, are identical within the limits of experimental error. In contrast to the above situation it is again seen[4-6] that the K_I values of the two bifunctional anionic competitive inhibitors, *i.e.*, β-(β-indole)-propionate and phenylacetate, in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM component of a THAM–HCl buffer are substantially greater than those observed in comparable systems 0.1 *M* in the phosphate component of a potassium phosphate buffer. While the K_I values observed in this study, *i.e.*, 11 ± 1.5 and 4.0 ± 0.4 × 10^{-3} *M* and 170 ± 15 and 60 ± 5 × 10^{-3} *M*, lead to differences which are but *ca.* 3-fold in both cases and are thus but *ca.* one-half of the 5–6 fold differences inferred from the earlier values of K_I[4-6,8] there can be no doubt that the observed differences are real and that they suggest a basic difference in the behavior of this class of competitive inhibitors in THAM–HCl and potassium phosphate buffers of *p*H 7.9 ± 0.1. Furthermore, with the above difference in the behavior of two representative bifunctional anionic competitive inhibitors of α-chymotrypsin in 0.02 *M* THAM–HCl and 0.1 *M* potassium phosphate buffers of *p*H 7.9 ± 0.1, which has now been verified by a new set of observations, we can reaffirm our earlier conclusion[9] that the different behavior of bi- and trifunctional anionic competitive inhibitors of this enzyme, with respect to the dependence or lack of dependence of the values of K_I upon the nature of the buffer components in two specified buffers, *cf.* Table III, provides an experimental basis for recognizing bi- and trifunctionality in anionic competitive inhibitors of the type $RCH_2CO_2^-$ and $R'CONHCHRCO_2^-$.

From the observations noted above it is not clear whether the different behavior of β-(β-indole)-propionate and of phenylacetate in 0.02 *M* THAM–HCl and 0.1 *M* potassium phosphate buffers of *p*H 7.9 ± 0.1 is due to a general ionic strength and/or a specific ion effect. To answer this question the inhibition of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 by potassium β-(β-indole)-propionate and by potassium phenylacetate was examined in systems containing varying amounts of the THAM–HCl and/or potassium phosphate buffer components and in one case added sodium chloride, *cf.* Table IV.

A summary of all available information relative to the dependence of values of K_I for β-(β-indole)-propionate and for phenylacetate upon the composition of the reaction system is presented in Table V. Examination of these data and of the plot presented in Fig. 1 indicates that there are at least two factors that appear to have an influence on values of K_I for the bifunctional anionic competi-

TABLE IV

K_I VALUES OF POTASSIUM β-(β-INDOLE)-PROPIONATE AND OF POTASSIUM PHENYLACETATE IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1 AS A FUNCTION OF THE NATURE AND CONCENTRATION OF SEVERAL BUFFER COMPONENTS[a]

Competitive inhibitor[c]	K_I[d]	E_I'[e]	I_I'
β-(β-Indole)-propionate[f]	7.6 ± 0.4	0.75	1.32
β-(β-Indole)-propionate[g]	4.7 ± 0.4	1.2	2.1
β-(β-Indole)-propionate[h]	7.5 ± 0.5	0.76	1.33
β-(β-Indole)-propionate[i]	8.0 ± 0.3	0.71	1.25
β-(β-Indole)-propionate[j]	7.6 ± 0.4	0.75	1.32
β-(β-Indole)-propionate[k]	6.6 ± 0.4	0.86	1.52
β-(β-Indole)-propionate[l]	5.1 ± 0.3	1.12	1.96
β-(β-Indole)-propionate[m]	3.2 ± 0.5	1.97	3.1
β-(β-Indole)-propionate[n]	2.5 ± 0.3	2.28	4.0
Phenylacetate[o]	110 ± 10	0.052	1.36

[a] Evaluated against acetyl-L-tyrosinamide with K_S = 32 × 10^{-3} *M*, [E] = 5.7 × 10^{-6} *M*[b], E_S' = 0.17 × 10^{-2} and S_S' = 0.625 to 1.25 unless otherwise noted. [b] Based upon an assumed molecular weight of monomeric α-chymotrypsin of 22,000 and a nitrogen content of 16.0%. [c] Evaluated with $[S]_0$ = 20, 25, 30 and 40 × 10^{-3} *M*, [I] = 10 × 10^{-3} *M*, *t* = 150 minutes with a total of 7 observations of extent of reaction within this time interval and with [E] = 0.200 mg. of protein-nitrogen per ml. of Armour preparation no. 00592 unless otherwise noted. [d] In units of 10^{-3} *M*. [e] In units of 10^{-2}. [f] System 0.4 *M* in the THAM component of a THAM-HCl buffer with total extent of reaction of 60.0, 57.5, 56.3 and 52.1%, respectively. [g] System 0.4 *M* in the THAM component of a THAM-HCl buffer, 0.4 *M* in added sodium chloride with total extent of reaction of 65.6, 62.7, 61.3 and 58.9%, respectively. [h] System 0.4 *M* in the THAM component of a THAM-HCl buffer, 0.0001 *M* in the phosphate component of a potassium phosphate buffer with total extent of reaction of 60.3, 57.9, 55.0 and 53.1%, respectively. [i] System 0.4 *M* in the THAM component of a THAM-HCl buffer, 0.001 *M* in the phosphate component of a potassium phosphate buffer with total extent of reaction of 62.1, 59.5, 57.5 and 54.4%, respectively. [j] System 0.27 *M* in the THAM component of a THAM-HCl buffer, 0.01 *M* in the phosphate component of a potassium phosphate buffer with total extent of reaction of 59.3, 57.2, 56.0 and 52.9%, respectively. [k] System 0.2 *M* in the THAM component of a THAM-HCl buffer, 0.04 *M* in the phosphate component of a potassium phosphate buffer with total extent of reaction of 58.0, 55.8, 52.2 and 51.8%, respectively. [l] System 0.0725 *M* in the phosphate component of a potassium phosphate buffer with total extent of reaction of 53.8, 52.1, 50.8 and 47.8%, respectively. [m] System 0.2 *M* in the phosphate component of a potassium phosphate buffer with total extent of reaction of 50.0, 47.6, 57.7 and 54.4%, respectively, and with $[S]_0$ = 5, 10, 30 and 40 × 10^{-3} *M*, [E] = 0.222 mg. protein-nitrogen per ml., *i.e.*, [E] = 6.3 × 10^{-5} *M*[b], E_S' = 0.2 × 10^{-2}, S_S' = 0.16 to 1.25, *t* = 110 minutes for values of $[S]_0$ = 5 and 10 × 10^{-3} *M* and 150 minutes for values of $[S]_0$ = 30 and 40 × 10^{-3} *M*. [n] System 0.4 *M* in the phosphate component of a potassium phosphate with total extent of reaction of 58.1, 56.2, 57.7 and 53.0% buffer, respectively. [o] System 0.3 *M* in the THAM component of a THAM-HCl buffer with total extent of reaction of 66.0, 65.3, 61.8 and 58%, respectively, and with [I] = 150 × 10^{-3} *M*.

tive inhibitors considered in this study. That one factor may be ionic strength and the other the nature of the ion species is seen from the observation that while an increase in ionic strength associated with an increasing concentration of the THAM–HCl buffer components causes a measurable decrease in the enzyme-inhibitor dissociation constants of this class of competitive inhibitors, the presence of significant amounts of potassium phosphate either alone or in the above buffer system produces a still greater decrease in the value

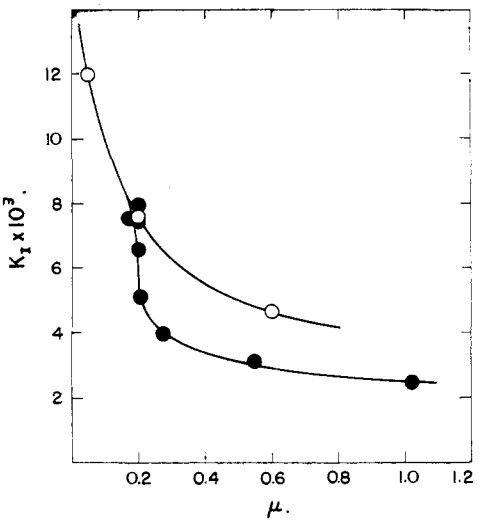

Fig. 1.—Effect of ionic environment upon the enzyme-inhibitor dissociation constants of β-(β-indole)-propionate; open circles THAM–HCl or THAM–HCl and sodium chloride, filled circles potassium phosphate with or without THAM–HCl.

of K_I when the comparisons are made for systems of comparable ionic strength.

TABLE V

EFFECT OF IONIC ENVIRONMENT UPON THE ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF TWO BIFUNCTIONAL ANIONIC COMPETITIVE INHIBITORS

Competitive inhibitor	THAM concn.[a]	Phos. concn.[b]	μ[c]	K_I[d]
β-(β-Indole-propionate[e]	0.02	0.00	0.05	11 ± 1.5
	.40	.00	.20	7.6 ± 0.4
	.40	.00	.60[f]	4.7 ± .4
	.40	.0001	.20	7.5 ± .5
	.40	.001	.20	8.0 ± .3
	.27	.01	.17	7.6 ± .4
	.20	.04	.20	6.6 ± .4
	.00	.0725	.20	5.1 ± .3
	.00	.10	.28	4.0 ± .4
	.00	.20	.55	3.2 ± .5
	.00	.40	1.09	2.5 ± .3
Phenylacetate[e]	.02	.00	0.16	170 ± 15
	.30	.00	.30[g]	110 ± 10
	.00	.10	.30[g]	60 ± 5

[a] Molar concentration of the THAM component of a THAM-HCl buffer of *p*H 7.9 ± 0.1 at 25°. [b] Molar concentration of the phosphate component of a potassium phosphate buffer at *p*H 7.9 ± 0.1 at 25°. [c] Ionic strength. [d] In units of 10^{-3} *M* for aqueous solutions at 25° and *p*H 7.9 ± 0.1. [e] Present as the potassium salt. [f] 0.4 *M* sodium chloride also present. [g] 0.15 *M* in potassium phenylacetate.

In passing from a system in which the ionic strength was 0.05 in the presence of a 0.02 *M* THAM–HCl buffer to one in which the ionic strength was 0.28 in the presence of a 0.1 *M* potassium phosphate buffer the value of K_I for potassium β-(β-indole)-propionate decreased from 11 ± 1.5 × 10^{-3} *M* to 4.0 ± 0.4 × 10^{-3} *M*, *i.e.*, *ca.* 64%. Similarly, in passing from a system in which the ionic strength was 0.16 in the presence of a 0.02 *M* THAM–HCl buffer to one in which the ionic strength was 0.3 in the presence of a 0.1 *M*

potassium phosphate buffer the value of K_I for potassium phenylacetate decreased from 170 ± 15 × 10^{-3} M to 60 ± 5 × 10^{-3} M, *i.e.*, *ca.* 65%. From these limited data it is not possible to decide whether the extent to which a value of K_I may be influenced by a change in ionic species or concentration is, or is not, independent of the specific nature of the bifunctional anionic competitive inhibitor.

The extent to which a value of K_I for a given inhibitor may be altered by a change in ionic strength and by a change in ion species may be seen from the experiments conducted with potassium phenylacetate. With this bifunctional anionic competitive inhibitor in systems containing THAM–HCl but no potassium phosphate an increase in the concentration of the THAM–HCl buffer components corresponding to an increase in ionic strength from 0.16 to 0.3 caused a decrease in the value of K_I from 170 ± 15 × 10^{-3} M to 110 ± 10 × 10^{-3} M, *i.e.*, *ca.* 35%, corresponding to a decrease in $-\Delta F^0$ of *ca.* 320 cal. per mole. However, in passing from a system containing the THAM–HCl buffer components in an amount corresponding to an ionic strength of 0.16 to one containing the potassium phosphate buffer components in an amount corresponding to an ionic strength of 0.3 the value of K_I was observed to decrease from 170 ± 15 × 10^{-3} M to 60 ± 5 × 10^{-3} M, *i.e.*, *ca.* 65%, corresponding to a decrease in $-\Delta F^0$ of *ca.* 670 cal. per mole. Thus, the presence of the 0.1 M potassium phosphate buffer components caused a decrease in $-\Delta F^0$, in addition to that associated with an increase in ionic strength caused by an increase in the concentration of the THAM–HCl buffer components, of *ca.* 350 cal. per mole.

Since there is reason to believe that there are one or more negatively charged groups at or near the catalytically active site of α-chymotrypsin, when this enzyme is present in aqueous solutions at 25° and *p*H 7.9 ± 0.1,[4-8,16,17] an increase in the ionic strength of the reaction medium would be expected to facilitate the approach of a negatively charged anionic competitive inhibitor to the negatively charged region of the catalytically active site of the enzyme. Thus, since combination would be enhanced the value of K_I would be reduced. While this effect has been observed with the two bifunctional anionic competitive inhibitors considered in this study the fact that the enzyme-inhibitor dissociation constants of the two trifunctional anionic competitive inhibitors that have been examined to date[9] appear to be uninfluenced by the presence of substantial amounts of added potassium phosphate[9] or of calcium chloride[17] suggests that even with what may be regarded, as a first approximation, as an ionic strength effect associated with an increased dielectric constant of the reaction medium there is a striking difference in the nature of the interaction of α-chymotrypsin with bifunctional and trifunctional anionic competitive inhibitors. It is not obvious at the present time as to why the K_I values of trifunctional anionic competitive inhibitors of α-chymotrypsin should be independent of the ionic strength of the reaction system particularly since it is known that the apparent protonation of one or more of the negatively charged groups which are present in the region of the catalytically active site of α-chymotrypsin does result in an expected decrease in the values of K_I for both bi- and trifunctional anionic competitive inhibitors of this enzyme.[5,17,18]

With regard to the possible nature of the specific ion effect noted earlier it may be assumed that the interaction of α-chymotrypsin with a bifunctional anionic competitive inhibitor, in aqueous solutions at 25° and *p*H 7.9 ± 0.1 in the absence of potassium phosphate, proceeds, in large part, *via* a R_2–ρ_2 interaction[19] with the R_3–ρ_3 interaction[19] being either small or of a negative character particularly in systems of low ionic strength. Thus, the principal attractive forces leading to combination of the enzyme with a bifunctional anionic competitive inhibitor of the type $R_2CH_2R_3$, where R_3 is a carboxylate group, would arise from a one center, *i.e.*, a R_2–ρ_2, interaction. However, if it is also assumed that there is a region of the active site of the enzyme, adjacent to the ρ_3 center, that is receptive to interaction with phosphate[20] then in the presence of phosphate and upon the approach of a bifunctional anionic competitive inhibitor a R_2–ρ_2 interaction would occur and the R_3 group, *i.e.*, the carboxylate group, would attempt to make its usual R_3–ρ_3 approach. However, the presence of combined phosphate near the ρ_3 center of the active site would provide an additional negative charge which would repel the negatively charged carboxylate group more effectively than would the negatively charged group or groups which are normally present at the catalytically active site and the inhibitor would be forced from its normal modes of combination into alternate modes of combination wherein the R_3 group could contribute more effectively to the total binding energy either in a positive or in a less negative sense. Thus phosphate combined with the site would supply the energy required to consummate alternative and more effective modes of combination which are denied to bifunctional anionic competitive inhibitors in the absence of phosphate. Presumably these alternative modes of combination would be unavailable to the trifunctional anionic competitive inhibitors because of steric reasons and thus this class of competitive inhibitors would be unaffected by the presence of phosphate in the reaction system.

For the case of immediate interest the interaction of enzyme, specific substrate and bifunctional anionic competitive inhibitor in the presence of phosphate may, in the first instance, be represented by equations 1 to 7 inclusive where B is taken to be the combining phosphate species. It is not necessary to consider equations such as $E_f + B_f \rightleftarrows EB$ since this implies competitive inhibition by B and it

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

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

(18) R. R. Jennings, Ph.D. Thesis, California Institute of Technology, Pasadena, California, 1954.

(19) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

(20) The term "phosphate" is used here in its broadest sense. While it is possible that $HPO_4^=$ may be the active species this point cannot be established because of restrictions arising from the necessity of maintaining the reaction systems at a definite *p*H, *i.e.*, 7.9 ± 0.1.

is known[9] that this situation does not obtain. It is also appropriate to recall[9] that the constant $K_S = (k_2 + k_3)/k_1$ is essentially independent of the ionic strength and of the concentration of B.

$$E_f + S_f \rightleftarrows ES \xrightarrow{k_3} E + P_{1f} + P_{2f} \quad (1)$$

$$E_f + I_f \rightleftarrows EI \quad (2)$$

$$B_f + E_f \rightleftarrows BE \quad (3)$$

$$B_f + ES \rightleftarrows BES \xrightarrow{k_3'} BE + P_{1f} + P_{2f} \quad (4)$$

$$BE + S_f \rightleftarrows BES \xrightarrow{k_3'} BE + P_{1f} + P_{2f} \quad (5)$$

$$BE + I_f \rightleftarrows BEI \quad (6)$$

$$B + EI \rightleftarrows BEI \quad (7)$$

If it is assumed that all equilibria are attained rapidly relative to the rate of appearance of the reaction products and that [S] >> [E], [B] > [E], $[S_f] \doteq [S]$; $[I_f] \doteq [I]$, $[B_f] \doteq [B]$, $d[ES]/dt \doteq 0$ and $d[BES]/dt \doteq 0$ then with $K_S = [E][S]/[ES]$, K_I^0[21] $= [E][I]/[EI]$, $K_{BE} = [B][E]/[BE]$, $K_{BES} = [B][ES]/[BES]$, $K_{BES'} = [BE][S]/[BES]$, $K_{BEI} = [BE][I]/[BEI]$ and $K_{BEI'} = [B][EI]/[BEI]$ it can be shown that $K_S K_{BES} = K_{BE} K_{BES'}$, $K_I^0 K_{BEI'} = K_{BE} K_{BEI}$ and $K_{BE} = K_S K_{BES}/K_{BES'} = K_I^0 K_{BEI'}/K_{BEI}$. From the relations $[E_f] = [ES]K_S/[S] = ([E] - [ES] - [EI] - [BE] - [BES] - [BEI])$ and $v_0 = -d[S]/dt = (k_3[ES] + k_{3'}[BES]) = (k_3 + k_{3'}[B]/K_{BES})[ES]$ we may derive equations 8, 9 and 10.

$$v_0 = \{(k_3 + k_3'[B]/K_{BES})[E]\}/\{1 + (K_S/[S])[1 + [I]/K_I^0 + [B]/K_{BE} + [B][I]/K_{BEI'}K_I^0] + [B]/K_{BES}\} \quad (8)$$

$$K_{BE} = [B]/\{[([E]/v)(k_3 + k_3'[B]/K_{BES}) - 1 - [B]/K_{BES}][[S]/K_S] - 1 - [I]/K_I^0 - [B][I]/K_{BEI'}K_I^0\} \quad (9)$$

$$K_{BEI'} = [B][I]/\{K_I^0\{[([E]/v)(k_3 + k_3'[B]/K_{BES}) - 1 - [B]/K_{BES}][[S]/K_S] - 1 - [I]/K_I^0 - [B]/K_{BE}\}\} \quad (10)$$

Since values of K_S are unaffected by the presence of B in the reaction system one is led to two conclusions, *i.e.*, either K_{BE} and K_{BES} are infinitely large relative to K_S and K_I^0 and hence there is little or no BE and BES formed, or K_S is equal to $K_{BES'}$ and there effectively is no difference between the path by way of ES to products and by way of BES to products. If one examines the latter case and assumes that K_S is equal to $K_{BES'}$ and that k_3 is equal to $k_{3'}$[22] then from the relation $K_{BE} = K_S K_{BES}/K_{BES'}$, K_{BE} may be considered as being equal to K_{BES}. Substituting K_{BE} for K_{BES} in equation 9 and solving for K_{BE} leads to equation 11. An approximate evaluation of equation 11, insofar

$$K_{BE} = \{[B][k_3 - (v/[E])[(K_S/[S]) + 1]]\}/\{-k_3 + (v/[E])[1 + (K_S/[S])(1 + [I]/K_I^0 + [B][I]/K_{BEI'}K_I^0)]\} \quad (11)$$

as the experimental data permitted, indicated that values of K_{BE} will be extremely large relative to values of K_S and K_I^0. Thus, it may be assumed as a reasonable approximation that values of K_{BE}, K_{BES} and K_{BEI} will be so large that they may be ignored for the case at hand. If the validity of this assumption is granted we may regard equations 3, 5 and 6 as being of no practical significance and represent the situation under discussion by equations 1, 2, 4 and 7 where equations 1 and 4 are to be regarded as being equivalent, *vide ante*. It is now possible to simplify equation 8 and to obtain equation 12 which may then be solved for $K_{BEI'}$ as in equation 13.

$$v_0 = k_3[E]/\{1 + (K_S/[S])(1 + [I]/K_I^0 + [I][B]/K_I^0 K_{BEI'}\} \quad (12)$$

$$K_{BEI'} = [B]/\{(K_I^0/[I])\{(k_3[E][S]/v) - [S](1/K_S) - 1 - [I]/K_I^0\}\} \quad (13)$$

The experimental data obtained for the system α-chymotrypsin–acetyl-L-tyrosinamide–β-(β-indole)-propionate in the presence of varying amounts of potassium phosphate in systems of comparable ionic strength, *cf.* Table V, were then evaluated with the aid of equation 13 and the known values for the various kinetic constants. A value of $K_{BEI'} = 150 \pm 50 \times 10^{-3}$ *M* was obtained.

It should be noted that meaningful values of $K_{BEI'}$ cannot be obtained for low values of B because the anticipated small differences in values of v_0 would be well within the limits of error associated with the evaluation of K_I^0. It will also be noted that the value of $K_{BEI'}$ is large relative to values by K_I^0. Consequently, contributions of BEI will be quite small for low values of B. This is in agreement with the experimental observations given in Table V.

It is now possible to relate changes in the values of K_I to changes in the concentration of B. In the absence of inhibition by the hydrolysis products, *i.e.*, for initial rates, the velocity expression is given by equation 14. From equation 12 we may obtain equation 15. By equating equations 14 and 15 and solving for K_I we may obtain equation 16.

$$v_0 = k_3[E][S]_0/\{K_S(1 + [I]/K_I) + [S]_0\} \quad (14)$$

$$v_0 = k_3[E][S]/\{K_S(1 + [I]/K_I^0 + [I][B]/K_I^0 K_{BEI'} + [S]\} \quad (15)$$

$$K_I = K_I^0 + [B]/K_{BEI'} \quad (16)$$

Equation 16 yields values of K_I which are in reasonable agreement with the experimentally determined values of K_I, *cf.* Table VI. Thus it may be seen that for the case at hand the argument given above provides a satisfactory description of the inhibition of the α-chymotrypsin catalyzed hydroly-

TABLE VI

ENZYME-INHIBITOR DISSOCIATION CONSTANTS FOR AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1

Inhibitor	[B][a]	K_I[b] Expt.	K_I[b] Calcd.[c]
β-(β-Indole)-propionate	0.00	7.6 ± 0.4	7.6
	.0001	7.5 ± .5	7.6
	.001	8.0 ± .3	7.6
	.01	7.6 ± .4	7.1
	.04	6.6 ± .4	6.0
	.0725	5.1 ± .3	5.1
	.10	4.0 ± .4	4.5
	.20	3.2 ± .5	3.3
	.40	2.5 ± .3	2.1
Phenylacetate	.1	60 ± 5	65

[a] [B] = total concentration of phosphate in *M*. [b] In units of 10^{-3} *M*. [c] From equation 16 and $K_I^0 = 7.6 \times 10^{-3}$ *M*.

(21) K_I^0 is herein defined as the value of K_I observed in the absence of B but in systems in which the ionic strength is maintained at a level equivalent to those in which K_I is evaluated in the presence of varying amounts of B.

(22) There is at present no information available as to the effect of varying amounts of B upon k_3 at constant ionic strength.

sis of acetyl-L-tyrosinamide by two representative bifunctional anionic competitive inhibitors in aqueous solutions at 25° and *p*H 7.9 ± 0.1 in the presence of varying amounts of potassium phosphate.

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Experimental[23]

Specific Substrate and Competitive Inhibitors.—Acetyl-L-tyrosinamide, colorless needles, m.p. 226–228°, $[\alpha]^{24}D$ + 51.8° (*c* 0.8%, in water) was prepared as described previously.[9] β-(β-Indole)-propionic acid, colorless needles, m.p. 133–134°, was recrystallized twice from a mixture of water and methanol. β-(β-Indole)-propionamide, fine, stunted colorless needles, m.p. 205–207°, was prepared as described previously.[4] Phenylacetic acid, shiny platelets, m.p. 77–78°, was recrystallized three times from a mixture of ethanol and water. Phenylacetamide, short, colorless needles, m.p. 157–158°, was prepared as described previously.[4] Tryptamine hydrochloride, short dense colorless prisms, m.p. 250–251°, was recrystallized twice from aqueous 5 *N* hydrochloric acid.

Enzyme Experiments.—The analytical procedure described previously[9] was employed without modification. The anionic competitive inhibitors were introduced into the reaction mixtures in the form of their potassium salts. The buffer compounds were prepared as before.[9] Crystalline bovine α-chymotrypsin Armour lot no. 00592 was employed in all experiments. The primary data were evaluated as before.[9]

(23) All melting points reported are corrected.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE, AND THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

The Availability of the Disulfide Bonds of Human and Bovine Serum Albumin and of Bovine γ-Globulin to Reduction by Thioglycolic Acid

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The extent of reduction of the disulfide bonds of human and bovine serum albumin and of bovine γ-globulin by thioglycolic acid at different *p*H values was investigated. Upon reduction human and bovine serum albumin acquired a maximum of one thiol group per molecule in the *p*H range 5.0 to 7.0. Beyond this range an increasing number of disulfide bonds became susceptible to reduction. In the case of bovine γ-globulin a maximum of one thiol group per molecule appeared upon reduction in the range of *p*H 3.5 to 5.0. With an increase in *p*H an increasing number of disulfide bonds could be reduced. The effect of *p*H on the number of disulfide bonds available for reduction was found to be reversible for bovine serum albumin and for bovine γ-globulin in the *p*H ranges of 1.2 to 10.2 and 5.0 to 10.2, respectively. In the three proteins investigated reduction of most of the disulfide bonds could be effected at *p*H values above 10 in the presence of guanidine. The conversion of unreactive disulfide bonds into reactive ones is explained as being due to changes in the configuration of the protein molecule, which may be either reversible or irreversible ones.

In contrast to the extensive literature dealing with the chemical reactivity of the thiol groups of native and denatured proteins,[1] only a few investigations on the reactivity of the disulfide groups of proteins have been published. Most of the work on the chemical reactivity of protein disulfide groups is concerned with their reduction which can be performed under mild experimental conditions. Reductions of the disulfide bonds of insulin,[2] keratin[3] and lactogenic hormone[4] have been reported. In the case of egg and serum albumin[5] and insulin[6] it was demonstrated that complete reduction of the disulfide bonds by thioglycolic acid can only occur after denaturation. The number of detectable –SH and S–S groups were found by Mirsky and Anson[7] to be closely linked with the extent of denaturation. On reversal of denaturation the number of detectable S–S groups decreased.

In the present article the reduction of the disulfide bonds of human and bovine serum albumin and of bovine γ-globulin by thioglycolic acid at different *p*H values will be described. The results obtained suggest that reversible as well as irreversible changes in the configuration of these proteins may occur.

Experimental

Materials.—**Human serum albumin** was prepared according to Cohn, *et al.*,[8] and crystallized three times in the presence of decanol. Its thiol content, determined by titration with methyl mercury nitrate (see below), was about 0.3 mole –SH per mole of albumin, assuming 65,000 as the molecular weight of the protein.[9] For the discussion below the value of 17 cystine residues per serum albumin molecule reported by Hughes[9] will be accepted.

Mercaptalbumin.—Mercury–mercaptalbumin dimer was prepared according to Hughes[10,11] and recrystallized five times before use. A pure aqueous solution of mercaptalbumin monomer was obtained from the mercury dimer according to the procedure of Dintzis.[12,13] Titration of the resulting concentrated mercaptalbumin solution with

(1) *Cf.* E. S. G. Barron, *Advances in Enzymology*, **11**, 201 (1951); H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944); F. W. Putnam, H. Neurath and K. Bailey, Editors, "The Proteins," Vol. IB, Academic Press, Inc., New York, N. Y., 1954, p. 807.

(2) V. du Vigneaud, A. Fitch, E. Pekarek and W. W. Lockwood, *J. Biol. Chem.*, **94**, 233 (1931); K. G. Stern and A. White, *ibid.*, **117**, 95 (1937).

(3) D. R. Goddard and L. Michaelis, *ibid.*, **112**, 361 (1935).

(4) H. Fraenkel-Conrat, M. E. Simpson and H. M. Evans, *ibid.*, **142**, 107 (1942); H. Fraenkel-Conrat, *ibid.*, **142**, 119 (1942).

(5) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **18**, 307 (1935).

(6) H. Lindley, THIS JOURNAL, **77**, 4927 (1955).

(7) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **19**, 427 (1936).

(8) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(9) *Cf.* W. L. Hughes; H. Neurath and K. Bailey, Editors, "The Proteins," Vol. IIB, Academic Press, Inc., New York, N. Y., 1954, p. 663.

(10) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947).

(11) W. L. Hughes, Jr., *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 79 (1949).

(12) H. M. Dintzis, Thesis, Harvard University, 1952.

(13) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).