suspension. The mixture is centrifuged after having stood several hours, with frequent stirring. The paste is resuspended in 40% ethanol, containing 5×10^{-4} M cysteine (or dialyzed against a buffer containing cysteine at this concentration), and finally dialyzed against a convenient buffer in order to render the preparation alcohol- and salt-free.

Acknowledgments.—I am indebted to the late Professor E. J. Cohn and to Professors J. L. Oncley and J. T. Edsall for helpful discussion and advice; and especially to Dr. W. L. Hughes, Jr., whose constant guidance and suggestions have been indispensable in carrying out this research.

BOSTON, MASSACHUSETTS

[CONTRIBUTION NO. 1870 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of L-Tyrosinhydroxamide in Aqueous Solutions at 25° and *p*H 6.9[1]

BY ROBERT J. FOSTER, ROBERT R. JENNINGS AND CARL NIEMANN[2]

RECEIVED JANUARY 4, 1954

The optimum *p*H for the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide in aqueous solutions at 25° has been found to be 6.95 ± 0.05. For the system α-chymotrypsin–L-tyrosinhydroxamide, in aqueous solutions 0.2 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, at 25° and *p*H 6.9, the value of $K_S = 41 \pm 2 \times 10^{-3}$ M and the value of $k_3 = 3.6 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. D-Tyrosinhydroxamide has been found to function as a competitive inhibitor in the above system and under the above conditions $K_I = 40 \pm 8 \times 10^{-3}$ M. An explanation is offered to account for the fact that the *p*H optimum for the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide lies in a more acid region than that of acetyl-L-tyrosinhydroxamide, or of acetyl-L-tyrosinamide, and for the fact that the value of k_3 for L-tyrosinhydroxamide is substantially less than that of acetyl-L-tyrosinhydroxamide at their respective *p*H optima.

The α-chymotrypsin catalyzed hydrolysis of L-phenylalaninamide, and of L-tyrosinamide, in aqueous solutions at 25° and *p*H 7.8, was first reported by Fruton and Bergmann[3] in 1942, and seven years later Kaufman and Neurath[4] found that L-tyrosine ethyl ester, in 30% aqueous methanol solutions at 25° and *p*H 7.8, was also slowly hydrolyzed in the presence of the same enzyme. In the following year Balls and his co-workers[5,6] noted that the apparent rate of the α-chymotrypsin catalyzed hydrolysis of L-tyrosine ethyl ester, in aqueous solutions at 25°, was approximately seven times faster at *p*H 6.25 than at *p*H 7.8, and that the optimum *p*H for this system was 6.25. At about the same time the Goldenbergs[7] reported that the apparent rate of the α-chymotrypsin catalyzed hydrolysis of L-phenylalanine ethyl ester, in aqueous solutions at 25°, was approximately nine times faster at *p*H 6.4 than at *p*H 7.85 and that the optimum *p*H for this system was 6.4 when determined with one analytical procedure and approximately 6.5 when determined with another. In a second communication[8] it was noted that the optimum *p*H for the α-chymotrypsin catalyzed hydrolysis of L-leucine ethyl ester, in aqueous solutions at 25°, also appeared to be dependent upon the analytical procedure used being 6.8 in one instance and 7.2–7.3 in another.

While there is no doubt that the optimum *p*H for the α-chymotrypsin catalyzed hydrolysis of L-tyrosine ethyl ester, in aqueous solutions at 25°, lies in a lower *p*H region than that observed for the comparable hydrolysis of acetyl-L-tyrosine ethyl ester[6] it is clear that there is need for considerably more data relative to the behavior of specific substrates containing α-amino, or α-ammonium, groups than is now available.

Since the hydrolysis of hydroxamides can be followed by a convenient and precise colorimetric procedure[9] and because data are available with respect to the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide in aqueous solutions at 25° and *p*H 7.6,[10,11] *i.e.*, the optimum *p*H for this system, we have, in this instance, determined the kinetics of the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide in aqueous solutions 0.2 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and at 25° and the optimum *p*H for this system. In the determination of the optimum *p*H of the above system two different types of buffers were employed, one, a 0.1 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and the other, a cacodylic acid–sodium cacodylate buffer 0.1 M in arsenic introduced as cacodylic acid.

It will be seen from the data presented in Fig. 1 that the dependence of the relative activity of the system under investigation upon *p*H can be represented by a fairly symmetrical curve with a maximum in the region of *p*H 6.9–7.0. Thus, in contrast to L-tyrosine ethyl ester and acetyl-L-tyrosine ethyl ester, where the difference in their respective *p*H optima is reported to be approximately 1.6 *p*H units,[6] the difference in the *p*H optima of L-tyrosin-

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

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

(3) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **145**, 253 (1942).

(4) S. Kaufman and H. Neurath, *Arch. Biochem.*, **21**, 437 (1949).

(5) E. F. Jansen, M. D. Fellows-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **185**, 209 (1950).

(6) E. F. Jansen, A. L. Curl and A. K. Balls, *ibid.*, **189**, 671 (1951).

(7) H. Goldenberg and V. Goldenberg, *Arch. Biochem.*, **29**, 154 (1950).

(8) H. Goldenberg, V. Goldenberg and A. D. McLaren, *Biochem. Biophys. Acta*, **7**, 110 (1950).

(9) B. M. Iselin, H. T. Huang and C. Niemann, *J. Biol. Chem.*, **183**, 403 (1950).

(10) D. S. Hogness and C. Niemann, THIS JOURNAL, **75**, 884 (1953).

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

hydroxamide and acetyl-L-tyrosinhydroxamide is less than one-half of the above value, *i.e.*, is only 0.6–0.7 of a *p*H unit. The reason for this rather striking difference in the behavior of comparable esters and hydroxamides is not immediately apparent.

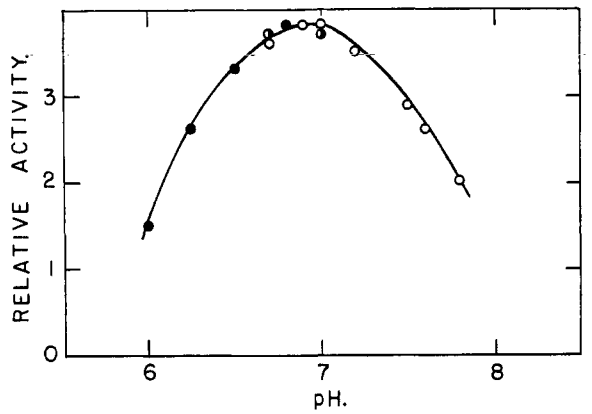

Fig. 1.—Relative activity *vs.* *p*H; $[S]_0 = 5 \times 10^{-3}$ *M* L-tyrosinhydroxamide; [E] = 0.104 mg. protein-nitrogen/ml. of α-chymotrypsin; $t = 25°$; ○, 0.1 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer; ●, 0.1 *M* cacodylic acid–sodium cacodylate buffer; ◑, 0.1 *M* tris-(hydroxymethyl)-aminomethane–cacodylic acid buffer.

It was expected from previous studies[10] that the initial stages of the α-chymotrypsin catalyzed hydrolysis of L-tryosinhydroxamide, under the conditions specified, would be described by equation 1 and, provided that E's < 0.1, that the rate of disappearance of the specific substrate would be given by equation 2 where $K_S = (k_2 + k_3)/k_1$.

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (1)$$

$$-d[S]/dt = k_3[E][S]/(K_S + [S]) \quad (2)$$

The initial velocities were determined, for various initial specific substrate concentrations and a constant enzyme concentration, from both zero and first-order plots using the method described by Jennings and Niemann[12] and the data so obtained are presented in Fig. 2 in the form of a v_0 *vs.* $v_0/[S]_0$ plot.[13–15] From the slope of this plot, which is equal to $-K_S$, and the ordinate intercept, which is equal to $k_3[E]$, it follows that for the system α-chymotrypsin–L-tyrosinhydroxamide in aqueous solutions 0.2 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and at 25° and *p*H 6.9 the value of $K_S = 41 \pm 2 \times 10^{-3}$ *M* and the value of $k_3 = 3.6 \pm 0.2 \times 10^{-3}$ *M*/min./mg. protein-nitrogen/ml.

Since the interpretation of the above kinetic constants requires knowledge of the nature of the substrate species present in aqueous solutions at *p*H 6.9 an attempt was made to determine the apparent dissociation constants of L-tyrosinhydroxamide. Potentiometric titraton of an aqueous solution 10^{-2} *M* in L-tyrosinhydroxamide hydrochloride with 10^{-2} *M* aqueous sodium hydroxide gave the titration curve reproduced in Fig. 3 and when the experimental data were analyzed by the method of Simms[16] three dissociation constants, corresponding to pK'_A values of 7.0, 9.2 and 10.0, respectively, were obtained. On the basis of previous experience[10,17] it is reasonable to assign the value of 10.0 to the ionization of the phenolic hydroxyl group, the value of 9.2 to the ionization of

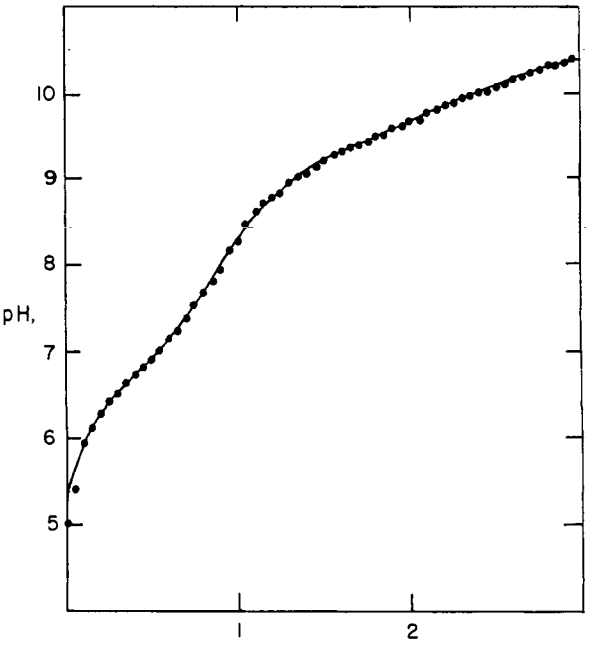

Fig. 2.—Titration of L-tyrosinhydroxamide hydrochloride with aqueous sodium hydroxide: initial concentration 6.67×10^{-3} *M*; final concentration 3.33×10^{-3} *M*; $t = 25°$.

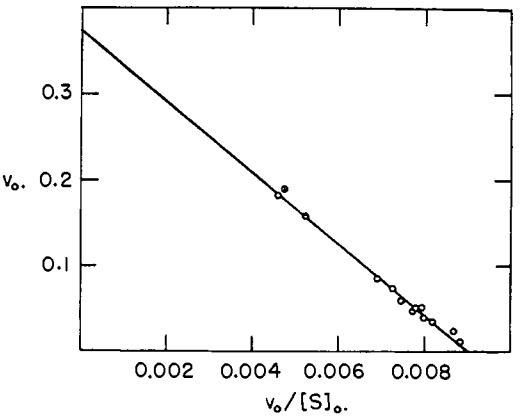

Fig. 3.—α-Chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide in aqueous solutions at 25° and *p*H 6.9: v_0 in units of 10^{-3} *M*/min.; $[S]_0$ in units of 10^{-3} *M*; [E] = 0.104 mg. protein-nitrogen/ml.; 0.2 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

(12) R. R. Jennings and C. Niemann, THIS JOURNAL, **75**, 4687 (1953).
(13) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).
(14) H. J. Hofstee, *Science*, **116**, 329 (1952).
(15) G. S. Eadie, *ibid.*, **116**, 688 (1952).
(16) H. Simms, THIS JOURNAL, **48**, 1239 (1926).
(17) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publ. Corp., New York, N. Y., 1943.

the hydroxamic acid moiety and the value of 7.0 to the ionization of the α-ammonium group. Thus, at the optimum pH for the system α-chymotrypsin–L-tyrosinhydroxamide, under the conditions previously specified, it appears that approximately 50% of the specific substrate is present in the form of a species with a net charge of +1 and 50% in the form of a species with a net charge of zero.

Before comparisons are made of the behavior of L-tyrosinhydroxamide with that of acetyl-L-tyrosinhydroxamide it should be noted that a re-evaluation of the primary experimental data collected by Hogness and Niemann,[10] through the use of the procedure described by Jennings and Niemann,[12] has led to a modest revision of the values of K_S and k_3 for the system α-chymotrypsin–acetyl-L-tyrosinhydroxamide in aqueous solutions 0.3 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and at 25° and pH 7.6. The earlier values of $K_S = 51 \times 10^{-3}$ M and $k_3 = 34 \times 10^{-3}$ M/min./mg. protein–nitrogen/ml.[10] are to be replaced by the revised values of $K_S = 45 \pm 5 \times 10^{-3}$ M and $k_3 = 32 \pm 3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. These latter values are in good agreement with the values of $K_S = 42 \pm 2 \times 10^{-3}$ M and $k_3 = 34 \pm 2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. reported by Foster and Niemann[11] on the basis of a series of independent experiments.

From the known kinetic constants of L-tyrosinhydroxamide and of acetyl-L-tyrosinhydroxamide it is seen that at the respective pH optima for the α-chymotrypsin catalyzed hydrolysis of these two specific substrates, the values of K_S are identical, within the limits of experimental error, whereas the value of k_3 of acetyl-L-tyrosinhydroxamide is approximately 10 times greater than that of L-tyrosinhydroxamide. While it is not possible, at the present time, to provide a rigorous explanation of the near identity of the K_S values of L-tyrosinhydroxamide and of acetyl-L-tyrosinhydroxamide, under the conditions previously specified, it is possible to offer a probable explanation of the differing pH optima of these two specific substrates and the lesser k_3 value of L-tyrosinhydroxamide.

If for a given specific substrate, in combination with the catalytically active site of the enzyme, the transition state arises through the operation of a mechanism which requires both an electrophilic and a nucleophilic group at the active site of the enzyme to be in the proper charge state, *e.g.*, the electrophilic group to be protonated and the nucleophilic group to be unprotonated[18] and if the protonated forms of each of these groups have pK'_A values of approximately 7.9 ± 0.1 then the dependence of the relative activity of the system upon pH should be represented by a symmetrical curve with a maximum at pH 7.9 ± 0.1 if it can be assumed that the relative activity is proportional to k_3/K_S, that K_S is not notably dependent upon pH, and that the system is not subject to perturbation by other ionization processes. The symmetrical pH–activity curves, with a maximum at pH 7.9 ± 0.1, that have been observed for acetyl-, chloroacetyl-, trifluoroacetyl- and nicotinyl-L-tyrosinamide,[19-21] for acetyl- and nicotinyl-L-phenylalaninamide,[22] and for acetyl-L-hexahydrophenylalaninamide[12] are consistent with the above expectation though it is clear that an expansion of the above hypothesis is required to account for the unusual pH–activity relationships exhibited by acetyl- and nicotinyl-L-tryptophanamide.[23]

For a specific substrate such as acetyl-L-tyrosinhydroxamide, which is capable of significant ionization to the corresponding hydroxamate ion at pH 7.9 ± 0.1, it was expected and it was found[10] that the pH optimum for the hydrolysis of this specific substrate was at a value less than pH 7.9 ± 0.1, *i.e.*, at pH 7.6, and that the activity of the system decreased more rapidly with an increase in pH on the alkaline side of the pH optimum than for a non-ionizing specific substrate such as acetyl-L-tyrosinamide. It is not obvious at present whether the coulombic repulsion that appears to be operative in univalent buffer systems at approximately pH 7.9, and to be responsible for the decreased affinity of the enzyme for negatively charged species at this pH[10,24] is due to a negative charge associated with the unprotonated nucleophilic group postulated above, or whether the negative charge is derived from another ionizable group at or neart he catalytically active site.

The presence of an electron releasing group in α-position to the polarized carbonyl group adjacent to the susceptible bond of the specific substrate would be expected to cause a substantial decrease in the reactivity of the susceptible bond over that obtaining for the case where no such groups were present. Since an amino group possesses a greater electron releasing potential than an ammonium group it is clear that with a specific substrate such as L-tyrosinhydroxamide, where the ammonium group has a pK'_A value of 7.0, that when the pH of the system is increased from pH 6 to the neighborhood of pH 8 the expected increase in the activity of the system associated with an increase in the degree of protonation of the electrophilic group at the catalytically active site will be opposed by a decrease in the activity of the system arising from an increase in the concentration of the amino form of the specific substrate at the expense of the more reactive ammonium form. Thus, as illustrated in part in Fig. 4, it is not unreasonable to anticipate that the pH optimum of L-tyrosinhydroxamide should lie in a region more acid than that of acetyl-L-tyrosinhydroxamide, and that the value of k_3 for the former specific substrate should be less than that of the latter at their respective pH optima.

From the data presented in Fig. 5 it appears that D-tyrosinhydroxamide functions as a competitive inhibitor in the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide and that the value of K_I for the system α-chymotrypsin-D-tyrosinhy-

(18) C. G. Swain and J. F. Brown, Jr., THIS JOURNAL, **74**, 2538 (1952).

(19) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).

(20) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, **73**, 3231 (1951).

(21) H. J. Shine and C. Niemann, *ibid.*, **74**, 97 (1952).

(22) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).

(23) H. T. Huang and C. Niemann, *ibid.*, **73**, 1541 (1951).

(24) H. T. Huang and C. Niemann, *ibid.*, **74**, 5963 (1952).

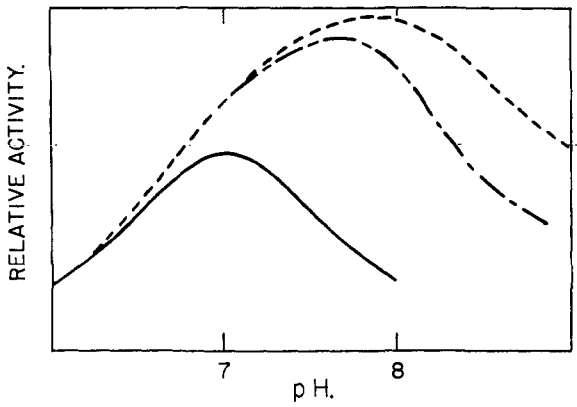

Fig. 4.—Relative activity *vs.* *p*H: —, L-tyrosinhydroxamide; – – –, acetyl-L-tyrosinhydroxamide; – – –, acetyl-L-tyrosinamide; ordinate values adjusted so as to achieve coincidence of all curves at *p*H 6.

droxamide in aqueous solutions 0.2 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, and at 25° and *p*H 6.9, is $40 \pm 8 \times 10^{-3}$ *M*. It has not been possible to obtain a more precise value for this constant with the analytical procedure used in this investigation because of the fact that since both the specific substrate and the competitive inhibitor are equally chromogenic one is confronted with the determination of small differences in the face of a relatively large blank value. Furthermore it is not possible to provide an interpretation of the K_I value, other than that it is composite in nature, since, as with the K_S value, it is not known to what extent each of the species present at *p*H 6.9 contributes to the observed value.

Experimental[25,26]

L-Tyrosinhydroxamide.[27]—To a solution of 7.65 g. (0.11 mole) of hydroxylamine hydrochloride in 200 ml. of methanol was added 110 ml. of 1.0 *N* methanolic sodium methoxide, the precipitated sodium chloride separated by centrifugation, the clear supernatant liquid cooled in an ice-bath and then slowly added to an ice-cold solution of 19.5 g. (0.11 mole) of L-tyrosine methyl ester in 200 ml. of dry methanol. The reaction mixture was allowed to stand at 4° for 3 days, then concentrated to near dryness by evaporation in a stream of dry air, the crystalline solid collected, dried, and recrystallized several times from hot water to give 12.5 g. (64%) of L-tyrosinhydroxamide, m.p. 161–162° with decomposition, $[\alpha]^{25}D$ 80° (*c* 0.3% in water).

Anal. Calcd. for $C_9H_{12}O_3N_2$ (196.1): C, 55.1; H, 6.1; N, 14.3. Found: C, 55.1; H, 6.0; N, 14.4.

D-Tyrosinhydroxamide.—Two grams of D-tyrosine methyl ester was treated as described above to give 1.0 g. (50%) of D-tyrosinhydroxamide, m.p. 161–162° with decomposition, $[\alpha]^{25}D$ −78° (*c* 0.3% in water).

Analytical Procedure.—In a series of preliminary experiments with solutions 0.02 *M* in ferric ion and 0.001 *M* in L-tyrosinhydroxamide it was found that the optical density at 505 mμ was strikingly dependent upon the nature of the other components present in the reaction mixture, *cf.* Table I. For example when the acid strength was high enough to suppress the hydrolysis of ferric ion, and the anionic species was perchlorate, the absorption at 505 mμ almost vanished, but if a small amount of dilute hydrochloric acid was added to the solution the optical density increased markedly. The observation that the addition of methanol

(25) All melting points are corrected.

(26) Microanalyses by Dr. A. Elek.

(27) K. G. Cunningham, G. I. Newbold, F. S. Spring and J. Stark, *J. Chem. Soc.*, 2091 (1949).

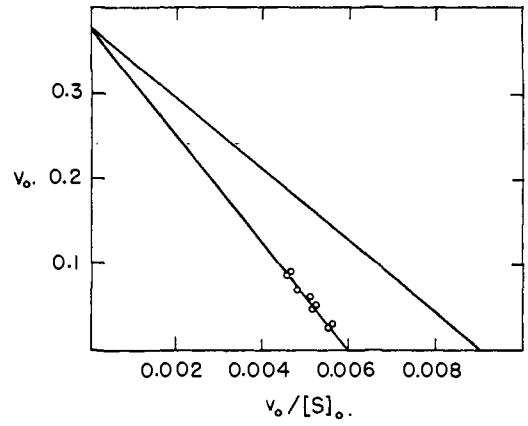

Fig. 5.—Competitive inhibition of the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide by D-tyrosinhydroxamide in aqueous solutions at 25° and *p*H 6.9; v_0 in units of 10^{-3} *M*/min.; $[S]_0$ in units of 10^{-3} *M*; [E] = 0.104 mg. protein-nitrogen/ml.; [I] = 20×10^{-3} *M*; 0.2 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer,

caused an increase in the optical density of the solution over that obtaining for the comparable aqueous system, *cf.* Table I, prompted a study of the effects produced by the addition of other diluents, *cf.* Table II, and it was found that in general a greater optical density was obtained when alcohols were so used. From these observations and the ones recorded in Table I the following analytical procedure was developed. A stock solution 0.2 *M* in ferric chloride, 0.2 *M* in hydrochloric acid and 50% (by volume) in methanol was prepared and immediately prior to a given experiment 1.0-ml. aliquots of this solution were introduced into a number of 10-ml. volumetric flasks followed by the subsequent addition of *ca.* 7.5 ml. of methanol to each flask.

TABLE I

EFFECT OF VARIOUS PARAMETERS ON THE OPTICAL DENSITY OF SOLUTIONS OF THE FERRIC–HYDROXAMIC ACID COMPLEX[a]

Methanol, volume %	$HClO_4$, *M*	HCl, *M*	Optical[b] density
0	0.8	0	0.050
0	.2	0	.176
0	.2	0.06	.508
70	.8	0	.470
80	.2	0	.790
85	0	0.08	1.040[c]

[a] Concentration of ferric ion 0.02 *M*, concentration of L-tyrosinhydroxamide 0.001 *M*. [b] $\log_{10} I_0/I$ for a path of 1 cm. at 505 mμ. [c] Solution also 0.02 *M* in tris-(hydroxymethyl)-aminomethane.

TABLE II

DEPENDENCE OF THE OPTICAL DENSITY OF SOLUTIONS OF THE FERRIC–HYDROXAMIC ACID COMPLEX UPON THE NATURE OF THE SOLVENTS[a]

Solvent	Optical[b] density	Solvent	Optical[b] density
Formamide	0.770	Propanol	0.985
Water	.656	Acetone	.353
Ethylene glycol	.818	*t*-Butyl alcohol	.988
Methanol	.970	Ethylene glycol mono-	
Ethanol	.990	benzyl ether	.818
Isopropyl alcohol	.980	Dioxane	.485

[a] Solutions prepared by the dilution of a mixture of 1.0 ml. of a solution 0.2 *M* in ferric chloride, 0.2 *M* in hydrochloric acid and 50 volume % in methanol and 1.0 ml. of an aqueous solution 0.01 *M* in L-tyrosinhydroxamide to 10.0 ml. with the indicated solvent. [b] log I_0/I for a path of 1 cm. at 505 mμ.

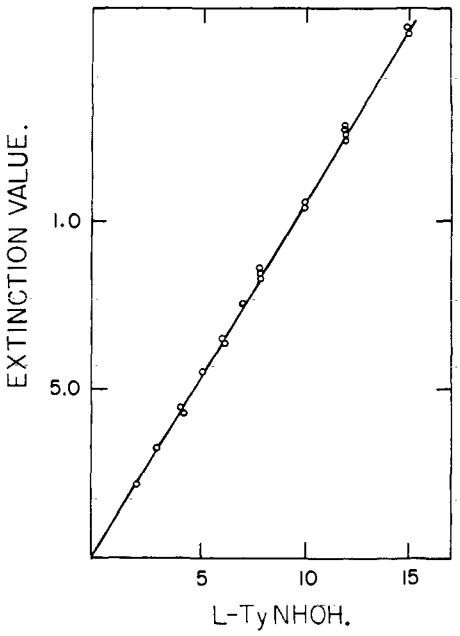

Fig. 6.—Extinction values for a 1-cm. path at 505 mμ. *vs.* concentration of L-tyrosinhydroxamide after reaction of the latter substance with the ferric chloride–hydrochloric acid reagent and subsequent dilution to 10 ml.; concentration of L-tyrosinhydroxamide in units of 10^{-3} *M* as present in the original reaction mixture.

At selected time intervals a 1.0-ml. aliquot of the reaction mixture was added to the contents of one of the above flasks, the solution made up to volume with methanol, and the optical density of the resulting solution, for a path of 1 cm. and at 505 mμ, determined in a model B Beckman spectrophotometer. A solution containing all of the components except the L-tyrosinhydroxamide, *i.e.*, the specific substrate, was used to zero the instrument. It will be seen from Fig. 6 that the dependence of the optical density upon the concentration of L-tyrosinhydroxamide was linear over the range of concentrations ordinarily used. When concentrations of L-tyrosinhydroxamide were used which were higher than those indicated on the abscissa of the plot given in Fig. 6, 2.5 ml. or 5.0-ml. aliquots of the stock solution were introduced into 25- or 50-ml. flasks and diluted to the appropriate volume after the addition of 1.0 ml. of the reaction mixture.

Enzyme Experiments.—The reaction mixtures used for the determination of the *p*H-activity relationship were either 0.1 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer or 0.1 *M* with respect to arsenic present as cacodylic acid in a cacodylic acid–sodium cacodylate buffer. In all of the kinetic studies the reaction mixtures were 0.2 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and possessed a *p*H of 6.9 ± 0.05 at 25 ± 0.1°, the temperature at which all measurements were made. The α-chymotrypsin employed was an Armour preparation, lot no. 90402, and the enzyme concentration in all experiments was equivalent to 0.104 mg. protein-nitrogen/ml. In view of the value of K_S that has been computed for the system in question there can be no doubt that with the above enzyme concentration zone A conditions[23] have been satisfied. The values of the constants K_S and k_3 were obtained from the primary experimental data as described previously.[12]

PASADENA 4, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF KANSAS SCHOOL OF MEDICINE]

Synthesis of Chloropyrimidines by Reaction with N-Chlorosuccinimide, and by Condensation Methods[1]

BY ROBERT A. WEST AND HAROLD W. BARRETT

RECEIVED DECEMBER 14, 1953

In glacial acetic acid, N-chlorosuccinimide can be used for nuclear substitution of uracil, thymine and derivatives of 2-thiouracil, and in chloroform plus benzoyl peroxide this same reagent can be used for substitution of an allylic methyl side chain. Theoretical considerations indicate that electrophilic attack should occur preponderantly at position 5 of the nucleus in 2-methylthiouracil and substances of this type. Two of the 5-chloro derivatives were obtained also by the base-catalyzed condensation of α-chloro-β-ketoesters with methylisothiourea sulfate.

Although the 5-iodo and 5-bromo derivatives of uracil and of the 2-alkylthio analogs are readily prepared,[2,3] considerable difficulties have been encountered in preparation of the 5-chloro derivatives. Chlorination of uracil gives a mixture of 5-chloro- and 5,5-dichloro-6-hydroxyuracil in water[4] and a small yield of 5-chlorouracil in glacial acetic acid.[3] In the latter solvent, 2-methylthiouracil also gives a small amount of the 5-chloro derivative, but the chief product is a salt which decomposes in the presence of moisture to liberate methyl mercaptan.[3,4] In order to obtain derivatives of this type in sufficient quantities for physiological testing, we have investigated the behavior of N-chlorosuccinimide (NCS) whose use was suggested by the unusual nuclear, as well as allylic, substitutions produced by the analogous N-bromosuccinimide.[4-8] N-Chloroacetamide and dichloramine-T were tested also, but were found to be ineffective for the chlorination of pyrimidines.

It was found that attack by NCS could be directed at will to the C_5 nuclear position, or to a methyl group in the 5- or 6-position, depending on the reaction conditions. Thus, when 2,6-dimethylthiouracil was treated with NCS in glacial acetic acid, nuclear attack occurred to produce the 5-chloro derivative. When the same pyrimidine was dissolved in chloroform containing benzoyl peroxide, it reacted with NCS to give 6-chloromethyl-

(1) This work was supported in part by a grant G-3195 from the National Institutes of Health, Public Health Service, and in part by a grant from the General Research Fund of the University of Kansas.

(2) T. B. Johnson and C. O. Johns, *Am. Chem. J.*, **34**, 186 (1905).

(3) H. W. Barrett, I. Goodman and K. Dittmar, THIS JOURNAL, **70**, 1753 (1948).

(4) H. L. Wheeler and T. B. Johnson, *Am. Chem. J.*, **31**, 603 (1904).

(5) H. Schmid, *Helv. Chim. Acta*, **29**, 1144 (1946).

(6) K. Dittmar, R. P. Martin, W. Herz and S. J. Cristol, THIS JOURNAL, **71**, 1201 (1949).

(7) R. Adams and A. W. Schrecker, *ibid.*, **71**, 1186 (1949).

(8) D. S. Tarbell, H. P. Hirschler and R. B. Carlin, *ibid.*, **72**, 3138 (1950).