phosphate buffer *p*H 7.4 and 10^{-4} *M* ethylenediaminetetraacetate.

Correlation of Loss of Catalytic Activity with Extent of Reaction with *p*-Mercuribenzoate.—For these experiments, from 1 to 5 × 10^{-6} *M* aldolase was incubated with 3 × 10^{-5} *M* *p*-mercuribenzoate and 0.15 *M* phosphate buffer, *p*H 7.0. At room temperature, only approximately 8 –SH groups per mole of aldolase reacted within a 3-hour period. Exposure at 37° for 1 hour was necessary to obtain reaction with 12 –SH groups and exposure for 3 hours resulted in reaction of 14 groups. Longer exposure periods usually resulted in appearance of turbidity from protein precipitation. Subsequent to the desired incubation with the *p*-mercuribenzoate, samples were diluted with water to give solutions containing 20 μg. of aldolase per ml., and the activity was determined by the usual procedure. No compensation was necessary for differences in unreacted *p*-mercuribenzoate in the diluted samples because the low residual concentrations did not affect the catalytic assay. Aldolase samples incubated at 37° for three hours in the phosphate buffer without addition of *p*-mercuribenzoate showed little or no loss of catalytic activity. The loss observed in the presence of *p*-mercuribenzoate could thus be ascribed to reaction of the enzyme with the mercurial.

Reversal of the *p*-Mercuribenzoate Inactivation of Aldolase.—Samples of 1 ml. total volume containing 1 × 10^{-6} *M* aldolase and 0.12 *M* phosphate buffer *p*H 7.2 with or without 3 × 10^{-5} *M* *p*-mercuribenzoate were incubated at 0 or 37° as indicated in Table II. Then either 0.2 ml. of water or of 0.05 *M* glutathione, *p*H 7, was added, the samples allowed to stand 20 minutes at room temperature, and placed in an ice-bath. Catalytic activity assays were then made on appropriate aliquots as described previously.

MINNEAPOLIS, MINN.

[CONTRIBUTION NO. 2077 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The α-Chymotrypsin-catalyzed Hydrolysis of a Series of Hydrazides. I. Determination of *p*H Optima and their Dependence upon Temperature[1]

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It has been found that the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide and of six α-N-acylated-L-tyrosinhydrazides can be followed quantitatively by the spectrophotometric determination of the liberated hydrazine in the form of a protonated bis-*p*-dimethylaminobenzalazine. With this procedure it has been shown that for aqueous solutions at 25° the *p*H optimum for the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide is 7.05 ± 0.15 and that the *p*H optima for the comparable reactions involving the six α-N-acylated-L-tyrosinhydrazides lie in a more basic region, *i.e.*, *ca.* 7.7 to 8.0. It also has been observed that the *p*H optima for the α-chymotrypsin-catalyzed hydrolysis of three representative α-N-acylated-L-tyrosinhydrazides are strikingly temperature dependent in the region between 25 and 40° and that an increase in temperature from 25 to 40° causes the *p*H optima to be shifted to a more acidic region, *i.e.*, from *ca.* 7.8 to *ca.* 6.8.

The knowledge that α-N-nicotinyl-L-tyrosinhydrazide and presumably α-N-acetyl-L-phenylalaninhydrazide are hydrolyzed in the presence of α-chymotrypsin[5-7] led us to consider the usefulness of the hydrazides of certain α-amino acids and acylated α-amino acids as specific substrates in studies involving the above enzyme. While MacAllister and Niemann[5] had followed the α-chymotrypsin-catalyzed hydrolysis of α-N-nicotinyl-L-tyrosinhydrazide, in aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the EDA[8] component of an EDA–HCl buffer, with the aid of a formol titration[9] and Goldenberg, Goldenberg and McLaren[6] had determined the extent of the α-chymotrypsin catalyzed hydrolysis of α-N-acetyl-DL-phenylalaninhydrazide, in aqueous solutions at 24.6° and *p*H 7.3 and 0.05 *M* in an unspecified phosphate buffer, with a Grassmann–Heyde titration[10] neither of these procedures was employed in the present study because it was anticipated that for many α-amino acid and α-N-acylated amino acid hydrazides any titrimetric procedure based upon the determination of liberated carboxyl groups would not be sufficiently sensitive to be used for determining their rates of hydrolysis by α-chymotrypsin.

Goldenberg, Goldenberg and McLaren[7] were aware of the limitations of the above titrimetric procedures when applied to the hydrazides and these investigators devised a colorimetric procedure for the qualitative recognition of the α-chymotrypsin-catalyzed hydrolysis of α-N-acetyl-DL-phenylalaninhydrazide, which was based upon the reduction of phosphomolybdate ion to the so-called molybdenum blue by the liberated hydrazine While it was stated[7] that this latter procedure was capable of detecting an extent of hydrolysis of 1 to 2% at specific substrate concentrations of 0.03 to 0.05 *M* no attempt appears to have been made to develop the procedure to the point where it could be used for quantitative measurements.

An alternative colorimetric procedure for determining the extent of the α-chymotrypsin-catalyzed hydrolysis of α-amino acid and α-N-acylated α-amino acid hydrazides, and one that promised exceptional sensitivity, was suggested by the observation of Pesez and Pelit[11] that hydrazine reacts with *p*-dimethylaminobenzaldehyde in aqueous acidic media to give a protonated bis-*p*-dimethylaminobenzalazine with an absorption maximum

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(5) R. V. MacAllister and C. Niemann, THIS JOURNAL, **71**, 3854 (1949).

(6) V. Goldenberg, H. Goldenberg and A. D. McLaren, *ibid.*, **72**, 5317 (1950).

(7) V. Goldenberg, H. Goldenberg and A. D. McLaren, *Biochem. Biophys. Acta*, **7**, 110 (1951).

(8) Ethylenediamine.

(9) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

(10) W. Grassmann and W. Heyde, *Z. physiol. Chem.*, **183**, 32 (1929).

(11) M. Pesez and A. Pelit, *Bull. soc. chim.*, 122 (1947).

in the region of 455 mμ. Therefore, a procedure for the spectrophotometric determination of hydrazine which was based upon the above reaction was developed and although it was anticipated, and in a sense improved, by Watt and Crisp[12] and by Wood[13] it was employed in its original form in all of the studies described in this communication. With the availability of this latter procedure, which was capable of determining quantities of hydrazine of the order of 10^{-6} to 10^{-7} M in contrast to the lower limits of 10^{-3} to 10^{-4} M encountered in the determination of carboxylate groups with the formol titration,[9] it was possible to determine the extent of the α-chymotrypsin-catalyzed hydrolysis of seven α-amino acid and α-N-acylated α-amino acid hydrazides to carboxylate ion and hydrazine under conditions that involved only a limited extent of hydrolysis.

The seven specific substrates employed in the present investigation were, respectively, L-tyrosinhydrazide and the formyl-, acetyl-, trimethylacetyl-, dichloroacetyl-, benzoyl-, and nicotinyl-α-N-acyl derivatives of L-tyrosinhydrazide. All of these compounds were prepared from the corresponding esters and were found to be stable in the absence of α-chymotrypsin under the conditions employed in the enzymatic studies. In every instance it was found possible to establish conditions that would permit the demonstration of an enzyme-catalyzed hydrolysis. In view of the fact that α-N-benzoyl-L-tyrosinhydrazide, a compound which was previously reported[14] to be ineffective as a competitive inhibitor of the α-chymotrypsin-catalyzed hydrolysis of benzoyl-L-tyrosinamide, or of benzoyl-L-tyrosine ethyl ester, has now been shown to be a specific substrate of this enzyme it is clear that the inference that benzoyl-L-tyrosinhydrazide is incapable of interacting with the catalytically active site of α-chymotrypsin can no longer be maintained.

In view of the general dependence of enzyme-catalyzed reactions upon hydrogen ion concentration the *p*H optima of systems containing α-chymotrypsin and the seven specific substrates listed above were determined for each enzyme–substrate pair in aqueous solutions at 25° and 0.02 M in the THAM[15] component of a THAM–HCl buffer.[16] The results obtained in these studies are given in Table I along with comparable values for the corresponding amides and hydroxamides.[17]

It will be seen from the values summarized in Table I that the *p*H optima for the six α-N-acylated-L-tyrosinhydrazides, in aqueous solutions at 25°, are identical, within the limits of experimental error, with those of the four acylated-L-tyrosinamides, which were studied under comparable conditions, and that in general the optimum *p*H with respect to the attainment of the maximum extent of reaction in aqueous solutions at 25° in a given time interval is approximately 7.8 for both the acylated-L-tyrosinamides and the α-N-acylated-L-tyrosinhydrazides. The observation that the *p*H optimum for the reaction involving acetyl-L-tyrosinhydroxamide, *i.e.*, 7.60 ± 0.05, lies in a more acidic region than that observed for the comparable reaction involving either acetyl-L-tyrosinamide, *i.e.*, 7.90 ± 0.10, or acetyl-L-tyrosinhydrazide, *i.e.*, 7.95 ± 0.20, is not unexpected since of these three specific substrates only acetyl-L-tyrosinhydroxamide is capable of partial ionization in the region of the *p*H optimum and to thus cause a shift of the *p*H optimum in the direction noted.[18]

TABLE I

*p*H OPTIMA AT 25°[a]

–CH($CH_2C_6H_4OH$)CO–	–NHOH	$–NH_2$	$–NHNH_2$
H_2N–	6.95 ± 0.05[b]	.....	7.05 ± 0.15
HCONH–	.....	.....	7.70 ± .20
CH_3CONH–	7.60 ± .05[c]	7.90 ± 0.10	7.95 ± .20
$C(CH_3)_3CONH$–	.....	.....	7.65 ± .20
CF_3CONH–	.....	7.85 ± .05	.....
$CH_2ClCONH$–	.....	7.75 ± .05	.....
$CHCl_2CONH$–	.....	.....	7.85 ± .20
$(C_6H_5)CONH$–	.....	.....	8.0 ± .20
β-(C_5H_4N)CONH–	.....	7.90 ± .05	7.80 ± .20

[a] With respect to the α-chymotrypsin-catalyzed hydrolysis of a series of derivatives of L-tyrosine in aqueous solutions 0.02 M in the THAM component of a THAM–HCl buffer unless otherwise noted. [b] 0.1 M THAM. [c] 0.3 M THAM.

From an argument[19] developed to explain the difference in *p*H optima for the α-chymotrypsin-catalyzed hydrolysis in aqueous solutions at 25° of acetyl-L-tyrosinhydroxamide, *i.e.*, 7.60 ± 0.05, and L-tyrosinhydroxamide, *i.e.*, 6.95 ± 0.05, it would be expected that the *p*H optimum for the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydrazide, *i.e.*, 7.95 ± 0.20, would lie in a more basic region than that observed for the comparable reaction with L-tyrosinhydrazide, *i.e.*, 7.05 ± 0.15. While this expectation has been realized it should be noted that Schwert[20] has suggested that the *p*H optimum reported[17,19] for the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydroxamide, in aqueous solutions at 25°, *i.e.*, 6.95 ± 0.05, is to be associated with a transpeptidation reaction rather than with the assumed hydrolytic reaction. Since the absence of pertinent information does not permit us at this time to affirm or deny what appears to us to be an unlikely possibility we have undertaken a study of the importance of transpeptidation reactions under the conditions ordinarily employed for the evaluation of the kinetic constants of α-chymotrypsin-catalyzed hydrolyses of α-amino acid esters, hydroxamides, amides and hydrazides. These studies are now in progress.

The observation that the *p*H optima for the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydrazide and of L-tyrosinhydroxamide,[17,19] in aqueous solutions at 25°, are identical, within the limits of experimental error, *i.e.*, *ca.* 7.0, and that this latter value is greater than the value of 6.25

(12) G. W. Watt and J. D. Crisp, *Anal. Chem.*, **24**, 2006 (1952).

(13) P. R. Wood, *ibid.*, **25**, 1879 (1953).

(14) S. Kaufman, H. Neurath and G. W. Schwert, *J. Biol. Chem.*, **177**, 793 (1949).

(15) Tris-(hydroxymethyl)-aminomethane.

(16) In a few instances a cacodylic acid–sodium cacodylate buffer which was 0.02 M in added cacodylic acid was employed in the region below *p*H 7.

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

(18) D. S. Hogness and C. Niemann, *ibid.*, **75**, 884 (1953).

(19) R. J. Foster, R. R. Jennings and C. Niemann, *ibid.*, **76**, 3142 (1954).

(20) G. W. Schwert, *Ann. Rev. Biochem.*, **24**, 99 (1955).

which has been reported[21,22] for the *p*H optimum for the equivalent reaction involving L-tyrosine ethyl ester raises the question as to whether the difference just noted is real or is the result of a variation in procedure.

We may assume that the ammonium group present in a monoprotonated α-amino acid ester, hydroxamide or hydrazide will have a pK'_A value of 7.4 ± 0.4.[19,23,24] Therefore, in aqueous solutions at 25° and between *p*H 6.0 and 9.0 one may expect to encounter significant amounts of the monoprotonated, *i.e.*, $H_3\overset{+}{N}CHRCOY$, and unprotonated, *i.e.*, $H_2NCHRCOY$, species of each of the above specific substrates[25] with the relative concentrations of the two species being dependent upon the *p*H of the particular system under investigation. Thus, with all three of the above specific substrates it is clear that the over-all dependence of relative activity upon *p*H is much more complicated than the case involving interaction of the enzyme with a single substrate species in systems of varying *p*H since in the former instance one is confronted not only with the added problem of multiple and competitive specific substrates[26] but also by the fact that their relative concentrations are varying with the *p*H of the systems. Consequently with situations of such complexity it is imperative that in the first instance significance be given only to those differences that can be observed through the use of comparable experimental procedures.

It will be noted that the *p*H optima for L-tyrosinhydrazide and L-tyrosinhydroxamide which are given above were evaluated under conditions where in each reaction system the extent of reaction arising from the interaction of the enzyme with all substrate species derived from either of the above added specific substrates was collectively determined. In contrast to these two cases the *p*H optimum for L-tyrosine ethyl ester which is given above was evaluated by a procedure which measured only the extent of reaction of the monoprotonated L-tyrosine ethyl ester in a given reaction system.

Balls and his co-workers[21,22,27] in their studies on α-chymotrypsin and modified α-chymotrypsin catalyzed hydrolyses of L-tyrosine ethyl ester employed an analytical procedure which was developed earlier[28] for following the enzyme-catalyzed hydrolysis of acylated α-amino acid esters. In this procedure, which may be represented by eq. 1 and 2, the reaction system was maintained at a constant, or nearly constant, *p*H by the frequent addition of standard alkali and the amount of the latter reagent added was taken to be proportional to the extent of reaction. However, in solutions

$$RCONHCHR'CO_2R'' + H_2O \longrightarrow RCONHCHR'CO_2^- + R''OH + H^+ \quad (1)$$

$$H^+ + OH^- \longrightarrow H_2O \quad (2)$$

containing added α-amino acid ester and in the *p*H region with which we are concerned one is confronted with a situation represented not only by eq. 3 and 2 but also by eq. 4. Therefore, when the

$$H_3{}^+NCHR'CO_2R'' + HOH \longrightarrow \overset{+}{H_3}NCHR'CO_2^- + R''OH + H^+ \quad (3)$$

$$H_2NCHR'CO_2R'' + HOH \longrightarrow \overset{+}{H_3}NCHR'CO_2^- + R''OH \quad (4)$$

procedure of Schwert, *et al.*,[28] is used with α-amino acid esters in the *p*H region where both monoprotonated and unprotonated species are present only the extent of reaction of the monoprotonated species can be determined.

Having established the fact that the procedure used by Balls and his co-workers[21,22,27] for the evaluation of the *p*H optimum of L-tyrosine ethyl ester differs in principle from those employed for the evaluation of the *p*H optima of L-tyrosinhydrazide and L-tyrosinhydroxamide the question that arises is whether the difference in *p*H optima noted above may or may not be due to the difference in procedures.

If it is assumed that the monoprotonated species derived from L-tyrosine ethyl ester is hydrolyzed at a faster rate than the corresponding unprotonated species[19] and that the pK'_A value of the monoprotonated L-tyrosine ethyl ester is *ca.* 7.3[29] it may be inferred that had Balls and his co-workers[21,22,27] employed a procedure that was comparable to those used for the evaluation of the *p*H optima of L-tyrosinhydrazide and L-tyrosinhydroxamide they would have observed with L-tyrosine ethyl ester that the relative activities in the region between *p*H 6.3 and 6.9 did not decrease, within the limits of experimental error, with increasing *p*H and thus would have obtained a *p*H activity relationship with a relatively flat maximum extending from *ca.* *p*H 6.3 to 6.9. Since the location of the *p*H optimum would be somewhat arbitrary in a case of this kind all that can be said with certainty is that the *p*H optimum evaluated by measurement of the cumulative extent of reaction embracing all reactive species would lie in a more basic region than the *p*H optimum evaluated by determining the extent of reaction of only the monoprotonated species. Thus, we may conclude that the difference in the *p*H optima of L-tyrosine ethyl ester and of L-tyrosinhydrazide and L-tyrosinhydroxamide which was noted earlier may not be real since the difference could arise either wholly or in part from a difference in procedures. In this connection it may be recalled that Goldenberg, Goldenberg and McLaren[7] have noted that the *p*H optimum for the α-chymotrypsin-catalyzed hydrolysis of L-leucine ethyl ester in aqueous solutions at 25° appears to

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

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

(23) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publ. Corp., New York, N. Y., 1948.

(24) R. J. Kerr, Ph.D. Thesis, Calif. Inst. Tech., Pasadena, Calif., 1957.

(25) With L-tyrosinhydroxamide the additional species $H_3\overset{+}{N}CHRCONHO^-$ and $H_2NCHRCONHO^-$ will be present in the more alkaline solutions. However, in systems more acid than *p*H 7.5 their contribution will be negligible.

(26) R. J. Foster and C. Niemann, THIS JOURNAL, **73**, 1552 (1951).

(27) E. F. Jansen, M. D. Fellows-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).

(28) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *ibid.*, **172**, 221 (1948).

(29) The pK'_A value of monoprotonated L-tyrosine ethyl ester does not appear to have been determined. However, the monoprotonated L-phenylalanine methyl ester is known to have a pK'_A value of 7.3.[14]

be *ca.* 6.8 when determined by the procedure used by Balls and his co-workers[21,22,27] for L-tyrosine ethyl ester and to be *ca.* 7.2 when determined by a procedure which would be expected to reveal the cumulative extent of reaction of both the unprotonated and monoprotonated L-leucine ethyl ester. Although it has been suggested[30] that the difference in *p*H optima noted above may be a consequence of a transpeptidation reaction we believe that the argument given above provides a more likely explanation of the observation of Goldenberg, Goldenberg and McLaren[7] and it may be added that the shift in the *p*H optima noted by these authors[7] is in the direction anticipated on the basis of this argument.

The absence of information with respect to the dependency of the *p*H optima of α-chymotrypsin-catalyzed hydrolyses upon temperature led us to determine the *p*H optima for the α-chymotrypsin-catalyzed hydrolysis of α-N-acetyl-, α-N-benzoyl- and α-N-nicotinyl-L-tyrosinhydrazide in aqueous solutions at 10, 25 and 40° and those of L-tyrosinhydrazide in aqueous solutions at 25 and 40°. It will be seen from the data summarized in Table II that the *p*H optima for the α-chymotrypsin-catalyzed hydrolysis of the above three α-N-acylated-L-tyrosinhydrazides in aqueous solutions at 10° are identical, within the limits of experimental error, with those observed at 25°. However, when the values observed at 25° are compared with those observed at 40° it is seen that an increase in temperature from 25 to 40° causes a shift in the *p*H optima from *ca.* 7.9 at 25° to *ca.* 6.8 at 40° for all three of the above α-N-acylated-L-tyrosinhydrazides. In contrast to the behavior of the α-N-acylated-L-tyrosinhydrazides it was found, *cf.* Table II, that the *p*H optimum for the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide in aqueous solutions at 25°, *i.e.*, 7.05 ± 0.15, is identical, within the limits of experimental error, with that observed in aqueous solutions at 40°, *i.e.*, 6.85 ± 0.15. It is noteworthy that in aqueous solutions at 40° the *p*H optimum for the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydrazide is practically the same as that observed for any one of the three α-N-acylated-L-tyrosinhydrazides considered in this study.

TABLE II

VARIATION OF *p*H OPTIMA WITH TEMPERATURE[a]

$-CH(CH_2C_6H_4OH)-CONHNH_2$	10°	25°	40°
H_2N	.....	7.05 ± 0.15	6.85 ± 0.15
CH_3CONH-	7.85 ± 0.25	7.95 ± .20	6.75 ± .15
$(C_6H_5)CONH-$	7.80 ± .25	8.0 ± .20	6.75 ± .20
β-$(C_5H_4N)CONH-$	7.80 ± .25	7.80 ± .20	6.75 ± .20

[a] With respect to the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide and of three α-N-acylated-L-tyrosinhydrazides in aqueous solutions 0.02 *M* in the THAM component of a THAM–HCl buffer.

When it is realized that the *p*H optimum of an enzyme-catalyzed reaction is a reflection of the effect of *p*H upon a relatively large number of independent reaction parameters, that each of these dependencies may in turn possess different temperature dependencies and that one may also encounter temperature dependencies which are independent of the *p*H of the reaction system it is clear that in the absence of knowledge of the magnitude and direction of each of the above dependencies one must regard the *p*H optimum and its dependence upon temperature simply as the summation of all of the above effects. Therefore, in view of the nature of the information reflected in knowledge of the *p*H optimum and its dependence upon temperature in the case of the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide and of α-N-acetyl-, α-N-benzoyl-, and α-N-nicotinyl-L-tyrosinhydrazide in aqueous solutions at 10, 25 and 40° one can only note that the practice of evaluating the thermodynamic constants of similar α-chymotrypsin catalyzed reactions[14,31] by studying such reactions at a constant *p*H, *i.e.*, 7.8, appears to contain more elements of uncertainty than those already envisaged[14,31] even though such reactions were studied in the temperature range from *ca.* 10 to 30° but in solutions containing 30% aqueous methanol.

Experimental[32,33]

L-Tyrosinhydrazide.[34]—This compound was prepared by the reaction of L-tyrosine methyl ester in methanol solution with hydrazine hydrate essentially as described[34] for the equivalent reaction of the ethyl ester. The product which crystallized from the reaction mixture was twice recrystallized from ethanol and dried *in vacuo* over phosphorus pentoxide to give L-tyrosinhydrazide, m.p. 193–194°. Curtius[34] gives a m.p. of 195.5° for the same compound and a m.p. of 171° for DL-tyrosinhydrazide.

Anal. Calcd. for $C_9H_{13}O_2N_3$ (195): C, 55.4; H, 6.7; N, 21.5. Found: C, 55.4; H, 6.8; N, 21.4.

α-N-Formyl-L-tyrosinhydrazide.—To a well-stirred solution of 10.5 g. of L-tyrosine ethyl ester, m.p. 108.5–109.5°, in 250 ml. of 80% formic acid was added 43 ml. of acetic anhydride at a rate such that the temperature of the reaction mixture remained below 70°. After the reaction mixture had stood overnight at room temperature the solution was evaporated to dryness *in vacuo*, the sirupy residue taken up in 200 ml. of ethyl acetate, the ethyl acetate solution successively washed with 3 *N* aqueous sodium hydroxide, 6 *N* aqueous hydrochloric acid and with water, dried over anhydrous potassium carbonate, the solvent removed *in vacuo* and the 7.1 g. of sirupy residue taken up in 20 ml. of methanol. To the boiling methanol solution was added 2.25 g. of hydrazine hydrate, the reaction mixture allowed to stand overnight prior to concentration, the concentrate allowed to crystallize, the crystalline product recrystallized from aqueous methanol and dried *in vacuo* over phosphorus pentoxide to give 1.7 g. of α-N-formyl-L-tyrosinhydrazide, m.p. 218° with decomp., $[\alpha]^{26}D$ +21.4° (in 30% aqueous ethanol).

Anal. Calcd. for $C_{10}H_{14}O_3N_3$ (224): C, 53.8; H, 6.0; N, 18.9. Found: C, 53.7; H, 6.0; N, 18.8.

α-N-Acetyl-L-tyrosinhydrazide.—This compound was obtained from L-tyrosine by the same procedure used by Hogness and Niemann[18] for the preparation of the D-isomer for which a m.p. of 236–236.5° was erroneously reported instead of the correct value of 226–226.5°. The crude α-N-acetyl-L-tyrosinhydrazide recovered from the reaction mixture was recrystallized twice from methanol and dried *in vacuo* over phosphorus pentoxide to give α-N-acetyl-L-tyrosinhydrazide, m.p. 227–228°.

Anal. Calcd. for $C_{11}H_{16}O_3N_3$ (237): C, 55.7; H, 6.4; N, 17.7. Found: C, 55.7; H, 6.3; N, 17.8.

α-N-Trimethylacetyl-L-tyrosinhydrazide.—A vigorously agitated solution of 10.5 g. of L-tyrosine ethyl ester in 300 ml. of ethyl acetate was cooled to −5° prior to the addition of 2.75 g. of trimethylacetyl chloride, b.p. 104–105°, pre-

(30) H. M. Green and H. Neurath in H. Neurath and K. Bailey, "The Proteins," Vol. IIB, Academic Press, Inc., New York, N. Y., 1954, p. 1104.

(31) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **182**, 577 (1950).

(32) All melting points reported are corrected.

(33) Microanalyses by Dr. A. Elek.

(34) T. Curtius, *J. prakt. Chem.*, [2] **95**, 349 (1917).

pared in 35% yield by the reaction of trimethylacetic acid with benzoyl chloride.[35] Two minutes after the addition of the above amount of acid chloride 50 ml. of 0.05 *N* aqueous potassium carbonate was added to the cold and vigorously agitated reaction mixture and the process repeated until four portions of acid chloride and of aqueous potassium carbonate solution had been introduced. The cold solution was then allowed to separate into two phases, the ethyl acetate phase collected, washed with 6 *N* aqueous hydrochloric acid and then twice with water prior to drying over anhydrous potassium carbonate. Removal of the solvent *in vacuo* gave 7.2 g. of a sirupy residue which was taken up in 20 ml. of methanol and allowed to react with 2 g. of hydrazine hydrate as described for the preparation of the α-N-formyl derivative, *vide ante*, to give 5.4 g. of the crude hydrazide which was recrystallized twice from aqueous methanol and dried *in vacuo* over phosphorus pentoxide to give 2.5 g. of α-N-trimethylacetyl-L-tyrosinhydrazide, m.p. 179–180°, $[\alpha]^{25}D$ +29° (in water).

Anal. Calcd. for $C_{14}H_{21}O_3N_3$ (247): C, 60.2; H, 7.5; N, 15.1. Found: C, 60.2; H, 7.5; N, 15.1.

α-N-Dichloroacetyl-L-tyrosinhydrazide.—L-Tyrosine ethyl ester, 10.5 g. in 300 ml. of ethyl acetate was acylated with 14.7 g. of dichloroacetyl chloride, b.p. 106–107.5°, and 200 ml. of 0.05 *N* aqueous potassium carbonate as described for the preparation of α-N-trimethylacetyl-L-tyrosine ethyl ester, *vide ante*, and the 7.7 g. of crude α-N-dichloroacetyl-L-tyrosine ethyl ester obtained as a gummy solid was taken up in 15 ml. of methanol and allowed to react with 1.8 g. of hydrazide hydrate to give 5.9 g. of the crude hydrazide which was recrystallized twice from aqueous methanol and dried *in vacuo* over phosphorus pentoxide to give 2.0 g. of α-N-dichloroacetyl-L-tyrosinhydrazide, m.p. 193–193.5°, $[\alpha]^{25}D$ +12.5° (in 40% aqueous ethanol).

Anal. Calcd. for $C_{11}H_{13}O_3N_3Cl_2$ (306): C, 43.2; H, 4.3; N, 13.7; Cl, 23.2. Found: C, 43.2; H, 4.2; N, 13.8; Cl, 23.3.

α-N-Benzoyl-L-tyrosinhydrazide.—This compound was prepared essentially as described by Bergmann and Fruton.[36] The crude hydrazide was recrystallized twice from methanol and dried *in vacuo* over phosphorus pentoxide to give α-N-benzoyl-L-tyrosinhydrazide, m.p. 247–248°. Bergmann and Fruton[36] report a m.p. of *ca.* 255°.

Anal. Calcd. for $C_{16}H_{19}O_3N_3$ (301): C, 64.2; H, 5.7; N, 14.0. Found: C, 64.2; H, 5.6; N, 14.1.

α-N-Nicotinyl-L-tyrosinhydrazide.—This compound was prepared as described by MacAllister and Niemann[6] from α-N-nicotinyl-L-tyrosine ethyl ester, m.p. 147–149°.[37] The crude hydrazide was recrystallized twice from methanol and dried *in vacuo* over phosphorus pentoxide to give α-N-nicotinyl-L-tyrosinhydrazide, m.p. 242–243°.[6]

Anal. Calcd. for $C_{15}H_{16}O_3N_4$ (300): C, 60.0; H, 5.4; N, 18.7. Found: C, 60.1; H, 5.4; N, 18.8.

Buffer Solutions.—Aqueous stock solutions were prepared from THAM, m.p. 169–169.5°, which had been recrystallized twice from aqueous methanol. These stock solutions were 0.20 *M* with respect to the THAM component and were adjusted to the desired *p*H by the dropwise addition of concd. hydrochloric acid at the temperature at which they were to be used and prior to adjustment to the final volume. In every case the *p*H was checked upon a 1:10 dilution of the above stock solutions and the *p*H readjusted if necessary. All reaction systems were 0.02 *M* with respect to the THAM component.

In a few cases a sodium cacodylate–cacodylic acid buffer, which was 0.02 *M* in cacodylic acid when present in the reaction system, was employed. Some evidence was obtained that a 0.02 *M* THAM–HCl buffer and a 0.02 *M* sodium cacodylate–cacodylic acid buffer of identical *p*H may not be exactly equivalent in the reaction systems considered in this communication. However, the differences observed with these two buffer systems were of minor significance.

***p*-Dimethylaminobenzaldehyde Reagent.**—The *p*-dimethylaminobenzaldehyde, m.p. 74.5–75°, was prepared from a reagent grade product by recrystallization from aqueous methanol or from a practical grade product by precipitation with aqueous sodium hydroxide from a hydrochloric acid solution followed by two recrystallizations from aqueous methanol. The reagent employed contained 1.0 g. of the aldehyde in 100 ml. of absolute ethanol. This solution was stable for periods up to 1 week when stored in sealed brown glass containers.

Enzyme Stock Solutions.—The aqueous enzyme stock solutions were prepared, usually in 5-ml. volumetric flasks, from crystalline bovine α-chymotrypsin, Armour preparations No. 10705 and 00592, but in no case was a stock solution used after it had stood at 25° for more than 1 hour. In every case the stock solutions were equilibrated for not less than 15 minutes at the temperature employed for the examination of a particular system.

A set of experiments were conducted to determine whether there was a significant change in activity when enzyme solutions were maintained at 40° for 1 hour by holding the enzyme in aqueous solutions at 40° and *p*H 7.8 and 0.02 *M* in the THAM component of a THAM–HCl buffer for periods up to 1 hour and then determining the activity at 25° and *p*H 7.8 using α-N-nicotinyl-L-tyrosinhydrazide as the specific substrate and comparing it with the activity of an equivalent enzyme solution that had been kept at 25° and *p*H 7.8 for the same period. The results obtained from these experiments gave no indication that there was a significant change in activity when the enzyme was maintained at 40° for 1 hour under the conditions specified.

Specific Substrate Stock Solutions.—Aqueous stock solutions of the various specific substrates were prepared at concentrations such as to permit the addition of the desired amount of specific substrate to the reaction system in the form of an 8.0-ml. aliquot.

Reaction System.—To a 10-ml. volumetric flask was added, at 25°, 8.0 ml. of the specific substrate stock solution and 1.0 ml. of the 0.2 *M* buffer stock solution and the mixture equilibrated for 30 minutes in a constant temperature bath maintained within ±0.1° of the selected temperature. At zero time a 1.0-ml. aliquot of the enzyme stock solution was added to the flask containing the buffered specific substrate solution and the solution mixed by gentle inversion six or seven times. Aliquots were then withdrawn from the reaction system at selected time intervals and analyzed as described below. A summary of representative reaction systems employed in this study is given in Table III.

TABLE III

REPRESENTATIVE REACTION CONDITIONS USED IN *p*H OPTIMUM STUDIES[a]

Hydrazide	*T*, °C.	[E], mg. P.N/ml.	$[S]_0 \times 10^4$, *M*	*t*, min.
L-Tyrosin-	25	0.104[b]	1.65	60
	40	.104[b]	1.65	60
α-N-Formyl-L-tyrosin-	25	.144[c]	0.12	135
α-N-Acetyl-L-tyrosin-	10	.104[b]	.45	60
	25	.104[b]	.45	60
	40	.104[b]	.49	15
α-N-Trimethylacetyl-L-tyrosin-	25	.144[c]	2.56	122
α-N-Dichloroacetyl-L-tyrosin-	25	.144[c]	0.60	40
α-N-Benzoyl-L-tyrosin-	10	.052[b]	.24	45
	25	.052[b]	.16	30
	40	.052[b]	.19	45
α-N-Nicotinyl-L-tyrosin-	10	.052[b]	.17	60
	25	.052[b]	.93	17
	40	.052[b]	.33	50

[a] In aqueous solutions 0.02 *M* in the THAM component of a THAM-HCl buffer except in a few cases below *p*H 7.0 where a sodium cacodylate–cacodylic acid buffer 0.02 *M* in cacodylic acid was employed. [b] Armour lot no. 10705. [c] Armour lot no. 00592.

Analysis of Reaction System.—At preselected time intervals an aliquot, usually 1.0 ml., was withdrawn from the reaction system and transferred to a 10-ml. volumetric flask containing 1.0 ml. of the *p*-dimethylaminobenzalde-

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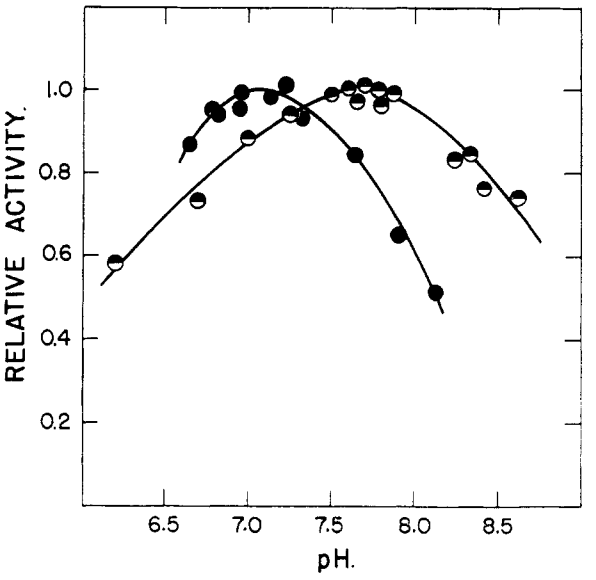

Fig. 1.—Relative activity *vs.* *p*H for the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide, ●, and of α-N-formyl-L-tyrosinhydrazide, ◓, in aqueous solutions at 25° and 0.02 *M* in the THAM component of a THAM–HCl buffer.

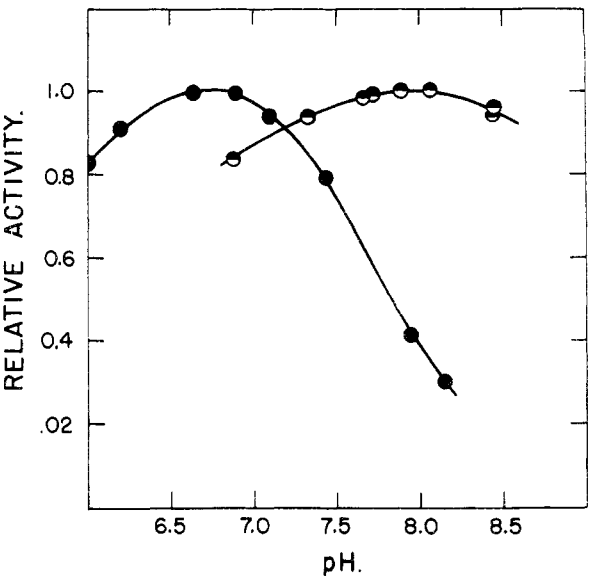

Fig. 2.—Relative activity *vs.* *p*H for the α-chymotrypsin-catalyzed hydrolysis of α-N-benzoyl-L-tyrosinhydrazide at 40°, ●, and at 25°, ◓, in aqueous solutions 0.02 *M* in the THAM component of a THAM–HCl buffer.

hyde reagent, 1.0 ml. of 1.72 *N* aqueous hydrochloric acid and *ca.* 5 ml. of water. The above solution was then made up to 10.0 ml. with water and allowed to stand for a period of 15–45 minutes before the optical density of the solution, contained in a 1-cm. quartz cell, was determined at 455 mμ using a Beckman Model B spectrophotometer equipped with a thermostated cell compartment maintained at 25°. It was found that the absorption at this wave length, *i.e.*, 455 mμ, followed Beer's law when the concentration of hydrazine was between 1 and 50 × 10^{-5} *M*. The size of the aliquot withdrawn from the reaction mixture was adjusted so the amount of hydrazine determined was between these limits.

With regard to blank corrections it should be noted that none of the specific substrates considered in this study were hydrolyzed in the absence of the enzyme under the conditions employed. However, a small blank correction was frequently made because of the presence of trace quantities of hydrazine in the specific substrates and because of a turbidity arising from the presence of a small amount of denatured enzyme in the solution of the azine.

Results.—The primary data obtained from analysis of the various reaction systems were recorded in terms of optical density, *i.e.*, extent of reaction, observed after a constant time interval for systems of varying hydrogen ion concentration with all other factors, *i.e.*, enzyme concentration, specific substrate concentration, time and temperature being maintained constant for any one specific substrate. Because the rather large variability in the susceptibility to hydrolysis of the various specific substrates resulted in a lack of uniformity, particularly with respect to enzyme and specific substrate concentrations, which had to be separately established for almost each specific substrate in order to obtain a significant extent of hydrolysis in a reasonable time interval, all of the primary data were normalized in order to facilitate presentation of the principal results of this study. In the normalization process the maximum optical density observed with any one specific substrate was assigned a value of unity and all other optical densities observed with the same specific substrate, where the only variable was the hydrogen ion concentration, were assigned values between zero and unity which were proportional to the respective observed optical densities relative to the observed maximum optical density. The plots given in Figs. 1 and 2 are representative examples of the derived data used for estimating the *p*H optima which are summarized in Tables I and II.

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