[CONTRIBUTION NO. 2404 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Re-examination of the α-Chymotrypsin Catalyzed Hydrolysis of α-N-Acetyl-L-tyrosinhydrazide[1]

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RECEIVED OCTOBER 15, 1958

An improved procedure for determining the rate of α-chymotrypsin catalyzed hydrolyses of α-amino acid and α-N-acylated α-amino acid hydrazides has been developed. A re-examination of the hydrolysis of α-N-acetyl-L-tyrosinhydrazide in the presence of the above enzyme has shown that the value of k_3 for a representative system is equal to or greater than that for a comparable system involving α-N-nicotinyl-L-tyrosinhydrazide. An explanation of this result, which is in marked contrast to that observed in the case of the corresponding amides, where the value of k_3 for the nicotinyl derivative is approximately twice that of the acetyl derivative, has been suggested. The role of product inhibition in enzyme catalyzed reactions has been considered.

Previous investigations[3-7] have shown that certain α-N-acylated-α-amino acid hydrazides are useful specific substrates of α-chymotrypsin principally because of the availability of an analytical procedure that is sufficiently sensitive to permit such reactions to be followed under conditions where the enzyme concentration is of the order of 10^{-5} M, the specific substrate concentration of the order of 10^{-3} M and the extent of reaction less than 10%.[4] However, in the course of such studies it became apparent that further exploitation of the hydrazide type of specific substrate could be facilitated by improvement of the original analytical procedure.[4] In the present study attention was first directed to this problem. With the development of an improved procedure it became possible to examine the α-chymotrypsin catalyzed hydrolysis of α-N-acetyl-L-tyrosinhydrazide with more confidence than was possible previously.[4] This reaction was of interest because of an earlier indication[4] that the k_3 value for a representative system was equal to or greater than that for a comparable system involving α-N-nicotinyl-L-tyrosinhydrazide[4,5] whereas with the corresponding amides, and for comparable systems, the k_3 value for the nicotinyl derivative is *ca.* twice that of the acetyl derivative.[8]

The original analytical procedure[4] was based upon conversion of the enzymatically liberated hydrazine to a protonated bis-*p*-dimethylaminobenzalazine and the colorimetric determination of the latter species. An examination of the absorption spectra of solutions 0.167 or 0.287 N in hydrochloric acid and containing *p*-dimethylaminobenzaldehyde and known amounts of hydrazine sulfate, both in the absence and presence of the components normally present in an enzymatic reaction system, *i.e.*, enzyme, specific substrate,[9] anionic hydrolysis product and a THAM[10]–HCl buffer,[11] established the fact that the position of the absorption maxima, observed at 455 mμ, was not affected by the presence of these latter components. However, under certain circumstances some of these components were found to contribute to the optical density, at 455 mμ, with all other factors being invariant. Therefore, it became desirable to establish a blank system that would lead to a zero-time optical density of essentially zero in order to avoid the earlier practice of making blank corrections for each experimentally determined point.[4,12]

In a series of experiments, conducted with solutions 0.167 or 0.287 N in hydrochloric acid and in which water, ethanol or an air path were used as blanks it became apparent that the aldehyde, the specific substrate and the enzyme were capable of contributing to the optical density observed at 455 mμ. The contribution arising from the specific substrate appeared to be due to occluded hydrazine salts since preparations that had been repeatedly recrystallized gave solutions that possessed little or no absorption at 455 mμ when corrected for the absorption of the aldehyde. The most important contribution arose from the turbidity produced when an aliquot of the enzyme solution was added to a solution of 1.0 ml. of the aldehyde reagent and 1.0 ml. of 1.67 or 2.87 N hydrochloric acid,[4] particularly when the more acidic solution was employed. It was found that dilution of either of the above solutions to *ca.* 80% of their final volume, *i.e.*, usually 10.0 ml., prior to the addition of the enzyme solution suppressed but did not eliminate the above effect. Considering all factors, including the possibility of a change in the nature of the buffer system, it was decided to establish a blank system 0.167 N in hydrochloric acid, *vide post*, and containing the aldehyde reagent, enzyme, specific substrate and buffer. The latter three species were present in the concentrations actually employed in the experiment of interest and were individually added to the diluted acidic aldehyde reagent. With a blank of this kind the zero-time correction was reduced to that of random error of *ca.* ± 0.004 optical density unit.

A second factor that was investigated was the dependence of the optical density of azine solutions,

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

(4) R. Lutwack, H. F. Mower and C. Niemann, *ibid.*, **79**, 2179, 5690 (1957).

(5) R. J. Kerr and C. Niemann, *ibid.*, **80**, 1469 (1958).

(6) R. R. Jennings, R. J. Kerr and C. Niemann, *Biochem. Biophys. Acta*, **28**, 144 (1958).

(7) W. E. M. Lands and C. Niemann, THIS JOURNAL, **81**, 2204 (1959).

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

(9) In this instance α-N-acetyl-L-tyrosinhydrazide.

(10) Tris-(hydroxymethyl)-aminomethane.

(11) These systems were static because of the acidity of the solutions.

(12) The empirical procedure for the evaluation of initial velocities developed by Booman and Niemann[13] requires a point for $t = 0$, and it is desirable that none of the points be subjected to individual correction if a valid estimate of variability is to be made.

(13) K. A. Booman and C. Niemann, THIS JOURNAL, **78**, 3612 (1956).

0.167 N in hydrochloric acid and prepared from the aldehyde reagent and known amounts of hydrazine sulfate, upon temperature. These studies showed that the optical density decreased with increasing temperature and for solutions with optical densities of less than 1.2 the decrease was approximately 0.46% per degree rise in temperature over the range from 15 to 40°. For solutions with optical densities from 1.4 to 1.6 and at temperatures in excess of 35° the decrease in optical density with increasing temperature was greater than that expected on the basis of the above relationship, possibly because of partial hydrolysis of the azine. It is obvious from these data that the temperature of the cell compartment of the spectrophotometer must be controlled, and as before[4] it was found convenient to thermostat it at 25.0°.

In the original procedure[4] it was recommended that the azine solutions be allowed to stand for a period of from 15 to 45 min. prior to the determination of optical densities. The minimum time requirement of 15 min. has been confirmed. However, it was noted in this study that after 30 min. the optical densities slowly increased with increasing time. Therefore, in the improved procedure the development time was standardized at 20 min.

Previously a color developing system that was 0.671×10^{-2} M in aldehyde and 0.172 N in hydrochloric acid was employed. On the basis of studies conducted with aldehyde concentrations of 0.671, 2.013 and 2.684×10^{-2} M and hydrochloric acid concentrations of 0.144, 0.167, 0.192, 0.240 and 0.287 N, it was concluded that the most satisfactory system with respect to sensitivity, adherence to a linear relationship between optical density and hydrazine concentration and minimal dependence of the optical density upon acid concentration was one which was 2.684×10^{-2} M in aldehyde, introduced as a 4% wt./vol. solution in absolute ethanol and 0.167 N in hydrochloric acid. The only disadvantage of this system was a consequence of its greater sensitivity relative to the one employed previously[4] in that dilution procedures had to be applied more frequently than before. However, if such dilution techniques were applied before an optical density of 1.0 to 1.2 was reached, no difficulty with stepped optical density–time relationships was encountered.

At fixed aldehyde and acid concentrations the relationship between optical density and concentration of hydrazine was found to be linear for optical densities of less than 1.2. However, for optical densities greater than this value, a negative departure from linearity was observed. When it developed that at least 55 to 60% of the observed departure from linearity was due to scattered light, it became apparent that the characteristics of the Beckman Model B spectrophotometer used in these studies had become a limiting factor. Therefore, with this instrument, or those of comparable characteristics, the dilution technique advocated previously is the only one that will ensure an extended linear relationship beyond that directly observed.[14]

There have been a number of discussions of the accuracy attainable in spectrophotometric analysis,[15-19] and it is recognized that minimum error occurs when the optical density has a value of 0.4343. However, in the case at hand it appears that the manipulation necessary to attain this value could lead to a greater error than that encountered in the use of the linear portion of the optical density–hydrazine concentration dependency, particularly when it is recalled that measurement of the optical density has to be made within a limited time interval. Therefore, with a prime requirement for simplicity and speed of operation, the latter procedure was used in the studies which follow.

α-N-Acetyl-L-tyrosinhydrazide was prepared as before[4] and the α-chymotrypsin catalyzed hydrolysis of this specific substrate, in aqueous solutions at 25.0° and pH 7.9 and 0.02 M in the THAM component of a THAM–HCl buffer, was examined with the aid of the improved analytical procedure. The pertinent details of these experiments are summarized in Table I. A least squares fit of the quantities $[S]_0/v_0$ and $[S]_0$ to equation 1 gave values of $K_S = 29.5 \pm 6.0 \times 10^{-3}$

$$([S]_0/v_0) = K_S/(k_3[E]) + [S]_0(1/k_3[E]) \quad (1)$$

M, $k_3 = 1.1 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml. and $k_3/K_S = 0.040 \pm 0.015$ min.$^{-1}$ (mg. protein-nitrogen/ml.)$^{-1}$. With $[E] = 4.1 \times 10^{-5}$ M,[20] $E_S' = 0.1 \times 10^{-2}$ and $S_S' = 0.015$ to 0.407. The above values of K_S and k_3 are to be preferred to those of $K_S = 22 \pm 8 \times 10^{-3}$ M and $k_3 = 0.7 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml. obtained earlier.[4]

It will be seen from the data given in Table I that in no case was the extent of hydrolysis greater than 8%. With a value of K_{P_1}, *i.e.*, the dissociation constant of the enzyme and α-N-acetyl-L-tyrosinate ion, of $110 \pm 30 \times 10^{-3}$ M[21] and a value of K_{P_2} so large as to be indeterminable,[22] it was assumed that inhibition of the hydrolytic reaction by the hydrolysis products would be of little or no consequence. However, since this problem has not been considered in detail it appeared worthwhile to determine whether or not the above assumption was a reasonable one.

For the case at hand we may write equation 2 which may be integrated to give equation 3. In the absence of product inhibition equation 2 is

$$-d[S]/dt = \{[E][S](k_3K_P/(K_P - K_S))\}/\{(K_S(K_P + [S]))/(K_P - K_S) + [S]\} \quad (2)$$

$$k_3[E]t = K_S(1 + [S]_0/K_P)\ln([S]_0/[S]_t) + (1 - K_S/K_P)([S]_0 - [S]_t) \quad (3)$$

(14) While the Beckman Model DU and particularly the Applied Physics Corporation (Cary) spectrophotometers have superior characteristics with respect to a greater freedom from scattered light, the convenience of the Beckman Model B spectrophotometer made it the instrument of choice for the enzymatic studies.

(15) F. Turjman and G. Lothian, *Proc. Phys. Soc. (London)*, **45**, 643 (1933).

(16) T. R. Hogness, F. P. Zscheile, Jr., and A. E. Sidwell, Jr., *J. Phys. Chem.*, **41**, 379 (1937).

(17) E. Sandell, "Colorimetric Determination of Traces of Metals," Interscience Publ., Inc., New York, N. Y., 1944, p. 49.

(18) G. H. Ayres, *Anal. Chem.*, **21**, 652 (1949).

(19) G. W. Watt and J. D. Chrisp, *ibid.*, **24**, 2006 (1952).

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

(21) R. J. Foster, H. J. Shine and C. Niemann, THIS JOURNAL, **77**, 2378 (1955).

(22) Unpublished observations of W. E. M. Lands and R. Lutwack.

reduced to equation 4 and equation 3 to equation 5.

$$-d[S]/dt = ([E][S]k_3)/(K_S + [S]) \quad (4)$$

$$k_3[E]t = K_S \ln([S]_0/[S]_t) + ([S]_0 - [S]_t) \quad (5)$$

It has been shown previously[23,24] that for a reaction described by equations 4 and 5 the initial velocities will be determined by the parameters $([S]_0 - [S]_t)$ and t_0', where $t_0'/t = f_0$ and f_0 is given by equation 6 in which the reaction parameter $P = ([S]_0 \ln([S]_0/[S]_t))/([S]_0 - [S]_t)$. Similarly for a reaction described by equations 2 and 3 we may write equation 7. Since $f_{0(P)} = t_0'_{(P)}/t$ and $f_0 = t_0/t_0'$ it follows that $t_0'_{(P)}/t_0' = f_{0(P)}/f_0$. Therefore, it is possible to evaluate the ratio $t_0'_{(P)}/t_0'$, *i.e.*, the ratio of the time required to attain a certain extent of reaction in the presence of product inhibition relative to that obtaining in the absence of such inhibition, as a function of extent of reaction for given values of K_S, K_P and $[S]_0$.[24] The ratio $f_{0(P)}/f_0 = t_0'_{(P)}/t_0'$ was evaluated by the procedure of Lands and Niemann[24] for reactions varying in extent from 5 to 80% for the case where $K_S = 30 \times 10^{-3}$ *M*, $[S]_0 = 100 \times 10^{-4}$ *M* and $K_P = 1$, 10, 50 and 100 $\times 10^{-3}$ *M*, *cf.*, Fig. 1, for the case where

$$f_{0(P)} = \{((K_S/[S]) + 1)/(1 - (K_S/K_P))/\{P\{((K_S/[S]_0) + (K_S/K_P))/(1 - (K_S/K_P))\} + 1\} \quad (7)$$

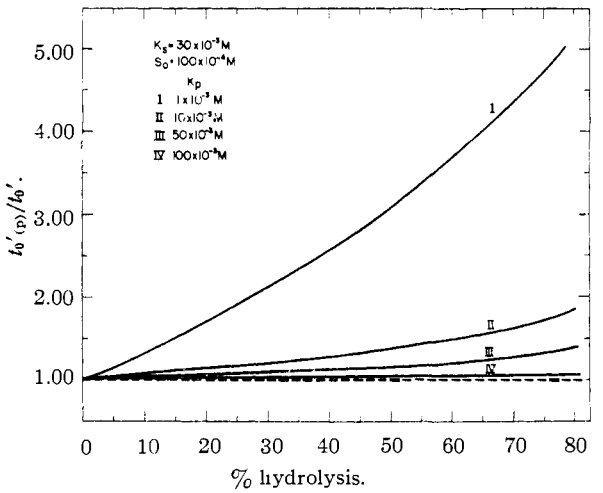

Fig. 1.—Dependence of ratio $t_0'_{(P)}/t_0'$ upon extent of reaction when $K_S = 30 \times 10^{-3}$ *M*. $[S]_0 = 100 \times 10^{-4}$ *M* and $K_P = 1$, 10, 50 and 100 $\times 10^{-3}$ *M*.

K_S 30 $\times 10^{-3}$ *M*, $K_P = 100 \times 10^{-3}$ *M* and $[S]_0 =$ 5, 25, 50, 100, 500 and 1000 $\times 10^{-4}$ *M*, *cf.* Fig. 2, and for the case where $K_S = 30 \times 10^{-3}$ *M*, $K_P =$ 10×10^{-3} *M* and $[S]_0 = 5$, 25, 50, 100, 500 and 1000×10^{-4} *M*.

It will be seen from Figs. 1 and 2 that with values of $K_S = 29.5 \times 10^{-3}$ *M*, $K_P = 110 \times 10^{-3}$ *M*, $[S]_0 = 4.5$ to 120 $\times 10^{-4}$ *M* and an extent of reaction of less than 8% one can ignore the consequences of product inhibition as was done in the present study. However, the information summarized in Figs. 1 to 3 inclusive emphasizes the fact that product inhibition cannot be ignored

(23) R. R. Jennings and C. Niemann, THIS JOURNAL, **75**, 4687 (1953).

(24) W. E. M. Lands and C. Niemann, *ibid.*, **77**, 6508 (1955).

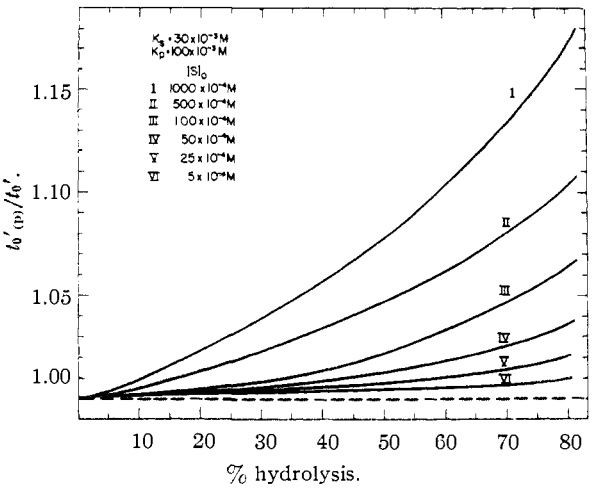

Fig. 2.—Dependence of the ratio $t_0'_{(P)}/t_0'$ upon extent of reaction when $K_S = 30 \times 10^{-3}$ *M*, $K_P = 100 \times 10^{-3}$ *M* and $[S]_0 = 5$, 25, 50, 100, 500 and 1000 $\times 10^{-4}$ *M*.

when a reaction is examined under conditions leading to extensive reaction, particularly when $[S]_0$ is large relative to K_S and $K_P \doteq K_S$. The necessity of employing relatively high values of $[S]_0$ and an extended course of reaction when evaluating K_P in the absence of added hydrolysis product[25,26] is forcefully illustrated in Figs. 2 and 3.[27]

TABLE I

THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF α-N-ACETYL-L-TYROSINHYDRAZIDE[a]

$[S]_0$[b]	v_0[c]	P_m[d]	Hydrol., %[e]	Dilution factor[f]
4.50	2.80 ± 0.09	2	7.8	1:10
7.51	4.74 ± .13	2	7.2	1:10
15.01	8.56 ± .14	2	7.5	1:10
22.52	11.91 ± .13	2	7.4	1:10
30.03	13.79 ± .10	2	6.2	1:10
37.54	15.94 ± .42	2	6.3	1:25
45.04	20.09 ± .23	2	6.2	1:25
52.55	22.55 ± .21	2	6.2	1:25
60.06	25.60 ± .08	3	6.0	1:25
67.56	29.74 ± .11	3	6.1	1:25
75.07	33.54 ± .54	3	6.1	1:25
82.58	35.63 ± 1.04	3	5.9	1:50
90.08	37.32 ± 0.28	2	5.9	1:50
105.10	44.85 ± 0.53	3	5.8	1:50
112.61	46.78 ± 1.15	3	5.8	1:50
120.11	50.79 ± 0.75	3	5.7	1:50

[a] In aqueous solutions at 25.0° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM–HCl buffer with [E] = 0.145 mg. protein-nitrogen per ml. of Armour preparation no. 00592. [b] In units of 10^{-4} *M*. [c] In units of 10^{-6} *M*/min. with the indicated probable error and determined from observations of the optical density at $t = 0$, 2, 4, 6, 8, 10, 12, 14 and 16 min. by the empirical procedure of Booman and Niemann.[13] [d] Degree of polynomial used for the evaluation of v_0. [e] Maximum extent of hydrolysis. [f] Extent of dilution of a 1.0-ml. aliquot of the 10.0-ml. reaction mixture for the determination of the optical density.

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

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

(27) In an isolated experiment Lands and Niemann[24] used the method of Foster and Niemann[25,26] in an attempt to simultaneously evaluate both K_S and K_P for the α-chymotrypsin catalyzed hydrolysis of α-N-acetyl-L-tyrosinhydrazide under conditions where $[S]_0 =$

There are now available reasonably reliable values of K_S and k_3 for comparable systems involving the interaction of α-chymotryspin with α-N-acetyl- and α-N-nicotinyl-L-tyrosinhydrazide and with the corresponding amides, *cf.*, Table II. While replacement of an acetyl group by a nicotinyl group causes a comparable decrease in the K_S values of both the amides and hydrazides, it is evident that such a structural modification has an entirely different effect upon the k_3 values, *i.e.*, in the amides the value of k_3 is increased to a substantial degree whereas in the hydrazides the value of this constant is either unchanged or decreased to a minor degree.[28] It is evident from these observations that even when the nature of the α-amino acid side chain remains invariant the dependence of values of k_3 upon the nature of the α-acylamino moiety may vary with the nature of the carboxyl function undergoing reaction in a way that would not be anticipated were the k_3 values largely determined by those factors that are operative in non-enzyme catalyzed reactions. However, if it is conceded that values of k_3 are indices not only of the intrinsic energetics of reactions involving the hydrolyzable bond but also of the probability of combination of the specific substrate with the active site of the enzyme in modes that can lead to a transition state,[4] it follows that the effect of a structural modification in one portion of a specific substrate molecule need not be limited to only one of the above two factors. With small molecules it is reasonable to expect that the nature of all structural elements may mediate to a marked degree any effect arising from a structural alteration at a point removed from the reactive bond particularly with regard to the entropy factor involved in combination in modes leading to the eventual production of reaction products.

Experimental[30,31]

Acid Reagent Solution.—One hundred and forty ml. of J. T. Baker's Analyzed hydrochloric acid (sp. gr. 1.186, 35.8%) was diluted to one liter with distilled water. A 1:10 dilution of this solution results in a final acid concentration of 0.167 *N*.

Aldehyde Reagent Solution.—*p*-Dimethylaminobenzaldehyde, m.p. 75.5–75.9°, was prepared by recrystallization of a reagent grade Matheson preparation from aqueous-methanol. Four grams of the aldehyde was dissolved in 100 ml. of absolute ethanol to give a solution that was stable for periods up to one week when stored in an amber glass bottle. One ml. of this solution when diluted 1:10 corresponds to a final aldehyde concentration of 2.684×10^{-2} *M*.

Hydrazine Solutions.—The approximate amount of Matheson reagent grade hydrazine sulfate was dissolved in distilled water. The exact hydrazine concentration was determined by a potassium iodate titration to an amaranth end-point as described by Penneman and Audrieth.[32]

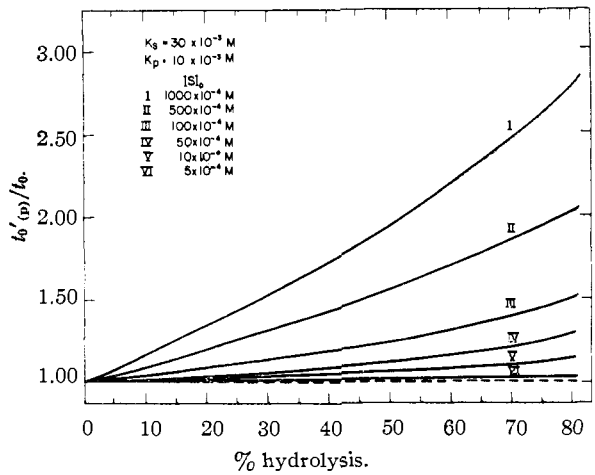

Fig. 3.—Dependence of the ratio $t_0'_{(P)}/t_0'$ upon extent of reaction when $K_S = 30 \times 10^{-3}$ *M*, $K_P = 10 \times 10^{-3}$ and $[S]_0 = 5, 10, 50, 100, 500$ and 1000×10^{-4} *M*.

Dependence of Optical Density upon Hydrazine Concentration.—Aliquots of standardized hydrazine solutions of varying concentration were added to 10.0 ml. G.S. volumetric flasks and were diluted to approximately 80% of the final volume. Equal volumes of the acid and aldehyde reagent solutions were mixed and a 2.0-ml. aliquot transferred to each of the volumetric flasks. The resulting solutions were equilibrated at 25.0 ± 0.1° for exactly 20 minutes, made up to volume and the optical densities determined for a 1 cm. path at 455 mμ using a Beckman Model B spectrophotometer equipped with a cell compartment maintained at 25.0 ± 0.1°. The blank was a solution containing only acid and aldehyde but in the same concentration as employed when hydrazine was present. The studies concerned with the determination of the optimum time of color development and the consequences of varying temperature, *vide ante*, were conducted essentially as described above but at a constant hydrazine concentration. The spectral characteristics of the various azine solutions were determined with the aid of a Model 11M Applied Physics Corporation spectrophotometer.

TABLE II

VALUES OF K_S AND k_3 FOR THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF α-N-ACETYL- AND α-N-NICOTINYL-L-TYROSINHYDRAZIDE AND THE CORRESPONDING AMIDES[a]

L-Tyrosine derivative	K_S[b]	k_3[c]	Ref.
α-N-Acetylamide	32 ± 4	2.4 ± 0.3	8
α-N-Nicotinylamide	12 ± 3	5.0 ± 1.0	8
α-N-Acetylhydrazide	29.5 ± 6.0	1.1 ± 0.2	..
α-N-Nicotinylhydrazide	8.0 ± 1.6[d]	0.84 ± 0.13	5

[a] In aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM-HCl buffer unless otherwise noted. [b] In units of 10^{-3} *M*. [c] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [d] Buffer 0.01 *M* in the THAM component.

α-N-Acetyl-L-tyrosinhydrazide.—Fifteen and two-tenths ml. of redistilled thionyl chloride (26.2 g., 0.22 mole) was added dropwise over a 30-minute interval to 100 ml. of absolute ethanol (1.6 moles) with continuous stirring and cooling. The reaction mixture was stirred for an additional 15 minutes whereupon 36.2 g. of L-tyrosine (0.20 mole) was added in several portions. The slurry was allowed to attain room temperature and then slowly warmed to 40° with stirring. After 4 hr. at 40° the clear orange solution was evaporated to dryness *in vacuo* and the residue dried at 100° for one hour to give 46.6 g. of crude L-tyrosine ethyl ester hydrochloride, m.p. 162–168°. The crude product was

24.54×10^{-4} *M* and the maximum extent of reaction was 10.7%. It is now evident that the values of $K_S = 60 \times 10^{-3}$ *M* and $K_P = 2 \times 10^{-3}$ *M* that were reported[24] are spurious and are a consequence of attempting an evaluation of this kind at too low a specific substrate concentration and too limited an extent of reaction.

(28) Of the eight α-N-acylated-L-tyrosinamides and the six α-N-acylated-L-tyrosinhydrazides that have been examined,[4,8,29] only two of the former, *i.e.*, the α-N-formyl and the α-N-carbethoxy derivatives, but the remaining five of the latter appear to have k_3 values that are less than those of the corresponding α-N-acetyl derivatives.

(29) D. T. Manning and C. Niemann, THIS JOURNAL, **80**, 1478 (1958).

(30) All melting points are corrected.

(31) Microanalyses by Dr. A. Elek.

(32) R. A. Penneman and L. F. Audrieth, *Anal. Chem.*, **20**, 1058 (1948).

suspended in 100 ml. of chloroform, the slurry added to 500 ml. of chloroform previously saturated with dry gaseous ammonia, the precipitated ammonium chloride removed by filtration, the filtrate evaporated to dryness *in vacuo* and the residue dried *in vacuo* over phosphorus pentoxide to give 29.6 *g.* of crude L-tyrosine ethyl ester; yield 59.4%. Acetylation of the crude ester with acetyl chloride, under Schotten–Bauman conditions, gave 29.0 g. (83%) of acetyl-L-tyrosine ethyl ester. This product was dissolved in absolute ethanol and the solution added to 3.8 g. of hydrazine in the same solvent. The reaction mixture was heated under refluxing conditions for 2 hr., cooled, the precipitated product collected, recrystallized twice from absolute methanol and dried *in vacuo* over phosphorus pentoxide to give 23.4 g. (83%) of α-N-acetyl-L-tyrosinhydrazide, m.p. 227–228°, $[\alpha]^{25.2}D$ 40.8 ± 0.6° (*c* 2.02%, in Methyl Cellosolve).

Anal. Calcd. for $C_{11}H_{15}O_3N_3$ (237): C, 55.7; H, 6.4; N, 17.7. Found: C, 55.6; H, 6.5; N, 17.8.

Enzyme Solutions.—An Armour preparation, lot no. 00592, was used throughout. The stock solutions were prepared as before[5] except that 50 mg. of the enzyme preparation was dissolved in 5.0 ml. of distilled water. One ml. of the enzyme stock solution diluted 1:10 led to a final concentration of 1 mg. per ml. or, when based upon a nitrogen content of 14.5%, to a concentration of 0.145 mg. of protein-nitrogen per ml. or 4.55×10^{-5} *M*.[20]

Buffer Solution.—A THAM–HCl buffer stock solution, 0.2 *M* in the amine component, was prepared as before.[5]

Enzymatic Reaction Systems and their Analysis.—The reaction systems were established essentially as described previously.[5] The analyses were conducted under conditions where the final concentration of aldehyde was 2.684×10^{-2} *M* and the hydrochloric acid concentration 0.167 *N*. In practice an acidic aldehyde reagent was prepared immediately before use by mixing equal volumes of the acid and aldehyde reagent solutions, *vide ante*. A 2.0-ml. aliquot of the acidic aldehyde reagent was introduced into a series of 10.0-ml. G.S. volumetric flasks and sufficient distilled water added to each flask to bring the volume to 9.0 ml. At selected time intervals, usually two minutes, a 1.0-ml. aliquot of the reaction mixture was transferred to a flask, the solution equilibrated at 25.0 ± 0.1° for exactly 20 minutes whereupon the optical density was determined at 455 mμ as indicated above except that in this instance the blank contained all of the components of the reaction and analyses systems other than the reaction products. The blank was prepared by diluting the acidic aldehyde reagent with the buffered specific substrate solution[5] and then adding the enzyme solution. When it became evident that the optical density would soon exceed a value of 1.1, larger volumetric flasks were substituted for the 10.0-ml. flasks used initially and the optical density corrected and recorded as its equivalent in a 10.0-ml. flask. Whenever this latter practice was followed, care was taken to maintain the final acid and aldehyde concentrations at a constant value by proportionally increasing the amount of acidic aldehyde reagent added to each flask, *i.e.*, 5.0 ml. to a 25.0 ml. and 10.0 ml. to a 50.0 ml. flask, and proportionally diluting the acidic aldehyde reagent before introducing the 1.0-ml. aliquot obtained from the reaction system. Further experimental details are summarized in Table I.

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[CONTRIBUTION NO. 2414 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Apparent Ionization Constants of a Series of Phenylalanine Derivatives[1]

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RECEIVED NOVEMBER 20, 1958

The apparent ionization constants of the α-ammonium groups present in DL-phenylalanine and its monoprotonated amide, thioamide, amidoxime, hydrazide, methyl ester and hydroxamide have been determined in aqueous solutions at 25.0 ± 0.1° and 0.05, 0.10 and 0.20 *M* in sodium chloride. The values of $pK^0_{A(NH_3^+)}$ were observed to increase from 6.78 ± 0.03 to 9.15 ± 0.01 in the order $-CONHOH < -CO_2CH_3 \leq -CONHNH_2 \doteq -C(NOH)NH_2 < -CSNH_2 < -CONH_2 \ll -CO_2^-$. Where comparison was possible the phenylalanine derivatives were found to have $pK'_{A(NH_3^+)}$ values that were 0.59 ± 0.04 of a *pK* unit lower than those of the corresponding glycine derivatives. The infrared spectra of all of the phenylalanine derivatives were determined for the solid in solid potassium bromide.

The use of amino acid derivatives, containing an α-amino or α-ammonium group, and an aromatic side chain, as specific substrates of α-chymotrypsin[3–7] has created a demand for knowledge of the apparent ionization constants of the α-ammonium groups present in these compounds.[7] In addition such data were required for an evaluation of the influence of the aromatic nucleus and the adjacent carboxyl function upon the above ionization constants.

Among the pertinent data that were available at the time this study was initiated were the pK'_A values of the α-ammonium group of glycine, 9.72[8]; alanine, 9.72[8]; phenylalanine, 9.12,[9] 9.13,[8] 9.15[10]; *o*-, *m*- and *p*-fluorophenylalanine, 9.01,[9] 8.98[9] and 9.05[9]; *o*-, *m*- and *p*-chlorophenylalanine, 8.94,[10] 8.91[10] and 8.96[10]; *p*-sulfamylphenylalanine, 8.64[10,11]; tyrosine, 9.11[8,11,12]; tryptophan, 9.39[8]; glycine methyl ester, 7.66[8]; glycine ethyl ester, 7.73[8]; alanine methyl ester, 7.80[8]; leucine methyl ester, 7.63[8]; methyl α-amino-*n*-butyrate, 7.71[8]; tyrosinhydroxamide, 7.0[5,11]; glycinamide, 7.93[8]; tryptophanamide, 7.5[14]; and glycinhydrazide, 7.69.[11,15] In order to provide a more systematic set of data it was decided to examine a series of phenylalanine derivatives in which the nature

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

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