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The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide in Aqueous Solutions at 25° and *p*H 7.6[1]

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From an analysis of the *p*H–activity curves of the systems α-chymotrypsin–acetyl-L-tyrosinamide and α-chymotrypsin–acetyl-L-tyrosinhydroxamide it has been concluded that in the latter system the active specific substrate is acetyl-L-tyrosinhydroxamide and not acetyl-L-tyrosinhydroxamate ion. At *p*H 7.6 and 25°, in aqueous solutions 0.3 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide have been found to be similar to those observed previously for this enzyme and specific substrates of the acylated α-amino acid amide type at *p*H 7.9 ± 0.1 and 25° in aqueous solutions 0.02 *M* with respect to the amine component of the same buffer system. Acetyl-D-tyrosine ethyl ester, acetyl-D-tyrosinhydrazide, acetyl-D-tyrosinhydroxamide and acetyl-D-tyrosinamide were found to be competitive inhibitors of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at *p*H 7.6 and 25°, under the conditions previously specified, and from these and other data it was concluded that acetyl-L-tyrosinamide and acetyl-L-tyrosinhydroxamide are hydrolyzed at the same active site of the enzyme.

It was anticipated from a previous study[4] that acetyl-L-tyrosinhydroxamide would be hydrolyzed in the presence of α-chymotrypsin and, as the first step in an investigation of the kinetics of hydrolysis of this specific substrate, the *p*H–activity curve for the system α-chymotrypsin–acetyl-L-tyrosinhydroxamide in a 0.3 *M* aqueous tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer was determined at 25°. It will be seen from the data summarized in Fig. 1 that the *p*H–activity curve for the above system has a rather sharp maximum at *p*H 7.6 and consequently all subsequent experiments were conducted at this *p*H.

A comparison of the *p*H–activity curve for the system α-chymotrypsin–acetyl-L-tyrosinhydroxamide with that for the system α-chymotrypsin–acetyl-L-tyrosinamide,[5] *cf.* Fig. 1, revealed that the two curves were identical, within the limits of experimental error, from *ca.* *p*H 6.5 to *p*H 7.6 but that at higher *p*H values the former system exhibited a lesser relative activity than did the latter.

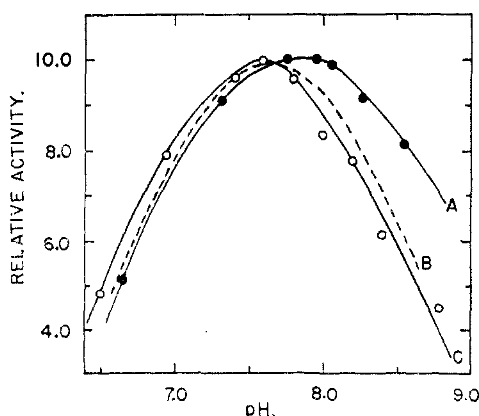

Fig. 1.—Relative activity *versus* *p*H: A, acetyl-L-tyrosinamide[5]; B, calculated for acetyl-L-tyrosinhydroxamide, *cf.* text; C, acetyl-L-tyrosinhydroxamide.

Although a quantitative explanation of the effect of *p*H on the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide cannot be given at the present time it is possible to account for the lower activity of the hydroxamide system, relative to the amide system, at the higher *p*H values.

Bergmann and Fruton[6] noted that the presence of a negative charge near the susceptible bond of a specific substrate caused a loss of substrate activity and it is now believed[7–10] that this effect is due to the presence of a negative charge at or near the catalytically active site of the enzyme. If it is assumed that acetyl-L-tyrosinhydroxamate ion, *i.e.* $[CH_3CONHCH(CH_2C_6H_4OH)CONHO]^-$, is inactive as a specific substrate for α-chymotrypsin then at the higher *p*H values an increasing fraction of the total amount of substrate added to the reaction system would be present in an inactive, charged form, thus causing a decrease in the activity of the system over and above that observed for a substrate which exhibits no such ionization, *e.g.*, the corresponding amide.

Since the above explanation rests upon knowledge of the pK'_A values of acetyl-L-tyrosinhydroxamide these values have been determined by potentiometric titration of an aqueous solution of this compound with 0.01 *N* aqueous sodium hydroxide, *cf.* Fig. 2, and have been found to be pK'_{A_1} 9.0 and pK'_{A_2} 10.2, respectively. It is reasonable to to associate the value of pK'_{A_2} 10.2 with the ionization of the phenolic hydroxyl group of the tyrosyl moiety since the $pK'_{A(OH)}$ values for tyrosine and glycyltyrosine have been found to be 10.07 and 10.40, respectively, and those of tyrosyltyrosine to be 9.80 and 10.26.[11] Therefore the value of pK'_{A_1} 9.0 can be taken as the apparent ionization constant of the hydroxamide moiety although it is true that this value is somewhat higher than would be expected on the basis of the ionization constants that have been reported for the simple acylhydrox-

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

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

(5) D. W. Thomas, R. V. MacAllister and C. Niemann, THIS JOURNAL, **73**, 1548 (1951).

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

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

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

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

(10) R. J. Foster, Ph.D. Thesis, Calif. Inst. Tech., Pasadena, 1951.

(11) 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.

amides, *i.e.*, $pK'_A \sim 7.5$.[12] However, it should be noted that these latter values were determined by a conductimetric procedure and therefore are of questionable accuracy.

From the pK'_A values, of 9.0 and 10.2, respectively, the fraction of the initial acetyl-L-tyrosinhydroxamide concentration which is present in the uncharged, or, on the basis of the preceding argument, in the active, form can be readily calculated as a function of the *p*H of the reaction system. Thus from *p*H 6.5 to *p*H 7.6, where maximum activity was observed, this fraction changes only slightly, dropping from 1.00 to 0.96. However, from *p*H 7.6, to *p*H 8.5, the fraction drops from 0.96 to 0.76, causing a greater loss of activity with increasing *p*H than might be expected of a non-ionizing substrate, such as the amide.

If it is assumed that the *p*H–activity curve for the un-ionized form of acetyl-L-tyrosinhydroxamide is identical within the limits of experimental error with that of acetyl-L-tyrosinamide then the initial velocities of hydrolysis of each of these substrates are given by equation (1)

$$v_0 = F(pH) \times C_S \times [S_{act}]_0 \qquad (1)$$

where F(*p*H) is a function of the *p*H and is not dependent upon the nature or concentration of either substrate; C_S is a constant dependent upon the nature of the substrate and where $F(pH) \times C_S$ is the apparent first-order rate constant for the reaction in question,[13] and $[S_{act}]_0$ is initial concentration of the active specific substrate. For acetyl-L-tyrosinamide it is assumed, with some justification, that within the *p*H range being considered $[S_{act}]_0 = [S]_0$, the initial substrate concentration, whereas for acetyl-L-tyrosinhydroxamide, where it is postulated that only the uncharged form is active, the concentration of the active specific substrate is given by equation (2)

$$[S_{act}]_0 = [S]_0 \frac{[H^+]^2}{[H^+]^2 + K_1[H^+] + K_1K_2} \qquad (2)$$

where K_1 and K_2 are the respective ionization constants of this substrate.

If the activity at a particular *p*H is defined as the ratio of the initial velocity at that *p*H to the initial velocity at the optimum *p*H, then for acetyl-L-tyrosinamide the activity, α_A, is given by equation (3)

$$\alpha_A(pH_i) = F(pH_i)/F(pH_{max}) \qquad (3)$$

where pH_{max} is the *p*H at which the maximum initial velocity is observed and pH_i is the *p*H under consideration. Similarly the activity of acetyl-L-tyrosinhydroxamide, α_H, is given by equation (4)

$$\alpha_H(pH_i) = F(pH_i)/F(pH_{max})\gamma \qquad (4)$$

where

$$\gamma = [1 + 10^{(pH_{max} - pK_1)} + 10^{(2pH_{max} - pK_1 - pK_2)}]/[1 + 10^{(pH_i - pK_1)} + 10^{(2pH_i - pH_1 - pK_2)}]$$

and the relation between α_H and α_A by equation (5).

$$\alpha_H(pH_i) = \alpha_A(pH_i)\gamma \qquad (5)$$

(12) E. Oliveri-Mandala, *Gazz. chim. ital.*, **40**, I, 102 (1910).

(13) It is also tacitly assumed that the initial substrate concentrations are sufficiently smaller than K_S so that the rate curves will be apparently first order over the time interval required to evaluate v_0; *cf.* Experimental section.

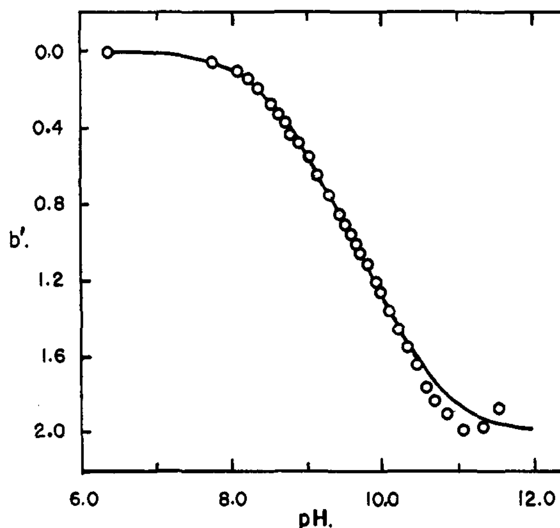
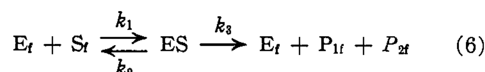


Fig. 2.—Titration data for acetyl-L-tyrosinhydroxamide and titration curve constructed on the basis of the constants pK'_{A1} 9.0 and pK'_{A2} 10.2.

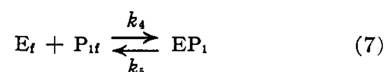
It should be noted that the above treatment contains the tacit assumption that the difference between F(*p*H) at pH_{max} for acetyl-L-tyrosinamide, *i.e.*, 7.8, and at pH_{max} for acetyl-L-tyrosinhydroxamide, *i.e.* 7.6, is negligible, as would be inferred from the 1% change in activity for acetyl-L-tyrosinamide between these two *p*H values; *cf.* Fig. 1.

A *p*H–activity curve for acetyl-L-tyrosinhydroxamide was calculated from that for acetyl-L-tyrosinamide with the aid of equation (5) and this curve is given in Fig. 1 along with the two experimentally determined curves. Because of the relatively good agreement between the experimental and calculated *p*H–activity curves for acetyl-L-tyrosinhydroxamide it appears reasonable to conclude that the lesser activity of acetyl-L-tyrosinhydroxamide at the higher *p*H values, relative to that of acetyl-L-tyrosinamide, is due to the fact that only the un-ionized form of the former compound can function as a specific substrate for α-chymotrypsin. However, in view of the assumptions made and the simplified treatment used, *e.g.*, no consideration was given to the probable inhibition of the hydrolysis of the un-ionized form of the specific substrate by the ionized form or to the possible hydrolysis of the ionized form at a reduced rate, the above conclusion should be regarded as a tentative one even though it appears to be supported by the observation that the α-chymotrypsin–benzoyl-L-tyrosinamide and the α-chymotrypsin–benzoyl-L-tyrosine ethyl ester systems exhibit indistinguishable *p*H–activity curves.[14]

For the reaction system



$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \qquad (6)$$



$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \qquad (7)$$

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

$$E_f + P_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_2 \quad (8)$$

$$E_f + I_f \underset{k_9}{\overset{k_8}{\rightleftharpoons}} EI \quad (9)$$

it can be shown[15] that when $d[ES]/dt \ll d[S]/dt$, $[S_f] \gg [ES]$, $[P_{1f}] \gg [EP_1]$, $[P_{2f}] \gg [EP_2]$, $[I_f] \gg [EI]$, and all components possess unit activity coefficients the velocity of the reaction is given by equation (10) which can be integrated to give equation (11). For the initial stages of the reaction and where there is no added inhibitor, *i.e.*, $[I] = 0$,

$$v = -\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S[1 + ([S]_0 - [S])(1/K_{P_1} + 1/K_{P_2}) + [I]/K_I] + [S]} \quad (10)$$

$$k_3[E]t = K_S[1 + [S]_0(1/K_{P_1} + 1/K_{P_2}) + [I]/K_I] \ln [S]_0/[S] + (1 - K_S/K_{P_1} - K_S/K_{P_2})([S]_0 - [S]) \quad (11)$$

equation (10) is reduced to the Lineweaver–Burk equation (12).[16] At a constant pH of 7.6, and a constant temperature of 25°, it was assumed that

$$1/v_0 = (K_S/k_3[E])(1/[S]_0) + (1/k_3[E]) \quad (12)$$

equation (12) was applicable to the initial stages of hydrolysis of acetyl-L-tyrosinhydroxamide since under these conditions practically all of the added substrate is present in an uncharged and hence an active form. From the $1/v_0$ *versus* $1/[S]_0$ plots given in Fig. 3 the mean values of K_S and k_3, for the system at pH 7.6 and 25° and 0.3 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, were found to be 51×10^{-3} M and 34×10^{-3} M/min./mg. protein-nitrogen/ml., respectively. If it is assumed that all of the protein-nitrogen present in the reaction mixture is α-chymotrypsin, that the molecular weight of the enzyme is 22,000[17,18] and that its nitrogen content is 16%,[7] then k_3 for the system α-chymotrypsin–acetyl-L-tyrosinhydroxamide, under the conditions previously specified, has the value of 2.0 sec.$^{-1}$.

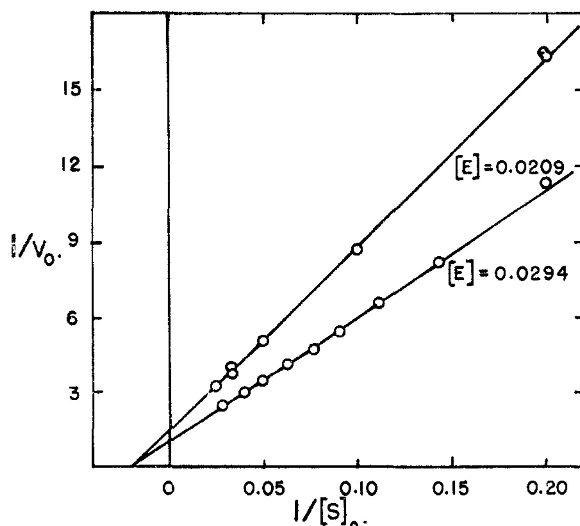


Fig. 3.—α-Chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at 25° and pH 7.6; v_0 in units of 10^{-3} M/min.; $[S]_0$ in units of 10^{-3} M; [E] in units of mg. protein-nitrogen/ml.; 0.3 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

The analysis of the $1/v_0$ *versus* $1/[S]_0$ plots given above tells us nothing in regard to possible competitive inhibition of the hydrolytic reaction by the reaction products. If no such inhibition occurred, and in the absence of an added competitive inhibitor, a plot of K_S (ln $[S]_0/[S]$) + ($[S]_0$ − [S]) *versus* time should give a straight line passing through the origin and having a slope k_3 [E]; *cf.* equation (11). The data given in Fig. 4 indicate, by the negative deviation of the experimental points from the expected straight line after approximately 35% hydrolysis, that there is inhibition by at least one of the hydrolysis products. This is to be expected since it has been found that acetyl-L-tyrosine functions as a competitive inhibitor in the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide, and has a K_{P_1} value of approximately 115×10^{-3} M.[5] If equation (11) is simplified by the condition of no added inhibitor and inhibition by only one of the hydrolysis products it follows that a plot of K_S (1 + $[S]_0/K_{P_1}$) (ln $[S]_0/[S]$) + (1 − K_S/K_{P_1}) ($[S]_0$ − [S]) *versus* time should give a straight line passing through the origin with slope k_3 [E]. Such a plot, based upon equation (11) with the condition that $K_S = 51 \times 10^{-3}$ M, $K_{P_1} = 115 \times 10^{-3}$ M, $K_{P_2} = \infty$, and $[I] = 0$, is given in Fig. 5 and it is seen that when the experimental data are so plotted no deviation from the expected linear relationship is observed. Thus in the absence of any evidence for the competitive inhibition of the hydrolytic reaction by the second hydrolysis product, *i.e.*, hydroxylamine or hydroxylammonium ion, equations (6) and (7) may

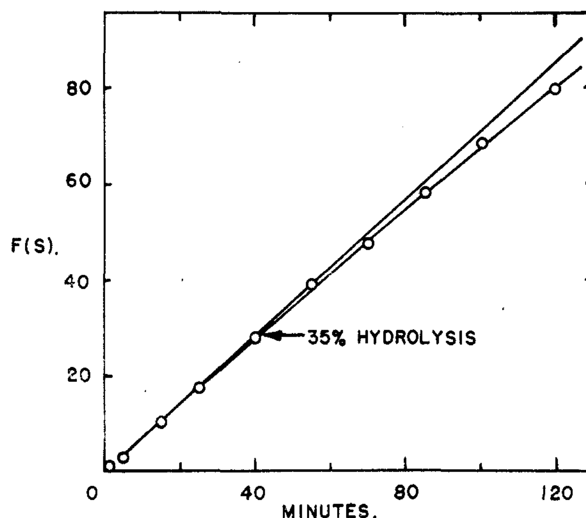


Fig. 4.—Plot of F(S) = 2.3 Ks log $[S]_0/[S]$ + ($[S]_0$ − [S]) in units of 10^{-3} M *versus* time for α-chymotrypsin and acetyl-L-tyrosinhydroxamide at 25° and pH 7.6; $[S]_0$ = 20×10^{-3} M; [E] = 0.0209 mg. protein-nitrogen/ml.

(15) *Cf.* H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951), for pertinent references and definition of symbols.

(16) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).

(17) G. W. Schwert and S. Kaufman, *J. Biol. Chem.*, **190**, 807 (1951).

(18) E. L. Smith, D. M. Brown and M. Laskowksi, *ibid.*, **191**, 639 (1951)

be taken to be an adequate kinetic formulation of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide under the conditions previously specified, provided that the assumptions involved in the derivation of the rate equations are valid.[19]

The fact that the enzyme concentration was never greater than 0.031 mg. of protein-nitrogen per ml., or *ca.* 8.8×10^{-6} *M*, and the substrate concentration was never less than 2×10^{-3} *M* indicates that the assumption that $[S_f] \gg [ES]$ must be valid as there is probably only one, at the most two, active sites per α-chymotrypsin molecule.[20-23] Similarly it must be concluded that after the initial rise of [ES] to its maximum, or steady state value, the assumption that $d[ES]/dt \ll d[S]/dt$ must hold. The fact that no initial period of increasing velocity was observed in the experiments used to determine the initial velocities, at the various specific substrate concentrations, indicates that there is negligible change in the specific substrate concentrations during the time interval required to reach the maximum value of [ES]. It is also apparent from the analysis summarized in Figs. 4 and 5, that the concentration of acetyl-L-tyrosine must be greater than 1×10^{-3} *M* before it need be considered in the formulation of the reaction kinetics, and that therefore the assumption that $[P_{1f}] \gg [EP_1]$ must also be valid in the range of $[P_{1f}]$ values that have an observable effect upon the reaction. Thus it may be concluded that the necessary assumptions involved in the derivation of the rate equations are valid for the experimental conditions used in this study and that the experimental data are in accord with the kinetic formulation given in the previous paragraph.

The fact that the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at *p*H 7.6 and 25° are similar to those noted previously for this enzyme and other synthetic specific substrates possessing but a single hydrolyzable bond,[5,7,15,24-26] prompted us to study the inhibition of the former reaction by acetyl-D-tyrosinamide, acetyl-D-tyrosinhydrazide, acetyl-D-tyrosinhydroxamide and acetyl-D-tyrosine ethyl ester. It was expected from previous observations[4,15,24-27] that these four compounds would function as competitive inhibitors in the above

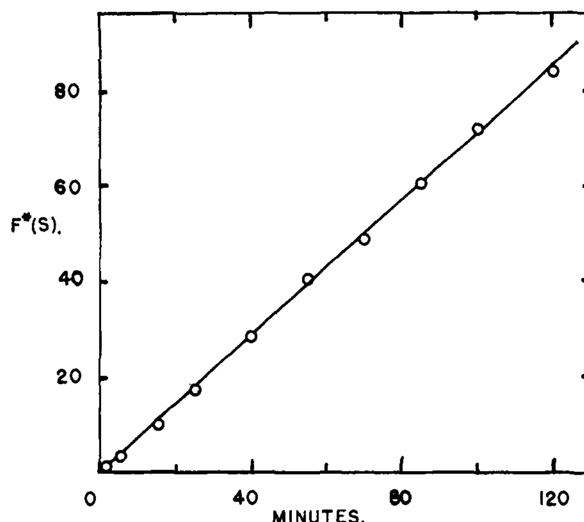


Fig. 5.—Plot of $F^*(S) = 2.3\ K_S\ (1 + [S]_0/K_{P_1}) \log [S]_0/[S] + (1 - K_S/K_{P_1})([S]_0 - [S])$ in units of 10^{-3} *M* *versus* time for α-chymotrypsin and acetyl-L-tyrosinhydroxamide at 25° and *p*H 7.6; $[S]_0 = 20 \times 10^{-3}$ *M*; [E] = 0.0209 mg. protein-nitrogen/ml.

reaction system and that their enzyme–inhibitor dissociation constants could be determined from $1/v_0$ *versus* $1/[S]_0$ plots based upon equation (11). The experimental data obtained in this study are summarized in Figs. 6–9 and in Table I, and it is seen from the plots given in Figs. 6–9 that there is no indication that the inhibition observed in all four cases is other than competitive. The validity of the assumption that $[I_f] \gg [EI]$, involved in the derivation of the rate equation for competitive inhibition by an added inhibitor, is apparent in all cases, since the total added inhibitor concentration was never less than 5×10^{-3} *M* whereas the enzyme concentration was in every case 0.0294 mg. protein-nitrogen per ml. or *ca.* 8.4×10^{-6} *M*.

TABLE I

ENZYME–INHIBITOR DISSOCIATION CONSTANTS OF SEVERAL COMPETITIVE INHIBITORS OF α-CHYMOTRYPSIN[a]

Inhibitor	K_I[b]	$-\Delta F°$[c]
Acetyl-D-tyrosine ethyl ester	4.0	3270
Acetyl-D-tyrosinhydrazide	6.8	2960
Acetyl-D-tyrosinhydroxamide	7.7	2890
Acetyl-D-tyrosinamide	11.2	2660

[a] At 25° and *p*H 7.6 in an aqueous tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer 0.3 *M* in respect to the amine component. [b] In units of 10^{-3} *M* with a maximum error of ±12%. [c] In cal./mole to the nearest 10 cal. with a maximum error of ±75 cal.

It is interesting to note that the K_I values of acetyl-D-tyrosinamide and acetyl-D-tyrosine ethyl ester which were evaluated against acetyl-L-tyrosinamide at *p*H 7.9 and 25° in a system 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, *i.e.* $12.0 \pm 1.0 \times 10^{-3}$ *M* and $3.5 \pm 0.5 \times 10^{-3}$ *M*, respectively,[5] are in good agreement with the values obtained in this study, *i.e.* $11.2 \pm 1.3 \times 10^{-3}$ *M* and $4.0 \pm 0.5 \times 10^{-3}$ *M*, at *p*H 7.6 and 25° in a system 0.3 *M* with respect to the amine component of the same buffer system.

(19) It should be emphasized that equation (11) with the condition that $K_S = 51 \times 10^{-3}$ *M*, $K_{P_1} = 115 \times 10^{-3}$ *M*, $K_{P_2} = \infty$, and [I] = 0, is a satisfactory rate equation only under the specified experimental conditions. Aside from the dependence of K_S and K_{P_1} upon *p*H one would have to consider, at *p*H values significantly higher than *p*H 7.6, the competitive inhibition of the hydrolytic reaction by acetyl-L-tyrosinhydroxamate ion; *i.e.*, [I] could no longer be taken as equal to zero and K_I would require evaluation.

(20) E. F. Jansen, M. D. Fellows-Nutting and A. K. Balls, *J. Biol. Chem.*, **179**, 201 (1949).

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

(22) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3228 (1951).

(23) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **50**, 672 (1952).

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

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

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

(27) H. T. Huang and C. Niemann, *ibid.*, **73**, 1555, 3223, 3228 (1951); **74**, 101 (1952).

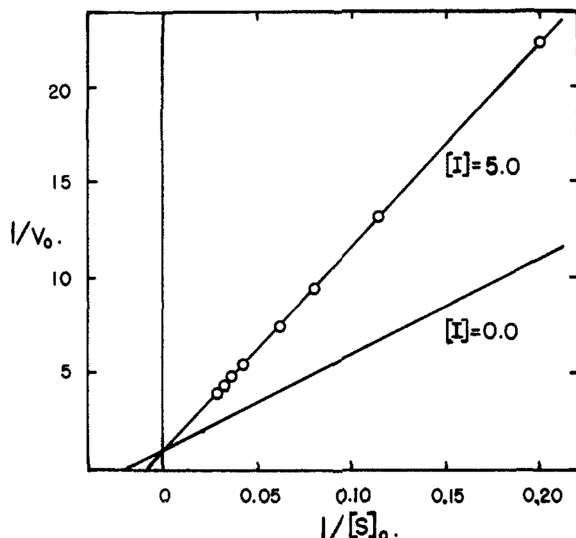


Fig. 6.—Inhibition of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at 25° and pH 7.6 by acetyl-D-tyrosine ethyl ester; v_0 in units of 10^{-3} M/min.; $[S]_0$ in units of 10^{-3} M; [I] in units of 10^{-3} M; [E] = 0.0294 mg. protein-nitrogen/ml.; 0.3 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

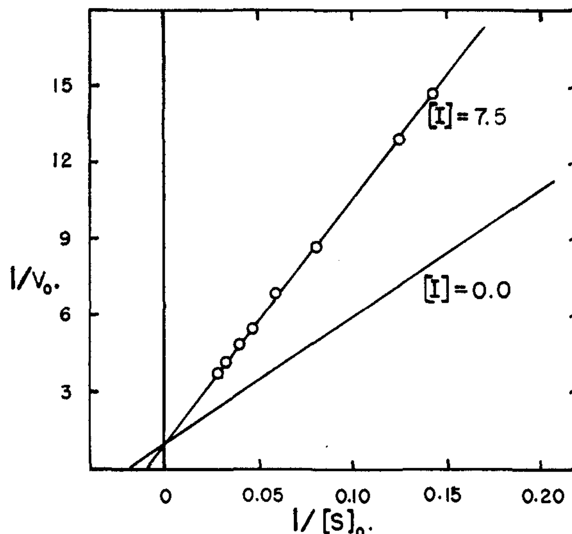


Fig. 8.—Inhibition of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at 25° and pH 7.6 by acetyl-D-tyrosinhydroxamide; v_0 in units of 10^{-3} M/min.; $[S]_0$ in units of 10^{-3} M; [I] in units of 10^{-3} M; [E] = 0.0294 mg. protein-nitrogen/ml.; 0.3 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

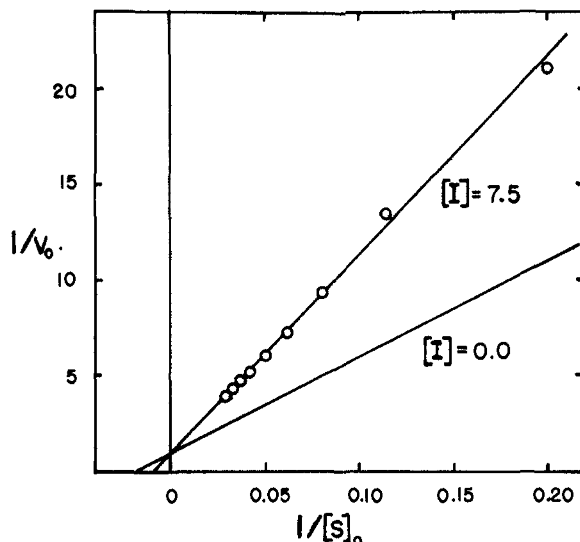


Fig. 7.—Inhibition of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at 25° and pH 7.6 by acetyl-D-tyrosinhydrazide; v_0 in units of 10^{-3} M/min.; $[S]_0$ in units of 10^{-3} M; [I] in units of 10^{-3} M; [E] = 0.0294 mg. protein-nitrogen/ml.; 0.3 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

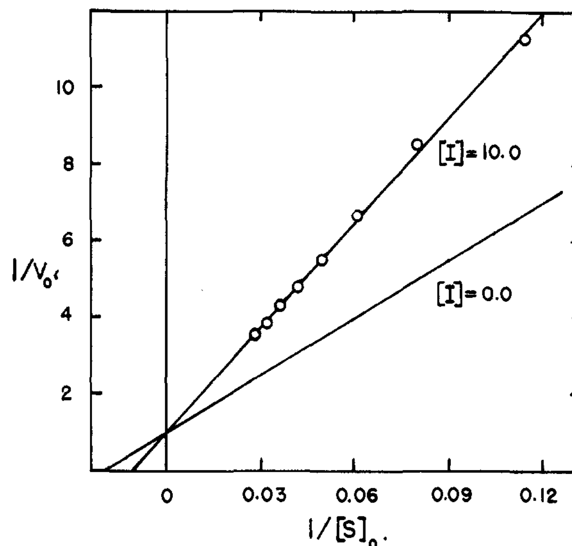


Fig. 9.—Inhibition of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at 25° and pH 7.6 by acetyl-D-tyrosinamide; v_0 in units of 10^{-3} M/min.; $[S]_0$ in units of 10^{-3} M; [I] in units of 10^{-3} M; [E] = 0.0294 mg. protein-nitrogen/ml.; 0.3 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

Aside from the knowledge that a fifteen-fold increase in the buffer concentration, and a decrease of 0.3 of a pH unit in the pH of the reaction system, caused no significant change in these two K_I values, these data provide substantial kinetic evidence that both acetyl-L-tyrosinamide and acetyl-L-tyrosinhydroxamide are hydrolyzed at the same catalytically active site.

For competitive inhibitors of the general formula $R_1CHR_2R_3$ where R_1 = acetamido and R_2 = p-hydroxybenzyl it is seen, from the data given in Table I, that with R_3 variant the affinity of α-chymotrypsin for inhibitors of the D-series is in the order $R_3 = -CO_2C_2H_5 > R_3 = -CONHNH_2 > R_3 = -CONHOH > R_3 = -CONH_2$ with a ratio of K_I values of 1.0:1.7:1.9:2.8, respectively. Since the ratio of the K_I values of acetyl-D-tryptophan ethyl ester (0.25×10^{-3} M[27]), acetyl-D-tryptophanhydrazide (0.75×10^{-3} M[27]) and acetyl-D-tryptophanamide (2.7×10^{-3} M[15]) is 1.0:3.0:10.8 it is apparent that, although the same order is observed when R_1 = acetamido and R_2 = β-indolylmethyl as that found when R_1 = acetamido and R_2 = p-hydroxybenzyl, the extent to which a

change in the nature of R_3 can influence the over-all combination process is clearly dependent upon the nature of R_2.

It is of interest to compare the effect that a variation in the nature of R_3 has upon the K_I values of competitive inhibitors possessing the D-configuration with its effect upon the K_S values of the corresponding enantiomophic L-specific substrates. The only data upon which such quantitative comparisons can be made at present relates to the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide and acetyl-L-tyrosinhydroxamide. The K_S values for these two specific substrates are 30.5×10^{-3} M,[5] and 51×10^{-3}, respectively, and the k_3 values are 2.4×10^{-3} M/min./mg. protein-nitrogen/ml.,[5] and 34×10^{-3} M/min./mg. protein-nitrogen/ml., respectively. The ratio of the K_S value of acetyl-L-tyrosinamide to that of acetyl-L-tyrosinhydroxamide is *ca.* 0.6 whereas the ratio of the K_I values of the corresponding D-enantiomorphs is *ca.* 1.5. It is not obvious whether the above relationship is due to the relatively large value of k_3 for acetyl-L-tyrosinhydroxamide or whether it really reflects a difference in the affinity of the enzyme for D- and L-enantiomorphs which varies with the nature of R_3. It appears that investigations which are now in progress in these laboratories will make it possible to decide which one of the above interpretations is correct.

Experimental[28,29]

Acetyl-D-tyrosine Ethyl Ester (I).—I, m.p. 96–97°, $[\alpha]^{25}D$ −24.8° (*c* 7% in ethanol) was prepared from acetyl-DL-tyrosine ethyl ester as directed by Thomas, MacAllister and Niemann[5]; lit.[5] m.p. 95–97°, $[\alpha]^{25}D$ −24.8° (*c* 7% in ethanol).

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.2; H, 6.8; N, 5.6.

Acetyl-D-tyrosinamide (II).—II, m.p. 225–226°, $[\alpha]^{25}D$ −51.1° (*c* 0.8% in water) was prepared by ammonolysis of I[5]; lit.[5] m.p. 225–226°, $[\alpha]^{25}D$ −49.4° (*c* 0.9% in water).

Anal. Calcd. for $C_{11}H_{14}O_3N_2$ (222); C, 59.4; H, 6.3; N, 12.6. Found: C, 59.5; H, 6.3; N, 12.6.

Acetyl-D-tyrosinhydrazide (III).—A solution of 6.0 g. of I in 15 ml. of absolute ethanol was slowly added to a boiling solution of 2.5 ml. of hydrazine hydrate in 5 ml. of absolute ethanol and this reaction mixture was heated under refluxing conditions for two hours. The precipitate which formed, after standing for three days at 4°, was recovered, recrystallized twice from water and dried *in vacuo* over phosphorus pentoxide to give 4.1 g. of III, m.p. 236–236.5°, $[\alpha]^{25}D$ −44.4° (*c* 0.4% in water).

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, 6.4; N, 17.6.

Acetyl-D-tyrosinhydroxamide (IV).—Eleven grams of I was dissolved in 22 ml. of 3.5 M hydroxylamine in methanol. This solution was cooled to 0°, 41 ml. of 1.3 N methanolic sodium methoxide added and the reaction mixture allowed to stand at 4° for five days. The crystalline precipitate which formed was recovered, washed with methanol and dried *in vacuo* over phosphorus pentoxide to give 7.5 g. of sodium acetyl-L-tyrosinhydroxamate (V), m.p. 182–183° with decomposition. Evaporation of the mother liquors to 40 ml. and addition of 20 ml. of 2.6 M hydroxylamine in methanol gave a second crop of 2.5 g. of V, m.p. 179–180° with decomposition. 7.5 g. of V and 3.6 g. of sodium bisulfate, previously dried *in vacuo* over phosphorus pentoxide for nine days (the apparent molecular weight of this sodium bisulfate was 124 as determined by titration with aqueous sodium hydroxide) were added to 180 ml. of absolute ethanol, the reaction mixture protected from the air, and shaken for 4.5 hours at 0°. The extremely fine precipitate of sodium sulfate which formed was removed and the filtrate evaporated *in vacuo* to give 6.4 g. of crystalline IV, m.p. 141–142° with decomposition. This material was recrystallized three times from water to give IV, m.p. 143–144° with decomposition, $[\alpha]^{25}D$ −38.5° (*c* 5% in water).

Anal. Calcd. for $C_{11}H_{14}O_4N_2$ (238): C, 55.5; H, 5.9; N, 11.8. Found: C, 55.6; H, 5.8; N, 11.7.

Acetyl-L-tyrosinhydroxamide (VI).—VI, m.p. 143–144°, $[\alpha]^{25}D$ +38.3° (*c* 5% in water) was prepared from acetyl-L-tyrosine ethyl ester, m.p. 96–97°, $[\alpha]^{25}D$ +24.6° (*c* 7% in ethanol), in the same manner and in approximately the same yield as was IV from I, except that isopropyl alcohol, as well as water, was used in the recrystallization of the product.

Anal. Calcd. for $C_{11}H_{14}O_4N_2$ (238): C, 55.5; H, 5.9; N, 11.8. Found: C, 55.5; H, 6.0; N, 11.6.

Buffer Solutions.—Technical tris-(hydroxymethyl)-aminomethane (Commercial Solvents) was recrystallized twice from aqueous methanol to give a product, large colorless rods, m.p. 169–169.5°. A stock solution 1.53 M with respect to the amine component was prepared by the addition of sufficient 3 N hydrochloric acid to an aqueous solution of the amine to give a solution of *p*H 7.62 at 25°. This stock solution was used in all studies conducted at *p*H 7.6, since it was found that in the presence of enzyme, substrate and inhibitor a 2.0:10.2 dilution of this stock solution gave a reaction mixture, 0.30 M in the amine component and of *p*H 7.60 ± 0.02 at 25°. A maximum *p*H change of only 0.05 of a *p*H unit was noted when as much as 0.015 M substrate was completely hydrolyzed. Other stock solutions 1.53 M with respect to the amine component were prepared in the same manner for studies at other *p*H values, *e.g.*, in the determination of the *p*H–activity curve.

Ferric Chloride Solutions.—A standard ferric chloride solution (solution A) was prepared as follows: 15.0 g. of reagent grade anhydrous ferric chloride was dissolved in 500 ml. of 1.29 N hydrochloric acid and diluted to 1 l. with absolute methanol. The ferric chloride solution used in the colorimetric determination of acetyl-L-tyrosinhydroxamide (solution B) was prepared by dilution of 100 ml. of solution A with 500 ml. of absolute methanol and sufficient water to make a total volume of 1 l. When kept in the dark, solution A was stable over a period of five months. Solution B was freshly prepared from solution A every few weeks and no instability was observed over this period. Stability of these solutions was measured by the intensity of the color formed with acetyl-L-tyrosinhydroxamide. The intensity of the color is sufficiently independent of the concentration of the ferric chloride so that a ±10% variation in this concentration does not cause an appreciable difference in the colorimeter reading for all concentrations of ferric chloride and acetyl-L-tyrosinhydroxamide used in this investigation; therefore it was possible, in spite of the deliquescence of the ferric chloride, to make up standard ferric chloride solutions which gave identical calibration curves in the colorimetric analysis of acetyl-L-tyrosinhydroxamide.

Enzyme Solutions.—Crystalline α-chymotrypsin (Armour, lot No. 90402), containing magnesium sulfate, was used in all of the experiments. Enzyme stock solutions were prepared daily and kept at 4°, except, for not more than four periods during the 24-hour interval, when they were brought to 25° for withdrawal of a 0.2-ml. aliquot. The enzyme solutions maintained a constant activity over this time interval; in fact the activity remained constant over a 48-hour interval when kept at 4°. The protein-nitrogen content of the enzyme solution was determined by the Kjeldahl method after precipitation with trichloroacetic acid.

Enzyme Reactions.—The substrate and buffer, or substrate, inhibitor and buffer, were added in solution to a 10-ml. G.-S. volumetric flask and the flask filled with water to the 10-ml. mark. The flask was then placed in a 25.00 ± 0.05° bath for at least 30 minutes. At minus 10 seconds from zero time, 0.2 ml. of enzyme solution was added and at zero time the flask was stoppered and gently inverted approximately 10 times. The flask was then replaced in the bath and 0.2-ml. aliquots were removed at convenient intervals and delivered into calibrated colorimeter tubes containing 10.0 ml. of ferric chloride solution B. The contents of the tubes were shaken vigorously and the intensity

(28) All melting points are corrected.

(29) The authors are indebted to Dr. A. Elek for the microanalyses reported in this section.

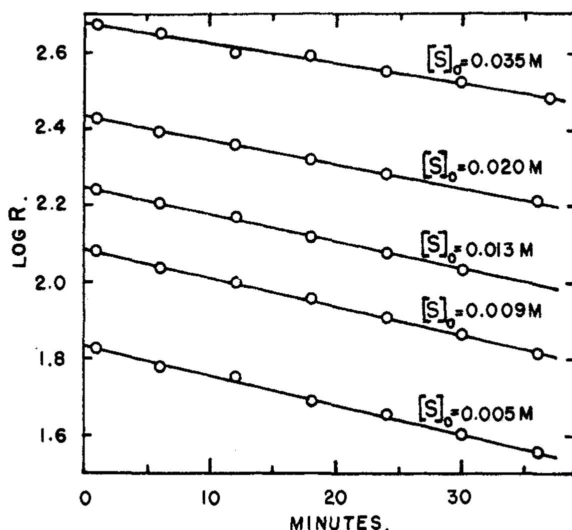


Fig. 10.—Estimation of initial velocities from apparent first-order plots; $[S]_0$ in units of 10^{-3} *M* acetyl-L-tyrosinhydroxamide; [E] = 0.0294 mg. protein-nitrogen/ml.; *R* = calorimeter reading; 0.3 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer at 25° and *p*H 7.6.

of the color determined with a Klett–Summerson photoelectric colorimeter equipped with a green filter (filter no. 54—transmission range *ca.* 500–570 mμ). A standard calibration curve was prepared from solutions containing varying amounts of acetyl-L-tyrosinhydroxamide in a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer 0.30 *M* in respect to the amine component. It was found that for concentrations of acetyl-L-tyrosinhydroxamide up to 0.050 *M* in the original solution the colorimeter readings were directly proportional to the concentration of the hydroxamide. The proportionality constant was found to be independent of the presence of α-chymotrypsin, acetyl-L-tyrosine, hydroxylamine, acetyl-D-tyrosine ethyl ester, acetyl-D-tyrosinamide and acetyl-D-tyrosinhydrazide, in amounts equal to or greater than the maximum amounts involved in the enzymatic reactions. The same proportionality constant was observed with acetyl-D-tyrosinhydroxamide, and this constant was found to be independent of the hydrogen ion concentration over the *p*H range from 6.5 to 9.1. The color formed, whether from the addition of aliquots from the enzyme reaction mixtures or from the solutions used for calibrations, was stable over a period of two hours after mixing. All readings were taken within this time interval and were reproducible to within ± 2%.

As the initial portion of the rate curves (20–30% hydolysis) were always observed to be first order, values of $\log_{10}R$ (*R* = colorimeter reading) were plotted against time (typical curves are given in Fig. 10) and the initial velocity at zero time determined from the apparent initial first-order rate constant and the initial substrate concentration. It should be pointed out that it is only necessary to know that the colorimeter readings are directly proportional to the acetyl-L-tyrosinhydroxamide concentration, since the value of the proportionality constant is not a factor in the determination of the apparent first-order constant.

Apparent Ionization Constants of Acetyl-L-tyrosinhydroxamide.—A 2.00-ml. aliquot of a 0.01002 *M* aqueous solution of acetyl-L-tyrosinhydroxamide was titrated with a 0.01075 *N* aqueous sodium hydroxide solution and the titration followed with a Beckman model G *p*H meter. The data so obtained was treated essentially as described by Simms[30] and from this treatment values of pK'_{A_1} and pK'_{A_2} of 9.0 and 10.2 were obtained. The calculated curve given in Fig. 2 is based upon these latter values. It is believed that the large deviation of the experimental points from this curve at *p*H values greater than 10.5 is due to the fact that a given error in the *p*H determination creates a larger and larger error in *b'* as the *p*H approaches a value of 12, and that at *p*H values greater than 10.5 the *p*H meter becomes inaccurate. A sodium ion correction was made according to the manufacturer's directions but this is clearly only an approximation for *p*H values above 10.5. Calculated curves based upon the use of pK'_A values that differed from those used for the construction of the curve given in Fig. 2 by ±0.1 of a *pK'* unit indicated that the pK'_A values given above, *i.e.*, 9.0 and 10.2, can be considered to be accurate to within ± 0.1 of a *pK'* unit. A duplicate experiment gave data that agreed with that given in Fig. 2 to within ± 0.03 of a *p*H unit and gave the same pK'_A values to within ± 0.1 of a *pK'* unit.

(30) H. Simms, THIS JOURNAL, **48**, 1239 (1926).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN]

Purification and Kinetics of β-D-Galactosidase from *Escherichia coli*, Strain K-12[1,2]

BY STEPHEN A. KUBY[3] AND HENRY A. LARDY

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β-D-Galactosidase has been purified about 100-fold from an extract of dried *Escherichia coli* cells by fractionation with methanol, $MnSO_4$, $(NH_4)_2SO_4$ and $Ca_3(PO_4)_2$. The effect of *p*H, substrate concn., type of substrate, temp., competitive and non-competitive inhibitors, and ionic environment on the catalytic activity of the purified enzyme have been investigated.

β-D-Galactosidase, an adaptive enzyme of *Escherichia coli*, strain K-12, has been studied in some detail in the crude state by Lederberg.[4] Cohn and Monod[5] purified a similar enzyme from *E. coli*, strain ML, and investigated many of its properties, especially the effects of ions on the rate of hydrolysis of β-D-galactosides.

It is the purpose of this paper to report a method of purification of β-D-galactosidase from *E. coli*, strain K-12, and to describe some of the catalytic properties of this purified enzyme.

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(2) Supported by grants from the United States Public Health Service and the University Research Committee.

(3) Predoctorate Fellow, National Institutes of Health.

(4) J. Lederberg, *J. Bact.*, **60**, 381 (1950).

(5) M. Cohn and J. Monod, *Biochim. Biophys. Acta*, **7**, 153 (1951).

Experimental

Analytical Methods.—The "chromogenic" substrate, *o*-nitrophenyl β-D-galactoside, introduced by Lederberg,[4] was used for following the purification of the enzyme.

Aliquots were withdrawn periodically from the reaction mixture and pipetted directly into a Na_2CO_3 solution (final molarity of Na_2CO_3, 0.1 *M*) to stop the enzymatic hydrolysis and to produce the maximum possible absorption due to the liberated *o*-nitrophenolate ion. *o*-Nitrophenol was determined colorimetrically using the Evelyn colorimeter with a 420 mμ filter. Although Beer's law is followed reasonably well over a fairly wide range of concentration, correc-