[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Thermodynamic Study of an Enzyme–Substrate Complex of Chymotrypsin. I[1,2]

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Experiments with O^{18}-enriched water have shown that chymotrypsin will catalyze the carboxyl oxygen exchange of acetyl-3,5-dibromo-L-tyrosine and hence this compound is a substrate for chymotrypsin. No net change occurs in the exchange reaction, making it possible to make direct equilibrium measurements of the enzyme-substrate binding. Using a radioactive bromine-labeled substrate and the equilibrium dialysis technique, the binding has been measured as a function of *p*H, temperature and substrate concentration. It is found that one substrate molecule is bound per chymotrypsin molecule of molecular weight 22,000. There is an apparent correlation between the variation of the thermodynamic functions of the enzyme substrate bond, and the activity of the enzyme as a function of *p*H.

Introduction

Current theories as to the nature of enzyme-catalyzed reaction include the formation of an intermediate enzyme–substrate complex, as first postulated by Michaelis and Menten.[3] Modern extension and elaboration of this theory have served to widen its scope and to yield good agreement with the experimentally observed kinetics of enzyme reactions under a wide variety of conditions. However, valuable as this concept of complex formation has been in kinetic interpretations, its presence has only recently been directly demonstrated for any enzyme.[4] Keilin and Mann and others have spectrophotometrically shown the existence of complexes of peroxides with catalase and peroxidase. Calculations of the free energy, entropy and enthalpy of the enzyme–substrate complex have rested upon ratios of rate constants which are resultants of several reactions that cannot be measured directly with the techniques available at this time. Consequently, calculation of the energetics of enzyme systems has necessarily been partly ambiguous and dependent on a number of approximations.[5]

In this study we have obtained thermodynamic functions of the enzyme–substrate complex under equilibrium conditions through the use of an isotopically labeled substrate and direct measurement of its binding by the enzyme molecule. The substrate used for the equilibrium experiments was acetyl-3,5-dibromo-L-tyrosine. That this compound could function as a substrate has been demonstrated by its enzyme-catalyzed oxygen exchange; furthermore, its carboxyl derivatives undergo the normal chymotrypsin-catalyzed reactions.

Preliminary Considerations.—An examination of the reactions catalyzed by α-chymotrypsin reveals that this enzyme will hydrolyze acetyl-L-tyrosine amide and acetyl-L-tyrosine methyl ester[5] and that it will synthesize acetyl-L-tyrosine anilide. Similar reactions have been established here for derivatives of 3,5-dibromo-L-tyrosine and the K_S and V_{max} determined for glycyl-3,5-dibromo-L-tyrosine amide and acetyl-3,5-dibromo-L-tyrosine amide. A single mechanism may be postulated for these diverse reaction, *i.e.*, the exchange of a group such as –OH, $-OCH_3$, $-S-C_2H_5$,[6] –NHR, $-NHNH_2$, –NHOH for another of this type. The free groups appear to be characterized primarily by one or more unshared electron pairs and when combined with hydrogen are weak acids. Thus the actual substrate becomes the acetyl-L-tyrosyl radical without the carboxylic hydroxyl group. The final product in such a series of exchange reactions with specific substrates will be determined by the free energy changes of the process. One consequence of such a postulate would necessarily be the enzyme-catalyzed exchange of the carboxyl hydroxyl group of acetyl-L-tyrosine with the hydroxyl group of the water solvent. This has been experimentally established in this work using O^{18} as a tracer.[7]

Since the mode of action of the enzyme toward each of these derivatives is essentially the same, it might be assumed that any binding properties that can be shown to be dependent on enzyme action can be obtained from the binding of any one of the derivatives. Utilizing acetyl-3,5-dibromo-L-tyrosine as a substrate for chymotrypsin, it becomes possible to measure enzyme–substrate equilibria in a static system, since no net reaction occurs in solutions of the free acid or salt of acetyl-3,5-dibromo-L-tyrosine. Measurements obtained in this manner should be characteristic of the enzyme–substrate system. True equilibrium constants and the thermodynamic data derivable from them can then be determined from the combined and uncombined substrate concentrations, limited to the extent that activities can be measured and standard states defined for both substrate and enzyme and that the Donnan equilibrium can be calculated or compensated for.

Results and Discussion

Kinetics.—In order to establish the susceptibility of 3,5-dibromo-L-tyrosine derivatives to chymotrypsin hydrolysis, acetyl-3,5-dibromo-L-tyrosine amide, acetyl-3,5-dibromo-L-tyrosine methyl ester and ethyl ester, and glycyl-3,5-dibromo-L-tyrosine amide were prepared. The enzyme catalyzed the hydrolysis of all these compounds although, due to solubility difficulties, kinetic measurements were obtained only with glycyl-3,5-dibromo-L-tyrosine amide and acetyl-3,5-dibromo-

(1) Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

(2) This paper was presented in part before the Biological Chemistry Division of the American Chemical Society, Chicago, Ill., Sept., 1950.

(3) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

(4) D. Keilin and T. Mann, *Proc. Roy. Soc.* (*London*), **B122**, 119 (1937); K. G. Stern, *J. Biol. Chem.*, **114**, 473 (1939); B. Chance, *ibid.*, **151**, 553 (1943); *Science*, **109**, 204 (1949).

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

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

(7) Since preparation of this manuscript, Sprinson and Rittenberg (*Nature*, **167**, 484 (1951)) have reported similar results.

L-tyrosine amide. The synthetic reaction was established by the formation of acetyl-3,5-dibromo-L-tyrosine anilide in small amounts from a mixture of acetyl-3,5-dibromo-L-tyrosine, aniline and chymotrypsin. Representative results of typical kinetic measurements calculated by the method of Lineweaver and Burk[8] are shown in Fig. 1 plotted as $1/V$ against $1/[S]_0$. From these data, the V_{max} for acetyl-3,5-dibromo-L-tyrosine amide was calculated to be 182 micromoles/ml./hour with a K_S of 0.08 M; for glycyl-3,5-dibromo-L-tyrosine amide, V_{max} was calculated to be 31 micromoles/ml./hour with a K_S of 0.05 M.

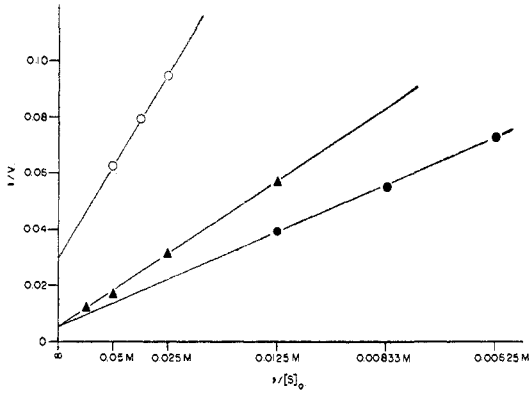

Fig. 1.—Activity of chymotrypsin: $1/V$ in micromoles/ml./hr./mg. N/ml.; $1/[S]_0$ in moles; enzyme concn. = 1 mg. N/ml.; pH 7.8; 5 min. values; buffer, tris-(hydroxymethyl)-aminomethane·HCl: ▲, glycyl-L-tyrosine amide acetate, T 25°; O, glycyl-3,5-dibromo-L-tyrosine amide HBr, T 25°; ●, acetyl-3,5-dibromo-L-tyrosine amide, T 40°.

Oxygen Exchange.—While no attempt was made to obtain kinetic data on the enzyme-catalyzed exchange of oxygen, the demonstration that the enzyme strongly increased the rate of the exchange reaction could be taken as meaning that acetyl-3,5-dibromo-L-tyrosine is a substrate for chymotrypsin. Accordingly, the amount of exchange in 24 hours of acetyl-3,5-dibromo-L-tyrosine with enriched water was determined for uncatalyzed, chymotrypsin-catalyzed and acid-catalyzed systems, and the results shown in Table I were obtained.

Two methods of obtaining carbon dioxide for isotope analyses were used. Both methods of decarboxylation clearly show a much higher rate of exchange for the enzyme-containing system over the uncatalyzed system. The decarboxylation reactions are not entirely satisfactory, however, and probably involve some oxygen interchange which would tend to lower the apparent amount of exchange. The value for the uncatalyzed exchange by the bromine method is in essential agreement with the work of Mears and Sobotka[9] who found zero exchange for tyrosine as well as several proteins in neutral solution.

The oxygen exchange of the free acid or salt can be predicted by the following argument. If the enzyme acts as a true catalyst and the catalyzed reaction is reversible, then it is necessary that the enzyme act on the products in the same manner that it had acted upon the original substrate. The reverse reaction must retrace the paths of the primary reaction. Thus, in the enzyme-catalyzed hydrolysis of an amide to an acid and ammonia, the reverse reaction must take place by combination of the acid and ammonia with the enzyme and lead to an activated complex identical with that obtained from the amide and water. In view of the apparent equivalence of the replacing and replaced groups, *e.g.*, (C_2H_5O–, C_2H_5S–, NHR–, OH–) on the substrate, any two different or two similar groups may be involved in the enzyme reaction. If the two groups were hydroxyls, then oxygen exchange may be expected. Fruton[10] and co-workers have reported an exchange of ammonia in the hydrolysis of hippuryl amide by papain where this mechanism may also apply.

While the theory does not actually specify that the replacement group must be bound by the enzyme, preliminary measurements by the authors have shown that tagged p-bromoaniline is bound by α-chymotrypsin. Also Laidler[11] has shown that water is bound to the active site of urease, indicating in these two cases that the replacement group is bound by the enzyme.

Enzyme–Substrate Equilibria.—The dialysis equilibrium technique of Klotz[12] was utilized for substrate binding measurements. Acetyl-L-tyrosine was brominated with Br_2 containing radioactive Br^{82} to produce the labeled substrate, acetyl-3,5-dibromo-L-tyrosine. The concentration of the substrate in the buffer and in the protein solution was determined from the radioactivity in each solution. The variation of the amount of substrate bound per gram of enzyme, as a function of substrate concentration, is shown in Fig. 2. The

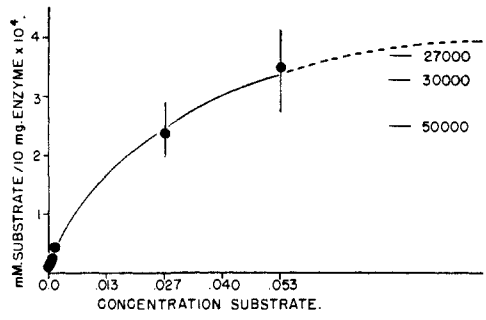

Fig. 2.—mM. × 10^4 of acetyl-3,5-dibromo-L-tyrosine bound per 10 mg. of chymotrypsin in 1% chymotrypsin solution.

theoretical amounts bound corresponding to one binding position per protein molecule for several published molecular weights are shown at the right of the figure. A plot Fig. 3 of the reciprocal quantities, *i.e.*, mg. protein/mM. bound substrate as a function of the reciprocal substrate concentration, for simple mass action behavior, should yield a straight line which may be extrapolated to give the molecular weight of the protein. Since the

(8) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).
(9) W. H. Mears and H. Sobotka, *ibid.*, **61**, 880 (1939).
(10) J. S. Fruton, *Yale J. Biol. Med.*, **22**, 263 (1950); R. B. Johnston, M. J. Mycek and J. S. Fruton, *J. Biol. Chem.*, **185**, 629 (1950); *ibid.*, **187**, 205 (1950).
(11) K. J. Laidler and J. P. Hoare, THIS JOURNAL, **71**, 2699 (1949).
(12) I. M. Klotz, F. M. Walker and R. B. Pivan, *ibid.*, **68**, 1486 (1946).

average precision of measurement of the total radioactivity was ±0.15% and, at the highest concentration of substrate, the amount bound was 0.66% of the total radioactivity, the extrapolated value of 22,000 is in good agreement with the probable best value for the molecular weight of chymotrypsin of 21,500.[13] This offers good evidence that there is one active center per chymotrypsin molecule. These data were obtained at *p*H 7.5 and 20°. At lower *p*H's, the insolubility of the substrate prevented accurate measurement of the effect of substrate concentration on binding.

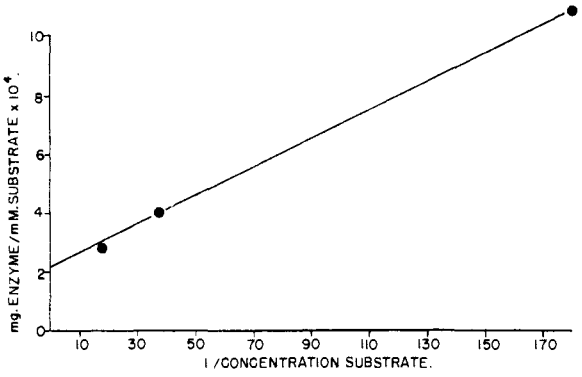

Fig. 3.—Mg. of enzyme binding 1 m*M*. substrate as function of reciprocal substrate concentration.

At substrate concentrations below 10^{-4} *M*, the amount of enzyme bound in the complex was found to be less than 1% of the enzyme in solution. Under these conditions, the dissociation constant for the binding reaction is approximately proportional to the reciprocal of the percentage of substrate bound; also, the per cent. bound is independent of the substrate concentration. The per cent. of substrate bound by 1% chymotrypsin solutions as a function of *p*H at 5 and 20° is shown in Fig. 4 for substrate concentrations below 10^{-4} *M*. Although it was desirable to measure binding above *p*H 8, autolysis of the protein molecule made experimental results meaningless. No measurements could be obtained at 20° above *p*H 7.6, while measurements at 5° yielded several points of doubtful precision between *p*H 7.6 and 8.0. A smooth curve has been drawn through the experimental points.

The activity maximum of chymotrypsin for this type of substrate is at about *p*H 7.8[5] and drops rapidly on either side. It is seen from the curve that the binding at this point is relatively low and is dropping rapidly toward higher *p*H's. The binding is temperature independent at low *p*H and as the enzyme becomes active the binding becomes temperature dependent, the dependence becoming quite large at the maximum *p*H that could be measured.

On the basis that the substrate combined with the enzyme only when a certain basic group was ionized, *i.e.*, combined with hydrogen ion, some attempts were made to fit mass action curves to the data. The two equilibrium equations

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

$$\frac{(E)(H^+)}{(EH^+)} = K_1 \quad (1)$$

$$\frac{(EH^+)(S)}{(EHS^+)} = K_2 \quad (2)$$

and the expressions for the total enzyme and substrate

$$T = E + EH^+ + EHS$$
$$R = S + EHS$$

can be combined to give the equation

$$\frac{(T - EHS)(R - EHS)}{EHS} = \frac{K_2(K_1 + H^+)}{H^+} \quad (3)$$

This expression, using constants calculated from the experimental data, did not fit the experimental curve. The multiplication of K_2 by a term $e^{-K_3\nu}$ (ν is the net charge on the enzyme) which would compensate for the change in charge of the protein molecule[14] did not particularly improve the fit of the curve. In addition to the ionization of the enzyme groups in the *p*H range studied, the substrate has groups of *pK* 3.2 and 6.6. The mathematical consideration of the *pK* 6.6 group along with the ionization of one enzyme group was also unsuccessful in fitting the curve. In view of the range of *p*H and the different types of groups involved it appears that more data is necessary before the function of the hydrogen ion can be determined.[15]

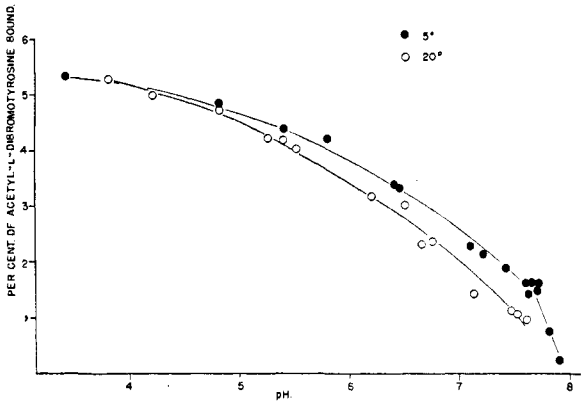

Fig. 4.—Percentage of acetyl-3,5-dibromo-L-tyrosine bound by 1% chymotrypsin solution as a function of *p*H; substrate less than 10^{-4} *M*.

Although the hydrogen ion is probably involved in the equilibria its exact function is not known; therefore, it was disregarded in all calculations of equilibrium constants and thermodynamic functions. Using the values 22,000 for the molecular weight of chymotrypsin and the per cent. substrate bound by a 1% enzyme solution, the apparent equilibrium constant may be calculated from equation (4)

$$K = \frac{\text{enzyme concn.} \times 100}{22{,}000 \times \%\ \text{bound}} \quad (4)$$

From the equilibrium constants and their variation with temperature, apparent values for the free energy, entropy and enthalpy change for the enzyme–substrate combination have been calcu-

(14) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(15) An entirely empirical equation involving three constants and fitting the data quite well is given for the 5° data: % binding = 4.2 + 8.9 (*p*H) − 0.16 (*p*H).[2]

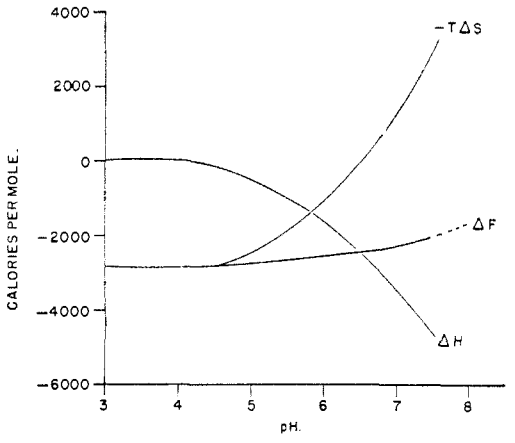

Fig. 5.—Variation of apparent thermodynamic functions of enzyme-substrate complex with *p*H. Free energy is average of 20 and 5° values and other functions calculated from change in equilibrium constant between above two temperatures.

lated. The results as a function of *p*H are shown in Fig. 5. It is seen that, throughout the *p*H range, the free energy of binding is relatively small and does not change greatly. In the low *p*H range, ΔH is 0 and the binding is primarily an entropy effect, characteristic of the binding of many small molecules by proteins.[16] As the *p*H is increased both the entropy and enthalpy rapidly become more negative, roughly parallel to the change in enzyme activity. The maximum value of the enthalpy (−5000 cal.) is close to that characteristic of imidazole groups[17] and, in addition, is in a *p*H region where the imidazoles are rapidly changing their degree of ionization.[18] Since Brand[19] has found two imidazole groups in chymotrypsinogen, the binding might be ascribed to ionized imidazole groups. However, the variation of binding extends over a longer *p*H region than could be expected on the basis of one or two groups. It is also true that the negative entropy and enthalpy calculated for the high *p*H region might indicate a strained state of the enzyme–substrate complex. At the same time the free energy of binding is low, allowing easy splitting of the substrate from the enzyme, factors of obvious advantage for a catalyst. This suggests that the binding change may be due to some more fundamental change in the enzyme structure than the ionization of a single group. At this time, however, any theory is largely speculative and further work is in progress to determine the nature of the variation of the thermodynamic quantities with *p*H and the properties of the enzyme–substrate bond.

Experimental

Enzyme.—The α-chymotrypsin utilized in this study was prepared from frozen bovine pancreas according to the method of Kunitz and Northrup.[20] The active enzyme was recrystallized three times, dialyzed at 5° until salt free, lyophilized and kept at 5°. Enzymatic activity was determined by a potentiometric formol titration of 0.2-ml. to 1.0-ml. aliquots of the buffered enzyme substrate mixtures. The aliquot was added to 1 ml. neutralized 37% formaldehyde in a 10-ml. beaker, Beckman type G electrodes inserted and the mixture stirred with a magnetic flea. Sodium hydroxide 0.01 *N* was added under the surface of the mixture to an end-point *p*H determined from titration curve of a 50% hydrolyzed sample of the mixture. Glycyl-L-tyrosine amide acetate yielded a V_{max} of 182 micromoles/ml./hour/mg. *N*/ml. with a K_S of 0.12 *M* (Fig. 1) comparable with previously published results. Electrophoretic analysis revealed the presence of only one component.

Substrates.—N-Acetyl-L-tyrosine derivatives were prepared by an improved procedure which yielded crystalline derivatives at each step. The key reaction was the acetylation of L-tyrosine ethyl and methyl esters to give N-acetyl-L-tyrosine ethyl and methyl ester.

N-Acetyl-L-tyrosine Ethyl Ester.—L-Tyrosine ethyl ester was prepared from the hydrochloride according to the procedure of Hillmann.[21] Ten and a half grams of L-tyrosine ethyl ester was added to 50 ml. of glacial acetic acid and 8 ml. of acetic anhydride. The mixture was heated to boiling for 1 minute and allowed to stand at room temperature for 1 hour. The solution was evaporated *in vacuo* to a sirup, taken up in ethyl acetate and evaporated *in vacuo* twice to remove excess acetic acid. The sirupy product was dissolved in 50 ml. of methyl alcohol and water added to turbidity. Upon scratching, crystallization took place. The crystals were filtered after 24 hours in the ice box; yield 12.6 g., m.p. 80–81°, 79[22]; $[\alpha]^{26}D$ +23.3° (*c* 2% in ethanol).

N-Acetyl-L-tyrosine methyl ester was prepared in a similar manner, 16.8 g. of the product being obtained from 15 g. of the free methyl ester, m.p. 118–118.5°, $[\alpha]^{25}D$ +26.8° (*c* 2% in ethanol). *Anal.* Calcd. for $C_{12}H_{15}O_4N$ (237.2); N, 5.9. Found: N, 5.8.

N-Acetyl-L-tyrosine amide was obtained in the usual manner with methanolic ammonia from either ester; m.p. 223–224°, $[\alpha]^{25}D$ +32.7° (*c* 2% in ethanol).[23]

N-Acetyl-L-tyrosine was prepared from either ester by dissolving in alcohol, adding two equivalents of 1 *N* sodium hydroxide and neutralizing with two equivalents of 1 *N* hydrochloric acid after 20 minutes. The solution was evaporated *in vacuo* to dryness and taken up in hot ethyl acetate; sodium chloride was filtered off and petroleum ether carefully added to the solution to turbidity. Crystallization took place in the ice-box; m.p. 152–153°, $[\alpha]^{25}D$ +59.6° (*c* 2% in ethanol).[24]

Glycyl-L-tyrosine Amide Acetate.—This substrate was prepared according to the procedure of Fruton and Bergmann.[25] In order to prevent the formation of a diketopiperazine, acetic acid was added after the reduction of carbobenzoxyglycyl-L-tyrosine amide was complete.

N-Acetyl-3,5-dibromo-L-tyrosine.—This compound was prepared by the bromination of N-acetyl-L-tyrosine. It was also prepared by de-esterification of acetyl-3,5-dibromo-L-tyrosine ethyl and methyl esters.

Twenty grams of N-acetyl-L-tyrosine was dissolved in 30 ml. of glacial acetic acid, and 28 g. of bromine in 60 ml. of glacial acetic acid was added to this solution in portions with cooling in ice over a 15-minute period. The mixture was kept at room temperature overnight and was then evaporated *in vacuo* to a sirup which was taken up in water and evaporated twice *in vacuo* to remove excess HBr and acetic acid. The resulting sirup was dissolved in 50 ml. of ethyl alcohol and water added to turbidity. Scratching induced crystallization and after standing overnight in the ice-box, crystals were filtered and dried; yield 26.1 g., sol. alcohol, dioxane, ethyl acetate, acetone; m.p. 115°. Recrystallization to constant m.p. from aqueous alcohol raised the melting point to 123–123.5°, $[\alpha]^{25}D$ +37.7° (*c* 2% in ethanol). *Anal.* Calcd. for $C_{11}H_{11}O_4NBr_2$ (381.0); N, 3.7; Br, 41.9. Found: N, 3.6; Br, 41.7.

This compound was also obtained in good yield by de-

(16) I. M. Klotz, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 97 (1949).

(17) E. J. Cohn, *Ergebnisse d. Physiol.*, **33**, 781 (1931).

(18) R. K. Cannan, A. C. Kibrick and A. H. Palmer, *J. Biol. Chem.*, **142**, 803 (1942).

(19) J. H. Northrup, M. Kunitz and R. M. Herriot, "Crystalline Enzymes," 2nd ed., Columbia U. Press, New York, N. Y., 1948.

(20) M. Kunitz and J. H. Northrup, *J. Gen. Physiol.*, **18**, 433 (1935); **19**, 991 (1936).

(21) G. Hillmann, *Z. Naturforsch.*, **1**, 682 (1946).

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

(23) S. Kaufman and H. Neurath, *ibid.*, **180**, 181 (1949).

(24) V. du Vigneaud and C. E. Meyer, *ibid.*, **98**, 295 (1932).

(25) J. S. Fruton and M. Bergmann, *ibid.*, **145**, 253 (1942).

esterification of N-acetyl-3,5-dibromo-L-tyrosine ethyl ester. Of the above ester, 12.3 g. was dissolved in 100 ml. of ethyl alcohol and 60 ml. of 1 *N* sodium hydroxide was added. After half an hour 60 ml. of 1 *N* hydrochloric acid was added, the solution diluted with water to turbidity, seeded and kept at 5° overnight. The product was filtered and dried *in vacuo;* yield 9.5 g., m.p. 123°.

N-Acetyl-3,5-dibromo-L-tyrosine Ethyl Ester.—3,5-Dibromo-L-tyrosine was prepared according to the method of Zeynek[26] and converted to the ethyl ester by the method of Abderhalden and Malin.[27] Acetic anhydride (5.5 ml.) was added to 13.2 g. of 3,5-dibromo-L-tyrosine ethyl ester dissolved in 75 ml. of glacial acetic acid. The mixture was heated to boiling for 1 minute, allowed to stand at room temperature 1 hour and then evaporated *in vacuo* to dryness when crystallization occurred. The solid material was dissolved in 75 ml. of hot ethyl acetate, petroleum ether added to a faint turbidity, the solution was seeded and allowed to crystallize at 5° overnight. The product was filtered and dried; yield 13.7 g., m.p. 140–141°, $[\alpha]^{25}D$ +15.2° (*c* 2% in ethanol). *Anal.* Calcd. for $C_{13}H_{15}O_4NBr_2$ (409.1): N, 3.4; Br, 39.1. Found: N, 3.4; Br, 39.2.

N-Acetyl-3,5-dibromo-L-tyrosine Methyl Ester.—3,5-Dibromo-L-tyrosine (13.6 g.) was suspended in 200 ml. of dry methanol and saturated with dry hydrochloric acid gas. The solution was evaporated *in vacuo* to dryness and the process repeated. The solid material was taken up in 50 ml. of methanol. Ether was added slowly to crystallization and the solid material filtered and dried after standing at 5° for 12 hours; yield 15 g., m.p. 203° $[\alpha]^{25}D$ +5° (*c* 2% in H_2O). *Anal.* Calcd. for $C_{10}H_{11}O_3NBr_2 \cdot HCl$ (389.5): N, 3.6. Found: N, 3.3.

The above ester hydrochloride was converted to the free ester in the usual manner in 75% yield; m.p. 199°, $[\alpha]^{25}D$ +8.3° (*c* 1.5% in pyridine). *Anal.* Calcd. for $C_{10}H_{11}O_3NBr_2$ (353.0): N, 4.0; Br, 45.3. Found: N, 3.8; Br, 45.3.

Six grams of 3,5-dibromo-L-tyrosine methyl ester was converted to the N-acetyl derivative by a procedure similar to that used for the ethyl ester; yield 4.2 g., m.p. 145°, $[\alpha]^{25}D$ +15.5° (*c* 2% in ethanol). *Anal.* Calcd. for $C_{12}H_{13}O_4NBr_2$ (395.0): N, 3.5; Br, 40.5. Found: N, 3.5; Br, 40.4.

N-Acetyl-3,5-dibromo-L-tyrosine Amide.—Eight grams of N-acetyl-3,5-dibromo-L-tyrosine methyl ester was added to 100 ml. of dry methanol saturated with dry ammonia at 0° and allowed to stand at room temperature 1 day. The solution was evaporated *in vacuo* to a sirup. The sirup was dissolved in 50 ml. of methanol and water added cautiously with scratching to facilitate crystallization. The product was filtered and dried after 1 day in the ice-box; yield 5.3 g., m.p. 240–241°, $[\alpha]^{25}D$ +24.4° (*c* 2% in ethanol). *Anal.* Calcd. for $C_{11}H_{12}O_3N_2Br_2$ (380.0): N, 7.4; Br, 42.0. Found: N, 7.4; Br, 42.1.

This compound could be prepared in a similar manner in a much lower yield from the ethyl ester.

Glycyl-3,5-dibromo-L-tyrosine Amide Hydrobromide.—Two grams of glycyl-L-tyrosine amide acetate was dissolved in 20 ml. of glacial acetic acid and a mixture of 3 g. of bromine in 10 ml. of glacial acetic acid added in portions with cooling in ice over half an hour. A gum precipitated from the solution. After 1 hour the mixture was evaporated *in vacuo* to a sirup, taken up in absolute ethyl alcohol, and evaporated *in vacuo* to remove traces of acetic acid. The sirupy residue was taken up in ethanol and ether added slowly with scratching to facilitate crystallization. The mixture was kept overnight at 5°, filtered and dried *in vacuo;* yield 2.8 g., m.p. 239°, dec., $[\alpha]^{25}D$ +17.4° (*c* 2% in ethanol). *Anal.* Calcd. for $C_{11}H_{14}O_3N_3Br_3$ (475.9): C, 27.6; H, 3.0; N, 8.8; Br, 50.4. Found: C, 28.0; H, 3.2; N, 8.6; Br, 50.4.

Radioactive N-Acetyl-3,5-dibromo-L-tyrosine.—This was obtained by a procedure very similar to that described previously for the direct bromination with inactive bromine. Pile bombarded NH_4Br (125 mg.) was treated with a chromic acid solution, the bromine produced carried off by an air stream bubbled through this solution and through a solution of 70 mg. of N-acetyl-L-tyrosine in 1 ml. of glacial acetic acid. The solution was heated in a steam-bath for 1 minute and then evaporated to dryness *in vacuo*. The product was crystallized from water, dissolved in 5 ml. of 0.04 *N* NaOH–0.25 *N* NaCl solution and placed on a 1-cm. diameter by 5-cm.-long Dowex-1-column in equilibrium with the same solvent. Elution was effected with the same solution. Two hundred milliliters of the peak of the eluate was acidified and extracted with ethyl acetate. The extract was washed with water, re-extracted into an aqueous sodium bicarbonate solution and this used as stock. Analysis for quantity was made spectrophotometrically in 0.008 *N* NaOH, 0.05 *N* NaCl solution at 2300 Å. The absorption spectrum was compared with the non-radioactive compound as a check for purity.

Oxygen Exchange.—Thirty milligrams of the sodium salt of N-acetyl-3,5-dibromo-L-tyrosine and 15 mg. of chymotrypsin was dissolved in 5 ml. of O^{18}-enriched water, the *p*H adjusted to 7.5 with NaOH and the mixture allowed to stand 16 hours at 20°. The solution was then boiled for 1 minute and the precipitated protein centrifuged off. An excess of silver nitrate solution was added precipitating the N-acetyl-3,5-dibromo-L-tyrosine silver salt which was washed, vacuum dried, and decarboxylated by two different methods. The first method was to heat the sample with a free flame under vacuum until it pyrolyzed. The carbon dioxide produced was passed through a Dry Ice trap and collected in a liquid nitrogen trap, volatile impurities being pumped off. The carbon dioxide was then sealed in glass tubes and analyzed in a mass spectrograph. This method gave a low yield, so another somewhat better method was tried. The tube containing the silver salt was evacuated, an excess (100 mg.) of Br_2 distilled in, the tube closed off and heated to 150° for 10 minutes. The volatile contents of the reaction tube were then passed through a −120° trap and condensed in a −190° trap, and the non-condensable material pumped off. The carbon dioxide was then passed over a red-hot silver spiral in a quartz tube to remove any Br_2 or HBr. After another Dry Ice–liquid nitrogen fractionation the carbon dioxide was sealed off for mass spectrographic analysis.

In the control no enzyme was added but other procedures were identical. The chymotrypsin was removed from solution by boiling to the extent that in the absence of substrate no precipitate was obtained with silver nitrate.

Equilibrium Experiments.—Buffer solutions of 0.1 *N* NaCl and 0.01 *M* phosphate ion adjusted to *p*H between 4 and 8 were used as solvents. Five milliliters of buffer containing 50 mg. of chymotrypsin, 0.1 mg. of active substrate and a variable amount of inactive substrate was placed in the inner compartment of cell, and 5 ml. of a similar solution minus enzyme was placed in the outer compartment. The cell was placed in the stirring rack at a 45° slope, with the cell axis at a small angle with the rotation axis in order to agitate the liquid as the cell revolved. Under the conditions of equal activity in each compartment equilibrium was obtained in less than 8 hours. Experiments in which all the substrate was placed in one or the other compartments gave similar values of binding; however, these took a somewhat longer time.

At the conclusion of the stirring, normally 16 hours, 4-ml. samples were pipetted from each compartment, placed in tared test-tubes, the solution weighed and then counted in the 100% geometry, γ-ionization chamber. A vibrating reed electrometer[28] was used to measure the voltages produced. The technique of counting was to measure the time (generally about 100 seconds) to build up 900 mv. on a 100 $\mu\mu$f. condenser in parallel with the ionization chamber. Four measurements each of the buffer and of the protein solution were made alternately. The specific radioactivities of the solution, *i.e.*, electrometer divisions per second, per gram of solution were computed, the excess radioactivity of the enzyme solution being attributed entirely to enzyme binding. The protein per gram of solution, and the binding per gram of protein were obtained from Kjeldahl nitrogen

TABLE I

EXCESS ATOM PER CENT. O^{18} IN SUBSTRATE

	Pyrolysis method	Bromination
Uncatalyzed	0.29	0.08
Enzyme catalyzed	.74, 0.75, 0.73	.47
Acid catalyzed	1.1	
Original water	1.1	

(26) R. Zeynek, *Z. physiol. Chem.*, **114**, 277 (1921).

(27) E. Abderhalden and H. Malin, *ibid.*, **178**, 266 (1928).

(28) P. C. Tompkins, L. Wish and W. T. Burnett, Jr., *Anal. Chem.*, **22**, 672 (1950).

analyses. The substrate concentration was determined from the amount originally added.

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Base-catalyzed Decomposition of α-(Benzene- and p-Toluenesulfonamido)-phenylacetyl Chlorides

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It has been observed that α-(benzenesulfonamido)-phenylacetyl chloride undergoes decomposition when treated with aqueous sodium hydroxide forming benzaldehyde, benzenesulfonamide and carbon monoxide. A possible mechanism for this reaction is presented and coördinated with the decomposition of the free acid in pyridine and acetic anhydride to diphenyl disulfide and carbon dioxide. Data on the preparation, properties and reactions of the previously undescribed α-(benzenesulfonamido)- and α-(p-toluenesulfonamido)-phenylacetyl chlorides and the corresponding amides are given.

In a previous communication we have reported[2] the unusual formation of diphenyl disulfide in the pyridine–acetic anhydride catalyzed decarboxylation of α-(benzenesulfonamido)-propionic acid. Since then we have observed a similar reaction with α-(benzenesulfonamido)-phenylacetic acid. This reaction was explained in terms of the rupture of the sulfur–nitrogen linkage in a hypothetical carbanion intermediate, $C_6H_5SO_2NHC\overset{\ominus}{H}(CH_3)$, to form the sulfinite ion and acetaldimine. This was visualized as a β-elimination type of reaction at a carbon–nitrogen bond. The cleavage products apparently undergo disproportionation and oxidation resulting in the reduction of the sulfinite ion to the disulfide. In continuing these studies we have also observed that aqueous sodium hydroxide cleaves α-(benzenesulfonamido)-phenylacetyl chloride with formation of benzenesulfonamide, benzaldehyde and carbon monoxide.

The α-(benzenesulfonamido)-phenylacetyl chloride and its p-toluene analog used in these studies were prepared using purified thionyl chloride, a procedure preferred to the phosphorus pentachloride method previously described.[3a,b] Attempts to analyze and characterize these acid chlorides were beset with unusual difficulties. The crude yellow solids showed wide melting point ranges that indicated presence of impurities. Although they are soluble in hydrocarbon solvents, attempts at purification by recrystallization were unsuccessful. Neutral equivalent values were uniformly high. The presence of a neutral impurity or decomposition product was indicated by the observation that part of the sample was insoluble in alkali at the end of the titration. It is to be noted that nitrogen analysis previously used to characterize arylsulfonamido acid chlorides[3a] is insufficiently precise to distinguish between the free acid and the acid chloride. On the basis of further observations on the reactions of these chlorides on heating or treatment with alkali, it is believed that these compounds are not stable enough to permit purification, analysis and identification by the usual methods. Both acid chlorides were converted in 50–70% yields to the previously undescribed amides by reaction with aqueous ammonium hydroxide at room temperature and the benzenesulfonamido acid chloride was hydrolyzed to the acid in 67% yield. The α-(benzenesulfonamido)-phenylacetyl chloride was used as the crude yellow solid obtained after thorough evacuation of the reaction mixture to remove sulfur dioxide, hydrogen chloride, and unreacted thionyl chloride.

The alkaline decomposition reactions were carried out under a variety of conditions as described in Table I. The benzaldehyde was extracted from the alkaline reaction mixture and isolated as its 2,4-dinitrophenylhydrazone. The benzenesulfonamide precipitated on acidification of the reaction mixture. Carbon monoxide was present in the gas evolved as evidenced by the combustibility and solubility in aqueous cuprous chloride of the gas. The yields of benzaldehyde, isolated as its 2,4-dinitrophenylhydrazone, and benzenesulfonamide, using different reagents, temperatures of 30° and nearly boiling, and concentrations of sodium hydroxide from 5–50%, are given in Table I. The yield of the aldehyde varies from 3 to 65% and the yield of the amide from 10–54%. The highest yields were obtained using hot 5% sodium hydroxide. No attempt has been made to determine the loss of benzaldehyde through the Cannizzaro reaction. On one run using the chloride obtained from 1.5 g. of acid and 5% hot sodium hydroxide 130 ml. of gas (uncorrected for air in the system) was collected. Of this, 55 ml. (48% of the theoretical amount of carbon monoxide) was soluble in acidic cuprous chloride.

The data given in this and the previous paper[2] show that α-(benzenesulfonamido)-phenylacetic acid derivatives undergo decomposition with the possibility of rupture at either the sulfur–nitrogen (a) or nitrogen–carbon (b) bond depending on

$$C_6H_5SO_2 \overset{(a)}{\text{——}} NH \overset{(b)}{\text{——}} CH(C_6H_5)COX$$

the conditions. This behavior is reminiscent of the "acid" and "ketone" cleavages of acetoacetic esters. To account for this phenomenon it is proposed that

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(2) R. H. Wiley and N. R. Smith, THIS JOURNAL, **73**, 4719 (1951).

(3) (a) H. E. Carter and J. Hinman, *J. Biol. Chem.*, **178**, 403 (1949). (b) H. Bovarnick and H. T. Clarke, THIS JOURNAL, **60**, 2426 (1938).