2. The homogeneity was checked by chromatography and countercurrent analysis.

3. Comirin is a peptide containing an ether-soluble moiety.

4. The antibiotic shows a striking apparent increase in activity on heating in acid solution.

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A New Method for the Determination of the Amidase Activity of Trypsin: Kinetics of the Hydrolysis of Benzoyl-L-Arginineamide

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The determination of the amidase activity of trypsin is complicated by the appreciable rate of self-digestion of the enzyme under optimum conditions for the hydrolysis. In order to obtain significant results it was necessary to find a kinetic procedure by which the hydrolysis could be followed rapidly relative to the rate of inactivation of the enzyme. In the course of tests of the formol titration method of Iselin & Niemann (1950) it was observed that the esterase activity of trypsin was unaffected by formaldehyde. Esterase activity was followed by the potentiometric titration of Schwert, Neurath, Kaufman & Snoke (1948) and of Schwert & Eisenberg (1949) with benzoyl L-arginine ethyl ester (BAEE) as substrate. The fact that the activity of trypsin is unaffected by formaldehyde (as determined by esterase activity) was made the basis of a potentiometric method for following the amidase activity. At the pH-activity optimum (pH 7.5–8.0), in the presence of formaldehyde the trypsin-catalysed hydrolysis of amides may be represented by the following equations:

Thus, in the case of trypsin, the hydrolysis of amides can be followed directly by measuring the

amount of oxonium ions liberated in the presence of formaldehyde. This method has been successfully carried out employing benzoyl-L-arginineamide (BAA) as substrate.

MATERIALS AND METHODS

Substrates. Benzoyl-L-arginineamide and ethyl ester were prepared from L-arginine hydrochloride (British Drug Houses Ltd., London) as described previously (Bergmann, Fruton & Pollok, 1939). Acetyl-L-phenylalanine (I) was prepared, according to the method of Huang & Niemann (1951), from L-phenylalanine (supplied by Roche Products Ltd., Welwyn, Herts). The ethyl ester was prepared by esterification of I in HCl-saturated ethanol at room temperature. After 2 days the reaction mixture was partially evaporated under vacuum and precipitated with water. The crude ester was recrystallized twice from hot water (pH 7).

Enzymes. Trypsin: the trichloroacetic acid precipitate of Northrop, Kunitz & Herriott (1948) was suspended in water and exhaustively dialysed against 0.01M-HCl until the protein all dissolved (about 3 days at 5°). Crystallization of this material resulted in no further increase in activity and hence the dialysed samples at pH 2 were stored and used in the rate experiments.

Chymotrypsin: chymotrypsin was prepared by Dr H. Gutfreund from the trypsin digest of a sample of chymotrypsinogen (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) by the method of Northrop *et al.* (1948). Before use a 0.1% solution was dialysed against 0.005M ethylenediamine (pH 7.5) solution.

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Method. The same procedure was followed in the measurement of all trypsin-catalysed rates. The reaction was carried out in a 20 ml. beaker immersed in a water bath maintained at $25.00 \pm 0.05^\circ$. In the beaker were placed 10 ml. of solution containing substrate, $0.1\text{M-Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer (pH 7.6) and 0.2M-NaCl . When the substrate was the amide $0.5\text{ ml. of } 40\% \text{ (w/v) formaldehyde}$ (neutralized to pH 7.5 with NaOH) was added in addition. This concentration of formaldehyde was found to be adequate (see Fig. 1). Approximately 0.1N-NaOH , standardized in terms of μ equivalents/micrometer division, was added to the reaction mixture by means of a hypodermic syringe firmly connected to a micrometer screw. The needle of the syringe dipped into the reaction mixture. The pH was measured with a model 505 pH meter (Pye Instrument Co., Cambridge). At zero time, $0.2\text{ ml. of enzyme solution}$ was added, the pH adjusted to slightly over 7.60 and the time at which the needle crossed the 7.60 mark recorded. Small amounts of base were added immediately following the time readings. Maximum deflexions were about 0.05 pH unit (no change in activity between pH 7.5 and 8.0 could be noted). The mixture was stirred throughout by bubbling through it CO_2 free nitrogen at 25° , saturated with water. Chymotrypsin-catalysed rates were followed similarly, with the exception that a $0.005\text{M ethylenediamine buffer}$ was used to follow the reaction at pH 7.90. Specific rates could be determined with a precision of $\pm 0.2\%$ (the mean deviation from least squares analysis) and accuracy of $\pm 1\%$ (the reproducibility of duplicate experiments).

Effect of formaldehyde on the trypsin- and chymotrypsin-catalysed rates of hydrolysis of esters

In the following trypsin experiments, the activity of the enzyme was determined.

(1) One ml. of 0.5% trypsin solution in 0.01N-HCl was mixed with $1\text{ ml. of } 40\% \text{ (w/v) formaldehyde}$ and allowed to stand at 25° for 2 min. The mixture was then diluted to $10\text{ ml. in a volumetric flask}$, and the activity tested using 0.2 ml. The procedure was repeated using lower formaldehyde concentration.

(2) The rate of hydrolysis of BAEE in the absence of formaldehyde was followed over a 4 min. period. One ml. of $40\% \text{ (w/v) formaldehyde}$ at pH 7.6 was then added, the pH brought rapidly back to 7.6 and the rate measurements continued. This procedure was repeated using $2\text{ ml. of } 40\% \text{ (w/v) formaldehyde}$.

(3) The rate of hydrolysis of $10\text{ ml. of } 0.02\text{M acetyl-L-phenylalanine ethyl ester (APEE)}$ by $0.04\text{ mg. of chymotrypsin}$ was followed in the absence and presence of 2% (w/v) formaldehyde.

Effect of formaldehyde concentration on the observed amidase activity

The hydrolysis of 0.044M-BAA was followed as described in the above method, except that $2.0\text{ ml. of formaldehyde}$ were used. 9.8 mg. of enzyme were used for each sample. The final concentration of BAA was adjusted to 0.044M since K_m was, at this stage, undetermined.

RESULTS AND DISCUSSION

As can be seen from Fig. 1, the rate of hydrolysis of BAEE is unaffected by formaldehyde and the rate

of observed BAA hydrolysis is insensitive to formaldehyde concentration. The rate of chymotrypsin-catalysed hydrolysis of acetyl-L-phenylalanine ethyl ester is materially reduced upon addition of formaldehyde. However, the residual activity appears to remain constant throughout the experiment; this probably indicates formation of a chemically altered but stable formol enzyme.

If S_0 is the original concentration of substrate at time t_0 , S its concentration at time t , and E_0 the total concentration enzyme, the integrated form of the Michaelis equation is

$$(S_0 - S) + K_m \ln S_0/S = k_3 E_0 (t_0 - t). \quad (1)$$

Examination of the curves in Fig. 2 indicates that the experimental plot of $(S_0 - S)$ against $(t_0 - t)$ deviates far more rapidly from linearity, especially at lower substrate concentrations, than is predicted by eqn. 1. This effect has been previously noted by Harmon & Niemann (1949), who ascribed it to a strong inhibition by product. Taking the concentra-

Fig. 1. Effect of formaldehyde on the rate of trypsin- (pH 7.60) and chymotrypsin- (pH 7.90) catalysed hydrolysis at 25° . $10\text{ ml. of } 3 \times 10^{-3}\text{M-BAEE}$ in $0.01\text{M phosphate buffer}$ and 0.2M-NaCl solution containing: $\bigcirc-\bigcirc$, no formaldehyde, $0.03\text{ mg. of trypsin}$; $\times-\times$, $0.03\text{ mg. of trypsin}$ previously treated with $20\% \text{ (w/v) formaldehyde}$; $\square-\square$, $0.03\text{ mg. of trypsin}$ and no formaldehyde for the first 4 min., then $4\% \text{ (w/v) formaldehyde}$; $\boxtimes-\boxtimes$, $10\text{ ml. of } 0.044\text{M-BAA}$ in $0.01\text{M phosphate buffer}$ and 0.2M-NaCl containing 3.3 mg. trypsin and the following concentrations of formaldehyde: $\bullet-\bullet$, $2\% \text{ (w/v)}$; $\times-\times$, $8\% \text{ (w/v)}$; $10\text{ ml. of } 0.02\text{M-APEE}$ in $0.05\text{M ethylenediamine buffer}$ and 0.2M-NaCl containing $0.04\text{ mg. chymotrypsin}$ and: $\triangle-\triangle$, no formaldehyde; $\diamond-\diamond$, $2\% \text{ (w/v) formaldehyde}$.

tion of the product as $(S_0 - S)$ and K_I as the enzyme-product dissociation constant gives

$$(1 - K_m/K_I)(S_0 - S) + K_m(1 + S_0/K_I) \ln S_0/S = k_3 E_0(t_0 - t). \quad (2)$$

Eqn. 2 shows that the rate of hydrolysis would decrease with time more rapidly the smaller is K_m/K_I . During the very early part of the reaction, as followed in these experiments, $\ln S_0/S$ differs negligibly from $(S_0 - S)/S_0$ so that eqn. 2 becomes:

$$\{(1 - K_m/K_I) + K_m(1 + S_0/K_I)/S_0\}(S_0 - S) = k_3 E_0(t_0 - t). \quad (3)$$

Eqn. 3 shows that, on the hypothesis of inhibition by the product, $(S_0 - S)$ should be linear against t , irrespective of the value of K_I , during the earliest part of the reaction. This was found, in fact, not to be the case. A smooth curve could be fitted to the product/time points and from this curve the rate was interpolated at mean times. A plot of rate versus time was constructed from the curve as shown in Fig. 3. It can be seen that the rate decreases linearly with time, the deceleration being a function of the initial substrate concentration.

The linear deceleration of the hydrolysis can be attributed to the self-digestion of the enzyme at the pH optimum. It was for this reason that the rate could not be readily followed by any method involving the removal of samples over periods longer than 10 min. This self-digestion appears to be inhibited by substrate. When benzoyl-L-arginine

ethyl ester was used as substrate in similar concentrations (0.01–0.003M), the rate was found to be constant over longer intervals (up to 30 min.). However, when this substrate concentration was of the order of 3×10^{-5} M deviations from linearity became marked. This suggested the possibility that the self-digestion process was a function of the fraction of sites saturated with substrate. This is borne out by the upper plot shown in Fig. 3 where the deceleration/(rate at zero time) is the same for 9.1×10^{-3} M-BAA and 3.1×10^{-5} M-BAEE. $K_m/[S_0]$ is equal to 0.34 in both cases. K_m of trypsin for BAEE (0.96×10^{-5} M) had been previously determined in this laboratory (Bernhard & Gutfreund, to be published).

K_m for the amide was determined by the usual method of Lineweaver & Burk (1934) at substrate concentrations between 10^{-2} and 10^{-3} M as illustrated in Fig. 4, and was found to be 3.1×10^{-3} M.

By extrapolation of the plot (Fig. 4) to infinite substrate concentration the maximum velocity was determined. $k_3[E_0] = 0.94 \times 10^{-6}$ moles/mg. enzyme nitrogen min. Assuming a molecular weight of 17 000 for trypsin (Gutfreund, 1954), the first-order specific rate was calculated. $k_3 = 0.043 \text{ sec.}^{-1}$.

The fact that trypsin activity is unaffected by formaldehyde is partially explicable in terms of its specificity. There is evidence that a positive charge on the substrate contributes to the specificity

Fig. 2. Hydrolysis of benzoyl-L-arginineamide at pH 7.60 and 25° (in solutions in 0.01M in phosphate buffer, 0.2M in NaCl and containing 3.3 mg. of trypsin) at concentrations of 2.96×10^{-3} M (lower curve), 9.1×10^{-3} M (middle curve) and 4.4×10^{-3} M (upper curve).

Fig. 3. Deceleration of the trypsin-catalysed hydrolysis in 0.2M-NaCl at pH 7.60 and 25°. ■—■, 2.96×10^{-3} M-BAA, 0.01M phosphate, 3.3 mg. trypsin; ○—○, 9.1×10^{-3} M-BAA, 0.01M phosphate, 3.3 mg. trypsin; ○—○, 3.1×10^{-5} M-BAEE, 0.01M phosphate, 0.016 mg. trypsin; v is the velocity at mean times obtained by point-to-point interpolation of product/time plots.

Fig. 4. Determination of K_m of benzoyl-L-arginineamide at pH 7.60 in 0.01M phosphate buffer, 0.2M-NaCl and 2% (w/v) formaldehyde. Each reaction mixture contained 3.3 mg. of enzyme. $[S_0]$ and v_0 are the respective initial substrate concentrations and rates. K_m was calculated from the slope by means of the equation

$$\frac{(V_{\max.})([S] \rightarrow \infty)}{v_0} = \frac{K_m}{[S_0]} + 1.$$

(Neurath & Schwert, 1950). It would be unlikely, therefore, that a free amine group (ammonium ion at pH 7.6) would be found in the region of the active site. It is of interest to note that acetylation of trypsin results in no loss of esterase activity (Jansen, Nutting, Jang & Balls, 1949).

SUMMARY

1. Although formaldehyde alters the chemical structure of trypsin it has no effect on the activity of the enzyme at the pH optimum.

2. A method for following the trypsin-catalysed hydrolysis of amides based on the above observation has been described.

3. The enzyme-substrate dissociation constant for benzoyl-L-arginineamide has been determined ($K_m = 3.1 \times 10^{-3}M$) as well as the first-order specific rate of decomposition of the enzyme-substrate complex ($k_3 = 0.043 \text{ sec.}^{-1}$).

4. The pronounced deceleration of trypsin-catalysed hydrolysis with time has been explained in terms of the self-digestion of the enzyme (a process which is inhibited by the substrate).

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The Amide Groups of Insulin

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Previous work has shown that insulin consists of two types of polypeptide chains the amino acid sequences of which have been determined by partial hydrolysis experiments (Sanger & Tuppy, 1951*a, b*;

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Sanger & Thompson, 1953*a, b*). For an assumed molecular weight of approximately 6000, which is the simplest chemical unit consisting of one phenylalanyl and one glycyl chain, there are six amide groups present (Rees, 1946). From a quantitative study of the products of reduction of insulin ester with lithium borohydride Chibnall & Rees (1952) showed that the three aspartic acids, one of which is