On Dielectric Constant and Enzymatic Kinetics

IV. Dipolar ions. Ester hydrolysis by trypsin and alpha chymotrypsin in glycine solutions

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ABSTRACT A study was made on the effect of glycine on systems involving trypsin and BAEE or TSAME on the one hand, or α-chymotrypsin with any of the substrates BAEE, TEE, or PEE, on the other. In all cases there was a linear relationship between the rate logarithm and the reciprocal of the dielectric constant of the glycine solution. The slopes were positive in the reactions of trypsin. In those catalyzed by α-chymotrypsin, the slopes were positive at pH 6.5 or lower, and negative at pH 7.5. However, the effects of glycine differ quantitatively from those of urea or other solvents. The presence of salt modifies somewhat the glycine effects. A low ionic strength increases the effect of glycine on trypsin, but if the inhibition caused by the ionic strength is relatively strong, the addition of glycine partially neutralizes the salt effect. Addition of salt to systems containing α-chymotrypsin always resulted in a diminished effect of glycine. An attempt is made to interpret the anomalies of glycine effects on the basis of its dipolar ion structure.

INTRODUCTION

Northrop in 1924 (10), applying the Donnan theory to the distribution of trypsin between molecules of undissolved gelatin and the surrounding solution, reached the conclusion that this enzyme behaved like a univalent positive ion. This conception of the ionic character of trypsin arose again recently

1 Throughout this paper the following abbreviations and symbols will be used:
$D$, dielectric constant.
$\Delta D/\Delta \epsilon$, dielectric increment.
$R/R_0$, relative rate of hydrolysis.
BAEE, benzoyl-$\epsilon$-arginine ethyl ester.
TSAME, $p$-toluenesulfonyl-$\epsilon$-arginine methyl ester.
TEE, $\epsilon$-tyrosine ethyl ester.
PEE, $\epsilon$-phenylalanine ethyl ester.

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when studies concerning the influence of dielectric strength on the kinetics of trypsin (3) and α-chymotrypsin (4) led to the conclusion that the former enzyme behaves like a positive ion, while the latter acts as a negative ion. Moreover, trypsin seems to maintain itself positively charged through the pH range of 5.5 to 8.5, while α-chymotrypsin appears positive from pH 5.5 to about 6.6 and then changes to negative (5). A further study on the effect of ionic strength on the kinetics of these two enzymes (6) added support to the assumption that the charges involved in the activity of trypsin and α-chymotrypsin are different.

All amino acids increase markedly the water dielectric constant (12). Their dielectric properties depend upon the structure of dipolar ions that they acquire in solution. As a matter of fact, most of the experimental support for the dipolar ion concept derived from measurements of dielectric constants.

Because of its positive dielectric increment, glycine must modify the rates in reactions catalyzed by trypsin and α-chymotrypsin in the same way that

**Figure 1.** Effect of glycine on the system BAEE-trypsin as a function of dielectric constant, in the presence of phosphate buffer (0.05 M KH₂PO₄-Na₂HPO₄). Curves: 1, pH 6.5, 6.6, and 6.7, slope = 1.07 ± 0.02; 2, pH 6.8, slope = 0.99 ± 0.02; 3, pH 6.9, slope = 0.43 ± 0.01; 4, pH 7.0, slope = 0.18 ± 0.01; 5, pH 7.4, slope = 0.07 ± 0.003; 6, pH 7.8, slope = 0.006 ± 0.002. Temperature, 25°C. Substrate concentration, 0.008 m. Enzyme concentration, 10 µg per ml.
urea does. However, since the presence of ions also alters the rate of the reactions referred to, and glycine has ionic structure, its effect might be different from that of urea or other non-ionic solvents which modify the dielectric constant. On the basis of these considerations, experiments were planned involving the addition of glycine to various systems such as ester-trypsin and ester-α-chymotrypsin.

**MATERIALS AND METHODS**

Trypsin and α-chymotrypsin were salt-free as well as three times crystallized preparations. The α-chymotrypsin sample was assayed for purity as described in an earlier communication (2) in order to eliminate the possibility of a trypsin contamination. The ester substrates were used as hydrochlorides. Glycine was an Eastman Kodak ammonia-free product which was further recrystallized three times from alcohol-water mixtures. A stock solution of glycine at the required pH was prepared every day. Its concentration was 2.21 M, so that when one-tenth of the final volume was
added to the test or titrating solution, the dielectric constant increased 5 units according to the value $\Delta D/\Delta C = 22.6$ given by Wyman and McMeekin (13) for glycine. The value $\Delta D/\Delta C = 2.7$ (12) was used for the calculation of dielectric constants of urea solutions.

The modified titrimetric determination of esterase activity was described in a former communication (3). When the experiments were conducted in phosphate buffer, the titrating solution used contained the same concentration of glycine as was used in the test. This was done because glycine, like alcohols, changes the dissociation of phosphate (3). The glycine-containing alkali solution was always freshly prepared by mixing the proper amounts of glycine stock solution, 0.4 N NaOH, and making up the volume. In the experiments without phosphate the titrating solutions were 0.1 N NaOH or KOH, the first being used in the presence of sodium salts, and the second when potassium salts had been added to the system.

The rates were measured as the amount of ester hydrolyzed after 5 minutes. This value was interpolated from the curve of alkali consumption against time, which in all cases was followed during 10 minutes. The relative rates given in the figures were

\[ \text{Figure 3. Effect of glycine on the system TSAME-trypsin at various pH values in the absence of phosphate buffer or added salt. All the rates are referred to the rate measured at 6.5 and } D = 79.6. \text{ The slope values are: pH 6.5, 1.54 ± 0.02; pH 7.0, 1.05 ± 0.03; pH 7.5, 0.46 ± 0.005; pH 8.0, -0.05 ± 0.003. Temperature, 25°C. Substrate concentration, 0.008 M. Enzyme concentration, 2.5 µg per ml.} \]
calculated with reference to the values obtained at the initial dielectric constant 78.5 (in water or phosphate buffer) or 79.6 (in 0.05 M glycine).

**EXPERIMENTAL**

Schwert and Eisenberg (11) failed to detect any change in the rate of hydrolysis of BAEE by trypsin at pH 7.8 in 0.05 M phosphate buffer when 0.1 M glycine was added to the medium. In view of the observation that apparently the positive charge of trypsin increases as pH decreases (6), it was thought that glycine might show some effect within some region of pH below 7.8. With this in mind, the effect of glycine on the system trypsin-BAEE was investigated at pH values from 6.5 to 7.8 in 0.05 M phosphate buffer. Fig. 1 represents the plots of the logarithm of relative rate against the reciprocal of dielectric constant. In all cases there was a linear relationship between these variables within the limits of dielectric constants 78.5 and 98.5 (0.88 M glycine). In an additional experiment at pH 6.5, not shown in the figure, it could be observed that higher concentrations of glycine fail to produce a greater inhibition. The maximum effect of glycine was produced at the pH values
6.5, 6.6, and 6.7. The experimental points of these fall in the same line with slope $1.07 \pm 0.02$. As pH increases the effect diminishes rapidly and becomes practically negligible at pH 7.8.

In order to investigate the influence of the buffer on the previous experiment, the hydrolysis of BAEE by trypsin at pH 6.5 was conducted in the absence of phosphate. Glycine at 0.05 M concentration was used as buffer in the control ($D = 79.6$). Curve 1 of Fig. 2 shows the resulting line with slope $0.30 \pm 0.01$. Since these values are small as compared to the previous one at the same pH (Fig. 1) and the only difference between these experiments is the presence of phosphate, a possible salt effect was considered. Accordingly,
the influence of several ions on the effect of glycine was studied. To begin with, 0.08 M sodium and potassium chloride (with the same ionic strength as 0.05 M phosphate buffer at pH 6.5) were used. Curves 2 and 5 (Fig. 2) show

![Figure 6](image_url)

**Figure 6.** Effect of glycine and urea on the α-chymotrypsin-catalyzed hydrolysis of TEE and PEE in the presence of added salt. TEE curves: 1, urea, pH 6.5, 0.08 M NaCl, slope = 1.19 ± 0.04; 2, glycine, pH 6.5, 0.08 M NaCl, slope = 0.12 ± 0.005; 3, glycine, pH 7.5, 0.08 M NaCl, slope = -0.62 ± 0.005; 4, urea, pH 7.5, 0.08 M NaCl, slope = -1.12 ± 0.01; 5, glycine, pH 7.5, 0.16 M NaCl, slope = -0.56 ± 0.02; 6, urea, pH 7.5, 0.16 M NaCl, slope = -0.87 ± 0.005. PEE curves: 1, urea, pH 6.25, 0.08 M KCl, slope = 1.52 ± 0.04; 2, glycine, pH 6.25, 0.08 M KCl, slope = 0.15 ± 0.01; 3, glycine, pH 7.5, 0.08 M KCl, slope = -0.74 ± 0.02; 4, urea, pH 7.5, 0.08 M KCl, slope = -1.24 ± 0.03. Temperature, 25°C. Substrate concentration, 0.016 M. Enzyme concentrations, the same as in Fig. 5.

the results; the initial points are at different levels but the slopes are equal and about 50 per cent greater than the one obtained when no salt was added. In the following experiment a mixture of 0.034 M NaCl and 0.016 M Na₂SO₄ was added to the system. In this, the proportion of uni- and divalent ions is the same as in the phosphate buffer at pH 6.5, KH₂PO₄ is substituted for by
NaCl, and Na₂HPO₄ by Na₂SO₄. Curve 3 (Fig. 2) shows that the initial inhibition is less marked than in the case of addition of 0.08 M KCl, but the slope value is slightly higher. The increase of NaCl concentration to 0.16 M (curve 4) gave rise to a further increase of slope value. However, in the presence of 0.16 M KCl, the slope value diminished, again reaching the original value of the experiment without added salt.

Fig. 3 shows the effect of glycine on the trypsin-catalyzed hydrolysis of TSAME at pH values ranging from 6.5 to 8.0. Neither phosphate nor neutral salt was added, the buffer being glycine itself. As in the case of BAEE hydrolysis, the slope values decrease as the pH increases. Nevertheless, the effect is stronger when TSAME is the substrate, the slope at pH 6.5 being about five times greater than with BAEE.

As a means for comparison of the effect of glycine on trypsin and α-chymotrypsin regardless of the substrate influence, advantage was taken of the observation that α-chymotrypsin can hydrolyze BAEE (2). Fig. 4 shows the effect of glycine on the hydrolysis of BAEE by trypsin and α-chymotrypsin at pH 7.5 either in the absence of salt or with 0.08 and 0.16 M KCl. Glycine increases the rate of hydrolysis by α-chymotrypsin but does not modify the rate significantly when trypsin is the catalyst. The presence of salt makes the slope values decrease slightly.

It was reported (5) that the effect of dielectric strength on ester hydrolysis by α-chymotrypsin is reversed around pH 6.6. For this reason, a comparative study was undertaken on the effect of glycine on the α-chymotrypsin-catalyzed hydrolyses of two of the water-soluble substrates of this enzyme. TEE and PEE, at pH 7.5, on the one hand, and at pH 6.5 or 6.25, on the other. Even in the case in which no foreign salt was added, the reaction mixtures contained some sodium or potassium and chloride ions resulting from the neutralization of the substrates with NaOH or KOH. The concentration of these ions represented as NaCl or KCl is 0.01 M at pH 7.5, 0.003 M at pH 6.5, and 0.002 M at pH 6.25. Fig. 5 compares the effects of glycine and urea in the absence of added salt and Fig. 6 shows the effects of these substances in the presence of added NaCl or KCl. In the case of urea, the dielectric constant was increased only from 78.5 to 88.5 (3.7 M urea), because greater concentrations produce a rapid fall of enzyme activity (5). It can be observed in both Figs. 5 and 6 that, notwithstanding quantitative differences, the effects of glycine and urea are very similar. As in the case of trypsin, the sensitivity of systems ester-α-chymotrypsin to urea and glycine effects varies with the substrate, being the greatest with PEE and the smallest with BAEE.

**DISCUSSION**

The present observations and earlier ones (5, 6) concerning the behavior of the trypsin- and α-chymotrypsin-catalyzed hydrolyses of BAEE show that
the trend of dielectric and salt effects is determined principally by the enzyme charges.

Even though the effects of glycine observed thus far maintain a relationship with the change in dielectric constant, they differ quantitatively from those produced by urea or other solvents (3, 4). However, if instead of the dielectric increment of urea 2.7 given by Wyman (12) and Dunning and Shutt (9), Devoto’s value 3.4 (7) were used, the difference between urea and glycine would diminish. Furthermore, glycine differs from the other solvents because of its dipolar ion structure. The structure of urea in solution has been a matter of discussion. In view of its positive dielectric increment, Devoto (8) suggested a true dipolar ion structure; but other authors, e.g. Wyman (12) do not think that the rise of water dielectric constant is sufficient to justify the dipolar ion structure of urea. Dunning and Shutt (9) determined the dielectric constants of glycine and urea solutions within wide ranges of pH while seeking an indication as to whether they were dipolar ions or not. They observed that the dielectric increment caused by urea was unaltered by change of pH from 2 to 11.5. On the contrary, the increment produced by glycine only remained unchanged between pH 4.5 and 7.5 falling when the pH was outside this range. The dielectric constant–pH curve of glycine parallels the one representing the concentration of dipolar ion as a function of pH. From these results, the authors concluded that urea is not a dipolar ion but that it is rather to be considered as having a large permanent dipole moment.

In a previous paper (6) it was suggested that the salt effects on the rate of ester hydrolysis by trypsin and α-chymotrypsin are probably a case of the phenomenon called by Brønsted “secondary salt effect” (1). This consists in a change of the degree of dissociation of ionizable groups produced by the presence of ions. The magnitude and sign of the effect depend on the charge of the base formed in the equilibrium of dissociation; the dissociation increases when the base is an anion and decreases when it is a cation. Presumably, the effect of dipolar ions should differ from that of salts. When they are attracted by the end of sign opposite to that of the charged group, the resulting ionic atmosphere would reinforce rather than weaken the central charge. When a positive base is involved in the dissociation equilibrium, such as that postulated for trypsin, the effect of glycine should be the opposite of that produced by a salt like NaCl. On the contrary, if an anionic base is formed in the equilibrium, such as in the case of α-chymotrypsin, the addition of either a salt or glycine would result in a similar effect. If mono- and dipolar ions coexist in solution they would compete in the formation of the ionic atmosphere.

The addition of glycine to a reaction system may give rise to three distinct effects: (a) the one just described due to the orientation of glycine ions around the charged groups. This would increase the activity of either trypsin or α-
chymotrypsin; (b) increase of dielectric constant, an effect which would

diminish the activity of trypsin and increase that of α-chymotrypsin at pH 7.5; and (c) if the system had been activated or inhibited by addition of

salts, this effect would be counteracted as glycine concentration increases

as a consequence of effects a and b. Under a given set of conditions, the

observed effect would be the algebraic sum of the separate effects. For trypsin,

where the positive charge seems to be weak or partially neutralized by a nega-

tive group at pH values above 7.0 (6), the sum of the first and second effects

might result in the low or null slope values observed. In the presence of salt

which decreases the first effect, the slopes would increase. However, when the

initial inhibition by salt is considerable, e.g. on addition of 0.16 M KCl, the

third effect, namely the neutralization of salt effects by glycine could be ob-

served. Quantitative differences in the effect of ions on trypsin have already

been observed (6); the degree of inhibition increases with the charge of the

anion and diminishes as the cation size or charge increases, e.g. sulfate is more
effective than chloride, and potassium more effective than sodium. The effect

of phosphate is stronger than the effects of the other salts. This might be due to

the fact that it is not a neutral salt and exerts effects in addition to those

corresponding to the general salt effect.

The effects of amino acids and salts on enzymic reactions offer a mechanism

of activation and inhibition complementary to pH. This result may be of in-

terest from the biological standpoint, inasmuch as pH variations in biological

media are quite restricted.

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