On Dielectric Constant and Enzymatic Kinetics

III. *Interrelationships of dielectric constant and pH*

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ABSTRACT The dielectric effects on trypsin and α-chymotrypsin activities have revealed that at pH 7.8 the active species of the former is the cation while that of the latter is the anion. The present study on the dielectric effects along the pH-activity curves shows that trypsin remains positive within the pH range of 5.5 to 8.5. Conversely, α-chymotrypsin is positive from pH 5.5 to 6.6, negative from 6.6 to about 8.1, and at pH 8.25 becomes positive again. The first point of inversion in charge sign shifts from 6.6 to 7.15 with the addition of 0.05 M phosphate buffer. The point of inversion does not seem to be modified significantly by changes in the substrate structure. At pH values near the point of inversion the plots of rate log *vs.* $100/D$ are broken lines formed by various straight portions, the slope of each varying progressively from a maximum positive to a maximum negative value. This suggests an effect of resonance possibly attributable to an imidazole group. As an attempt to explain the two observed points of sign inversion in α-chymotrypsin, the possibility is suggested that different enzyme configurations are disclosed by the combined action of pH and dielectric constant. On this theoretical basis, it is feasible that more than one isoionic point exists.

INTRODUCTION

It was reported previously that the rate of ester hydrolysis by trypsin (5) or α-chymotrypsin (6) is modified by changes in the medium dielectric strength. By comparison with non-enzymatic reactions, it might be inferred from the behavior of these two enzymes that at pH 7.8 the active form of trypsin is the cation and that of α-chymotrypsin is the anion. These observations are in harmony with two earlier findings: (*a*) Northrop (16) deduced, by Donnan equilibrium considerations, that trypsin behaves like a univalent positive

Received for publication, January 18, 1960.

ion, and (*b*) Foster and Niemann (7), in their experiments on the effect of inhibitors on α-chymotrypsin at pH 7.9 ± 0.1, reached the conclusion that in this enzyme there should be a negatively charged grouping next to or at the active site. Recently (6), we discussed the finding that the apparent charge signs of trypsin and α-chymotrypsin at pH 7.8 are in agreement with some of the various data of their isoelectric points found in the literature.

With the hope of detecting changes in the structure and charge induced by pH on the enzyme molecules, dielectric effects on trypsin and α-chymotrypsin with simultaneous pH variations were studied. The present paper deals with the results of this investigation.

MATERIALS AND METHODS

The source of the synthetic substrates used was the same as in previous work (5, 6). Trypsin and α-chymotrypsin were salt-free and three times crystallized preparations.

The chemicals employed for modifying the dielectric constant of water were of the best grade available and further purified by redistillation or recrystallization.

The procedure for measuring activity was described in an earlier communication (5). The rates of hydrolysis are given in 10^{-6} mols of ester hydrolyzed per milliliter in an initial interval of 5 minutes. The hydrolysis course was followed in every case for a period of 10 minutes.

RESULTS

The activity of trypsin with the substrates BAEE and TSAME[1] at three D values was measured within the pH range of 5.5 to 8.5. The solvents used were 0.6 M acetone (D = 76.5), water (D = 78.5), and 0.74 M urea (D = 80.5). Such low concentrations of urea and acetone were chosen in order to minimize specific effects. Fig. 1 shows that the three curves in each case are approximately parallel.

Likewise, the activity of α-chymotrypsin with ATEE and TEE as substrates was determined. With the former substrate, which is not soluble in water, only two D values were studied: 76.5 and 80.5. In contradistinction to trypsin, the pH-activity curves at different D values are not parallel but

[1] Throughout this paper the following abbreviations and symbols will be used:
BAEE, benzoyl-L-arginine ethyl ester.
TSAME, *p*-toluenesulfonyl-L-arginine methyl ester.
ATEE, acetyl-L-tyrosine ethyl ester.
TEE, L-tyrosine ethyl ester.
PEE, L-phenylalanine ethyl ester.
TrEE, L-tryptophane ethyl ester.
D, dielectric constant.
R, rate of hydrolysis in a given medium.
R_o, rate of hydrolysis in aqueous media.

they cross each other at pH values 6.9 and 7.1 respectively (Fig. 2). For the purpose of investigating whether the substrate plays a role in the observed inversion of dielectric effects, two more substrates were tested: (*a*) PEE, which like TEE is water-soluble, and (*b*) the trypsin substrate BAEE in cross-reaction with α-chymotrypsin. A similar phenomenon was observed

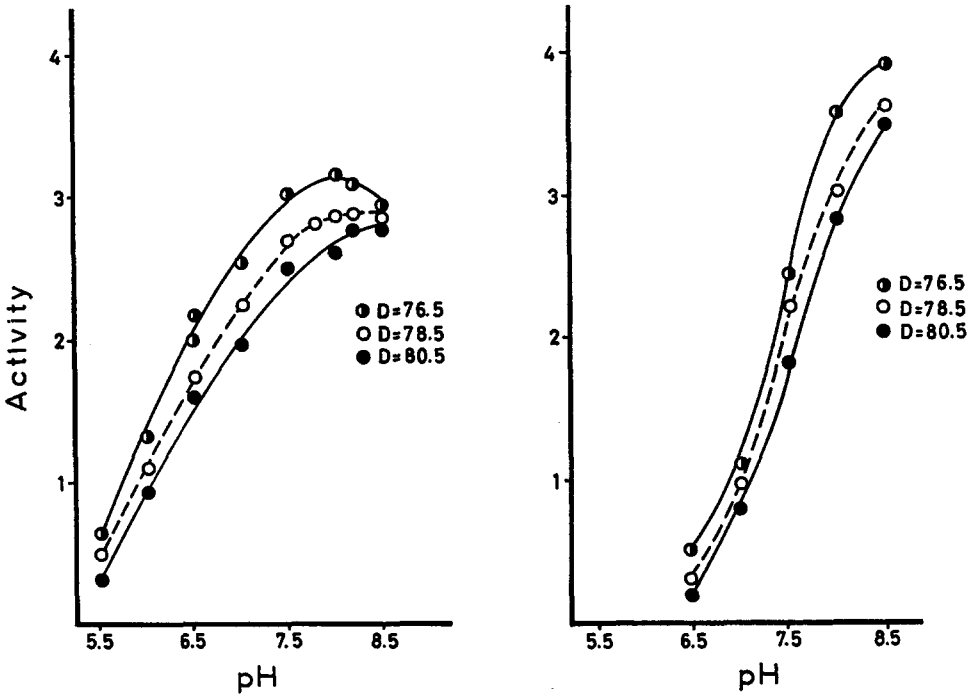

FIGURE 1. Effect of small changes in the medium dielectric constant on the activity of trypsin at different pH values. Enzyme concentrations, 1.45 μg. N per ml. with BAEE and 0.41 μg. N per ml. with TSAME. Substrate concentration, 0.008 M. Temperature, 25°C. Buffer, 0.05 M KH_2PO_4–Na_2HPO_4. Solvents, 0.6 M acetone (D = 76.5), water (D = 78.5), and 0.74 M urea (D = 80.5).

and the curves of activity in different dielectric constants intersect at pH values 7.15 and 7.3 respectively (Fig. 3).

Since the inversion of dielectric effects on α-chymotrypsin activity seems to be independent of the substrates, only one of these, PEE, was employed to investigate whether the linear relationship log of rate against reciprocal of D observed previously at pH 7.8 with TrEE (6) is also maintained at a pH lower than the point of intersection. For this purpose, urea was used to modify the medium dielectric constant at pH values 6.5 and 7.5. The choice of urea was founded on the previous observation (6) that the reaction rates of the system α-chymotrypsin–TrEE in urea solutions at pH 7.8 hold a rela-

tionship with the dielectric constant within the D range of 78.5 to 92.5 (0 to 5.19 M urea). Again, no specific effects are noticeable within the mentioned range.

The plots of log of the relative rate of hydrolysis *versus* $100/D$ at the two pH values, one lower (6.5), and the other higher (7.5) than the point of intersection, are straight lines within D ranges of 78.5 to 90.5 (0 to 4.45 M urea) and 78.5 to 89.5 (0 to 4.08 M urea) respectively. However, the slopes

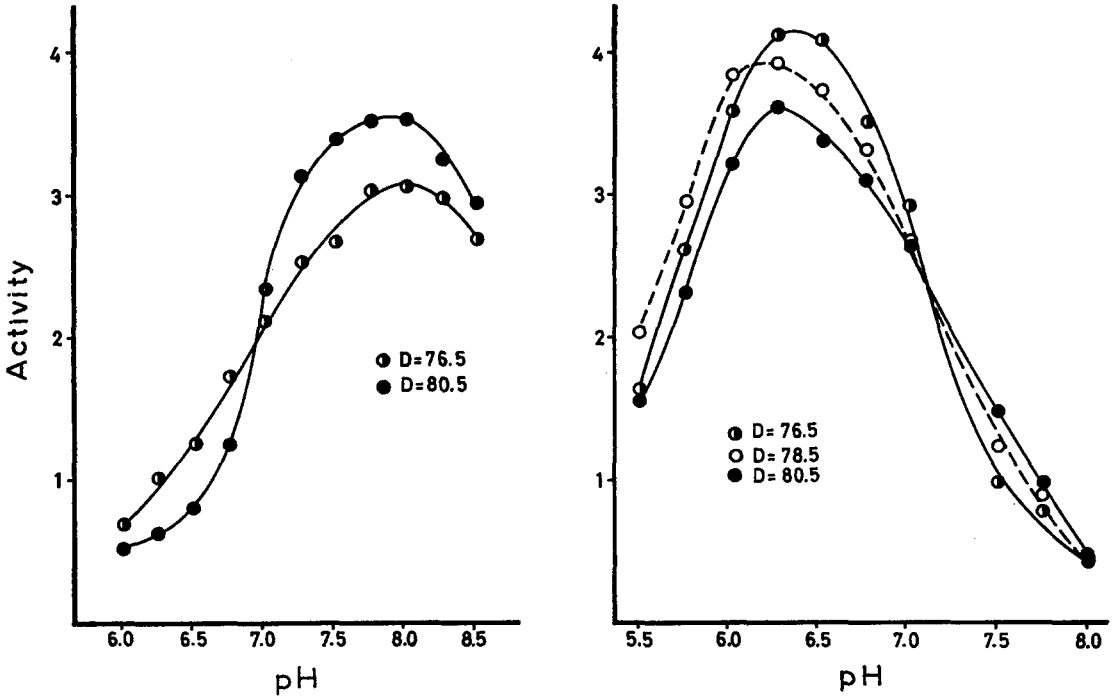

FIGURE 2. Effect of changes in the medium dielectric constant on the activity of α-chymotrypsin on ATEE and TEE at different pH values. Enzyme concentrations, 0.47 μg. N per ml. with the first substrate and 3.1 μg. N per ml. with the second. Substrate concentrations, 0.005 M and 0.016 M respectively. Temperature, 25°C. Buffer, 0.05 M KH_2PO_4–Na_2HPO_4. Solvents, 0.6 M acetone (D = 76.5), water (D = 78.5), and 0.74 M urea (D = 80.5).

are opposite in sign: 0.66 ± 0.03 at pH 6.5, and −1.05 ± 0.02 at pH 7.5. After D = 90.5 or 89.5 in either case the curves bend gradually up to a limit of D = 92.5 (5.19 M urea). From D = 93.5 onward, there is a steep fall in the activity on both sides (Fig. 4).

All the experiments just described were carried out in solutions of phosphate buffer at concentration 0.05 M. In order to ascertain any influence of the buffer on the observed phenomenon, the pH-activity curves of α-chymotrypsin

–PEE were determined again at three dielectric constants but without buffer. The required pH of the solutions was attained by the addition of the proper amount of 0.1 N sodium hydroxide solution. As can be observed in Fig. 5, the inversion of dielectric effects persists in the absence of phosphate. The point of crossing in this case is pH 6.6 approximately.

The observation that the hydrolysis of PEE by α-chymotrypsin is affected in two opposite ways by changes in D depending on pH, on the one hand,

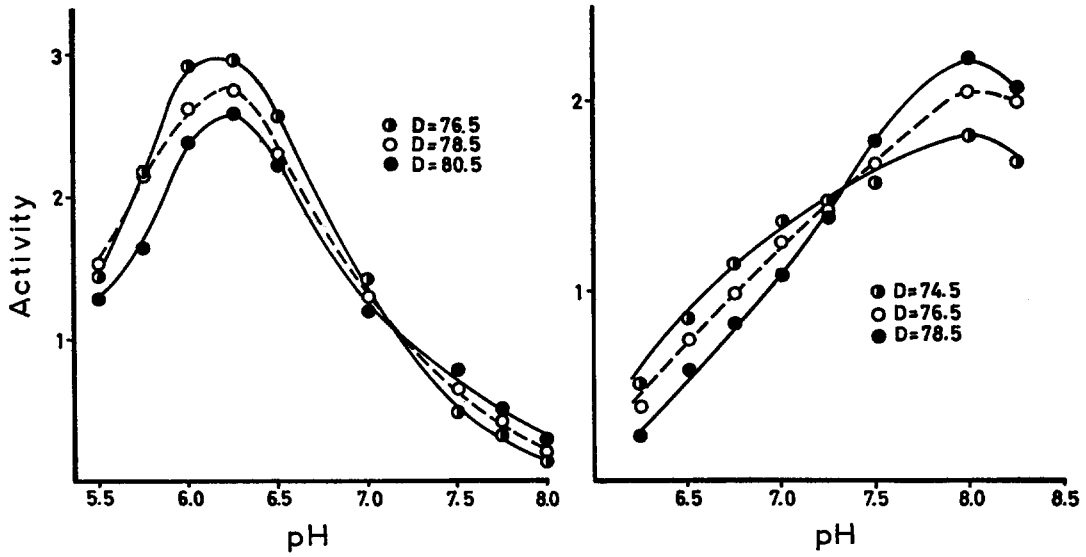

FIGURE 3. Activity-pH curves of the systems α-chymotrypsin–PEE and α-chymotrypsin –BAEE at three different medium dielectric constants. Enzyme concentrations, 6.2 μg. N per ml. with PEE and 46.5 μg. N per ml. with BAEE. Substrate concentrations, 0.016 M and 0.020 M respectively. Buffer, 0.05 M KH_2PO_4–Na_2HPO_4. Temperature, 25°C. Solvents, 1.2 M acetone (D = 74.5), 0.6 M acetone (D = 76.5), water (D = 78.5), and 0.74 M urea (D = 80.5).

and that these effects are well defined at points approximately equidistant from the intersection, on the other, presented the problem of whether the effects changed abruptly at the crossing point or showed states of transition around this pH. To elucidate this question, experiments were done involving variation of dielectric constant from 78.5 to 92.5 at pH values ranging from 6.25 to 7.75 with the system α-chymotrypsin–PEE, in the absence of added buffers. The D value of 92.5 was chosen as an upper limit because it corresponds to the maximum concentration of urea at which no rapid falls in the activity of α-chymotrypsin were previously induced. The curves representing these experiments are given in Fig. 6. It can be observed that starting from 6.75 up to 7.75, all the lines have a common initial portion with negative

slope -1.02 ± 0.05. At pH 7.5 and 7.75 the lines are straight up to D = 90.5, at which point they descend abruptly. The lines corresponding to pH values 7.25, 7.0, and 6.75 change their course at a lower level each time, that is at about the following D values: 85.5, 83.5, and 82.5 respectively. Those of pH 7.25 and 7.0 show two points of inflection, the second being at D =

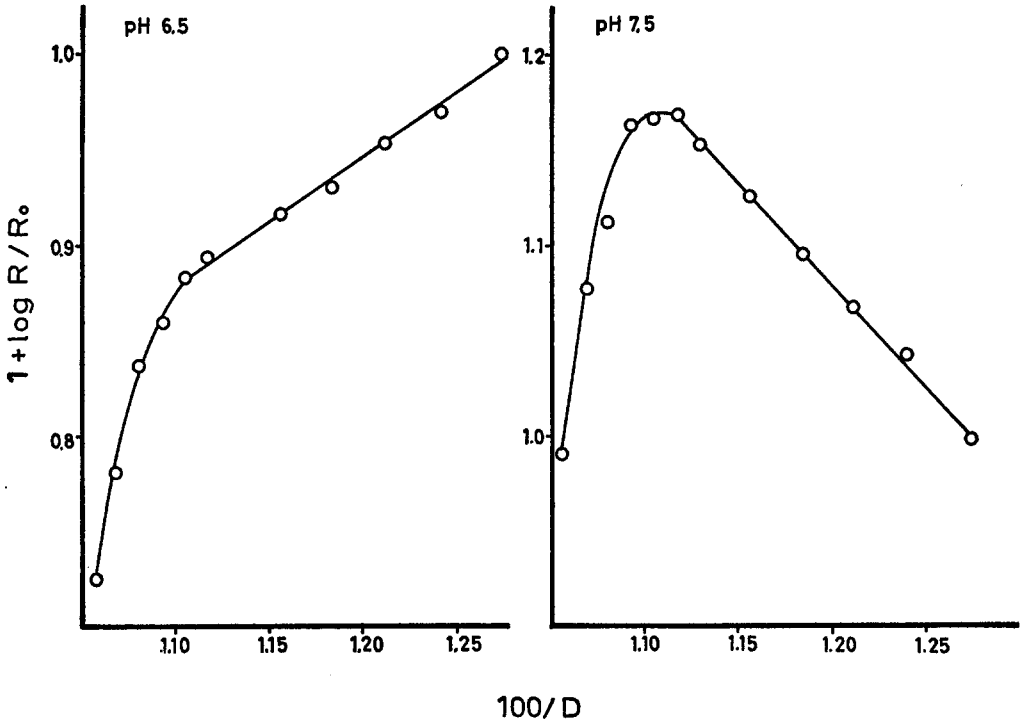

FIGURE 4. Effect of the variation in the medium dielectric strength on the hydrolysis of PEE by α-chymotrypsin at pH values of 6.5 and 7.5. Enzyme concentrations, 6.2 μg. N per ml. at pH 6.5 and 12.4 μg. N per ml. at pH 7.5. Substrate concentration, 0.016 M. Temperature, 25°C. Buffer, 0.05 M KH_2PO_4–Na_2HPO_4. Solvents, urea solutions varying in concentration from 0 to 5.93 M (D = 78.5 to 94.5). The straight portions of the lines have the slope values 0.66 ± 0.03 (pH 6.5), and -1.05 ± 0.02 (pH 7.5), and intersections 0.15 ± 0.03 (6.5), and 2.34 ± 0.01. (pH 7.5).

90.5. The curve of pH 6.75 has three points of inflection at D = 82.5, 86.5, and 90.5. At pH 6.6, the slope of the initial portion has turned positive (0.33 ± 0.02) but is less steep than that of the line corresponding to pH 6.25 (1.08 ± 0.06). At this last pH, the slope has reached a numerical value equal to that of pH 7.75 although opposite in sign.

In the same way as in the above experiments, the effect of variation in D was studied at pH values higher than 7.75 (Fig. 7). At pH 8.0 the slope of the line remains negative but has a smaller value, and at pH 8.25 it becomes

again positive and shows a point of inflection at $D = 80.5$. The final bending observed in most of the other curves is absent here; the line is continued through $D = 80.5$ to 92.5. This final portion has a slope 1.52 ± 0.03. More alkaline pH values were not tested because of the possibility of substrate autolysis.

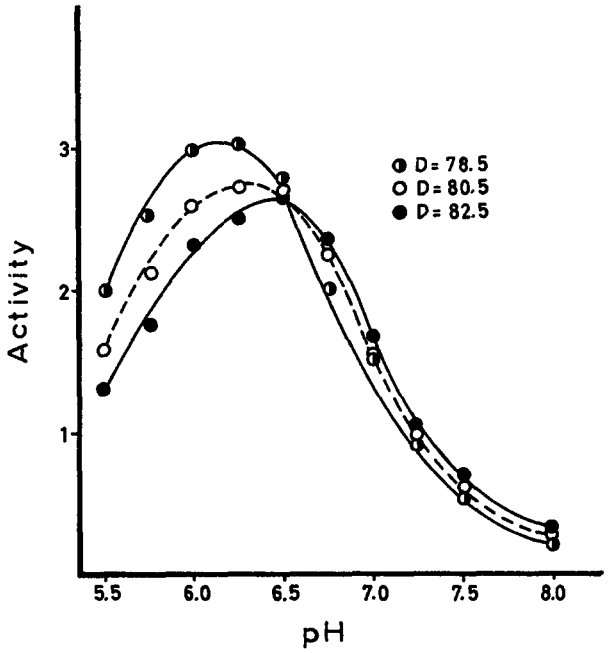

FIGURE 5. Activity-pH curves of the system α-chymotrypsin–PEE at three distinct medium dielectric constants in absence of added buffer. Enzyme concentration, 6.2 μg. N per ml. Substrate concentration, 0.016 M. Solvents, water ($D = 78.5$), 0.74 M urea ($D = 80.5$), and 1.48 M urea ($D = 82.5$). Temperature, 25°C.

DISCUSSION

The addition of organic solvents or solutes to modify the dielectric constant in a reacting system may give rise to additional effects of non-electrostatic character which also alter the rate of reaction. Amis (1) pointed out that when one refers to these as "specific effects" to differentiate them from the dielectric effects, he is really confessing his ignorance of their nature. Until recently the impossibility of discriminating electrostatic contributions from those of other types was considered the principal inconvenience in attempts made to ascertain the influence of the medium dielectric constant on enzymatic reactions (13). However, as has been stated by the authors (6), it is feasible to distinguish both types of effects by using as many substances as possible to modify D. The comparison of the rates measured in the various

solvents, when plotted as a function of D, shows that most points follow the same trend, while others deviate markedly. It might be presumed that those falling in one line regardless of solvent are not affected significantly by effects other than the dielectric, which is common to all. The best coincidence is observed in the points which correspond to relatively low concentrations of substances. Based on such experience, acetone and urea were used in this

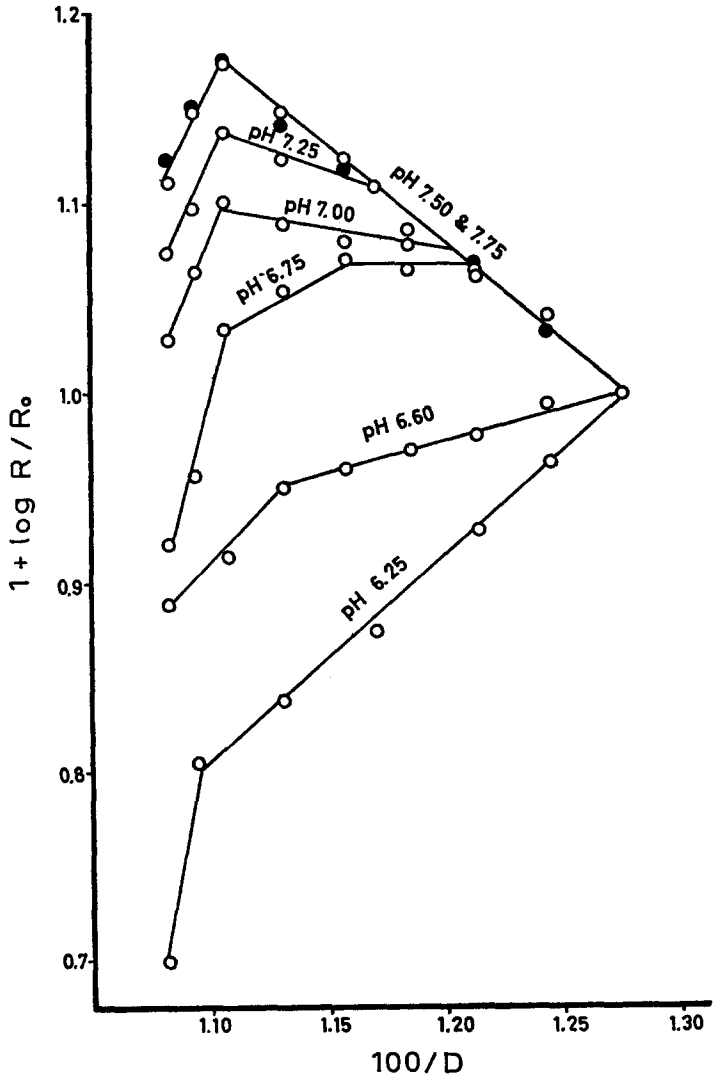

FIGURE 6. Effect of the variation in the medium dielectric constant on the system α-chymotrypsin–PEE at pH values ranging from 6.25 to 7.75. Enzyme concentration, 6.2 μg. N per ml. Substrate concentration, 0.016 M. No buffer added. Solvents, 0 to 5.19 M urea. Temperature, 25°C.

study of dielectric effects on trypsin and α-chymotrypsin at different pH values.

From the results obtained with trypsin it might be deduced that along the pH range of 5.5 to 8.5 this enzyme does not undergo any change in its charge sign. Under the specified experimental conditions, it seems to remain positively charged through the range of pH investigated. On the contrary, α-chymotrypsin shows an inversion of dielectric effects around pH 7. Accord-

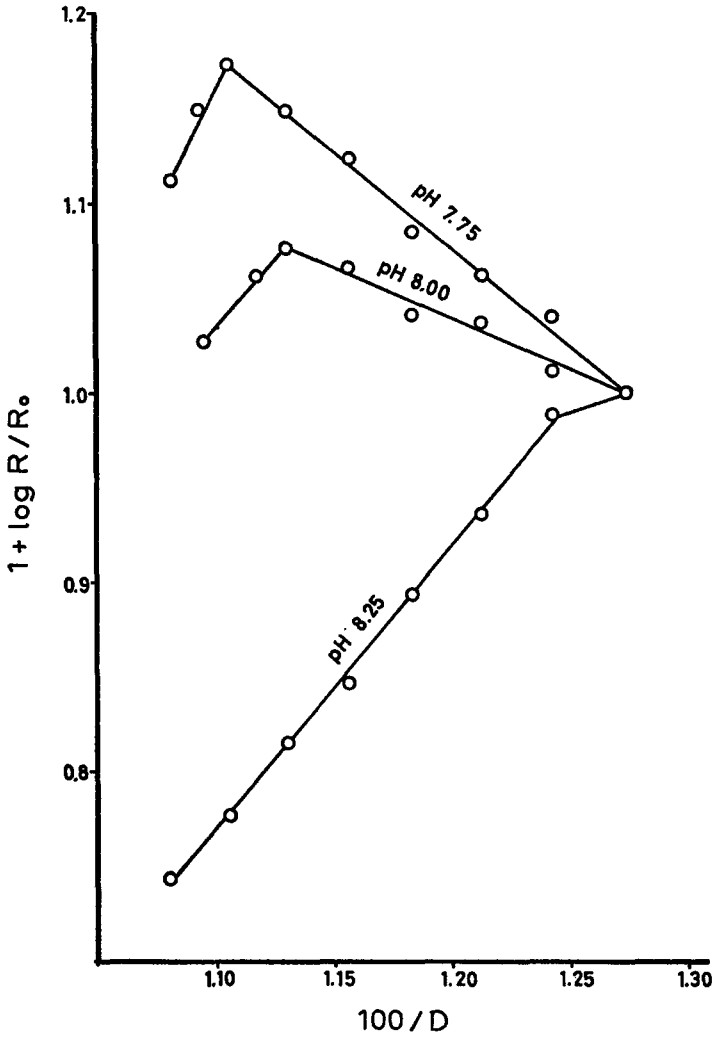

FIGURE 7. Effect of the variation in the medium dielectric constant on the system α-chymotrypsin–PEE at pH values ranging from 7.75 to 8.25. Enzyme concentration, 15.5 μg. N per ml. Substrate concentration, 0.016 M. No buffer added. Temperature, 25°C. Solvents, 0 to 5.19 M urea.

ing to the mathematical expressions deduced by Amis (2) to account for the behavior of non-enzymatic reactions, the hydrolysis of esters by α-chymotrypsin adopts the course of a reaction cation-dipole in the region of pH below 7 approximately, and that of a reaction anion-dipole at pH values between 7 and 8. Somewhere between pH 8 and 8.25 the enzyme seems to undergo another change in the charge sign, so that at pH 8.25 the reaction is again like one of a positive ion and dipolar molecule. These observations agree with the suggestion first made by Neurath and Schwert (15) that possibly a negative charge is present at or near the catalytically active site in α-chymotrypsin (they worked at pH 7.8). Further evidence of this negative charge was obtained by Foster and Niemann (7) on the basis of the comparative affinity of α-chymotrypsin for charged and non-charged inhibitors at pH values 6.9 and 7.9. These authors observed that the negative charge referred to is particularly evident at pH 7.9 and seems to be largely lost at pH 6.9. This last value lies within those obtained here for the points of change in the charge sign of the enzyme with the various substrates (6.6 for PEE without phosphate, 6.9 to 7.3 with phosphate buffer).

The dielectric effects are similar in all reactions involving α-chymotrypsin studied here. In the first place, the reaction rate diminishes as D increases, then at a pH which varies with the 4 substrates studied from 6.9 to 7.3 (in 0.05 M phosphate buffer) the effect is reversed; *i.e.*, the rate increases as D increases. By comparison of the curves obtained with ATEE and BAEE on the one hand, and TEE and PEE on the other, it is obvious that the presence of a free ammonium group in the latter substrates shifts the optimum pH of activity from 8 to 6.25. Nevertheless the points of intersection do not appear to be influenced significantly, in spite of the presence of different ionizable groupings in the substrates: phenolic hydroxyl in ATEE and TEE, ammonium in TEE and PEE, and guanidinium in BAEE. This suggests that the electrostatic attractions which are modified by the medium dielectric strength, are exerted mainly through the positively or negatively charged molecule of the enzyme.

In the reaction α-chymotrypsin–PEE, the linear relationship log of rate *versus* reciprocal of D is followed at both pH values 6.5 and 7.5, in accordance with Amis' (2) equations for ion-dipole reactions. At pH 6.5 the slope is positive as predicted for reaction cation-dipole, and at pH 7.5 the slope is negative as would be expected for an anion-dipole reaction.

The point of crossing seems to change somewhat in the presence of phosphate buffer. With PEE, this point was shifted from pH 6.6 to 7.15 by addition of 0.05 M phosphate. The plots log of rate against $100/D$ with and without phosphate also show a dissimilarity: after reaching the maximum (enhancing or delaying) effect, the curves of the experiments with phosphate

bend gradually (within the D range of 89.5 or 90.5 to 92.5), while in the absence of added buffer, the lines are broken in angles (*cf.* Figs. 4, 6, and 7).

The two observed points at which the enzyme appears to change its charge sign suggest two interpretations: The first possibility is that the isoionic point of the enzyme is that of the first intersection; that is, *ca.* 6.6. Kunitz and Northrop (11) found the isoelectric points of crystalline chymotrypsinogen and α-chymotrypsin to be 5 and 5.4 respectively by their method of microcataphoresis. More recently, Ingram (9) working in Kunitz' laboratory, determined again the isoelectric point of chymotrypsinogen by a method based on the Donnan equilibrium, and obtained a value of 6.3 (in either dilute acetate or phosphate buffer). Even though he did not report a value for the isoelectric point of α-chymotrypsin, if it were assumed that there is a difference between the isoelectric points of the enzyme and its precursor similar to that in the method of microcataphoresis, the resultant value would approximate the data obtained here for the first intersection. On the supposition that 6.6 is the isoionic point of α-chymotrypsin, it must be admitted that the positive charge which the enzyme acquires at pH 8.25 is due to the appearance of a positive grouping which was masked in more acid media. The mechanism of unmasking might be similar to the one proposed for explaining the liberation of masked titratable groups in globin by action of dilute acid (*cf.* Steinhardt and Zaiser (17)). The change in dielectric strength at pH 8.25 might cause one "trigger" group to break and liberate one or more positively charged groupings.

The second possibility might be that the isoionic point of the enzyme is higher than pH 8.25, and that in the pH region from 6.6 to 8 a negative charge appears. This assumption is in agreement with the isoelectric point values of chymotrypsin obtained by electrophoresis by Anderson and Alberty (3) and Kubacki *et al.* (10). The data reported by these authors range from pH 8.1 (in 0.1 M buffer) to 8.6 (in 0.01 M buffer). If such is the case, the enzyme charge must change to negative at a more alkaline pH. Unfortunately, this could not be proved because most esters autolyze under these conditions. Van Vunakis and Herriott (quoted by Herriott (8)) observed that a comparison of the titration curves of chymotrypsinogen and α-chymotrypsin over the range of pH 5.4 to 7.4 shows the enzyme to possess one more titratable group per molecule than its precursor. This group showed a midpoint at pH 6.8, which is near pK of histidine imidazole (pH 7.0). On the other hand, examination of the graphs which represent the dielectric effects on PEE hydrolysis by chymotrypsin at pH values ranging from 6.25 to 7.75 (Fig. 6) draws attention to the following: (*a*) at the two extreme pH values the slopes are equal in magnitude but with opposite sign, and (*b*) at intermediate pH values, the lines appear broken in various straight portions, the slope of each varies

progressively in magnitude and sign from one limit to the other. Such behavior suggests that the observed effect might be due to resonance, a phenomenon present in the imidazole ring. As Barnard and Stein (4) have pointed out in a review of the biological properties of imidazole, the coincidence in this ring of a secondary and a tertiary nitrogen which may be mutually interconverted bestows on it special properties. The possibility of resonance electron shifts in the ring is enhanced in acid media. A histidine side chain has been involved as an essential group for the catalytic function of a number of enzymes, α-chymotrypsin among them. Evidence in support of this idea is collected in the review of the literature by Barnard and Stein (4) and by Neurath (14).

According to present ideas, proteins are susceptible to wide variations in structure. Many proteins, *i.e.* those so called "motile" proteins (α-chymotrypsin has been included in this group (12)), may undergo reversible unfolding reactions. This makes possible the coexistence of various forms at equilibrium. Experimental evidence that this phenomenon of "motility" is present in insulin and poly-D-L-alanine has been provided by Linderstrøm-Lang (12) who used a method of deuterium exchange. On the other hand, there is a notable discrepancy among the various isoelectric points of chymotrypsinogen or α-chymotrypsin as measured by different procedures. For instance, the following data have been reported for the isoelectric point of chymotrypsinogen: 5.0 by microcataphoresis (11), 6.3 by Donnan equilibrium (9), and 9.1 (10) to 9.5 (3) by electrophoresis. Such wide variation hardly can be attributed to experimental error. It is suggested that, on the basis of the motile properties of these proteins and under the conditions prevailing in the methods referred to, a distinct structure might dominate in each case. It might be further speculated that successive variations of pH and dielectric constant, as in the present study, may induce configurational changes in α-chymotrypsin, so that as one progresses in the pH–activity–D curves, distinctly predominant forms are being evolved. Some of these may coincide with those appearing separately and which are responsible for the various isoelectric points determined. The concept of motility renders theoretically possible the existence of more than one isoelectric point in a single molecule of α-chymotrypsin. This seems to be not the case with trypsin, at least within the ranges of pH and D studied.

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