

# Effect of the Medium Dielectric Strength on the Activity of Alpha Chymotrypsin

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**ABSTRACT** The rates of hydrolysis of TrEE, TEE, and ATEE<sup>1</sup> by  $\alpha$ -chymotrypsin were determined in media of variable dielectric strength. Many substances which modify the dielectric constant of the medium, exert additional specific effects on the reaction rate, noticeable at more or less elevated concentrations. Notwithstanding, it is possible to differentiate the dielectric and specific effects by comparing the rates in solvents of distinct nature at relatively low concentrations. Thus, the effect of varying the dielectric strength could be studied within wider ranges ( $\Delta D = 20$  with TrEE and *ca.* 28 with ATEE) than in the previous study of trypsin ( $\Delta D = 12$ ). The dielectric effect on  $\alpha$ -chymotrypsin is the opposite of that observed with trypsin. In both cases there is a linear relationship between the logarithm of the rate of hydrolysis and the reciprocal of the dielectric constant. The slope is negative with  $\alpha$ -chymotrypsin and positive with trypsin. According to expressions relating the dielectric constant to the rate in non-enzymatic reactions, the behavior of  $\alpha$ -chymotrypsin is like that of a negative ion, while trypsin behaves as a positive ion. The enzyme activity appears to depend upon the arrangement of charges in the enzyme and substrate molecules, rather than on the presence of certain atomic groupings in the substrate.

## INTRODUCTION

Evidence was given in an earlier communication (8) that the rate of ester hydrolysis by trypsin varied inversely to the medium dielectric strength. This reaction seems to follow the same law as a non-enzymatic reaction ion-dipole. In the present study, observations were made on the influence of the variation

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<sup>1</sup> Throughout this paper the following abbreviations will be used:

TrEE, L-tryptophane ethyl ester.

TEE, L-tyrosine ethyl ester.

ATEE, acetyl-L-tyrosine ethyl ester.

BAEE, benzoyl-L-arginine ethyl ester.

BAME, benzoyl-L-arginine methyl ester.

TSAME, *p*-toluenesulfonyl-L-arginine methyl ester.

*D*, dielectric constant.

$\Delta D$ , change in dielectric constant.

$\Delta E$ , energy of activation.

$\Delta E_c$ , Coulombic energy of activation.

*R*, rate of hydrolysis in a given medium.

*R*<sub>0</sub>, rate of hydrolysis in aqueous media (*D* = 78.5 at 25°C.).

in dielectric strength on the hydrolyses of esters by alpha chymotrypsin, under conditions which minimize the specific effects of the solvents. Several investigators (4, 10, 11) had previously attempted kinetic studies of the hydrolyses of various esters by alpha chymotrypsin in media of various dielectric constants, but unfortunately interference by specific interactions with the solvent was not eliminated. The substrates most commonly used for chymotrypsin are not soluble in water. On the other hand, the reactions involving this enzyme were found to be even more sensitive to the specific effects than those in which trypsin intervenes. Then, the difficulty arose that the dielectric range available for the intended study was so reduced as to preclude useful results. Nevertheless, the problem could be solved by the use of two water-soluble substrates: TrEE and TEE. Furthermore, the observation that ATEE is soluble in urea, thiourea, and dimethylurea solutions, though insoluble in water, made it possible to widen the dielectric range for this ester to a more acceptable value.

### *Materials and Methods*

All esters used were of Mann preparations. The alpha chymotrypsin sample was the same one which was assayed for purity as described in a former publication (7).

The method of esterase activity described by Schwert *et al.* (17) and modified by the authors (8) was used to determine the reaction rates. These were calculated as the amount of ester hydrolyzed in  $10^{-6}$  mols in an initial interval of 5 minutes.

The preselected dielectric constants were obtained by adding various substances at the required concentrations to the aqueous media according to the available data cited in the previous paper (8).

The accuracy and sensitivity of the determinations could be improved by using the amplified scale of a Beckmann pH meter model GS.

### RESULTS

On the basis of the previous observations with trypsin (8), the behavior of alpha chymotrypsin with regard to the change in dielectric constant was then investigated. The initial experiments were performed with one of the water-soluble esters, TrEE. This was advantageous because of the possibility of obtaining the relative rates with reference to a water control. Fig. 1 shows the effect of various concentrations of urea and symmetrical dimethylurea on the rate of hydrolysis of TrEE by alpha chymotrypsin. With urea the rate increased up to a limit value corresponding to a dielectric constant 92.5. Afterwards, it diminished slowly at first, then abruptly. With dimethylurea the rate increased progressively until the dielectric constant reached a value of 86.5, after which it began to decrease. The increase in rate observed with dimethylurea was less marked than with urea. In the case of urea there was a linear

ratio between the logarithm of the rate and the reciprocal of the dielectric strength within the range of 78.5 (aqueous medium) to 92.5 (5.19 M urea).

In order to ascertain whether the increased rate in these experiments was due either to the increase in dielectric strength or to some other effect, determinations were made in media of lower dielectric constants (mixtures of

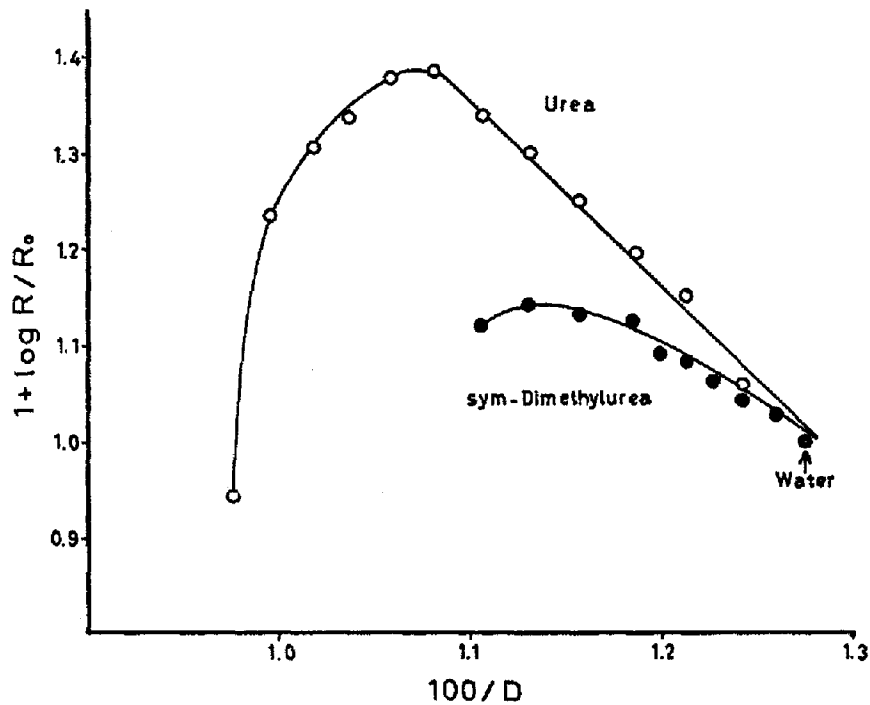


FIGURE 1. Effect of the variation in the medium dielectric strength, by addition of urea or dimethylurea, on the rate of hydrolysis of TrEE by alpha chymotrypsin. Temperature, 25°C., pH 7.8. Ester concentration, 0.02 M. Enzyme, 15.5  $\mu$ g. N per ml. Ionic strength, 0.14.

water with alcohols or dioxane). The reaction rate diminished in these media. At isodielectric concentrations, the results obtained with the various solvents agreed, within experimental error, in a dielectric range of 78.5 to 72.5. Beyond this value, the rates observed in the individual solvents differed more widely, probably owing to the specific effects. The average values of the rates measured in seven solvents were treated statistically together with those determined in the urea solutions to obtain the best fit for the line to be passed through them. Fig. 2 shows that all the points fell in the same line which has a negative slope  $-1.93 \pm 0.04$ , and intersection  $3.48 \pm 0.04$ .

The chymotryptic hydrolysis of the second substrate, TEE, responded to the change in dielectric strength in the same way as that of TrEE. The rates

measured in isodielectric solutions of isopropanol, tertiary butanol, acetone, and dioxane are in accord among themselves within the dielectric range of 78.5 to 74.5. That the specific effects are minimum with these solvents may be inferred from the observation that the standard deviations are practically equal for all the points (Fig. 3 A). The slope of the line was  $-2.45 \pm 0.04$ ,

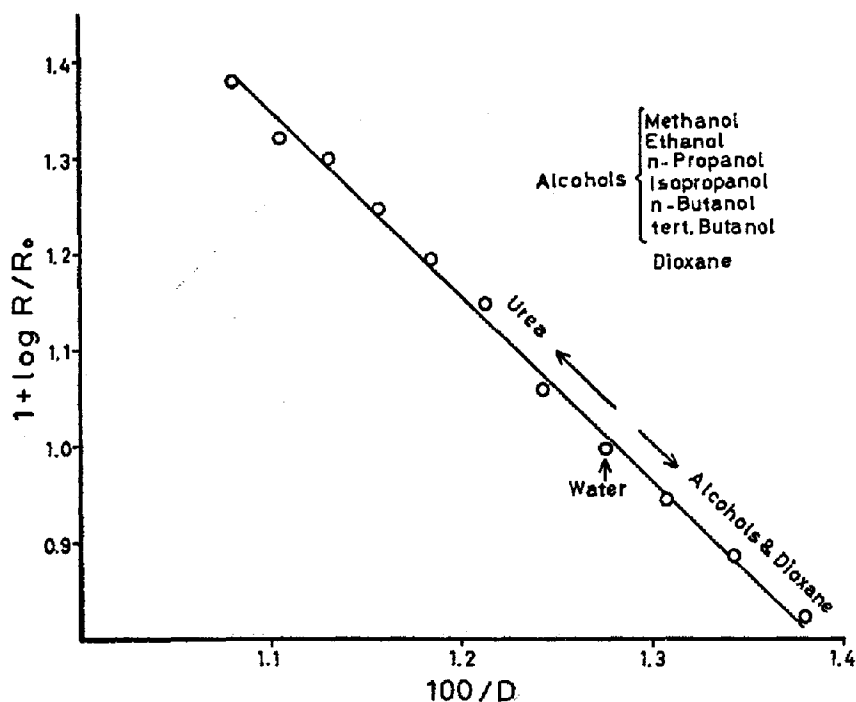


FIGURE 2. Effect of the variation in the medium dielectric strength, by addition of urea, alcohols, or dioxane, on the rate of hydrolysis of TrEE by alpha chymotrypsin. The line was fitted by the least square method. Temperature, 25°C., pH 7.8. Ester concentration, 0.02 M. Enzyme concentration, 15.5  $\mu$ g. N per ml. Ionic strength, 0.14.

and the intersection  $4.10 \pm 0.05$ . Conversely, it is evident from Fig. 3 B that ethanol, normal propanol, and normal butanol exert specific effects, because the standard deviations increased as the concentration of alcohol increased. The slope of the least square line was  $-3.69 \pm 0.09$ , and the intersection  $5.69 \pm 0.12$ .

The third substrate studied was ATEE, one of those most commonly used with chymotrypsin. It is insoluble in water and soluble in mixtures of organic solvents with water, solvency time decreasing with the proportion of solvent in the solution. The solvation of small amounts of this ester in relatively low concentrations of solvent was attained by prolonged stirring. By the same procedure ATEE could be dissolved in urea, thiourea, and dimethylurea solutions. This permitted the enlargement of the dielectric range of the determina-

tions to a value of *ca.* 28. The spread of the results with ATEE was greater than in the previous experiments with TrEE or TEE. This might be due to the impossibility of referring the data to a water control, or to some uncertainty concerning the complete solvation of the substrate in dilute solvent. Owing to these observations and in order to get results more representative

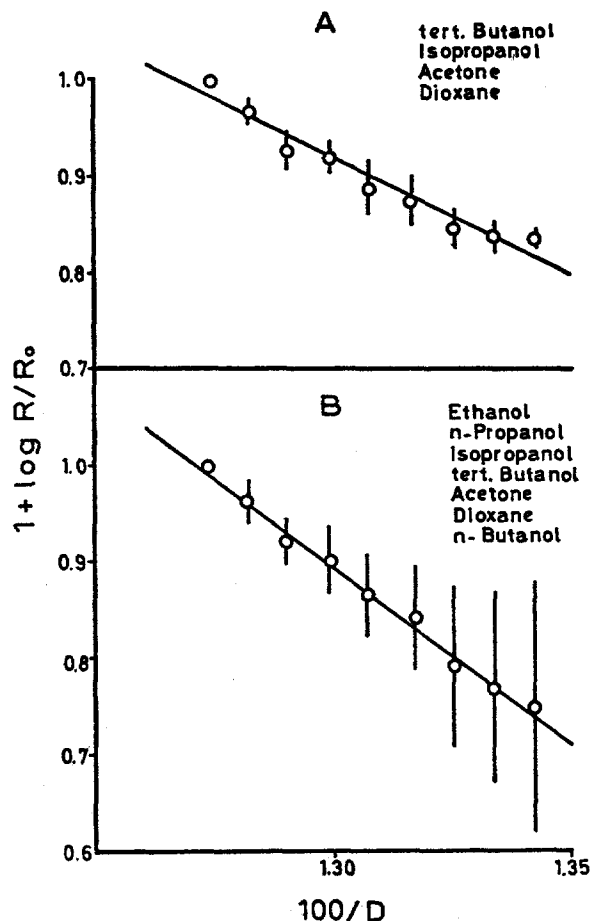


FIGURE 3. Effect of the variation in dielectric strength of the medium on the rate of hydrolysis of TEE by alpha chymotrypsin. A, average and standard deviations of data given by the four solvents indicated. B, average and standard deviations of rates measured in the solvents used in A, plus ethanol, normal propanol, and normal butanol. Both plots are the least square lines. Temperature, 25°C., pH 7.8. Ester concentration, 0.02 M. Enzyme concentration, 15.5  $\mu$ g. N per ml. Ionic strength, 0.14.

of the relationship between the rate of hydrolysis and the dielectric constant, a larger number of solvents was used than with the other two substrates. Some of the solvents were tested at various concentrations, from the minimum required for dissolving the ester to the maximum which did not appear to pro-

duce a significant specific effect. The least square line fitting the experimental points was plotted in Fig. 4. The slope value, like the slope values of TEE and TrEE, was negative,  $-2.21 \pm 0.08$ . The intersection was  $4.33 \pm 0.11$ . The correlation coefficient  $-0.98$  indicates a nearly perfect functional relationship.

The coulombic energies of activation for TEE and TrEE were determined

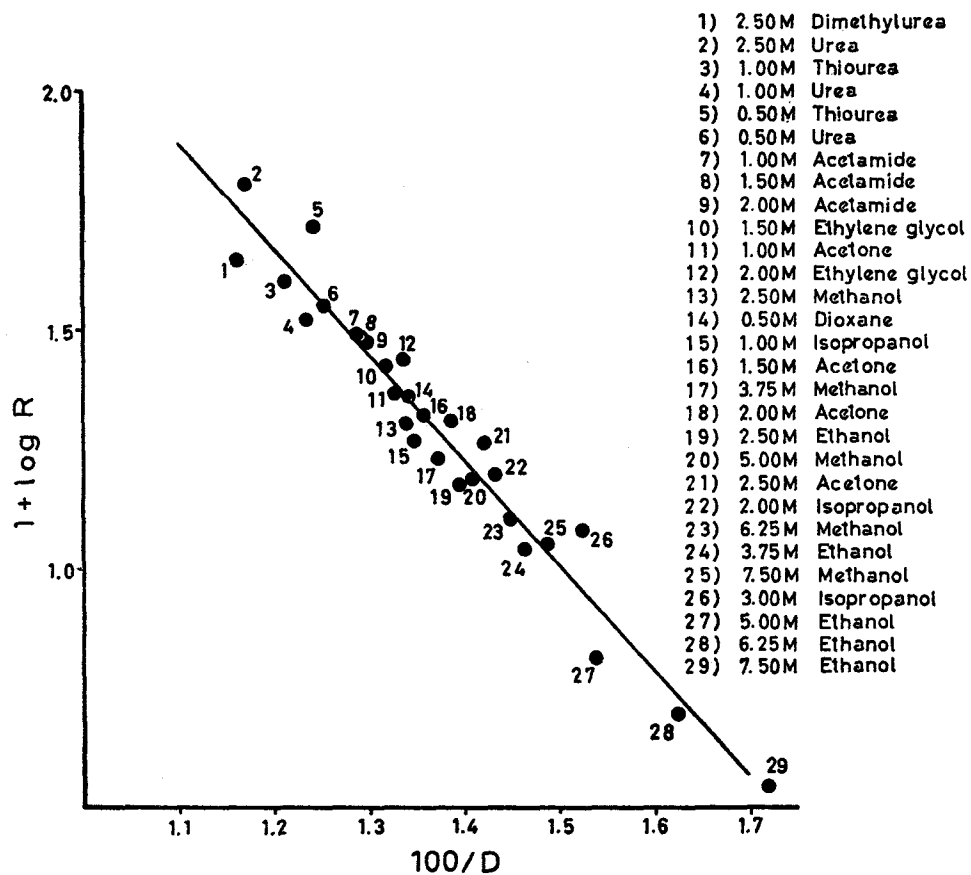


FIGURE 4. Effect of the variation in dielectric strength, by addition of various solvents, on the rate of hydrolysis of ATEE by alpha chymotrypsin. The line was fitted by the least square procedure. Temperature, 25°C., pH 7.8. Ester concentration, 0.005 M. Enzyme concentration, 0.47  $\mu$ g. N per ml. Ionic strength, 0.14.

in the same way as with trypsin (8). In the first place, the rates of hydrolysis were measured at two temperatures: 10° and 25°C. in isodielectric solutions. With these data, the total energy of activation for a chosen dielectric strength was calculated in the usual manner. The procedure was then repeated in media of a different dielectric strength, and the coulombic energy of activation calculated by subtracting energies of activation at the two distinct di-

electric constants (Table I). The phenomenon previously observed with trypsin of the different trends in the coulombic energies of activation for the two substrates TSAME and BAEE (8) appeared also with TEE and TrEE.

## DISCUSSION

The rates of hydrolysis of TrEE, TEE, and ATEE by alpha chymotrypsin varied with the medium dielectric strength. Of the various substances employed to modify the dielectric constant of water, some exerted specific effects more marked than the others. The extent of such effects varied not only with the solvent but also with the substrate. However, it was possible to obtain in-

TABLE I  
COULOMBIC ENERGIES OF ACTIVATION OF TEE AND TrEE

Substrate	<i>D</i>	Solvent	$\Delta E$ (cal.)	$\Delta E_c$ (cal.)	$\Delta E_c/\Delta D$
TEE	73.5	Isopropanol	3416	-1833	-367
	78.5	"	1583		
	73.5	Dioxane	3872	-1717	-343
	78.5	"	2155		
TrEE	72.5	Ethanol	93	3388	339
	82.5	Urea*	3481		
	70.0	Methanol	641	3352	394
	78.5	"	3993		
	73.5	Dioxane	36	1282	256
	78.5	"	1318		

\* At 25°C. the solvent was urea solution and at 10°C., ethanol solution.

formation about the relationship between the rate and the dielectric constant regardless of specific solvent actions by using a variety of solvents. Most rates thus obtained lay along a line when plotted in function of the dielectric strength, indicating that the same effect in every case is responsible for the variation in rate. In the investigations concerning the influence of the dielectric strength on chymotrypsin kinetics made prior to this work, only mixed methanol-water solvents were used (4, 10, 11). Under these conditions, the results can scarcely be considered as representative of the dielectric effect only. According to Laidler, the information given by those experiments was not very reliable (14). If a single solvent is used in increasing proportions, sooner or later its specific effects will become significant. Thus, there is no way of separating the dielectric and specific effects. On the contrary, by the comparison of the rates in solvents of distinct nature, it is possible to distinguish the two effects. When significant specific interactions are involved, the experi-

mental points depart markedly from the trend followed by most of the data; *e.g.*, the effect of dimethylurea shown in Fig. 1 and the effects of ethanol, *n*-propanol, and *n*-butanol shown in Fig. 3 B.

By the procedure above indicated, involving the use of rather low concentrations of various solvents, it was possible to investigate the effect of the variation in dielectric strength upon the rates of hydrolysis of TrEE and ATEE by chymotrypsin within wider dielectric ranges ( $\Delta D = 20$  and 28 respectively) in comparison with the range in which the trypsin kinetics was studied ( $\Delta D = 12$  (8)).

Urea, in concentrations not higher than 5.19 M, seems to exert no other effect on the hydrolysis of TrEE by chymotrypsin than that due to the increase in dielectric strength. It might be supposed that the further diminution of rate is caused by denaturation of the enzyme.

For the three substrates studied, TrEE, TEE, and ATEE, the plots of the logarithm of the rate against the reciprocal of the dielectric constant were straight lines with negative slopes. The magnitude of the slopes was similar in the three cases. This indicates approximately equal sensitivities of the three reactions to the dielectric effect.

According to the expression:

$$\ln k'_D = \ln k'_\infty + \frac{z\epsilon\mu}{DkT\tau^2}$$

deduced by Amis (1) to account for the relationship between the rate and the dielectric constant in a non-enzymatic reaction ion-dipole, the hydrolyses of esters by chymotrypsin respond like the reaction of a negative ion with a dipolar molecule. This behavior differs from that observed with trypsin, which acts like a positive ion (8).

In order for trypsin to be positively charged in solution its isoelectric point must be higher than the pH of the solution (7.8 in this case). Northrop and Kunitz (16) found the isoelectric point of trypsin to be approximately 7 when determined by the electrophoretic mobilities of collodion particles suspended in a solution of this enzyme. Bier and Nord (5) obtained the datum 10.8 by standard electrophoresis. According to Hartman, Bateman, and Edelhoch (9), the discrepancy between these results might be due to the formation, in the first case, of an inactive product at the interface between solid trypsin and the aqueous solution. This product differs electrophoretically from active trypsin and its mobilities coincide with those of the collodion particles. The isoelectric point of alpha chymotrypsin was determined by cataphoresis (5.4) by Kunitz and Northrop (13) and by electrophoresis (8.1 to 8.6) by Anderson and Alberty (2) and Kubacki *et al.* (12). The present observation that alpha chymotrypsin behavior is like that of a negative ion would be consistent with Kunitz and Northrop's datum but not with the data given by the other



authors. Anyway, the pH of the test is not far from the isoelectric point obtained by electrophoresis, and it is possible that the isoelectric point varies with the dielectric constant of the medium.

The chymotryptic hydrolyses of esters are reactions more sensitive to the dielectric effect than the hydrolysis of BAEE by trypsin, as can be deduced from the slope values, which in the first cases reach points approximately twice as great as in the second example.

The previous suggestion of the authors (8) concerning the possibility that the dielectric constant is one of the factors which regulate the equilibrium among the various forms of trypsin (active, inactive, denatured), might be valid also for chymotrypsin. The dielectric effect is opposite with the two enzymes; while the activity of chymotrypsin increases with the dielectric

TABLE II  
COMPARISON OF APPROXIMATE RELATIVE ACTIVITIES OF  
TRYPSIN AND ALPHA CHYMOTRYPSIN UPON THEIR OWN  
AND OTHER "SPECIFIC" SUBSTRATES

Substrate	Trypsin	Alpha chymotrypsin	Reference No.
BAEE	40	1	7
BAME	50	1	7
TSAME	6000	1	7
ATEE	1	100	*
TEE	1	5	*
TrEE	1	5	*

\* Unpublished experiments.

strength, that of trypsin diminishes as the dielectric constant increases. The magnitude of the dielectric effect on chymotryptic activity is very notable. For example with ATEE, a change of 28 in the dielectric constant gave rise to an increase in rate of more than 20-fold.

The coulombic energies of activation of the substrates TrEE and TEE resulted with opposite sign. The same phenomenon was observed in the hydrolyses of BAEE and TSAME by trypsin (8). As was then said, the significance of these results is obscure. It is possible that either the dielectric effect or the specific effects of solvents are different with each substrate when the temperature is varied.

The knowledge acquired through the use of synthetic substrates for proteolytic enzymes, that each enzyme hydrolyzes preferentially substrates with a determinate structure, gave rise to the idea of a structural specificity. However, such specificity is not very strict. Cross-reactions of trypsin or alpha chymotrypsin with the "specific" substrates of each other are not infrequently observed. Table II shows that each enzyme is more active when it hydrolyzes

its own substrates than when it acts upon the substrates of the other. Proof has already been given (7) that the cross-reactions of chymotrypsin with trypsin substrates cannot be due to a contamination of the first enzyme with the second. The difference of activities varies with the substrate, being more obvious with TSAME. The differences observed with BAEE, BAME, TEE, and TrEE are not so great and might be compared to those existing among the various "specific" substrates when acted upon by their own "specific" enzyme (Table III). These differences in activity are apparent not only with synthetic substrates, but also with proteins. Table IV shows that gelatin is hydrolyzed faster by trypsin than by chymotrypsin. The opposite occurs with native egg albumin, and casein is hydrolyzed more or less at the same speed by the two enzymes. On the basis of these observations it is difficult to maintain that the affinity of the enzyme for its substrates is a matter of structural requirements exclusively.

The reactions involving trypsin or chymotrypsin show a higher sensitivity to the variation in dielectric strength of the medium as compared with many non-enzymatic reactions. This suggests that the electrostatic forces between

TABLE III  
COMPARISON OF RELATIVE ACTIVITIES OF TRYPSIN AND ALPHA  
CHYMOTRYPSIN ON SOME "SPECIFIC" SUBSTRATES

Trypsin			
BAEE	BAME	TSAME	Reference No.
1	1	25	7
Alpha chymotrypsin			
TEE	TrEE	ATEE	Reference No.
1	1	30	*

\* Unpublished experiments.

TABLE IV  
COMPARISON OF RELATIVE ACTIVITIES OF TRYPSIN AND ALPHA  
CHYMOTRYPSIN ON PROTEINS

Protein	Trypsin	Alpha chymotrypsin	Reference No.
Gelatin	60	1	6, 7*
Native egg albumin	1	30	3
Casein	1	1	6, 7

\* Value obtained by direct titration. Northrop (15) pointed out that when the determination is carried out by formol titration, the ratio of activities is 100:1, and by viscosity changes 10:1.

enzyme and substrate play a preponderant role in the reaction. Again, since trypsin acts like a positive ion and alpha chymotrypsin like a negative ion, the greater or lesser activity of these enzymes would depend upon the arrangement of charges in the substrate molecule rather than on the presence of certain atomic groupings. In the case of proteins, the distribution of charges would be more important than their total number.

#### CORRECTION

In Vol. 42, No. 3, January 20, 1959, pages 630 and 631, Tables III and IV,  $\Delta E$  values appear with negative sign. They should be positive. Values of  $\Delta E_c$  and  $\Delta E_c/\Delta D$  for BAEE must be positive and those of TSAME negative. In the second column (Table III) the order of  $D$  values must be: 78.5, 73.5, 78.5 73.5. The head at the top of the fifth column (Table III) must be:  $\Delta E_c$  ( $\Delta E_{78.5} - \Delta E_{73.5}$ ).

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