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DIELECTRIC EFFECTS AND TRANSESTERIFICATION REACTIONS CATALYZED BY TRYPSIN*

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SUMMARY

1. A comparative study has been made of the effect of two solvent systems, aqueous acetone and *n*-propanol, on the trypsin-catalyzed hydrolysis of L-lysine methyl ester (LME).

2. The use of acetone, which does not compete with water as an acceptor of the acyl group, made it possible to ascertain that the rate of this reaction depended on the gross dielectric constant of the medium in the same manner as the rates previously observed in the trypsin-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) and *p*-toluenesulfonyl-L-arginine methyl ester (TAME), *viz.*, the reaction was accelerated in media of low dielectric constant and decelerated in media of high dielectric constant.

3. The effect of *n*-propanol was converted progressively from inhibitory to enhancing as pH increased, passing through a transition between pH 7 and 7.5.

4. A trypsin-catalyzed transesterification reaction took place between LME and *n*-propanol which resulted in the formation of L-lysine propyl ester. Similar transesterification products of BAEE or TAME could not be detected.

5. The predominance of the transesterification effect of alcohols over the dielectric one at low pH values was apparently related to the degree of ionization of the alpha ammonium group of LME.

INTRODUCTION

It has been shown that the logarithm of the rate constant of tryptic hydrolysis of either benzoyl-L-arginine ethyl ester (BAEE) or *p*-toluenesulfonyl-L-arginine methyl ester (TAME) increases linearly as a reciprocal function of the medium di-

Abbreviations: LME, L-lysine methyl ester; BAEE, L-arginine ethyl ester; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

* Some of these results were included in a review on solvent effect on enzymic reactions presented at the International Symposium on "Enzymic Aspects of Metabolic Regulation" held at Mexico City from November 28 to December 1, 1966.

electric constant (D) (ref. 1). The value $\partial \log k/\partial (1/D)$ varies somewhat with the solvent system utilized, *e.g.*, alcohols are more effective than dioxane in accelerating the tryptic hydrolysis of BAEE[1,2]. However, in the trypsin-catalyzed hydrolysis of L-lysine methyl ester (LME) at pH 6.2, the liberation of titratable acid is slower in the presence of alcohols because of transesterification reactions which take place between LME and the alcohol present[3]. It appeared of interest to investigate the cause of the different behavior in alcoholic solutions of the trypsin-catalyzed hydrolyses of the N-substituted arginine derivative substrates on the one hand and that of LME on the other.

METHODS

Trypsin was a twice crystallized, salt-free preparation. The enzyme, as well as the substrates LME dihydrochloride, BAEE hydrochloride and TAME hydrochloride, were purchased from Nutritional Biochemicals Corp. The molecular weight of trypsin was taken as 24 000 (DIXON AND NEURATH[4]). Acetone and *n*-propanol were of the best grade commercially available and further redistilled.

The rates of hydrolysis were measured by the method of titration to a constant pH at 25±0.01°. The volume of titrant (0.05 M NaOH) was recorded as a function of time with the aid of a Radiometer model TTT 1c pH-stat equipped with a thermostated reaction vessel and a SBR2 recorder. The volume of the reaction mixture was 2 ml. The use of buffers was avoided because of the effect of alcohols on them[1]. At pH values of 8 or higher the rates were corrected for non-enzymic hydrolysis; the maximum value of this amounted to $8.6 \cdot 10^{-8}$ equivalents per ml per min at pH 10. The concentrations of acetone and *n*-propanol required to attain the desired D values were calculated from the data reported by ÅKERLÖF[5].

The presence of transesterification products in hydrolysates containing *n*-propanol or acetone was investigated by one-dimensional descending paper chromatography in *n*-butanol–pyridine–acetic acid–water[3]. Lysine derivatives were revealed with ninhydrin and arginine derivatives with Sakaguchi's reagent[6].

RESULTS AND DISCUSSION

In order to investigate the dependence of the trypsin-catalyzed hydrolysis of LME on the gross dielectric constant at pH 6.2, the rate of the reaction was measured in aqueous acetone at various D values. The choice of acetone was on the basis that it cannot compete with water as an acceptor of the acyl group. Fig. 1a shows the plot of the logarithm of the initial rate of hydrolysis relative to that in water ($D = 78.5$ at 25°, ref. 5) *versus* $1/D$. This is linear within the D range 78.5 to 74.5 and its slope, 120.5±4.5 (S.D.) is very close to the one formerly obtained for the effect of D as modified by 18 distinct additives on the trypsin-catalyzed hydrolysis of BAEE at pH 7.8, *viz.* 117±5 (S.D.) (CASTAÑEDA-AGULLÓ AND DEL CASTILLO[1]). No such relationship was observed in the effect of *n*-propanol and the rate of appearance of acid diminished as the concentration of alcohol increased (Fig. 1b). Chromatographic analysis revealed the presence of L-lysine propyl ester in the hydrolysates containing *n*-propanol. In the aqueous acetone samples the only spots which appeared where those of lysine and unhydrolyzed LME.

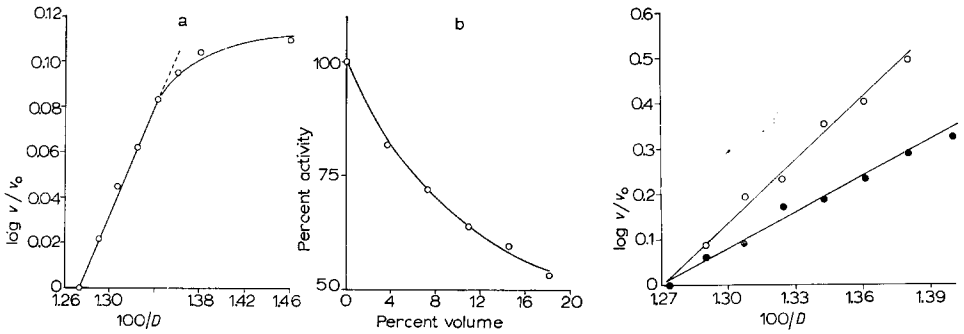


Fig. 1. Trypsin-catalyzed hydrolysis of LME in the presence of acetone (curve a) and *n*-propanol (curve b) at pH 6.2 and 25°. The former is represented as a function of the macroscopic dielectric constant (D) of the medium. The reaction mixture contained initially 0.0125 M LME, 0.005 M NaCl and $8.3 \cdot 10^{-7}$ M trypsin.

Fig. 2. Effect of *n*-propanol on the trypsin-catalyzed hydrolysis of BAEE (filled circles) and TAME (open circles) at pH 6.2 and 25° as a function of the medium gross dielectric constant. The reaction mixture contained initially 0.0125 M substrate. The enzyme concentration was $1.25 \cdot 10^{-6}$ M for BAEE and $4.17 \cdot 10^{-7}$ M for TAME.

The effect of *n*-propanol on the trypsin-catalyzed hydrolyses of BAEE and TAME at pH 6.2 (Fig. 2) was enhancing and the linear relationship between the logarithm of the rate and the reciprocal of D was obeyed in the same manner as that previously observed at pH 7.8 (ref. 1). The respective slope values, 257.3 ± 13.0 and 463.3 ± 20.5 (S.D.), indicate that the dielectric effect is greater when TAME is the substrate and that this increases as pH decreases. The possible products of transesterification between the above substrates and *n*-propanol, *i.e.* benzoyl-L-arginine and *p*-toluenesulfonyl-L-arginine propyl esters could not be detected by paper chromatography.

Since the inhibition by *n*-propanol was observed only when LME was the substrate, a possible inference is that the effect was due to some feature of this ester. One distinctive characteristic of LME as compared to BAEE and TAME is the free alpha ammonium group. If this has some bearing, the effect of *n*-propanol would

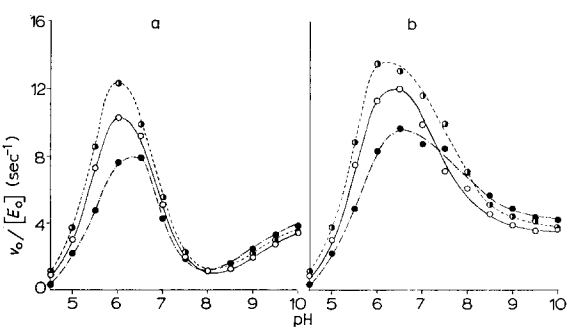


Fig. 3. Comparative effect of acetone at $D = 75.5$ (half filled circles) and *n*-propanol at $D = 75.5$ (filled circles) on the trypsin-catalyzed hydrolysis of LME at 25° as a function of pH. Control: in aqueous solution, $D = 78.5$ (open circles). Curve a, observed data. Curve b, data corrected for pK_a shifts. Substrate concentration, 0.0125 M .Enzyme concentration, $3.12 \cdot 10^{-6}$ M.

change in some way with pH in the vicinity of the pK_a of this group. Accordingly, an experiment was carried out involving a comparison of the effects of *n*-propanol and acetone at isodielectric concentrations as a function of pH from 4.5 to 10. Fig. 3a was plotted using the rates measured in the three solvent systems. The pH–rate profiles appeared to consist of two portions, a bell-shaped curve with maximum at pH 6 and an ascending curve starting after a minimum at pH 8. The effect of acetone was enhancing through the whole pH range but that of *n*-propanol was inhibitory below pH 8, nil at this pH, and enhancing in the alkaline region.

Corrections for pK shifts. Since the substitution of the carboxyl group of an alpha amino acid makes the pK_a of the amino group shift approximately 2 units (ref. 7), some of the protons released by enzymic action will be bound to the amino group of the product as a result of the increased basicity. Accordingly, the titration values will be lower than the actual rates, the fraction titrated depending on the respective differences between pK_a of either the ester or the amino acid and pH. The rates measured in aqueous solution were corrected for this pK_a shift (Fig. 3b) assuming values of 7 and 9 for LME and lysine, respectively. (SCHMIDT, KIRK AND APPLEMAN[8] have reported 8.95 for the pK_a of the alpha amino group of lysine.) Upon correction the observed minimum at pH 8 disappears. At this point, only 18.2% of the protons set free in the reaction can be titrated.

In addition to the pK_a shift due to the conversion of the ester to amino acid, the effect of the solvent on the dissociation constants must be considered. It is known that the dielectric constant influences dissociation equilibria which involve a change in the number of ions. A lower dielectric constant makes the strength of both acids and bases decrease. Thus, when D changes from 78.5 to 75.5, the pK_a of the alpha ammonium group of LME should diminish in alkaline media where the equilibrium is not isoelectric:

$$\overset{+}{H_3N}\text{—R—}NH_2 + HOH \rightleftharpoons R(NH_3^+)_2 + OH^-$$

According to BORN's equation[9,10] the change in the standard free energy per mole of electrolyte transferred from a medium of dielectric constant D_1 to another of dielectric constant D_2 would be:

$$\Delta F^0 = \frac{N z_A z_B \varepsilon^2}{2}\left(\frac{1}{D_2} - \frac{1}{D_1}\right)\left(\frac{1}{r^+} + \frac{1}{r^-}\right) \qquad (1)$$

in which N stands for Avogadro's number, z_A and z_B for the ionic valencies, ε for the electronic charge, and r^+ and r^- for the radii of the cation and anion. From Eqn. 1 the change in pK_a can be calculated:

$$\Delta pK_a = pK_{D_2} - pK_{D_1} = \frac{z_A z_B \varepsilon^2}{4.6kT}\left(\frac{1}{D_1} - \frac{1}{D_2}\right)\left(\frac{1}{r^+} + \frac{1}{r^-}\right) \qquad (2)$$

In Eqn. 2, k is Boltzmann's constant. If we ascribe the values $6.2 \cdot 10^{-8}$ cm to r^+ and $0.5 \cdot 10^{-8}$ cm to r^-, $\Delta pK_a = -0.26$ for $D_1 = 78.5$ and $D_2 = 75.5$ at 25°. The radial values are taken from the review on ionic size by STERN AND AMIS[11], the first being that of methyl adipate with approximately the same size as LME, and the second the average of the two more reasonable values—according to the above authors—of the various given for OH^-.

The change of pK_a computed from Born's equation seems somewhat high as

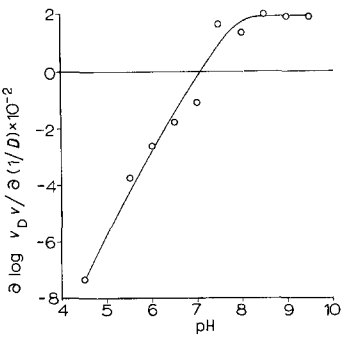
compared to data in literature for the observed effect of alcohols on the pK_a of various amines. For instance, GUTBEZAHL AND GRUNWALD[12] determined the acid dissociation constants of aniline, methylaniline and dimethylaniline in aqueous ethanol solutions at various concentrations of alcohol and found that pK_a was abated from 0.22 to 0.26 units in 20% (by weight) ethanol with respect to the respective values in aqueous solution at 25°. At this temperature the dielectric constants of water and 20% ethanol are 78.5 and 67.0, respectively (ÅKERLÖF[5]). Assuming a linear relationship between pK_a and the reciprocal of D in agreement with Born's equation (this is true only at low concentrations of alcohol), a value $\Delta pK_a = -0.05$ or -0.06 can be interpolated for $D = 75.5$. Even taking into account that z_A for LME is 2 instead of 1 for the former compounds, -0.26 still seems rather large for LME. The uncertainty lies in the values assumed for r^+ and r^-. Ionic radii are difficult to ascertain and published data differ widely depending on the experimental method and equations employed to compute them[11]. Instead of ionic radii some theories of ion pairs involve one ion-size parameter, the "contact distance", *viz.* the distance between the positive and negative centers of charge when the ions are in contact. In chemical kinetics the quantity r is utilized, this being the distance of closest approach of the charges at the moment of the reaction. If we substitute

$$\frac{1}{r} \text{ for } \frac{1}{2}\left(\frac{1}{r^+} + \frac{1}{r^-}\right)$$

in Eqn. 2 and give r the value $2 \cdot 10^{-8}$ cm obtained by AMIS[13] using the coulombic energy approach for a pair constituted of an organic ion and hydroxyl ion, the result is $\Delta pK_a = -0.12$, a figure which seems reasonable. Assuming this change, the pK_a of LME in a solvent of $D = 75.5$ would be 6.88.

While on decreasing D the pK_a of the alpha ammonium of LME also decreases, that of lysine should increase because a diminished D results in an increase of both the pK_a of acidic and basic groups of dipolar ions. As far as we know no data are available of lysine pK_a values in mixed solvents. Data taken from ROBINSON AND STOKES[10] indicate that at 25° the pK_2 of glycine changes from 9.780 in aqueous solution to 9.907 in 20% (by weight) dioxane ($D = 60.8$ according to ÅKERLÖF AND SHORT[14]). Interpolating ΔpK_a for a change of D from 78.5 to 75.5, we obtain $+0.02$. Supposing the same change in lysine, the curves of acetone and *n*-propanol in Fig. 3 were plotted with the data corrected for pK_a shifts using the values 7 for LME below pH 7, 6.88 for the same compound in the alkaline region and 9.02 for lysine. Even though these values may be different from the actual ones, the real situation would not differ very much from that depicted in Fig. 3b. From the point of view of the present investigation what really matters is the change in the trend of the effect of *n*-propanol between pH 7 and 7.5, that is, near the pK_a of the alpha ammonium group of the substrate. When the dissociation of this group falls to 24% or less, the trypsin-catalyzed hydrolysis of LME behaves similarly to the hydrolyses of BAEE or TAME in so far as the effect of *n*-propanol is concerned. This can be clearly seen in Fig. 4. At pH 4.5, $\partial \log v_0 / \partial (1/D)$ has a large negative value, but as pH increases the value changes and becomes positive at about pH 7.0. In the pH range 7.5–9.5, the effect remains sensibly constant.

It is also noteworthy that the pH–rate profile around pH 7, usually ascribed to the dissociation of an imidazolium group, is inverted in the hydrolysis of LME with



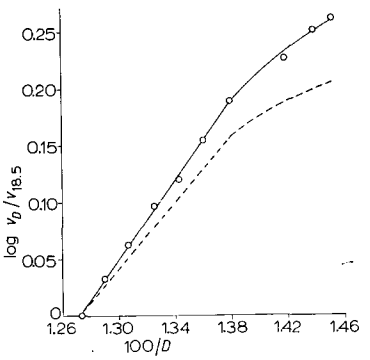


Fig. 4. Change in the rate logarithm of the trypsincatalyzed hydrolysis of LME per unit of reciprocal dielectric constant in aqueous *n*-propanol as a function of pH (data corrected for pK_a shift). Conditions as in Fig. 3.

Fig. 5. Effect of *n*-propanol on the trypsin-catalyzed hydrolysis of LME at pH 9 and 25° as a function of the macroscopic dielectric constant Circles and continuous line, data corrected for pK_a shifts. Broken line, observed data. Details as in Fig. 3.

respect to that of the trypsin-catalyzed hydrolysis of BAEE or TAME which is ascending (CASTAÑEDA-AGULLÓ AND DEL CASTILLO[15]). The same occurs with the hydrolyses of esters catalyzed by alpha chymotrypsin: those substrates with a free alpha ammonium group show descending pH-rate profiles near pH 7, whereas the N-acylated substrates produce ascending curves in this region of pH (ref. 15). BENDER AND KÉZDY[16] postulated a mechanism for alpha chymotrypsin catalysis in which an important role is attributed to the unionized imidazole. However, from the above observations it is possible to suggest that for substrates with a positive charge in the alpha position the situation is reversed and the active species of the imidazole would be the protonated one.

A quantitative study of the effect of *n*-propanol on the trypsin-catalyzed hydrolysis of LME at pH 9 revealed a dependence on D of the same type as the one previously observed with acetone at pH 6.2. Fig. 5 shows the plot of the logarithm of the relative rate *vs.* $1/D$ either with the observed data (dotted line, slope 147 ± 2.7 S.D.) or with the figures corrected for pK_a shifts (slope 176.5 ± 3.1 S.D.). The following formula derived from Eqn. 2 was used to calculate ΔpK_a for LME at each D value from 78.5 to 68.5,

$$\Delta pK_a = -3.1 \left(\frac{78.5 - D_2}{D_2} \right) \quad (3)$$

and the empirical Eqn. 4 based on data from ROBINSON AND STOKES[10] served for the computation of ΔpK_a of the alpha ammonium of lysine:

$$\Delta pK_a = 0.45 \left(\frac{78.5 - D_2}{D_2} \right) \quad (4)$$

Chromatographic analysis of the hydrolysates after 5 min of enzymic action indicated that in spite of the change in the trend of the effect of *n*-propanol, transesterification took place to some extent, so that L-lysine propyl ester was among the products of the reaction. Nevertheless, under these conditions, the predominant effect

of the alcohol was that related to the variation in macroscopic dielectric constant. In contrast with these results, tryptic hydrolysates of BAEE or TAME in aqueous *n*-propanol or *n*-butanol solutions developed solely the spots corresponding to benzoyl-L-arginine, *p*-toluenesulfonyl-L-arginine and the remaining BAEE or TAME. However, the possibility that transesterifications occurred between either BAEE or TAME and alcohols cannot be excluded. It has been shown[3] that the various esters formed as a result of trypsin-catalyzed transesterification between LME and alcohols, *i.e.*, lysine *n*-propyl, ethyl, *n*-butyl and hydroxyethyl esters, also function as substrates for trypsin but are hydrolyzed more slowly than LME. This is the reason why the transesterification reaction competes successfully with the hydrolytic reaction. Again, if the transesterification product were a better substrate than the original one, it would be more difficult to detect.

From the results presented herewith it may be concluded that (a) the presence of an alpha ammonium group in the substrate, for some reason which at present is not clear, makes the transesterification effect of alcohols predominate over the dielectric one, and (b) the behavior of the trypsin-catalyzed hydrolysis of LME, which initially appeared as anomalous from the standpoint of the influence of the medium dielectric strength, is in agreement with previous observations of the authors about the solvent effects on tryptic esterolysis[1,2,15].

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