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The Mechanism of Hydrolysis of Amino Acyl RNA*

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The hydrolyses of leucyl RNA and leucine ethyl ester are proportional to hydroxide ion concentration below *p*H 8 and above *p*H 10, with a plateau between these values. The *p*H rate profiles for hydrolysis of the two compounds are strictly parallel between *p*H 6 and *p*H 11, leucyl-RNA reacting thirty times as rapidly as leucine ethyl ester. The rate behavior indicates a double mechanism of saponification, involving hydroxide attack on both the amino acid ester and its conjugate acid.

The chemical properties of amino acid esters have attracted increasing interest since the discovery that such esters are intermediates in protein synthesis (Hoagland *et al.*, 1957; Zachau *et al.*, 1958). The high free energy of hydrolysis of amino acid esters of soluble RNA at physiological *p*H (Berg *et al.*, 1961; Lipmann *et al.*, 1959; Allen *et al.*, 1960; Leahy *et al.*, 1960) has been shown to be a general property of amino acid esters and arises from destabilization, by the positively charged α-ammonium group, of the ester relative to the acid released by hydrolysis (Jencks *et al.*, 1960).

In experiments requiring the removal of amino acids from sRNA by treatment with alkali it was observed that in the region between *p*H 8 and 10, which is most commonly employed, the rate of hydrolysis increases with *p*H but not in proportion to the concentration of alkali.

A detailed study of the *p*H dependence of the rate of hydrolysis of leucyl-RNA and of leucine ethyl ester was undertaken in order to obtain information about the mechanism of amino acid ester hydrolysis.

Experimental Procedure

L-Leucine ethyl ester hydrochloride was prepared by the method of Greenstein and Winitz (1961) and gave mp 134° (uncorrected). Soluble RNA was prepared from *E. coli* strain B by phenol extraction and isopropanol fractionation, and was enzymatically esterified with L-leucine-1-C^{14} (20 mc/mmole; New England Nuclear Corp.) according to published procedures (Zubay, 1962). The product contained one mole of amino acid per 700 moles of nucleotide, based on a measured optical density of 2.00 for a 0.01% solution of sRNA (measured at 260 mμ, *p*H 7.0) and an average molecular weight of 240 per nucleotide.

Buffer solutions for hydrolysis contained potassium carbonate, tris(hydroxymethyl)aminomethane hydrochloride, potassium phosphate or potassium acetate buffers, 0.10 or 0.05 M, together with sufficient potassium chloride to make the ionic strength 0.30.

The hydrolysis mixture for leucyl-RNA contained 5 μg of RNA per ml of buffer solution. Aliquots of 0.25 ml removed at various time intervals were cooled and mixed, first with 0.12 ml of a solution containing 1% yeast sodium nucleate (Schwarz BioResearch, Inc.) and 4% potassium acetate, and second with 0.9 ml, 0.5 M HCl in ethanol. After removal of the RNA by centrifugation in the cold, aliquots of the clear supernatant were removed for radioactivity determination of the amino acid released.

In the case of leucine ethyl ester the reaction mixture contained 2.5 μmole of ester hydrochloride per ml of buffer solution. Aliquots of 1 ml were removed at timed intervals and the disappearance of hydroxylamine-reacting material was determined by the method applied to glycine ethyl ester by Jencks *et al.* (1960); the time allowed for reaction with hydroxylamine before addition of ferric chloride was extended to 20 minutes for maximum color development.

The *p*H determinations were carried out before and after reaction with a Radiometer Model 25 *p*H meter and showed that negligible change in *p*H occurred during the course of the reactions.

All rate measurements were made in duplicate and reactions were followed for at least two half-times with at least four measurements and usually at least ten. Typical observations on leucyl-RNA are recorded in Figure 1. Good first-order kinetics were obtained in all cases, and apparent first-order rate constants were calculated from the equation $k_{obs} = 0.693/t_{1/2}$. A small correction for buffer catalysis was made by extrapolation to zero buffer concentration using the values obtained in 0.10 and 0.05 M buffer, yielding corrected observed rate constants at zero buffer concentration and ionic strength 0.30. The required correction for buffer catalysis never exceeded 10% of the observed rate and was usually much less.

Results

In Figure 2 the observed rates of hydrolysis of leucyl-RNA and of leucine ethyl ester at 37° and ionic strength 0.30, extrapolated to zero buffer concentration, are plotted as a function of *p*H. This type of rate profile is consistent with a mechanism involving attack by hydroxide ion on the conjugate acid of the ester at neutral *p*H, superseded in importance at high *p*H by hydroxide attack on the free ester. At the plateau, the reaction between species of opposite charge ap-

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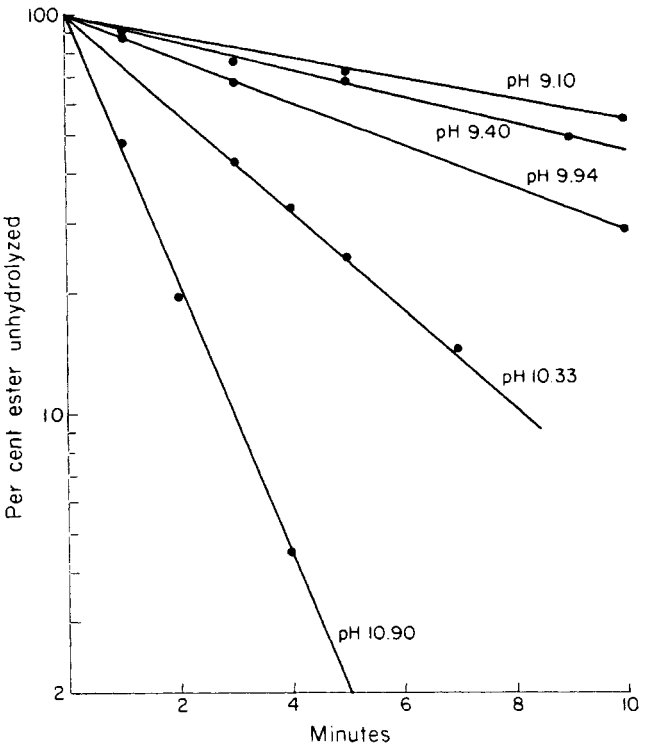

FIG. 1.—Hydrolysis of leucyl-RNA at 37°, ionic strength 0.30, in 0.10 M potassium carbonate buffers.

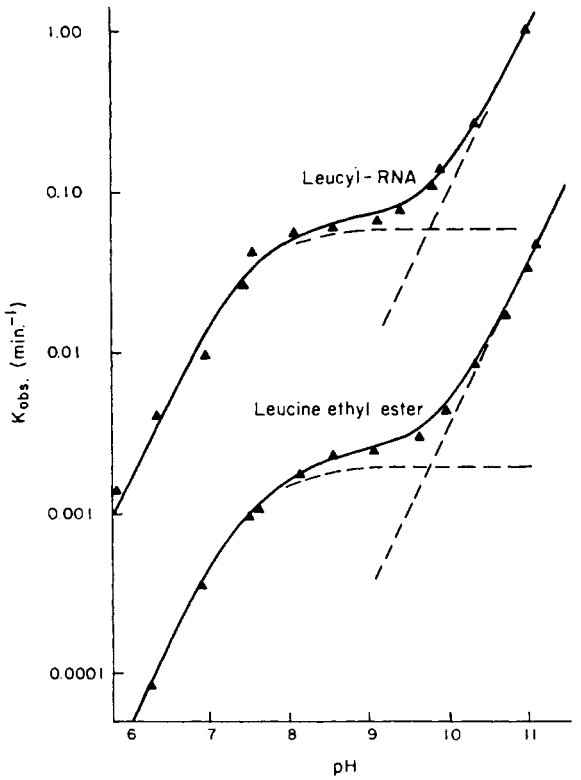

FIG. 2.—Rate of hydrolysis of leucyl-RNA and of leucine ethyl ester, extrapolated to zero buffer concentration, as a function of pH, at 37° and ionic strength 0.30. The break in the curves between pH 8 and pH 10 represents the shift from rate-determining hydroxide attack on the ammonium acid ester cation to rate-determining attack on the free amino acid ester. Solid lines are calculated from rate equations presented in the text.

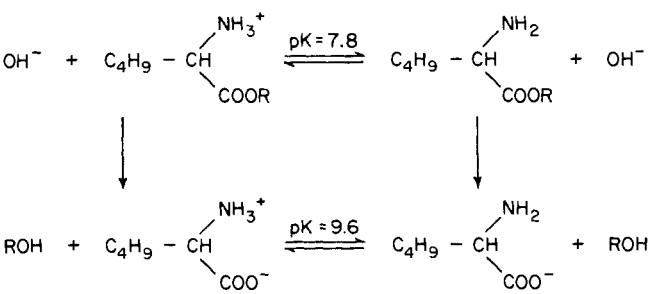

FIG. 3.—Mechanism of saponification of leucine esters.

proaches a constant rate, since above the pK of the amino group the concentration of protonated ester falls off in inverse proportion as the hydroxide ion concentration increases.

To determine rate constants for the two types of attack, it was noted that in the neutral and in the strongly alkaline regions the rate of hydrolysis is proportional to hydroxide ion concentration. The two rate constants calculated from these respective slopes are true rate constants, since at these extremes neither rate expression contributes appreciably to the slope from which the other is calculated. The inflection in both logarithmic plots at pH 7.8 indicates the pK_a of the conjugate acid of the ester. Theoretical rate profiles were calculated from the rate equations, substituting for [ester-H$^+$] the fraction of the ester protonated at each pH value, and substituting for [ester] the fraction present as free base.

The hydrolyses of the two esters follow the rate laws:

$$k_{\text{leu-RNA}} = 150{,}000[OH^-][\text{ester-H}^+] + 900[OH^-][\text{ester}]$$

$$k_{\text{leu-eth}} = 5{,}000[OH^-][\text{ester-H}^+] + 35[OH^-][\text{ester}]$$

assuming a pK of 7.8 for both amino acid esters. The solid lines in Figure 2 are calculated from these equations, while the dashed lines represent the contributions of the two modes of attack. The pK of 7.8 indicated by the experimental data is in good agreement with a measured pK of 7.80 for leucine ethyl ester hydrochloride at 37° and ionic strength 0.30, determined separately by the conventional thermodynamic method. The over-all mechanism is illustrated in Figure 3.

In concentrated salt solutions the rate of the reaction near neutrality was found to be depressed, whereas the rate of the reaction at high pH was unaffected. At pH 8.0 an increase in ionic strength from 0.25 to 1.0 by addition of KCl resulted in a 26% decrease in the rate of hydrolysis of leucyl-RNA, while no measurable change occurred at pH 10.5. This is consistent with the dominant modes of attack at the two pH values.

The postulated attack of hydroxide ion on the protonated ester cannot be distinguished kinetically from an attack by water on the free ester. However water, if it were acting as a nucleophile, would be expected to attack the protonated ester much more rapidly than the free ester. No such reaction is observed.

DISCUSSION

Hydroxide ions attack leucyl-RNA thirty times more rapidly than leucine ethyl ester throughout the pH range studied. This rate difference can be mainly attributed to the presence of a *cis*-hydroxyl group on the ribose to which leucine is esterified in sRNA. Similar rate enhancements with the introduction of a *cis*-hydroxyl group have been observed in the hydrolysis of cyclopentanyl acetate and dichloroacetate (Bruice and Fife, 1962), cholestan-3β-ol acetate (Kupchan *et al.*, 1962), and the glycine ester of 3-hydroxytetrahydrofuran (Zachau and Karau, 1960). Although

factors other than neighboring hydroxyl assistance may enhance the reactivity of amino acid esters of sRNA, over that of simpler amino acid esters, the rate difference noted in the present comparison is of the order expected.

The rate of hydrolysis of leucyl-RNA at *p*H 7.25 is approximately twice the rate of hydrolysis of valyl-RNA at that *p*H, as recorded in the careful study of Coles *et al.* (1962) on the hydrolysis of a number of amino acid esters of yeast RNA. The somewhat more rapid hydrolysis of leucyl-RNA may be ascribed to the fact that valine, though less bulky than leucine, contains five more hydrogen atoms attached to γ-carbon atoms in a position to interfere with nucleophilic attack on the ester linkage (Newman, 1950). Rate constants at a single *p*H value have been recorded for the hydrolysis of glycine ethyl ester in aqueous solution (Jencks and Carriuolo, 1961), and for the hydrolysis of phenylalanine ethyl ester in 85% ethanol (Bender and Turnquest, 1955).

It is apparent that the hydrolysis of amino acid esters even below neutral *p*H is more properly termed a saponification. Protonation of the amino group results in a 150-fold increase in the rate of hydroxide attack on both esters. Ågren *et al.* (1961) have reported a similar *p*H dependence in the hydrolysis of ethyl salicylate, which loses a proton at *p*H 10 to form the anionic ester. The 1000-times greater rate of hydrolysis in strong alkali of *t*-butyl betainium chloride as compared with *t*-butyl dimethyl glycinate (Westheimer and Shookhoff, 1940), and the 50-fold decrease in the rate of hydroxylaminolysis of the alanine ester of glycol near neutrality when the amino group is converted to the carbobenzyloxy derivative (Shabarova *et al.*, 1962) are other examples. There are also a number of examples of enhancement of the rate of hydrolysis of carboxylic acid derivatives by introduction of a positively charged group in the acylated portion. Some *p*H rate profiles similar to those under present consideration have been observed for a number of dialkyl amino alkyl esters of carboxylic acids by Ågren *et al.* (1961) and by Hansen (1962). Hydroxide ions attack acetyl choline much more rapidly than ethyl acetate (Butterworth *et al.*, 1953); in like manner, the rate of hydroxide attack on acetylimidazole is increased 450-fold by introduction of a positive charge in 1-acetyl-3-methylimidazolium chloride (Wolfenden and Jencks, 1961). The absence of a marked plateau in a plot of the rate data of Coles *et al.* (1962) against *p*H for aspartyl- and glutamyl-RNA may be due to a peculiarity in the mechanism of hydrolysis of esters of dicarboxylic amino acids or to the requirement for strict control of ionic strength in these salt-sensitive reactions.

Since protonation of a free α-amino group greatly heightens the reactivity of the ester linkage, it would be expected that a growing peptide chain esterified at the carboxyl end with sRNA ought to be much more stable to hydrolysis than aminoacyl-RNA, since the peptide ester is not protonated. (It has recently been reported, Gilbert, 1963, that phenylalanine esters of soluble RNA are hydrolyzed at approximately seven times the rate of hydrolysis of the corresponding esters of polyphenylalanine at *p*H 8.8 and 30°.) This device might help to avert the "wasting" of partly formed peptide chains in protein synthesis. The chemical hydrolysis of aminoacyl-RNA, which might occur to some extent *in vivo* at physiological *p*H, would not create a serious problem since activating enzymes and high energy phosphate are constantly available for resynthesis of the esters.

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