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Effect of Alkylguanidines and Alkylamines on Trypsin Catalysis*

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ABSTRACT: A series of alkylguanidine hydrochlorides has been prepared, and their inhibitory action against trypsin catalysis has been studied. Inhibition constants obtained have indicated alkylguanidines are stronger inhibitors than the corresponding amines. Methyl- and ethylguanidines increase the rate of the hydrolysis of acetylglycine ethyl ester catalyzed by trypsin seven- and twofold, respectively. However, the extents of the activation are less than that obtainable with ethylamine. The dissociation constant of the methylguanidine–trypsin complex in the activation is identical with the inhibition constant, indicating that binding of the recognition site of enzyme causes the activation. The activation of the acetylglycine ethyl ester hydrolysis by methylguanidine and ethylamine has been studied as a function of temperature and pH. Results have been obtained which indicate that a shift of the pH–activity profile to a lower pH is a factor contributing to the apparent increase in the rate in the activated catalysis. However, the major factor causing the increased rate seems to be an increase in the entropy of activation and not a decrease in the heat of activation, as suggested by a temperature independence of the relative increment of the rate of the acetylglycine ethyl ester hydrolysis in the activation experiment using methylguanidine and ethylamine as activators.

It has been found that trypsin is inhibited by alkylamines at pH 6.6 (Inagami, 1964). Trypsin has also been found to catalyze the hydrolysis of a nonspecific substrate Ac-Gly-OEt[1] (Inagami and Mitsuda, 1964). The rate of this nonspecific catalysis is increased considerably by methyl- and ethylamines whereas their higher homologs, 1-propyl-, and *n*-butylamines, inhibit even the nonspecific catalysis of the Ac-Gly-OEt hydrolysis (Inagami and Murachi, 1964). The activation by the methyl-, and ethylamines has been considered as the indication of the role played by the basic side chain of a specific substrate of trypsin. However, the maximum rate obtained by such an activation was considerably

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Ac-Gly-OEt, acetylglycine ethyl ester; Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester.

TABLE I: Alkylguanidine Hydrochlorides, Melting Points, Elemental Composition, and Solvents for Recrystallization.

Alkyl Group	Mp (°C) (not cor)	Formula (mol wt)	Calcd (%)			Found (%)			Solvent for Recrystzn-
			C	H	N	C	H	N	
Ethyl	124–125	$C_3H_{10}ClN_3$ (123.6)	29.15	8.16	34.00	28.97	8.04	34.06	Ethanol–ether
1-Propyl	52.5–53.5	$C_4H_{12}ClN_3$ (136.7)	34.91	8.79	30.54	33.01	8.80	29.60	Butanol
n-Butyl	69.5–70.0	$C_5H_{14}ClN_3$ (151.6)	39.60	9.31	27.71	39.84	9.40	27.84	Butanol
Benzyl	176–177	$C_8H_{12}ClN_3$ (185.7)	51.75	6.52	22.64	51.98	6.74	22.72	Ethanol
Cyclohexyl	227–228[a]	$C_7H_{16}ClN_3$ (177.7)	47.32	9.08	23.65	47.17	9.05	23.62	Ethanol

[a] Literature value is 244–226° (Cockburn and Bannard, 1957).

lower than the level obtained by specific ester substrates. In the study of amidine compounds as inhibitor for trypsin, Mares-Guia and Shaw (1965) have shown that methylguanidine is bound to the enzyme more strongly, with a K_i value of 0.036 M, than methylamine (K_i = 0.3 M) or ethylamine (K_i = 0.06 M) (Inagami, 1964). When alkylamines are used in the pH region where the enzyme activity is at the maximum, they are susceptible to many chemical reactions such as acylation, alkylation, and guanidination, used for modification of trypsin. Moreover these amines could be involved in the trypsin catalysis as an acyl acceptor resulting in the formation of alkylamides from substrate at a pH above 8, where the concentration of the un-ionized species of the amines will not be negligible. Therefore, alkylamines are not of much practical value as protectors of the active site or as inhibitors for a kinetic study.

Alkylguanidines seemed to present a promising possibility to be used in place of the alkylamine. They are inert at a neutral pH to mild reagents used for the protein modification. The stronger affinity of methylguanidine to trypsin over methyl-, or ethylamines was suggestive of a possibility that a more extensive activation of the Ac-Gly-OEt hydrolysis could be attained by the alkylguanidine. Therefore a systematic study on the effect of alkylguanidines on the trypsin-catalyzed hydrolysis of both Ac-Gly-OEt and Bz-Arg-OEt has been carried out.

The activation of the Ac-Gly-OEt hydrolysis by alkylamines has been found to be due to the increased catalytic rate and not due to the increased affinity of Ac-Gly-OEt to trypsin (Inagami and Murachi, 1964). As reported here the alkylguanidines also increase the catalytic rate of the Ac-Gly-OEt hydrolysis. The increase of the catalytic rate could be attributed to a variety of mechanisms, both chemical and kinetic. It could be due to a shift of the pH profile of the catalytic rate, since the rate measurement was made at a single pH of 6.6 where the profile was fairly strongly dependent upon pH. The decreased free energy of activation accompanying the activation could be due to a decrease in the heat of activation or an increase in the entropy of activation or both. The activation must be due to the increased chemical reactivity of catalytically important group. In the present study the kinetic aspect of these alternative possibilities has been investigated. Studies on the chemical aspect will be reported separately.

TABLE II: The Rate Constant and Michaelis–Menten Constant of Bz-Arg-OEt Hydrolysis by Trypsin in the Presence of 0.2 M KCl.

	Temp (°C)	K_m (μM)	k_{cat} (sec^{-1})
pH 6.6	34.6	26	10.8
	25.0	17	6.0
	15.4	14	2.5
pH 8.0	34.6	17	13.8
	25.0	12	8.1
	15.4	6.6	4.4

Materials

Trypsin (beef pancreatic) was a twice-recrystallized, salt-free, lyophilized preparation (TRL) from Worthington Biochemical Corp., Freehold, N. J. It was used without further purification. Determination of protein concentration was based on an $E^{1\%}_{280}$ of 14.4 (Davie and Neurath, 1955) and a molecular weight of 24,000 (Cunningham, 1954).

Ac-Gly-OEt was prepared from acetylglycine and anhydrous methanol using thionyl chloride as previously (Inagami and Mitsuda, 1964). Bz-Arg-OEt was purchased from Mann Research Laboratories, Inc., New York, N. Y. *Methylamine hydrochloride*, *ethylamine hydrochloride*, *methyl-*, *ethyl-*, *1-propyl-*, *n-butyl-*, *benzyl-*, and *cyclohexylamines* were obtained from Eastman Organic Chemicals, Rochester, N. Y. *Methylguanidine hydrochloride* and *S-methylisothiourea sulfate* were purchased from K & K Laboratories, Inc., Plainview, N. Y.

Ethyl-, *1-propyl-*, *n-butyl-*, *cyclohexyl-*, and *benzylguanidine hydrochlorides* were prepared through their respective sulfates, which were synthesized from the corresponding amines and *S*-methylisothiourea sulfate by an extension of the method developed for the synthe-

TABLE III: The Inhibition Constants of Alkylguanidines in the Trypsin-Catalyzed Hydrolysis of Bz-Arg-OEt.

pH	Temp (°C)	K_i (mM) of Alkylguanidines[a]					
		Methyl	Ethyl	1-Propyl	*n*-Butyl	Benzyl	Cyclohexyl
6.6	34.6	14	2.6	0.90	2.1	9.8	5.6
	25.0	11	2.0	0.69	1.7	7.4	4.4
	15.4	8.8	1.5	0.55	1.3		3.6
8.0	34.6						
	25.0	7.0	1.4	0.53	1.3		
	15.4						
		K_i (mM) of Alkylamines[b]					
6.6	25	260	62	8.7	1.7	0.60	

[a] Obtained at ionic strength 0.2. [b] Taken for comparison from Inagami (1964), except for the value for methylamine, which was redetermined in this study. These values were obtained at ionic strength 0.1.

TABLE IV: Thermodynamic Constants of Alkylguanidine Binding to Trypsin Determined from the Temperature-Dependent Change of Inhibition Constant at pH 6.6.

Alkylguanidines	Methyl	Ethyl	1-Propyl	*n*-Butyl	Benzyl	Cyclohexyl
$\Delta H°$[a] (kcal mol^{-1})	−4.4	−4.8	−4.5	−4.5	−4.8	−4.3
$\Delta F°$[b] (kcal mol^{-1})	−2.7	−3.7	−4.3	−3.8	−2.9	−3.2

[a] Heat of binding at 25°. [b] Standard free energy of binding at 25°.

sis of methylguanidine sulfate by Phillips and Clarke (1923). The sulfates crystallized upon cooling the guanidination mixture or after evaporation of water. However, cyclohexylguanidine sulfate was very soluble in water and crystallization occurred only after the addition of ethanol and cooling. These sulfates were converted into their respective hydrochlorides by passing through a column of Amberlite IRA-410 in the chloride form. Conversion of benzylguanidine sulfate was carried out by treating its hot aqueous solution with $BaCl_2$. Cyclohexylguanidine hydrochloride was prepared similarly and purified by the method of Cockburn and Bannard (1957). The uncorrected melting points, the calculated and experimental elemental compositions, and solvents used for recrystallization are listed in Table I.

Methods

Determination of the initial rate of the hydrolysis of Ac-Gly-OEt and Bz-Arg-OEt was made using a Radiometer pH statrecorder assembly, TTT 1a-SBR2. The ionic strength of the reaction solution was always 0.2. When the alkylamine or alkylguanidine hydrochloride was used at a concentration less than 0.2 M, KCl was added to make the total salt concentration 0.2 M. In the absence of the amine or guanidine, 0.2 M KCl was used. When Bz-Arg-OEt was used as substrate at a concentration above 0.01 M, the amount of KCl was adjusted to maintain the constancy of the ionic strength.

The inhibition constant, K_i, for an alkylamine and an alkylguanidine was obtained in a series of competitive inhibition experiment as described previously (Inagami, 1964). In each series of the inhibition experiments the inhibitor concentration [I] was fixed, while the concentration [S] of substrate, Bz-Arg-OEt, was changed. The initial rate, v, was plotted against v/[S] according to Eadie (1942). K_i was computed from the slope of the plot and K_m obtained separately, using eq 1, which is

$$K_i = \frac{[I]K_m}{K_m + \text{slope}} \quad (1)$$

derived from the equation for competitive inhibition as described previously (Inagami, 1964).

The dissociation constant of an enzyme–alkylamine or guanidine complex, K_a,[2] was also determined from the activation experiment using Ac-Gly-OEt as substrate by the method described previously (Inagami and Murachi, 1964). At a given concentration [A] of the alkylamine or alkylguanidine used as activator for the Ac-Gly-OEt hydrolysis, the initial rate, v, will be higher

[2] Note that K_a is a dissociation constant inspite of the suffix a, which indicates that the constant derives from an activation experiment.

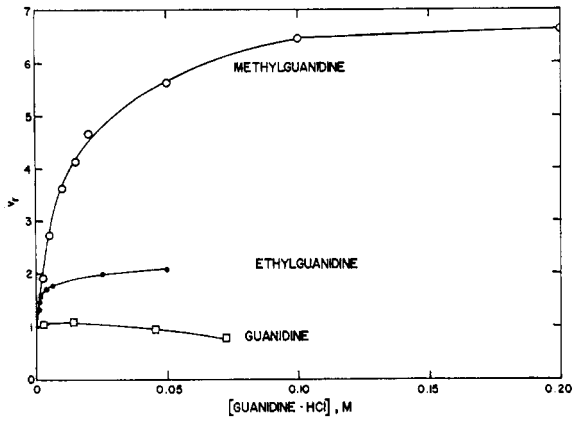

FIGURE 1: Effect of guanidine hydrochlorides on the rate of hydrolysis of Ac-Gly-OEt at pH 6.6, 25°, and ionic strength 0.2. The relative rate, v_r, is unity in the absence of the guanidines. The Ac-Gly-OEt concentrations were 0.406 M for methylguanidine hydrochloride, and 0.35 M for both ethylguanidine and guanidine hydrochlorides. Trypsin (10 mg) was used in a reaction mixture of 10 ml.

than the initial rate, v_0, observed in the absence of the activator. The ratio, v/v_0, which is defined as relative rate, v_r, is given by eq 2, where $v_{r\,max}$, is the limiting value

$$v_r = -K_a(v_r - 1)/[A] + v_{r\,max} \qquad (2)$$

of v_r at an infinite concentration of the activator. The negative slope of the plot of v_r *vs.* $(v_r - 1)/[A]$ gives K_a, and the intercept with the ordinate gives $v_{r\,max}$. The initial concentration of Ac-Gly-OEt is kept constant throughout one series of experiments.

Results

The dissociation constant of the alkylguanidine–trypsin complex was determined as the competitive inhibition constant, K_i, from the experiments in which Bz-Arg-OEt was used as substrate, using eq 1. The K_m values for Bz-Arg-OEt were obtained at an ionic strength of 0.2 maintained by KCl as listed in Table II. The K_i values thus determined are listed in Table III. The values for the corresponding alkylamines taken from previous work (Inagami, 1964) are also listed for comparison. As expected, the alkylguanidines bind to trypsin considerably stronger than the alkylamines. The heat of binding was computed from the data by Arrhenius plots. Results are shown in Table IV.

Activation of the Ac-Gly-OEt Hydrolysis. Methylguanidine increases the rate of the Ac-Gly-OEt hydrolysis by trypsin approximately by a factor of 7 at pH 6.6 and ionic strength 0.2 as shown in Figure 1. Ethylguanidine activates the reaction only very little and guanidine has no effect. 1-Propyl- and *n*-butylguanidine inhibited the Ac-Gly-OEt hydrolysis.

The plot of v_r against $(v_r - 1)/[\text{alkylguanidine}]$ according to eq 2 gave the K_a and $v_{r\,max}$ values. Example of the plot for methylguanidine is shown in Figure 2. A similar experiment was repeated with ethylamine (Figure 3),

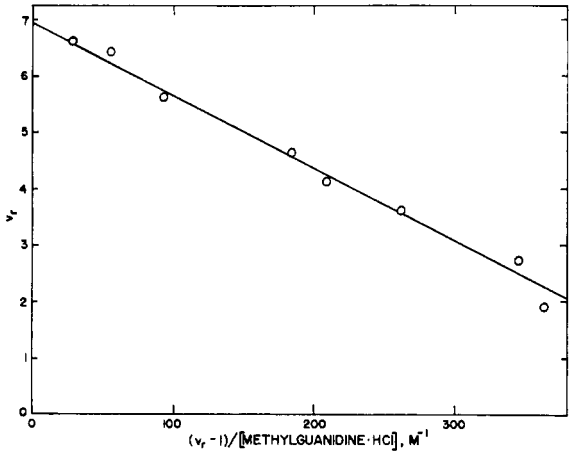

FIGURE 2: Activation of the trypsin-catalyzed hydrolysis of Ac-Gly-OEt by methylguanidine hydrochloride at pH 6.6, 25°, and ionic strength 0.2, plotted according to eq 2 in the text. The Ac-Gly-OEt concentration, 0.406 M; trypsin, 10 mg in the 10-ml reaction mixture.

TABLE V: Comparison of the Dissociation Constants of Trypsin–Alkylguanidine Complex Obtained from the Activation of the Ac-Gly-OEt Hydrolysis, K_a, and from the Inhibition of the Bz-Arg-OEt Hydrolysis, K_i.[a]

	Methylguanidine (mM)		Ethylguanidine (mM)	
Temp (°C)	K_a	K_i	K_a	K_i
35	18	13	3.0	2.6
30	15			
25	13	11	2.0	2.0
20	13			2.6

[a] pH 6.6, ionic strength 0.2.

TABLE VI: The Maximum Relative Activation, $v_{r\,max}$,[a] Obtained with Methylguanidine and Ethylamine in the Ac-Gly-OEt Hydrolysis by Trypsin at pH 6.6 and Ionic Strength 0.2.

	$v_{r\,max}$ (obsd)		$v_{r\,max}$ (cor)[b]	
Temp (°C)	Methylguanidine	Ethylamine	Methylguanidine	Ethylamine
35	6.8	14.8	4.3	11.5
30	7.2	16.4	4.2	12.9
25	6.9	14.5	4.2	10.8
20	7.9	16.8	4.1	11.1

[a] Obtained by eq 2. Ac-Gly-OEt concentration was kept constant within a series of experiment approximately at 0.3 M. [b] Corrected for pK shift. For the method of correction, see text.

TABLE VII: The pH Profile of the First-Order Rate Constant of Ac-Gly-OEt Hydrolysis by Trypsin.[a]

Temp (°C)	K^b and pK^c			
	No Addition[d]	Methylguanidine[e]	Ethylguanidine[f]	Ethylamine[g]
35	6.75 (17.8)	6.33 (46.7)		6.56 (27.6)
30	6.85 (14.1)	6.41 (38.8)		6.66 (21.8)
25	6.90 (12.6)	6.51 (30.9)	6.46 (34.7)	6.69 (20.4)
20	7.06 (9.11)	6.58 (26.3)		6.77 (17.0)

[a] In each experiment the Ac-Gly-OEt concentration was fixed approximately at 0.3 M. [b] The ionization constant was computed considering a sigmoidal pH profile of the catalytic rate constant as a titration curve for the ionization of a single group with the ionization constant K; the $K \times 10^8$ value is given in parentheses in molar concentration. [c] pK = $-\log K$; the pK values are given by unparenthesized numbers. [d] In 0.2 M KCl. [e] In 0.1 M methylguanidine hydrochloride and 0.1 M KCl. [f] In 0.1 M ethylguanidine hydrochloride and 0.1 M KCl. [g] In 0.1 M ethylamine hydrochloride and 0.1 M KCl.

since in the previous experiment (Inagami and Murchi, 1964), the ionic strength was not kept constant as the concentration of ethylamine hydrochloride was changed. The K_a values obtained in this experiment are compared with the K_i values obtained in the inhibition experiment using Bz-Arg-OEt as substrate (Table V). In every case K_a and K_i agree reasonably well, indicating that the binding of the alkylguanidine at the same site is causing the activation in the Ac-Gly-OEt hydrolysis and the competitive inhibition against the Bz-Arg-OEt hydrolysis.

The $v_{r\,max}$ values obtained with methylguanidine hydrochloride and ethylamine hydrochloride are listed in the left half of Table VI. It will be noted that the values change very little with temperature. $v_{r\,max}$ obtained with methylguanidine is approximately half of the value obtained with ethylamine. This was contrary to the expectation that the stronger binding methylguanidine would exert a stronger activating effect.

The pH Profile. It has previously been noted that the pH profile of Ac-Gly-OEt hydrolysis by trypsin is sigmoidal resembling a single group titration curve with a pK of approximately 7 (Inagami and Mitsuda, 1964). The activation of this reaction by methylguanidine and ethylamine was found to shift the sigmoidal pH–rate curve to a lower pH. The pK value of the curve was obtained from a series of rate determinations at a fixed initial substrate concentration over pH 5.8–7.2, instead of determining k_{cat} at each pH by the Eadie plot (Eadie, 1942). This procedure was justified by the previous finding that the K_m value was practically constant over the pH range investigated (Inagami and Mitsuda, 1964) and that the activation by alkylamines does not affect the K_m value (Inagami and Murachi, 1964). This was also confirmed by spot checks in the present study. For example, the K_m of the Ac-Gly-OEt hydrolysis in the presence of 0.2 M methylguanidine at 25° was 1.2 M at pH 7.0, 1.2 M at pH 6.6, and 1.3 M at pH 6.0. In the actual determinations the concentrations of both methylguanidine and ethylamine were 0.1 M, which is sufficiently high to saturate the enzyme since the K_a value for the former is 13 mM and that for the latter is 2 mM as shown in Table V. The ionization constant, K, for the single group whose ionization would supposedly give rise to the pH profile, such as those shown in Figure 4, were obtained from the negative slope of the plot of $[H^+] \times$ (observed initial rate) *vs.* observed initial rate and are presented in parentheses in Table VII. The pK values obtained from K are also shown in the same table. It is clearly seen that the activators lower the pK by as much as 0.4 pH unit. Such a shift alone can partly account for the activation of the Ac-Gly-OEt hydrolysis by either an alkylamine or guanidine, as will be discussed later.

Discussion

Binding of Alkylguanidines. The comparison of the K_i values shown in Table III between an alkylguanidine

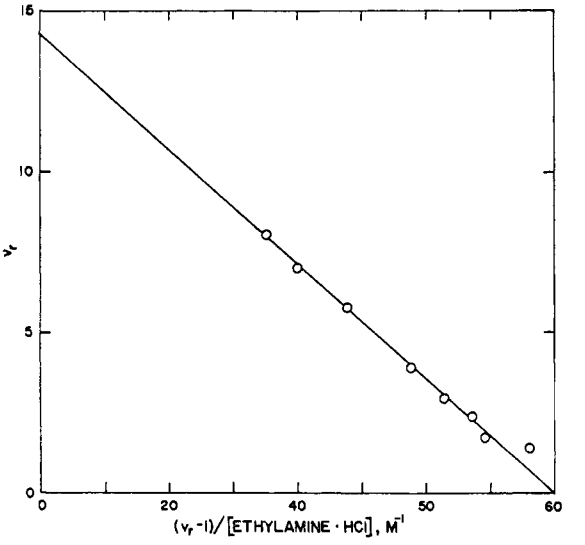

FIGURE 3: Activation of the trypsin-catalyzed hydrolysis of Ac-Gyl-OEt by ethylamine hydrochloride at pH 6.6, 25°, and ionic strength 0.2, plotted according to eq 2 in the text. Ac-Gly-OEt concentration, 0.371 M; trypsin concentration, 10 mg in 10 ml.

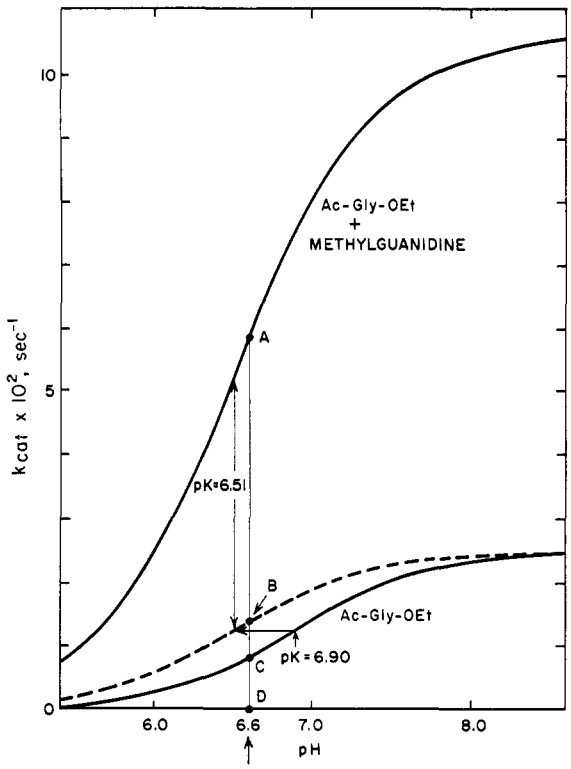

FIGURE 4: Illustration of the relationship between the activation and the shift of pH profile of the rate constant of Ac-Gly-OEt hydrolysis caused by 0.1 M methylguanidine hydrochloride.

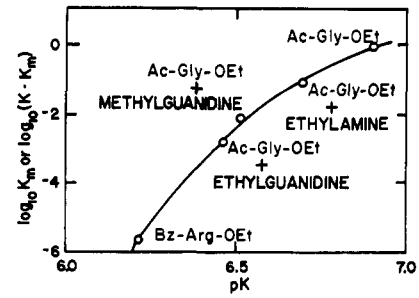

FIGURE 5: Relationship between log K_m or log $K \cdot K_m$ and pK of the pH profile of k_{cat} of the trypsin-catalyzed hydrolysis of Bz-Arg-OEt, Ac-Gly-OEt, and Ac-Gly-OEt in the presence of ethylamine and methylguanidine.

and -amine with the same alkyl group clearly indicates that the former have lower K_i values except for the *n*-butyl compounds. Since no matter what catalytic mechanism for the enzyme is assumed, the kinetically obtained K_i can be shown to be identical with the dissociation constant of the enzyme–inhibitor complex, one can conclude that alkylguanidines are more tightly bound to trypsin than alkylamines. The K_i of 7 mM obtained for methylguanidine at pH 8, 25°, and ionic strength 0.2 in the present study is far too much lower than 36 mM, obtained by Mares-Guia and Shaw (1965) in 0.05 M Veronal buffer at pH 8.15, 15°, with 2-*N*-benzoyl-DL-arginine naphytylamide as substrate, to be explained by the difference in temperature or pH. The effect of ionic strength does not seem to account for such a difference because the K_i or K_a value increases as ionic strength is elevated. For example, at pH 6.6 and 25° the K_a values were found to be 12, 13, and 19 mM at ionic strengths 0.1, 0.2, and 0.4, respectively. It could be due to the buffer effect unless 2-*N*-benzoyl-DL-arginine naphytylamide had some unexpected effect on the inhibitory binding of methylguanidine. The reasonable agreement between K_a obtained from the activation experiment and K_i from the competitive inhibition experiment is an indication that an alkylguanidine binds to the same site of trypsin to exert the inhibition and activation. The relatively lower K_i value and the fact that the alkylguanidine is completely in the protonated form near pH 8 is of greater practical value for use as protector of trypsin against autolysis, although the more strongly binding benzamidine reported by Mares-Guia and Shaw (1965) will be of even greater value. The heat of binding, $\Delta H°$, of alkylguanidines to trypsin is again not dependent upon the alkyl group just as in the case of alkylamines (Inagami and Mitsuda, 1964). The heat factor, therefore, seems to be the contribution from the coulombic force. The average of the heats of binding for the alkylguanidines, -4.5 kcal mol^{-1}, is smaller than -12 kcal mol^{-1} of the alkylamines. This could be due to the larger size of the guanidine group which places the center of its positive charge farther away from the negative charge in the recognition site of trypsin. The smaller contribution of the heat term to the binding must be overcome by a much larger entropic contribution from the guanidine group in order to explain the stronger binding by alkylguanidines. In the alkylamine series addition of one methylene group had a uniform decrease in $\Delta F°$ by 1 kcal mol^{-1}. On the other hand, the difference between the standard free energy of binding, $\Delta F°$, between methyl-, and ethylguanidines is 1 kcal mol^{-1}, but between ethyl- and 1-propylguanidine the difference is only 0.7 kcal mol^{-1}. In the next step to *n*-butyl compound the direction is reversed. It looks as if the 1-propyl and *n*-butyl groups extend beyond the hydrophobic region of the recognition site of trypsin, making the binding less strong than expected from a full contribution of their alkyl chains to hydrophobic interaction.

The Activation of Ac-Gly-OEt Hydrolysis. It had been hoped that the stronger binding methylguanidine would result in a more extensive activation of the Ac-Gly-OEt hydrolysis, so that the maximum catalytic rate obtained by such an activation would be closer to the value observed with a specific substrate such as Bz-Arg-OEt. The result obtained is contrary to such an expectation. An explanation to this observation may be that the stronger binding may make the conformation of the catalytic site too rigid, thus making it less accessible to Ac-Gly-OEt, even if the conformation of the catalytic site may be the one which is correct or more efficient for catalysis.

The activation experiment with ethylamine had been carried out previously at a fixed concentration of KCl (Inagami and Murachi, 1964), rather than at the fixed ionic strength. When experiments with ethylamine were repeated at a fixed ionic strength of 0.2, made up by ethylamine hydrochloride and KCl, a somewhat higher

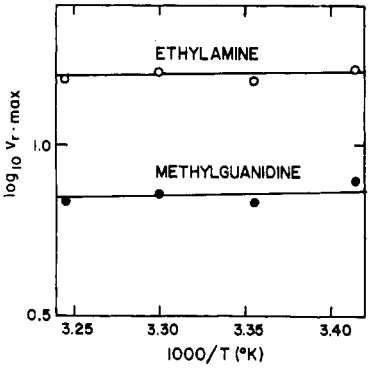

FIGURE 6: The Arrhenius plots for the maximum relative activation, $v_{r\,max}$ uncorrected for the shift of pH profile. The $v_{r\,max}$ values are for the activation of the trypsin-catalyzed hydrolysis of Ac-Gly-OEt by methylguanidine (filled circles) and ethylamine (open circles) at pH 6.6 and ionic strength 0.2.

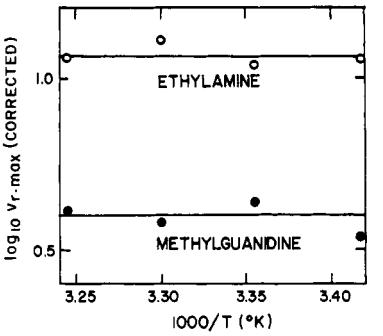

FIGURE 7: The Arrhenius plots for the maximum relative activation, $v_{r\,max}$, corrected for the shift of pH profile as described in the text. Conditions are identical with Figure 6.

activation v_r of 15 as compared with the previous value of 12 was obtained.

The shift of pH profile of the rate of Ac-Gly-OEt hydrolysis to a lower pH was observed when an alkylamine or an alkylguanidine was added as activator. An example in which methylguanidine was used at 25° is illustrated in Figure 4. The lower sigmoidal, solid curve of pK = 6.90 was obtained with 0.351 M Ac-Gly-OEt and 0.2 M KCl in the absence of methylguanidine. When half the KCl was replaced with 0.1 M methylguanidine (which is enough to saturate the binding site) while the Ac-Gly-OEt concentration is kept at 0.351 M, the activation occurred to the level of the top solid curve with pK shifted to 6.51. The broken line curve represents a hypothetical situation in which the lower curve represents a hypothetical situation in which the lower curve is shifted from pK = 6.90 to 6.51 without a change in its height. When rate is observed at a single pH of 6.6, the distance $\overline{AC}$ is the observed increment or v_r should be the ratio $\overline{AD}/\overline{CD}$. However, since the p$K$ of the profile is shifted from 6.90 to 6.51, the increment $\overline{BC}$ is due to the pK shift and $\overline{AB}$ is the true activation. Therefore, a true or corrected v_r is the ratio of $\overline{AD}/\overline{BD}$. Such a correction can be readily made when the distance $\overline{BD}$ is obtained by multiplying the ratio $(K' + [H^+])/(K + [H^+])$ to the rate observed at pH 6.6, where K' is the dissociation constant for the original profile with Ac-Gly-OEt alone, K is the dissociation constant for the shifted (broken line) profile, and $[H^+]$ is the hydrogen ion concentration for pH 6.6. The $\overline{AD}$ is experimentally determined in an activation experiment. The v_r (corrected) = $\overline{AD}/\overline{BD}$ is obtained and is shown in the right half of Table VI.

The pH profile shift has an interesting relationship with K_m values. Bz-arg-OEt which has the lowest K_m value has the lowest pK value of 6.25 (Gutfreund, 1955). In Figure 5, log K_m is plotted against the pK of the pH profile of k_{cat}. For the Ac-Gly-OEt hydrolysis activated by an alkylamine or -guanidine, K_mK_a is used in place of K_m alone, where K_m is for Ac-Gly-OEt. This is based on the assumption that the free-energy changes of binding of Ac-Gly-OEt and the activator are additive. Although K_m for an ester substrate of trypsin may not be a simple dissociation constant of the enzyme–substrate complex (Bender and Kézdy, 1965), the two functions are closely related. The smooth curve of Figure 5 suggests that the strength of the substrate binding affects the pH profile of the ionization of a group in the catalytic site, presumably an imidazole group. Such a smooth curve could not be drawn when log k_{cat} was plotted in place of log K_m. The correlation between log K_m and pK may be considered simply as a result of the coulombic effect of the positive charge of the substrate on the imidazole group. However, a similar relationship was observed with several ester substrates of chymotrypsin,[3] which suggests that the shift of the pH profile involves something other than electrostatic interaction.

The temperature dependence of v_r provides perhaps the most interesting information on the mechanism of the activation by alkylamines and -guanidines. The data in Table VI indicate that the relative increment of the rate due to the activation by methylguanidine or ethylamine is not affected by temperature. When the Arrhenius plots of the uncorrected v_r were made as shown in Figure 5, both lines obtained had zero slopes. Similar plots with the corrected v_r also gave flat lines. These results indicate that the activation is not due to the change in the heat of activation but it is exclusively due to an increase in the entropy of activation.

Previously the activation of the trypsin-catalyzed hydrolysis of Ac-Gly-OEt by methylguanidine or ethylamine has been found to be due to an increase in k_{cat} but not due to the increased affinity (Inagami and Murachi, 1964). Now it has been found that the increase in k_{cat} is partly due to a shift in the pH profile, but that it is mainly due to an increase in the entropy of activation and that the contribution from a decrease in the heat of activation is negligible.

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[3] Unpublished observation of T. Inagami.

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Steroid–Protein Interaction at Sites Which Influence Catalytic Activity*

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ABSTRACT: The effects of 14 steroids (representing major classes in terms of chemical structure and biological activity) on the catalytic activity of 6 enzymes have been studied. Activities of fumarase, lactate dehydrogenase, and isocitrate dehydrogenase were essentially unchanged (<5%) in the presence of steroid concentrations as high as 1.3×10^{-4} M. On the other hand, activities of bovine liver glutamate dehydrogenase, glucose 6-phosphate dehydrogenase from bovine corpus luteum, and rabbit liver aldehyde dehydrogenase were inhibited by a variety of steroids. With the latter three enzymes, inhibition was effective immediately on addition of steroid, reversible on dilution, and not competitive in regard to substrate and cofactor. The experimental data were found to fit a mathematical model applied to determine the association constant, K_A, for steroid–protein interaction at those sites which influence catalytic activity. In the case of glucose 6-phosphate dehydrogenase, the reversible combination of a single steroid molecule with the sensitive site was sufficient to completely eliminate activity at the affected catalytic site. Further, different steroids effect inhibition by binding at identical sensitive sites. Findings are similar in the case of aldehyde dehydrogenase. With glutamate dehydrogenase, the data are consistent with a mechanism whereby the combination of two steroid molecules with the sensitive site is necessary to completely eliminate activity, although a mechanism with interacting inhibitor binding sites (allosteric) would give similar results.

Determination of K_A values of the several steroids tested indicates that the sensitive sites of glutamate dehydrogenase have the highest affinity for estradiol-17β while those of glucose 6-phosphate dehydrogenase have the highest affinity for Δ^5-3β-hydroxy steroids and those of aldehyde dehydrogenase have the highest affinity for 3-keto steroids.

Characterization of the interaction of steroids with appropriate receptor sites on macromolecules which ultimately mediate the biological response lies at the heart of current investigation dealing with the mechanism of hormone action. Several models of steroid allosteric effects on the activity of key enzymes have been presented. Three important examples are glutamate dehydrogenase (Yielding and Tomkins, 1960; Tomkins *et al.*, 1961; Warren *et al.*, 1964), glucose 6-phosphate dehydrogenase (Marks and Banks, 1960; McKerns and Kaleita, 1960; Nielson and Warren, 1965; Warren and Betz, 1965), and aldehyde dehydrogenase (Maxwell and Topper, 1961).

This investigation was designed to provide a general, quantitative evaluation of the steroid binding sites of these and various other enzymes. By determination of steroid–enzyme association constants with a kinetic method, we thought to selectively evaluate steroid binding at those sites which influence catalytic activity. We considered the important aspects of these steroid–protein interactions to be: (1) the affinity of steroid binding, (2) the stoichiometry of steroid binding, (3) whether various steroids bind at identical sites on a given enzyme, and (4) binding as a function of steroid structure.

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