[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, THE OHIO STATE UNIVERSITY)

Behavior of Dowex-50 as an Aminopeptidase

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Dowex-50 requires a free amino group for its catalytic activity. At 104° Dowex-50 splits glycylglycine very rapidly compared to acetylglycine. Aspargine is also split more rapidly by Dowex-50 than is acetamide. Because of the complex formed between the amino group of the substrate and the charged sulfonate group of the resin, the peptide bond is placed under a strain which permits easier splitting of the bond. The formation of the resin–substrate complex can be mathematically treated in the same manner as is used for the treatment of enzyme systems. The nature of the substrate, temperature, *p*H of the medium and inhibitors affect the dissociation constant of the resin–substrate complex.

Introduction

Steinhardt and Fugitt[1] have shown that the rates of protein hydrolysis by dilute acids depend not only on temperature and acidity, but also on the nature of the anion of the acid used. They demonstrated that certain water-soluble high molecular weight sulfonic acids, at concentration levels of less than 0.2 *M*, are more effective catalysts than the common mineral acids. These workers thought that catalysis was due to the increased basicity of the amide and peptide bonds as a result of a combination of these bonds with the large anion of the catalyst. Previous reports[2,3] from this Laboratory have shown that the insoluble resin Dowex-50 is an excellent catalyst for hydrolyzing some proteins. In understanding the catalytic action certain questions come to mind. Is it acting as an insoluble "strong acid," a surface active solid, or a highly hydrated polymer showing certain membrane phenomena? Why does the resin have more effect on some proteins and peptides than on others? The present work is concerned with requirements for and the mechanism of hydrolysis of the peptide linkage by Dowex-50 and indicates that this sulfonated polystyrene resin shows some properties similar to certain enzymes.

Experimental Procedure

Treatment of Resin.—Dowex-50, a polystyrenesulfonic acid ion-exchange resin, of 200–400 mesh size and approximately 12% cross linked was used in this work. Before use, the resin was treated as described by Paulson, *et al.*[2] The resin was dried at room temperature for 16 hours and then stored in a rubber-stoppered bottle. The resin contained 36.94% water as determined by drying in an oven at 105° for 24 hours and had 5.05 meq. of hydrogen ions per gram dry resin as determined by titration of hydrogen ions replaced by an excess of sodium chloride.

Substrates.—These were all commercial samples. Gelatin: moisture, 10.18%; ash, 1.46%; nitrogen, 18.48; glycylglycine: nitrogen, 21.27%; R_f value in water saturated phenol, 0.32.

The method of hydrolysis, removal of hydrolysis products and the measurement of the extent of hydrolysis has been described previously.[3] 0.1000 gram of Dowex-50 equivalent to 0.318 mmole of acid and 5.0 ml. of a standard solution of the substrate were used in each tube. In the hydrolysis with hydrochloric acid, the resin and water were replaced by 5 ml. of 1.081 *N* or 0.0636 *N* hydrochloric acid. In the studies on the rate of hydrolysis of acetylglycine, glycylglycine, asparagine and acetamide and on the effect of *p*H on the rate of hydrolysis of glyclyglycine, 0.0379 meq. of the substrate was used in each tube. The substrate concentration was varied over a large range in studies on the dissociation constant of the resin–substrate complex.

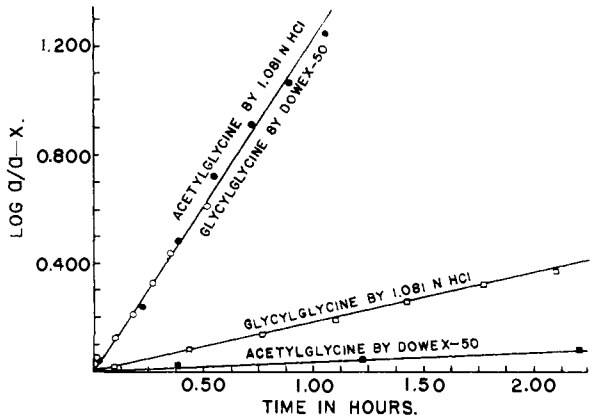


Fig. 1.—The rate of hydrolysis of glycylglycine and acetylglycine with Dowex-50 and with 1.081 *N* HCl at 104.0°.

Results and Discussion

Hydrolysis of Acetylglycine and Glycylglycine.—Acetylglycine and glycylglycine were hydrolyzed by Dowex-50 (equivalent to 0.0636 *N* acid) and by 1.081 *N* hydrochloric acid at 104.0°. The results are shown in Fig. 1 and Table I and indicate that using 1.081 *N* hydrochloric acid acetylglycine was hydrolyzed 6.6 times more rapidly than glycylglycine.

This shows the retarding effect a free amino group has on the rate of hydrolysis of the peptide bond by hydrochloric acid. On the other hand glycylglycine is hydrolyzed by the resin 34.5 times as rapidly as acetylglycine. It should be noted that some of the hydrolysis of acetylglycine attributed to Dowex-50 is due to autocatalysis.[4] If this is corrected for, glycylglycine is split about 66 times faster than acetylglycine. It may be assumed that the splitting of acetylglycine is due to the hydrogen ions contributed by the resin and the anion of the resin does not contribute to the rate of hydrolysis. Dowex-50 was only 0.50 times as effective in splitting acetyglycine as an equivalent amount of hydrochloric acid.

Hydrolysis of Asparagine and Acetamide.—Hydrolyses of asparagine and acetamide by Dowex-50 (equivalent to 0.0636 *N* acid) and by 0.0636 *N* hydrochloric acid were carried out at 104.0°. The results in Table I show that asparagine is hydrolyzed by Dowex-50 23.1 times more rapidly than with an equivalent amount of hydrochloric

(1) J. Steinhardt and C. H. Fugitt, *J. Research Natl. Bur. Standards*, **29**, 315 (1942).

(2) J. C. Paulson, F. E. Deatherage and E. F. Almy, THIS JOURNAL, **75**, 2039 (1953).

(3) J. R. Whitaker and F. E. Deatherage, *ibid.*, **77**, 3360 (1955).

(4) J. R. Whitaker, Ph.D. Dissertation, The Ohio State University, 1954.

TABLE I

RATE CONSTANTS, HALF-LIVES AND RELATIVE RATES FOR HYDROLYSES OF SEVERAL SUBSTANCES AT 104.0°

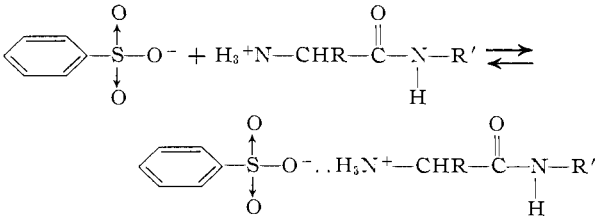
Substrate	Medium	$K \times 10^2$, l. mole^{-1} hr.$^{-1}$	Half-life (hr.)	Relative rate, K_r/K_a	Relative rate, Dowex-50	Relative rate, acid
Glycylglycine	Dowex-50	4510 ± 80	0.242		1	
	1.081 *N* HCl	40.1 ± 0.6	1.60	112		1
Acetylglycine	Dowex-50	132 ± 6	8.26		0.029	
	1.081 *N* HCl	265 ± 5	0.242	0.498		6.61
Asparagine	Dowex-50	11,100 ± 700	0.0982		2.46	
	0.0636 *N* HCl	480 ± 10	2.27	23.1		
Acetamide	Dowex-50	2330 ± 100	0.468		0.517	
	0.0636 *N* HCl	4150 ± 50	0.263	0.561		

acid. In contrast, Dowex-50 splits acetamide only 0.56 times as fast as an equivalent amount of hydrochloric acid. Asparagine is split 4.8 times faster by Dowex-50 than is acetamide. The amino group is in the β-position with respect to the amide group of asparagine so the effect of the formation of the resin–asparagine complex on the rate of hydrolysis would be less than if the amino group were in the α-position.

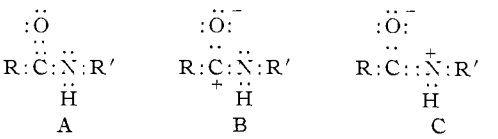
The presence of an amino group α to the peptide bond increases the rate of hydrolysis by Dowex-50 by a factor of 66 as shown by comparison of the rates of hydrolysis of glycylglycine and acetylglycine while the presence of an amino group β to the amide group increases the rate of hydrolysis by Dowex-50 by a factor of 4.8 as shown by comparison of the rates of hydrolysis of asparagine and acetamide. This is another example of the well-known fact that as one moves further from the acid group with the substituent atom or radical the effect of substitution decreases very rapidly.

The above data on the rates of hydrolysis of acetylglycine, glycylglycine, asparagine and acetamide support the hypothesis that a free amino group must be present on the substrate if the anion of Dowex-50 is to be catalytically active and that a complex is formed between the amino group of the substrate and the charged sulfonate group of the resin before splitting is produced. Another point which supports the proposed mechanism is the relative rates found for resin and acid hydrolysis and reported in another paper.[3] For example, consider the case of DL-valyl-DL-isoleucine which is more stable to hydrochloric acid than many peptides. The carbon skeleton of this molecule appears to almost completely block the entrance of hydrogen ions to the peptide bond. Consequently the much larger sulfonic acid group would have little chance of penetrating the barrier and there should be a large difference in the relative rates of hydrolysis this peptide and glycylglycine with Dowex-50 and with hydrochloric acid. But the relative rates are similar for the Dowex-50 and the acid-catalyzed reactions; the relative rates at 104.0° are 0.0086 and 0.0091, respectively. These observations together with the fact that acetylglycine is not readily split by Dowex-50, leads one to conclude that the point of attachment of the resin to the substrate cannot be at the peptide bond.

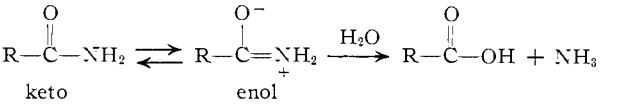
Proposed Mechanism of Resin Catalysis.—The following mechanism of resin-catalyzed protein hydrolysis is presented for consideration. A free charged amino group must be available to the resin if it is to be catalytically active. The sulfonic acid group of the resin combines with the amino group of the substrate to form the resin–substrate complex



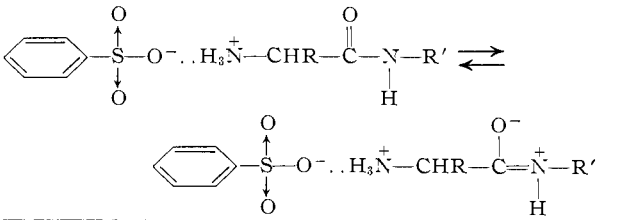
Because of the electronegativity of oxygen, the bonding electrons between the carbon atom of the carbonyl group and the nitrogen atom of the peptide bond will be shared. This decreases the basicity of the nitrogen atom



It has been shown[5] that the hydrolysis of the amide bond depends upon the formation of the enol form

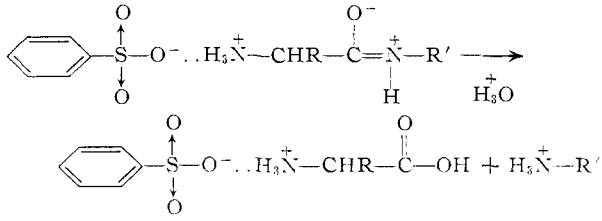


Substituted amides also undergo this keto–enol tautomerism.[6] Corey and Donohue,[7] from the results of X-ray analysis as well as other data available, assign a value of 1.32 Å. for the distance between the C and N of the peptide bond. This corresponds to about 50% double bond characteristics. Any electronegative group attached to the α-carbon atom of the amide or peptide linkage would shift the equilibrium in favor of the enol form.



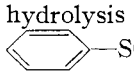
(5) V. K. Krieble and K. A. Holst, THIS JOURNAL, **60**, 2976 (1938).
(6) W. D. Kumler and C. W. Porter, *ibid.*, **56**, 2549 (1934).
(7) R. B. Corey and J. Donohue, *ibid.*, **72**, 2899 (1950).

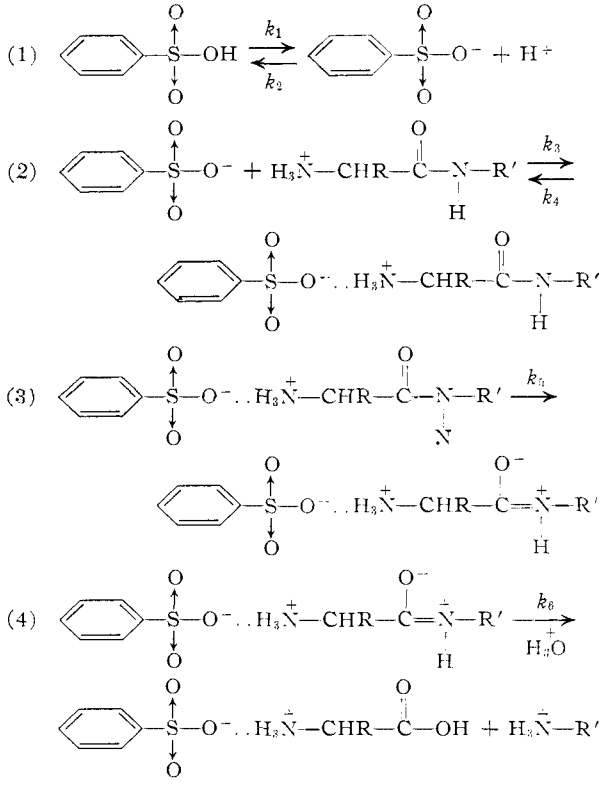
The enol form would then be readily split

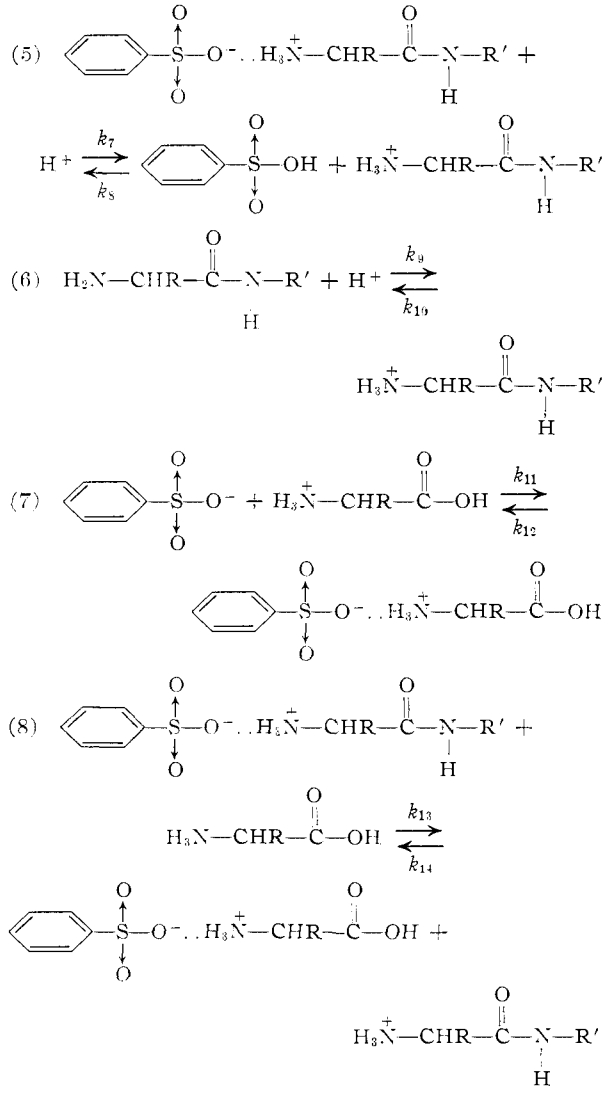


The factors which tend to labilize the C–N linkage of the peptide bond are (a) loss of the stabilizing resonance of the molecule and (b) the positive inductive effect of the charged sulfonate group of the resin molecule. The electronegativity of the oxygen atoms of the charged sulfonate group distorts the electronic configuration of the peptide bond in favor of the enol form. The catalytic activity of the resin should be further increased by an increase in the electronegativity of the resin. This may be produced by the addition of electronegative groups to the benzene ring. For example, a dinitrostyrenesulfonic acid polymer should be a better catalyst for hydrolysis then Dowex-50.[3]

Theoretical Treatment of the Resin–Substrate Complex.—An examination of the data available on the effect of Dowex-50 on the peptide bond shows that its action appears to be similar to the action of enzymes on their substrates. The hypothesis was then advanced that the kinetics that apply to enzymatic reactions should also apply to the action of Dowex-50 in splitting the peptide bond.

Assume the following reactions take place in the hydrolysis of the substrate by the resin and let  be a unit of the resin polymer.





(9) No equation can be written for this reaction but assume the following to be true:

$$RS + (n-1)S \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} RS_n \text{ (inactive)}$$

Reactions 1, 5 and 6 will depend upon the pH of the medium and will be constant for a single pH. Reactions 7 and 8 will depend upon the concentration of the inhibitory products and their effect on the rate of hydrolysis will increase as hydrolysis proceeds. But initially the inhibitory effect is zero and will be small for some time. A discussion of the effect of inhibitors will be given later.

Consider reactions 2, 3, 4 and 9. Let

(R) = total concn. of resin
(S) = substrate concn.
(RS) = substrate-resin complex concn. (active)
(RS_n) = substrate-resin complex concn. (inactive)

$$K_r = \frac{k_{16}}{k_{15}} \text{ and } K_r = \frac{k_4}{k_3}$$

then (R) − (RS) − (RS_n) = concn. of free resin

Following, by analogy, the derivation of Michaelis and Menten[8] the final equation is

(8) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

$$K_r = (S)\left[\frac{V}{v} - 1\right] - \frac{(S)^n}{K_r} \quad (1)$$

It is best to take the reciprocal of both sides of equation 1[9]

$$\frac{1}{v} = \frac{K_r}{V} \times \frac{1}{(S)} + \frac{1}{V} + \frac{\frac{(S)^n}{K_r}}{V(S)} \quad (2)$$

A better estimation of V and K_r can be made from some data if both sides of equation 2 are multiplied by (S).[9]

$$(S)/v = \frac{K_r}{V} + \frac{1}{V}[(S)^n/K_I + (S)] \quad (3)$$

A plot of (S)/v against (S) yields a curve which becomes concave upward at high values of (S) where $(S)^n/K_I$ is no longer negligible. But at low values of (S) the term $[(S)^n/K_I]/V(S)$ becomes negligible and the equation

$$\frac{1}{v} = \frac{K_r}{V}\frac{1}{(S)} + \frac{1}{V} \quad (4)$$

may be derived from reactions 1, 2 and 3 where v is the observed velocity of hydrolysis, V is the maximum velocity attainable, (S) is the substrate concentration and K_r is the dissociation constant of the resin–substrate complex.

Equation 4 was derived assuming that there is no inhibition of the reaction by the products. But the resin does bind the products of the hydrolysis. All of the amino acids with the exception of aspartic and glutamic acid are held by the resin and must be eluted with suitable reagents. It is assumed that the active center of the resin is the sulfonic acid group on the benzene ring and this would be the position tied up by the amino acids and other products of the reaction. So the inhibition would be competitive. The equation

$$\frac{1}{v_1} = \frac{1}{V_1}\left[K_r + \frac{K_r(P)}{K_p}\right]\frac{1}{(S)} + \frac{1}{V_1} \quad (5)$$

may be shown to apply. V_1 represents the maximum velocity and v_1 the observed velocity in the presence of the inhibitory products, K_p is the dissociation constant of the inhibitory products–resin complex and (P) is the concentration of the inhibitory products. In the hydrolysis of a protein the concentration of the inhibitory products (P) is not a constant but increases with increasing hydrolysis and is equal to $S_0 - S$, where S_0 is the initial substrate concentration and S is the substrate concentration at time t. If this value is substituted for (P) in equation 5 and $-\frac{dS}{dt}$ for v, the equation can then be integrated to give the form

$$t = \frac{K_pK_r + K_rS_0}{V_1K_p}\ln\frac{S_0}{S} + \frac{K_p - K_r}{V_1K_p}(S_0 - S) \quad (6)$$

The behavior of the terms involving K_p in equation 6 is interesting at low values of $S_0 - S$. As $S_0 - S$ approaches zero, each of these terms also approaches zero. By expansion of the logarithmic term in Maclaurin's series, however, it may be shown that the sum of these terms constitutes a second-order infinitesimal with respect to $S_0 - S$. This relationship is another way of stating that early in the reaction inhibitory effects due to the split products are negligible.

(9) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

Effect of Substrate Concentration on Rate of Hydrolysis.—The effect of substrate concentration on the rate of hydrolysis of glycylglycine and gelatin with Dowex-50 was determined. The plot of substrate concentration *versus* velocity of hydrolysis for gelatin is shown in Fig. 2. The velocity of hydrolysis is expressed in milliliters of 0.01 N sodium hydroxide per 1.38 hours. The hydrolysis of gelatin is first order with respect to substrate concentration over the substrate range of 0.00 to 5.50 g. per liter and zero order with respect to substrate concentration over the substrate range of 5.50 to 18.00 g./l. Above a substrate concentration of 18.00 g. per liter the velocity of hydrolysis decreases and according to the data would be zero at a substrate concentration of 88.0 g. per liter. This is due to inhibition of hydrolysis by the gelatin. In the case of glycylglycine the reaction is first order with respect to substrate concentration over the substrate range 0.0000 to 0.0273 mole per liter and zero order over the substrate range of 0.0273 to 0.0546 mole per liter. The velocity of hydrolysis decreases above a substrate concentration of 0.0546 mole per liter.

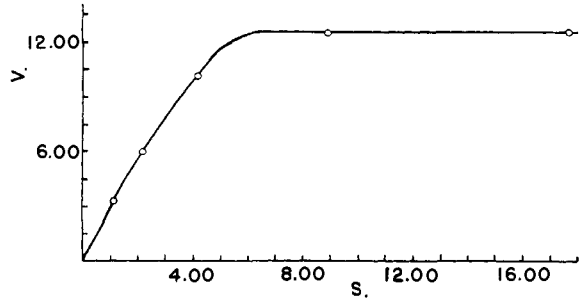


Fig. 2.—The effect of gelatin concentration on the velocity of hydrolysis with Dowex-50 at 103.0°.

Determination of the Dissociation Constant of the Resin–Substrate Complex.—Experiments were carried out to determine the dissociation constant of the resin–substrate complex. As pointed out in the above discussion the substrate has an inhibitory effect on the rate of hydrolysis and this effect increases with increasing substrate concentration so that it must be corrected for. It was also stated that when the substrate–resin ratio is small the term $[(S)^n/K_I]/V(S)$ approaches zero and V and K_r may be calculated from the simpler equation $1/v = K_r/V(S) + 1/V$. There will be inhibition of the rate of hydrolysis by the split products but this is also small near the beginning of hydrolysis. In experiments where the dissociation constant, K_r, of the resin–substrate complex was being determined the smallest ratio of resin to gelatin was 4.5 to 1 on a weight basis and for resin to glycylglycine it was 4.2 to 1 on a molar basis.

The dissociation constant K_r and V, the maximum velocity, were determined by the least squares method of analysis of the data according to equation 4. Plots of $1/v$ *versus* 1/(S) are shown in Fig. 3 for gelatin. K_r and V were determined in the absence and presence of added 0.0500 N

hydrochloric acid. K_r, calculated for gelatin in the absence and presence of 0.0500 N hydrochloric acid, was found to be 12.0 g. per liter and 8.68 g. per liter, respectively, measured after 1.38 hours of hydrolysis at 103°. V, the maximum velocity, for gelatin in the absence and presence of acid was found to be 37.1 ml. per 1.38 hours and 21.0 ml. per 1.38 hours, respectively. The values calculated for glycylglycine: absence of acid, $K_r = 4.07 \times 10^{-2}$ mole per liter measured after 0.217 hour of hydrolysis at 104.0°, V = 18.3 ml. per 0.217 hour; presence of acid, $K_r = 7.59 \times 10^{-2}$ mole per liter measured after 0.217 hour of hydrolysis at 104.0° and V = 14.5 ml. per 0.217 hour. These values are readily reproducible by identical experiments.

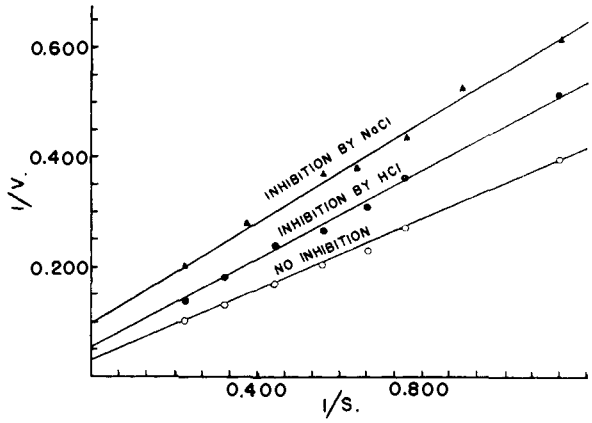


Fig. 3.—Plot of the reciprocals of the velocity of hydrolysis of gelatin *versus* the reciprocals of gelatin concentration in grams/liter. Inhibition of hydrolysis with 0.05 N HCl and 0.03 N NaCl.

Effect of *p*H on Rate of Hydrolysis.—The effect of added acid on the rate of hydrolysis of glycylglycine by Dowex-50 is shown in Fig. 4. Table II

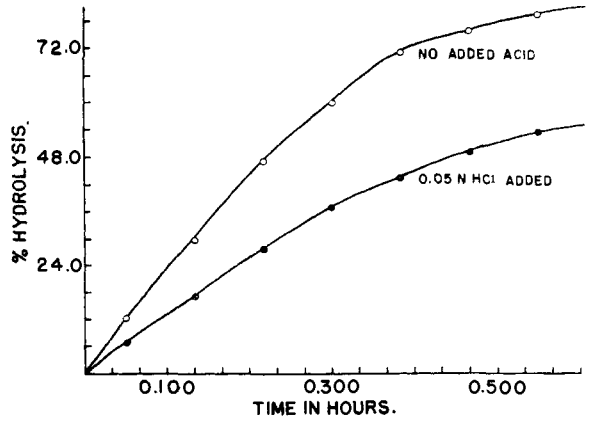


Fig. 4.—The effect of added 0.05 N HCl on the rate of hydrolysis of glycylglycine with Dowex-50 at 104.0°.

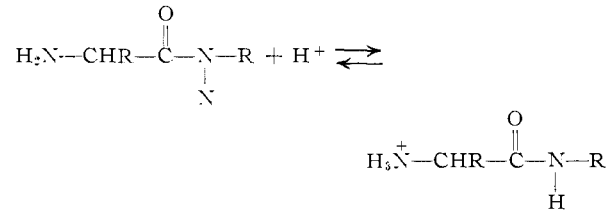
shows the effect of added acid on the rate of hydrolysis of gelatin, glycylglycine and casein by Dowex-50. There is a marked decrease in the rate of hydrolysis of glycylglycine and gelatin in the presence of 0.0500 N hydrochloric acid. As postulated above, it is believed that acid has three

TABLE II

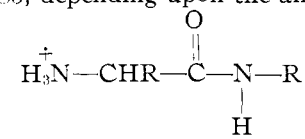
EFFECT OF ADDED ACID ON RATE OF HYDROLYSIS AT 104.0° WITH DOWEX-50 (0.1000 G. RESIN/5.0 ML.)

Gelatin Hydrolysis, %			Glycylglycine Hydrolysis, %			Casein Time, 2.22 hr.	
Time (hr.)	Dowex-50	0.0500 N HCl added	Time (hr.)	Dowex-50	0.0500 N HCl added	[H⁺], moles/l. × 10²	Hydrolysis, %
0.217	27.2	13.1	0.050	12.4	7.20	0.00	25.2
0.550	39.2	21.6	.133	30.0	17.6	0.10	27.2
0.884	50.6	34.3	.217	47.4	28.3	0.50	35.6
1.22	59.4	41.6	.300	60.1	36.9	1.00	43.0
1.55	62.9	48.2	.384	71.2	43.6	2.00	46.6
1.88	65.0	53.7	.467	75.6	49.0	3.50	43.7
2.22	66.2	58.5	.550	79.1	53.2	5.00	42.1

effects on the rate of hydrolysis. (a) It reacts with the substrate in the following manner



Since the *p*H of the hydrolysates are in the range of 1.00–3.85, depending upon the amount of added acid, the



form is the predominant one and is the form in which the amino group is attached to the resin. (b) The addition of acid also markedly decreases the dissociation of the resin. The *p*H of suspension of the acid form of Dowex-50 is 3.85, which means that a large proportion of the potentially ionizable groups of the resin are not dissociated. However, at *p*H 3.85, the amino groups of amino acids and proteins are almost completely protonated. A decrease in the *p*H of the system below 3.85 would not measureably affect the relative concentrations of charged and uncharged amino groups but would change very markedly the dissociation of the resin. Thus the addition of acids decreases the effective resin concentration. It is postulated that the anion of the sulfonic acid is the active catalyst and it acts upon the ammonium form of the substrate. (c) The acid also decreases the concentration of the resin–substrate complex in a second way by tending to displace the substrate from the resin. This would make K_r larger. This is borne out by the data on the glycylglycine–resin complex but not on the gelatin–resin complex.

Effect of Inhibitors on Rate of Hydrolysis.—The effect of added glycine and lysine·HCl on the velocity of hydrolysis of gelatin is shown in Fig. 5. It was found that lysine·HCl has a more pronounced effect on the velocity of hydrolysis than does glycine, which is to be expected. It should be noted that only the first two amounts of glycine added are in the range of the substrates used in any of the above experiments provided the substrate gave only glycine on hydrolysis and was completely hydrolyzed.

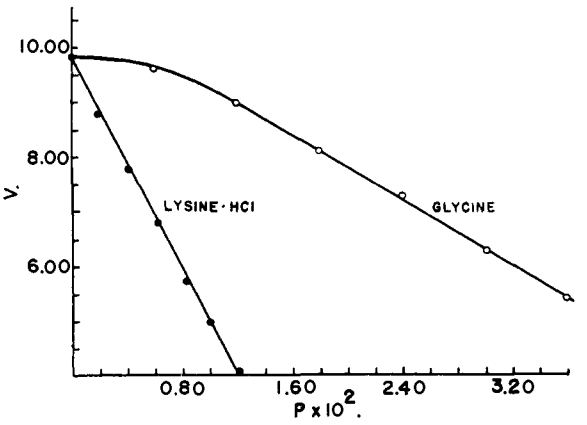


Fig. 5.—The effect of added glycine and lysine·HCl on the rate of hydrolysis of gelatin by Dowex-50 at 103.0°.

The effect of the addition of sodium chloride on the velocity of hydrolysis of gelatin is shown in Fig. 3. There is a marked decrease in the velocity of hydrolysis. When the data were plotted as in Fig. 3 it was found that there was a change in both the slope and intercept of the line from the uninhibited hydrolysis. But the change in the intercept is much larger than the change in the slope. There are three effects from the added sodium ions. (a) There is a reaction with the resin to form the sodium form which is inactive for splitting the peptide linkage, (b) the resin–substrate complex is decomposed by the sodium ions and (c) hydrogen ions are displaced from the resin by the sodium ions decreasing the *p*H of the medium.

In the foregoing discussion the mechanism has been in part developed on the basis of the charged sulfonate group being in solution. The resin is not in solution in a strict sense but seems to be acting as an aminopeptidase. It is impossible to define precisely the nature of the resin but it is of interest to note that it may be treated also as a surface active solid.

Effect of Temperature on the Adsorption of Glycine by Dowex-50.—Another factor that has an effect on K_r is the temperature. It was found that the amount of adsorption decreased as the temperature increased. The data were analyzed according to the adsorption equation of Langmuir

$$\frac{C}{x/m} = \frac{1}{\alpha} + \frac{\beta}{\alpha} \times C$$

where C is the concentration of the solution in equilibrium with the surface layer expressed in grams per liter; x is the mass, in grams, of substance adsorbed; m is the mass, in grams, of the adsorbing material; α and β are constants. α and β were determined by the analysis of the data according to the least squares method. The adsorption at low glycine concentrations deviated from the Langmuir adsorption equation.

The values calculated for β were used to determine the heat of adsorption of glycine by Dowex-50 by use of the relationship

$$\log \frac{\beta_2}{\beta_1} = \frac{\Delta H}{2.3R}\left[\frac{T_2 - T_1}{T_2 \times T_1}\right]$$

ΔH, the heat of adsorption, was calculated by the least squares treatment of the data (Fig. 6) and found to be -1864 cal. over the temperature range 25.3 to 103.0°.

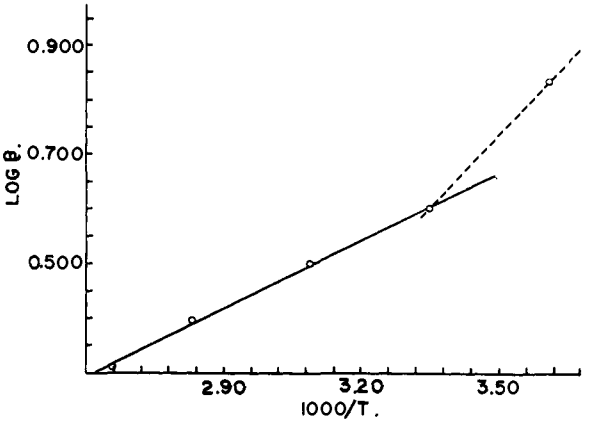


Fig. 6.—The effect of temperature on the adsorption of glycine by Dowex-50.

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