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

Hydrolysis of Proteins and Dipeptides by Ion-exchange Resin Catalysis

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Dowex-50, a polystyrenesulfonic acid ion-exchange resin is superior to Nalcite-HCR, Dowex-50-NO_2, Zeo-Rex, Dowex-50-NH_2, Amberlite IR-105, Dowex-30, Amberlite IRC-50 and Nalcite-SAR for the hydrolysis of glycylglycine. Dowex-50 was found to show more specificity in the hydrolysis of proteins than did 1.081 *N* hydrochloric acid. The kinetics of the hydrolyses by 1.081 *N* hydrochloric acid and by Dowex-50 of glycylglycine, DL-alanylglycine, glycyl-L-leucine, glycyl-DL-valine, DL-leucylglycine, DL-alanyl-DL-asparagine, prolyl-L-tyrosine and DL-valyl-DL-isoleucine were studied at 104.0°. The relative rates by resin and by acid are similar, yet Dowex-50 is 115 times more effective than an equivalent amount of hydrochloric acid in the hydrolysis of most of the dipeptides. The kinetics of the hydrolyses by Dowex-50 of glycylglycine, DL-alanylglycine, glycyl-L-leucine, glycyl-DL-valine and DL-leucylglycine were studied over a range of temperatures. The ΔH^* values (enthalpies) ranged from 20.7 to 22.5 kcal. mole^{-1} and the ΔS^* values (entropies) from -8.77 to -14.1 cal. deg.$^{-1}$ mole^{-1}. The catalytic efficiency of Dowex-50 is due to a marked increase in ΔS^* as compared with hydrochloric acid since there are no significant differences in ΔH^* for the resin- and acid-catalyzed reactions.

Introduction

It has been demonstrated by Underwood and Deatherage[1] that casein and the water-soluble proteins of coffee are hydrolyzed by Dowex-50. Later it was found that casein is essentially completely hydrolyzed in 92 hours by being refluxed with a 5 to 1 ratio of resin to protein in 0.05 *N* hydrochloric acid.[2] The amount of amino acid destruction was similar to that caused by 6 *N* hydrochloric acid even though there was no humin formation. Several other cation-exchange resins were studied and Amberlite IRC-50, a carboxylic acid type resin was less effective than Dowex-30, Amberlite IR-105 and Zeo-Rex, which are phenolsulfonic acid type resins.[3]

The anion-exchange resins, Amberlite IH-4B and XE-67, weak base resins, and Dowex-2, a strong base resin, gave little or no hydrolysis after 150 hours at reflux temperatures. Gelatin and casein are very rapidly hydrolyzed by Dowex-50 while ovalbumin, bovine serum albumin and lactalbumin are more resistant to hydrolysis. It also was found that aspartic acid is preferentially released in the hydrolysis of bovine serum albumin and edestin at 100° with Dowex-50.[4] The rates of appearance of the amino acids were found to conform to that of acid hydrolysis. The present work is concerned with a comparison of the efficiencies of Dowex-50 and 1.081 *N* hydrochloric acid in splitting the peptide linkage.

Experimental Procedure

Treatment of Resins.—Before use, the resins were treated as described by Paulson, *et al.*[3] The moisture content of each resin was determined by drying in an oven at 105° for 24 hours. The acid-equivalence of each resin was determined by either of two methods, (a) replacement of the hydrogen ions by 10% sodium chloride or (b) reaction of the resin with an excess of a standard acid or base and back titration. By the first method, a weighed amount of resin was placed in a 100-ml. volumetric flask and 10% sodium chloride added to the mark. After standing for 2 hours with frequent mixing, an aliquot was removed and titrated with standard carbonate-free sodium hydroxide to the phenolphthalein end-point. The first method was used for determination of the hydrogen ion content of Dowex-50, Dowex-50-NO_2, Nalcite-HCR, Dowex-30, Zeo-Rex and Amberlite IR-105. The second method was used for Dowex-50-NH_2, Amberlite IRC-50 and Nalcite-SAR.

Preparation of Nitro and Amino Forms of Dowex-50.—Two modified forms of Dowex-50 were prepared. The nitrated form was prepared by refluxing 12.0 g. of air-dried resin for 3 hours with 20 ml. of concentrated nitric acid and 17 ml. of concentrated sulfuric acid in a 100-ml. round-bottomed flask equipped with an air condenser. The temperature was 84.0°. The mixture was partially cooled, then poured on to chipped ice. The bright yellow resin was washed with distilled water by repeated suspension and decantation and finally on a buchner funnel under suction until the wash water was neutral to methyl orange. It was then air-dried for 16 hours at room temperature. The amino form of Dowex-50 was prepared by reduction of 6.0 g. of the nitrated Dowex-50 with 12.5 g. of stannous chloride and 50 ml. of concentrated hydrochloric acid for 2 hours at 84.0°. The resin was then washed free of excess acid. Both resins were then regenerated with 4 *N* hydrochloric acid as described above. These resins will be designated as Dowex-50-NO_2 and Dowex-50-NH_2.

Substrates.—The casein was isolated from skim milk according to Cohn and Hendry.[5] The other substrates were commercial samples. The moisture contents were determined by drying at 105° for 24 hours. The ash contents were determined after ignition at 555° for 16 hours and nitrogen was determined by the micro-Kjeldahl method proposed by Miller and Houghton.[6] The R_f values of the dipeptides were determined by paper partition chromatography[7] and the dipeptides found to be free of amino acids. DL-Valyl-DL-isoleucine was obtained as its carbobenzoxy derivative. The carbobenzoxy group was removed by hydrogenolysis with palladium as the catalyst.[8]

Hydrolysis Procedure.—Hydrolysis was carried out in sealed Pyrex test-tubes, 20 × 150 mm. in size. A weighed amount of resin (0.318 mmole with respect to hydrogen ion content) and 5.0 ml. of a standard solution of the substrate were used in each tube. Where the substrate was not soluble in the solvent, the correct amount of the substrate was weighed into each tube and then 5.0 ml. of the solvent added. In each 5 ml. of solution there were 0.0379 milliequivalent of dipeptide or 0.00667 g. of protein. In the hydrolysis with hydrochloric acid, the resin and water were replaced by 5 ml. of 1.081 *N* acid. The hydrolyses of the dipeptides by resin were carried out in a water solution while 0.0500 *N* hydrochloric acid served as the solvent for the hydrolysis of the proteins by the resin. The tubes were sealed and the hydrolyses were carried out for the desired time in an oven in which the temperature could be controlled to ±0.2°. In order to assure good contact between the resin and substrate the tubes were rotated at 10 r.p.m. by use of a specially designed rack rotated by a motor. The tubes were removed at intervals and placed in an ice-bath.

Removal of Hydrolysis Products from Resin.—After hydrolysis the tubes were opened by pinching off the tip. The

(1) G. E. Underwood and F. E. Deatherage, *Science*, **115**, 95 (1952); *Food Research*, **17**, 425 (1952).

(2) J. C. Paulson, Ph.D. Dissertation, The Ohio State University, (1952).

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

(4) J. C. Paulson and F. E. Deatherage, *J. Biol. Chem.*, **205**, 909 (1953).

(5) E. J. Cohn and J. L. Hendry, in Blatt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943.

(6) L. Miller and J. A. Houghton, *J. Biol. Chem.*, **159**, 373 (1945).

(7) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

(8) B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951).

contents were washed from the tubes with 30 ml. of 10% sodium chloride into 100-ml. beakers. The mixture was allowed to stand for 2 hours to allow sufficient time for replacement by sodium ions of the amino acids which are bound to the resin. The determination of the extent of hydrolysis was then carried out as described below. The resin was not removed from the solution as it does not interfere in any way with the formol titration. (Dowex-50-NH_2 and Nalcite-SAR must be removed before titration because of the presence of amino groups.) Titration of known mixtures of amino acids showed that this method gave satisfactory results, within 0.2%.

Extent of Hydrolysis.—The modified form of the Sorensen formol titration proposed by Dunn and Loshakoff[9] with some modification was used to determine the extent of hydrolysis. Titrations were made with 0.01 N carbonate-free sodium hydroxide using a Beckman glass electrode pH meter to determine the end-point. A 37.5% formaldehyde solution of commercial grade treated with basic magnesium carbonate[10] was adjusted to pH 8.0 just before use.

To carry out a titration, the mixture consisting of the hydrolysate, 10% sodium chloride and the resin (or acid) was carefully adjusted to pH 8.0 by the addition of carbonate-free sodium hydroxide. The total volume was approximately 35 ml. To this 15 ml. of the 37.5% formaldehyde was added and the mixture titrated back to pH 8.0 with standard 0.01 N sodium hydroxide delivered from a microburet. During the titration, which required about 10 minutes, the mixture was stirred continuously by a magnetic stirrer. The final concentration of formaldehyde was approximately 9%. Blank determinations were run on all reagents before and after the titration of a set of samples. Standard curves were prepared for all the dipeptides. Mixtures of the dipeptide and its constituent amino acids were prepared to represent different degrees of hydrolysis and the titration carried out under the conditions described above.

Hydrolysis of all of the peptides was considered complete after treatment with 6 N hydrochloric acid at 105° for 24 hours except DL-valyl-DL-isoleucine which was hydrolyzed for 60 hours. All of the proteins were hydrolyzed for 24 and 60 hours. The titration values after 60 hours were lower than those for 24 hours because of destruction of some of the amino acids. The titration values obtained for the 24 hour hydrolyses are used in calculating the per cent. hydrolysis according to the equation, % hydrolysis = $[(t_t - t_0)/(t_e - t_0)] \times 100$, where t_t is the titration value at time t, t_0 is the titration value initially and t_e is the titration value at complete hydrolysis.

Ammonia produced from DL-alanyl-DL-asparagine during hydrolysis was removed from the resin by treating the hydrolysis mixture with 10% sodium chloride as before. After 2 hours the mixture was then made neutral to methyl orange with sodium hydroxide and then quantitatively transferred to a micro-Kjeldahl distillation apparatus. One gram of magnesium oxide (carbonate-free) was added and the ammonia was steam distilled into 20 ml. of a 2% boric acid containing 5 drops of methyl red-brom cresol green indicator and then titrated with 0.01 N hydrochloric acid. Preliminary determination showed that ammonia is not liberated from asparagine by this procedure.

Results and Discussion

Hydrolysis of Glycylglycine with Several Resins.—In order to determine which resin to use in this work, the action of a number of resins on glycylglycine at 104° was measured. A dipeptide was chosen because in the hydrolysis of a protein it is usually the hydrolysis of the dipeptides that requires the longest time. The same amount of each of the resins with respect to its hydrogen ion equivalence was used. However, the pH of the mixture varied depending on the resin being used. The phenolsulfonic acid resins are more acidic than the sulfonic acid resins. The pH cannot be controlled by the addition of a buffer because the cations inactivate the resin which is only active in the hydrogen form.

(9) M. S. Dunn and A. Loshakoff, *J. Biol. Chem.*, **113**, 359 (1936).

(10) M. C. Daring, U. S. Patent 1,925,795 (Sept. 5, 1933); *C. A.*, **27**, 5483 (1933).

The results, shown in Figs. 1 and 2, indicate that Dowex-50 is superior to the other resins tested. The rate of hydrolysis of glycylglycine by Dowex-50-NO_2 is very rapid initially but slows down after several minutes. Perhaps some species of the nitrated resin are very efficient catalysts but since they occur in limited amounts, they are soon saturated with the inhibitory product, glycine, and thus inactivated. The sulfonic acid resins, Dowex-50 and Nalcite-HCR hydrolyze glycylglycine at a faster rate than the phenolsulfonic acid resins, Zeo-Rex, Amberlite IR-105 and Dowex-30. The difference in pH explains part of this difference but not all of it. The pH of the hydrolysates containing the sulfonic acid resins is 3.85 while the pH of the hydrolysates containing the phenolsulfonic acid resins range from 3.43 for Zeo-Rex to 2.92 for Dowex-30. It has been found that the addition of acid decreases the rate of hydrolysis of glycylglycine and gelatin with Dowex-50.

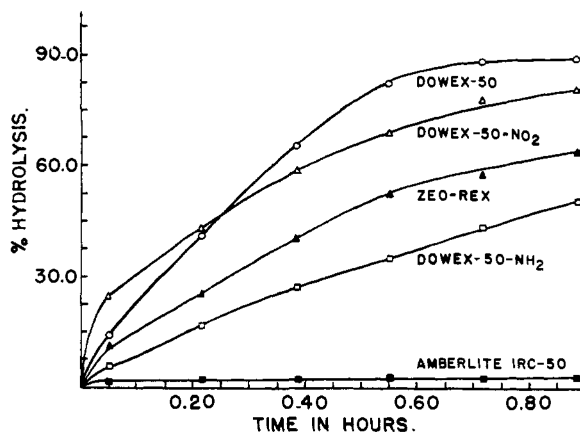


Fig. 1.—The rate of hydrolysis of glycylglycine with several resins at 104.0°.

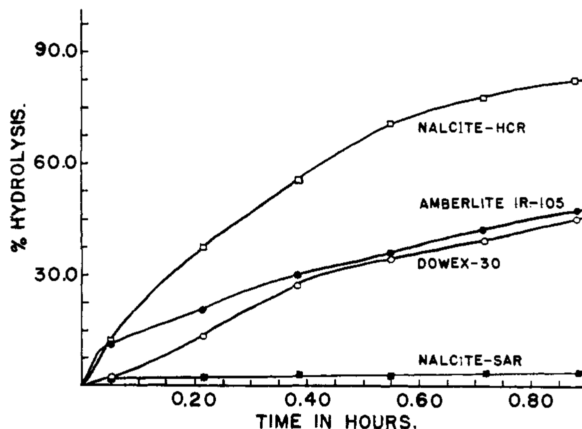


Fig. 2.—The rate of hydrolysis of glycylglycine with several resins at 104.0°.

Hydrolysis of Several Proteins.—The rates of hydrolysis of gelatin, edestin, casein, bovine serum albumin and ovalbumin by Dowex-50 (equivalent to 0.0636 N HCl) and 1.081 N hydrochloric acid were determined at 104.0° and the results are shown in Figs. 3 and 4.

The rate of hydrolysis decreases in the order: gelatin, edestin, bovine serum albumin, casein and

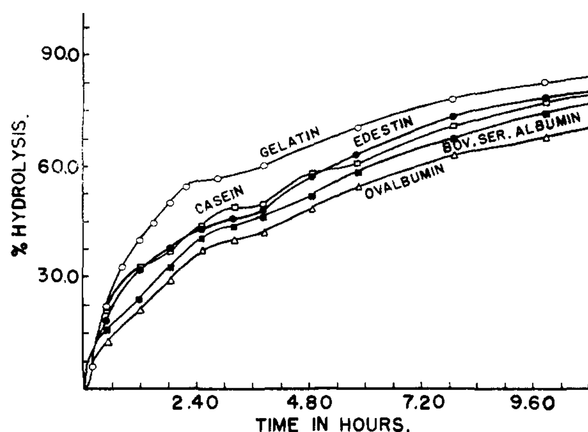


Fig. 3.—The rate of hydrolysis of several proteins with 1.081 N HCl at 104.0°.

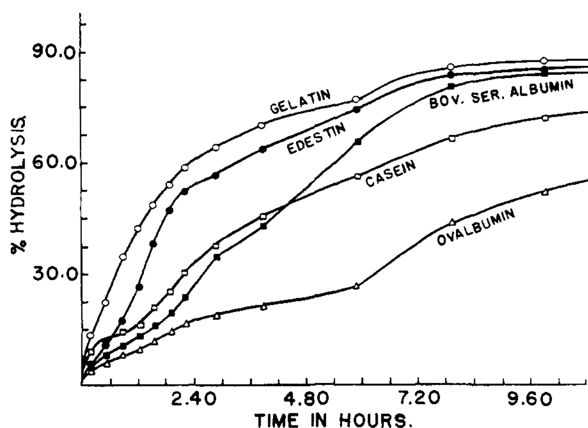


Fig. 4.—The rate of hydrolysis of several proteins with Dowex-50 at 104.0°.

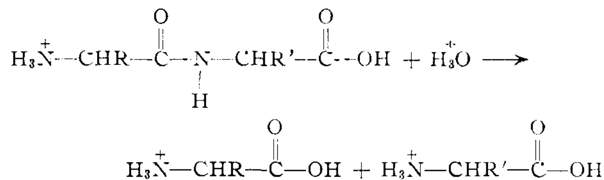
ovalbumin for Dowex-50. The same order is true for the hydrolysis by 1.081 N hydrochloric acid except the position of bovine serum albumin and casein are reversed. It was also found that during the initial stages of hydrolysis, the rate of hydrolysis by 1.081 N hydrochloric acid was much faster than with Dowex-50 but as the hydrolysis continued the reverse was true. Dowex-50 and 1.081 N hydrochloric acid hydrolyzed gelatin at about the same rate up to 50% hydrolysis but then Dowex-50 was more effective in the later stages. The decrease in the rate of hydrolysis by acid is to be expected as the dipeptide bonds are about eight times more resistant to hydrolysis than the same bonds would be if they were not adjacent to free amino groups.[11] It is suggested that the rate of hydrolysis by Dowex-50 is slower than with 1.081 N hydrochloric acid initially because there are not many free amino groups available to the resin. As the hydrolysis continues, more and more amino groups become available to the resin and the rate of hydrolysis increases. The hydrolysis that takes place initially is partially obscured by the hydrolysis of the amide groups of glutamine and asparagine.

Dowex-50 appears to be much more specific than 1.081 N hydrochloric acid for the hydrolysis of a protein. With 1.081 N hydrochloric acid, hydrolysis rates were comparable for all five of the proteins. On the other hand, there are large differences in the ability of Dowex-50 to hydrolyze the different proteins.

During the hydrolysis of bovine serum albumin and ovalbumin with Dowex-50 there are periods when the hydrolysis is very rapid and other periods when it comes almost to a stand-still. The hydrolysis of bovine serum albumin is rather slow up to about 25% but then it becomes very rapid and at 9.88 hours it is almost as completely hydrolyzed as gelatin and edestin. Ovalbumin also shows this rapid increase in the rate of hydrolysis after about 25%. The importance of the stepwise type of hydrolysis for isolation of split products from partial hydrolysates is obvious. It was reported by Waldschmidt-Leitz and Zinnert[12] that while the hydrolysis of clupein with 0.1 N hydrochloric acid or sulfuric acid proceeds uniformly right through to the amino acids, the hydrolysis with 0.1 N alkylsulfonic acids and alkyl sulfates proceeds with a number of reaction steps during which the hydrolysis nearly comes to a stand-still. The stepwise hydrolysis of bovine serum albumin and ovalbumin with Dowex-50 would be expected to be more pronounced as the temperature of hydrolysis is lowered.

Hydrolysis of Several Dipeptides.—The hydrochloric acid and Dowex-50 hydrolyses of the following dipeptides were studied at 104.0°: glycylglycine, DL-alanylglycine, glycyl-L-leucine, glycyl-DL-valine, DL-leucylglycine, DL-alanyl-DL-aspartic acid, prolyl-L-tyrosine and DL-valyl-DL-isoleucine.

The hydrolysis of a dipeptide in a solution of a strong acid may be represented as



$$H_3\overset{+}{N}-CHR-\overset{O}{\overset{\|}{C}}-\underset{H}{\underset{|}{N}}-CHR'-\overset{O}{\overset{\|}{C}}-OH + H_3\overset{+}{O} \longrightarrow$$

$$H_3\overset{+}{N}-CHR-\overset{O}{\overset{\|}{C}}-OH + H_3\overset{+}{N}-CHR'-\overset{O}{\overset{\|}{C}}-OH$$

One $H_3{}^+O$ is consumed for each peptide bond that is split. When the ratio of hydrogen ions to dipeptide is large, however, the concentration of the acid can be considered to be constant. In these experiments the ratio of acid to dipeptide was 143 and the rate of hydrolysis should be expressed by the first-order rate equation

$$Kt = \ln \frac{1}{1 - x} \quad (1)$$

where K is the rate constant and x is the fraction of hydrolysis at time t. In the calculation of the rate constant the rate equation was divided by the hydrogen ion concentration of the solution

$$K = \frac{2.303}{(H^+)t} \log \frac{1}{1 - x} \quad (2)$$

In calculating the rate constants of the reactions with Dowex-50, the hydrogen-ion concentration in the rate expression was replaced by 1000 $n_H m_R/V$ where n_H is the number of equivalents of hydrogen ion in a gram of resin, m_R is the mass of the resin and V is the volume of the solution. The standard state to which the activation entropies and en-

(11) L. Lawrence and W. J. Moore, THIS JOURNAL, **73**, 3973 (1951).

(12) E. Waldschmidt-Leitz and F. Zinnert, *Macromol. Chem.*, **6**, 272 (1951).

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
DL-Alanylglycine	Dowex-50	2770 ± 60	0.394		0.614	
	1.081 N HCl	24.8 ± 0.4	2.59	112		0.618
DL-Alanyl-DL-aspartic acid	Dowex-50	2710 ± 60	0.402		.601	
	1.081 N HCl	86.1 ± 0.8	0.745	31.5		2.15
Glycyl-L-leucine	Dowex-50	2230 ± 50	0.489		.494	
	1.081 N HCl	19.1 ± 0.6	3.36	117		0.476
Glycyl-DL-valine	Dowex-50	2450 ± 100[a]	0.642		.376	
	1.081 N HCl	13.6 ± 0.3	4.71	126		.339
DL-Leucylglycine	Dowex-50	1010 ± 30	1.08		.224	
	1.081 N HCl	8.68 ± 0.10	7.39	116		.216
Prolyl-L-tyrosine	Dowex-50	180 ± 3	6.06		.040	
	1.081 N HCl	4.66 ± 0.15	13.8	38.6		.116
DL-Valyl-DL-isoleucine	Dowex-50	38.6 ± 2.8	28.2		.0086	
	1.081 N HCl	0.363 ± 0.014	177	106		.0091

[a] Calculated according to second-order rate equation $\times 10^2$.

TABLE II

RATE CONSTANTS, ENTHALPIES AND ENTROPIES OF ACTIVATION FOR HYDROLYSES OF SEVERAL DIPEPTIDES

Substrate	$K \times 10^1$, l. mole^{-1} hr.$^{-1}$ 57.9°	76.6°	104.0°	ΔH^*, kcal. mole^{-1}	$-\Delta S^*$, cal. deg.$^{-1}$ mole^{-1}	ΔF^* (104°), kcal. mole^{-1}
Glycylglycine	6.35 ± 0.07	44.2 ± 1.0	451 ± 8	22.2	8.77	25.5
DL-Alanylglycine	3.99 ± .12	27.5 ± 0.5	277 ± 6	22.1	10.1	25.9
Glycyl-L-leucine	4.13 ± .02	25.7 ± 0.4	223 ± 5	20.7	14.1	26.0
Glycyl-DL-valine		25.4 ± 1.1[a]	245 ± 10[a]	21.0	14.0	26.3
DL-Leucylglycine	1.36 ± .10	9.19 ± 0.11	101 ± 3	22.5	11.1	26.7

[a] Calculated according to the second-order rate equation $\times 10^2$. All other calculated according to the first-order rate equation.

thalpies in Table II refer is therefore one equivalent of hydrogen ion per liter of solution.

Figure 5 shows data for the hydrolysis of several dipeptides with 1.081 N hydrochloric acid plotted according to the first-order rate law. Similar plots

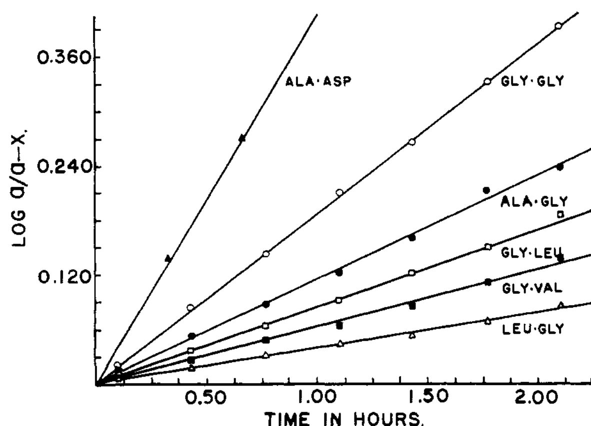


Fig. 5.—The rate of hydrolysis of several dipeptides with 1.081 N HCl at 104.0°.

are shown in Fig. 6 for the hydrolysis of several dipeptides by Dowex-50. Table I gives the rate constants, half-lives and relative rates for the dipeptide at 104°.

The rate of hydrolysis of glycyl-DL-valine with Dowex-50 does not follow the first-order rate law

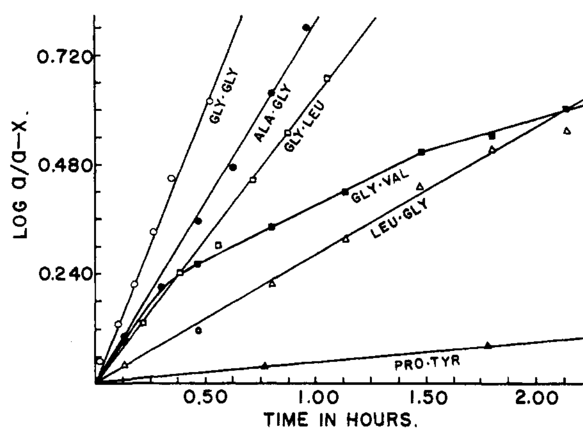


Fig. 6.—The rate of hydrolysis of several dipeptides with Dowex-50 at 104.0°.

as is shown in Fig. 6 but does follow the second-order equation

$$K = \frac{1}{t} \times \frac{x}{100 - x} \times \frac{1}{100} \quad (3)$$

where K is the rate constant and x is the percentage of hydrolysis at time t. The hypothesis was advanced that the resin is capable of distinguishing between the two isomers of glycylvaline and that one isomer is split more rapidly than the other. Neither the D nor the L isomer was available, but glycyl-DL-leucine and glycyl-L-leucine were. It was hoped that a difference in the rate of hydrolysis

of these two substances would be found; however, none was found at 81.0°. It is possible that the carbon chain of leucine does not interfere with the formation of the resin–dipeptide complex as much as the carbon chain of valine.

The rate constant was calculated for each dipeptide by taking the average of the values calculated from the per cent. hydrolysis found at a given time. The standard deviation, s, was calculated by the equation

$$s = \sqrt{\frac{d^2}{n(n-1)}} \quad (4)$$

where d is the deviation of $K_1, K_2 \ldots . K_n$ from the mean value of K and n is the number of determinations in that run.

It was found that 0.1000 g. of Dowex-50 (equivalent to 0.0636 N hydrochloric acid) hydrolyzes most of the dipeptides about 6.6 times as rapidly as 1.081 N hydrochloric acid. However, when the difference in the normalities of the acids are taken into account Dowex-50 is found to hydrolyze most of the dipeptides 115 times as rapidly as an equivalent amount of hydrochloric acid. DL-Alanyl-DL-aspartic acid and prolyl-L-tyrosine were hydrolyzed only 31.5 and 38.6 times as rapidly by Dowex-50 as by an equivalent amount of hydrochloric acid.

The relative rates of hydrolysis, expressed as the ratio of the rate constant of the dipeptide to the rate constant of glycylglycine, of the dipeptides are about the same for both acid and resin hydrolysis. DL-Alanyl-DL-aspartic acid and prolyl-L-tyrosine are exceptions. These relative values are also close to those reported by Synge.[13]

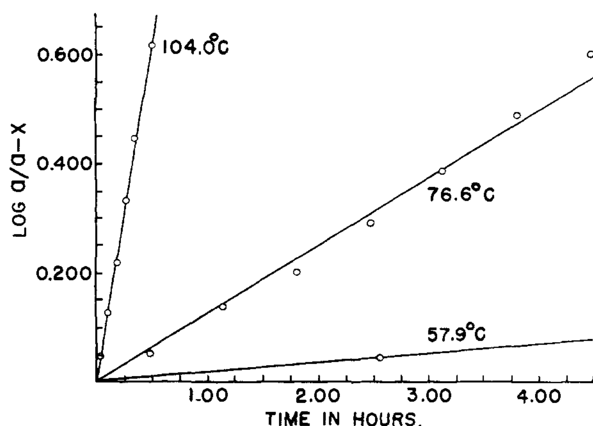


Fig. 7.—The effect of temperature on the rate of hydrolysis of glycylglycine with Dowex-50.

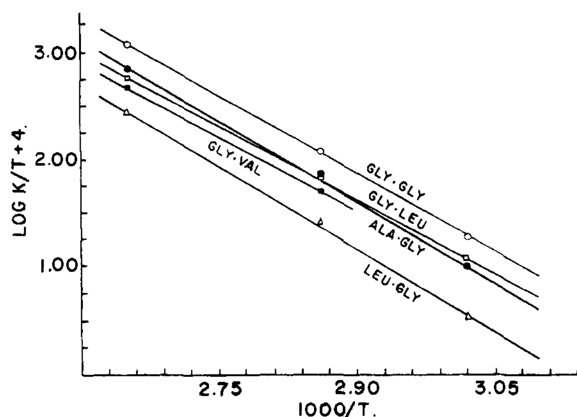
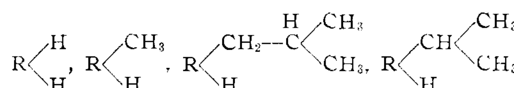


Fig. 8.—Plots of log K/T *versus* $1/T$ for the Dowex-50 hydrolyses of several dipeptides.

This work shows that the addition of an R group, where R represents a carbon chain, to glycylglycine has an effect on the rate of hydrolysis of the peptide bond. It takes only 0.242 hour to hydrolyze half of the glycylglycine with Dowex-50 at 104° but 28.2 hours for DL-valyl-DL-isoleucine, a change of 116 times caused by the addition of two R groups to glycylglycine. The addition of an R group to the residue whose carboxyl group forms part of the peptide bond has more effect on the rate of hydrolysis than if the same R group is added to the residue whose amino group forms part of the peptide bond (compare glycyl-L-leucine and DL-leucylglycine). The most stable peptides appear to be those containing valine. The bulky CH_3–CH–CH_3 group is close to the main peptide chain and effectively prevents the approach of hydrogen ions from a fairly wide angle. In leucine, the CH_3–CH–CH_3 group is one carbon atom further from the peptide bond so that leucyl peptides are less stable than the corresponding valyl peptides.

The effect of the carbon skeleton on the rate of hydrolysis of the dipeptides by hydrochloric acid and by Dowex-50 were found to increase in the order



where R is the remainder of the peptide molecule. This is the same order reported by Levene, *et al.*[14]

Effect of Temperature on the Rate of Hydrolysis of Several Dipeptides by Dowex-50.—The effect of temperature on the rate of hydrolysis of glycylglycine, DL-alanylglycine, glycyl-L-leucine, glycyl-DL-valine and DL-leucylglycine with Dowex-50 has been measured. The rate studies were carried out at 104.0, 76.6 and 57.9°. Figure 7 shows the effect of temperature on the rate of hydrolysis of glycylglycine. Table II contains a summary of the rate constants, as well as the enthalpies, entropies and free energies of activation. The plots of log K/T *versus* $1/T$ for the different dipeptides are shown in Fig. 8. The values for ΔH^* and ΔS^* were obtained by the least squares analysis of the data according to the equation

$$K = \frac{kT}{h} e^{-\Delta H^*/RT} e^{\Delta S^*/R} \quad (5)$$

where K is the specific reaction rate constant, k is the Boltzmann constant, h is the Planck constant, T is the absolute temperature, R is the gas contant, ΔS^* is the entropy of activation and ΔH^* is the enthalpy of activation. The free energy of activation, ΔF^*, is calculated from the equation

$$\Delta F^* = \Delta H^* - T\Delta S^* \quad (6)$$

The enthalpies of activation for the resin-catalyzed hydrolysis are 1 to 2 kcal. mole^{-1} higher than those reported for acid hydrolysis while the entro-

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pies of activation for the resin-catalyzed hydrolysis are 13 to 18 cal. deg.$^{-1}$ mole^{-1} higher than for acid hydrolysis.[11] Thus the greater efficiency of the resin is explained by the increase in the entropy of activation as compared with the entropy of activation of the acid-catalyzed reaction. This increase in the entropy of activation is probably due to the fixation of the dipeptide molecule to the resin particle.

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The Evaluation of the Enzyme–Inhibitor Dissociation Constants of α-Chymotrypsin and Several Pairs of Charged and Uncharged Competitive Inhibitors at *p*H 7.9 and 6.9[1,2]

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The enzyme–inhibitor dissociation constants of α-chymotrypsin and several pairs of charged and uncharged competitive inhibitors of this enzyme which were evaluated previously in aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM–HCl buffer now have been evaluated in aqueous solutions at 25° and *p*H 6.9 and 0.2 *M* in the THAM component of a THAM–HCl buffer. A comparison of these two sets of dissociation constants has led to the suggestion that the development of a negative charge in the environment of the catalytically active site of the enzyme particularly at *p*H 7.9 is responsible for the lesser affinity of α-chymotrypsin at *p*H 7.9 than at *p*H 6.9 for certain negatively charged competitive inhibitors of this enzyme. The effect of adding phosphate ion to the above systems has been discussed.

In 1937, Bergmann and Fruton[4] reported that α-chymotrypsin, acting in aqueous solutions *M*/15 in a phosphate buffer and at 40° and *p*H 7.6–7.8, hydrolyzed carbobenzoxy-L-tyrosylglycinamide, to give carbobenzoxy-L-tyrosine and glycinamide, but did not hydrolyze carbobenzoxy-L-tyrosylglycine. On the basis of the above evidence these authors concluded that the enzyme was incapable of causing the hydrolysis of specific substrates in which a carboxyl group was bonded to the non-carbonyl carbon atom immediately adjacent to the nitrogen atom involved in the susceptible peptide bond. This conclusion was substantiated further by the subsequent report[5] that α-chymotrypsin, acting in aqueous solutions *M*/15 in a phosphate buffer,[6] but at 25° and *p*H 7.1–7.5, hydrolyzed carbobenzoxy-L-phenylalanylglycinamide but did not hydrolyze carbobenzoxy-L-phenylalanylglycine, and by the claim[7] that carbobenzoxy-L-glutamyl-L-tyrosylglycinamide and L-glutamyl-L-tyrosylglycinamide were hydrolyzed in the presence of α-chymotrypsin but that carbobenzoxy-L-glutamyl-L-tyrosylglycine was not.[8] In 1950, Neurath and Schwert,[9] recognizing that the carboxyl group in question would be completely ionized in aqueous solutions in the region of *p*H 7–8, concluded, on the basis of the above evidence, that the presence of a negative charge near the susceptible bond caused a loss in substrate activity.

The first suggestion that a negative charge was present at or near the catalytically active site of the enzyme and that an electrostatic repulsion could arise from the interaction of this negative charge with that present in a competitive inhibitor containing a carboxylate group was offered by Neurath and Schwert[9] on the basis of experiments described by Kaufman and Neurath[10,11] which were conducted at 25° and *p*H 7.8 in the presence of a 0.1 *M* phosphate buffer when aqueous solutions were employed, or a 0.045 *M* phosphate buffer when the solvent system was 30% aqueous methanol. However, the experiments of Kaufman and Neurath[10,11] do not provide a direct and unambiguous demonstration that the affinity of α-chymotrypsin, when evaluated in aqueous solutions at *p*H 7.8 and in the presence of a phosphate buffer, for a competitive inhibitor containing a negatively charged carboxylate group is substantially less than for an uncharged competitive inhibitor which possesses the same structural features except for the replacement of the negatively charged carboxylate group by an uncharged group of approximately the same volume. The only uncharged competitive inhibitors which were studied by Kaufman and Neurath[10,11] were DL-1-phenyl-2-acetamidobutanone-3 and DL-1-*p*-hydroxyphenyl-2-acetamidobutanone-3 and the inhibition constants determined were for the DL-mixtures. It was a tenuous comparison of these composite inhibition constants with those of aceturic acid, hippuric acid, acetyl-DL-methionine, benzoyl-DL-methionine, benzoyl-D-, L- and DL-phenylalanine, and presumably that of O,N-diacetyl-L-tyrosine, that led Neurath and Schwert[9] to the conclusion that replacement of a carboxylate group by an acetyl group results in an increased affinity of the enzyme for the inhibitor, under the conditions previously specified, rather than a direct comparison of, for example, acetyl-L-phenylalanine with

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(2) *Cf.* R. J. Foster, Ph.D. Thesis, Calif. Inst. Tech., Pasadena, Calif., 1952.

(3) To whom inquiries regarding this article should be sent.

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(8) The authors have been informed by J. S. Fruton that these experiments were conducted in aqueous solutions at 38–39° and *p*H 7.6 and *M*/15 in a phosphate buffer and that in the experiment with carbobenzoxy-L-glutamyl-L-tyrosylglycinamide the specific substrate was initially present in suspension.

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