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THE HYDROLYSIS OF PROTEINS*

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SUMMARY

Experiments were made to investigate the effect of temperature on the hydrolysis reaction in aqueous 6 *N* HCl with a view to developing a rapid hydrolysis procedure. The maximum yield for all of the protein amino acids was obtained at 145° ± 2° for the minimal time of 4 h. Essentially equivalent hydrolysis of ribonuclease was achieved at the two different hydrolysis conditions, *i.e.*, 110° ± 1° for 26 h, or 145° ± 2° for 4 h. The yields obtained were in good agreement.

INTRODUCTION

The particular method used for the hydrolysis of proteins prior to an amino acid analysis is of considerable importance since some amino acids are preferentially destroyed and the hydrolysis of others is incomplete. In view of the high precision attained in the gas–liquid chromatographic (GLC) analysis of amino acid mixtures, the nature of the hydrolytic conditions plays an increasingly important role and can be easily evaluated. The speed, precision, and accuracy of the GLC methods developed by GEHRKE *et al.*[1–7] make possible a thorough investigation of the various parameters involved in the quantitative hydrolysis of different proteins and their compositional characterization.

A hydrolysis reagent of broad specificity is required to break all of the possible peptide bonds which are found in natural products of varying complexity. The particular reagent used must be capable of cleaving all peptide bonds in a protein. Further, the peptide bonds must be accessible to the hydrolytic agent; however, two features of protein structure present difficulties in this respect. First, there is steric hindrance due to the bulky side chains of the aliphatic amino acids, and secondly, the macromolecular structure, *i.e.* that due to secondary and tertiary bonding of the protein,

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prevents complete hydrolysis. The degree to which a protein molecule can unfold is limited by its secondary and tertiary structure; therefore, the hydrolysis reagent may react rapidly on one part of a protein molecule and slowly on another. This is evidenced by the number of different hydrolysis methods that are reported.

Differences in the stability of the various functional groups of amino acids necessitate a compromise among several experimental conditions in order to achieve the optimum hydrolysis of the protein. MOORE AND STEIN[8] reported that the best "all around" hydrolysis can be achieved by reaction for 24 h with 6 *N* HCl at 110° under conditions rigorously excluding oxygen, non-protein substances, and metals. Oxygen can be excluded by using a sealed tube hydrolysis technique. Generally, acid hydrolysis will yield over 95% recovery for aspartic and glutamic acids, proline, glycine, alanine, methionine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. However, tryptophan is completely destroyed, whereas 5–15% of threonine and serine are destroyed. Extrapolation to "zero-time" of hydrolysis can be done, but requires several different times of hydrolysis for each sample. GLC makes studies of "zero-time" hydrolysis practical. The peptide bonds of valine, isoleucine, and leucine are quite stable and thus a longer hydrolysis time is required to obtain maximum yield for these amino acids. WHITFIELD[9] has studied this problem and explained it in terms of steric factors. Extending the hydrolysis time to 70 h gives maximum yields[8] for these three amino acids. This, of course, results in lower yields for the other amino acids as compared to a 24 h hydrolysis time. As yet, no satisfactory method has been found for tryptophan, except alkaline, or enzymatic hydrolysis[8].

The purpose of this research was to study the rates and yields of protein hydrolysis and to determine the optimum reaction conditions which would give maximum yields of all twenty of the protein amino acids in the shortest possible time, using ribonuclease as a representative protein.

LITERATURE REVIEW

BRACONNOT[10], in 1820, first used sulfuric acid for the hydrolysis of a protein. The use of HCl as a hydrolytic agent was introduced by BOPP[11] in 1849. The hydrolytic agent commonly used today is HCl since the rate of peptide bond cleavage is increased in HCl over what it would be in sulfuric acid of equal concentration. An added advantage of HCl is that it can easily be removed from an amino acid mixture by evaporation. Protein samples are usually hydrolyzed with 2.5–5000 times their weight of 6 *N* HCl under reflux for 18–24 h.

The method of MACPHERSON[12] is generally recommended for large protein samples (*ca.* 0.2 g or larger). A protein sample which has been equilibrated under atmospheric conditions is weighed into a suitable round-bottomed flask which is fitted with a condenser. Concentrated A.R. HCl (36 w/w%) is added (*ca.* 20 ml/g protein), the protein is dissolved on a water bath at 35–40°, then sufficient hot doubly distilled water is added to bring the concentration of HCl to 20 w/w%. The solution is boiled gently under reflux for 24 h. The excess of HCl is removed under a partial vacuum and the sample is diluted to a suitable volume with 0.1 *N* HCl. An aliquot of this solution is then removed for classical amino acid analysis or GLC amino acid analysis.

The method of MOORE AND STEIN is in common use. A sample of air-dried or

lyophilized protein is placed in a 16 × 135 mm heavy-walled Pyrex tube (Corning No. 9860). The protein is suspended in 1 ml of 6 *N* HCl (a 1:1 dilution of concentrated reagent HCl with doubly distilled water). The sample is frozen by placing in a bath of ethanol and solid carbon dioxide. After freezing, the sample container is evacuated to below 50 μ, then sealed under vacuum. The hydrolysis is conducted at 110° ± 1° for 20 h or 70 h. Excess HCl is removed under vacuum at 40–45°, the sample is diluted to a known volume, and aliquots are removed for analysis. This technique or some modification of it is presently the preferred method for the hydrolysis of protein samples.

A serious problem associated with the acidic hydrolysis of proteins is the partial decomposition of some of the amino acids. The destruction of tryptophan is almost complete and a considerable loss of cysteine may occur. The breakdown of the other amino acids generally occurs to a lesser degree.

REES[13] reported in 1946 that hydrolysis with 6 *N* HCl for 24 h leads to a recovery of only 89.5% for serine and 94.7% for threonine. CORFIELD AND ROBSON[14] reported a 14% loss of serine in the hydrolysis of salmine. REES[13] and HIRS *et al.*[15] found the rate of decomposition of serine and threonine to vary with the purity of the HCl used in the acidic hydrolysis. However, an accurate determination of the threonine and serine content can be made by extrapolation to "zero-time" of hydrolysis if data are available for several different hydrolysis times. Examples of this technique were included in publications by HARFENIST[16] in 1953, by SMITH AND STOCKELL[17] in 1954, by HIRS *et al.*[15] in 1954, and by NOLTMANN *et al.*[18] in 1962.

There is a possibility that proline is degraded during acid hydrolysis. ELLIOT *et al.*[19] and ZUBER AND JAQUES[20] both suggested an empirical formula of $Arg_2Phe_2Pro_2Gly \cdot Ser$ for the peptide bradykinin from results based on amino acid analyses after acidic hydrolysis. BOISSONMAS *et al.*[21], however, synthesized bradykinin and found that the actual structure corresponded to the formula $Arg_2Phe_2Pro_3Gly \cdot Ser$. The variance between the formula determined from amino acid analysis and the actual formula may be due to the decomposition of proline during the acid hydrolysis prior to analysis.

LUGG[22] observed that pure tyrosine was not affected by heating it in acid at 100° for 20 to 30 h. LIGHT AND SMITH[23], however, reported that tyrosine was completely destroyed during the acid hydrolysis of the peptide Ala · Val · Gly · Tyr. SHEPHERD *et al.*[24] also obtained low recoveries of tyrosine from several peptides. This destruction was reduced but not eliminated when the samples were hydrolyzed under a nitrogen atmosphere. The decomposition of tyrosine may involve aspartic acid since tyrosine was quantitatively recovered from the peptide Val · Tyr · Pro but not from Val · Tyr · Pro · Asp. MUNIER[25] reported that tyrosine may be converted to 3-chlorotyrosine during hydrolysis by reacting with traces of chlorine in the HCl. This reaction could not, however, account entirely for the losses observed by HIRS *et al.*[15].

A large concentration of carbohydrates in the hydrolysis medium may seriously reduce certain amino acids. TRISTRAN[26] noted that arginine was extensively destroyed during acidic hydrolysis in the presence of carbohydrates with the amount of destruction being proportional to the concentration of carbohydrates, and BAILEY[27] reported losses of methionine as high as 20% in samples which were high in carbohydrate content. OSONO *et al.*[28] found that refluxing methionine with 10% HCl

resulted in the production of some homocystine, homocysteine, and glycine. LUGG[29] observed only a slight loss of cystine during acidic hydrolysis in the absence of carbohydrate; however, losses of 6 to 7% were noted in the presence of carbohydrates.

Lysine is considered to be the most stable of the diamino acids, but ISHII[30] reported a loss of 3% when lysine was heated at reflux with 20% HCl. The reported degradation products were aspartic acid, glycine, glutamic acid, and α-aminoadipic acid.

Steric hindrance by bulky side chain residues results in the slow release of some amino acids, particularly valine and isoleucine. Kinetic studies, by SYNGE[31] in 1945, and by HARRIS *et al.*[32] in 1956, clearly indicated hindrance by valine, leucine, alanine, and isoleucine and the yields for these amino acids which have been hydrolyzed for varying lengths of time were found to be a function of time. An accurate value for each of these amino acids can be determined by plotting yield as a function of hydrolysis time, and by drawing tangents to the maximums in the curves, then extrapolating to "zero-time".

The rates of decomposition of the amino acids during acidic hydrolysis are dependent on several factors including: the concentration of the hydrolyzing acid, the purity of the acid used, the time and temperature of hydrolysis, the presence of carbohydrates, aldehydes or metal impurities. Current methods represent a compromise among the several considerations mentioned above. The most common methods for the hydrolysis of proteins are outlined in two excellent review articles by LIGHT AND SMITH[33] and by MOORE AND STEIN[8].

Because of the considerable time required for the hydrolysis of proteins by the reported methods and the losses involved, it was considered highly important to investigate other procedures. From studies directed toward this goal, this research reports on a rapid hydrolysis method which can be completed in 4 h.

EXPERIMENTAL

Apparatus

A Varian Aerograph Model 2100 gas chromatograph with a four-column oven bath, four flame ionization detectors, two dual differential electrometers, and equipped with a Varian Model 20 recorder, and a Packard Instruments Co. Model 7300 dual column gas chromatograph with hydrogen flame detectors and equipped with a Honeywell Electronik 16 strip chart recorder were used. Peak areas were determined with a digital readout integrator, Infotronics Model CRS 104.

Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser, and a Welch Duo-Seal vacuum pump.

Pyrex 2.5 × 20 cm screw top culture tubes with teflon-lined caps (Corning No. 9826) were used as the reaction vessels for the hydrolysis.

Reagents

All reagents used were those specified by GEHRKE *et al.*[2].

The 6 *N* HCl solution in water was prepared by distilling a 6 *N* solution in an all-glass system and then adjusting the concentration of the constant boiling HCl to 6 *N* by the addition of doubly distilled water.

The 6 *N* HCl solution in *n*-butanol was prepared by bubbling anhydrous HCl into anhydrous *n*-butanol.

Ribonuclease A from bovine pancreas, crystallized five times, Type I-A, protease free, essentially salt free, activity of 70 Kunitz units per mg, was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Chromatography

The columns and chromatographic conditions were those as specified in ref. 2.

HYDROLYSIS METHODS

Hydrolysis of samples in 6 N HCl in water for GLC analysis

(1) Accurately weigh 10.0 mg of dry protein (ribonuclease) into the pyrex screw top with teflon-lined cap culture tube. (2) Flush tube with filtered N_2. (3) Add 10.0 ml of 6 *N* HCl in water to each tube. (4) Flush each tube again with N_2. (5) Place teflon-lined cap on each tube and heat at 110° ± 1°, or 145° ± 2° in an oil bath for the specified time. (6) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (7) Add an appropriate quantity of I.S. (0.50 mg butyl stearate, dissolved in 1 ml of BuOH–HCl). (8) Add 1.5 ml of *n*-butanol 3 *N* in HCl per 1.0 mg of total amino acids, ultrasonic mix for 15 sec, esterify at 100° for 35 min, then dry at 60° under partial vacuum, and acylate as described in ref. 2.

Hydrolysis of samples in 6 N HCl in water for analysis by both GLC and classical ion exchange

(1) Accurately weigh 25.0 mg of dry protein (ribonuclease) into a large culture tube. (2) Flush tube with filtered N_2. (3) Add 25.0 ml of 6 *N* HCl in water to each tube. (4) Flush each tube again with N_2. (5) Place teflon-lined cap on each tube and heat at 110° ± 1°, or 145° ± 2° in an oil bath for the specified time. (6) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (7) Accurately pipet 20.0 ml of 0.1 *N* HCl into each of the samples to dissolve the amino acid residue. Mix each sample thoroughly. (8) Draw a 5.0 ml aliquot of each sample and place in a $\bar{\$}$ 125 ml flat-bottom boiling flask for GLC analysis, or analyze by classical ion exchange. (9) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (10) Add an appropriate quantity of I.S. (0.50 mg butyl stearate, dissolved in 1 ml of BuOH–HCl). (11) Add 1.5 ml *n*-butanol 3 *N* in HCl per 1.0 mg of total amino acids, ultrasonic mix for 15 sec, esterify at 100° for 35 min, then dry at 60° under a partial vacuum, and acylate as described in ref. 2.

Hydrolysis of samples by 6 N HCl in n-butanol

(1) Accurately weight 10.0 mg of dry protein (ribonuclease) into a large culture tube. (2) Flush tube with filtered nitrogen gas. (3) Add 15.0 ml of *n*-butanol 6 *N* in HCl (1.5 ml of BuOH–HCl per 1.0 mg of protein). (4) Flush reaction vessel again with filtered N_2. (5) Place teflon-lined cap on each tube and heat at 110° ± 1°, or 145° ± 2° in an oil bath for the specified time. (6) Add an appropriate quantity of I.S. (0.50 mg butyl stearate, dissolved in BuOH–HCl). (7) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (8) Acylate as described by GEHRKE *et al.*[2].

RESULTS AND DISCUSSION

According to the direct esterification procedure reported by ROACH AND GEHRKE[6] the amino acids are esterified in *n*-butanolic HCl prior to their acylation with trifluoroacetic anhydride. The present procedure for the GLC analysis of a protein sample requires that the protein sample be hydrolyzed in 6 *N* HCl in water, dried, and then esterified with *n*-butanol 3 *N* in HCl. If complete hydrolysis of the protein were achieved in *n*-butanolic HCl, one of the steps in the GLC analysis of a protein hydrolysate could be eliminated since the *n*-butyl esters of the amino acids would be formed during the hydrolysis of the protein. Thus, studies were made to investigate the yields of hydrolysis of a model protein, ribonuclease, in 6 *N* HCl in *n*-butanol.

Samples of ribonuclease were hydrolyzed at 110° ± 1° in *n*-butanol 6 *N* in HCl for varying lengths of time. The experimental results from the GLC analysis of this hydrolysate are presented in Table I. A sample of ribonuclease was also hydrolyzed at 110° ± 1° in water 6 *N* in HCl for varying lengths of time. The GLC data are presented in Table II. Much higher yields were obtained for the samples hydrolyzed in aqueous 6 *N* HCl. Also, decomposition of all of the amino acids became a serious problem at hydrolysis times longer than 44 h with *n*-butanol 6 *N* in HCl, and very poor results were obtained on hydrolysis for 4 h to 10 h (Table I).

Since *n*-butanol 6 *N* in HCl was found to be unsuitable for the hydrolysis of proteins, it was decided to investigate the effect of temperature on the hydrolysis reaction in aqueous 6 *N* HCl. Samples were hydrolyzed at 110° ± 1°, 145° ± 2°, and 175° ± 5° for 1 to 10 h. The optimum hydrolysis temperature in terms of rate of hydrolysis and a minimum of decomposition was found to be at 145° ± 2° for 4 h.

TABLE I

AMINO ACID ANALYSIS OF RIBONUCLEASE AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed in a closed tube with *n*-butanol 6 *N* in HCl for the specified time at 110° ± 1°. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Analyses by GLC.

Amino acid	*Yield (w/w%)*[a]				
	4 h	*10 h*	*24 h*	*44 h*	*70 h*
Alanine	2.26	3.61	6.54	9.14	3.31
Valine	0.81	1.72	3.69	5.27	3.48
Glycine	0.73	0.84	1.59	2.06	1.01
Isoleucine	0.27	0.47	0.73	1.23	1.50
Leucine	0.28	0.58	1.95	2.22	1.47
Proline	1.45	1.87	3.62	3.90	2.75
Threonine	1.34	1.59	2.59	2.20	1.24
Serine	3.53	3.48	3.98	3.29	1.81
Methionine[b]	—	—	—	—	—
Phenylalanine	0.61	0.94	2.52	2.90	2.36
Aspartic acid	2.93	4.33	8.52	7.00	3.80
Glutamic acid	3.06	4.31	8.15	7.10	4.56
Tyrosine[b]	—	—	—	—	—
Lysine	1.90	3.06	6.94	5.76	2.17

[a] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml of *n*-butanol 6 *N* in HCl).

[b] The peaks obtained for methionine and tyrosine were too small to allow an accurate determination.

TABLE II

AMINO ACID ANALYSIS OF RIBONUCLEASE AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed in a closed tube with 6 *N* HCl for the specified time at 110° ± 1°. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Analyses by GLC.

Amino acid	*Yield (w/w%)*[a]					
	9½ h	*26 h*	*35 h*	*48 h*	*81 h*	*116 h*
Alanine	7.06	7.12	7.35	7.46	7.57	7.51
Valine	4.33	6.43	6.90	7.28	7.56	7.52
Glycine	1.60	1.65	1.66	1.66	1.69	1.68
Isoleucine	0.81	1.50	1.69	1.99	2.39	2.59
Leucine	1.83	1.91	1.96	1.99	2.02	2.01
Proline	2.78	3.09	3.12	3.14	3.19	3.17
Threonine	6.91	7.35	7.71	7.72	7.58	7.27
Serine	9.91	10.04	9.96	9.80	9.20	8.32
Methionine	3.47	3.39	3.36	3.44	3.17	2.93
Phenylalanine	2.59	3.05	3.17	3.22	3.25	3.29
Aspartic acid	13.23	13.39	13.74	13.97	13.96	13.99
Glutamic acid	11.13	12.08	12.43	12.04	12.49	12.33
Tyrosine	5.74	6.56	6.63	6.59	6.26	6.06
Lysine	8.88	10.15	10.44	10.59	10.61	10.75

[a] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml 6 *N* HCl).

TABLE III

AMINO ACID ANALYSIS OF RIBONUCLEASE AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed in a closed tube with 6 *N* HCl for the specified time at 145° ± 2°. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Analyses by GLC.

Amino acid	*Yield (w/w%)*[a]						
	2 h	*4 h*	*5 h*	*6 h*	*7 h*	*8½ h*	*9 h*
Alanine	7.30	7.28	7.24	7.37	7.40	7.54	7.45
Valine	6.25	7.15	7.16	7.31	7.41	7.54	7.55
Glycine	1.69	1.17	1.66	1.72	1.67	1.71	1.71
Isoleucine	1.40	1.94	2.07	2.23	2.26	2.53	2.55
Leucine	1.99	1.98	1.98	2.04	2.00	2.02	2.07
Proline	3.18	3.19	3.15	3.16	3.14	3.15	3.16
Threonine	7.73	7.62	7.52	7.36	7.11	7.18	7.17
Serine	10.25	9.66	9.40	8.82	8.52	8.15	8.01
Methionine	3.54	3.36	3.29	3.11	3.06	2.77	2.24
Phenylalanine	3.16	3.26	3.22	3.29	3.22	3.42	3.38
Aspartic acid	13.83	13.60	13.66	13.76	13.60	13.81	13.97
Glutamic acid	11.79	11.88	11.97	12.00	11.75	12.05	12.29
Tyrosine	6.57	6.80	7.00	7.26	7.29	6.17	6.47
Lysine	10.29	10.37	10.45	10.42	10.10	10.27	10.65

[a] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml 6 *N* HCl).

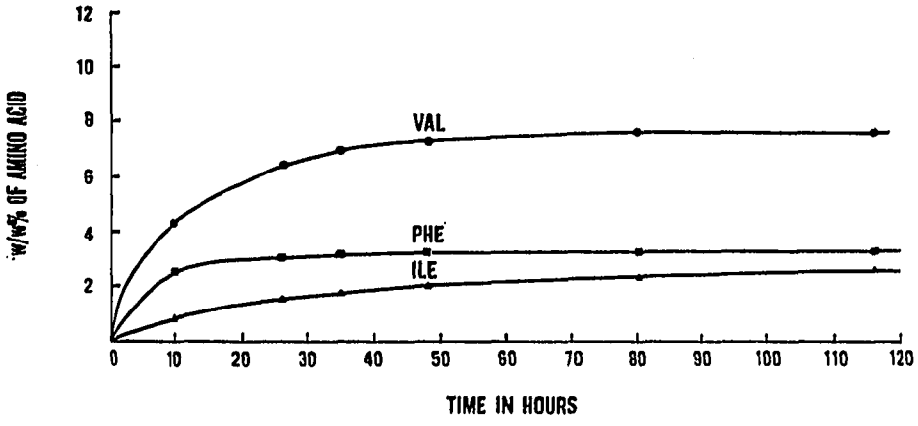

Fig. 1. Yields of the amino acids valine, isoleucine and phenylalanine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 110° ± 1°.

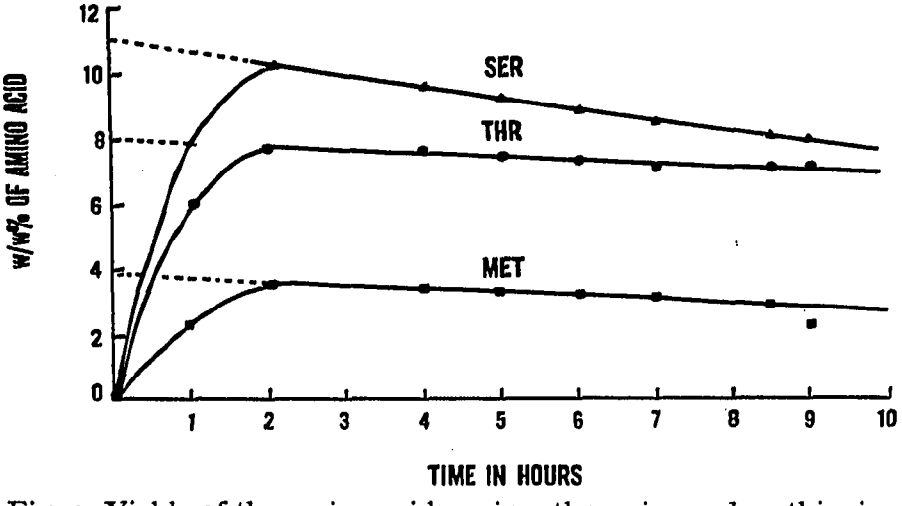

Fig. 2. Yields of the amino acids serine, threonine and methionine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 145° ± 2°.

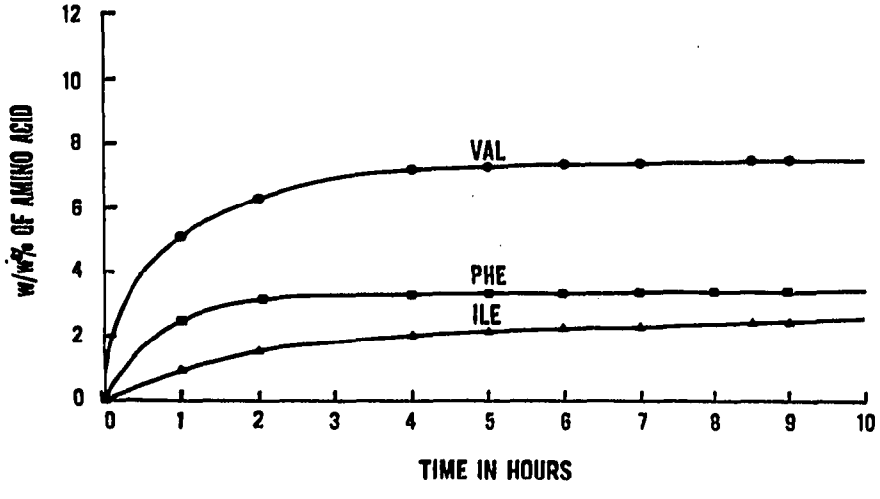

Fig. 3. Yields of the amino acids valine, isoleucine and phenylalanine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 145° ± 2°.

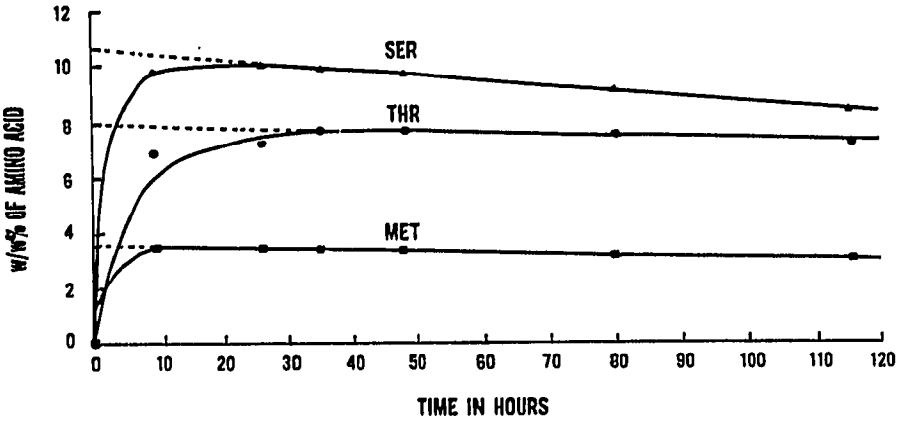

Fig. 4. Yields of the amino acids serine, threonine and methionine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 110° ± 1°.

The GLC data in Table III were obtained from the analysis of samples of ribonuclease which had been hydrolyzed at 145° ± 2° for periods of time from 2 to 9 h. These data indicate that approximately equivalent hydrolyses of a protein can be achieved in 4 h at 145° ± 2° and in 24 h at 110°.

MOORE AND STEIN[8] reported that the best "all around" hydrolysis can be achieved with 6 *N* HCl for 24 h at 110° under conditions rigorously excluding oxygen, non-protein substances, and metals. These experimental conditions were selected to give maximum total recovery of the amino acids. Certain of the amino acids, however, undergo serious decomposition and the hydrolysis of others is incomplete. Maximum values for isoleucine, leucine, valine, and phenylalanine can be obtained from a plot of yield *versus* hydrolysis time. Also, an extrapolation to "zero-time" can be made to determine more accurate values for those amino acids which undergo serious decomposition, *i.e.* threonine, serine, and methionine.

Plots of yield *versus* hydrolysis time at 110° ± 1° for valine, isoleucine, phenylalanine, threonine, serine, and methionine from ribonuclease are shown in Figs. 1

TABLE IV

SELECTED AMINO ACIDS FROM THE AMINO ACID ANALYSIS OF RIBONUCLEASE

Amino acid	*Yield (w/w%)*		
	110° for 24 h	*145° for 4 h*	*Literature value*[15]
Valine	7.54[a]	7.55[a]	7.49
Isoleucine	2.54[a]	2.59[a]	2.67
Threonine	8.00[b]	8.00[b]	8.90
Serine	10.70[b]	11.05[b]	11.40
Methionine	3.54[b]	3.83[b]	4.00
Phenylalanine	3.30[a]	3.40[a]	3.51

[a] Values obtained by drawing a tangent to the maximum in a plot of yield of amino acid *versus* time of hydrolysis to obtain the maximum values.

[b] Values obtained by extrapolating to "zero-time" a plot of yield of amino acid *versus* time of hydrolysis.

TABLE V

AMINO ACID ANALYSIS OF BOVINE SERUM ALBUMIN AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed with 6 *N* HCl for the specified time at 145° ± 2° in a closed tube, with norleucine as internal standard. Analyses by classical ion exchange.

Amino acid	*Yield (w/w%)*					
	2 h	*4 h*	*6 h*	*8 h*	*9 h*	*24 h*[a]
Alanine	4.95	5.00	5.14	4.83	4.91	4.76
Valine	4.15	4.46	5.10	4.79	4.89	4.56
Glycine	2.05	2.25	2.11	2.02	1.97	1.97
Isoleucine	1.18	2.12	2.27	2.16	2.22	2.04
Leucine	11.45	11.91	12.30	11.91	11.70	11.42
Proline	4.72	4.23	4.34	4.73	3.84	3.76
Threonine	4.97	5.07	5.05	4.91	4.93	4.97
Serine	3.64	3.73	3.47	3.08	3.40	3.55
Methionine	0.74	0.81	0.68	0.72	0.72	0.70
Phenylalanine	6.50	6.88	6.83	6.53	6.45	6.59
Aspartic acid	9.43	9.49	9.62	9.42	9.18	9.02
Glutamic acid	15.82	15.79	16.13	15.91	15.56	15.49
Tyrosine	5.57	5.92	5.34	5.38	5.15	5.53
Lysine	11.51	11.88	12.01	11.73	11.76	11.56
Histidine	4.00	4.43	4.28	4.16	4.06	4.09
Arginine	5.84	6.10	6.00	5.73	5.87	5.83
Tryptophan	—	—	—	—	—	—
Half-cystine	5.93	6.08	5.84	5.96	5.51	5.86

[a] Hydrolyzed with 6 *N* HCl for 24 h at 110° ± 1° in a closed tube, norleucine as internal standard. Analyses by classical ion exchange.

TABLE VI

RECOVERY OF AMINO ACIDS FROM A STANDARD MIXTURE

0.4 mg of *each* amino acid + 10 ml 6 *N* HCl heated for the specified time at 145° ± 2°. Each value represents an average of two independent analyses. Analyses by GLC.

Amino acid	*Recovery (%)*					
	2 h	*4 h*	*6 h*	*7 h*	*8 h*	*9 h*
Alanine	100.6	101.8	96.9	95.6	95.5	93.1
Valine	101.2	100.9	94.1	92.9	91.8	89.4
Glycine	100.6	99.7	95.5	92.9	90.3	87.7
Isoleucine	101.8	102.9	95.8	92.3	89.9	87.6
Leucine	101.2	98.8	94.9	92.8	91.5	89.2
Proline	99.4	94.6	89.9	88.7	89.9	88.9
Threonine	98.6	93.2	90.4	86.3	84.9	84.6
Serine	99.3	90.5	87.6	84.7	81.8	78.8
Methionine	98.1	90.6	79.2	73.6	71.7	75.5
Hydroxyproline	97.4	94.7	89.5	85.5	84.2	83.8
Phenylalanine	102.3	97.7	95.5	94.1	93.2	90.9
Aspartic acid	101.9	98.9	97.6	96.3	95.5	95.4
Glutamic acid	101.6	100.7	100.6	99.7	99.4	97.2
Tyrosine	102.6	100.0	98.7	94.2	93.5	90.9
Lysine	100.0	97.4	94.8	93.4	89.5	92.1
Arginine	95.2	88.1	85.7	76.2	77.1	65.2
Histidine	101.1	97.5	97.4	97.3	97.3	88.4
Cystine	102.8	97.1	100.0	85.7	77.1	74.3

and 4. Similar plots for hydrolysis of ribonuclease at 145° ± 2° are included in Figs. 2 and 3. Maximum values for threonine, serine, and methionine were obtained by extrapolating to "zero-time" as shown, whereas the maximum values for valine, isoleucine, and phenylalanine were obtained from the maximumin the curves. Maximum values for the protein amino acids from ribonuclease obtained in this way are included in Table IV. The agreement of the maximum values from the 110° curves with the corresponding values at 145° ± 2° indicates that hydrolysis under both sets of conditions gave essentially the same results. Also, both sets of data are in excellent agreement with the literature values.

After developing the set of "hydrolysis conditions" (145° ± 2° for 4 h) using ribonuclease as a model protein, several other proteins were then hydrolyzed under the same conditions and analyzed. The data for one of the proteins, bovine serum albumin, analyzed by classical ion exchange are given in Table V. The good agreement of the data for hydrolysis for 4 h at 145° ± 2° with 24 h at 110° shows that the hydrolysis of these proteins can be conducted equally well under these two sets of conditions.

Recovery data for GLC analysis of mixtures of the protein amino acids which had been heated for varying times at 145° ± 2° are included in Table VI. The recovery for all of the amino acids except arginine was excellent with a hydrolysis time of 2 h. On hydrolysis for 4 h, the losses in some cases were significant. Low recoveries were obtained for proline, threonine, serine, methionine, hydroxyproline, and arginine. Plots of yield *versus* hydrolysis time are required to obtain accurate results for these amino acids.

CONCLUSIONS

The use of *n*-butanol 6 *N* in HCl as a protein hydrolysis reagent would obviate one of the steps in the reported[2] GLC analysis of proteins since the *n*-butyl esters of the amino acids would be formed during the hydrolysis. Thus, these studies were initiated to investigate the yields on hydrolysis of a model protein, ribonuclease, in 6 *N* HCl in *n*-butanol. However, this reagent was found to be unsatisfactory since the rate of hydrolysis was much slower in this medium than it was in 6 *N* HCl in water, and the rates of decomposition of the amino acids were considerably faster.

Since *n*-butanol 6 *N* in HCl was found to be unsuitable for the hydrolysis of proteins, experiments were made to investigate the effect of temperature on the hydrolysis reaction in aqueous 6 *N* HCl with a view to developing a rapid hydrolysis procedure. The maximum yield for all of the protein amino acids was obtained at 145° ± 2° for the minimal time of 4 h. Essentially equivalent hydrolysis of ribonuclease was achieved at the two different hydrolysis conditions, *i.e.* 110° ± 1° for 26 h, or 145° ± 2° for 4 h. The yields obtained were in good agreement.

Plots of yield *versus* hydrolysis time *must* be made for *each* amino acid to obtain the best possible values for the amino acid composition of a protein. These plots are then extrapolated to "zero-time" for those amino acids which undergo decomposition; for those amino acids which are difficult to hydrolyze, the values are obtained from the maximum in the curves. Several protein samples must be hydrolyzed at different times to obtain all the data necessary to construct these plots. Therefore, a rapid hydrolysis method is needed which gives maximum values for the amino acids with a minimum of decomposition. Recovery studies as a function of hydrolysis time at

145° were made using standard amino acid mixtures; essentially complete recovery was obtained on hydrolyzing for 2 to 4 h. Pro, Thr, Ser, Met, OHPro, and Arg were the most sensitive to heat. This reported procedure allows one to rapidly hydrolyze several samples of a protein at 145° ± 2° and then to conveniently obtain the data by GLC for plotting these curves. The agreement among the data for the two different hydrolysis temperatures and times conclusively demonstrates that such plots can be made. Of special interest is a comparison of the data obtained for ribonuclease and bovine serum albumin for the 4 h hydrolysis at 145° ± 2° with the 24 h hydrolysis at 110° ± 1°. In almost every case a higher recovery was obtained for the 145° ± 2° hydrolysis.

With this method a protein can be essentially completely hydrolyzed in 4 h with a minimum of decomposition of the amino acids. Rapid hydrolysis of proteins coupled with quantitative GLC analysis of amino acids provides a powerful tool in protein research, biochemical, and nutritional investigations.

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