to 17 g. of anhydrous hydrogen fluoride at 0°. The content of the copper flask was then poured onto ice. The hydrocarbon layer was separated, washed with potassium hydroxide, water, dried over calcium chloride and distilled. Three grams of (I) was obtained, boiling at 161° (7.5 mm.), $n^{20}D$ 1.5579.

The infrared and ultraviolet absorption spectra were taken.

Two-tenths gram of (I) was nitrated with 5 ml. of nitrating mixture consisting of 2 vol. 96% sulfuric acid and 1 vol. of 72% nitric acid. The nitro compound obtained was crystallized from a solution of ethanol and chloroform. It melted at 251°, and did not depress the melting point of the tetranitro derivative of the $C_{20}H_{24}$ hydrocarbon.

Dimerization of 1-Methyl-4-isopropenylbenzene

A. Dimethyl-*p*-tolyl-carbinol.—The carbinol was prepared from 256 g. (1.5 mole) of *p*-bromotoluene and 81 g. (1.4 mole) of acetone via a Grignard reaction according to the procedure of Sabatier and Marat.[13] The carbinol distilled at 73° (2.5 mm.), $n^{20}D$ 1.5168; yield 66%.

B. 1-Methyl-4-Isopropenylbenzene.—Forty-three grams of the carbinol was passed at 350° during a period of one hour over 40 cc. of activated alumina of a 10–12 mesh size. The hydrocarbon distilled at 82° (21 mm.), $n^{20}D$ 1.5350; yield was over 80%.

C. Polymerization of 1-Methyl-4-isopropenylbenzene.—Eight grams of 1-methyl-4-isopropenylbenzene dissolved in 7 g. of methylcyclohexane was added with agitation to 10 ml. of anhydrous hydrogen fluoride placed in a copper beaker. The mixture was stirred for fifteen minutes and then poured onto 15 g. of ice precooled to −40°. The hydrocarbon layer was separated, diluted with ether, washed with aqueous potassium hydroxide, dried, and distilled. Three grams of a hydrocarbon boiling at 171° (5 mm.) was obtained; $n^{20}D$ 1.5545, melting point 33°. According to ultraviolet analysis it consisted of 1,3,3,6-tetramethyl-1-*p*-tolylindan (Compound I).

Anal. Calcd. for $C_{20}H_{24}$: C, 90.85; H, 9.15. Found: C, 91.03; H, 9.11.

(13) P. Sabatier and M. Murat, *Compt. rend.*, **156**, 184 (1913); *Ann. Chim.*, [9] **4**, 253 (1915).

On nitration with nitrating mixture consisting of 2 vols. of sulfuric acid and 1 vol. of nitric acid it formed a tetranitro derivative, which after two crystallizations from ethanol–chloroform solution melted at 248°, and did not depress the melting point of the tetranitro derivative prepared from a known sample of Compound I.

Acknowledgment.—We are indebted to Mr. Don Strehlau for some of the derivatives, to Mrs. Margaret Ledyard for the elementary analyses and to Dr. W. S. Gallaway of the Universal Oil Products Company for the infrared analyses.

Summary

The reaction of *p*-cymene with trimethylethylene, methylcyclohexene, dihydrolimonene, 1-octene, and cyclohexene in the presence of either sulfuric acid or hydrogen fluoride was investigated.

Hydrogen transfer occurs when *p*-cymene reacts with the first three olefins; the products resulting from such reaction consist of isopentane, methylcyclohexane, and *p*-menthane, respectively, and 1,3,3,6-tetramethyl-1-*p*-tolylindan which is formed in each case.

1,3,3,6-Tetramethyl-1-*p*-tolylindan was synthesized.

A mechanism for the hydrogen transfer reaction is proposed:

The following new compounds and their derivatives were prepared: (a) 4-methyl-4-*p*-tolyl-2-pentanone, (b) 4-methyl-2,4-di-*p*-tolyl-2-pentanol, (c) 1,3,3,6-tetramethyl-1-(4-methylcyclohexyl)-hexahydroindan, (d) 1,1,3,5-tetramethylindan, and (e) 1,1,3,5-tetramethylhexahydroindan.

EVANSTON, ILLINOIS RECEIVED NOVEMBER 17, 1947

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

The Acid Hydrolysis of Egg Albumin. I. Kinetic Studies

BY HENRY B. BULL AND J. WILFRID HAHN

It becomes increasingly clear that a central problem of protein chemistry is the localization of the amino acid residues in the peptide chains, and it is possible that study of the hydrolysis of proteins might shed light on this problem. While the literature on protein hydrolysis is extensive, there are certain features of this reaction which have not been explored. The present paper reports the results of an investigation of the hydrolysis of purified hen's egg albumin at 30°, at 45° and at 60° by hydrochloric acid. The amino nitrogen, the free amino acids, the ammonia, the material insoluble in trichloroacetic acid as well as the heat coagulable material have been determined, and an interpretation of these results is suggested.

Experimental

One volume of a solution of crystalline albumin prepared from fresh chicken eggs by the method of Kekwick and Cannan[1] was added to two volumes of concentrated hydrochloric acid. The flask containing the reaction mixture was securely stoppered and placed in a water-bath at the desired temperature and rotated occasionally. A single, clear, homogeneous phase resulted in a few minutes. At intervals, 20-cc. aliquots were removed and neutralized with powdered sodium bicarbonate, and the turbid solutions resulting made up to 50 cc. with water. Ammonia, free amino acids and amino nitrogen were determined on these dilutions.

Material precipitable by trichloroacetic acid was measured on 5-cc. aliquots of the reaction mixture which were diluted to 50 cc. without neutralization. Five cc. of this dilution was treated with 10 cc. of a 4% trichloroacetic acid solution, and after fifteen minutes filtered and the total nitrogen of the filtrate determined.

The heat coagulable material was determined by adjusting a 5-cc. aliquot of the hydrolyzate to about *p*H 4.5, heating on a boiling water-bath for five minutes, diluting to 50 cc. and filtering. Total nitrogen was run on the filtrate.

(1) Kekwick and Cannan, *Biochem. J.*, **30**, 227 (1936).

On the basis of total nitrogen in egg albumin,[2] the reaction mixture contained 4.25 g. of protein per 100 cc. The hydrochloric acid during the first part of the reaction was 7.95 *N* as determined by titration with standard base.

The ammonia was obtained by a steam distillation in the Pregl micro-Kjeldahl apparatus, using magnesium oxide as the alkalizing agent.

The amino nitrogen was measured in the Van Slyke volumetric apparatus by the Kendrick–Hanke modification,[3] and the reaction time was ten minutes. An ammonia correction was determined by the method of Irving, Fontaine and Samuels[4] and this correction, which amounted to 55% of the ammonia, was applied to the amino nitrogen values.

To calculate the rate of hydrolysis of peptide bonds from the amino nitrogen, it was necessary to assume that the bonds involving proline and hydroxyproline were hydrolyzed at a rate proportional to their amounts[5] in egg albumin.

The free amino acids released were measured by the ninhydrin titrimetric method at *p*H 2.5.[6] Aspartic acid yields carbon dioxide from both its carboxyl groups, and the ninhydrin values are too large by this amount. Following Frost and Heinsen,[7] we have assumed that aspartic acid is liberated at a rate proportional to its relative concentration[8] in the protein, and the ninhydrin values corrected on this basis.

Controls were prepared by neutralizing 13.5 cc. of concentrated hydrochloric acid with sodium bicarbonate and then adding 6.67 cc. of the mother protein solution. Control values have been subtracted from each determination to give the groups actually produced by the hydrolysis.

Results

Tables I, II and III show the quantities of ammonia, amino nitrogen and free amino acids produced by acid hydrolysis at 30°, at 45° and at 60° expressed in milliequivalents per gram of egg albumin. From the average residue weight for egg albumin and on the basis of 4 peptide chains in this protein,[8] there are about 8.89 milliequivalents of peptide bonds in one gram of protein. Percentage hydrolysis was calculated using this value after the proline correction had been applied.

Table IV shows the percentage of the total nitrogen in solution after treatment with trichloroacetic acid by the technique described above. Also shown is the per cent. of the total nitrogen in solution after isoelectric heat coagulation. The agreement between these sets of data is by no means perfect. We are inclined to believe that

TABLE I

MILLIEQUIVALENTS OF AMMONIA, AMINO NITROGEN AND FREE AMINO ACIDS PER GRAM OF EGG ALBUMIN PRODUCED BY ACID HYDROLYSIS AT 30°. ALSO SHOWN ARE THE PERCENTAGES OF PEPTIDE BONDS HYDROLYZED

Time in hours	NH_3	Amino nitrogen: Corrected for NH_3	Amino nitrogen: Corrected for NH_3 and proline	Amino nitrogen: Per cent. hydrolysis	Free amino acids: Uncorrected	Free amino acids: Corrected for aspartic acid
2	0.05	..	..	..	...	...
4	.09	0.17	0.18	2.0	...	...
9	.21	0.73	0.76	8.5	0.019	0.017
22	.36	1.59	1.66	18.6	.152	.142
29.5	.50	1.86	1.94	21.8	.248	.232
48.5	.60	2.50	2.61	29.3	.357	.334
72.25	.66	3.02	3.15	35.4	.515	.482
120	.70	3.44	3.59	40.4	.935	.875
144	.71	3.88	4.04	45.4	.930	.870
195	.69	4.32	4.50	50.5	1.14	1.07
240	.72	4.42	4.62	52.0	1.36	1.27
289	.72	4.84	5.04	56.5	1.54	1.44
335	.71	4.96	5.17	58.1	1.81	1.69

TABLE II

MILLIEQUIVALENTS OF AMMONIA, AMINO NITROGEN AND FREE AMINO ACIDS PER GRAM OF EGG ALBUMIN PRODUCED BY ACID HYDROLYSIS AT 45°. ALSO SHOWN ARE THE PERCENTAGES OF PEPTIDE BONDS HYDROLYZED

Time in hours	NH_3	Amino nitrogen: Corrected for NH_3	Amino nitrogen: Corrected for NH_3 and proline	Amino nitrogen: Per cent. hydrolysis	Free amino acids: Uncorrected	Free amino acids: Corrected for aspartic acid
0.5	0.03	..	..	..	...	...
1.0	.10	0.51	0.53	6.0	0.071	0.066
1.5	.15	.57	.59	6.6	...	...
2.0	.20	.81	0.84	9.5	.038	.035
3.0	.30	1.00	1.04	11.7	.140	.131
3.1	.32	1.06	1.11	12.5	.102	.096
4.0	.37	1.14	1.19	13.4	.179	.167
6.1	.50	1.66	1.73	19.4	.236	.221
10.0	.62	1.93	2.01	22.6	.338	.316
24.0	.70	3.20	3.14	35.3	.812	.760
30.5	.70	3.76	3.92	44.0	.965	.903
48.0	.65	4.51	4.70	52.9	1.38	1.29
77.0	.68	5.13	5.35	60.0	1.88	1.76
103.0	.72	5.54	5.77	64.8	2.60	2.43
127.0	.69	5.60	5.84	65.5	2.75	2.57
145.0	.72	5.68	5.92	66.5	...	...

TABLE III

MILLIEQUIVALENTS OF AMMONIA, AMINO NITROGEN AND FREE AMINO ACIDS PER GRAM OF EGG ALBUMIN PRODUCED BY ACID HYDROLYSIS AT 60°. ALSO SHOWN ARE THE PERCENTAGES OF PEPTIDE BONDS HYDROLYZED

Time in hours	NH_3	Amino nitrogen: Corrected for NH_3	Amino nitrogen: Corrected for NH_3 and proline	Amino nitrogen: Per cent. hydrolysis	Free amino acids: Uncorrected	Free amino acids: Corrected for aspartic acid
0.30	0.12	..	..	..	0.050	0.047
.50	.17	0.85	0.88	9.9	.056	.052
.75	.29	0.95	0.99	11.1	.118	.110
1.00	.36	1.35	1.41	15.8	.087	.081
1.50	.48	1.83	1.91	21.4	.206	.193
2.00	.57	2.11	2.20	24.7	.224	.210
2.50	.59	2.30	2.39	26.8	.330	.319
3.00	.63	2.62	2.73	30.7	.387	.362
4.08	.65	2.94	3.06	34.4	.516	.483
5.00	.66	3.24	3.37	37.8	.610	.571
6.00	.68	3.53	3.68	41.4	.718	.672
7.00	.68	3.88	4.04	45.4	.855	.800

(2) Chibnall, Reese and Williams, *Biochem. J.*, **37**, 354 (1943).
(3) Kendrick and Hanke, *J. Biol. Chem.*, **117**, 161 (1937).
(4) Irving, Fontaine and Samuels, *Arch. Biochem.*, **4**, 347 (1944).
(5) Calvery, *J. Biol. Chem.*, **94**, 613 (1931).
(6) Van Slyke, MacFadyen and Hamilton, *ibid.*, **141**, 627 (1941).
(7) Frost and Heinsen, *ibid.*, **161**, 517 (1945).
(8) Chibnall, *Proc. Roy. Soc. (London)*, **B131**, 152 (1942).

the isoelectric heat coagulation is more nearly a measure of the intact protein present in the hydrolysate than is trichloroacetic acid precipitation.

TABLE IV

PER CENT. OF THE TOTAL NITROGEN IN THE FILTRATE AFTER PRECIPITATION BY 2.67% TRICHLOROACETIC ACID (TCA) AND BY ISOELECTRIC HEAT COAGULATION (IHC)

30° Hydrolysis			45° Hydrolysis			60° Hydrolysis		
Time in hours	% Nitrogen TCA	IHC	Time in hours	% Nitrogen TCA	IHC	Time in hours	% Nitrogen TCA	IHC
2	8.27	8.64	0.5	7.46	10.10	0.25	18.70	..
4	21.70	25.50	1.0	22.65	31.65	.316	24.40	..
6	...	39.80	1.5	38.50	45.00	.50	43.7	50.0
8	...	51.70	2.0	47.50	55.40	.75	61.2	67.6
9	49.20	...	3.0	67.30	73.30	1.00	71.3	75.9
10	...	62.0	4.0	72.30	78.20	1.50	81.3	80.5
24.5	78.50	...	6.0	...	90.6	2.00	89.5	90.7
			6.1	81.40	...			

Discussion

The liberation of ammonia follows the kinetics of a first order reaction, and the rate constants for this reaction were 0.039, 0.22 and 0.79 mole per hour per mole at 30, at 45 and at 60°, respectively. These rates were proportional to 0.72, 0.72 and 0.71 millimole ammonia per gram of protein at 30, at 45 and at 60°, respectively. The values compare favorably with that reported by Chibnall.[4] The energies of activation were 22,100 calories and 17,700 calories for the temperature ranges 30–45 and 45–60°, respectively.

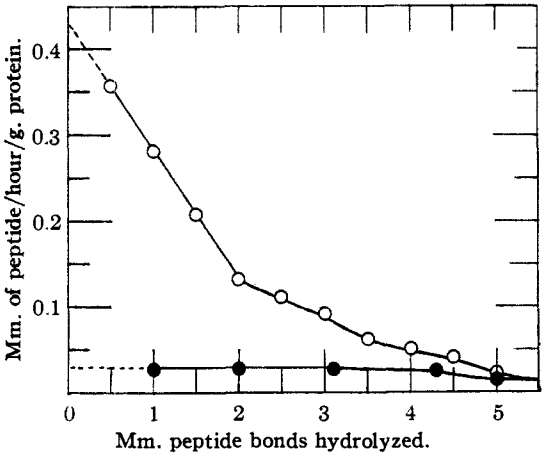

Fig. 1.—Rates of hydrolysis in millimoles of peptide bonds per hour per gram of protein. Open circles represent total peptide bonds and filled circles the liberation of free amino acid, 45° hydrolysis.

For a molecular weight of 45,000 for egg albumin, there are about 400 peptide bonds per molecule and, accordingly, there are potentially a large number of simultaneous reactions to consider. This is a complex problem, and its complete solution must await the future. It is not surprising that none of the measures of reaction velocity, with the exception of the ammonia liberation, follows with exactness any simple kinetic relation, and we do not regard speculation on the order of these reactions as profitable at the present.

The peptide bonds hydrolyzed and the amino acids liberated were plotted against the time of hydrolysis and the rates of rupture of the peptide bonds obtained from the slopes of the curves as a function of the bonds split. Shown in Fig. 1 is a plot of these rates at 45° against the extent of hydrolysis. The rates at 30° and at 60° exhibit the same qualitative behavior as is shown in Fig. 1.

From Figure 1, it appears that there is a fast reaction whose rate is 6 to 7 times greater than the slow reaction which accompanies it, and that the fast reaction is exhausted after a fraction of the peptide bonds have been hydrolyzed.

As a measure of the initial rates of reaction, we have determined the slopes of the tangents drawn from the origin for the various amount–time curves. From these rates we have calculated the ratio of the initial rate of hydrolysis of total peptide bonds and the initial rate of liberation of amino acids to the initial rate of destruction of protein as judged by isoelectric heat coagulation. We have calculated the energies of activation from these initial rates. These results are shown in Table V.

The average ratio of the initial rate of hydrolysis of peptide bonds to initial rate of destruction of protein is 56.5, and the corresponding ratio for the rate of liberation of amino acids is 4.58. When the intact protein is measured by the trichloroacetic acid precipitation, the total peptide ratio is about 70 and the free amino acid ratio is about 6.

Shown in Fig. 2 is a plot of the number of millimoles of peptide bonds hydrolyzed per gram of egg albumin against the fraction of the protein hydrolyzed. It will be noted that the experimental points lie on lines which apparently tend to converge into a single straight line during the first part of the reaction. As the reactions proceed toward completion, the curves swing upward rather sharply. Expressing the slope of the linear portion of the curve in peptide bonds hydrolyzed per gram molecular weight of egg albumin, we find

TABLE V

INITIAL RATES OF HYDROLYSIS IN MILLIMOLES PER HOUR PER GRAM OF PROTEIN, THE RATIO OF INITIAL RATES TO THE INITIAL RATE OF DESTRUCTION OF PROTEIN AND THE ENERGIES OF ACTIVATION

Temp., °C.	Free amino acids			Total peptide			Protein	
	Rate	Ratio to protein	Energy of activation	Rate	Ratio to protein	Energy of activation	Rate	Energy of activation
30	0.0066	4.55		0.074	50.7		0.00146	
45	.0294	4.23	19,100	0.43	61.1	22,500	.00695	20,000
60	.120	4.95	19,700	1.40	57.6	16,700	.0241	17,500

that 55 peptide bonds and 4.3 free amino acids are liberated for each egg albumin molecule destroyed. These values compare favorably with those given in Table V.

As can be seen in Table V the energies of activation for the hydrolysis of peptide bonds, the liberation of free amino acids and the destruction of protein are all of the same order of magnitude, and the conclusion is drawn that the destruction of the protein molecule is initiated by the hydrolysis of a single peptide bond, and the simultaneous activation of two or more peptide bonds is not necessary. It is to be noted that while the energy of activation for the liberation of amino acids is substantially constant over the temperature interval, the energy required for the activation of bonds leading to the destruction of the protein as well as the energy involved in the hydrolysis of total peptide bonds decreases significantly with increasing temperature, reflecting the existence of a complex reaction. Probably of great significance for the problem of hydrolysis of peptide bonds is the resonance energy of this bond. It can be estimated from bond distances that the peptide bond has about 45% double bond character and the activation of this bond, no doubt, blocks the resonance and weakens the bond by the extent of the resonance energy. It should be noted that the hydrolysis of a peptide bond is an exothermic reaction[9] yielding between 2,000 and 4,000 calories per mole. Whether or not the release of this energy would be sufficient to start an energy chain is not known.

In light of the above results and discussion, we suggest that the most reasonable interpretation of the acid hydrolysis of egg albumin is as follows: There exist two classes of peptide bonds in the protein as far as ease of hydrolysis is concerned. About 56 peptide bonds out of the 400 in the protein molecule can be hydrolyzed rapidly. The fragments produced by this initial split of the molecule are then further hydrolyzed, but at much slower rates. The rate at which a peptide bond is split is probably determined by the nature of the amino acid residues next to this bond.

(9) Haugaard and Roberts, THIS JOURNAL, **64**, 2664 (1942).

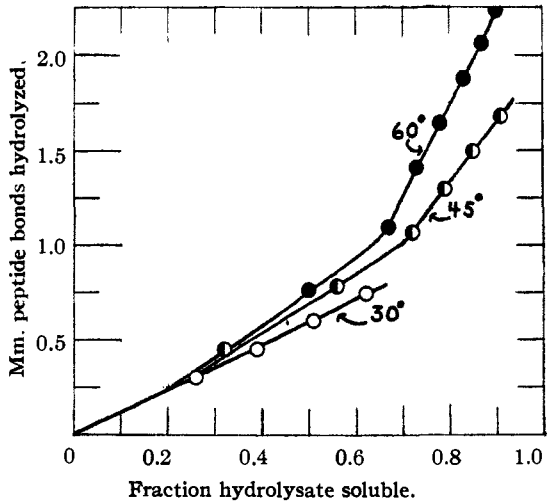

Fig. 2.—Millimoles of peptide bonds hydrolyzed per gram of egg albumin plotted against the fraction of protein not heat coagulable at the isoelectric point. Open circles 30° hydrolysis, half circles 45° hydrolysis and filled circles 60° hydrolysis.

Acknowledgment.—It is a pleasure to acknowledge the generous support afforded this research by Corn Products Refining Company.

Summary

1. The hydrolysis of egg albumin by hydrochloric acid has been studied at 30, at 45 and at 60°.

2. The amino nitrogen, the free amino acids and the ammonia liberated have been determined. The material soluble in trichloroacetic acid as well as the amount which cannot be heat coagulated at the isoelectric point have been measured.

3. The hydrolysis of a protein is not a simple kinetic process. About 56 of the peptide bonds in an egg albumin molecule are rapidly hydrolyzed, but the remaining bonds are hydrolyzed at a much lower rate.

CHICAGO 11, ILLINOIS RECEIVED DECEMBER 6, 1947