[CONTRIBUTION FROM THE CHEMICAL LABORATORY, UNIVERSITY OF CALIFORNIA, SOUTHERN BRANCH]

THE LIBERATION OF CARBON DIOXIDE, AMMONIA AND AMINO NITROGEN FROM CASEIN BY ACID HYDROLYSIS

BY MAX S. DUNN

RECEIVED JUNE 1, 1925 PUBLISHED OCTOBER 6, 1925

Extensive studies upon the rates of liberation of ammonia and amino nitrogen by the hydrolysis of proteins have been made[1] but the investigation of carbon dioxide, another product of the hydrolytic cleavage of proteins, is incomplete.

That carbon dioxide is a product of protein hydrolysis was first reported by Thiele[2] in 1867. More recently Habermann and Ehrenfeld,[3] Mörner,[4] Lippich,[5] and Johnson[6] have studied the carbon dioxide obtained by the acid and alkaline hydrolysis of proteins. However, the rate of liberation of this product when proteins are hydrolyzed needs further investigation.

The object of this study was to measure the rate at which carbon dioxide is liberated from a protein by acid hydrolysis and to compare the rates at which carbon dioxide, ammonia and amino nitrogen are cleaved by acid hydrolysis from the same samples of a protein.

Experimental Part

Casein was prepared according to the procedure of Van Slyke and Bosworth,[7] the treatment with ammonium oxalate being omitted.

The acid hydrolysis of casein was accomplished under the following uniform conditions. Exactly 5 g. of air-dried casein was emulsified with 50 cc. of distilled water. To this emulsion was added 50 cc. of 0.1 *N* sodium hydroxide solution and the mixture was allowed to stand, during occasional stirring, until practically complete solution of the casein had occurred. The casein solution was transferred to a 1-liter Pyrex flask and to it added 25 cc. of 36 *N* sulfuric acid dissolved in enough distilled water to bring the total volume of liquid in the flask to 250 cc. The contents of the flask were at once thoroughly mixed by shaking. Hydrolysis was obtained by refluxing on an electric plate for intervals of from five to 30 hours. In all cases excepting for the 24- and 30-hour periods, hydrolysis was continuous. Hydrolysis time was considered to begin when the solutions reached the boiling point.

The carbon dioxide from the control solution and that evolved during hydrolysis of the casein were collected in each case in 0.1 *N* barium hydroxide solution contained in three traps connected in series to the top of the reflux condenser. To insure complete removal of this product, air freed from carbon dioxide and ammonia was allowed to

[1] See Vickery [*J. Biol. Chem.*, **53**, 495 (1922)] for a review of papers upon the rates of liberation of ammonia and amino nitrogen by the hydrolysis of proteins.

[2] Thiele, *Chem. Zentr.*, 12 (new ser.), 385 (1867).

[3] Habermann and Ehrenfeld, *Z. physiol. Chem.*, **30**, 453 (1900).

[4] Mörner, *ibid.*, **34**, 207 (1901–02).

[5] Lippich, *ibid.*, **90**, 441 (1914).

[6] Johnson, *Chem. News*, **113**, 127 (1916).

[7] Van Slyke and Bosworth, *J. Biol. Chem.*, **14**, 203 (1913).

pass through the experimental and control solutions and through the barium hydroxide solution in the traps at the rate of one or two bubbles per second. At the end of hydrolysis the unneutralized barium hydroxide was washed from the traps with distilled water and, without removing the precipitated barium carbonate, was titrated with 0.1 *N* sulfuric acid, using phenolphthalein as an indicator. This method was used by Constantino[8] and it proved to be satisfactory in the present work.

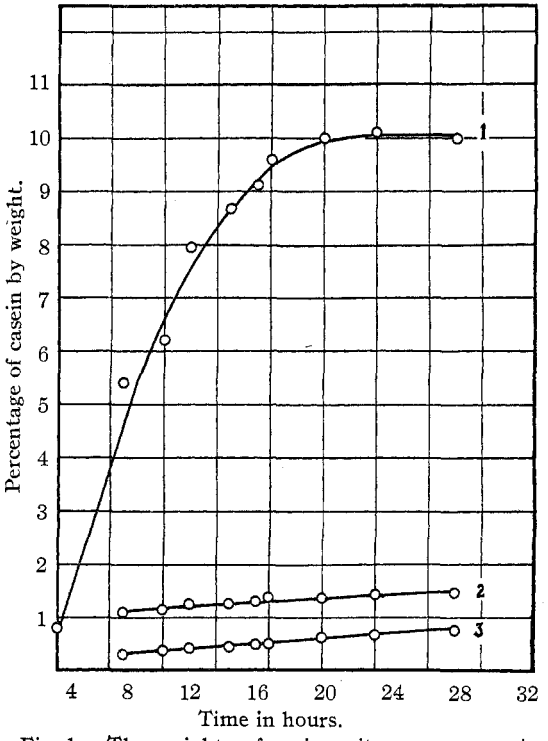


Fig. 1.—The weights of amino nitrogen, ammonia and carbon dioxide liberated from 5g. samples of casein and calculated as percentage of casein are plotted as the ordinate against the time in hours. 1, Amino nitrogen; 2, ammonia; 3, carbon dioxide.

It was found, within the experimental error, that barium hydroxide could be recovered from the three traps by washing with distilled water until the washings failed to give a color with phenolphthalein. When 80 minutes was the time of heating and aspiration, the carbon dioxide liberated from a sample of pure sodium carbonate was quantitatively retained by the barium hydroxide in the three traps.

The ammonia and amino nitrogen of the acid hydrolysates of casein were determined by the methods of Van Slyke.[9]

For each hydrolysis 5.00g. samples of casein, containing 14.51% of nitrogen, were used. The carbon dioxide from the experimental flask was caught in 35.0 cc., and from

[8] Constantino, *Atti accad. Lincei*, [II] **28**, 118 (1919); *C. A.*, **14**, 1465 (1920).

[9] Van Slyke, *J. Biol. Chem.*, **10**, 15 (1911); **9**, 185 (1911).

TABLE I

CARBON DIOXIDE, AMMONIA AND AMINO NITROGEN LIBERATED FROM CASEIN BY SULFURIC ACID HYDROLYSIS

Hydrolysis time Hrs.	Carbon dioxide in terms of Casein %	Carbon dioxide in terms of Total nitrogen %	Ammonia in terms of Casein %	Ammonia in terms of Total nitrogen %	Amino nitrogen in terms of Casein %	Amino nitrogen in terms of Total nitrogen %
0	..	..	..	..	0.80	5.7
5	0.31	2.01	1.10	7.6	5.48	36.2
8	.39	2.70	1.18	8.1	6.25	44.1
10	.41	2.81	1.24	8.5	8.00	55.1
13	.42	2.87	1.28	8.8	8.75	60.0
15	.51	3.48	1.32	9.1	9.20	63.4
16	.53	3.53	1.36	9.4	9.52	67.8
20	.64	4.41	1.42	9.8	10.10	70.0
24	.71	4.89	1.46	10.0	10.20	70.3
30	.81	5.58	1.52	10.5	10.10	70.0

the control flask in 15.0 cc. of 0.1 *N* barium hydroxide solution. Three traps were used in each case to contain the barium hydroxide. For the determination of ammonia, samples of acid hydrolysate equivalent to 0.5 g. of casein were used and distillation at 35 mm. for 60 minutes was carried out. The reaction time of 20 minutes, used in all cases for the determination of amino nitrogen,[10] was that shown to be ample for the observed temperatures.

Discussion

Amino nitrogen from the acid hydrolysis of casein becomes constant at approximately the level found by Van Slyke[11] and by Osborne and Guest.[12] The time for complete hydrolysis, about 20 hours, is less than that reported by either of the authors mentioned. However, it has been shown by Vickery[1] that the time for complete hydrolysis varies with the character and concentration of the acid used. Also, in the present work the rate of hydrolysis may have been influenced by the current of air used to effect removal of the carbon dioxide.

The results of Pittom[13] and those of Vickery[1] show that ammonia increases rapidly during the first hours of hydrolysis, while according to Denis ammonia values remain constant during hydrolysis of from 15 to 70 hours with various concentrations of either sulfuric or hydrochloric acid. Values for the ammonia obtained from the acid hydrolysis of casein by certain other workers are not in harmony with those of Denis. By acid hydrolysis for 10 to 48 hours Van Slyke[11] finds that "ammonia reaches no maximum, but increases the longer hydrolysis is continued." The values of Henriques and Gjaldbäk[14] indicate an increasing liberation of ammonia when casein is hydrolyzed under pressure. In the present

[10] Dunn and Schmidt, *ibid.*, **53,** 401 (1922).

[11] Van Slyke, *ibid.*, **12,** 295 (1912).

[12] Osborne and Guest, *ibid.*, **9,** 333 (1911).

[13] Pittom, *Biochem. J.*, **8,** 157 (1914).

[14] Henriques and Gjaldbäk, *Z. physiol. Chem.*, **67,** 8 (1910).

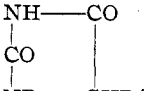
study ammonia was found to increase from 1.10 to 1.52% of casein during hydrolysis of from five to 30 hours.

Johnson[6] obtained 0.35% of the weight of casein as carbon dioxide by acid hydrolysis. Lippich[5] did not study casein but reported from 0.18 to 0.37% of carbon dioxide obtained from various proteins by acid hydrolysis for eight to 27 hours. The results of the present experiments indicate that carbon dioxide from the acid hydrolysis of casein increases from 0.31 to 0.81% of the casein during five to 30 hours.

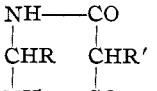
What is the source of the carbon dioxide and the ammonia that result from acid hydrolysis of casein? As is well known, a large part of the ammonia comes from decomposition of the amides of the dicarboxylic acids, glutaminic and aspartic. Decomposition of amino acids has been suggested as a possible explanation of the extra ammonia but there is considerable evidence,[9,14,15] that many of the amino acids of casein are stable in acid solution at the boiling temperature. Osborne, Leavenworth and Brautlecht[16] state that "none of the amino acids which are known products of protein hydrolysis yield any ammonia by long boiling with hydrochloric acid and cannot, therefore, be considered as contributing any part of the ammonia formed from proteins by acid hydrolysis."

By inspection of the curves and the table it is seen that the ammonia liberated at the beginning of the hydrolysis is in excess of the carbon dioxide. This portion of the ammonia may be considered as being formed from amide decomposition. Because amino nitrogen apparently becomes practically constant, while both ammonia and carbon dioxide reach no maximum but increase at approximately the same rates, it would seem that at least a part of the last two substances may come from the breakdown of some complex structure present in the protein molecule. According to current conceptions,[5,6,15,17] protein structure may include such groups as uramino acids, uramino acid anhydrides (hydantoins) and peptide anhydrides (cyclic diacipiperazines).

Of the groups that have been suggested, the following structures would seem to represent the most likely sources of carbon dioxide and of ammonia not otherwise accounted for.



I



II

[15] Andersen and Roed-Müller, *Biochem. Z.*, **70**, 442 (1915). Hoffman and Gortner, THIS JOURNAL, **44**, 341 (1922).

[16] Osborne, Leavenworth and Brautlecht, *Am. J. Physiol.*, **23**, 180 (1908–09).

[17] Johnson and Daschavsky, *J. Biol. Chem.*, **62**, 197 (1924). Pictet and Cramer, *Helvetica Chim. Acta*, **2**, 188 (1919); *C. A.*, **13**, 1076 (1919). Abderhalden, *Z. physiol. Chem.*, **128**, 119 (1923); **131**, 281, 284 (1923). Abderhalden and Komm, *ibid.*, **139**, 147, 181 (1924).

According to Johnson and Bates[18] the products of hydrolysis of Structure I would be carbon dioxide, ammonia and R—NH—CHR'—COOH.

Summary

1. The amino nitrogen, carbon dioxide and ammonia liberated from casein by sulfuric acid hydrolysis during intervals of from five to 30 hours have been determined.

2. Amino nitrogen from the acid hydrolysis of casein was found to become constant after about 20 hours.

3. The total ammonia liberated by acid hydrolysis is in excess of the carbon dioxide at the beginning, but after five hours these substances appear to be evolved at approximately the same rates.

4. Structures are presented representing the possible sources of the carbon dioxide and of part of the ammonia obtained by the acid hydrolysis of casein.

LOS ANGELES, CALIFORNIA

THE DIMETHYLTIN GROUP AND SOME OF ITS REACTIONS

BY CHARLES A. KRAUS AND WILLARD N. GREER

RECEIVED JUNE 6, 1925 PUBLISHED OCTOBER 6, 1925

Many of the less electropositive elements exhibit marked amphoteric properties.[1] With the exception of hydrogen, these elements have valences greater than unity, and in case part of these valences are satisfied by hydrocarbon groups, the number of amphoteric valences is less than the maximum. An element of valence n yields a univalent group $R_{n-1}M^n$. Such a group may appear either as positive or as negative ion, or as neutral group.[2] Common examples of groups of this type are triphenylmethyl, trimethyltin, triethyl-lead, etc.

We should also expect to obtain divalent groups of the type $R_{n-2}M^n$, which should act as divalent positive or negative ions and should be capable of existing in the neutral state. The diphenyltin group, $(C_6H_5)_2Sn$,[3] has been obtained in the neutral condition, as has also the diethyltin group, $(C_2H_5)_2Sn$.[4] Compounds of the type R_2SnX_2, in which X is a halogen, are well known and their behavior in solution gives indication of the existence of a divalent cation, R_2Sn^{++}.[5] Nothing is known thus far of compounds of the type N_2SnR_2, where N is a strongly positive element and SnR_2 acts as a divalent anion. It is the purpose of the present investiga-

[18] Johnson and Bates, THIS JOURNAL, **38**, 1087 (1916).

[1] Kraus, *ibid.*, **44**, 1216 (1922); *Trans. Am. Electrochem. Soc.*, **45**, 175 (1924).

[2] Kraus, THIS JOURNAL, **46**, 2196 (1924).

[3] Krause, *Ber.*, **53**, 173 (1920).

[4] Frankland, *Ann.*, **85**, 338 (1853).

[5] Zelinsky and Krapiwin, *Z. physik. Chem.*, **21**, 46 (1896).