far prepared differentiates them from the aromatic azoxy compounds. However, this may be due to absence of conjugation. As a ring structure for the azoxy group in aliphatic compounds is not inconsistent with the data on molecular refraction, one must admit of this possibility, although it requires the assumption of a special parachor value for this ring.

The author wishes to thank Professor J. G. Aston for suggesting this problem.

Summary

1. Ethyl α-azoxyisopropyl ketone and α-azoxyisopropyl isobutyl ketone were prepared by reduction of the corresponding *bis*-nitroso compounds with stannous chloride in concentrated hydrochloric acid.

2. The molecular refractions and parachors of three liquid aliphatic azoxy compounds have been determined and yield a constant value for the azoxy groups.

3. There is no evidence that the azoxy group in aliphatic azoxy compounds has the unsymmetrical open chain structure rather than that of a three-membered ring.

STATE COLLEGE, PENNA. RECEIVED JUNE 9, 1938

[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY, UNIVERSITY OF NORTH CAROLINA, AND THE CHICAGO MEDICAL SCHOOL]

Studies on Proteins in Liquid Ammonia. IV. On the Enzymatic Hydrolysis of Proteins Reduced by Metallic Sodium in Liquid Ammonia[1]

BY EVAN W. MCCHESNEY[2] AND RICHARD G. ROBERTS

Previous papers of this series[3] have shown that, of the reactions of proteins in liquid ammonia, perhaps the most interesting is that with metallic sodium. The proteins behave as acids, hydrogen gas is liberated, and sodium salts are formed. From these salts the sodium can be displaced by the addition of ammonium salts such as the chloride or sulfate. During the reaction with sodium reducible groups in the proteins, such as the —S—S— linkages in cystine, undergo reduction. This reaction has been demonstrated by du Vigneaud, Audrieth and Loring.[4]

Goddard and Michaelis[5] have shown that if keratins, such as wool, are treated with sodium thioglycolate in aqueous solution, they undergo reduction of the —S—S— linkages of the cystine residues, forming "kerateines" which are digestible by pepsin and trypsin, even if the resultant —SH groups are reoxidized during the course of the isolation of the kerateine. They postulate that the —S—S— groups act as "very firmly established cross links uniting the elementary fibers of polypeptide chains," and that reduction of the —S—S— groups destroys the fibrous pattern of the protein. Routh[6] has confirmed these observations and has extended them to show that powdered wool, produced by prolonged grinding of wool in a ball mill, is digested about half as fast as casein under comparable conditions.

It occurred to us that it would be of interest to determine whether proteins subjected to reduction by metallic sodium in liquid ammonia are altered with respect to their digestibility by enzymes, and to study proteins of several types, including silk fibroin. This protein is an albuminoid and has been shown[3a] to react rapidly with sodium in liquid ammonia to give a product soluble (as the free acid) in both ammonia and water. Its behavior should be of interest by comparison to wool, since the presence of cystine in the molecule has not been demonstrated.

In this work five substances have been studied: peptone, a substance previously digested by pepsin; egg albumin, a protein not readily digested by trypsin; casein, a protein readily digested by trypsin; wool, an albuminoid containing cystine; and silk fibroin, an albuminoid not containing cystine.[7]

Materials.—Commercial Witte's peptone, egg albumin (Merck impalpable powder), casein (Hammarsten), washed sheep's wool, and silk fibroin (prepared from silk noils supplied by the Cheney Bros. Silk Mills, South Man-

(1) Presented at the Dallas meeting of the American Chemical Society, April 19, 1938.

(2) Present address: Winthrop Chemical Co., Rensselaer, N. Y.

(3) (a) McChesney and Miller, THIS JOURNAL, **53**, 3888 (1931); (b) Miller and Roberts, *ibid.*, **56**, 935 (1934); (c) Roberts and Miller, *ibid.*, **58**, 309 (1936).

(4) Du Vigneaud, Audrieth and Loring, *ibid.*, **52**, 4500 (1930).

(5) Goddard and Michaelis, *J. Biol. Chem.*, **106**, 605 (1934); **112**, 361 (1935).

(6) Routh, *J. Biol. Chem.*, **123**, Proc., civ (1938).

(7) Analysis by the Sullivan method showed that cystine is entirely lacking in silk fibroin.

chester, Conn.). The trypsin used was a commercial product (Digestive Ferments Co., Trypsin, 1:110).

Methods.—For the reduction experiments, 10 g. of the protein (dried for forty-eight hours at 80°) was suspended in about 200 cc. of liquid ammonia (dried over sodium) and 3 g. of metallic sodium was added. The reaction was allowed to proceed for two hours, then the equivalent amount of ammonium chloride (7 g., C. P. grade) was added. The ammonia was allowed to evaporate through a mercury seal, and the product was collected. It was then ground in a mortar, and dried in a desiccator over concentrated sulfuric acid until no odor of ammonia could be detected.

Half of the product was now dissolved, or suspended, in 100 cc. of water. A portion of 1 cc. was removed, and the amount of acid or alkali needed to bring the whole solution to a reaction just faintly acid to phenolphthalein was determined and added. Trypsin, 0.2 g., was added, and enough water to make a total volume of 200 cc. Amino nitrogen was immediately determined by the method of Van Slyke, after which toluene was added as a preservative, and the solution was incubated at 37°. Amino nitrogen was determined at intervals of one, three, seven, and fourteen days thereafter. Total nitrogen in solution was determined by the Kjeldahl method at the end of the fourteenth day.

The control experiments were of two types. In one type the native protein, 5 g., was brought into solution at *p*H 8, 2.5 g. of sodium chloride (C. P.) and 0.2 g. of trypsin were added, followed by enough water to make a total volume of 200 cc. The digestion and analyses were run as before. In the second type of control (referred to in the table as the "NH_3 control"), metallic sodium, 3 g., was dissolved in about 200 cc. of liquid ammonia and the exact equivalent of ammonium chloride (as judged by the disappearance of the blue color) was added immediately. Then the protein, 10 g., was added and the ammonia was allowed to evaporate. The product was powdered, dried over sulfuric acid, and analyzed as before.

Experimental Data.—The results are shown most conveniently in the form of tables.

TABLE OF RESULTS

	Amino nitrogen					
Days	Mg./cc.	% total sol. N	Mg./cc.	% total sol. N	Mg./cc.	% total sol. N
	Peptone		Reduced peptone		Casein	
0	0.63	18	0.71	21	0.34	9
1	1.32	37	1.38	39	1.54	40
3	1.54	43	1.42	41	1.70	44
7	1.63	46	1.42	41	1.84	48
14	1.63	46	1.52	43	1.86	49
Total N in soln. end digest, mg./cc.	3.53		3.44		3.83	
	Reduced casein		Reduced silk fibroin		NH_3 control, Silk fibroin	
0	0.10	11	0.50	13	0.08	7
1	.27	30	.84	22	.24	21
3	.28	30	.93	25	.25	22
7	.29	31	1.09[8]	29	.30	27
14	.35	38	1.00	27	.33	29
Total N in soln. end digest, mg./cc.	0.92		2.77		1.13	

(8) Evidently analytical error.

	"Egg albumin"		NH_3 control, "Egg albumin"		Reduced "Egg albumin"	
0	0.07	3	0.10	4	0.31	11
1	.13	5	.32	13	.45	16
3	.20	7	.46	18	.55	19
7	.25	9	.73	28	.64	22
14	.32	11	1.05	41	.66	23
Total N in soln. end digest, mg./cc.	2.85		2.56		2.87	

	Reduced wool		NH_3 control, wool	
0	0.11	4	0.01	4
1	.58	22	.05	22
3	.71	27	.06	27
7	.86	32	.07	32
14	.92	35	.07	32
Total N in soln. end digest, mg./cc.	2.64		0.22	

Discussion of Results

It is apparent that reduction of small molecules of the order of polypeptides, as in peptone, does not affect their digestibility by trypsin appreciably. Any changes such as tautomerization which occur during the reaction with sodium, would be expected to be reversed when the sodium is neutralized and the product is dissolved in water. Furthermore, the treatment with sodium would not be expected to alter significantly the structure of the molecules, their solubility, or the degree of their dispersion. For casein the results are somewhat difficult to interpret. The protein becomes more insoluble in water (at *p*H 8) as a result of the treatment with sodium in liquid ammonia, or with liquid ammonia alone, and its digestibility is decreased markedly in spite of the fact that the amount of the enzyme is greater relative to the amount of protein in solution. This would seem to indicate that the treatment in some manner causes the molecule to become more compact: decreases the extent of dispersion.

Egg albumin seems to be partly denatured by treatment with liquid ammonia since it is not completely soluble in water after having been suspended in ammonia. However, the treatment seems to open up the molecule in some way, since the increase in digestibility is very striking. The increase in the digestibility of reduced albumin probably, therefore, can be attributed to the action of the ammonia, not the sodium. The reason for the difference between the behavior of the two albumins (one reduced, one not, both ammonia-treated) is not apparent.

The amount of digestion of native wool and silk fibroin by trypsin is of course practically negligible. Both ot these become digestible when treated with sodium in liquid ammonia, the results for wool being particularly striking. In the case of silk fibroin, it is evident that some dispersion occurs in both the reduced and the ammonia-control samples. The rate of digestion of the material actually in solution in the water is practically the same in both cases. The greater dispersion of the reduced material is probably due to some alteration in structure during the reaction with sodium. Sufficient information on the structure of silk fibroin is not available to make it possible to suggest what this alteration may be. However, if the molecular weight is as great as postulated by Bergmann and Niemann[9] (217,000), then it would seem as if some of the cross ties between the polypeptide chains must be ruptured. Since there are no —S—S— linkages, the rupturing would have to be (on the basis of present knowledge) by a reaction of the nature of ammonolysis.

The results with wool would be anticipated, perhaps, from the work of Goddard and Michaelis. Wool is only slightly dispersed in ammonia, and the product of the ammonia treatment is only slightly dispersed in water. However, that material which is dispersed digests about as rapidly as the reduced wool. The wool which is subjected to reduction is much more highly dispersed in water (not completely, however), probably due to reduction of the —S—S— linkages and consequent rupture of the cross ties between the polypeptide chains, hence digests rather rapidly.

It is worthy of note that in every case in which the protein was treated with sodium, the ratio of amino nitrogen to total nitrogen is increased (except in wool where the figures for the control are too small to be significant). This is strikingly demonstrated in the case of egg albumin where the native protein and the ammonia control each show about 4% of nitrogen in the amino form, while the reduced sample shows 11%. This would seem to indicate that some reaction of the nature of ammonolysis is occurring, with the rupture of some peptide bonds.

Acknowledgment.—We wish to thank Dr. W. D. Block of the University of Michigan for running the cystine analysis on silk fibroin.

Summary

The digestibility (by trypsin) of a number of proteins has been studied after treatment with sodium in liquid ammonia and compared with their behavior in the native state (or after treatment with liquid ammonia alone). Peptone is not affected by the treatment, and the digestibility of casein is decreased. The digestibilities of egg albumin, silk fibroin, and wool are increased by the treatment with sodium in liquid ammonia. Controls treated only with liquid ammonia appear to digest as rapidly as the reduced materials when one considers the smaller amount of the protein dispersed by the ammonia in *the cases of silk fibroin and wool.*

(9) Bergmann and Niemann, *J. Biol. Chem.*, **122**, 581 (1938).

CHAPEL HILL, N. C. RECEIVED MAY 27, 1938