The more important products have been identified, and the approximate proportions in which they were formed have been determined.

DURHAM, NORTH CAROLINA RECEIVED JULY 21, 1939

[CONTRIBUTION FROM THE LAKESIDE LABORATORIES, MILWAUKEE, AND THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY OF THE CHICAGO MEDICAL SCHOOL]

Studies on Proteins in Liquid Ammonia. V. Reaction of Sodium in Liquid Ammonia with Peptones and Related Substances[1]

BY CLEMMY O. MILLER AND RICHARD G. ROBERTS

From previous work[2,3] reported in this series, it is known that certain amino acids, peptides, proteins and related substances are acidic in liquid ammonia. A preliminary report[4] on peptones studied under similar conditions has been made. In this paper we have compared the reactions of Armour's Meat Peptone, Merck's Meat Peptone and Witte's Peptone when metallic sodium is added to them in liquid ammonia by determining the hydrogen evolved from them, and correlating it with their nitrogen content. This method is also used to follow the course of an acid digestion of silk fibroin through several of its peptones, since it reveals the presence of certain compounds and reactive groups that do not affect a Sörensen formol titration or a Van Slyke amino nitrogen determination greatly. It has been noted by others[5] that certain procedures carried out in non-aqueous solvents can reveal groups that do not ordinarily dissociate in aqueous solutions.

Experimental

Method.—The manner of drying the liquid ammonia over sodium and of drying the peptones and related substances has been described previously, as has also been the apparatus for collecting and analyzing the hydrogen.[2,3] The silk peptones were prepared by treating silk fibroin with 70% sulfuric acid at room temperature for one, two, three, four and ten days according to the method described by Morrow[6] for the preparation of four day silk peptones. The synthetic silk peptone was simply a mixture of glycine, alanine and tyrosine made up in the proportions found in silk fibroin.

(1) Some of the data presented here were collected in the laboratories of the Northwestern University Medical School.

(2) C. O. Miller and R. G. Roberts, THIS JOURNAL, **56**, 935 (1934).

(3) R. G. Roberts and C. O. Miller, *ibid.*, **58**, 309 (1936).

(4) R. G. Roberts and C. O. Miller, *Proc. Soc. Exptl. Biol. Med.*, **30**, 821 (1933).

(5) E. J. Cohn, *Ann. Rev. Biochem.*, **4**, 141 (1935).

(6) C. A. Morrow, "Biochemical Laboratory Methods," John Wiley and Sons, Inc., New York, N. Y., 1927.

Discussion

Our work on amino acids showed that a linear curve rising at a forty-five degree angle was obtained when the varying amounts of sodium added were plotted against the hydrogen evolved for one nitrogen equivalent of the substances used. With dipeptides such as glycylglycine or glycyl-*dl*-alanine the curve is linear, but the slope is less than for the amino acids. The curve with proteins can be divided into three segments, one and three roughly paralleling the base line, while segment two roughly parallels the rising curve of the amino acids. Protein curves in general resemble a one-day digestion silk peptone (Fig. 1, curve III). Diketopiperazine gives a curve similar to the proteins except that segment two rises only about 50% as high on the average as the latter. In general the curves of peptones resemble the curve of diketopiperazine. As shown in Fig. 1 Armour's peptone (curve I) gives a smooth curve, while Witte's peptone (curve II) gives sharp segments as if a definite amount of sodium or hydrogen had to be absorbed before a definite quantity of acidic hydrogen could be liberated. For one to one ratios of sodium to nitrogen, Merck peptone is relatively more acidic than either Armour or Witte peptone. Curve III of the one-day digestion silk peptone resembles the curve of silk fibroin; it is still acting like a protein. By the second day, however, the acidity has dropped to that which is characteristic of peptones and the three-day digestion gives a typical peptone curve (IV). By the fourth day the digest has become increasingly acidic again, about equal to that of the one-day digest. The ten-day digest is more acidic than the four-day digest, and gives a curve characteristic of amino acids (V). The ten-day digest seems to be the limit of acidity for a silk fibroin hydrolyzed by sulfuric acid, since a mixture (glycine 53.3%, alanine 31.1% and tyrosine 15.6%), the proportions found in silk fibroin, and

the ten-day digest have the same acidity in liquid ammonia.

The acidic properties of silk fibroin, some of its peptones, and mixtures of its constituent amino acids with themselves and with diketopiperazine can be compared by taking the moles of hydrogen evolved from one nitrogen equivalent of these substances when they are treated with an excess of sodium in liquid ammonia. (For the meaning of above terms, see legend of Fig. 1.) Silk fibroin would start at a high level (0.339) remain at 0.294 for the one-day digest, pass through a minimum for the two-day (0.134) and three-day (0.144) digests and approach a maximum in the four-day (0.320) and in the ten-day (0.359) digests and synthetic mixture (0.358) which is of the same order of acidity as the original silk fibroin (0.339). That other mixtures of amino acids do not evolve the same quantity of hydrogen as the theoretical maximum (0.580) is shown by the equimolar mixture of glycine and tyrosine (0.385). The very marked effect of diketopiperazine in reducing the amount of hydrogen evolved is shown by the equimolar mixture of glycylglycine and diketopiperazine (0.021). That these last two substances tend to form a new compound is made evident by a change in the crystalline form, the solubility and the melting point of the new product. Tyrosine and glycine react in this way also since they do not liberate hydrogen additively when mixed, and likewise form a new compound as described above and before the sodium is added. Another interpretation of these results is that the rings present are more easily reduced by hydrogen in the presence of another acidic substance. However, Roberts and Horvitz have shown recently[7,8] by bio-assay of blood pressure in dogs that adrenalin forms a fairly stable conjugate with glycine in liquid ammonia that will not form at all in water.

If one follows the digestion of a protein by the methods of Sörensen or of Van Slyke, the carboxyl and amino groups would be found to increase even if some diketopiperazine were liberated or were formed during the process. However, in liquid ammonia the acidity as determined by the method described here would not increase, but would actually decrease during digestion if diketopiperazine were liberated or formed during the process of acid digestion or the following amonolysis, since it would combine in liquid ammonia with the amino acids or peptides present. One interpretation of our data would indicate that diketopiperazines are liberated or formed during an acid digestion up to the three-day period, after which they are broken down to form peptides and amino acids.

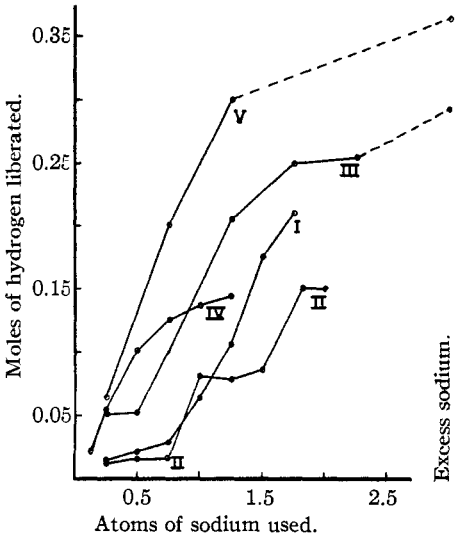

Fig. 1.—Curve I, Armour meat peptone; Curve II, Witte peptone; Curve III, 1-day digestion silk peptone; Curve IV, 3-day digestion silk peptone; Curve V, 10-day digestion silk peptone. In practice 1-g. samples were used, the sodium was added in small pieces and the hydrogen evolved varied from a few cc. to 0.1 l. or more. For convenience in plotting, the sodium is shown in gram atoms (23 g.) and the hydrogen in gram moles (2 g.) for 1 equivalent of nitrogen (14 g.) of the peptones. For a peptone containing 16% nitrogen by the method of Kjeldahl this would represent a sample of 62.5 g. The excess sodium represents an amount necessary to keep the liquid ammonia solution blue after the evolution of hydrogen has ceased. The excess may vary.

The relatively high acidity of native proteins in liquid ammonia is rather puzzling. However, this might be due to the acidity of the peptide hydrogen in long chains, if they are bent or folded in such a way as to bring phenyl or carbonyl groups into the immediate neighborhood of the nitrogen to which the replaceable hydrogen is attached. We did not find the peptide hydrogen of glycylglycine or glycyl-*dl*-alanine to be acidic. It might also come from other groups such as the hydrogen of the tertiary carbon in leucine or the hydrogen of the phenolic group in tyrosine. We have found both leucine and tyrosine to be more acidic in liquid ammonia than glycine or alanine. In his work with hydrocarbons, Wooster[9] found that

(7) R. G. Roberts and H. J. Horvitz, *Am. J. Physiol.*, **119**, 391 (1937).

(8) R. G. Roberts and H. J. Horvitz, *Trans. Illinois State Acad. Sci.*, **31**, 141 (1938).

(9) C. B. Wooster, *Chem. Rev.*, **11**, 1 (1932).

hydrogen attached to a tertiary carbon atom is not replaceable by sodium in liquid ammonia unless at least two phenyl groups are attached to the same carbon. Hydrogen might also be evolved from side chains attached to the longer peptide chains or from diketopiperazines. We regard the variation in acidity as due to the nature of the starting material, and to the conditions of the digestion. We think of both proteins and peptones as being built up of peptides and diketopiperazines with the peptones containing a relatively large amount of diketopiperazines.

Summary

1. A study of the reaction of sodium in liquid ammonia with certain peptones has been made.

2. Peptones are acidic in liquid ammonia, and liberate hydrogen when sodium is added to them.

3. Peptones are more acidic in liquid ammonia than diketopiperazine, but less acidic than proteins or amino acids.

4. Silk peptones prepared by digesting silk fibroin for ten days pass through a minimum acidity on the second and third days.

5. Peptones are more closely related to diketopiperazine than are proteins in their acid reactions in liquid ammonia.

6. Peptones react in liquid ammonia as if they contained relatively more diketopiperazines than do proteins.

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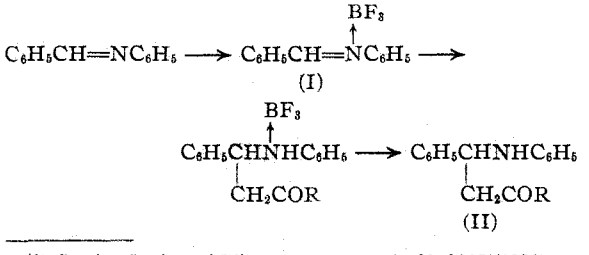
[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Reactions of Anils. II. Addition of Methyl Ketones to Benzalaniline in the Presence of Boron Fluoride

BY H. R. SNYDER, H. A. KORNBERG AND J. R. ROMIG

In connection with a study of the reactions of anils,[1] it became desirable to effect the addition of methyl ketones to these substances. The reaction has been known for several years,[2] but in many cases it is a very slow process. Boron fluoride would be expected[3] to combine with an anil to produce the linkage $>C=N\rightarrow BF_3$, in which the double bond must be highly polarized because of the charge on the nitrogen atom. As a consequence of the increased polarization the complex should undergo addition reactions more readily than the anil. These predictions now have been realized. The coördination compound (I) is obtained readily and its rapid reaction with methyl ketones affords a convenient method of preparing amino ketones corresponding to (II).



(1) Snyder, Levin and Wiley, THIS JOURNAL, **60**, 2025 (1938).

(2) Mayer, *Bull. soc. chim.*, [3] **31**, 953, 985 (1904); [3] **33**, 157, 498 (1905); [4] **19**, 452 (1916).

(3) Kraus and Brown, THIS JOURNAL, **51**, 2690 (1929).

Procedure. Preparation of Benzalaniline–Boron Fluoride.—To a solution of 2.86 g. of benzalaniline in 155 cc. of dry ether was added 2 cc. of boron fluoride etherate. The precipitate which appeared was separated; analysis showed it to be high in boron. The mother liquor, after standing for several hours in a closed flask, deposited yellow needles, m. p. 135–145° (dec.). The product was analyzed for boron by the hydrogen peroxide oxidation method of Snyder, Kuck and Johnson.[4]

Anal. Calcd. for $C_{13}H_{11}NBF_3$: B, 4.35. Found: B, 4.44.

Addition of Methyl Ketones to Benzalaniline–Boron Fluoride.—One and one-half grams of the coördination compound was dissolved in 25 cc. of acetone. The solution warmed slightly. After a few minutes water was added and the mixture was cooled. The colorless needles of 1-anilino-1-phenylbutan-3-one were collected and recrystallized three times from dilute alcohol; yield, 0.5 g., m. p. 87–88°.

Anal. Calcd. for $C_{16}H_{17}ON$: N, 5.86; mol. wt., 239. Found: N, 5.86, 5.90; mol. wt., 251.

Substitution of ethyl methyl ketone for acetone gave 1-anilino-1-phenylpentan-3-one.[2] When the experiments were repeated with benzalaniline instead of the boron fluoride complex the anil was recovered.

For the preparation of amino ketones by this method the isolation of the coördination compound (I) is unnecessary. The reaction is effected more conveniently by adding boron fluoride etherate to a solution of the anil in an excess of the

(4) Snyder, Kuck and Johnson, THIS JOURNAL, **60**, 110 (1938).