sium cyanate were slowly added during constant stirring. After several days' standing the cooled reaction mixture failed to show any separation of product. A few drops of hydrochloric acid were then added to secure the decomposition of all cyanate and the solution subjected to distillation with steam to remove the acetic acid. Upon evaporation of the residuum in flask to small volume there soon appeared a considerable quantity of fine, colorless needles in clusters. Recrystallization of this substance from alcohol gave a pure product melting at 226°. This ethyl-phenyldiketo-cyanidine is readily soluble in glacial acetic acid, fairly soluble in alcohol, acetone, chloroform, benzene, ethyl acetate or water, crystallizing from each; it is slightly soluble in ether and insoluble in ligroin.

Calc. for $C_{11}H_{13}O_2N_3$: C, 60.27; H, 5.98; N, 19.18. Found: C, 60.21; H, 6.19; N, 19.27.

This diketo-sym-triazine gives a flocculent precipitate with silver nitrate. As previously stated, this substance corresponds closely in its properties to those of the methyl keto-triazine of Ostrogovich—a compound prepared by condensing urea with acetylurethane. The compounds are closely similar but in the one only a tetrahydro ring is present, whereas in the case we describe the completely reduced or hexahydro ring is at hand.

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THE FORMS OF NITROGEN IN PROTEIN-FREE MILK.[1]

BY CORNELIA KENNEDY.

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Since the use of so-called protein-free milk as an adjuvant to isolated proteins to furnish the inorganic elements of the diet[2] has been questioned[3] because of the unknown nature of its nitrogen content, it seemed advisa-

[1] Published with the approval of the Director as Paper No. 148, Journal Series, Minnesota Agricultural Experiment Station. Presented before the Division of Biological Chemistry of the American Chemical Society, at the Cleveland meeting, September, 1918.

[2] T. B. Osborne, and L. B. Mendel, "Feeding Experiments with Isolated Food-Substances," *Carnegie Inst. Publication* **156**, 2, 82 (1911); "Relation of Growth to Diet," *J. Biol. Chem.*, **15**, 318 (1913); "Influence of Butter-Fat on Growth," *Ibid.*, **16**, 426 (1913); "Influence of Fats on Growth," *Ibid.*, **17**, 404 (1914); "Protein Minima for Maintenance," *Ibid.*, **22**, 256 (1915).

[3] E. V. McCollum, N. Simmonds and W. Pitz, "Is Lysine the Limiting Amino Acid in the Proteins of Wheat Maize or Oats?" *Ibid.*, **28**, 485 (1916); J. C. Drummond, "The Growth of Rats upon Artificial Diets Containing Lactose," *Biochem. J.*, **10**, 91 (1916); H. H. Mitchell and R. A. Nelson, "Preparation of Protein-Free Milk," *J. Biol. Chem.*, **23**, 461 (1915).

ble to determine the nature and complexity of any nitrogen present in it, in peptid or protein form. It is evident that in a diet in which the efficiency of a protein is to be determined, the presence of unknown nitrogenous material amounting to 02.1 g. in each 100 g. of food[1] must be taken into consideration. Although this quantity is small and there are proteins which are known to be inefficient when fed with this amount of protein-free milk, it may, however, be the factor determining success when fed with other proteins.

Experimental.

The samples of protein-free milk used in the following determinations were obtained by precipitating approximately 40 liters of fresh centrifuged milk diluted with 8 liters of distilled water with conc. hydrochloric acid, care being taken not to pass by more than a few cc. the point at which the curd began to appear: A clear filtrate was generally obtained by filtering through several thicknesses of fine cheesecloth. A small portion was always tested with acid for further precipitation but no precipitate was obtained in any case. The filtrate was then boiled for $^1/_2$ minute, cooled and filtered by suction through a paper pulp filter. The filtrate was water clear. This was carefully neutralized with sodium hydroxide and evaporated to dryness at a temperature of 60–70°. The pale yellow mass thus obtained was ground to a very fine powder in a ball-mill. Five samples of protein-free milk were thus prepared from milk obtained at different times from the same herd.

In the following determinations 100 g. of material was dissolved in water and made to a volume of 1000 cc. For the most part samples representing 10 g. of protein-free milk were used for each determination, but as in a few cases it was necessary to use samples representing 5 g. all calculations were made on a 5 g. basis.

As a preliminary test, the amino nitrogen of the protein-free milk was determined both before and after acid hydrolysis. The results of this determination indicated that there was present in solution either unprecipitated proteins or peptids of considerable size, the average increase in amino nitrogen after hydrolysis being 4.5 times greater than the amount obtained before hydrolysis. Moreover, the trace of ammonia nitrogen present in the original protein-free milk was greatly increased by hydrolysis. The increase in both amino and amide nitrogen, as shown in Table I, leads to the conclusion that there are conjugated amino acids present in protein-free milk. These amino acids may be present in milk due to the action of a proteolytic enzyme or they may be formed by the action of acids and heat in the precipitation of the casein. Sherman, Berg, Cohen

[1] Based upon 28 g. of protein-free milk, containing 75% nitrogen, in 100 g. of total food.

and Whitman[1] show that proteolytic products are produced in milk by bacteria. Olson[2] shows that a new protein obtained from the centrifugal slimes collected from separations contains a proteolytic enzyme. Moreover, he shows that many of the bacterial forms present in the udders of cows are of a proteolytic type and would therefore lead to the production of enzymes in the milk before it was drawn. As the milking periods of most dairy cows are from 10 to 12 hours apart this would give ample time for bacterial enzymes to bring about changes in the proteins of milk. Palmer[3] states in his investigations on milk proteins that the proteins which invariably remain after the coagulable proteins have been removed are probably merely the residues of albumen and globulin from the original milk which have been so changed during the precipitation of casein by acid as to render them non-coagulable by heat.

TABLE I.

Showing the Change in Amino and Amide Nitrogen in Protein-Free Milk[4] Due to Acid Hydrolysis.

No. sample.	1. %	2. %	3. %	4. %	5. %	Av. %
Total N in protein-free milk.....	0.71	0.73	0.80	0.74	0.78	0.75
Amide N before hydrolysis.....	0.0042	...	Trace	Trace	Trace	Trace
Amide N after hydrolysis.....	15.27	14.31	14.93	20.62	7.12	14.45
Amino N before hydrolysis.....	4.44	6.13	8.05	3.61	11.75	6.79
Amino N after hydrolysis.....	20.59	40.16	27.82	17.70	34.12	28.07
Amino N % increase due to hydrolysis.....	...	...	...	...	...	314.

The distribution of nitrogen in percentages of total nitrogen is shown in Table II. The quantity of nitrogen present was so small that all of the fractions of Van Slyke's method[5] were not determined. In addition, the presence of carbohydrates will interfere with a true nitrogen distribution but all of the analyses are comparative and sufficiently accurate for the purpose in hand.

[1] H. C. Sherman, W. H. Berg, L. J. Cohen and W. G. Whitman, "Ammonia in Milk and Its Development during Proteolysis under the Influence of Strong Antiseptics," *J. Biol. Chem.*, **3**, 172 (1907).

[2] G. A. Olson, "Milk Proteins," *Ibid.*, **5**, 270–277 (1908).

[3] L. S. Palmer, "A Study of Heat Coagulable and Water-Soluble Protein of Cow's Milk," Missouri Agr. Sta., *Bull.* **151**, 37 (1917).

[4] Method of preparation of protein-free milk in Samples 3 and 4 was slightly changed from general method of preparation as given above. In 3 in the precipitation of casein just enough acid was used to cause formation of curd, then filtered, boiled, refiltered and without neutralization evaporated to dryness. In 4 after neutralization the sample was boiled. A heavy precipitate formed which was filtered off, solution then evaporated to about $1/2$ its volume; as a heavy flocculent precipitate had formed, it was again filtered and evaporated to dryness.

[5] D. D. Van Slyke, "The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids," *J. Biol. Chem.*, **10**, 15 (1912).

TABLE II.

Showing the Nitrogen Distribution of the Different Samples of Protein-Free Milk after Acid Hydrolysis.

Sample.	1. %.	2. %.	3. %.	4. %.	5. %.	Av. %.
Ammonia N	15.27	14.31	14.93	20.62	7.12	14.45
Humin N	22.15	23.47	18.40	30.46	23.91	23.67
Total N of bases	7.66	10.00	14.61	9.43	14.93	11.32
Total N of filtrate from bases	52.19	56.67	63.43	55.52	64.88	58.53
Amino N of filtrate	34.08	32.03	25.54	12.58	37.20	28.28
Amino N of bases	3.22	5.04	4.45	1.48	3.28	3.50

The first conclusion to be drawn from this table is that protein-free milk is variable in composition, the amide nitrogen varying from a minimum of 7.12% of the total nitrogen to a maximum of 20.62%, the nitrogen precipitated by phosphotungstic acid from 7.66 to 14.93%, and the nitrogen in the filtrate from the bases from 52.19 to 64.88%. The high values for the humin nitrogen are what one would expect from the fact that large amounts of carbohydrate are present[1] and have no appreciable significance as regards the presence or absence of particular amino acids. The values for the humin nitrogen are higher than those obtained by Gortner[1] when fibrin was hydrolyzed in the presence of 3 times its weight of cellulose or by Hart and Sure[2] who hydrolyzed casein in the presence of 5 times its weight of various carbohydrates. The larger amount found in my experiments is easily explained by the enormous quantity of lactose in proportion to the total nitrogen present, for it has been shown[1] that the humin nitrogen curve of protein hydrolysis in the presence of carbohydrate will rise quite rapidly at first and then flatten out with no indication of ever reaching a straight line maximum when increasing amounts of carbohydrate are added.

However, taking the table as a whole, the values of the different fractions are distributed in a manner so closely resembling a protein hydrolysate that there can be no doubt that they are derived from protein or peptid material.

In order to gain further information as to the form in which the nitrogen was present an aqueous solution containing 100 g. of protein-free milk per liter was treated with the usual protein precipitants, *i. e.*, acid mercuric nitrate, phosphotungstic acid and trichloroacetic acid. In each test 50 cc. of the above protein-free milk solution was precipitated by one cc. of acid mercuric nitrate, by 10 cc. of 10% phosphotungstic acid, the protein-free milk solution being first acidified with 10 cc. of conc.

[1] R. A. Gortner, "The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. II. Hydrolysis in the Presence of Carbohydrates and of Aldehydes," *J. Biol. Chem.* **26**, 177–204 (1916).

[2] E. B. Hart and B. Sure, "The Influence of Carbohydrates on the Accuracy of the Van Slyke Method in the Hydrolysis of Casein," *Ibid.*, **28**, 244 (1916).

hydrochloric acid and with 2.5 g. of trichloroacetic acid, respectively. The amounts of nitrogen removed calculated in per cent. of the total nitrogen originally present are shown in Table III.

TABLE III.

The Amount of Nitrogen Removed on Precipitation.

Sample.	1. %	2. %	3. %	4. %	5. %	Av. %
Acid mercuric nitrate	...	47.93	35.82	42.53	39.06	41.33
Phosphotungstic acid	...	49.83	36.07	27.76	37.40	37.76
Trichloroacetic acid	...	15.21	20.14	16.71	8.39	15.11

From this table it is seen that acid mercuric nitrate removes slightly larger amounts of nitrogenous material than does phosphotungstic acid. Precipitation by the copper sulfate method as modified by Blish[1] gave unreliable results owing, doubtless, to the large amount of salts and reducing sugar present in the material, which prevented the complete precipitation of cupric sulfate by sodium hydroxide, inasmuch as after the salts and sugars were removed by dialysis a sharp end-point for the precipitation could be obtained.

A solution representing 100 g. of protein-free milk was precipitated with phosphotungstic acid, the precipitate thoroughly washed with hydrochloric phosphotungstic acid solution, decomposed with barium hydroxide and made up to volume. Amino nitrogen was determined on this solution both before and after acid hydrolysis. The average of several determinations showed that the amino nitrogen was increased by hydrolyzing from 10.59% of the nitrogen precipitated by phosphotungstic acid to 53.21% thus showing the presence of conjugated amino groups in this fraction. That conjugated amino groups are present in the protein-free milk is further proved by the increase in amino nitrogen after acid hydrolysis as shown in Tables I and II. This fact is again substantiated by the following experiment: 50 g. of protein-free milk was dissolved and dialyzed in a collodion sack for 96 hours, first against running water, and the last 24 hours against distilled water, in order to remove salts, sugars and any dialyzable nitrogenous compounds. The dialyzate was made alkaline (0.5%) and digested with trypsin at 37°, using toluene as a preservative. After 4 days' digestion the ammonia was removed by distillation with calcium hydroxide under reduced pressure and the amino nitrogen determined. The following values were obtained:

Before digestion, 5.12% amino N in total N
After digestion, 11.49% amino N in total N

The increase in amino nitrogen in this experiment is considerably less than that due to acid hydrolysis of undialyzed material. Nevertheless the increase is significant and shows that the peptid linking must be present.

[1] M. J. Blish, "A Study of the Non-Protein Nitrogen of Wheat Flour," *J. Biol. Chem.*, 33, 553 (1918).

Summary.

1. When prepared under carefully controlled conditions protein-free milk is variable in composition.

2. Amino nitrogen determinations before and after acid hydrolysis and the nitrogen distribution indicate that either unprecipitated protein or peptids of considerable size are present in protein-free milk.

3. By precipitation with acid mercuric nitrate or phosphotungstic acid a little less than $^1/_2$ of the nitrogenous compounds is removed. Examination of the phosphotungstic acid precipitate indicates that this reagent removes only non-amino nitrogen.

4. Further proof that conjugated amino groups are present in protein-free milk is shown by the increase in amino nitrogen after tryptic digestion.

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THE RELATIONS BETWEEN THE CHEMICAL STRUCTURES OF CARBONYL DERIVATIVES AND THEIR REACTIVITIES TOWARD SALTS OF SEMICARBAZIDE.

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Qualitative differences in the behavior of ketones and aldehydes towards reagents have been known for many years. The formation of silver mirrors from aldehydes, and the insolubility of the compounds of potassium hydrogen sulfite with aldehydes and ketones of the type R–CO–M, have long been used to detect and to separate them. According to E. Fischer,[1] α-ketonic acids react with phenylhydrazine in the presence of an excess of hydrochloric acid, while β- and γ-ketonic acids and fatty ketones do not. Von Pechmann[2] placed the reactivity of α-diketones towards phenylhydrazine and hydroxylamine between that of the monoketones and the aldehydes.

The writer[3] showed that Fischer's observations regarding the β- and γ-ketonic derivatives are inaccurate, since levulinic acid gives a hydrazone with the reagent, and also acetoacetic ester,[4] although in the latter case the product undergoes a spontaneous condensation to the salt of the corresponding pyrazolone; further, that ketones react slowly, but aldehydes quickly, which may be used to detect and

[1] *Ann.*, **236**, 146 (1886).
[2] *Ber.*, **22**, 2116 (1889).
[3] *J. prakt. Chem.*, [2] **44**, 114 (1891).
[4] *Ibid.*, [2] **45**, 587 (1892).