Fractions III and IV crystallized when cool. They were dissolved in ether and the ether solution was shaken with 10% sodium hydroxide. The sodium hydroxide solution yielded 1.6 g. of benzylcyanoacetic acid; the ether solution gave 4.9 g. of dibenzylcyanoacetic acid methyl ester, which crystallized when cold. It was recrystallized from an ether-ligroin mixture, coming out as large, six-sided, white plates melting at 78–79°.

Calc. for $C_{18}H_{17}NO_2$: C, 77.43; H, 6.09; N, 5.02. Found: C, 77.81 and 77.69; H, 6.83 and 6.42; N, 5.11.

The methyl ester was also prepared by treatment of the silver salt of dibenzylcyanoacetic acid (*q. v.*) with methyl iodide.

The study of the derivatives of cyanoacetic acid is being continued.

DECATUR, ILL.

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A COMPARATIVE STUDY OF AERATION AND HEAT DISTILLATION IN THE KJELDAHL METHOD FOR THE DETERMINATION OF NITROGEN.

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In 1903, Folin[1] described a method for the determination of ammonia in urine based upon its removal by passing a rapid current of air through the solution under suitable conditions, absorbing the ammonia in acid, and titrating the excess of acid.

P. A. Kober,[2] in 1908, proposed the use of aeration for the separation of ammonia in all Kjeldahl determinations in place of the ordinary heat distillation and described the apparatus and method to be employed. He quoted experiments made by Dr. Kristeller on the estimation of the ammonia in an ammonium chloride solution, three by aeration and two by heat distillation, with excellent agreement.

In the following year, Sebelien[3] suggested the same method for all Kjeldahl determinations. He worked with ammonium salts only and studied the conditions necessary for the removal and absorption of the ammonia.

Davis[4] found aeration to give unsatisfactory results in the determination of nitrogen in cottonseed meal and proposed to heat the solutions during aeration. Gill and Grindley[5] confirmed the work of Davis with cottonseed-meal, but obtained results agreeing with heat distillation for a number of other substances.

[1] *Z. physiol. Chem.*, 33, 161 (1903).
[2] THIS JOURNAL, 30, 1 (1908).
[3] *Chem. Ztg.*, 33, 795 (1909).
[4] THIS JOURNAL, 31, 556 (1909).
[5] *Ibid.*, 31, 1249 (1909).

In a second paper[1] Kober gave further details for carrying out the method and recorded three results comparing the aeration and heat distillation procedures with magnesium phosphate present, showing practically perfect agreement, while in a third paper[2] some of the questions involved were discussed further and a number of experiments using the aeration procedure given.

Dillingham,[3] following Kober's directions, found with ammonium sulfate that from 3 to 15% of the ammonia remained in the residues after aeration, and that this could be obtained by subsequent heat distillation.

In Kjeldahl determinations made in this laboratory, the aeration procedure has heretofore been used. It was observed recently, in the analysis of certain proteins in this way, that the nitrogen contents were much less than those recorded in the literature. These differences led to a careful study of the factors involved and ultimately to a comparison of the aeration and heat distillation procedures, the results of which are communicated in this paper.

The main point to be emphasized in this study is that in the determination of the ammonia by aeration, not only were the results obtained compared with the theoretical nitrogen contents of pure substances wherever possible and with the results obtained by heat distillation, but every solution which was aerated was then submitted to heat distillation. It may be stated in advance that the results showed the aeration method to be unreliable when compared with the direct heat distillation method.

The conditions stated by Kober to be most suitable were strictly adhered to in carrying out the aeration experiments. The substances given in Table I, dissolved in 20-25 cc. water, were digested with 20 cc. conc. sulfuric acid, 10 g. potassium sulfate, and 0.2 g. crystallized copper sulfate, either for an hour after charring had disappeared or, with urea and uric acid where there was little charring, for an hour after the appearance of dense white fumes. The mixtures were then diluted with 80 cc. of water and 75 cc. of sodium hydroxide[4] were added with cooling in the aeration apparatus. Aeration was allowed to proceed for two

[1] THIS JOURNAL, 32, 689 (1910).

[2] *Ibid.*, 35, 1594 (1913).

[3] *Ibid.*, 36, 1310 (1914).

[4] Kober recommended 75 cc. NaOH solution, sp. gr. 1.46–1.48 (48° Bé. obtained in steel drums) for 20 cc. conc. sulfuric acid. He found 52 cc. of this alkali to neutralize 20 cc. of the acid. In the experiments given in Table I, three different solutions of alkali were used. They were made up from sodium hydroxide, electrolytic lumps. Expts. 15, 16, 18, 19, 23, 24, 27, 28, 30, 31, 33, 34, 36, 37, 39, 40 were performed with alkali, sp. gr. 1.461, 46.0 cc. of which were required to neutralize 20 cc. of the conc. sulfuric acid (sp. gr. 1.84 at 13°); Expts. 1, 2, 5, 6, 9, 10, 12, 13, 21, 22, 42, 43, with alkali, sp. gr. 1.444, 49.2 cc. neutralizing 20 cc. of the acid; and Expt. 4, with alkali, sp. gr. 1.401, 55.6 cc. neutralizing 20 cc. of the acid.

hours or some minutes longer and the excess acid then titrated, using alizarin sulfonic acid as indicator. The alkaline mixtures, from the aeration, were then diluted to 300 cc. with distilled water and distilled by heat as in the customary Kjeldahl procedure. Suitable blanks were run frequently in order to eliminate errors which might arise from distilled water, sulfuric acid, etc. A total volume of 400 to 600 liters of air were stated to be sufficient in the aeration for the average amount of ammonia. In order to be certain that these conditions were adhered to, an apparatus was constructed similar to the crude one recommended and the rate of aeration determined immediately before and after each experiment. This ranged from 280 to 390 liters of air per hour for the different experiments. In any one set there was often a difference even up to 30% in testing before and after the run. Taking the mean of the two values in these cases, in general the rate of aeration as measured for the two hours or longer was 330–340 liters per hour.

In the direct heat distillation experiments, the mixtures after digestion were diluted to about 225 cc., 75 cc. of the NaOH solution, sp. gr. 1.444 or 1.461, added, and distilled in the customary way into standard acid.

In Table I are shown the results obtained for a number of nitrogenous substances. Column 1 gives the substance studied, and for simple substances the calculated percentage of nitrogen; Column 2, the number of the experiment; Column 3, the number of cc. of 0.1 N acid neutralized by the ammonia driven over in the aeration; Column 4, the number of cc. of 0.1 N acid neutralized by the ammonia driven over by heat distillation from the solutions which had just been aerated; Column 5, the number of cc. of 0.1 N acid neutralized by the ammonia obtained by heat distillation directly without aeration, that is to say the results as in the ordinary Kjeldahl method; Columns 6–9, the percentages of nitrogen found; Column 6 by the aeration; Column 7 by the heat distillation following aeration; Column 8 the sum of Columns 6 and 7; Column 9 by the direct heat distillation alone. The substances were dried in an electric thermostat at 100–105° to constant weight before analysis. The antitoxin preparations were obtained from Dr. E. J. Banzhaf of the Research Laboratory of the New York Health Department. The castor bean globulin was prepared in the course of an investigation on lipase.

The results given in this table require little comment. For only two substances, the second tetanus antitoxin preparation and tyrosine, are the values found by aeration alone in any way satisfactory. Here the percentage of nitrogen was increased 0.10 to 0.27 by subsequent heat distillation. For the rest of the substances, the nitrogen contents by the aeration procedure were found to be in error (low) by amounts ranging from 0.30 to 1.97%. The sum total of nitrogen found by aeration and subsequent heat distillation agreed very satisfactorily in every case with

TABLE I.—COMPARISON OF AERATION AND HEAT DISTILLATION IN KJELDAHL DETERMINATIONS.

Substance.	Expt. no.	Gram taken.	Cc. 0.1 *N* acid used. Aeration.	Then distn.	Distn. alone.	Percentage nitrogen found. Aeration.	Then distn.	Sum.	Distn. alone.
Casein (Merck)	1	0.1519	15.85	0.52	...	14.62	0.48	15.10	...
	2	0.1530	16.00	0.56	...	14.65	0.51	15.16	...
	3	0.1549	...	..	16.72	...	..	...	15.12
Antitoxin preparation (meningitis)	4	0.1496	13.72	0.65	...	12.85	0.61	13.46	...
	5	0.1532	14.29	0.34	...	13.07	0.31	13.38	...
	6	0.1512	14.04	0.37	...	13.01	0.34	13.35	...
	7	0.1518	...	..	14.61	...	..	...	13.48
	8	0.1515	...	..	14.44	...	..	...	13.35
Antitoxin preparation (tetanus)	9	0.1503	14.16	0.51	...	13.20	0.47	13.67	...
	10	0.1512	14.44	0.32	...	13.38	0.30	13.68	...
	11	0.1510	...	..	14.71	...	..	...	13.65
Antitoxin preparation (tetanus)	12	0.1489	14.42	0.26	...	13.56	0.24	13.80	...
	13	0.1511	14.53	0.24	...	13.47	0.23	13.70	...
	14	0.1518	...	..	15.01	...	..	...	13.85
Antitoxin preparation (streptococcus)	15	0.1520	14.23	0.36	...	13.12	0.33	13.45	...
	16	0.1507	14.05	0.52	...	13.06	0.50	13.56	...
	17	0.1503	...	..	14.60	...	..	...	13.61
Castor-bean preparation (globulin)	18	0.1535	18.04	0.65	...	16.46	0.59	17.05	...
	19	0.1525	18.20	0.41	...	16.75	0.38	17.11	...
	20	0.1517	...	..	18.45	...	..	...	17.04
Hexamethylenetetramine (39.99%)	21	0.1016	27.53	1.43	...	37.96	1.97	39.93	...
	22	0.1011	27.93	0.78	...	38.70	1.08	39.78	...
	23	0.1016	...	..	28.86	...	..	...	39.79
Uric acid (33.33%)	24	0.1519	35.63	0.45	...	32.86	0.41	33.27	...
	25	0.1512	35.35	0.60	...	32.78	0.56	33.34	...
	26	0.1525	...	..	36.25	...	..	...	33.30
Glycylglycine (21.21%)	27	0.1501	22.23	0.58	...	20.75	0.54	21.29	...
	28	0.1517	22.48	0.55	...	20.76	0.51	21.27	...
	29	0.1516	...	..	22.92	...	..	...	21.18
Alanyl glycine (19.18%)	30	0.1499	20.12	0.37	...	18.81	0.35	19.16	...
	31	0.1515	20.50	0.33	...	18.91	0.30	19.21	...
	32	0.1518	...	..	20.76	...	...	...	19.20
Tyrosine (7.74%)	33	0.2016	10.71	0.39	...	7.44	0.27	7.71	...
	34	0.2018	10.86	0.15	...	7.54	0.10	7.64	...
	35	0.2030	...	..	11.09	...	..	...	7.66
Glycine (18.67%)	36	0.1518	19.52	0.69	...	18.02	0.64	18.66	...
	37	0.1513	19.76	0.39	...	18.29	0.36	18.65	...
	38	0.1512	...	..	20.10	...	..	...	18.63
Alanine (15.73%)	39	0.1508	16.33	0.55	...	15.17	0.51	15.68	...
	40	0.1525	16.85	0.30	...	15.48	0.28	15.76	...
	41	0.1513	...	..	17.00	...	..	...	15.74
Urea (46.66%)	42	0.1028	32.79	1.40	...	44.69	1.91	46.60	...
	43	0.1004	32.24	1.11	...	44.99	1.55	46.54	...
	44	0.1025	...	..	34.08	...	..	...	46.58

the nitrogen content found by direct heat distillation in the customary way.

A number of results were obtained with different protein substances in which the heat distillation and aeration methods were used, but in which the solutions after aeration were not heat distilled. The differences ranged for 0.40 to 1.50% in the amounts of nitrogen present, the results by aeration being smaller in every case. As these results add nothing essential to the results given in Table I, they need only be referred to in connection with pointing out the unreliability of the aeration procedure.

Although the scope of this paper does not include the study of the factors upon which the accuracy of the aeration procedure might depend, but only the practical usefulness of the method, some of the results obtained with ammonium sulfate (without digesting) may be of interest and are shown in Table II. The headings of the columns taken in connection with the footnotes and the description of Table I explain the experiments. The rates of aeration are again in the neighborhood of 330 liters per hour and were fairly uniform.

TABLE II.—AERATION AND HEAT DISTILLATION WITH AMMONIUM SULFATE.

				Cc. 0.1 *N* acid used.			Percentage nitrogen found.			
Mixture.	Expt. no.	H_2SO_4. cc.	NaOH. cc.	Aeration.	Then distn.	Distn. alone.	Aeration.	Then distn.	Sum.	Distn. alone.
A	45	..	25	14.17	0.87	...	19.75	1.21	20.96	...
	46	..	35	14.61	0.58	...	20.37	0.81	21.18	...
	47	..	45	14.98	0.19	...	20.89	0.27	21.16	...
	48	..	55	15.15	0.06	...	21.12	0.08	21.20	...
B	49	20	75	14.60	0.48	...	20.35	0.67	21.10	...
	50	20	75	14.83	0.20	...	20.68	0.28	20.96	...
	51	20	75	14.88	0.21	...	20.75	0.29	21.04	...
	52	20	75	14.87	0.18	...	20.73	0.25	20.98	...
	53	20	75	14.81	0.25	...	20.65	0.35	21.00	...
C	54	..	..	...	..	15.22	...	..	...	21.07
D	55	20	75	14.77	0.36	...	20.67	0.50	21.17	...
	56	15	75	14.82	0.34	...	20.74	0.48	21.22	...
	57	10	75	14.89	0.26	...	20.84	0.36	21.20	...

Mixture A.—Total volume 175 cc. No H_2SO_4 or K_2SO_4, 25 cc. $(NH_4)_2SO_4$ solution (= 0.1005 g. $(NH_4)_2SO_4$). Different amounts NaOH solution, sp. gr. 1.444.

Mixture B.—Total volume 175 cc. 20 cc. conc. sulfuric acid, 10 g. K_2SO_4, 0.2 g. $CuSO_4.5H_2O$, 25 cc. $(NH_4)_2SO_4$ solution (= 0.1005 g. $(NH_4)_2SO_4$). NaOH solution, Expts. 49, 50, 51, sp. gr. 1.444; Expts. 52, 53, sp. gr. 1.470.

Mixture C.—0.1012 g. $(NH_4)_2SO_4$, 20 cc. conc. sulfuric acid, 10 g. K_2SO_4, 0.2 g. $CuSO_4.5H_2O$.

Mixture D.—Total volume 175 cc. Different amounts sulfuric acid, 10 g. K_2SO_4, 0.2 g. $CuSO_4.5H_2O$, 25 cc. $(NH_4)_2SO_4$ solution (= 0.1001 g. $(NH_4)_2SO_4$). 75 cc. NaOH solution, sp. gr. 1.461.

With Mixture A and different amounts of NaOH solution, practically complete distillation of the ammonia took place with 55 cc. of alkali.

With less alkali, distillation was incomplete, although no acid was present originally in the solution. With Mixture B, the distillation was incomplete with 75 cc. alkali, sp. gr. 1.444 or 1.470, sulfuric acid and potassium sulfate having been added to the solution in amounts corresponding to digestion mixtures. With Mixture D the presence of different quantities of sulfuric acid originally, made only a comparatively small difference in the error of the aeration procedure.

Conclusion.

The aeration procedure in the ordinary Kjeldahl method for nitrogen very often gives inaccurate and therefore unreliable results, and should not be used.

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STUDIES ON ENZYME ACTION. CORRECTIONS.

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In the course of the study of lipolytic actions, some of the results of which were communicated in previous papers, a number of solid preparations were described and analyzed. The nitrogen contents were determined by the Kjeldahl method with the aeration procedure recommended by Kober. The unreliability of this procedure was pointed out in the preceding paper[1] and it is the object of this paper to correct the nitrogen results given in former papers on the basis of the customary heat distillation procedure in the Kjeldahl method.

The esterase preparations from castor beans,[2] water extract of castor beans, dialyzed, filtered clear, precipitated with acetone, designated E II gave 16.2% nitrogen for the dried material whether dialyzed 5 or 20 hours, while the ash-free material gave 17.0% nitrogen (as against 15 7 and 16.3% by the aeration procedure). The lipase preparations[3] L I, saturated NaCl extraction of castor beans residues after water extractions, dialysis, filtration and washing with acetone, gave 16.8% nitrogen for the dried material, and 17.9% for moisture and ash-free material (17.0 by aeration). L II, obtained similarly with 1.5 *N* NaCl solution, gave 17.1% for moisture-free or 17.9% for ash- and moisture-free material (16.7 by aeration). L II reprecipitated from NaCl solution, gave 18.2% for dried or 18.4% for dried and ash-free substance (17.6% by aeration). The lipase preparation from soy beans,[4] preparation by water extraction

[1] Cf. also Dillingham, THIS JOURNAL, 36, 1310 (1914).

[2] XII paper, THIS JOURNAL, 37, 223 (1915).

[3] *Ibid.*, p. 227.

[4] XIII paper, THIS JOURNAL, 37, 651 (1915).