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A NEW DIRECT NESSLERIZATION MICRO-KJELDAHL METHOD AND A MODIFICATION OF THE NESSLER-FOLIN REAGENT FOR AMMONIA

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RECEIVED MARCH 31, 1924 PUBLISHED SEPTEMBER 5, 1924

The method here given avoids the troublesome separation of silicon dioxide so common in the Folin-Denis[1] procedure and also shortens the time of digestion.

The New Kjeldahl Method

1. Reagents: (a) A 30% solution of hydrogen peroxide (Merck's Superoxol or Kahlbaum's Perhydrol). (b) A 1:1 solution of sulfuric acid; to distilled water add an equal volume of concd. c. p. sulfuric acid. (c) The modified Nessler-Folin reagent.

Dissolve 22.5 g. of iodine in 20 cc. of water containing 30 g. of potassium iodide. After the solution is complete add 30 g. of pure metallic mercury, and shake the mixture well, keeping it from becoming *hot* by immersing in tap water from time to time. Continue this until the supernatant liquid has lost all of the yellow color due to iodine. Decant the supernatant aqueous solution and test a portion by adding a few drops thereof to 1 cc. of a 1% soluble starch solution. Unless the starch test for iodine is obtained the solution may contain mercurous compounds. To the remaining solution add a few drops of an iodine solution of the same concentration as employed above, until a faint excess of free iodine can be detected by adding a few drops thereof to 1 cc. of the starch solution. Dilute to 200 cc. and mix well.

To 975 cc. of an accurately prepared 10% sodium hydroxide solution now add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to clear by standing.

This solution is to be used in the proportion of 10 cc. per 100 cc. of solution to be nesslerized, except in special cases where a great excess of acid is present, as in the direct nesslerization methods. In such methods one should aim to add an amount of the reagent sufficient to ensure the same alkalinity in the unknowns as in the standards.

This modified reagent is an improvement over the Folin-Nessler reagent in that the solution never separates a dark green precipitate of mercurous compounds and also that it is not likely to cause turbidity when added to the ammonia solutions. The reagent is very slightly more sensitive than the original and the nesslerized solutions remain clear for days.

(d) Stock and standard ammonium sulfate solutions in 0.05 N sulfuric acid; these are made according to the usual Folin procedure.

Procedure

The diluted urine, blood filtrate, etc., representing 0.3 to 1.0 milligram of nitrogen, is measured into a 20 × 2.5 cm. Pyrex test-tube. To this is

[1] Folin and Denis, *J. Biol. Chem.*, **26**, 473 (1916).

added 1 cc. of the 1:1 sulfuric acid and the tube is then heated over a free flame (and shaken) or on the sand-bath until the water has been removed. The tube is then heated over the micro-burner until filled with dense, white fumes of sulfuric acid. After it has been allowed to cool for 15 to 30 seconds, 1 to 5 drops of the 30% hydrogen peroxide solution are added and the heating is continued over the micro-burner. In case the material remains colorless after it has again been heated until white fumes form, gentle boiling is continued for two to five minutes. If the fluid again becomes discolored, the addition of several drops of 30% hydrogen peroxide solution and the heating are repeated. After complete digestion and cooling, the solution is transferred to a 100cc. volumetric flask and diluted to about 75 cc. This is cooled, 15 cc. of the modified Nessler reagent is added, and the mixture is at once diluted to 100 cc., mixed well and after five to twenty minutes compared with the standard. The standard is prepared by mixing 1.5 to 5 cc. of the usual standard ammonium sulfate solution (representing 0.3 to 1 mg. of nitrogen) and 1 cc. of the 1:1 sulfuric acid solution in a 100cc. volumetric flask, diluting to about 75 cc., adding 15 cc. of the modified Nessler reagent and diluting as usual. In case the hydrogen peroxide gives an appreciable blank a correction should be made or the peroxide should be properly redistilled from a slightly acid solution before it is used.

A factor which must be carefully controlled is the volume of Nessler reagent added, because the variation in alkalinity affects the intensity of the color. It requires approximately 8.3 cc. of the modified Nessler reagent (containing 8.4% of sodium hydroxide) to neutralize the acid used in the digestion. If now an additional 6.7 cc. of Nessler reagent is added for every 100 cc. of final volume nesslerized, very nearly the same alkalinity will be obtained in every dilution, that is, a titratable alkalinity equivalent to about 0.56% sodium hydroxide. The most satisfactory results are obtained by always preparing the standards containing 1 cc. of the 1:1 sulfuric acid in exactly the same volumes as the unknowns. By so doing the alkalinities and excess of reagent are so nearly identical that theoretical values are easily obtained. When the nesslerization is conducted in a 50 cc. volume 12 cc. of the Nessler reagent should be used, and the standard should similarly be prepared in a 50cc. volume.

Application of the Method

Tables I, II and III give the results obtained by this method with various types of substances of biological importance.

The comparative results obtained by the two macro-Kjeldahl methods conclusively prove the value of hydrogen peroxide in the oxidation of organic matter in concd. sulfuric acid. The authors do not recommend the use of this new macro procedure in place of the usual macro-Kjeldahl

TABLE I

ANALYSIS OF A HYDROLYZED PROTEIN SOLUTION STANDARDIZED BY MEANS OF THE KJELDAHL-GUNNING MACRO-KJELDAHL METHOD

Volume taken Cc.	Nitrogen present by macro-method Mg.	Nitrogen found by the new method Mg.
0.3	0.145	0.131
.5	.240	.228
.2	.097	.103
.8	.390	.379
1.2	.580	.567

TABLE II

ANALYSES OF PURE SUBSTANCES

Substance	Weight taken G.	Nitrogen Calcd. %	Nitrogen Found %
Uric acid	0.001	33.33	33.80
			33.20
			33.60
Acetanilide	.003	10.36	10.11
			10.09
Tryptophan	.002	13.72	13.63
			13.63
Histidine $(HCl)_2$	.00433	18.42	18.46
			18.10
Caffeine	.0010	28.87	28.40
			28.62
Strychnine	.0050	8.38	8.01
			8.04

TABLE III

COMPARATIVE RESULTS BY THE KJELDAHL-GUNNING, A MODIFIED MACRO-KJELDAHL AND THE NEW MICRO-KJELDAHL METHODS

	Distillation macro methods			New micro method	
	Kjeldahl-Dunning digestion 5 cc. taken; 3½ hours of digestion	$H_2SO_4 + H_2O_2$ digestion 5 cc. taken; 1 hour of digestion			
Material	N %	Colorless Mins.	N %	Taken Cc.	N %
Milk	0.516	10	0.514	0.2	0.500
	.517	10	.596	.2	.488
Urine	.717	5	.694	.1	.745
	.714	5	.728	.1	.747
				.2	.743
				.2	.747
	2.5 cc. taken		2.5 cc. taken		
Blood	2.62	20	2.64	.05	2.45
	2.56	20	2.56	.05	lost
	2.56			.10	2.57
				.10	2.58

because the reagent is rather costly as compared with potassium sulfate and copper sulfate. We do, however, consider the use of hydrogen peroxide very desirable in preventing the troublesome foaming so common with substances high in carbohydrates and fats. The time is a very serious factor here. Frequently it is necessary to digest filter paper in biological analyses. We have been able to digest completely 2 sheets of 12.5cm. filter paper in 25 cc. of concd. sulfuric acid in 30 minutes by the addition of 30% hydrogen peroxide.

We have convinced ourselves that ammonia in the absence as well as in the presence of chloride is not lost in this digestion. We are also certain that the changes in alkalinity or in concentration of sulfate brought about by differences in lengths of time of digestion are negligible factors.

Summary

1. The addition of 30% hydrogen peroxide to a solution of organic matter in concd. sulfuric acid causes a very rapid oxidation with complete retention of the nitrogen as ammonia.

2. A new micro-Kjeldahl method involving this action has been devised. It is the most rapid method yet reported.

3. The use of hydrogen peroxide is also recommended for the macro-Kjeldahl estimation on substances high in carbohydrates.

4. The results obtained by the application of this reagent to the micro- and macro-Kjeldahl estimations upon urine, milk, blood and pure substances are very satisfactory.

5. A modified Nessler-Folin reagent is described which does not cause the turbidity so frequently and easily obtained when the regular Folin reagent is used.

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STUDIES IN THE DIPHENIC ACID SERIES. II

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RECEIVED MARCH 31, 1924 PUBLISHED SEPTEMBER 5, 1924

In a previous paper[1] we pointed out that when phenol and diphenic anhydride are heated together in the presence of fuming stannic chloride, two isomeric condensation products are formed. One of these, phenoldiphenein (I), dissolves in sodium hydroxide solution with the development of a yellow color. If phenoldiphenein has a structural formula similar to that of phenolphthalein, the appearance of the solution of its sodium salt is anomalous.

[1] THIS JOURNAL, **45**, 3071 (1923).