Determination of Nikethamide with Cyanogen Bromide and Barbituric Acid

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Two procedures are presented for the determination of nikethamide which involve its reaction with cyanogen bromide and barbituric acid. The first method is highly specific while the second is more rapid but slightly less specific. These methods have the advantage of being simpler, more rapid, and more specific than the present N.F. procedure.

WHILE testing various pyridine derivatives in the cyanogen bromide-barbituric acid method recently developed in this laboratory for the determination of niacinamide (1), it became evident that under certain conditions the diethylamide of nicotinic acid (nikethamide) also produced a pink color with these reagents which might be useful for its estimation.

The present N.F. method (2) involves a titration of the diethylamine after digestion and distillation without differentiating other nitrogen-containing compounds. Since this procedure was not specific it was decided to investigate the color reaction. This paper describes two possible methods for determining nikethamide and the studies that were made in developing these methods.

METHOD

Reagents.—*Cyanogen Bromide,* 10%.—Prepare and use under hood. Do not let cyanogen bromide or solution come in contact with skin. Dissolve a weighed amount of cyanogen bromide in warm water at about 40°. Allow to come to room temperature and dilute to 10% solution. Store in refrigerator and bring to room temperature before using.

Potassium Phosphate, Monobasic, 3%.—Dissolve 15 Gm. KH_2PO_4 in water to make 500 ml.

Potassium Phosphate, Monobasic, 0.3%.—Dilute 100 ml. of 3% KH_2PO_4 to 1000 ml. with water.

Barbituric Acid Buffered Solution, Saturated (2%).—Prepare volume required for each batch of assays by adding 100 ml. of 3% KH_2PO_4 to each 2 Gm. of reagent grade barbituric acid and shaking vigorously at intervals for about 1 hour. Filter before using.

Assay.—*Nikethamide Standard Solution.*—Dilute an aliquot of nikethamide N.F. 25% standard[1] with 0.3% KH_2PO_4 to 3.0 mcg. per ml.

Assay Solution.—Dilute to 3 mcg. per ml. with 0.3% KH_2PO_4.

Reaction.—Run in duplicate. To 1.0 ml. of standard or assay solution in colorimeter tube, add 0.5 ml. cyanogen bromide, mix, stopper, and let stand 10 minutes. Add 10 ml. barbituric acid, mix, and let stand 5 minutes. Place tubes in water bath at 50° for 25 minutes and then transfer to another bath at 20° for 5 minutes. Within the next 15 minutes set spectrophotometer at 0 absorbance at 560 mμ with a standard blank in which cyanogen bromide is replaced by water and measure absorbance of reaction product.

Calculations.—Mcg. per aliquot = $Au \times 3/As$, where Au = absorbance of unknown and As = absorbance of standard.

RAPID METHOD

Dilute standard with 0.3% KH_2PO_4 to 1.0, 1.5, 2.0, 2.5, and 3.0 mcg. per ml., respectively, and dilute sample to about 2 mcg. per ml. To 1.0-ml. standard solution or assay solution (in duplicate) in colorimeter tube, add 0.5 ml. cyanogen bromide, mix, stopper, and let stand 10 minutes. Add 10 ml. of barbituric acid, mix by swirling, and read maximum absorbance which remains stable from 10 to 15 minutes after addition of barbituric acid. Set spectrophotometer at 0 absorbance at 505 mμ with a standard blank in which cyanogen bromide is replaced by water. Plot absorbance of standard *vs.* concenration and draw line of best fit but do not consider the origin as a point in drawing the line. Read from curve the number of mcg. per ml. corresponding to absorbance of sample.

EXPERIMENTAL RESULTS AND DISCUSSION

Factors Affecting the Reaction.—Modifications of the niacinamide method previously presented (1) were thoroughly investigated. The optimum time of cyanogen bromide reaction was 10 (±1) minutes. When the reaction was conducted at room temperature, maximum absorbance was obtained at 505 mμ 10 minutes after addition of barbituric acid. There was also a minor peak at 560 mμ which reached its maximum in about 2.5 hours. Development of the latter peak could be accelerated by incubating 25 minutes at 50° after allowing the barbituric acid reaction to proceed 5 minutes at room temperature. The use of other temperatures offered no advantages for this determination.

Concentration of Nikethamide.—At 560 mμ there was a linear relation between absorbance and concentrations up to 10.0 mcg. which passed through

TABLE I.—PERCENTAGE OF ABSORBANCE OF NIACIN DERIVATIVES IN COMPARISON TO NIKETHAMIDE (100%)

Substance	Regular Method,[a] %	Rapid Method,[b] %
Nikethamide	100	100
Niacin	none	0.5
Niacinamide	none	7.4
Pyridine-3-sulfonic acid	1.2	1.9
Nicotinic acid dimethylamide	none	9.1
Nicotinic acid methylamide	none	3.6
Nicotinic acid ethylamide	1.2	1.4
Nicotinic acid methyl ester	none	2.5
Nicotinic acid ethyl ester	none	2.5

[a] Substance tested at 3 mcg. [b] Substance tested at 2 mcg.

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[1] Proposed standard, containing 25% nikethamide according to the N.F. requirements (2).

the origin. At 505 mμ, concentrations from 1.0 to 3.5 mcg. gave a straight line which did not pass through the origin. In this latter case a standard curve was used to calculate the results. The mean recoveries at different levels were 100.4 ± 2.7% for 7 determinations with the first method and 100.1 ± 2.5% for 6 determinations with the second method.

Reaction of Related Compounds.—To test the specificity of the methods, compounds most likely to react similarly to nikethamide were assayed at the 3-mcg. level for the regular method and at 2 mcg. for the rapid method. Results are reported in Table I in terms of percentage of absorbance of related compound in comparison to nikethamide. For the regular method, the highest percentage was 1.2% for pyridine-3-sulfonic acid and nicotinic acid ethylamide; other substances tested gave no absorbance. For the rapid method, the highest percentages were 9.1% for nicotinic acid dimethylamide and 7.4% for niacinamide; others were below 4%. The fact that nikethamide is practically the only compound giving a colored product seems to be due (*a*) to the different rate of reaction of various pyridine derivatives with cyanogen bromide and (*b*) to the difference in stability of the colored compound formed upon reaction of the pyridinium derivative with barbituric acid unsubstituted in the 5 position.

It may be concluded that the regular method is more specific than the rapid method although the latter would be quite satisfactory for most routine checking purposes.

REFERENCES

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Citrinin from *Penicillium steckii* Zaleski

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The antibiotic substance isolated from cultured *Penicillium steckii* Zaleski was identified as citrinin.

PENICILLIUM STECKII Zaleski[1] was shown to have antibiotic activity against *Staph. aureus* and *Ps. pyocyanes* (1). It was later found that the same strain formed a penicillin-like antibiotic (2). In this paper the isolation, characterization, and crystallization of the antibiotic is described and it is confirmed that the antibiotic is citrinin.

The organism *Penicillium steckii* Zaleski was cultivated in Czapek-Dox solution containing 0.25% yeast extract; the active principle from the potent greenish culture broth was isolated by chloroform, and purified by adsorption chromatography on acid-washed alumina. The antibiotic was crystallized in prismatic needles and in plates from chloroform and petroleum ether mixture. The crystalline sample, melting at 165–169° (with decompn.), is readily soluble in chloroform and acetone. LD_{50} is 100 mg. The determination of its functional groups and the studies of its physicochemical properties show that the antibiotic is citrinin.

EXPERIMENTAL

Culture Media.—Czapek-Dox solution; sucrose, 20 Gm.; sodium nitrate 2.0 Gm.; potassium dihydrogen phosphate, 1 Gm.; potassium chloride, 0.5 Gm.; magnesium sulfate, heptahydrate, 0.5 Gm.; ferrous sulfate, heptahydrate, 0.01 Gm.; water to 1 L. The only source of nitrogen in the medium is sodium nitrate which corresponds to 0.3296 Gm. of nitrogen per L. Czapek-agar medium was the best for sporulation.

Production and Isolation of the Antibiotic on Czapek-Dox Solution.—For the production of the antibiotic, the surface culture was found to be better than the shake culture. Of the different carbon sources such as glucose, sucrose, lactose, and galactose, sucrose was the best. Grain extract, beef extract, and yeast extract were found to have a stimulatory effect both on the growth of the organism and on the production of the antibiotic.

Czapek-Dox solution containing 0.25% yeast extract was distributed in 50-ml. amounts in 250-ml. conical flasks which were plugged with cotton and sterilized at 15 lb. pressure for 20 minutes. The contents of the flask were then inoculated with spore-suspension in water, prepared from Czapek-agar slopes of *Penicillium steckii*, one flask being sown from each slope. The flasks were incubated at room temperature for 4 days. The culture fluids were separated from the mycelia by decantation. The filtrates were then extracted with an equal volume of chloroform. For the isolation of the antibiotic present in the mycelia, the thoroughly washed mycelia were crushed with sand and extracted with alcohol. The extracts were evaporated to dryness at 50° under reduced pressure, taken into chloroform, and chromatographed on acid-washed alumina to remove the coloring matter. The antibiotic was crystallized from chloroform and petroleum ether mixture. Repeated crystallization gave lemon-yellow prismatic needles, sometimes lemon-yellow plates, melting at 165–169° with decomposition.

The purified product is readily soluble in chloroform and acetone, very sparingly soluble in water. It is soluble in alcohol, aqueous alkali, less soluble in ether, still less in petroleum ether.

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[1] *Penicillium steckii* Zaleski was isolated from local soils and has been identified by Dr. A. H. S. Brown and deposited in Herb IMI as 61422.