THE DETECTION OF AMMONIA PRODUCTION BY BACTERIA IN AGAR SLANTS

P. ARNE HANSEN

New York State Agricultural Experiment Station, Geneva, New York

Received for publication, August 26, 1929

The production of ammonia from various substrata serves, in many cases, as an important character in the identification of species of bacteria. The detection of ammonia as used by Thomas (1912), was first recommended by Ayers, Rupp, and Mudge (1921) and later by Hucker and Wall (1922) as having certain advantages over Nessler's reagent. The reagents necessary in the Thomas test may be used in the presence of glucose, protein, and other organic compounds which generally make up the nutrient media in common use in the laboratory. In the Manual of Methods for Pure Culture Study of Bacteria by the Society of American Bacteriologists the reagents for the Thomas test are listed as follows: I. A 5 per cent solution of phenol. II. A solution of sodium hypochlorite containing 1 per cent available chlorine. To obtain this amount of available chlorine the solution should be so adjusted that 1 cc. of it should neutralize 2.82 cc. of a N/10 solution of sodium thiosulphate (i.e., 24.8 grams to the liter), titrating with starch as an indicator in the presence of acetic acid and potassium iodide.

In carrying out this technic in the laboratory, difficulty is often encountered in maintaining a sodium hypochlorite of the proper concentration of available chlorine. Hypochlorite preparations are rarely stable and gradually undergo changes with the loss of available chlorine. For the Thomas test, stock solutions of chlorinated lime are sometimes used similar to the solutions available as disinfectants. It is necessary to standardize these hypochlorite solutions before use in order to adjust the final solution to the proper concentration. Frequently it is found that

the available chlorine in these solutions has been reduced so much that the solution is useless, as an insufficient amount not only causes inconvenience but has a marked effect upon the results. Thomas has suggested that other oxidizing agents may be of use in place of the hypochlorite. This encouraged the author to try the use of a substitute for the hypochlorite which would be easy to prepare and would not require standardization. After repeated trials such a solution was found to be one containing hypobromite. This reagent is prepared by adding 35 cc. of 2N sodium hydroxide to 100 cc. of saturated bromine water. At first it may seem of no advantage to use such an unstable compound as hypobromite. However, the hypobromite solution is easily made up, and a saturated solution of bromine is sufficiently constant to warrant its use as a routine reagent. The solubility of bromine in grams per 100 cc. water is:

	grams per 100 cc.
At 15°C	3.65
At 20°C	3.58
At 25°C	3.48

As the solubility does not vary greatly at these temperatures a solution of bromine in water at "room temperature" does not need standardization for the purpose under discussion. A solution containing 3.5 grams of bromine in a volume of 135 cc. will titrate per 1 cc. 3.24 cc. N/10 thiosulphate. Acceptable bromine water for the purpose of this test may be made as follows: In a glass stoppered bottle, distilled water is shaken with sufficient bromine to leave an undissolved excess. The bottle is cooled in running tap water. If cold water is used (see table above) no difficulties will arise. Without any special precautions these hypobromite solutions were found to titrate approximately 3.2 cc. Small variations do not affect the results. The sodium hydroxide must of course be added to the bromine water immediately, as otherwise some bromine will escape. The hypobromite reagent is a yellow solution, does not have a strong odor, and keeps for some time. A solution which at first titrated 3.15 N/10 thiosulphate per cubic centimeter showed a titration of 3.05 after three weeks.

In the Thomas test, other phenols may be substituted for the recommended phenol solution. Cresol gives a similar reaction but there appears to be no advantage in using it in place of phenol. However, thymol seems to be of great interest. When a solution of thymol in sodium hydroxide is added to a weak[1] solution of an ammonium salt, mixed well, a hypobromite solution added and the whole left for 20 minutes, a blue color appears which is lighter or darker according to the amount of ammonia present. When a few cubic centimeters of ether are added and shaken with the mixture, the ether absorbs the color with a resultant beautiful red-violet shade. When allowed to stand for a few seconds the ether collects upon the surface of the mixture and makes the reaction very striking. The bromine compounds which are simultaneously formed do not render the reaction indistinct, especially when ether is used.

The mixture may have a green color caused by a mixture of the blue dye from the reaction and the yellow color of the bromine compounds. This green color may indicate a positive reaction, but the reaction appears more definite when the blue color is extracted as mentioned above.

The reagents for this test are:

I. Thymol	2 grams
Sodium hydroxide 2 N	10 cc.
Water	90 cc.
II. Bromine water	100 cc.
Sodium hydroxide 2 N	35 cc.

The test may be carried out as follows:

One cubic centimeter of reagent I is added to 5 cc. of the liquid (which must not be acid) to be tested for ammonia and thoroughly mixed. One cubic centimeter of reagent II is then added and shaken and allowed to stand for 15 to 20 minutes, a small amount of ether added, shaken well and allowed to stand until the ether rises to the top. It is important to add the two solutions sepa-

[1] A strong solution gives a development of nitrogen.

$$3\,NaBrO + 2NH_3 \rightarrow 3NaBr + 3H_2O + N_2$$

rately and not to add the ether before the color has had time enough to develop.

A comparison was made between this technic (D), the Thomas test (A) as described in the Manual of Methods for Pure Culture Study, procedure (B) in which the hypobromite reagent was substituted for the hypochlorite solution, and a procedure (C) in which the thymol solution was used as a first reagent and the original hypochlorite solution as the second solution.

The methods were tried upon 5 cc. of solutions of NH_4Cl, CH_3NH_3Cl, and NH_2CH_2COOH. In order to be comparable the solutions were standardized to the same content of NH_2 per

TABLE 1

A comparison of the sensitivity of the various procedures studied

PROCEDURE	HIGHEST DILUTION WHICH GAVE A POSITIVE REACTION WITH:		
	Ammonium chloride	Methyl-ammonium chloride	Glycine
A	1–100,000*	1–100,000	1–10,000
B	1–500,000	1–10,000	1–5,000
C	1–10,000	1–5,000	1–5,000
D	1–1,000,000	1–50,000	1–100,000

* Very faint reaction in 1–500,000.

5 cc. The following dilutions of NH_2 were used: 1/100, 1/1,000, 1/5,000, 1/10,000, 1/500,000, 1/1,000,000.

In the case of the dilution of 1/100 the solution to be tested must have an alkaline reaction as the salts used in the tests shown above are acid in reaction and act as buffers. The glycine in all cases failed to give a reaction in strong solutions.

The procedure which uses a solution of phenol and the hypobromite (B) seemed to be the most promising according to these results. It was more sensitive towards ammonia than the Thomas test (A) and is not affected so much by methylamine. The original Thomas reaction (A) gave as pronounced a reaction with methylamine as with ammonia, although the color was not the same, being more greenish in the case of the former, and developed more slowly. In practice, however, it was difficult to distinguish

the two shades of color. The color in using the hypobromite solution (B) is not sky-blue like that of the Thomas reaction (A), but tends to a greenish blue, although it is just as easy to recognize. This particular practice (B), although applicable under certain conditions is unfortunately frequently difficult to handle. The color may disappear upon standing. It cannot be recommended unless further studies show it to be useful.

The substitution of the thymol for the phenol, but without substituting the hypochlorite by the hypobromite (C) has no especial advantage and has several disadvantages so that it may be dropped without further discussion.

The use of both the thymol and the hypobromite (D) is the method which seemed to be the best. It was more sensitive than the Thomas test towards ammonia, and less to methylamine. It is a little more sensitive than the Thomas test (A) towards glycine (the only disadvantage). This reaction is so extremely sensitive that even very small traces of ammonia may be detected. It may therefore be wise only to acknowledge distinct reactions as positive. Besides these advantages, it also has the advantage of eliminating the hypochlorite reagent as mentioned above.

All tests have to be applied in an alkaline solution or in a solution which is not more acid, than the alkaline reagent will neutralize. The well known Nessler's reagent must also be applied in an alkaline or neutral solution. The amount of alkali which must be added depends upon the acidity and the buffer action of the medium. For example, a 3.3 per cent aqueous solution of ammonium chloride (5 cc. were used for test) did not give any reaction with three (A) (C) (D) of the procedures, unless enough sodium hydroxide was added to give an alkaline reaction.

None of the nitrogen free organic compounds that have been tried gave any reaction with these reagents. The behavior of a primary amine has been described, and, of the amino-acids, glycine alone gave a distinct reaction. Alanine gave a faint reddish shade. Leucine, phenyl-alanine, tyrosine, asparaginic acid, asparagine, glutaminic acid, tryptophane, and cystine did not give any reaction. Neither did trimethylamine, aniline, pyridine, nor uric acid. A number of peptones were tried. In

some cases a reaction was obtained and upon further study certain brands of peptone were found not to be ammonia free.

When this test is to be applied in bacteriological technic, it is preferable to use agar slants as recommended by Hucker and Wall (1922). The organisms which are to be tested any be grown upon the following medium:

	per cent
Peptone	4.0
Glucose	0.2
Dipotassium phosphate	0.5
Agar	1.5
Water.	
Adjusted to pH 7.5.	

One of the great advantages of using agar slants for certain biological tests is that the growth products are collected in a small space, causing the reaction to be more pronounced as well as permitting short incubation. Another advantage in the use of agar slants is that the reagents are not directly mixed with the culture media. In this case, the reaction is more distinct and sensitive and produces the correct pH in the reagents without neutralizing the medium. After sufficient incubation the test is carried out as described above (p. 225). The agar is not mixed with the thymol solution although it may be desirable to mix the growth on the surface with the reagents.

A number of strains of bacteria have been tested for ammonia production from pepton using this agar and the thymol hypobromite test.

1 *Bacterium coli* Lehmann and Neumann	
1 *Bacterium fluorescens-liquefaciens* Flügge	
6 *Bacterium prodigiosum* Lehmann and Neumann	
2 *Tetracoccus liquefaciens* Orla-Jensen	
1 *Streptococcus liquefaciens* Orla-Jensen	
1 *Bacillus albolactis* Migula, No. 3	American Type Culture Collection
1 *Bacillus cereus* Frankland, No. 21	
1 *Bacillus flavus* Ford, No. 58	
1 *Bacillus mesentericus* Trevisan, No. 76	
1 *Bacillus mycoides* Flügge, No. 80	
1 *Bacillus subtilis* Cohn, No. 102	
1 *Bacillus vulgatus* Trevisan, No. 123	

All of these strains gave a distinct reaction after incubation at their optimum temperature for 3 days.

This test may also be applied in a liquid medium of the formula given above, the agar being omitted. A longer incubation period is necessary in order to produce sufficient ammonia for the test. The medium is neutralized before adding the reagents. Thymol blue may be used as an indicator, and does not interfere with the reaction when ether is used. In an alkaline mixture this indicator is blue and this color is, of course, no indication of ammonia. Fortunately it cannot be shaken out with ether, so that an appearance of a red color in the ether is an indication of ammonia.

SUMMARY

A method for detecting ammonia, particularly to be used on agar slant cultures, is given. 1 cc. of a solution of thymol and 1 cc. of a hypobromite solution are added successively to the culture and allowed to act for 20 minutes. If ammonia is present the mixture becomes blue or greenish blue. The blue color may be extracted by means of ether in which it is soluble, resulting in a deep red-violet color. The reaction is also given by certain aliphatic amines and by glycine. It has the advantage over the Thomas test of not using a hypochlorite. The hypobromite is more easily prepared fresh and of definite strength each time it is used. This is done by mixing bromine water with sodium hydroxide. Like the Thomas test only weak solutions give the ammonia reaction.

REFERENCES

AYRES, S. H., RUPP, P., AND MUDGE, C. S. 1921 The production of ammonia and carbon dioxide by streptococci. Jour. Inf. Dis., **29**, 235–260.

HUCKER, G. J., AND WALL, W. A. 1922 The use of agar slants in detecting ammonia production and its relation to the reduction of nitrates. Jour. Bact., **7**, 515–518.

THOMAS, P. M. 1912 Sur une réaction colorée de l'ammoniaque. Bulletin Société chimique, Ser. 4, **11**, 796–799.