THE REDUCTION OF NITRATES TO NITRITES BY SALMONELLA PULLORUM AND SALMONELLA GALLINARUM[1]

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The results of investigations dealing with the ability of *Salmonella pullorum* and *Salmonella gallinarum* to reduce nitrates to nitrites in a nitrate-peptone solution do not agree. Rettger and Harvey (1908) reported reduction by both organisms. Bushnell, Hinshaw and Payne (1926), Kaupp and Dearstyne (1927), Wallace and Neave (1927), and Tittsler (1928) reported reduction by *Sal. pullorum*. Hadley, Elkins and Caldwell (1918) found no reduction by either organism. Gage (1922), and Hadley, Caldwell, Elkins and Lambert (1917) reported no reduction by *Sal. pullorum*. Kaupp (1927) states that nitrates are reduced by *Sal. gallinarum* but not by *Sal. pullorum*. Bergey's Manual (1925) characterizes both organisms as negative to the nitrate test. Mallmann (1925) in an extensive study of *Sal. pullorum* found considerable variation. In 1924 his cultures gave a "weak positive test," but in 1925 some "were strongly positive, while others were weakly positive" and still others "were positive in only one tube." Perhaps the differences in results were due to differences in the media or methods of testing for nitrites; however, no definite conclusions can be drawn since no statements were made concerning these points except in the reports of Wallace and Neave (1927) and Tittsler (1928). The assumption that a "Standard Nitrate-Peptone Solution" was used would be erroneous, as evidenced by the report of Conn and Breed (1919) which

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is supported by an unpublished review of textbooks and laboratory manuals in bacteriology by the author. This review shows that the amounts of ingredients recommended vary greatly: peptone, from 0.1 to 1.0 per cent; potassium nitrate, from 0.02 to 1.0 per cent, while Chester (1901) specifies 0.002 per cent sodium nitrate; meat extract, although not included in most formulae, is specified in some as 0.3 per cent, and sodium chloride, used in only two formulae, as 0.5 per cent. Most formulae call simply for peptone, while three specify Witte's, one Parke-Davis and one Merck's. Similar variations are noted in the amounts of reagents employed in testing for nitrites. Some procedures call for only "a few drops" while others call for 2 cc. each of sulphanilic acid and alpha-naphthylamine solutions.

Although the ability to reduce nitrates to nitrites is not employed in Bergey's (1925) classification to differentiate species within the genus *Salmonella*, it is used so extensively in other instances that consideration of these variations in media and methods of testing for nitrites is imperative. The investigation reported here was begun in 1927 to determine: first, if *Sal. pullorum* and *Sal. gallinarum* reduce nitrates to nitrites; second, to study variations between strains, and third, to study the adaptability of various nitrate-peptone media for this test.

During the progress of this study Wallace and Neave (1927) recommended the use of dimethyl-alpha-naphthylamine in place of alpha-naphthylamine in testing for the presence of nitrites; therefore, a comparative study was made of these two tests.

TECHNIQUE

Cultures

Two hundred and seventy-five strains of *Sal. pullorum* and ten strains of *Sal. gallinarum* were tested. The *Sal. pullorum* cultures came from widely separated sources and were of widely varying dates of isolation; 225 were isolated by the author over a period of five years and 50 were secured from eight different Agricultural Experiment Stations. Of the total number, 230 were isolated from chicks, 18 from septicemic infections in adult fowls, 15 from the ovaries of carriers, and 12 from eggs. Both the so-called

aerogenic and anaerogenic types were represented. The *Sal. gallinarum* cultures were also from various sources, nine having been secured from four Agricultural Experiment Stations, and one isolated by the author.

Media

During the progress of this study many nitrate-peptone formulae were employed. In all cases, except those noted, Difco-peptone, C.P. nitrite-free potassium nitrate, Liebig's meat extract and distilled water were used. All media were sterilized at 15 pounds for twenty minutes.

Testing for nitrites

Tests for nitrites were made after three to four days incubation at 37°C., using 1 cc. sulphanilic acid and 0.5 cc. of either alpha-naphthylamine or dimethyl-alpha-naphthylamine. The appearance of a red color with either test was considered to be indicative of nitrites.

EXPERIMENTAL

One hundred and fifty strains of *Sal. pullorum* and nine strains of *Sal. gallinarum* were tested in 1927, using a nitrate-peptone solution made of Difco peptone 1.0 gram, potassium nitrate, 0.2 gram, and distilled water, 1 liter. Alpha-naphthylamine was used in testing for nitrites. All cultures reduced nitrates.

In 1928 these cultures and 50 additional strains of *Sal. pullorum* were tested under the same conditions, with similar results. Equally good results were obtained in a duplicate series of cultures where dimethyl-alpha-naphthylamine was used in place of alpha-naphthylamine.

In 1929 the same cultures, and also 75 strains of *Sal. pullorum* and one strain of *Sal. gallinarum* which had been isolated recently, were tested in standard nutrient broth plus 0.1 per cent potassium nitrate. Reduction of nitrate was obtained in every case. Therefore, both species were considered capable of reducing nitrates to nitrites in both of the above media. However, no explanation was at hand to account for the irregular results en-

countered by Mallman (1925) or the negative results of Gage (1922) and Hadley *et al* (1917, 1918). In view of the fact that so many formulae of nitrate-peptone solutions have been recommended, a study was made of various brands and amounts of

TABLE 1

The effect of various kinds and amounts of peptone and nitrate upon the character of growth and reduction of nitrate by Sal. pullorum and Sal. gallinarum

MEDIA				CHARACTER OF GROWTH	RESULTS	
Kind of peptone	Grams per Liter				Alpha-naphthylamine Test	Dimethyl-alpha-naphthylamine Test
	Peptone	KNO_3	Meat extract			
Difco	1.0	0.2		+++	Good—very clear	Good—light
	1.0	1.0		+++	Good—very clear.	Good—light
	1.0	2.0		+++	Good—clear	Good—clear
	1.0	5.0		+++	Good—very dark	Good—clear
	1.0	10.0		+++	Good—very dark	Good—clear
	0.5	1.0		+	Good—very clear	Poor—very light
	1.0	1.0		+++	Good—very clear	Good—light
	2.0	1.0		+++	Good—very dark	Good—clear
	4.0	1.0		++++	Very dark—precipitate	Very good—dark but clear
	8.0	1.0		++++	Very dark—precipitate	Very good—dark but clear
Proteose	1.0	1.0		++++	Good—very dark	Very good—clear
Witte	1.0	1.0		+	Some negative—irregular	Poor—very irregular
	2.0	1.0		++	Some negative—irregular	Poor—very irregular
	5.0	1.0		+++	Poor—irregular	Poor—irregular
	10.0	1.0		+++	Poor—irregular	Poor—irregular
	5.0	1.0	3.0	+++	Good—clear	Good—light
Armours	2.0	1.0		++++	Good—very dark	Very good—clear
Difco	5.0	1.0	3.0	++++	Negative or very strong—then faded	Very good—dark but clear
		1.0	3.0	+++	Good—clear	Good light
Difco	5.0		3.0	++++	Negative	Negative

peptone, various amounts of potassium nitrate, and of the influence of meat extract. In order not to make this portion of the study unnecessarily cumbersome, only ten strains of *Sal. pullorum* and three strains of *Sal. gallinarum* were used.

The formulae of media, types of growth and average results are summarized in table 1. These results show that both species reduced nitrate in a medium composed of from 0.5 to 8 grams of Difco peptone and from 0.2 to 10 grams of potassium nitrate per liter. It was noted that the greater the amount of peptone the greater was the amount of growth and the stronger the nitrite test. Both Difco proteose peptone and Armour's peptone gave equally good results. However, Witte's peptone not only gave poorer growth but either negative or irregular nitrite tests. A solution of 5 grams of Witte's peptone, I gram of potassium nitrate and 3 grams of Liebig's meat extract per liter gave approximately the same results as 1 gram of Difco peptone and 1 gram of potassium nitrate. These irregular results with Witte's peptone are in accord with the findings of Mallman (1925).

A nitrate-peptone solution composed of 10 grams of peptone and 0.02 gram of sodium nitrate per liter as recommended by Chester (1901) gave negative nitrite tests. However, good tests were obtained when 2 grams of sodium nitrate per liter were used. Perhaps the negative results obtained by a few investigators were due to the use of Chester's formula.

It was noted in the course of the study that 1 cc. of sulphanilic acid and 0.5 cc. of either alpha-naphthylamine or dimethyl-alpha-naphthylamine was sufficient to give a strong color; in fact only a few drops of alpha-naphthylamine were needed.

A comparison of the tests in which alpha-naphthylamine and dimethyl-alpha-naphthylamine were used favored the latter in that it always gave a clearer color, without any precipitate, while it never faded in solutions which contained meat extract.

DISCUSSION OF RESULTS

These results indicate that both the negative and irregular results of some investigators may have been due to either the use of Witte's peptone or of Chester's formula.

Although slight quantitative differences between strains were noted, they were not sufficient to be of importance except when Witte's peptone was used. Since some strains were negative and

others very irregular with this peptone, it is the author's opinion that it should not be used. The results obtained with various amounts of diffco peptone show reduction of nitrate in various degrees. The amount of peptone which is desirable appears to be determined rather by the intensity of color desired in the nitrite test than by the qualitative results to be expected. The use of 2 grams of peptone and 0.2 gram of potassium nitrate per liter gave the most satisfactory results from the author's viewpoint. This was especially true when alpha-naphthylamine was used.

The use of standard nutrient broth plus one gram of potassium nitrate, as recommended by the Manual of Methods (1923), did not give satisfactory results with alpha-naphthylamine, as there was either an immediate fading or no appearance of color at all. However, when dimethyl-alpha-naphthylamine was used the color was permanent and satisfactory. The use of dimethyl-alpha-naphthylamine is highly recommended, especially to those who wish to use the standard broth-nitrate solution, because of the permanent color produced by it and the lack of appreciable precipitate. Attention is called to the slower development of color.

The results of a few tests showed that two days incubation at 37°C. were sufficient to give a strong nitrite test when 2 grams of Difco peptone were used.

CONCLUSIONS

1. Both *Sal. pullorum* and *Sal. gallinarum* reduce nitrates to nitrites.
2. No appreciable difference exists between strains.
3. Witte's peptone is not suitable for use in a nitrate-peptone solution.
4. A solution composed of 2 grams of Difco peptone, 0.2 gram of potassium nitrate and 1 liter of distilled water is satisfactory for these species.
5. Dimethyl-alpha-naphthylamine is superior to alpha-naphthylamine in testing for nitrites.

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