# NITRATE, NITRITE AND INDOLE REACTIONS OF GAS GANGRENE ANAEROBES[1]

R. W. REED

*Queen's University, Kingston, Canada*

Received for publication January 21, 1942

In an attempt to work out a scheme for the rapid identification of the gas gangrene organisms (Reed and Orr, 1941), many of the published reports of nitrate reduction and indole formation by this group of organisms were found to be contradictory. The nature of the contradictions suggested that this was largely the result of faulty interpretation of results so clearly described for the nitrate reduction of bacteria in general by Conn (1936).

## NITRATE REDUCTION

Woods (1938) found that washed suspensions of *Clostridium welchii* catalyzed the reduction of $NO_3$, $NO_2$ and $NH_2OH$ to $NH_3$ by molecular hydrogen. Moreover, during the reduction of $NO_3$ to $NH_3$ he demonstrated the appearance and disappearance of $NO_2$. This made it probable that tests for $NO_2$ alone in growing cultures of this and related species would give little indication of the nitrate-reducing action of the organisms. A somewhat more detailed examination has therefore been made.

Cultures were tested for nitrite production in the following medium, Reed and Orr (1941):

| | |
|---|---|
| Bacto tryptone | 20 grams |
| $Na_2HPO_4$ | 2 grams |
| Glucose | 1 gram |
| Agar | 1 gram |
| $KNO_3$ | 1 gram |
| Water | 1000 cc. |

This was adjusted to pH 7.6 and autoclaved in deep tubes. In other experiments the medium without $KNO_3$ was tubed and autoclaved and filtered aqueous solutions of $KNO_3$ or $KNO_2$ added subsequently.

Qualitative tests for nitrites were made in the usual manner with Tittsler's (1930) sulphanilic acid, dimethyl-a-naphthylamine reagent. Where the qualitative nitrite reaction was negative, tests were made for nitrate by adding zinc dust, as suggested by Zo Bell (1932), to reduce remaining nitrate to nitrite. A quantitative nitrite reaction was devised by modifying the qualitative reaction. Aqueous solutions of $KNO_2$ were made up to contain 0.005 to 0.15 mg. per cent. To 5 ml. amounts, 1 ml. of Tittsler's mixed sulphanilic acid and dimethyl-a-naphthylamine reagent was added. After 15 minutes, readings were then made on a photoelectric colorimeter (Cenco photelometer with a No. 2 filter). The results

[1] Part of an investigation aided financially by the Canadian National Research Council.

were plotted on semi-log paper against the corresponding concentration of nitrite to form a basis for comparison with cultures. Cultures to be tested were centrifuged until clear and the supernatant diluted in water, usually 1–10, which largely obliterated the pale colour of the peptone solution. The test reagent was then added, readings made as in the case of the controls, concentrations read from the graph and multiplied by the dilution of the culture.

Twenty-one species belonging to the gas gangrene group of the genus *Clostridium* were grown in this medium and tested qualitatively for nitrites. Only five

TABLE 1

*Qualitative determinations of nitrites and nitrates on cultures of twenty-one species of the genus Clostridium in a medium containing 100 mg. per cent $KNO_3$*

| | SPECIES | NO. OF STRAINS | TESTS FOR $NO_2$ | | | TESTS FOR $NO_3$ | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 day | 2 days | 5 days | 1 day | 2 days | 5 days |
| Group I—$NO_3$ reduced rapidly, $NO_2$ present | *C. welchii* | 8 | + | + | + | Obscured by + $NO_2$ test | | |
| | *C. fallax* | 1 | + | + | + | | | |
| | *C. tertium* | 4 | + | + | + | | | |
| | *C. septicum* | 5 | + | + | + | | | |
| | *C. aerofoetidum* | 2 | + | + | + | | | |
| Group II—$NO_3$ reduced rapidly, $NO_2$ absent | *C. sordelli* | 4 | − | − | − | Tr. | − | − |
| | *C. bifermentans* | 2 | − | − | − | − | − | − |
| | *C. novyi* | 7 | − | − | − | Tr. | − | − |
| | *C. difficile* | 1 | − | − | − | Tr. | Tr. | − |
| | *C. paraputrificum* | 2 | − | − | − | Tr. | − | − |
| | *C. butyricum* | 4 | − | − | − | Tr. | Tr. | − |
| | *C. sporogenes* | 4 | − | − | − | Tr. | − | − |
| | *C. tetanomorphum* | 2 | − | − | − | Tr. | − | − |
| | *C. tetani* | 3 | − | − | − | Tr. | − | − |
| | *C. capitovalis* | 2 | − | − | − | Tr. | − | − |
| Group III—$NO_3$ reduced slowly or not reduced, $NO_2$ absent | *C. carnis* | 1 | − | − | − | + | + | + |
| | *C. histolyticum* | 4 | − | − | − | + | + | + |
| | *C. tyrosinogenes* | 2 | − | − | − | + | + | + |
| | *C. sphenoides* | 2 | − | − | − | + | + | + |
| | *C. cochlearium* | 5 | − | − | − | + | + | + |
| | *C. multifermentans* | 2 | − | − | − | + | + | + |

of the twenty-one species, *C. welchii*, *C. fallax*, *C. tertium*, *C. septicum* and *C. aerofoetidum*, as indicated in table 1, gave positive nitrite reactions. This is in agreement with most of the recorded findings, (Spray, 1936; Reed and Orr, 1941). But, as also indicated in table 1, ten of the twenty-one species, so break down the nitrate added to the medium that none is evident by the zinc-dust nitrite test. Cultures of the remaining six species (table 1), give positive tests for nitrate but show no accumulation of nitrites.

When quantitative tests for nitrite were made on cultures of the five species which gave positive nitrite tests (table 2) only small concentrations were found.

In the medium originally containing 100 mg. per cent of nitrate after 5 day's growth of *C. welchii* or *C. fallax*, less than 1 mg. per cent of nitrite was present and in cultures of *C. septicum* and *C. aerofoetidium* only about 10 mg. per cent of nitrite was present. This suggests that the $NO_2$ is itself being rapidly transformed and that the difference between this group (group I, table 1) in which $NO_2$ accumulated in measurable amounts and the next group (group II, table 1) in which no $NO_2$ could be detected is purely quantitative.

Quantitative nitrite tests of cultures of the twenty-one species after 1 to 5 days' growth in the peptone medium to which 10 mg. per cent of $NaNO_2$ was added, gave further evidence of the nature of the reaction (table 3). The almost complete disappearance of this amount of nitrite, probably by reduction to ammonia, from cultures of the five species which give positive qualitative nitrite tests when grown in a nitrate medium (group I, table 3) must indicate that ordinarily the reduction of nitrate to nitrite by these species is more rapid than the further reduction of nitrite. However, since the amount of nitrite

TABLE 2

*Quantitative $NO_2$ in mg. per cent. Cultures in peptone medium containing 100 mg. per cent of $KNO_3$. Determinations were made at 1, 2 and 5 days after inoculation*

| SPECIES | MG. PER CENT OF NITRITE | | |
|---|---|---|---|
| | 1 day | 2 days | 5 days |
| *C. welchii* | 0.20 | 0.51 | 0.58 |
| *C. fallax* | 1.20 | 0.63 | 0.66 |
| *C. tertium* | 3.10 | 3.20 | 4.10 |
| *C. septicum* | 10.80 | 11.70 | 12.35 |
| *C. aerofoetidum* | 9.85 | 10.10 | 12.80 |
| Sterile medium | 0 | 0 | 0 |

which accumulates is very small (table 2) it is quite likely that under different circumstances the rate of the two reactions may be equalized, in which case the qualitative tests for nitrite will become negative. This probably accounts for the considerable confusion which occurs in the literature.

Cultures of the ten species in group II, table 3 in media initially containing nitrate give *negative* qualitative reactions for both nitrates and nitrites. When grown in the peptone solution to which 10 mg. per cent of $NaNO_2$ is added the species of group II, like those of group I, show rapid breakdown of the nitrite. It therefore follows that when these species are grown in nitrate medium the rate of reduction of $NO_3$ to $NO_2$ must be equal to, or less than, the rate of reduction of $NO_2$. Here too is a likely source of error. It may be anticipated that under other circumstances the rates of the two reactions, relative to each other, may alter, in which case positive $NO_2$ reactions might be observed.

The species which make up the last group (group III, table 3) produce slow or negligible reduction of nitrite. Since in a nitrate-containing medium these species give negative qualitative reactions for $NO_2$ (table 1) and strongly positive

nitrate tests, it must be concluded that the reduction of $NO_3$ is also slow or does not occur.

### INDOLE FORMATION

Hertzfeld and Klinger (1915) demonstrated a quantitative transformation of tryptophane to indole in cultures of *Escherichia coli*, due presumably to the enzyme tryptophanase recently described by Happold and Hoyle (1935). On

TABLE 3

*Quantitative $NO_2$ in mg. per cent. Cultures in peptone medium containing 10 mg. per cent $NaNO_2$. Determinations made at 1, 2, and 5 day intervals after inoculation*

| | SPECIES | MG. PER CENT OF NITRITE | | |
|---|---|---|---|---|
| | | 1 day | 2 days | 5 days |
| Group I—reduce $NO_3$ more rapidly than $NO_2$ | *C. welchii* | Tr. | 0 | 0 |
| | *C. fallax* | 0 | 0 | 0 |
| | *C. tertium* | 1.07 | 0.55 | 0.80 |
| | *C. septicum* | 0 | 0 | 0 |
| | *C. aerofoetidum* | 0 | 0 | 0 |
| Group II—reduce $NO_2$ more rapidly than $NO_3$ | *C. sordelli* | 0 | 0 | 0 |
| | *C. bifermentans* | 0 | 0 | 0 |
| | *C. sporogenes* | 1.15 | 0.56 | 0 |
| | *C. novyi* | 0.42 | 0.23 | 0 |
| | *C. tetani* | 1.43 | 0 | 0 |
| | *C. tetanomorphum* | 0.05 | 0 | 0 |
| | *C. difficile* | 0 | 0 | 0 |
| | *C. paraputrificum* | 0 | 0 | 0 |
| | *C. butyricum* | 1.15 | 0 | 0 |
| | *C. capitovalis* | 5.30 | 1.50 | 0.05 |
| Group III—reduce $NO_3$ and $NO_2$ slowly or not at all | *C. carnis* | 6.90 | 4.30 | 3.80 |
| | *C. histolyticum* | 7.05 | 7.20 | 4.60 |
| | *C. tyrosinogenes* | 8.25 | 5.90 | 6.30 |
| | *C. sphenoides* | 8.70 | 7.50 | 7.00 |
| | *C. cochlearium* | 8.80 | 7.10 | 7.55 |
| | *C. multifermentans* | 9.45 | 8.10 | 7.70 |
| | Sterile medium | 9.40 | 8.30 | 7.80 |

the other hand, Happold and Hoyle (1936) have shown that *E. coli*, growing in a synthetic medium, slowly decomposes indole. It has also been shown by Sasaki (1923) that *B. subtilis*, and by Supniewski (1924) that *Pseudomonas aeruginosa* oxidizes indole to anthranilic acid, and by Gray (1928) that *Pseudomonas indoloxidans* will oxidize indole to indigotin. While it is unlikely that the anaerobic bacteria will oxidize indole, these observations do suggest a possible breakdown of indole as it is formed.

Indole determinations have been made on cultures of species of the genus

*Clostridium* after one to ten days' growth in the following medium, Reed and Orr (1941):

| | *grams* |
|---|---|
| Bacto tryptone | 20 |
| $Na_2HPO_4$ | 5 |
| Glucose | 1 |
| Agar | 1 |
| Sodium thioglycollate | 1 |
| Water | 1000 ml. |

The medium was adjusted to pH 7.6 and autoclaved in deep tubes. For some experiments this was modified by the addition of an aqueous solution of indole,

TABLE 4

*Indole reactions of 17 species of Clostridia when grown for 1 to 10 days in a tryptophane medium without added indole and with 2 mg. and 10 mg. per cent of indole*

| | SPECIES | NO. OF STRAINS | MEDIUM CONTAINING NO ADDED INDOLE | | | | MEDIUM CONTAINING 2 MG. PER CENT INDOLE | | | | MEDIUM CONTAINING 10 MG. PER CENT INDOLE | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 day | 2 days | 5 days | 10 days | 1 day | 2 days | 5 days | 10 days | 1 day | 2 days | 5 days | 10 days |
| Group I—indole accumulates | *C. sordelli* | 3 | + | + | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | ++++ |
| | *C. bifermentans* | 2 | + | + | + | ± | ++ | ++ | + | + | +++ | +++ | ++ | + |
| | *C. capitovalis* | 2 | + | + | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | ++++ |
| | *C. sphenoides* | 2 | + | + | + | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ |
| Group II—indole used as rapidly as formed | *C. welchii* | 3 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | +++ | ++ | + | + |
| | *C. sporogenes* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *C. novyi* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++ | + | + | 0 |
| | *C. tetani* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 |
| | *C. carnis* | 1 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | ++ | ++ | 0 | 0 |
| | *C. fallax* | 1 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | ++ | ++ | + | 0 |
| | *C. septicum* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++ | ++ | + | + |
| | *C. multifermentans* | 2 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | ++ | ++ | + | + |
| | *C. histolyticum* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++ | ++ | + | + |
| | *C. tetanomorphum* | 2 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | ++ | ++ | + | 0 |
| | *C. difficile* | 1 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | + | 0 | 0 | 0 |
| | *C. aerofoetidum* | 2 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | ++ | ++ | + | 0 |
| | *C. paraputrificum* | 2 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | ++ | ++ | + | 0 |
| | Sterile medium | | 0 | 0 | 0 | 0 | ++ | ++ | ++ | ++ | +++ | +++ | +++ | ++ |

sterilized by filtration, to provide 2 mg. per cent or 10 mg. per cent of added indole.

Qualitative tests for indole were made on cultures after 1, 2, 5 and 10 days' incubation with Fellers and Clough's (1925) modification of Ehrlich's reagent.

A summary of the results of these tests on cultures of several strains of seventeen species grown in the tryptophane medium without added indole, with 2 mg. per cent, and 10 mg. per cent of indole are shown in table 4. From the first column it is apparent that indole accumulates in measurable amounts in cultures in the tryptophane medium of only four of the seventeen species tested, *C. sordellii*, *C. bifermentans*, *C. capitovalis*, and *C. sphenoides*. This is in agreement with previous reports (Spray, 1936; Reed and Orr, 1941).

The remaining thirteen species tested, it will be observed from the table, do not accumulate measurable amounts of indole in the tryptophane medium. But, it is also apparent from the table, that indole added to the medium in which these organisms are grown rapidly disappears. There is a great difference, however, in the rate of disappearance. In the medium with 2 mg. per cent of added indole, five species bring about its disappearance in twenty-four hours and all thirteen species in two days. In the media with 10 mg. per cent of indole, *C. sporogenes* causes complete disappearance in twenty-four hours, but in cultures of four species, detectable amounts are still present after ten days' incubation.

It seems probable therefore that all thirty-three cultures belonging to the seventeen species tested produce indole from tryptophane, but that all except four species either utilize or break down indole as rapidly as formed.

It is apparent from the qualitative reactions indicated in the table that a species like *C. bifermentans* breaks down or utilizes indole but that ordinarily the rate of indole formation from tryptophane is more rapid than the rate of disappearance. Under other conditions it is possible that the rates of the two reactions may be equalized. Conversely *C. welchii* brings about the disappearance of indole more slowly than most of the other species grouped with it, yet rapidly enough to prevent the accumulation of indole from tryptophane. A slight modification in the rate of either of these reactions would probably result in indole accumulation.

## CONCLUSIONS

It is shown that the gas gangrene species of the genus *Clostridium* fall into three groups in respect to the reduction of nitrates and nitrites: (1) Five species which reduce both $NO_3$ and $NO_2$ but in which the rate of reduction of $NO_3$ is ordinarily more rapid than the reduction of $NO_2$; as a result qualitative reactions for $NO_2$ are generally positive. (2) Ten species which also reduce both $NO_3$ and $NO_2$ but in which the rate of reduction of $NO_2$ is equal to or greater than the rate of $NO_3$ reduction. As a result qualitative tests for $NO_2$ are ordinarily negative. (3) Six species which fail to reduce both $NO_3$ and $NO_2$ or reduce them at an equally slow rate, so that qualitative tests for $NO_2$ are regularly negative.

It is also shown that these species fall into two groups in respect to indole formation: all species tested break down or utilize indole but (1) four species grown in a tryptophane medium give positive indole tests in which the rate of indole formation is greater than the rate of indole breakdown (2) thirteen species in which the rate of indole formation is equal to or less than the rate of indole breakdown.

## REFERENCES

CONN, H. J. 1936 On the detection of nitrate reduction. J. Bact., **31**, 225–233.

FELLERS, C. R., AND CLOUGH, R. W. 1925 Indole and skatole determinations on bacterial cultures. J. Bact., **10**, 105–133.

GRAY, P. H. H. 1928 The formation of indigotin from indole by soil bacteria. Proc. Roy. Soc., London, **102**, 263.

HAPPOLD, F. C., AND HOYLE, L. 1935 The coli-tryptophane-indole reaction: I. Enzyme preparations and their action on tryptophane and some indole derivatives. Biochem. J., **29,** 1918–1926.

HAPPOLD, F. C., AND HOYLE, L. 1936 The coli-tryptophane-indole reaction: II. The non-production of tryptophanase in media containing glucose. Brit. J. Exptl. Path., **17,** 136–143.

HERTZFELD, E., AND KLINGER, R. 1915 Quantitative Untersuchungen über den Indol- und Tryptophanumsatz der Bacterien. Zentr. Bakt., Parasitenk., I, orig., **76,** 1.

REED, G. B., AND ORR, J. H. 1941 Rapid identification of gas gangrene anaerobes. War Medicine, **1,** 493–510.

SASAKI, T. 1923 Über die Bildung des Anthranilsäure aus *l*-tryptophan durch Subtilisbacterien. J. Biochem., **2,** 251.

SPRAY, R. S. 1936 Semisolid media for the cultivation and identification of the sporulating anaerobes. J. Bact., **32,** 135–155.

SUPNIEWSKI, J. 1924 Der Stoffwechsel der Zyklischen Verbindungen bei *B. pyocyaneus*. Biochem. Z., **146,** 522.

TITTSLER, R. P. 1930 The reduction of nitrates to nitrites by *Salmonella pullorum* and *Salmonella gallinarum*. J. Bact., **19,** 261–267.

WOODS, D. D. 1938 The reduction of nitrate to ammonia by *Clostridium welchii*. Biochem. J., **32,** 2000–2012.

ZO BELL, C. E. 1932 Factors influencing the reduction of nitrate and nitrite by bacteria in semi-solid media. J. Bact., **24,** 273–281.