SPECIATION WITHIN THE GENUS BRUCELLA

III. Nitrate Reduction and Nitrite Toxicity Tests

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Reduction of nitrate has been employed by several workers as a test for distinguishing species in the genus *Brucella* (*cf* Huddleson, 1943). We examined this test briefly during our studies on the dye sensitivity of brucellae (Pickett *et al.*, 1952) and concluded that it should serve at least as a useful guide for identification of the species. However, further study disclosed that the outcome of qualitative nitrate reduction tests is determined not only by the species but also by the nature and concentration of reductant in the test medium, the initial concentration of nitrate, the amount of inoculum, and the period of incubation. With these considerations in mind, we attempted to develop a nitrate reduction test which would serve as a useful adjunct for identification of these species. Though this attempt did not reach fruition, these studies did lead to the development of an alternative "nitrite toxicity" test which conveniently distinguishes *Brucella melitensis*.

METHODS

Inocula. The routine growth medium was Albimi's brucella agar enriched with thiamin, nicotinamide, and hemin (Liberman and Pickett, 1952). Working suspensions of cells for experimental inocula were obtained from forty-eight hour slants which had been incubated at 35 C under 10 per cent carbon dioxide. The growth on these slants was suspended in sterile distilled water, and the concentration of such suspensions was determined photometrically.

Nitrate reduction tests. Several media and inocula were examined during exploratory experiments. The former included phosphate buffer, pH 6.5 to 7.5, 0.02 M, with glucose, glycerol, or lactate as reductant; Albimi's C peptone, 1 and 2 per cent, both with and without 0.5 per cent glucose; and Albimi's brucella broth. The inocula (0.1 ml in 1.0 ml medium) varied in concentration from 1×10^8 to 500×10^8 cells per ml. The procedure selected as most satisfactory for nitrate reduction tests was as follows: (1) Nitrate medium—0.05 per cent sodium nitrate in 2 per cent of Albimi's C peptone, 1 ml in a 12 by 100 mm tube. (2) Inoculum—0.1 ml of a stock suspension containing 5×10^9 bacteria per ml. (3) Incubation—35 C, aerobic. (4) Nitrite reagent—0.5 per cent sulfanilic acid and 0.5 per cent α-naphthylamine in 1 per cent acetic acid. (5) Nitrite detection—one 4 mm loop of nitrate broth culture was added to one drop of nitrite reagent. Both faintly pink and frankly red tests were recorded as positive. Tests were made after 6, 24, 30, and 48 hours of incubation; cultures which were nitrite negative at 48 hours were tested for nitrate by depositing a few mg of granular zinc and six drops of nitrite reagent directly into the culture tube.

Nitrite toxicity tests. Nitrite tablets were prepared and employed according to the procedure already described for carbamate tablets (Pickett *et al.*, 1953). Initial trials with tablets containing 10, 5, 4, 2, 1, and 0.5 per cent of sodium nitrite led to the selection of 2 per cent nitrite as that concentration which gave most satisfactory results.

RESULTS

Nitrate reduction. Figures 1 and 2 illustrate the type of results which were obtained from exploratory experiments with representative typical strains of *Brucella abortus* and *B. melitensis*. It is apparent from figure 1 that, with relatively large inocula (50×10^8 cells), both species are "nitrite positive" as judged by qualitative nitrite tests made at any time between 2 and 48 hours of incubation. However,

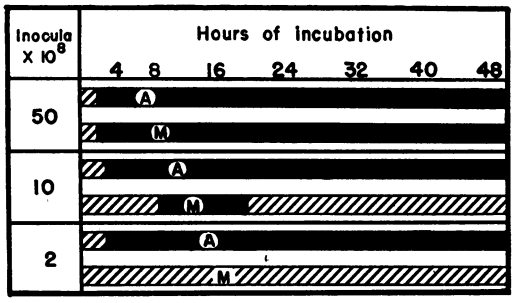

Figure 1. The effect of concentration of inoculum (cells per 0.1 ml) on the nitrite test. Medium, 0.05 per cent sodium nitrate in 2 per cent of Albimi's C peptone, pH 7.0; incubation, 35 C, aerobic; A, *Brucella abortus*, strain A9; M, *Brucella melitensis*, strain M3; crosshatch, nitrite negative; black, nitrite positive.

with smaller inocula (10 × 10^8 cells) the results obtained with *B. melitensis* will depend upon the time of test, and with still smaller inocula (2 × 10^8 cells) the nitrite test appears to distinguish these two species. The importance of initial nitrate concentration, as well as size of inoculum and time of test, is illustrated in figure 2, from which it may be seen that *B. abortus*, as well as *B. melitensis*, appears to be "nitrite negative" when a medium containing 0.01 per cent nitrate is employed and the test is made after 48 hours' incubation. These results clearly indicate that both species decompose both nitrates and nitrites, that the species differ in relative enzymic activities, and that these differences may not be manifest under conditions of large inocula or high initial concentration of nitrate.

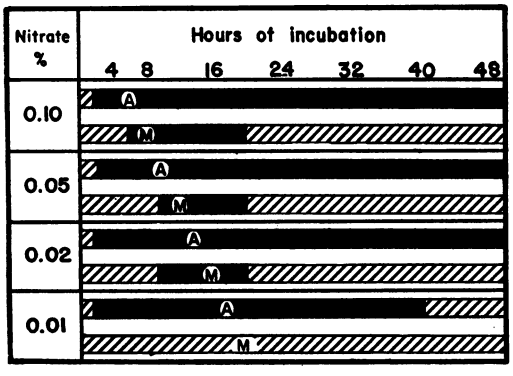

Figure 2. The effect of initial concentration of nitrate on the nitrite test. Inoculum, 1 × 10^9 cells; other experimental conditions and notations as in figure 1.

TABLE 1

Nitrate reduction tests on 82 strains of brucellae

SPECIES	NUMBER OF STRAINS	NUMBER NITRITE POSITIVE AT:			
		6 hr	24 hr	30 hr	48 hr
Typical					
Brucella abortus	21	17	17	13	8
Brucella melitensis	18	9	4	3	2
Brucella suis	24	18	13	11	9
Atypical					
Brucella abortus	7	5	5	4	1
Brucella melitensis	4	0	0	1	0
Brucella suis	8	5	6	7	5

The experimental conditions which appeared best to distinguish strains of *B. melitensis*, namely 0.05 per cent nitrate in 2 per cent Albimi C peptone and an inoculum of 5 × 10^8 cells, were employed in the examination of additional strains of brucellae (table 1). Strains which give slightly or frankly atypical differential dye, sulfide, urease, and carbamate tests (Pickett *et al.*, 1953) were included intentionally among the 82 which were examined. These data show that under the experimental conditions employed here, and when the nitrite test is performed at 24 hours, *B. melitensis* is usually nitrite negative while *B. abortus* and *B. suis* are usually nitrite positive. At this time the per cent of strains which gave positive nitrite tests were 18, 79, and 59, respectively. At 48 hours, however, the corresponding figures were 9, 32, and 44. At best, therefore, qualitative nitrate reduction tests on brucellae provide only suggestive information in regard to species.

Nitrite toxicity. The results obtained during examination of the nitrate reduction test suggested that metabolically formed nitrite may accumulate and inhibit decomposition of this compound (figure 1). The data also suggested the following relationship among the variables where the lengths of the arrows are approximately proportional to the relative enzymic activities and the number of diagonal bars indicate relative inhibition by nitrite:

B. abortus: NO_3^- → NO_2^- → [?]

B. melitensis: NO_3^- ⟶ NO_2^- —/-/-/-/-/⟶ [?]

B. suis: NO_3^- → NO_2^- —/⟶ [?]

TABLE 2

Nitrite toxicity tests on 70 strains of brucellae

GROUP	STRAINS +/STRAINS TESTED*		
	Brucella abortus	*Brucella melitensis*	*Brucella suis*
Typical strains	0/13	26/29	0/11
Slightly atypical	0/3	2/2	0/4
Frankly atypical	0/4	3/3	0/1
Totals	0/20	31/34	0/16

* A positive test indicates complete inhibition of growth in a zone extending 4 mm or more beyond the margin of the 4 mm, 50 mg, 2 per cent nitrite tablet.

Nitrite toxicity was examined therefore to determine whether it could be employed as a reliable means for distinguishing these species. The results summarized in table 2 show that *B. melitensis* is distinguished by this test; all 36 strains of *B. abortus* and *B. suis* gave negative nitrite toxicity tests, and only 3 of the 34 strains of *B. melitensis* failed to give positive tests.

DISCUSSION

Identification of species within the genus *Brucella* has long been a problem because of their close similarity in biochemical and serological tests. We have been attempting for some time to evaluate the several procedures which have been employed for this purpose and have already found the differential dyes of Huddleson and hydrolysis of urea to be practical and reliable tests for the identification of typical and slightly atypical strains. Conversely, no satisfactory correlation was found between these (and serological) tests and sulfide production.

Upon turning to an examination of the nitrate reduction test, we first attempted to employ the semisolid nitrate media of Zobell and Meyer (1932) but obtained neither reproducible nor differential results by their procedures. Subsequent attempts to employ nitrate broth media soon disclosed the relatively complex nature of the test when it is applied to species which reduce both nitrates and nitrites, and for which metabolically formed nitrite may somewhat unpredictably affect the over-all reaction. Pertinent data, accumulated during our examination of this test, on which further consideration of the problem should be based may be summarized as follows:

(1) All three species decompose both nitrates and nitrites. The reductant may be glucose or lactate in phosphate buffer, pH 6.5 to 7.0, 0.02 M; or peptone (1 or 2 per cent Albimi C). Satisfactory test conditions were not provided by glycerol in phosphate buffer, by glucose or lactate in phosphate buffer at pH 7.5, or by Albimi's brucella broth. Two per cent of Albimi's C peptone at pH 6.8 to 7.0 was the most satisfactory basal medium examined, and supplementing the 2 per cent peptone with 0.5 per cent glucose (as additional reductant) did not influence favorably the results obtained.

(2) Under favorable experimental conditions there is a significant difference among the species in respect to the amount of metabolically formed nitrite which accumulates in a nitrate broth medium, and this appears to be referable more to the relative nitritase activities than to the nitratase activities. Nitrite accumulation is in the order *B. abortus* > *B. suis* ≧ *B. melitensis*. It follows that *negative* nitrite tests at appropriate time intervals might contribute toward identification of brucellae. This conversion from "nitrite positive" to "nitrite negative" was shown in figures 1 and 2.

(3) Under the unfavorable experimental conditions of a large inoculum and a high initial concentration of nitrate, sufficient nitrite will accumulate in the medium to inhibit nitritase activity. The species differ in their sensitivity to nitrite, toxicity being in the order *B. melitensis* > *B. suis* ≧ *B. abortus*. Failure to appreciate this nitrite toxicity, particularly in the instance of *B. melitensis*, can lead to apparently contradictory results (figures 1 and 2).

With the above points in mind we attempted to establish experimental conditions which would distinguish clearly between *B. melitensis* and the other two species. However, when additional strains of brucellae were examined (table 1), the nitrate reduction test which was finally adopted did not give results that assisted materially in their identification. Hence it now appears to us unlikely that the classical nitrite test can be adapted as a tool for speciation within this genus because the variables are, in a sense, too great to encompass the variations found within each species.

Since the nitrate reduction test was unsatisfac-

tory for our purposes, we chose as an alternative to utilize only one of the variables, nitrite toxicity. Thus, it was possible to establish a reproducible and relatively simple test which gives results that correlate well with other characteristics of the brucellae and which distinguishes between *B. melitensis* and the other two species. This test appears to be particularly helpful in the examination of strains which give atypical results in dye and carbamate sensitivity tests. The four frankly atypical strains of *B. abortus*, for example (table 2), are acutely sensitive to all differential dyes and had been identified provisionally by urease and serological tests (Pickett *et al.*, 1953); the results obtained here in the nitrite toxicity tests therefore advantageously support our original species designation.

SUMMARY

Reduction of nitrate by brucellae was examined with the hope that a reliable test which would help to differentiate the species in this genus could be developed. All three species were shown to reduce both nitrate and nitrite, and significant differences among the species in rates of reduction were observed. However, variations among strains within each species, both in rates of reduction of these substrates and in sensitivity to metabolically formed nitrite, were of such magnitude that no satisfactory differentiation of species could be made with a qualitative nitrate reduction test. The frank and consistent sensitivity of *Brucella melitensis* to nitrite was developed therefore into a simple and reliable nitrite toxicity test which distinguishes strains of this species.

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