COMPARATIVE METABOLISM OF SPECIES AND TYPES OF ORGANISMS WITHIN THE GENUS *BRUCELLA*

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The classification of organisms in the genus *Brucella* recognizes three species. Huddleson (1957) expanded this classification by subgrouping the species into types in order to accommodate taxonomically the increased number of organisms reported to be atypical of a species (Wilson, 1933; Renoux and Carrere, 1952; Renoux, 1952*a*, *b*; Cruickshank, 1954; Huddleson and White, 1954; Pickett *et al.*, 1953; Pickett and Nelson, 1955).

Meyer and Cameron (1957, 1958) reported that typical organisms of each of the three species displayed distinctive and characteristic metabolic patterns under manometric conditions on substrates of amino acids and carbohydrates. Gram-negative microorganisms that are morphologically similar to brucellae, one of which is tentatively classified within this genus (Buddle and Boyes, 1953), could be differentiated from brucellae by its oxidative metabolism (Meyer and Cameron, 1956). Organisms that have been classified within the genus, but which are atypical of the existing species (Stoenner and Lackman, 1957), can also be identified by this method (Cameron and Meyer, 1958). It was therefore considered highly desirable to extend these investigations to include all atypical organisms (types) within each species in order to elucidate, if possible, the metabolic features that constitute an atypical organism. It was further believed that an investigation of this nature might help determine some of the fundamental biological relationships that must surely exist between organisms within a genus. This paper, then, is a report upon the comparative metabolism of all species and types within the genus *Brucella*.

MATERIALS AND METHODS

The cultures of brucellae employed in this investigation were obtained from a number of laboratories. Insofar as it was possible to ascertain, the source, brief history, and species identification of the culture at time of receipt, are presented in table 1.

Organisms were used only as smooth forms, as determined by the methods of Braun and Bonestell (1947) and White and Wilson (1951). Tryptose agar (Difco), dispensed in Roux flasks, slants, and plates, was used as the growth medium. Conventional procedures as recommended by the World Health Organization Expert Committee on Brucellosis (1953) were used for species identification.

To obtain resting cell suspensions for manometry, Roux flasks containing tryptose agar were inoculated with 2.5 ml of a saline suspension of the desired culture, incubated 24 hr at 37.5 C, and the resultant growth harvested, washed, and suspended in Sorenson's 0.06 M phosphate buffer at pH 7.0. Cell concentrations were adjusted on a Coleman spectrophotometer and cellular nitrogen determined by a micro-Kjeldahl technique. Complete details for cell preparations have been described earlier by Cameron and Meyer (1953, 1955).

The substrates were dissolved in Sorenson's 0.06 M phosphate buffer and where necessary, the pH of the solution was adjusted to 7.0 by the addition of sodium hydroxide. Conventional manometric techniques were employed to determine oxygen uptake (Umbreit *et al.*, 1957). Each flask contained 1.0 ml of cell suspension, 0.5 ml solution containing 5 mg of the desired substrate, 1.4 ml buffer, and 0.1 ml of 20 per cent KOH. Endogenous respiration rates were determined for each experiment and all experiments were repeated on several harvestings of cells from various lots of media. The figures given in the results are ($Q_{O_2}N$) values with the endogenous rates subtracted.

RESULTS

The comparative effect of basic fuchsin and thionin upon the growth of all types of organisms within the *Brucella* species is shown in table 2,

TABLE 1

Source, history, and identification of brucella cultures

Culture No.	Species	Source and History of Culture
19	*B. abortus*	U. S. Dept. of Agr. Standard vaccine strain.
2308	*B. abortus*	U. S. Dept. of Agr. Standard challenge strain.
53	*B. abortus*	Univ. of Calif. at Davis. Recent isolate from milk.
544	*B. abortus*	Designated as a reference strain by World Health Organization.
57	*B. suis*	Isolated from blood culture of human case of brucellosis by Calif. State Dept. of Public Health. Maintained on tryptose agar since 1952.
55	*B. suis*	Isolated from aborted swine fetus by Univ. of Calif. at Davis. Maintained as stock culture on liver agar and tryptose agar since 1930.
4103	*B. melitensis*	Isolated from blood culture of human case of brucellosis by Calif. State Dept. of Public Health. Maintained on tryptose agar since 1953.
281	*B. melitensis*	Isolated from a sheep in South Africa. Obtained as lyophilized culture from Dr. C. M. Carpenter.
Sc-3a-20	*B. abortus*	Isolated from human case of brucellosis by Kiser Laboratories in 1952. Lyophilized culture obtained from Dr. M. J. Pickett. Described as acutely dye sensitive, atypical *B. abortus*.
Sc-3a-103	*B. abortus*	Isolated from human case of brucellosis by Holdeman, Communicable Disease Center in 1952. Lyophilized culture obtained from Dr. M. J. Pickett. Described as acutely dye sensitive, atypical *B. abortus*.
Sc-4m-6	Species intermediate	Isolated in northern France. Lyophilized culture obtained from Dr. M. J. Pickett. Described as *B. abortus* antigenically, *B. melitensis* in dye behavior, *B. abortus-B. melitensis* intermediate in fermentation tests.
Sc-4m-40	Species intermediate	Isolated in Switzerland. Lyophilized culture obtained from Dr. M. J. Pickett. Same characteristics as Sc-4m-6.
Sc-3a-105	Species intermediate	Obtained as lyophilized culture from Dr. M. J. Pickett. History and source not known.
Sc-3a-106	Species intermediate	Isolated by Dr. Thiago de Mello, Institute Oswaldo Cruz in 1952. Lyophilized culture obtained from Dr. M. J. Pickett.
Sc-3a-128 Sc-3a-131 Sc-3a-132	Species intermediate	Originally isolated in England (see Cruickshank, 1954). Obtained as lyophilized cultures from Dr. M. J. Pickett.
Sc-4m-31 Sc-4m-33	Species intermediate	Originally isolated at the Univ. of Pisa, Santo Padre. Obtained as lyophilized cultures from Dr. M. J. Pickett. Described as *B. melitensis* antigenically and in dye behavior, *B. abortus* in fermentation tests.
Sc-4m-47	Species intermediate	Isolated from a goat in Iran.
Sc-4m-48	Species intermediate	Isolated from a sheep in Iran. Obtained as lyophilized cultures from Dr. M. J. Pickett. Described as *B. abortus-B. suis-B. melitensis* intermediates.
Sc-3s-25	*B. suis* atypical	Originally isolated by the laboratory of the Indiana State Board of Health in 1952. Obtained as lyophilized culture from Dr. M. J. Pickett. Described as fuchsin-fast *B. suis*.
Sc-3s-102	Species intermediate	Isolated by the Wadsworth Veterans Hospital in 1954. Obtained as lyophilized culture from Dr. M. J. Pickett. Described as *B. suis-B. melitensis* intermediate.
Sc-4m-8	*B. suis* atypical	Isolated from human case by Holdeman, Communicable Disease Center in 1952. Obtained as lyophilized culture from Dr. M. J. Pickett. Described as fuchsin-fast *B. suis*.
1813	Danish *B. suis*	Obtained from Dr. I. F. Huddleson.
5K33 5E1266	*B. neotomae*	Obtained from Dr. H. G. Stoenner. Cultures are separate isolations from pooled viscera of wood rats.

TABLE 2

Brucella cultures in table 1 classified into species and types by comparative growth on basic fuchsin and thionin; hydrogen sulfide production also shown

Culture No.	Dye Conc			H_2S Production	Species and Type
	Basic fuchsin	Thionin			
	10*	10*	50*		
544	+	−	−	++	*B. abortus* type I
19	+	−	−	++	
2308	+	−	−	++	
53	+	−	−	++	
Sc-3a-20	−	−	−	++	*B. abortus* type II
Sc-3a-103	−	−	−	++	
Sc-4m-6	+	+	−	++	*B. abortus* type III
Sc-4m-40	+	+	−	+	
Sc-3a-105	+	+	−	+	
Sc-3a-106	+	+	−	+	
Sc-3a-128	+	+	−	−	
Sc-3a-131	+	+	−	−	
Sc-3a-132	+	+	−	−	
4103	+	+	−	−	*B. melitensis* type I
281	+	+	−	−	
Sc-4m-31	+	+	−	−	
Sc-4m-33	+	+	−	−	
Sc-4m-47	+	+	−	−	
Sc-4m-48	+	+	−	−	
57	−	+	+	++++	*B. suis* type I
55	−	+	+	++++	
1813	−	+	+	−	*B. suis* type II
Sc-3s-25	+	+	+	−	*B. suis* type III
Sc-3s-102	+	+	+	−	
Sc-4m-8	+	+	+	−	
5K33	−	−	−	++++	*B. neotomae*
5E1266	−	−	−	++++	

* Micrograms of dye per ml of media.

as is the hydrogen sulfide production. Although the majority of brucellae organisms will show a species specific pattern of growth that is compatible with other identifying characteristics, it is evident that there are also growth patterns shared by types classified as belonging to different species. It may be observed from table 2 however, that all types within the *Brucella suis* species will grow on five times greater concentrations of thionin than will any of the other organisms within this genus.

Table 3 shows the oxidative metabolism of the species and types within this genus on substrates of amino acids and carbohydrates. Included in this table are only those substrates which contributed to species and type differentiation. The following substrates were also investigated: D- and L-alanine, L-aspartic acid, L-proline, adonitol, fructose, glucose, and D-xylose. The oxidative pattern of species types on substrates in the Krebs cycle (acetate, α-ketoglutarate, citrate, lactate, malate, pyruvate, oxalacetate, and succinate) was similar to that previously reported for the three species (Meyer and Cameron, 1958).

As may be seen from table 3, discrete quantitative and qualitative metabolic differences exist between the three species and also between most of the types within the species on selected amino acid and carbohydrate substrates.

Brucella abortus type I shows the dye growth and metabolic patterns that are characteristic for this species. *B. abortus* type II fails to grow on basic fuchsin. However, its metabolic pattern is the same as that of type I. *B. abortus* type II is the only type within a species for which both basic fuchsin and thionin are bacteriostatic, and is also the only type within a species that, although showing altered dye behavior, retains a metabolic pattern that is characteristic for the species. *B. abortus* type III grows in the presence of both dyes. By its metabolic pattern it can be distinguished from the other two types of *B. abortus* in its failure to oxidize L-arabinose, D-galactose, and D-ribose.

Brucella melitensis type I can readily be distinguished from types I and II of *B. abortus* by the differences that exist in their metabolism of carbohydrates, and from all types of *B. suis* in both carbohydrate and amino acid metabolism. However, *B. melitensis* type I and *B. abortus* type III, which are identical in their dye growth, are also identical in their metabolic patterns.

B. suis is remarkably different in its metabolic pattern, both in carbohydrate and amino acid utilization, from all types of *B. abortus* and *B. melitensis*. The three types within this species

TABLE 3

Comparative oxidative rates (Qo_2N) on amino acid and carbohydrate substrates by species and types in the genus Brucella

Species and Type	Culture No.	Substrates								
		L-Arginine	L-Asparagine	DL-Citrulline	L-Glutamic acid	L-Lysine	DL-Ornithine	L-Arabinose	D-Galactose	D-Ribose
B. abortus type I	544	17	151	40	280	20	50	78	150	180
	19	40	198	50	490	6	40	45	50	158
	2308	18	182	30	296	0	30	56	112	285
	53	18	120	0	420	0	0	122	156	263
B. abortus type II	Sc-3a-20	15	101	20	285	10	17	85	127	228
	Sc-3a-103	6	133	0	398	0	0	54	61	231
B. abortus type III	Sc-4m-6	0	250	0	258	0	0	0	10	30
	Sc-4m-40	0	133	5	180	0	0	8	4	0
	Sc-3a-105	0	107	0	206	0	0	0	0	0
	Sc-3a-106	10	50	20	110	10	18	0	5	0
	Sc-3a-128	0	24	0	112	0	0	0	10	0
	Sc-3a-131	6	85	10	151	20	0	0	0	0
	Sc-3a-132	10	102	0	120	30	0	12	0	0
B. melitensis type I	4103	10	166	0	310	10	0	0	10	19
	281	0	222	0	213	0	0	4	0	10
	Sc-4m-31	10	90	5	100	15	10	0	0	0
	Sc-4m-33	15	106	0	112	10	19	0	0	0
	Sc-4m-47	0	90	0	120	0	0	10	8	40
	Sc-4m-48	8	111	5	163	0	10	20	14	0
B. suis type I	57	106	0	115	60	90	158	320	210	228
	55	107	0	137	34	150	230	384	317	370
B. suis type II	1813	50	130	118	135	14	153	33	52	360
B. suis type III	Sc-3s-25	52	0	98	60	145	115	48	8	349
	Sc-3s-102	85	0	108	50	70	130	30	25	326
	Sc-4m-8	66	0	100	58	83	106	20	30	342
B. neotomae	5K33	0	91	0	469	30	0	363	365	33
	5E1266	0	84	0	255	29	0	192	379	295

can also be differentiated metabolically. *B. suis* type I characteristically utilizes L-arginine, DL-citrulline, L-lysine, DL-ornithine, L-arabinose, D-galactose, and D-ribose. It consistently displays low oxidative rates on L-glutamic acid, and does not oxidize D- or L-asparagine. *B. suis* type II does not oxidize lysine and will oxidize L-glutamic acid and L-asparagine. It can be further distinguished from type I by its very low uptake rates on L-arabinose and D-galactose. A unique feature of this organism is found in its dye characteristics, in that it is the only type within a species that retains the patterns associated with the species, whereas all other types show an alteration in this characteristic.

B. suis type III displays the same amino acid metabolic pattern as type I, but differs in its very low oxygen uptake on the carbohydrate substrates of L-arabinose and D-galactose.

Brucella neotomae will not grow in the presence of either basic fuchsin or thionin, and has a metabolic pattern that is distinctive from the three species and all types. *B. neotomae* has characteristics in its amino acid metabolism

that are similar to *B. abortus* and *B. melitensis*, and completely dissimilar to *B. suis*. However, in carbohydrate metabolism it is like *B. suis* in its utilization of L-arabinose and D-galactose. Its utilization of D-ribose varies from culture to culture.

DISCUSSION

The species which constitute the genus *Brucella* are remarkably homogeneous. As proliferating cells, the species can be distinguished only by a limited number of biochemical tests that measure quantitative differences. Serological identity is also measured quantitatively by agglutinin adsorption techniques. This species homogeneity poses a taxonomic question as to whether sufficient differences exist between the organisms to justify species status. Renoux (1952*a*), after studying 2598 brucellae cultures, reported that the tests ordinarily used for distinguishing the species do not provide a sound biological basis for species differentiation. He proposed, since only quantitative biochemical differences existed between the species, that this genus be considered a single species and be sub-grouped into varieties. The question of species status has been further intensified by the reports of investigators who have found that many organisms display biochemical and serological identities which do not coincide with each other. Cruickshank (1954) called these cultures *B. melitensis*, but stipulated that they behaved biochemically as *B. abortus* and only serologically as *B. melitensis*. Pickett and Nelson (1955), referred to such cultures as species intermediates and urged that a decision be made as to whether they be classified on their biochemical or their serological characteristics. Renoux (1952*b*), reporting on a small group of organisms that displayed dual identities, proposed that cultures displaying such behavior be considered a new species, *Brucella intermedia*. These 18 cultures were serologically *B. abortus*, but were *B. melitensis* according to dye differentiation and hydrogen sulfide production. If species status were to be accorded this group of organisms, the natural corollary would then be another new species to accommodate cultures that were serologically *B. melitensis* and biochemically *B. abortus*. The total number of possible combinations that could ensue, and would have to be accorded species rank, is unthinkable.

The significance of the problem of classifying organisms that fail to conform to species standards has perhaps been magnified in the literature. Cruickshank (1954) and Spink (1956), from careful literature reviews, estimated that less than 10 per cent of the organisms in this genus were atypical. The metabolic patterns would indicate that rather than considering these organisms to be atypical of a species, they should more appropriately be considered as transitional strains. One of the most outstanding examples of the transitional nature of organisms that are types within a species is *B. suis* type II (Danish strain).

In a comparative study of the biochemical characteristics of 444 brucellae cultures, including Danish porcine strains, Meyer and ZoBell (1932) and ZoBell and Meyer (1932) concluded that this strain of *B. suis* was transitional between *B. abortus* and *B. suis*. The metabolic pattern shows definitively its transitional characteristics. *B. suis* type II oxidized the amino acid substrates of L-arginine, DL-citrulline, and DL-ornithine that are distinguishing of *B. suis*. However, it also oxidized L-asparagine and L-glutamic acids, both of which are oxidized rapidly by *B. abortus* and *B. melitensis*. That it is transitional between *B. abortus* and *B. suis* rather than *B. melitensis* and *B. suis* may be observed in its carbohydrate metabolism. It oxidized D-ribose, which *B. melitensis* does not, and approximated the rates of *B. abortus* type II in its utilization of L-arabinose and D-galactose.

Further evidence that strains showing altered biochemical behavior are transitional organisms may be observed by comparing their patterns of growth on basic fuchsin and thionin with their metabolic patterns on carbohydrate substrates. Organisms that have the ability to grow in the presence of both basic fuchsin and thionin are not able to utilize the substrates of L-arabinose and D-galactose. This combination of characteristics clearly distinguishes *B. melitensis* from *B. abortus* and *B. suis*. *B. abortus* type III displays this identical combination of characteristics and can be distinguished from *B. melitensis* only by quantitative serological techniques. In *B. suis* type III, the drop in the oxidative rates on these substrates is very pronounced. Whether the phenomenon of growth on both dyes and inability to oxidize L-arabinose and D-galactose is one of cause and effect or is a

simultaneous but unrelated occurrence cannot be deduced from these data. The point of interest here is the close biological features of these three types.

The metabolism of *B. neotomae* (Stoenner and Lackman, 1957) has been discussed in detail previously by Cameron and Meyer (1958). This organism has metabolic features that show it to be transitional between existing species of *Brucella*, being similar to *B. suis* in its carbohydrate utilization, carbon dioxide requirements, and hydrogen sulfide production. It is, however, similar to *B. abortus* and *B. melitensis* in its amino acid utilization.

SUMMARY

The differential effect of thionin and basic fuchsin upon the growth of organisms within the genus *Brucella* has been the most valuable technique for species identification. The original classification of the genus *Brucella* into three species was, in fact, based upon their growth pattern on these dyes by Huddleson in 1929. The existence of three definable species was serologically confirmed by Wilson and Miles in 1932. Organisms that fail to conform biochemically or serologically to species criteria can now be accommodated taxonomically in the enlarged schema. That this classification is biologically sound is reflected in the differing metabolic patterns of the organisms within the species and types, especially when correlated with the bacteriostatic action of the dyes and H_2S production.

REFERENCES

Braun, W. and Bonestell, A. E. 1947 Independent variation of characteristics in *Brucella abortus* variants and their detection. Am. J. Vet. Research, **8**, 386–390.

Buddle, M. B. and Boyes, B. W. 1953 A *Brucella* mutant causing genital disease of sheep in New Zealand. Australian Vet. J., **29**, 145–153.

Cameron, H. S. and Meyer, M. E. 1953 Comparative metabolic studies on the genus *Brucella*. II. Metabolism of amino acids that occur in the urea cycle. J. Bacteriol., **67**, 34–37.

Cameron, H. S. and Meyer, M. E. 1955 Synthesis of amino acids from urea by the genus *Brucella*. Am. J. Vet. Research, **16**, 149–151.

Cameron, H. S. and Meyer, M. E. 1958 Metabolic studies on *Brucella neotomae* (Stoenner and Lackman). J. Bacteriol., **76**, 546–548.

Cruickshank, J. C. 1954 Observations on *Brucella* species based on the examination of 800 strains. J. Hyg., **52**, 105–118.

Huddleson, I. F. 1929 The differentiation of the species of the genus *Brucella*. Mich. State Univ. Agr. Expt. Sta., Tech. Bull. No. **100**.

Huddleson, I. F. 1957 In R. S. Breed, E. G. D. Murray, and N. R. Smith: *Bergey's manual of determinative bacteriology*, pp. 404–406, 7th ed. The Williams & Wilkins Co., Baltimore.

Huddleson, I. F. and White, E. A. 1954 Isolation of *Brucella abortus* type II (Wilson) from the milk of dairy cows on one herd in Michigan. Michigan State Coll. Vet., **14**, 120–122.

Meyer, K. F. and Zobell, C. E. 1932 Metabolism studies on the *Brucella* group. IV. The bacteriostatic action of dyes. J. Infectious Diseases, **51**, 72–90.

Meyer, M. E. and Cameron, H. S. 1956 Studies on the etiological agent of epididymitis in rams. Am. J. Vet. Research, **17**, 495–497.

Meyer, M. E. and Cameron, H. S. 1957 Species metabolic patterns in morphologically similar gram negative pathogens. J. Bacteriol., **73**, 158–161.

Meyer, M. E. and Cameron, H. S. 1958 Species metabolic patterns within the genus *Brucella*. Am. J. Vet. Research, **19**, 754–758.

Pickett, M. J. and Nelson, E. L. 1955 Speciation within the genus *Brucella*. IV. Fermentation on carbohydrates. J. Bacteriol., **69**, 333–336.

Pickett, M. J., Nelson, E. L., and Liberman, J. D. 1953 Speciation within the genus *Brucella*. II Evaluation of differential dye, biochemical, and serological tests. J. Bacteriol., **66**, 210–219.

Renoux, G. 1952*a* La classification des *Brucella*. Remarques a propos de l'identification de 2598 souches. Ann. inst. Pasteur, **82**, 289–299.

Renoux, G. 1952*b* Une nouvelle "espece" de *Brucella: Br. intermedia*. Ann. inst. Pasteur, **83**, 814–815.

Renoux, G. and Carrere, L. 1952 De la valeur des caracteres d'identification des *Brucella*. Ann. inst. Pasteur, **82**, 277–288.

Spink, W. W. 1956 *The nature of brucellosis*. The University of Minnesota Press, Minneapolis.

Stoenner, H. G. and Lackman, D. B. 1957 A new species of *Brucella* isolated from the desert wood rat, *Neotoma Lepida* Thomas. Am. J. Vet. Research, **18**, 947–951.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. 1957 *Manometric techniques and re-*

lated methods for the study of tissue metabolism. Burgess Publishing Co., Minneapolis.

WHITE, P. G. AND WILSON, J. B. 1951 Differentiation of smooth and nonsmooth colonies of *Brucella*. J. Bacteriol., **61,** 239–240.

WILSON, G. S. 1933 The Classification of the *Brucella* group: a systematic study. J. Hyg., **33,** 516–541.

WILSON, G. S. AND MILES, A. A. 1932 The serological differentiation of smooth strains of the *Brucella* group. Brit. J. Exptl. Pathol., **13,** 1–13.

World Health Organization 1953 Joint FAO/WHO Expert Committee on Brucellosis. Second Report FAO Agricultural Studies, No. **24,** 22–27.

ZOBELL, C. E. AND MEYER, K. F. 1932 Metabolism studies on the *Brucella* group. VI. Nitrate and nitrite reduction. J. Infectious Diseases, **51,** 99–108.