SPECIATION WITHIN THE GENUS BRUCELLA

II. Evaluation of Differential Dye, Biochemical, and Serological Tests

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Of the several biochemical tests which have been utilized for speciation within the genus *Brucella*, the "urea test" is one for which there is considerable contradiction in the literature. More specifically, there have been conflicting reports concerning the relative urease activity of *Brucella melitensis*, whereas there appears to be general agreement that *Brucella suis* rapidly hydrolyzes urea and that *Brucella abortus* hydrolyzes urea very slowly. Our brief observations on this test and its correlation with the differential dye pattern (Pickett *et al.*, 1952) led us to suspect that it should be a reliable means for distinguishing *B. suis* from the other two species. Further examination of the urea test has confirmed our original impression that it is a distinctly valuable tool for characterizing the species within this genus. This conclusion implies, however, that the test be conducted only (1) with frankly smooth strains, as established by careful examination of colonial morphology and by nonagglutinability in acriflavine, (2) with a well buffered, peptone-free, urea medium, e.g., the medium of Rustigian and Stuart (1941) but not that of Christensen (1946) or Ferguson and Hook (1943), and (3) with a carefully standardized inoculum.

Our observations during this study have disclosed an urgent need for reevaluation and standardization of methods for speciation within this genus. Accordingly, we have examined several tests, in addition to urea, and have attempted to evaluate their relative merits for the identification of brucellae. Of these, the carbamate test of Renoux (1952*a*) has been found most helpful. Finally, the definitive role of specific agglutination with absorbed sera has not been questioned; however, since this fails to distinguish between *B. suis* and *B. abortus* and since completely satisfactory absorbed sera are not prepared readily, we have attempted to determine with what assurance one may safely neglect such serological examination of recently isolated brucellae.

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MATERIALS AND METHODS

Routine cultures. All glassware was acid-cleaned (hot sulfuric-nitric). The routine growth medium was Albimi's brucella agar enriched with thiamin, nicotinamide, and hemin (Liberman and Pickett, 1952). All cultures, unless otherwise indicated, were incubated at 35 C under 10 per cent carbon dioxide.

Working suspensions of brucellae. The growth on a forty-eight hour slant was suspended in sterile distilled water to give 5×10^9 bacteria per ml. Such stock suspensions were used on the day of preparation as inocula for tubes of urea medium, slants (to test for CO_2-dependence and for H_2S production), differential dye plates, and carbamate test plates.

Differential dye tests. The general procedure was that already described for thionin, basic fuchsin, crystal violet, and pyronin (Pickett *et al.*, 1952). Additional dyes examined were eosin Y, safranin O, rose bengal, and azure A.

Carbamate tests. Several representative strains of brucellae were examined according to the procedure of Renoux (1952*a*) with satisfactory results. For convenience, however, subsequent tests were made with the reagent tableted, as with the dyes, and with the sodium diethyldithiocarbamate at a concentration (weight:weight) of 1:200. For the test, one-quarter of a petri plate was inoculated uniformly with one 2 by 10 mm loopful of a stock suspension of brucellae. A carbamate tablet then was placed on this inoculated quadrant, and the plate was incubated (without prior overnight refrigeration as with the dyes) for two days. The results obtained from these tests are

expressed as the width, in mm, of the outer zone of inhibited growth.[4]

Urea tests. The double strength urea medium, maintained at 4 C over chloroform and not sterilized before use, was similar to that of Rustigian and Stuart (1941). It contained 4 per cent urea, 4.8 per cent $Na_2HPO_4 \cdot 12H_2O$, 1.8 per cent KH_2PO_4, 20 mg per cent of yeast extract (Difco), and 2 mg per cent phenol red. For the test, 0.5 ml of this medium and 0.5 ml of stock suspension were placed in a 12 by 100 mm tube; this mixture was incubated aerobically at room temperature and was observed for color change at 5 to 10 minute intervals during the following two hours. The test was then read at irregular intervals through a total incubation period of forty-eight hours.

Tests for hydrogen sulfide production. A slant (2 ml of medium per 12 by 100 mm tube) was inoculated with one 4 mm loopful of stock suspension. Sulfide production was detected by inserting a "lead acetate strip" under the cotton plug. A new strip was inserted daily through six days' incubation.

Serological tests. Absorbed *B. abortus* (abs-A) and *B. melitensis* (abs-M) antisera were prepared by appropriate treatment of the respective raw sera with heterologous antigens (*B. melitensis* and *B. abortus*, respectively). The stock absorbed sera were diluted with normal serum so each had a 1:320 homologous titer and 1:40 to 1:80 heterologous titer. Each serological test was set up according to the following protocol (volumes are in ml):

	Tube no.					
	1	*2*	*3*	*4*	*5*	*6*
abs-M serum, 1:20	0.5	0.1				
abs-A serum, 1:20			0.5	0.1		
raw-A serum, 1:100					0.5	
acriflavine, 0.1%						0.1
saline		0.4		0.4		0.4
antigen	0.5	0.5	0.5	0.5	0.5	0.5

[4] In sterile control quadrants there are four rings or zones surrounding each tablet. Inhibition occurs with nearly all strains of *B. melitensis* and *B. suis* in the first wide zone; inhibition is variable in the second and third zones; most strains of brucellae show enhanced growth in the fourth zone; and only strains of *B. melitensis* are strongly inhibited beyond the fourth zone.

The raw-A serum was one used routinely for diagnostic agglutinations and had a homologous titer of 1:5,120. The antigen for each test was obtained by adding 0.35 ml of 10 per cent NaCl and 0.35 ml of 5 per cent phenol to 2.8 ml of stock *Brucella* suspension (thus giving a 0.1 per cent suspension in phenolized saline). The tubes were incubated at 56 C for 18 hours before reading (Feinberg and Wright, 1951). Any strain giving a positive acriflavine test (Braun and Bonestell, 1947) was considered, for the purposes of this study, to be a nonsmooth brucella variant.

RESULTS

Urea and dye patterns. Table 1 summarizes the data obtained from an examination of 232 strains of smooth brucellae. Of these, 199, or 86 per cent of those examined, gave urea and dye tests which were completely typical for the species (groups Ia through III). An additional 15 strains (groups IV, V, and VI) were slightly atypical in respect to one or more dyes, but the over-all dye pattern, in conjunction with the urea test, readily permitted species designations. Therefore, it was possible, by reference only to the urea test and the dye pattern, to define the species for 92 per cent of the 232 smooth strains examined.

We may note here that one purpose of this study was to determine with what frequency one may hope to define a strain in this genus by reference only to its dye pattern and urease activity. The data in table 1 provide at least an approximate answer to this question. As a corollary to this question one must, in turn, ask with what frequency these two tests, particularly if apparently completely typical for a given species, would be contradicted by additional tests. We have not examined all of the smooth strains indicated in table 1 in an attempt to answer this question. For other purposes, however, we have performed one or more additional tests with 137 strains which gave "typical" urea and dye tests, and in no instance was evidence obtained that these two tests had been misleading. Data in support of this argument may be found in the several tables below.

Additional characterizing tests were sought for the 15 strains of groups IV, V, and VI (table 1) and particularly for the 18 atypical strains of group VII. These tests and their evaluation will be presented before turning to further consideration of the atypical strains.

Carbamate test. A tableted reagent for this test, rather than the dipping of a paper disc into a solution of the reagent as recommended by Renoux, appeared desirable both for better agreement in replicate tests and also because of the lability of this reagent when in aqueous solution. Trial tablets were prepared therefore at dilutions of 1:100, 1:200, and 1:800. These were tested both with and without overnight refrigeration of the prepared plates before incubation. The 1:200 tablets were found to give zones of suitable diameter for quarter-plate tests (figure 1), and the results were interpreted no less readily than with the 1:100 tablets. The zones were considerably larger when the plates were refrigerated before incubation, but the results were defined usually less readily than when the prerefrigeration was omitted. The carbamate test was performed with 113 strains of typical and slightly atypical brucellae; in no instance were the results in disagreement with those obtained from urea and dye tests. The results obtained with representative typical strains are given in table 2.

TABLE 1

Urea and dye sensitivity tests on 232 strains of smooth brucellae

GROUP	SPECIES	NUMBER OF STRAINS	UREA*	SENSITIVE TO†:			
				T	F	V	P
Ia	*Brucella abortus*, CO_2-independent	41	−	+	−	−	−
Ib	*Brucella abortus*, CO_2-dependent	33	−	+	−	−	−
II	*Brucella melitensis*	32	−	−	−	−	−
III	*Brucella suis*	93	+	−	+	+	+
IV	*Brucella abortus*	8	−	(slightly atypical)			
V	*Brucella melitensis*	2	−				
VI	*Brucella suis*	5	+				
VII	*Brucella abortus*	10	(atypical)				
	Brucella melitensis	3					
	Brucella suis	5					

* +, urea medium alkalinized in less than 40 min; −, medium still acid or neutral at 100 min.

† T, thionin, 1:800; F, basic fuchsin, 1:200; V, crystal violet, 1:400; P, pyronin, 1:8,000; +, zone of inhibition 4 mm or more; −, zone of inhibition less than 4 mm.

Additional dye tests. The data of Cameron and Meyer (1952) suggested the use of additional, or alternate, dyes for distinguishing the species in the genus *Brucella.* Several of the dyes examined by them were tested therefore with representative typical strains. The dyes first were screened by the paper disc procedure, and those which gave promising results were reexamined by the tableted procedure. Of the dyes tested by the latter method, eosin Y was too water soluble and inadequately inhibitory to be of practical value. Rose bengal at 1:100 dilution was more inhibitory for *B. suis* than for the other two species, but the difference was not sufficient to recommend this dye for differential purposes. Azure A (National Aniline, 82 per cent dye content) was tested at dilutions of 1:100, 1:200, 1:500, 1:800, and 1:1,000. The most promising concentration appeared to be 1:1,000 since at this level only strains of *B. melitensis* were not inhibited appreciably. That is, it appeared that azure A at 1:1,000 could be employed as a supplementary test for thionin insensitive strains of *B. abortus* or thionin sensitive strains of *B. melitensis.* Safranin O (National Aniline, 95 per cent dye content), like azure A, showed rather consistent selection, in this instance only strains of *B. suis* being appreciably inhibited at a dye dilution of 1:100. The results obtained from an examination of these two dyes are presented in table 2.

Hydrogen sulfide production. The position of this test as it applies to characterizing the brucellae has been stated succinctly by Wilson and Miles (1946), and the data presented in table 3 are in agreement with their conclusions. The strains of brucellae included in table 3 are segregated according to whether they are typical or atypical in respect to their urea and dye sensitivity tests. These data show that under the experimental conditions employed here this test

(1) for typical strains does not contribute significantly toward identification of species in this genus, and (2) frequently fails, with slightly or frankly atypical strains, to reinforce tentative conclusions drawn from the urea and dye tests.

Atypical strains. Table 4 presents quantitative data obtained from the slightly atypical strains of groups IV, V, and VI, table 1. None of these strains presented a real problem since the overall dye pattern, in conjunction with the urea test, permitted ready designation of species. It should be noted here that the five strains of *B. suis* listed in table 4 were relatively fuchsin resistant, and therefore the omission of crystal violet and pyronin from the dye sensitivity tests would have led to the obtaining of a "*B. melitensis* dye pattern" for each. Four of these strains, in addition, produced only a trace of hydrogen sulfide. However, all four were typical of *B. suis* in respect to their antigenic, urea, and carbamate characteristics.

Ten of the atypical strains listed in table 5, like all the slightly atypical strains of table 4, presented no serious problem in respect to designation of species. Five of these (A19, A20, A45, A102, and A103) were acutely dye sensitive and therefore were similar in this respect to certain strains described by Huddleson (1943). With these five strains the differential dye test neither contributed to nor contradicted other tests employed for distinguishing the species. Similarly, the carbamate test gave results which were frankly atypical for brucellae (a wide and continuous zone of inhibition surrounding the tablet) and therefore did not tend to contradict other, and quite representative, tests. The slow hy-

Figure. 1 Carbamate tests with representative strains of brucellae. Reading clock-wise from upper right quadrant: *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, and control.

TABLE 2

Carbamate, azure A, and safranin O reactions of representative smooth brucellae

STRAIN	SPECIES	DYE SENSITIVITY (MM)*		CARBAMATE (MM)†
		A	S	
A14	*Brucella abortus*, CO_2-independent	8	2	0
A17		17	3	0
A29		11	2	0
A32		14	3	0
A35	*Brucella abortus*, CO_2-dependent	12	3	0
A36		10	1	0
A37		13	2	0
A39		12	2	0
M2	*Brucella melitensis*	2	2	3
M3		1	1	3
M32		0	0	2
M42		1	1	2
S17	*Brucella suis*	6	8	0
S24		8	7	0
S36		7	8	0
S42		8	9	0

* A, azure A, 1:1,000; S, safranin O, 1:100.
† Sodium diethyldithiocarbamate, 1:200.

TABLE 3

Hydrogen sulfide production by 103 typical and 20 atypical strains of smooth brucellae

SPECIES	NUMBER OF STRAINS	NUMBER SHOWING POSITIVE TEST AFTER SPECIFIED DAYS OF INCUBATION					
		1	2	3	4	5	6
Typical							
Brucella abortus	24	16	22	22	24	23	24
Brucella melitensis	35	3	6	8	6	7	5
Brucella suis	44	36	39	39	40	41	41
Atypical							
Brucella abortus	7	2	6	6	6	6	5
Brucella melitensis	2	1	2	2	2	2	2
Brucella suis	11	2	2	6	10	9	10

drolysis of urea and the prolonged production of hydrogen sulfide offered strong evidence that all were strains of *B. abortus*. It is clear, however, that only the serological data, in conjunction with the urea tests, provided the information required for satisfactory speciation of these strains.

With strains M8, S19, S25, S86, and S87, the dye tests considered alone conformed to the *B. melitensis* pattern. The urea tests, however, were those indicative of *B. suis*; all gave negative carbamate tests; all were moderately sensitive to safranin; and three of the five produced an abundance of hydrogen sulfide. These five strains present instances in which (1) the urea test was very helpful (and also reliable, as shown by the serological data); (2) the carbamate test also contributed toward recognition of the species; (3) the sulfide test supported other data with three of the five strains; (4) the supplementary dye, safranin O, served to warn that the results obtained with the four classical dyes might be atypical; and (5) in the absence of serological data, reliance on urea, dye, and carbamate tests would still permit the designation "dye resistant *B. suis*".

TABLE 4

Quantitative results from urea, dye, and carbamate tests on slightly atypical smooth brucellae

STRAIN	SPECIES	UREA	DYE SENSITIVITY (MM)				CARBAMATE (MM)
			T	F	V	P	
		min					
A15	*Brucella abortus**	115	10	5	5	0	0
A24	*Brucella abortus**	210	13	3	7	0	0
A56	*Brucella abortus**	240	11	5	6	1	0
A57	*Brucella abortus**	210	12	2	4	5	0
A58	*Brucella abortus**	180	19	1	6	0	0
A60	*Brucella abortus**	120	12	5	3	0	0
A66	*Brucella abortus**	110	12	5	4	0	0
A86	*Brucella abortus**	210	12	2	6	0	0
M13	*Brucella melitensis*	245	5	3	0	0	3
M52	*Brucella melitensis*	200	4	2	0	9	2
M11	*Brucella suis*	20	0	3	6	8	0
M12	*Brucella suis*	20	3	3	5	5	0
M15	*Brucella suis*	30	0	2	9	6	0
M24	*Brucella suis*	10	2	2	5	8	0
S66	*Brucella suis*	15	2	2	6	8	0

* Of the eight strains of *Brucella abortus* listed here, only A86 was CO_2-dependent.

Strain A90, of recent human origin (California), reacted in the dye test like a moderately sensitive strain of *B. melitensis*; insensitivity to azure A, a positive carbamate test, very slow hydrolysis of urea, and negative sulfide tests amply sup-

ported this conclusion, and the serological data were confirmatory.

Strains M6, M36, and M40 had the antigenic characteristics of typical "American" strains of *B. abortus*, but all gave typical *B. melitensis* reactions in the dye and carbamate tests. Strain M40 (isolated in Switzerland) gave sulfide tests characteristic of *B. melitensis*; strains M6 (isolated in Northern France) and M36 (of human origin, isolated in Iran) produced an abundance of hydrogen sulfide. Two additional strains received fron Brazil, but not included in table 5 because of incomplete data covering their origin, were similar to strains M6, M36, and M40. All five strains have been designated tentatively *B. abortus* on the basis of their antigenic and urease characteristics although it is quite clear that these strains give carbamate and dye tests which would immediately mark them *B. melitensis*. Similar discrepancies between antigenic and biochemical characteristics have been reported for strains isolated in southeastern France (*cf* Wilson and Miles, 1946). It may be noted, finally, that all showed completely stable characteristics when subjected to repeated plating on our brucella agar medium and on brucella agar containing 1 mg per cent crystal violet.

Both strain M47 (caprine origin) and strain M48 (ovine origin) were isolated in Iran; both showed the rapid hydrolysis of urea which is typical for strains of *B. suis*, but both behaved like *B. melitensis* in all other tests. Both were replated repeatedly with no change in characteristics. Among 37 strains of *B. melitensis* and 103 strains of *B. suis* examined, these two strains therefore represent the only exceptions to the rule that only those of the latter species are strongly urea positive.

TABLE 5

The characteristics of 16 frankly atypical smooth brucellae

STRAIN	SPECIES	SEROLOGY*					UREA	DYE SENSITIVITY (MM)†						CARBAMATE (MM)	H_2S ON DAY:					
		1	2	3	4	5		T	F	V	P	A	S		1	2	3	4	5	6
							min													
A19	*Brucella abortus*	+	−	+	+	+	110	15	8	12	12	10	11	3	−	+	+	+	+	+
A20	*Brucella abortus*	+	−	+	+	+	200	15	15	12	10	15	10	3	−	+	+	+	+	+
A45	*Brucella abortus*	+	−	+	+	+	400	12	4	9	8	20	3	1	+	+	+	+	+	+
A102	*Brucella abortus*	+	−	+	+	+	210	8	15	9	10	19	15	3	−	+	+	+	+	+
A103	*Brucella abortus*	+	−	+	+	+	180	12	13	10	13	9	9	3	+	+	+	+	+	−
M8	*Brucella suis*	+	−	+	+	+	25	2	2	4	5	4	3	0	−	−	−	+	−	+
S19	*Brucella suis*	+	−	+	+	+	20	0	0	0	1	6	3	0	+	+	+	+	+	+
S25	*Brucella suis*	+	−	+	+	+	25	0	0	2	5	5	4	0	+	+	+	+	+	+
S86	*Brucella suis*	+	−	+	+	+	20	2	0	0	0	1	3	0	−	−	+	+	+	+
S87	*Brucella suis*	+	−	+	+	+	20	1	1	0	0	0	3	0	−	−	+	+	+	−
A90	*Brucella melitensis*	+	+	+	−	+	360	6	3	4	4	1	0	2	−	−	−	−	−	−
M6	*Brucella abortus*	+	−	+	+	+	180	3	0	0	0	1	2	3	+	+	+	+	+	+
M36	*Brucella abortus*	+	−	+	+	+	150	2	0	0	0	0	0	3	−	+	+	+	+	+
M40	*Brucella abortus*	+	−	+	+	+	300	2	0	0	0	2	1	3	−	−	−	−	−	+
M47	*Brucella melitensis*	+	+	+	−	+	30	2	0	0	0	0	0	2	−	−	−	−	−	−
M48	*Brucella melitensis*	+	+	+	−	+	30	1	0	1	0	1	0	2	−	−	−	−	−	−

* Tubes no. 1 through 5 of the serology protocol.

† The dye tablets previously employed, see tables 1 and 2.

Strains with questionable species designation. Several strains of brucellae received under title of *B. abortus* (strain A16, through strain S89, table 6) gave no reactions, except abundant sulfide production, which characterize this species. They were, rather, quite typical strains of *B. suis*. Another 15 strains of *B. suis* were received as *B. melitensis*, though all unquestionably fell into the *B. abortus-B. suis* group antigenically. Four of these 15 (strains M10, S91, S92, and S98) were typical strains of *B. suis*, and two (strains M7 and M9) resembled *B. melitensis* only in their feeble production of sulfide. An additional five of these strains (M11, M12, M15, M16, and M24) were relatively fuchsin resistant and therefore might be classified as *B. melitensis*

if only thionin and fuchsin sensitivity and sulfide production were employed for their characterization. Even in the absence of serological data, however, these five strains are unquestionably *B. suis* as judged by their four-dye sensitivity, clerical error since none of its characteristics would tempt one to title it *B. melitensis*.

Nonsmooth strains. Twenty-six strains examined during the course of these studies gave positive acriflavine tests despite the fact that all

TABLE 6

Twenty-eight strains of smooth brucellae with characteristics atypical for their original species designations

STRAIN	SPECIES DESIGNATION		AGGLUTINATED BY*:		UREA	DYE SENSITIVITY (MM)†						CARBAMATE (MM)	H_2S ON DAY:					
	Original	Revised	M	A		T	F	V	P	A	S		1	2	3	4	5	6
					min													
A16	*Brucella abortus*	*Brucella suis*	x	x‡	20	1	11	6	6	4	6	0	+	+	+	+	+	+
A23	*Brucella abortus*	*Brucella suis*	x	x	20	2	12	9	11	7	4	0	−	+	+	+	+	+
A38	*Brucella abortus*	*Brucella suis*	x	x	20	0	10	8	8	7	7	0	+	+	+	+	+	+
A61	*Brucella abortus*	*Brucella suis*	x	x	15	3	10	7	7	4	9	0	+	+	+	+	+	+
A65	*Brucella abortus*	*Brucella suis*	x	x	15	1	12	9	7	9	9	0	−	+	+	+	+	+
A68	*Brucella abortus*	*Brucella suis*	x	x	15	2	12	8	8	6	8	0	+	+	+	+	+	+
A69	*Brucella abortus*	*Brucella suis*	x	x	20	1	10	8	8	7	8	0	+	+	+	+	+	+
S12	*Brucella abortus*	*Brucella suis*	x	x	25	0	5	7	5	6	8	0	+	+	+	+	+	+
S13	*Brucella abortus*	*Brucella suis*	x	x	25	0	5	7	5	6	7	0	+	+	+	+	+	+
S81	*Brucella abortus*	*Brucella suis*	x	x	11	0	13	8	5	5	8	0	+	+	+	+	+	+
S88	*Brucella abortus*	*Brucella suis*	x	x	30	2	10	7	6	9	7	0	+	+	+	+	+	+
S89	*Brucella abortus*	*Brucella suis*	x	x	30	0	9	7	7	8	8	0	+	+	+	+	+	+
M7	*Brucella melitensis*	*Brucella suis*	−	+	35	1	7	11	7	5	3	0	−	−	−	−	−	−
M8	*Brucella melitensis*	*Brucella suis*	−	+	20	2	2	4	5	4	3	0	−	−	−	+	−	+
M9	*Brucella melitensis*	*Brucella suis*	−	+	20	2	7	9	8	4	3	0	−	−	−	−	−	+
M10	*Brucella melitensis*	*Brucella suis*	−	+	35	0	11	6	8	5	7	0	−	+	+	+	+	+
M11	*Brucella melitensis*	*Brucella suis*	−	+	20	0	3	6	8	6	3	0	−	−	−	+	−	+
M12	*Brucella melitensis*	*Brucella suis*	−	+	20	3	3	5	5	4	4	0	−	−	+	+	+	+
M15	*Brucella melitensis*	*Brucella suis*	−	+	20	0	2	9	6	1	2	0	−	−	−	+	+	+
M16	*Brucella melitensis*	*Brucella suis*	−	+	25	1	4	7	5	3	3	0	−	−	−	+	+	+
M24	*Brucella melitensis*	*Brucella suis*	−	+	10	2	2	5	8	4	2	0	−	−	−	−	+	+
S86	*Brucella melitensis*	*Brucella suis*	−	+	20	2	0	0	0	1	3	0	−	−	+	+	+	+
S87	*Brucella melitensis*	*Brucella suis*	−	+	20	1	1	0	0	0	3	0	−	−	+	+	+	−
S91	*Brucella melitensis*	*Brucella suis*	−	+	30	0	8	9	8	7	8	0	+	+	+	+	+	+
S92	*Brucella melitensis*	*Brucella suis*	−	+	30	0	10	7	7	4	8	0	+	+	+	+	+	+
S98	*Brucella melitensis*	*Brucella suis*	−	+	25	0	10	12	8	4	9	0	+	+	+	+	+	+
S25	*Brucella melitensis*	*Brucella suis*	−	+	25	0	0	2	5	5	4	0	+	+	+	+	+	+
M28	*Brucella melitensis*	*Brucella abortus*	−	+	250	6	0	0	0	2	0	0	+	+	+	+	+	+

* M and A are tubes no. 2 and 4, respectively, of the serology protocol.

† The dye tablets previously employed, see tables 1 and 2.

‡ Not done.

urea, and carbamate reactions. The remaining four of these 15 strains (M8, S25, S86, and S87) have already been discussed in connection with tables 4 and 5. All were sufficiently dye resistant to resemble *B. melitensis* in this test. All, however, gave typical *B. suis* reactions in urea and carbamate tests. The final strain (M28) listed in table 6 may be dismissed as an instance of had appeared previously to be colonially smooth. Reexamination by oblique light (Henry, 1933) disclosed that they were indeed nonsmooth, and most showed the dissociative characteristics of the "intermediate" brucella variant (*cf* Braun, 1950). Smooth clones were obtained then from several of these by repeated plating and careful selection of colonies. In other instances, however,

repeated plating yielded only progressively less smooth clones. The biological characteristics of this latter group are being made the subject for further study.

DISCUSSION

It is now well established that brucellae have a marked propensity for undergoing dissociation and that several of the dissociants closely resemble smooth brucellae in their microscopic and colonial morphology. It has been shown also that certain of the dissociants respond quite differently to a given environment than do the smooth brucellae from which they were derived (Braun, 1950). The reactions of these "pseudosmooth" variants in hydrogen sulfide, urea, carbamate, and dye sensitivity tests have not yet been thoroughly investigated, but we must anticipate that they may be significantly altered. Certainly, until such data are available, we should accept with considerable reservation all conclusions which are based on the examination of brucellae which were not first rigorously screened, i.e., for which the colonial morphology (as seen by obliquely transmitted light) and the stability in acriflavine have not been examined.[5] During our limited examination of several such nonsmooth strains we have noted, for example, that the oxidase and urease activities are considerably weaker, and dye sensitivity decreased as compared with the homologous smooth brucellae.

The sphere which defines a species of microorganism is of human construction and must often be of quite arbitrarily determined size and rigidity. It should always be a unit which is both practical and functional, and also amenable to redefinition and modification according to the dictates of current knowledge and need. Renoux and his associates (Renoux and Carrère, 1952; Renoux, 1952*b*) have urged that delineation of species always be based on qualitative, not quantitative, differences. We agree that this is desirable but seriously doubt that it is a practical approach to the problem. A more practical approach, we believe, is to recognize that apparently related strains of microorganisms usually represent clones which have stemmed from a common progenitor. The clones may differ both quantitatively and qualitatively; the problem is to segregate or group the clones in a recognizable and functional manner. Particularly in the instance of brucellae an additional problem appears, namely an intrastrain variation. That is, any given strain may be quite unrecognizable when it has dissociated toward the mucoid, intermediate, or dwarf morphology. It is essential then that each strain be presented under the common denominator, smooth, before its other characteristics are examined in an attempt to assign it to a group. We feel that there are three relatively distinct groups in the genus *Brucella*, that each group is relatively homogeneous, and that each group therefore may permissibly be given a species designation. Within each group, just as with other genera, there will be found strains which show "atypical" characteristics; these irregularities, however, need not detract seriously from the utilitarian value of such species designations, particularly for epidemiological purposes.

We have attempted to determine those characteristics of smooth brucellae which will best delineate the three species. Our data reaffirm that the dye sensitivity tests are the most useful tool for this purpose. Both the urea and carbamate tests correlate well with dye sensitivity and serological tests, and both should be included in descriptions covering the characteristics of these three species. With these two tests, no irregularities were found among 197 strains of brucellae received from laboratories in Mexico and the United States. Among 35 strains received from Africa, Asia, and Europe, three were found which gave the biochemical tests (including carbamate and urea) associated with *B. melitensis* but which fell into the *B. abortus-B. suis* antigenic group. These strains clearly present a taxonomic problem, but this situation is not unique for the genus *Brucella* (*cf* Ewing *et al.*, 1952). It is apparent, however, that the global distribution and incidence of such strains should be determined, and that a decision should be made regarding whether the species designation for these shall be based on antigenic or on biochemical characteristics. Only two strains of *B. melitensis* (both isolated in Iran), among 37 smooth and 6 intermediate strains examined, rapidly hydrolyzed urea. Fifteen additional

[5] During this study we have found the acriflavine test and careful colonial examination to be much superior to salt or heat agglutination, triphenyltetrazolium chloride media, or crystal violet staining (White and Wilson, 1951) for the recognition of nonsmooth brucellae.

strains of brucellae, received as *B. melitensis* from laboratories in the Americas, were strongly urea positive, but all were strains of *B. suis* as judged by both their antigenic and biochemical characteristics.

Four additional tests—CO_2-dependence, azure A and safranin O sensitivity, and hydrogen sulfide production—also were examined; none satisfactorily delineated these species. CO_2-dependent strains invariably have those characteristics which are associated with *B. abortus*, but many CO_2-independent strains also have the same characteristics. This is precisely what one would predict from the data of Marr and Wilson (1950). Azure A and safranin O sensitivity tests, like sulfide tests, sometimes provide helpful but far from definitive data insofar as they identify a species; all three tests, however, might well be used in the examination of atypical strains. The former dye is particularly helpful in that it supplements the one other dye (thionin) to which strains of *B. abortus* are sensitive.

Some measure of the relative merits of the tests employed during this study may be gained from a summary of those instances in which there was disagreement among tests. Thus, the number of strains encountered in this study for which the following tests would not readily have permitted satisfactory designation of species (excluding the three peculiar *B. abortus-B. melitensis* strains of table 6) were, approximately: thionin and fuchsin only, 33; thionin, fuchsin, and sulfide, 32; four dyes, 24; four dyes and sulfide, 23; four dyes and urea, 20; four dyes and carbamate, 17; four dyes, urea, and carbamate, 5. It is clear that difficulty will be encountered frequently when only thionin and fuchsin tests or thionin, fuchsin, and sulfide tests are employed for identifying brucellae. The other two dyes (i.e., crystal violet and pyronin) should be included also, and the use of carbamate and urea frequently will permit interpretation of slightly irregular dye sensitivity tests. By using the four dyes, urea, and carbamate for identification of brucellae, the inclusion of agglutination with absorbed sera should be required only for acutely dye sensitive strains.

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SUMMARY AND CONCLUSIONS

Two hundred and fifty-eight strains of brucellae, isolated from man and animals in Europe, Asia, Africa, and the Americas, were screened by the acriflavine agglutination test; twenty-six of these gave positive tests and therefore were considered unsuitable for inclusion in an examination of the biochemical, dye sensitivity, and antigenic characteristics of smooth brucellae.

All of the 232 smooth strains were tested for urease activity and for sensitivity to thionin, basic fuchsin, crystal violet, and pyronin. Both representative and atypical strains were examined also in respect to (1) sulfide production, (2) sensitivity to diethyldithiocarbamate when this was employed as a tableted "carbamate" reagent, and (3) sensitivity to azure A and safranin O.

Two hundred and fourteen of the 232 smooth strains fell into three well defined groups, corresponding to the three generally recognized species of *Brucella*, on the basis of the thionin, fuchsin, crystal violet, pyronin, urea, and carbamate tests. Five strains were acutely sensitive to both carbamate and the dyes and therefore required serological examination for their identification. Three strains gave sensitivity and biochemical tests indicative of *Brucella melitensis* but reacted serologically like *Brucella abortus*.

Both carbamate and urea are practical and reliable tests for identifying strains of brucellae; with but rare exceptions, only strains of *B. melitensis* give positive carbamate tests, and only strains of *Brucella suis* are strongly urea positive.

Tests for production of hydrogen sulfide were found not to contribute significantly toward identification of species in the genus *Brucella*.

Azure A and safranin O, particularly the former, were found to be useful adjuncts to the traditional four dyes for the identification of atypical strains of brucellae.

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