

THE EFFECT OF IONIC Mn AND Mg ON THE VARIATION OF BRUCELLA ABORTUS¹

LEONARD J. COLE AND WERNER BRAUN

*Department of Veterinary Science, University of California, and
Camp Detrick, Frederick, Maryland*

Received for publication June 22, 1950

Although the modifying effect of environmental factors on bacterial variation (dissociation) has been widely studied (Braun, 1947), there has been a paucity of data concerning the influence of the trace metals on this phenomenon. In the course of an investigation of factors that influence variation in *Brucella abortus*, our attention was directed to the possible role of the trace metal ions by the finding that during prolonged growth of smooth cultures of *B. abortus* in broth no nonsmooth variants (e.g., R, M) could be observed when 0.03 M Na-pyrophosphate was added to the medium. In contrast, similar cultures without pyrophosphate showed a progressive establishment of nonsmooth variants (mutants). Since pyrophosphate forms complexes with many metal ions and is known to inhibit certain enzymes (Sumner and Somers, 1947), the present study was undertaken in an attempt to elucidate the mechanism of action of pyrophosphate as well as of trace metals in bacterial variation.

METHODS

A description of the procedures employed in the study of variation (dissociation) in *B. abortus* has been published previously (Braun, 1946). The method is based on the determination of the dissociation index (D.I.)² of a clone (2583) of *B. abortus* (originally isolated from strain 19, U. S. Bureau of Animal Industry), cultured in buffered beef extract broth (pH 6.8) under standardized conditions.

The substances to be investigated were sterilized by Seitz filtration and added to test tubes containing 5 ml sterile broth. The tubes were then seeded with approximately 4×10^7 cells, incubated at 37 C for 10 days, and finally streaked on "2-1" agar plates on which the percentage of smooth and nonsmooth colonies was ascertained after 4 days' incubation at 37 C. All figures for D.I. represent the average of at least triplicate determinations.

The rough variant (7748R) used in the experiments with mixtures of rough and smooth types was derived from strain 19, *B. abortus*. Bacterial growth was assayed by plate counts (viable counts) made in the usual manner, and by direct

¹ This paper is based upon work sponsored in part by the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland, under Contract No. W-18-108-CM-97 with the University of California.

² D.I. is the percentage of nonsmooth types that have established themselves during 10 days in originally smooth cultures as indicated by the percentage of nonsmooth colonies observed on agar plates made from 10-day-old broth cultures.

counts (total counts) in a Petroff-Hauser counting chamber or by turbidimetric measurements in a Klett photoelectric colorimeter, using a blue filter.

EXPERIMENTAL RESULTS

The results of a series of experiments with $\text{Na}_4\text{P}_2\text{O}_7$ are summarized in table 1. In the presence of 0.013 M or more of $\text{Na}_4\text{P}_2\text{O}_7$, no "dissociation" of the smooth clones of *B. abortus* occurred, whereas control cultures without pyrophosphate showed 19 per cent nonsmooth types after 10 days (D.I. = 19). The same re-

TABLE 1
The effect of pyrophosphate on the establishment of nonsmooth variants in broth cultures of smooth B. abortus and its reversal by Mg and Mn

ADDED TO BUFFERED BEEF EXTRACT BROTH	AMOUNT ADDED TO 5 ML OF BROTH	DISSOCIATION INDEX	10TH DAY BACTERIAL COUNTS (BILLIONS)	
			Viable	Total
	<i>ml</i>			
$\text{Na}_4\text{P}_2\text{O}_7$ (0.33 M).....	0.2	0		
$\text{Na}_4\text{P}_2\text{O}_7$ (0.33 M).....	0.5	0	0.45	1.0
(Broth controls).....	—	19	0.71	1.05
$\text{Na}_4\text{P}_2\text{O}_7$ (0.33 M).....	0.6	0	0.41	
$\text{Na}_4\text{P}_2\text{O}_7$ (0.33 M).....	0.6	8	0.57	
+ Mn^{++} (0.005 M).....	0.1			
$\text{Na}_4\text{P}_2\text{O}_7$ (0.33 M).....	0.6	20	0.63	
+ Mn^{++} (0.01 M).....	0.3			
$\text{Na}_4\text{P}_2\text{O}_7$ (0.33 M).....	0.6	7	0.63	
+ Mn^{++} (0.1 M).....	0.3			
$\text{Na}_4\text{P}_2\text{O}_7$ (0.33 M).....	0.6	13	—	
+ Mg^{++} (0.1 M).....	0.2			
Mn^{++} (0.005 M).....	0.1	25	0.70	
Mn^{++} (0.01 M).....	0.3	52	0.58	
Mn^{++} (0.1 M).....	0.3	33	0.72	
(Broth controls).....	—	22	0.55	
Na-glycerophosphate (0.33 M).....	0.1	33		
Na-glycerophosphate (0.33 M).....	0.2	37		
Na-glycerophosphate (0.33 M).....	0.5	34		
(Broth controls).....	—	29		

sults were obtained both with unneutralized (pH 9.0) and neutralized (pH 7.2) solutions and also with autoclaved pyrophosphate, whereas glycerophosphate and inorganic orthophosphate failed to produce this effect. Although the total bacterial count was not inhibited by effective pyrophosphate concentrations, the number of viable organisms was appreciably reduced. It is to be noted that the "dissociation-suppressing" action of pyrophosphate could be antagonized by the simultaneous addition of small amounts of MnSO_4 , MnCl_2 , or MgSO_4 , but not by Na_2SO_4 or K_2SO_4 . As little as 5×10^{-4} M Mn was effective. Indeed, the dissociation index could be increased at will, depending upon the quantity of

Mn⁺⁺ added to the culture tube. Thus the addition of 0.3 ml of 0.01 M Mn⁺⁺ to the medium increased the D.I. nearly threefold.³

In view of these results, it was decided to repeat the experiments, using a synthetic medium in which the Mn concentration could be controlled. For this purpose, the synthetic, chemically defined medium of Gerhardt and Wilson (1948) was employed. This medium consists of 0.30 per cent DL-asparagine, 0.50 per cent lactic acid, 3.0 per cent glycerol, 0.75 per cent NaCl, 1.0 per cent K₂HPO₄, 0.01 per cent Na₂S₂O₃, 10.0 μg per ml Mg⁺⁺, 0.10 μg per ml Fe⁺⁺, 0.10 μg per ml Mn⁺⁺, thiamine, nicotinic acid, Ca-pantothenate, and biotin. The pH was

TABLE 2

The effect of metal ions upon the establishment of nonsmooth variants in smooth cultures of B. abortus grown in synthetic medium

ADDED TO 5 ML OF SYNTHETIC MEDIUM	AMOUNT OF CATION ADDED	DISSOCIATION INDEX	BACTERIAL GROWTH (TURBIDITY)*
	<i>ml</i>		
Na ₄ P ₂ O ₇ (0.33 M)—0.5 ml.....	—	0	31.5
—0.2 ml.....	—	0	30.5
Mn ⁺⁺ (0.01 M).....	0.5	39	33.0
	0.2	46	32.0
Mg ⁺⁺ (0.1 M).....	0.2	32	31.0
	0.1	44	—
(Control).....	—	16†	29.0
Mn-deficient synthetic medium.....	—	0	31.5
Mn-deficient medium + Mn ⁺⁺ (0.01 M).....	0.3	11	36.0
Mn-deficient medium + Mg ⁺⁺ (0.1 M).....	0.1	10	29.5
Mn-deficient medium + Co ⁺⁺ (0.01 M).....	0.2	—	0
	0.1	—	0
	0.05	0	—

* Klett reading.

† Average D.I. of 10 cultures.

adjusted to 6.8. A Mn-deficient medium containing all of the constituents above except Mn was also prepared.

Table 2 presents some typical data obtained with the synthetic medium. The D.I. of *Brucella* grown in this medium was more constant than that in beef extract broth. In the presence of 0.03 M pyrophosphate, nonsmooth types did

³ The addition of higher concentrations of Mn or Mg salts produced toxic effects and consequently lower D.I.'s (cf. lines 7 and 11, table 1), thereby masking the specific effects detectable in the presence of nontoxic cation concentrations. Because of the uncontrolled trace metal "contamination" of the ingredients of complex media results obtained after the addition of Mn or Mg salts may differ between different batches of broth; e.g., 0.3 ml of 0.1 M MnSO₄ added per 5 ml of broth may produce toxic effects in one batch of broth but not in another one.

not establish themselves, whereas the control cultures had a D.I. of 16. The striking enhancement of the D.I. produced by the addition of small amounts of Mn^{++} is evident, as is also the absence of nonsmooth variants in Mn-deficient synthetic medium. The latter effect could be reversed by the addition of either Mn^{++} or Mg^{++} to the Mn-deficient medium, but not by Co^{++} . The question arises at this point whether the enhancement of the D.I. by Mn^{++} and Mg^{++} may be attributed to a growth stimulation induced by these substances. The data in table 1 and table 2 make it appear improbable that such is the case. The only instance of definite growth stimulation was that obtained after the addition of Mn to Mn-deficient synthetic medium.

In order to explain the absence of nonsmooth types in 10-day-old cultures containing pyrophosphate, it could be assumed that the smooth to nonsmooth change is prevented, that the establishment of nonsmooth types is selectively inhibited, or that a combination of these processes is in operation. In an attempt

TABLE 3

The influence of pyrophosphate upon the establishment of rough types in 10-day-old broth cultures of B. abortus inoculated with various mixtures of smooth and rough cells

	INOCULUM											
	2% R + 98% S			4% R + 96% S			10% R + 90% S			100% S		
Ml of 0.33 M $Na_4P_2O_7$ added to 5 ml of broth.....	—	0.2	0.5	—	0.2	0.5	—	0.2	0.5	—	0.2	0.5
Per cent of rough types in 10-day-old cultures.....	10*	6	5	17	11	6	36	14	12	7	0	0

* All figures represent averages of triplicate determinations.

to clarify this point, media with and without pyrophosphate were inoculated with mixtures of smooth and rough clones of *B. abortus* containing known percentages of rough and smooth organisms. The cultures were treated as before, and the percentage of rough types was determined 10 days after inoculation.

From the results of these experiments, summarized in table 3, it may be noted that, even though the increase of rough types was less in pyrophosphate-containing media than in unsupplemented media, the percentages of rough types in $Na_4P_2O_7$ were higher after the 10-day growth period than they were in the original inoculum.

Thus only an incomplete selective effect was demonstrable in these experiments, and it remained to be determined whether this incomplete effect might have been due to the ability of rough types to increase initially in these mixed cultures, regardless of the presence of pyrophosphate, by utilizing stored materials.⁴ If this was true, the fate of rough types in mixed cultures should be dif-

⁴ Subsequent information (cf. Goodlow, Mika, and Braun, *J. Bact.*, *this issue*) indicates that it is not the utilization of stored materials of rough types that may be involved but rather the utilization of stored materials of smooth types, which, even in the presence of $Na_4P_2O_7$, may lead to an initial accumulation of certain metabolites creating conditions that will favor the establishment of rough types.

ferent if smooth and rough cells were grown separately in pyrophosphate-containing media prior to mixed inoculation into fresh media with pyrophosphate. Broth containing 0.013 M or 0.03 M $\text{Na}_4\text{P}_2\text{O}_7$ was, therefore, inoculated with mixtures of smooth and rough cells that had previously been grown separately for 5 days in media containing 0.03 M $\text{Na}_4\text{P}_2\text{O}_7$. When such cultures were sampled after 10 days it was found that the percentage of rough types did not exceed that present in the inoculum; in fact it had decreased (table 4). Thus, under these conditions a selective effect against rough types in the presence of pyrophosphate and smooth cells is demonstrable, and this selective effect is strong enough to explain the inability of nonsmooth variants to establish themselves in smooth cultures containing pyrophosphate.

TABLE 4

The influence of pyrophosphate upon the establishment of rough types in 10-day-old broth cultures of B. abortus inoculated with mixtures of "pyrophosphate-grown" smooth and rough cells

	INOCULUM					
	1% R 99% S			7% R + 93% S		
Ml of 0.33 M $\text{Na}_4\text{P}_2\text{O}_7$ added to 5 ml of broth.....	—	0.2	0.5	—	0.2	0.5
Per cent of rough types in 10-day-old cultures.....	7*	<1	1	13	5	4

* All figures represent averages of triplicate determinations.

DISCUSSION

The present data suggest that the concentration of ionic Mn or Mg, or both, in the culture medium of *B. abortus* is an important environmental determinant of the percentage of nonsmooth variants that may establish themselves during 10 days of growth in originally smooth broth cultures (D.I.). Not only can the dissociation index in a given culture of *B. abortus* be made to increase two- or threefold by suitable addition of Mn or Mg ions, but this effect is elicited also without any marked growth stimulation of the bacterial population. Furthermore, the ability of Na-pyrophosphate to suppress the establishment of nonsmooth types in originally smooth cultures appears to be attributable to its ability to form metal complexes with Mn and Mg, since these ions can reverse the pyrophosphate effect.

Other instances of similar relationships between pyrophosphate and ionic Mg or Mn may be cited. Thus, the enzyme isocitric dehydrogenase requires Mn or Mg ions for its activity. When 0.01 M pyrophosphate is added, the enzyme is inhibited because the free Mg or Mn ions are bound by the pyrophosphate. This inactivation can be abolished by the addition of 5×10^{-4} M Mn^{++} (Adler, Euler, Gunther, and Plass, 1939). Likewise, Warburg and Christian (1942) have shown that yeast zymohexase is inactivated by 0.02 M pyrophosphate. Activity could be regenerated by traces of divalent metals.

The enhancement by Mn or Mg of the establishment of nonsmooth types in smooth cultures, and the selective suppression of nonsmooth variants in the absence of these ions, may constitute an indirect rather than a direct effect upon nonsmooth variants. This is suggested by the recent results of Goodlow, Mika, and Braun (1950), who demonstrated that the accumulation of alanine as a metabolic end product in smooth broth cultures creates conditions favorable for the establishment of spontaneously arising nonsmooth variants. It is conceivable that Mn or Mg, or both, are necessary for the activation of enzymatic processes involved in the production of alanine. The absence of these activators, in turn, may inhibit alanine accumulation and thereby deprive the nonsmooth types of conditions that favor their establishment when they arise in or are mixed with smooth populations. This is also indicated by the fact that there is no significant effect upon the growth of pure R populations in pyrophosphate broth (Goodlow and Mika, unpublished data).

In this connection it is interesting to note that Mn and Mg ions have been shown to be essential for phosphatase activity (Green and Colowick, 1944), for the reaction between phosphopyruvate and adenosine diphosphate in cell-free bacterial suspensions (Boyer, Lardy, and Phillips, 1943), and for the oxidation of pyruvic acid in *Lactobacillus delbrueckii* (Lipmann, 1937) and *Escherichia coli* (Krebs, 1937). Also of possible interest is the recent work of Webb (1948), who has shown that Mg^{++} is required for the cell division mechanism of *Clostridium welchii*. In the absence of Mg^{++} this microorganism produces filamentous forms that revert to cells of normal morphological appearance on subculture to a medium containing free Mg^{++} ions. Finally, Pollock and Wainwright (1948) have demonstrated a specific stimulating effect of Mg^{++} on nitratase adaptation of a gram-negative coliform bacterium, "1433." According to these authors, Mg^{++} is concerned at some point in the chain of reactions that constitute the phenomenon.

SUMMARY

In the presence of 0.013 M or more of Na-pyrophosphate the establishment of nonsmooth variants in growing smooth broth cultures of *Brucella abortus* is selectively suppressed. This effect, produced in complex media as well as in a simple synthetic medium, can be antagonized by the simultaneous addition of small amounts of Mn^{++} or Mg^{++} . It was shown that the concentration of ionic Mn^{++} or Mg^{++} is an important environmental determinant of the dissociation index (i.e., the percentage of nonsmooth variants that may establish themselves during 10 days of growth in originally smooth broth cultures). The effect of pyrophosphate may, therefore, be attributable to its ability to remove free Mn^{++} and Mg^{++} from the media by forming metal complexes with these ions. The probable mechanism responsible for the effect of these environmental agents upon population changes has been discussed.

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