METABOLISM AND POPULATION CHANGES IN *BRUCELLA ABORTUS*

II. Terminal Oxidation and Oxygen Tension in Population Changes

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The numerous investigations of population changes in aging liquid cultures of *Brucella* species have been reviewed in the preceding paper. Other environmental factors greatly influence population changes but have received insufficient attention since the comprehensive study of Braun (1946) who demonstrated the effects of inoculum size, pH, O/R potential and other conditions on population changes in *Brucella abortus*. The results of renewed interest in these factors have been published recently in connection with a study on the effects of antisera upon population changes (Braun *et al.*, 1955). In addition it was observed (Mika *et al.*, *unpublished data*) that the extent and rate of population changes were strongly controlled by surface to volume ratios. Braun's earlier observations (1946) showed that no population changes occur on the surface of solid media, and that agitation of a tube of liquid culture medium prior to sampling for colonial variants so altered conditions in the tube that it could not be compared after additional incubation to a tube which had remained undisturbed for the total period of incubation. Such observations indicated the probable importance of oxygenation in influencing population changes. This paper presents more critical evidence obtained in studies specifically designed to test such effects of oxygen tension on population changes.

MATERIALS AND METHODS

The methods employed have been fully described in the preceding paper (Altenbern *et al.*, 1956). Conventional Warburg manometric techniques were used where indicated.

RESULTS

Since oxygenation reduced population changes effectively it became desirable to test the effects of hydrogen acceptors other than oxygen. Initially, nitrate ion, which is reduced by *Brucella* species was employed. The addition of potassium nitrate to liquid synthetic medium prior to inoculation significantly reduced population changes compared to the control value. The effect occurred in other nonsynthetic liquid media such as Albimi broth, buffered beef extract broth and a casein hydrolyzate medium (table 1). It is known that both methylene blue and resazurin are rapidly reduced by Brucella cells, and either dye proved effective in reducing population changes (table 2). Attempts to reverse such effects by adding sodium thioglycolate were unsuccessful since the extreme toxicity of this compound, and other sulfhydryl compounds, interfered with growth of smooth type cells in concentrations necessary for the production of lowered oxygen concentration.

To strengthen further the evidence for the selective function of hydrogen acceptors, the growth of a smooth and a nonsmooth *Brucella abortus* clone was determined in media containing nitrate. The nonsmooth clone was isolated from old cultures of the smooth type. The growth of the smooth strain was very greatly enhanced by nitrate whereas the nonsmooth cells were stimulated to a lesser degree (figure 1).

An effort was made to determine the effects of reduced oxygen tension on both population changes and growth. Reduction in available oxygen was accomplished by physical means since chemical anaerobiosis was unsuccessful. Tubes of liquid Gerhardt-Wilson medium were inoculated with smooth cells and placed in large vacuum desiccators. Air pressure in the desiccators was reduced with a vacuum pump to various values. After incubation for 14 days, these cultures were examined for nonsmooth variants. Incubation of originally smooth cultures in liquid synthetic medium in pressures of air ranging from 720 to 50 mm Hg had a profound affect on the rate and extent of population change (figure 2). The effect was obtained with

four separate smooth isolates of *B. abortus* strain 19.

Turbidimetric determination of growth of smooth and nonsmooth isolates in G.W. medium showed that nonsmooth types grew at a lesser rate than smooth types at atmospheric pressure, but at low air pressure, the nonsmooth type demonstrated greater growth rate than the smooth cells (table 3). The rate of diffusion of dissolved oxygen and the rate of consumption of oxygen by hydrogen transport mechanisms of the cell probably determined the time at which nonsmooth mutant types became selectively

TABLE 1

The effect of potassium nitrate on population changes of originally smooth cultures of Brucella abortus strain 19 in various liquid media

Concentration of KNO_3	Medium			
	Gerhardt-Wilson	Buffered beef extract broth	Albimi broth	Partially hydrolyzed casein medium
per cent	*per cent nonsmooth types*			
0	47	10	95	96
0.01	54	0	77	80
0.02	45	0	32	—*
0.03	9	0	—*	78
0.04	1	0	27	—*
0.07	0	0	10	70
0.10	—*	0	1	38

*—Conditions not tested.

Each tube was inoculated with 0.1 ml of a saline suspension of smooth *B. abortus* strain 19. Incubated at 37 C for 14 days. Results are averages of triplicate tubes. Figures represent per cent of nonsmooth types in population.

TABLE 2

The effect of methylene blue and resazurin on population changes of originally smooth cultures of Brucella abortus strain 19 in liquid Gerhardt-Wilson medium

Methylene Blue Concentration	Nonsmooth types	Resazurin Concentraition	Nonsmooth types
μg/ml	*per cent*	*μg/ml*	*per cent*
0	47	0	13
0.2	9	0.1	13
0.5	15	0.2	1
1.0	0	0.5	1
		1.0	0.1
		1.5	0

Each tube inoculated with 0.1 ml of a saline suspension of smooth *B. abortus* strain 19. Incubated at 37 C for 14 days. Results are averages of triplicate tubes.

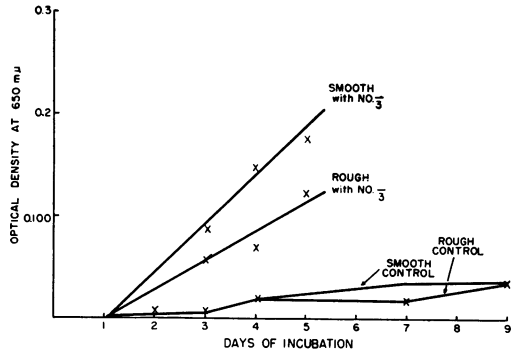

Figure 1. Growth of smooth and rough types of *Brucella abortus* strain 19 in liquid synthetic medium with and without potassium nitrate.

Final concentration of KNO_3 was 0.07 per cent. Results are averages of triplicate determinations. Incubation temperature, 37 C.

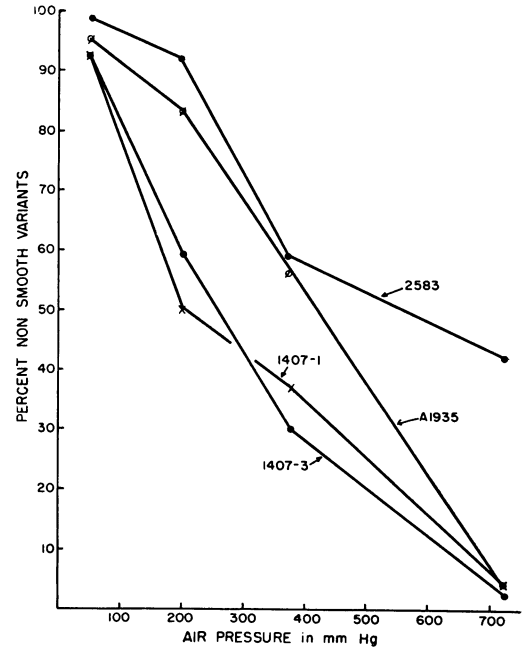

Figure 2. Population changes of isolates of smooth *Brucella abortus* strain 19 in liquid synthetic medium incubated under various pressures of air.

All results are averages of triplicate determinations. Incubated at 37 C for 14 days.

favored and began to constitute a gradually increasing percentage of the culture's viable cells.

Therefore, the effect of reduced pressure on population changes in very shallow layers was determined. Tubes of liquid G.W. medium were inoculated with a mixture of 95 per cent smooth cells and 5 per cent nonsmooth cells and the tube contents were poured aseptically into petri dishes which were then incubated in desiccators under reduced air pressure as previously described. No change in rate or degree of population change could be detected until the pressure of air had been reduced to 50 mm Hg (table 4). Although the factor of diffusion of dissolved oxygen has not been eliminated, it has been minimized and the experiment shows that the critical concentration of oxygen below which nonsmooth types are favored must be quite low.

On the basis of the foregoing results it could be predicted that population changes might be accelerated by decreasing the time required to produce large numbers of cells, mostly of the smooth type, which would lead to more rapid oxygen depletion and selection of nonsmooth types. Figure 3 demonstrates the direct proportionality between inoculum size and degree of population changes at the time of sampling. The increased number of nonsmooth cells introduced initially by a larger inoculum is insignificant since it has been shown that mixed inocula consisting of as much as 5 per cent nonsmooth cells yield results only slightly different from results obtained with an all-smooth inoculum. Identical results concerning the relationship between inoculum size and population changes were reported by Braun (1946).

Investigations of differences in hydrogen transport mechanism between smooth and nonsmooth type cells have revealed that smooth cells have a lesser affinity for sodium azide than nonsmooth cells. Azide inhibition of lactate oxidation by smooth and derived nonsmooth cells was measured for four sets of smooth and nonsmooth (rough) organisms. Three of these pairs were

TABLE 3

Growth rates of smooth and nonsmooth Brucella abortus strain 19 in liquid Gerhardt-Wilson medium during incubation under various air pressures

	Air Pressure During Incubation (mm Hg)		
	720	375	50
	optical density		
Smooth cells	0.068	0.079	0.002
Rough cells	0.034	0.083	0.023

Figures represent changes in optical density at 650 mμ per 24 hr during period of linear growth rate (0 to 4 days). Results are averages of triplicate tubes. Incubated at 37 C.

TABLE 4

The effect of pressure of air during incubation on population changes in cultures of Brucella abortus strain 19 grown in shallow layers of liquid Gerhardt-Wilson medium

	Pressure of Air (mm Hg)				
	50	100	200	375	720
Per cent nonsmooth cells.	48	11	17	9	13

Incubated at 37 C for 13 days. Inoculum consisted of 95 per cent smooth cells and 5 per cent rough cells.

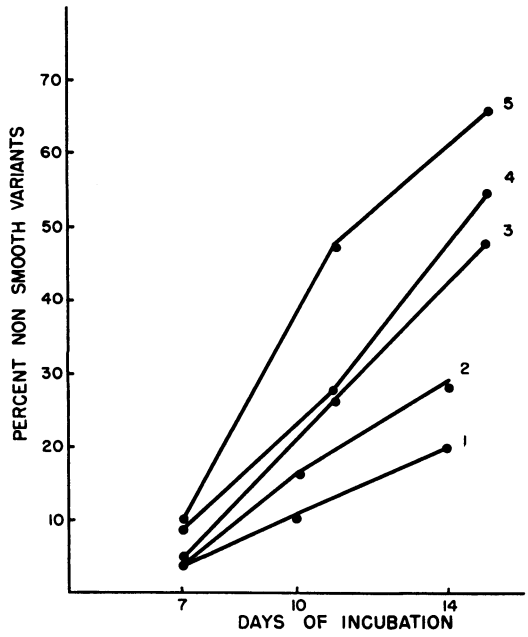

Figure 3. The effect of inoculum size on the rate and extent of population changes in originally smooth cultures of *Brucella abortus* strain 19 in liquid synthetic medium.

All results are averages of triplicate determinations. Incubated at 37 C for times indicated. Final cell concentrations per ml at times of inoculation were: *1*, 6 × 10^6; *2*, 11 × 10^6; *3*, 22 × 10^6; *4*, 55 × 10^6; *5*, 110 × 10^6.

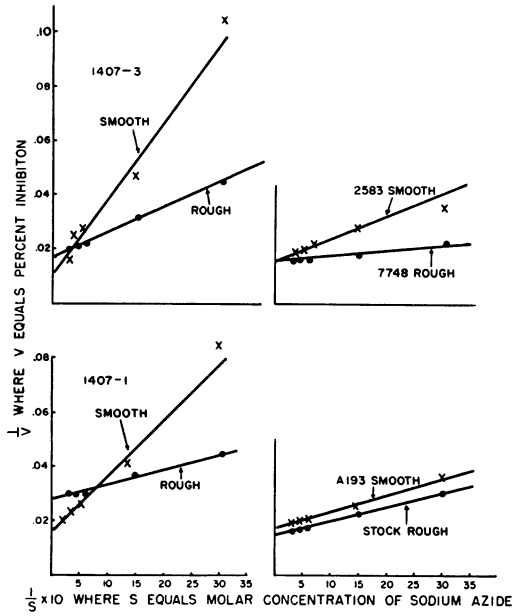

Figure 4. Azide inhibition of lactate oxidation by smooth-nonsmooth pairs of *Brucella abortus* strain 19.

Standard manometric techniques employed. Flasks contained 1.0 ml of cells (2–3 mg N), 1.1 ml of 0.1 M phosphate buffer, pH 7.4, 0.2 ml of 20% KOH in center cell, 0.2 ml of 0.1 M lactate, pH 7.4 and 0.5 ml of sodium azide in 7.4 buffer. Lactate tipped in at 20 min; azide tipped at 70 min. Percentage inhibition calculated from linear rates of oxygen uptake. Temperature 36.8 C. Gas phase was air.

closely related; i.e., the nonsmooth clone employed was isolated from an aged culture of the smooth type, while one pair consisted of smooth and nonsmooth cells which were "unrelated" and had been maintained as stock cultures for many months. Data portrayed in figure 4 show that, in the three pairs of smooth and nonsmooth cells which were related, nonsmooth cells exhibited greater affinity for azide than did smooth cells, whereas, in the smooth-nonsmooth pair which was unrelated, the azide affinities were identical. Similar results were obtained when cyanide was employed as the inhibitor. Appropriate experiments demonstrated that after use of mixed inocula (95 per cent smooth cells and 5 per cent nonsmooth cells) of the three related smooth-nonsmooth pairs, population changes in liquid G.W. medium involved primarily the selective establishment of nonsmooth types corresponding to the nonsmooth cells used in the inoculum. Population changes occurring in cultures grown from a mixed inoculum of the unrelated smooth-nonsmooth pair involved the selective establishment of novel intermediate types whereas the rough cells of the inoculum did not increase in percentage and did not possess any selective advantage over the smooth type employed. It appears valid on the basis of general enzyme kinetics to assume that affinities for oxygen parallel those observed for sodium azide. The data demonstrating the effects of aeration and the supply of hydrogen acceptors other than dissolved oxygen in suppressing population changes indicate more efficient use of hydrogen acceptors by rough cell types than by the smooth types. Such a concept is supported by the studies on azide inhibition discussed above.

DISCUSSION

The foregoing results strongly indicate that oxygen or the supply of other hydrogen acceptors is a critical factor in population changes of *B. abortus* from smooth to nonsmooth under the conditions employed. Similar control of population changes may apply to comparable systems. It is obvious that any condition which alters energy-yielding or other synthetic mechanisms in one type more strongly than in another type will provide conditions favoring growth of the less inhibited type. The fact that the results of oxygen deficiency and of addition of other hydrogen acceptors can be duplicated in several synthetic or complex nonsynthetic media indicates that other nutritional factors are of minor importance compared to oxygen supply. With rapid aeration (shaking machine or Warburg apparatus), growth rates and Q_{O_2} values for various substrates are nearly the same for both smooth and rough organisms. Reduction in the concentration of hydrogen acceptor creates conditions favoring the growth and viability of rough organisms. The selective value of rough cells under such conditions may stem from the probable greater affinity for oxygen in the terminal hydrogen transport. Experiments with lactate oxidation in evacuated Thunberg tubes using methylene blue as the hydrogen acceptor have revealed no differences in K_M values for methyene blue between smooth and rough cells indicating

that the observed difference in azide (oxygen) affinity represent true differences in the cytochrome components of the two cell types.

The recent discussion of cytochrome pigments in bacteria (Clark *et al.*, 1955) contains material of possible relevance to the observations on population changes. It is conceivable that an efficient pathway for hydrogen transport other than cytochrome exists for rough organisms whereas smooth organisms are dependent predominantly on cytochrome systems when grown in the presence of iron. Investigation by Waring *et al.* (1953) has revealed that cultivation of smooth *Brucella* species in iron-free medium results in moderately decreased growth and markedly decreased population change. These results may indicate that, in the absence of iron, cytochrome pigments are not formed by either smooth or rough organisms and that smooth cells rely on other methods of hydrogen transport which provide them with greater selective advantage. Experiments with pyrophosphate (Cole and Braun, 1950) and Versene (Braun and Matney, unpublished data) have indicated the same relationships.

It should be stated that investigations such as those concerned with factors controlling population changes necessarily involve a fair amount of time and, under the conditions employed here, the utilization of cells from repeated transfers of stock strains. In the course of such studies, repetitions of certain experiments designed to demonstrate absolute quantitative metabolic differences between smooth and rough cells frequently yielded different values; however, relatively comparable results always were obtained. It is probable that the repeated transfers and manipulations of cultures resulted in orthoselection; i.e., otherwise innocuous genetic changes modifying processes which determine absolute selective values.

Finally, the studies of population changes reported here, coupled with recent data concerning the role of oxygen in mixed bacterial cultures (Charlton, 1955) and in human malignancy (Warburg, 1956) indicate the increasing recognition of the important position of oxygen in general cellular differentiation.

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SUMMARY

Population changes from smooth to nonsmooth type cells in originally smooth cultures of *Brucella abortus* strain 19 in a liquid defined medium are suppressed by the addition of hydrogen acceptors such as nitrate ion, methylene blue, and resazurin. Potassium nitrate in a concentration of 0.07 per cent stimulated the growth of smooth type cells to a greater degree than rough type cells.

Incubation of smooth cultures under reduced air pressure greatly accelerated population changes. It has been determined that under reduced pressure of air, rough types grow more rapidly than smooth types.

Increases in the size of the smooth inoculum increase the rate and extent of population changes. The rough type cells, which constitute the majority of the viable population after prolonged incubation of originally smooth cultures, possess a greater affinity for sodium azide than the smooth parent cells.

The implications of these results with regard to the probable causative mechanism of population changes are discussed.

ADDENDUM

After these manuscripts were prepared, Dr. Werner Braun called to our attention a paper by Sanders and Huddleson (Am. J. Vet. Research, **17**, 324–330, 1956) in which the critical role of oxygen in population changes of Brucella cultures was clearly recognized.

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