METABOLISM AND POPULATION CHANGES IN *BRUCELLA ABORTUS*

I. Roles of Alanine and Pantothenate in Population Changes

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The studies of Goodlow *et al.* (1950) established the concurrence of population changes of strains of brucella in liquid synthetic medium with the accumulation of alanine. D-Alanine added to the culture medium accelerated population changes whereas L-alanine had no effect except in very high concentrations (Goodlow *et al.*, 1951). The configuration of asparagine used as a nitrogen source in the synthetic medium significantly affected population changes (Goodlow *et al.*, 1952) and pantothenic acid suppressed such changes when the inoculum had been grown in pantothenate-free medium (Mika *et al.*, 1954).

These data indicated a selective establishment of spontaneously arising nonsmooth mutants with increased alanine resistance, suggesting a direct relationship between alanine accumulation and population changes. However, alanine appears in detectable concentrations in culture filtrates only after conditions selectively favoring nonsmooth types have been established and the quantity of alanine produced even after prolonged incubation is considerably less than the amount of added D-alanine required to alter the rate and extent of population changes. In this and the following paper, it will be shown that other conditions, notably oxygen deficiency, are the major selective factors governing population changes.

MATERIALS AND METHODS

Isolates of *Brucella abortus* strain 19 were maintained on modified tryptose agar. Cells for inocula were prepared by suspending the growth from a 48-hr slant of the organism on modified tryptose agar (Goodlow *et al.*, 1950) or albimi agar in sterile 0.1 M phosphate buffer and adjusting with the same buffer to a density of approximately 1×10^8 cells per ml. Tubes containing 10 ml of synthetic Gerhardt-Wilson medium (Gerhardt and Wilson, 1948), were inoculated with 0.1 ml of the cell suspension and incubated at 37 C. After various times of incubation, cultures were thoroughly agitated and a loopful of culture was streaked on 2–1 agar (Goodlow *et al.*, 1950). Colonial variants were detected and counted with obliquely transmitted light. When various additions were made to the medium prior to inoculation, double strength Gerhardt-Wilson medium was prepared and 5-ml amounts were placed in tubes. Desired amino acids or peptides dissolved in distilled water were added and the total volume was adjusted to 10 ml with distilled water. L- and D-Alanine were obtained from Nutritional Biochemicals Company. L-Alanyl-L-valine and L-alanyl-L-leucine were synthesized by the organic chemistry section at this installation. Procedures for obtaining large numbers of cells have been previously described (Altenbern and Ginoza, 1954).

Vitamin assays were performed with dehydrated assay media (Difco) and the appropriate organism.

Amino acid concentrations were determined by the quantitative paper chromatography method of Housewright and Thorne (1950). Pyruvate was determined by the method of Friedmann and Haugen (1943).

RESULTS

Initially, studies were conducted to determine whether L-alanine would influence accelerated population changes induced by the addition of D-alanine to the liquid synthetic medium. Series of tubes of liquid Gerhardt-Wilson medium were prepared with varying concentrations of L- and D-alanine, and inoculated with smooth *B. abortus* strain 19 (strain no. 2583). After 10, 14, and 19 days of incubation at 37 C, samples from triplicate tubes of each concentration of amino acids were streaked on 2–1 agar, incubated for 4 or 5 days, and the percentage of colonial variants was determined.

In these experiments, the inertness of L-alanine in modifying population changes was confirmed. In contrast, D-alanine promoted

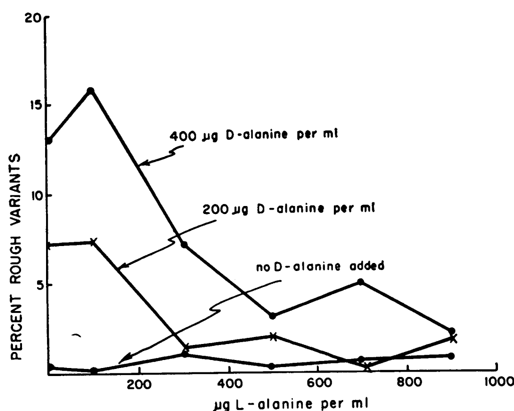

Figure 1. L-Alanine reversal of D-alanine acceleration of population changes of originally smooth cultures of *Brucella abortus* strain 19 in liquid synthetic medium.

All results are averages of triplicate tubes. Incubation time was 14 days at 37 C.

population changes in proportion to the amount originally added to the medium. This acceleration of population changes by D-alanine was competitively antagonized by L-alanine (figure 1).

The foregoing results suggested that L-alanyl dipeptides would noncompetitively antagonize the promotion of population changes by D-alanine. Series of tubes of Gerhardt-Wilson medium containing varying amounts of D-alanine and either L-alanyl-L-leucine or L-alanyl-L-valine were prepared and inoculated with smooth *B. abortus* strain 19 (2583). After 10, 14, and 19 days of incubation, triplicate tubes were analyzed for colonial variants. It was found that both L-alanyl dipeptides reversed the action of D-alanine (table 1). Further investigation revealed that reversal of D-alanine effects by these two compounds may proceed by two separate mechanisms. L-Alanyl-L-leucine was found to inhibit growth of the nonsmooth type of *B. abortus* strain 19 (strain no. 7748 which was derived from smooth 2583) whereas L-alanyl-L-valine had no noticeable inhibitory effect on such cells (table 2). Thus, it appears that L-alanyl-L-valine reverses the action of D-alanine by true, noncompetitive antagonism but L-alanyl-L-leucine antagonizes acceleration of population changes by a suppression of growth of nonsmooth types. However, even in the latter case, noncompetitive reversal of D-alanine activity may also be operative.

The competitive nature of the antagonism between L- and D-alanine and the apparent noncompetitive reversal of D-alanine activity by L-alanyl dipeptides resemble the work on D-alanine inhibition of *Lactobacillus casei* and its reversal of L-alanine and L-alanyl dipeptides by Kihara and Snell (1952).

The suggestion that D-alanine may be synthesized by these organisms, thus creating a selective

TABLE 1

The effect of L-alanyl-L-leucine and L-alanyl-L-valine on population changes of cultures of smooth Brucella abortus strain 19 in liquid Gerhardt-Wilson medium in the presence and absence of D-alanine

D-Alanine	L-Alanyl-L-leucine (µg/ml)			
	0	100	200	400
	Percentage of Cells*			
µg/ml				
0	16	5	4	1
200	79	35	2	3
400	72	38	7	7
	L-Alanyl-L-valine (µg/ml)			
	0	100	200	400
0	13	15	14	13
200	22	28	17	20
400	50	18	21	23

* All figures represent percentage of nonsmooth cells in the culture and are averages of triplicate determinations. Incubated at 37 C for 14 days. Inoculum contained less than 0.01 per cent nonsmooth variants.

TABLE 2

The effect of L-alanyl-L-leucine on growth of rough Brucella abortus strain 19 in liquid Gerhardt-Wilson medium in the presence and absence of D-alanine

D-Alanine	L-Alanyl-L-leucine (µg/ml)			
	0	100	200	400
	Optical densities*			
µg/ml				
0	0.104	0.072	0.052	0.047
200	0.117	0.074	0.066	0.046
400	0.090	0.062	0.051	0.043

* Figures are optical densities of cultures at 650 mµ after incubation for 14 days at 37 C. All data are averages of triplicate determinations.

environment favoring nonsmooth types, assumed an efficient biochemical mechanism for production of D-alanine. Therefore, several possible routes of D-alanine synthesis have been explored. Alanine is synthesized mainly or entirely in this organism by transamination reactions (Altenbern and Housewright, 1953). Filtrates from smooth cell suspensions synthesizing alanine from L-glutamic acid and sodium pyruvate were incubated with a D-amino acid oxidase preparation from pig kidney employing standard manometric technique. The filtrate contained approximately 500 μg alanine per ml. There was no oxygen uptake noted from as much as 2 ml of filtrate. The same amount of filtrate did not inhibit the oxidation of an authentic specimen of D-alanine. Filtrates from aged cultures containing 90–100 μg of alanine per ml gave similar results. Attempts to demonstrate an alanine racemase with whole cells of either smooth or rough types failed, confirming the report of Marr and Wilson (1954). Neither smooth nor nonsmooth cells possessed enzymes capable of transaminating D-amino acids with pyruvate. A variety of conditions was tested in an effort to detect β-decarboxylation of D-aspartic acid yielding D-alanine by either smooth or rough types but negative results were obtained.

Pantothenate metabolism. A study of the constituents of liquid Gerhardt-Wilson medium during growth of smooth and rough types showed few alterations in the medium. In general, alanine became detectable at 8 days of incubation. Pyruvate accumulated rapidly and become detectable after 24 hr of growth. Asparagine was continually hydrolyzed, releasing free aspartic acid. When asparagine was present as the racemate or D-isomer, pantothenate concentration remained unchanged. However, when L-asparagine was employed as the nitrogen source, smooth cultures produced excess pantothenate whereas rough cultures utilized the pantothenate of the medium (table 3). Therefore several corresponding smooth-rough types (i.e., smooth parent type and rough type derived from a given parent smooth strain during population changes in liquid synthetic medium) were examined for differences in pantothenate metabolism which might support previously published data concerning the effect of pantothenate and its analogues on population changes. Determination of the Michaelis constants (K_M) in dialyzed sonic extracts of smooth and rough types for potassium and for β-alanine in the coupling reaction producing pantothenate (Ginoza and Altenbern, 1955) from these precursors revealed no differences in enzyme characteristics. The K_M for potassium pantoate was 3.3×10^{-2} M for the smooth type and 2.9×10^{-2} M for the rough type, measured at pH 8.0. Dialyzed sonic extracts of both smooth and rough cells gave identical Michaelis constants for β-alanine, about 4×10^{-5} M.

TABLE 3

Concentration of ingredients of Gerhardt-Wilson medium after growth of smooth and nonsmooth cells of Brucella abortus strain 19

Asparagine Isomer	Colony Type	Concentration (µg/ml) after 17 Days of Incubation				
		Aspartate	Alanine	Pantothenate	Niacin	Pyruvate
L	smooth	168	67	0.11	0.17	129*
DL	smooth	391	98	0.06	0.18	125*
D	smooth	315	79	0.047	0.22	120*
L	rough	157	trace	0.029	0.19	350
DL	rough	360	64	0.052	0.18	55
D	rough	204	trace	0.052	0.22	112
Uninoculated medium		trace	0	0.040	0.20	0

* Denotes values obtained after incubation for 10 days.

Pantothenate, added to undialyzed sonic extracts, rapidly becomes unavailable for assay by *Lactobacillus arabinosus*. The rate and amount of disappearance of free pantothenate are dependent upon ATP, indicating incorporation of pantothenate into higher conjugates which are not detectable by *L. arabinosus* assay. Determination of Michaelis constants for pantothenate in this reaction revealed no differences between sonic extracts of smooth and rough cells (about 8.3×10^{-6} M).

Subsequently, possible differences between intact smooth and rough cells in uptake and assimilation of pantothenate were studied. Washed cell suspensions of equal optical density were inoculated in equal amount into flasks containing pantothenate solutions in buffer. The flasks were incubated at 37 C on a reciprocating shaker. Samples were withdrawn after various times of incubation, centrifuged to remove cells,

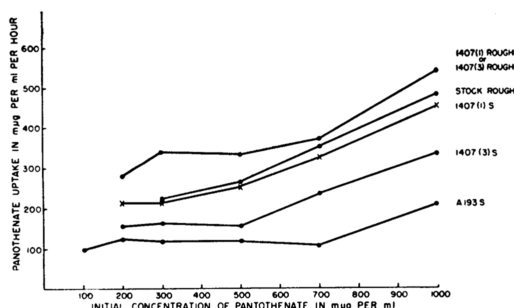

Figure 2. The uptake of pantothenate by smooth and rough cells of *Brucella abortus* strain 19.

Each flask contained 5 ml cells (8.2 mg N) in 0.1 M phosphate buffer, pH 7.4, 2 ml of pantothenate solution of varying concentration and 13 ml of buffer. Shaken at 37 C on reciprocating shaker.

and the supernatant fluids assayed for remaining pantothenate. The results (figure 2) indicate that the rate of pantothenate uptake (maximum rate attained) for any particular initial pantothenate concentration was greater for the rough type than for the smooth type, suggesting that the pantothenate concentration of synthetic Gerhardt-Wilson medium (40 mμg per ml) is less than optimal after long incubation for the smooth types and, consequently, favors nonsmooth types. Clone A193 S was subsequently found to be an exceptionally smooth type which is selectively favored over the rough types in liquid aged culture, thus corresponding to the behavior of S′ types reported by Goodlow *et al.* (1951).

Finally, the effect of surplus pantothenate in suppressing population changes suggested that synthesis of the vitamin by smooth types was inhibited, whereas nonsmooth types were unaffected or inhibited to a lesser extent. It has been shown that the β-alanine moiety of pantothenate can arise from L-asparagine but not from D-asparagine, and that D-asparagine inhibits this reaction (Altenbern and Ginoza, 1954). Since liquid synthetic Gerhardt-Wilson medium contains DL-asparagine as a nitrogen source, the degree of D-asparagine inhibition of pantothenate synthesis by smooth cells was compared to that of rough cells. Pantothenate synthesis by smooth types was inhibited to a slightly greater extent by D-asparagine than pantothenate synthesis by rough types. However, the significance of this difference remains questionable.

DISCUSSION

These data demonstrate that added L-alanine is capable of antagonizing the selective effects of added D-alanine, whereas comparable concentrations of L-alanine fail to affect the extent and rate of population changes occurring in nonsupplemented cultures. This difference minimizes the possible role of naturally occurring alanine in population changes. There remains a great deal of evidence that D-alanine is indeed selective and does promote population changes. However, the possibility that smooth types normally synthesize and excrete D-alanine, and thereby create selective conditions favoring nonsmooth types, may be questioned. Resting cell experiments have shown that alanine produced by transamination contains no detectable amounts of the D-isomer. Also, the possibility of a D-amino acid transaminase of the type reported by Thorne and Molnar (1955) was tested and eliminated. A racemase for alanine in *B. abortus* has been described by Marr and Wilson (1954). However, demonstration of this racemase required sonic extracts and an unusually high concentration of pyridoxal phosphate. These authors found no racemase activity in whole cells with or without pyridoxal phosphate, but they did find that DL-alanine accumulated during L-glutamate oxidation by whole cells, thus effectively demonstrating the intracellular activity of this enzyme.

In addition to the previous conclusions that naturally accumulating alanine may represent a major selective factor, it has been suggested that such effects may involve differential inhibition of pantothenic acid synthesis or utilization. The evidence for such conclusions was the finding that pantothenic acid affected population changes provided the smooth inoculum had been passed through several transfers in pantothenate-free medium. All present efforts to detect, in resting cells or cell free extracts, pronounced rate differences between smooth and rough cells in pantothenate synthesis, uptake of pantothenate, or incorporation of pantothenate into higher compounds were unsuccessful. Therefore, it may be suggested that growth of the inoculum in pantothenate-free medium in the previous studies selectively damaged smooth cells, and

that such damage can be repaired when the cells subsequently are provided with adequate pantothenate.

The above considerations in regard to selective effects in aging smooth cultures require an alternative explanation for the known sequence of events. The relatively large concentration of pyruvate in the culture adequately provides the carbon skeleton for alanine which accumulates during population changes. It is known that resting cell suspensions of smooth type cells in the presence of pyruvate will synthesize alanine slowly by transamination with intracellular amino acids. This may account for the appearance of alanine in culture filtrates at times of gradual establishment of nonsmooth types. In paper II we will discuss evidence suggesting that the population changes in aging cultures may be due primarily to oxygen deficiency.

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SUMMARY

The selective action of D-alanine in promoting population changes from smooth to nonsmooth types in originally smooth cultures of *Brucella abortus* strain 19 in liquid synthetic medium was antagonized competitively by L-alanine. Two L-alanyl dipeptides also antagonized the selective properties of D-alanine, apparently noncompetitively. One of these dipeptides, L-alanyl-L-valine reversed the activity of D-alanine which suppresses growth of smooth type cells, while the other dipeptide, L-alanyl-L-leucine, exerted its antagonistic effects by suppression of spontaneously arising nonsmooth mutant types. There was no effect of L-alanine in suppressing population changes in cultures not supplemented with D-alanine. These data contradict the hypothesis that metabolically produced D-alanine is a major selective factor which promotes population changes.

During growth in Gerhardt-Wilson synthetic medium containing L-asparagine as the nitrogen source, smooth type cells synthesized and excreted pantothenic acid whereas rough type cells utilized the preformed pantothenate present in the medium. There were no quantitative differences between cell free extracts of smooth and rough cells in the kinetics of pantothenate synthesis or metabolism. Whole rough type cells exhibited a greater rate of pantothenate uptake than did smooth type cells. D-Asparagine inhibition of pantothenate synthesis from pantoyl lactone and L-asparagine was greater for smooth cells than for rough cells.

The relevance of these findings to the known sequence of events in population changes from smooth to nonsmooth types is discussed.

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