THE NATURE OF THE EFFECT OF α-ALANINE ON POPULATION CHANGES OF BRUCELLA[1]

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Previous studies have related population changes in originally smooth cultures of brucella to the extracellular accumulation of the metabolite α-alanine (Goodlow *et al.*, 1950; Altenbern and Housewright, 1951; Braun, 1952). Alanine resistant, usually nonsmooth mutants, occurring at a frequency of approximately 1 in 10^7 cells (Braun and Ciaccio, 1952), established themselves in such cultures as soon as the alanine concentration had reached a critical level. By adding D- or DL-alanine to cultures at time of inoculation with alanine susceptible smooth cells, such naturally occurring phenomena could be reproduced at an early point in the culture's growth; L-alanine proved inactive (Goodlow *et al.*, 1951*a*). However, even after the initial addition of very large amounts of D- or DL-alanine, smooth cells were not inhibited until the culture was approximately 48 hours old. This indicated that the inhibitory effects of α-alanine might not be direct but that alanine might interfere with the synthesis or utilization of a substance vital for the multiplication of alanine susceptible cells. The delayed inhibitory effects of this amino acid could then be due to the prolonged utilization of stored intermediates of such vital substances. Dr. Werner Maas suggested to us that α-alanine may interfere with pantothenic acid (PA) synthesis, as also indicated by previous observations with yeast (Nielsen *et al.*, 1944). Following this lead, population studies with brucella were initiated to test whether the effects of α-alanine may have a similar relationship to pantothenic acid synthesis or utilization. Previous investigators have claimed that pantothenic acid or its precursors, pantoic acid (or its lactone) and β-alanine, are required for growth of brucella (see Hoyer, 1950).

The following data will describe the results of population studies in which the possible relationship between pantothenic acid synthesis and α-alanine inhibition of brucella was investigated.

[1] Portions of this paper were presented at the 1951 SAB meeting.

MATERIALS AND METHODS

The majority of experiments were performed with *Brucella abortus*, strain 6232 (CO_2 dependent); but in some experiments *Brucella abortus*, strain 19, and *Brucella suis*, strain PSIII, also were employed. Standardized suspensions of smooth clones of these strains (Goodlow *et al.*, 1950) were inoculated into flasks or tubes containing, respectively, 100 ml or 10 ml of Gerhardt-Wilson medium (Gerhardt and Wilson, 1948). Plate counts and the percentage of nonsmooth variants were determined according to previous descriptions (Goodlow *et al.*, 1950) after various periods of incubation at 37 C. α-Alanine was employed in the DL-form.

RESULTS

Addition of pantothenate. The effects of increased concentrations of pantothenate upon population changes in nonaerated test tube cultures inoculated with smooth (S) cells of either *B. suis* or *B. abortus*, strain 19, were first investigated. As usual, a significant number of nonsmooth mutants established themselves in the control cultures within ten days of incubation. These control cultures contained 0.04 μg per ml of calcium pantothenate as prescribed in the standard Gerhardt-Wilson medium. In contrast, in cultures to which increased amounts of pantothenate had been added, such population changes were suppressed (table 1). The amount of pantothenate required to overcome the alanine effect differed depending on the strain of *Brucella* employed; the addition of 0.2 μg per ml of calcium pantothenate significantly suppressed population changes of *B. abortus*, whereas at least 12 μg per ml were required for *B. suis* cultures. These effects of pantothenate upon population changes in initially smooth cultures

TABLE 1

The effect of increase in calcium pantothenate concentration upon population changes of Brucella

Amount of Pantothenate Added to Gerhardt-Wilson Medium	Average Percentage of Nonsmooth Types after 15 Days in Originally Smooth Cultures of:	
	Brucella abortus, strain 19	*Brucella suis*
μg/ml		
0.2	3	54
1.2	2	38
4.0	2	45
8.0		44
12.0		12
none	61	64

were not associated with any change of growth rates of the smooth cells. Similar results were obtained in experiments with the CO_2 dependent *B. abortus*, strain 6232, which was used in most of the previous alanine studies and in all additional experiments reported here.

Depletion of intracellular pantothenic acid. To determine whether the normally observed initial resistance of S populations to alanine may be associated with the utilization of preformed, intracellularly stored pantothenic acid inocula for flask cultures were prepared from pantothenate deficient S cells that had been transferred five times at 24 hour intervals through pantothenate deficient Gerhardt-Wilson medium. The control cultures were inoculated with cells that had been grown on pantothenate-rich tryptose agar. As shown in figure 1, pronounced dependence of population changes on pantothenic acid levels again became apparent: cultures inoculated with "deficient" cells displayed far more pronounced population changes than cultures inoculated with nondeficient cells. The establishment of alanine resistant mutants also was more pronounced in the presence of DL-alanine in pantothenate deficient media compared to the extent of population changes of similar inocula in pantothenate deficient media without alanine.

The fact that even in pantothenate-free media growth and population changes occurred eventually implied that S cells can continue growth in the absence of added pantothenic acid. This was further confirmed by the observation that, following five daily transfers through pantothenate-free media, contained in test tubes, S cells ceased to grow but were able to reinitiate growth 72–96 hours later. No evidence could be obtained which would indicate that such cells, capable of renewed growth without extracellular pantothenate, represent mutants; and it must be concluded that pantothenic acid stimulates growth but is not an absolute growth requirement for brucella. Subsequent studies with resting cells of *B. abortus*, strain 19 (Altenbern and Ginoza, 1954), demonstrated even more decisively that *B. abortus* can synthesis pantothenic acid.

Cultural conditions also strongly affected the dependence of growing smooth *B. abortus*, strain 6232, cells on preformed pantothenate; temporary cessation of growth in pantothenate-free media, observed consistently after five daily transfers of smooth cells through media in test tubes, did not occur when smooth cells were transferred as much as twelve times through similar media in flasks. In addition to daily transfers in deficient media, aeration of resting cells and aeration of cells growing in deficient cultures for various periods of time were used in attempts to deplete cells of stored pantothenic acid. The results were similar to those already described: depending on the period of aeration, growth either continued after transfer or ceased only temporarily. This ability of smooth cells to reinitiate or to continue growth even in pantothenate-free media, presumably by slow synthesis of pantothenic acid, seriously interfered with the unequivocal demonstration of relationships among growth, pantothenic acid synthesis, and inhibitory effects of α-alanine. These complications should be kept in mind in inspecting the results of growth studies with smooth cells in

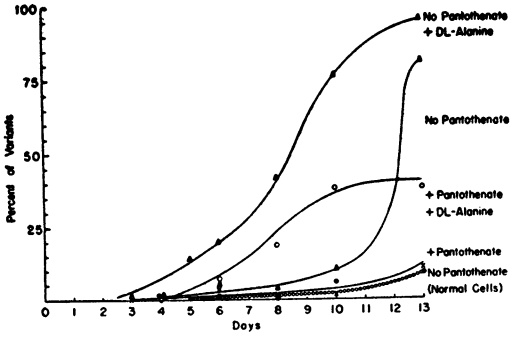

Figure 1. Population changes in various media inoculated with smooth *Brucella abortus*, strain 6232, cells previously transferred in calcium pantothenateless Gerhardt-Wilson medium.

flask cultures presented in figure 2. They show that partially depleted smooth cells grow more slowly in deficient media than in pantothenate containing media. However, for the present analysis the result with pantothenic acid deficient smooth cells in pantothenateless, α-alanine containing media is particularly significant: although growth occurs in these cultures within four days after inoculation, it can be accounted for by the multiplication of nonsmooth, alanine resistant mutant cells. These mutants, subsequently isolated from cultures that have undergone population changes, show a significantly shorter period of inhibition of growth in pantothenateless, α-alanine containing media. Thus, both population and growth studies further indicated a relationship between pantothenic acid and the effects of α-alanine.

Studies with intermediates of pantothenic acid synthesis. Since pantothenic acid could alleviate the effects of α-alanine on population changes, pantoic acid and β-alanine, the two moieties of pantothenic acid, were also tested. Flasks and test tubes containing Gerhardt-Wilson medium supplemented with different amounts of either β-alanine, pantoyl lactone, or calcium pantothenate (used on an equimolar basis) were inoculated with either pantothenate deficient smooth cells (previously transferred through pantothenate-free medium in test tubes) or "normal" smooth cells from tryptose-agar slants. In addition one series of cultures contained 200 μg of α-alanine per ml. The levels of added pantothenate used extended from 0.01 μg to 40.0 μg/ml, β-alanine from 0.03 μg to 11.0 μg/ml, and pantoyl lactone from 0.06 μg to 23.0 μg/ml. Population changes and growth (viable cells) were checked 2, 4, 6, 10, and 17 days after inoculation. (The term growth, as used in the following paragraphs, will refer to the increase in the total number of viable cells independent of the ratio of smooth to nonsmooth cells in this total.) Some representative results are shown in table 2. In the case of "normal" cells all three compounds were active in suppressing population changes in cultures with or without added α-alanine. In contrast, in cultures inoculated with pantothenate deficient cells only the addition of pantothenate tended to suppress population changes, whereas β-alanine or pantoyl lactone failed to reduce the extent of population changes. The viable counts were uniform among the various cultures and, therefore, are not presented in the tabulation of results.

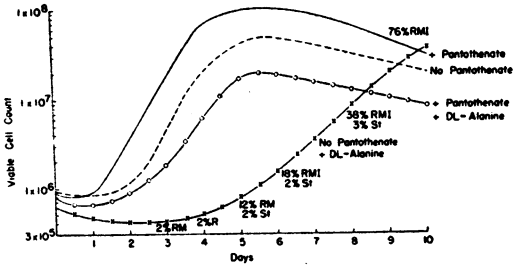

Figure 2. Growth in various media inoculated with smooth *Brucella abortus*, strain 6232, cells previously transferred in calcium pantothenateless Gerhardt-Wilson medium.

TABLE 2

The effects of pantothenate and its precursors upon population changes in originally smooth Brucella abortus cultures (flasks) started with or without the addition of DL-alanine

Pantothenate-free Gerhardt-Wilson Medium Supplemented with:	μg/ml	Average Percentage Nonsmooth Types after 10 Days in Originally Smooth Cultures Inoculated with:	
		"Normal cells"	Pantothenate deficient cells
no DL-alanine added:			
—	—	65	60
Calcium pantothenate	4.0	12	30
β-Alanine	1.1	20	73
Pantoyl lactone	2.3	29	70
with 200 μg/ml DL-alanine:			
—	—	90	100
Calcium pantothenate	4.0	17	34
β-Alanine	1.1	37	99
Pantoyl lactone	2.3	14	90

The effects of salicylate, 2-chloro-4-aminobenzoic acid, and pantothenic acid analogues. In studies with *Escherichia coli* mutants, Maas (1952) demonstrated that salicylate interferes with pantothenic acid synthesis, more specifically with pantoic acid synthesis. Since in the brucella studies α-alanine appeared to act by antagonism with pantothenic acid, salicylate might be expected to produce the same effects as α-alanine upon population changes. This was found to be the case. Salicylate added to liquid cultures in concentrations above 100 μg/ml significantly

enhanced population changes, and when incorporated into soft Gerhardt-Wilson agar (0.75 per cent agar) 0.12 mg of sodium salicylate per ml completely inhibited the growth of smooth cells but did not interfere with the growth of nonsmooth mutants or highly alanine resistant S′ mutants. Because of this parallel activity of α-alanine and salicylate, it became possible to use soft Gerhardt-Wilson agar containing salicylate as an effective screening medium for the determination of mutation rates from the smooth to the more alanine and salicylate resistant nonsmooth types (Braun and Ciaccio, 1952; Braun, 1953).

King *et al.* (1948) have reported that the inhibition of growth which takes place when *E. coli* cells are incubated with 2-chloro-4-aminobenzoic acid (CAB) can be reversed by pantothenic acid. In view of this relationship, the effect of 2-chloro-4-aminobenzoic acid upon growth and population changes of *B. abortus* in pantothenate-free media was tested in tube and flask cultures. Inocula were prepared either from pantothenate deficient cells or from "normal" cells previously exposed, under strong aeration, to 2-chloro-4-aminobenzoic acid in phosphate buffer at 35 C for two hours ("preadaptation"). With either type of inoculum initially smooth cultures displayed a considerable retardation of growth when the concentration of 2-chloro-4-aminobenzoic acid was 500 μg/ml or higher and showed a corresponding suppression of population changes (table 3). Unlike α-alanine or salicylate, 2-chloro-4-aminobenzoic acid appeared to have a general inhibitory effect on *B. abortus;* even nonsmooth mutants present in these 2-chloro-4-aminobenzoic acid cultures were inhibited to the same extent as smooth cells and did not establish themselves selectively.

Several analogues of pantothenic acid and its precursors also were tested for their effects, namely β-amino propiophenone, an analogue of β-alanine, tauryl pantothenone (Snell, 1941), and phenyl pantothenone, both of which interfere with the utilization of pantothenic acid (Novelli and Lipmann, 1948), and DL-[(N-pantoyl) N tauryl] *p*-anisidide, which also has been claimed to be an antagonist of pantothenic acid (Winterbottom *et al.*, 1947). Two types of inocula were prepared as described for 2-chloro-4-aminobenzoic acid above, and the cells were added to pantothenate-free Gerhardt-Wilson medium containing various concentrations of the analogues. Tauryl pantothenone, at 1.4 μg to 1,000 μg/ml, had no effect in any of the experiments. Phenyl pantothenone at 40 and 80 μg/ml had no effect on growth and population changes, whereas it completely inhibited growth at concentrations of 250 μg/ml and above. β-Amino propiophenone in concentrations up to 10 μg/ml had no effect, but it produced considerable inhibition of growth and *enhanced* population changes when present in concentrations of 20 or 40 μg/ml (see table 3) and completely inhibited growth and population changes at concentrations above 40 μg/ml. It proved far more toxic for "preadapted" cells than for cells harvested from a tryptose-agar slant or from daily transfers through pantothenate-free media. DL-[(N-pantoyl) N tauryl] *p*-anisidide had no effect when cultures containing up to 500 μg/ml were inoculated with pantothenate deficient

TABLE 3

The effects of DL-*alanine, β-amino propiophenone, and 2-chloro-4-aminobenzoic acid (CAB) on growth and population changes in Brucella abortus cultures*

Days after Inoculation	Control: Pantothenate-free Gerhardt-Wilson Medium		+ DL-Alanine 500 μg/ml		+ Amino Propiophenone 40 μg/ml		+ CAB 1,000 μg/ml	
	% nonsmooth	Viable count × 10^7	% nonsmooth	Viable count × 10^7	% nonsmooth	Viable count × 10^7	% nonsmooth	Viable count × 10^7
0	5	0.003	5	0.003	5	0.003	5	0.003
1	4	0.013	4	0.001	4	0.017	2	0.009
2	16	0.14	28	0.04	18	0.06	5	0.027
3	24	4.4	28	1.9	34	0.13	4	0.045
4	29	9.7		8.7	63	0.20	4	0.11
5	44	17.0	59	11.8	69	0.21	3	0.10
6	56	17.9	84	15.0	75	0.11	9	0.12
7	59	15.0	100	15.0	98	0.12	7	0.092

TABLE 4

Summary of the effects of various analogues

Analogue	Effect on Growth	Effect on Population Changes	Remarks
β-Amino propiophenone	+	+	
Phenyl pantothenone	+	−	
Tauryl pantothenone	−	−	
DL-[(N-pantoyl) N tauryl] *p*-anisidide	−	−	With pantothenate deficient cells
DL-[(N-pantoyl) N tauryl] *p*-anisidide	−	+	With nondeficient cells

cells, whereas as little as 4 μg/ml enhanced population changes without inhibiting growth of inocula of nondeficient smooth cells. These results are summarized in table 4.

The effects of coenzyme A. Since pantothenic acid is a component of coenzyme A, the effects of the latter were tested with the aid of two preparations: (a) a coenzyme A extract with 75 per cent activity (obtained from the Pabst Laboratories), and (b) a crude coenzyme A extract from brucella prepared according to Kaplan and Lipmann (1948). Both preparations at 4 μg/ml failed to show any effects.

Alanine resistance of streptomycin resistant mutants. Streptomycin resistant mutants furnished additional but more indirect evidence regarding a relationship between α-alanine effects and pantothenic acid. Most streptomycin resistant smooth mutants (S/sr) isolated from *B. abortus*, strain 6232, proved more resistant to the inhibitory effects of α-alanine and, accordingly, tended to display lesser population changes, and remained unaffected by increasing α-alanine levels or reduced pantothenate supply under cultural conditions under which their more streptomycin and alanine susceptible parent types tended to undergo rapid population changes (table 5). This increased stability of populations of streptomycin resistant smooth cells, comparable to that previously observed with alanine resistant S′ mutants (Goodlow *et al.*, 1951*b*), expressed itself in both the absence and presence of noninhibitory concentrations of streptomycin in the medium (table 6). But whereas streptomycin resistant mutants frequently showed increased resistance to alanine, none of a number of mutants isolated for increased resistance to alanine (S′) displayed any increase in resistance to streptomycin. These observations fit in well with the results reported for relationships between streptomycin resistance and pantothenic acid in *E. coli:* Wyss and Schaiberger (1953) found that some streptomycin resistant strains contain increased amounts of pantothenic acid and increased activity of a pantothenate synthesizing enzyme, while Lichstein and Gilfillan (1951) observed earlier that inhibition by strep-

TABLE 5

Population changes after six days in originally smooth cultures of several streptomycin resistant mutants in the presence of various levels of DL*-alanine and pantothenate. All inocula were prepared from pantothenate deficient cells.*

Inoculum	Calcium Pantothenate in the Medium	Amounts of DL-Alanine Added (μg/ml)			
		0	200	300	500
S	+	25*	80	95	95
S	−	45	92	95	98
S/sr A	+	10	5	5	7
S/sr A	−	7	6	8	10
S/sr B	+	17	17	24	23
S/sr B	−	23	22	22	37
S/sr C	+	50	50	50	50
S/sr C	−	47	50	50	44

* Percentage nonsmooth types.

TABLE 6

Population changes in cultures started with S, S′, or S/sr cells in the presence or absence of streptomycin (50 μg/ml) and DL*-alanine (200 μg/ml)*

Inoculum	Added to Basal Gerhardt-Wilson Medium	Percentage Nonsmooth Types after:			
		2 days	4 days	6 days	8 days
S	—	1	3	10	38
S′	—	1	0	0	1
S/sr	—	1	1	1	6
S	Stm		no growth		
S′	Stm		no growth		
S/sr	Stm	1	2	4	11
S	DL-alanine	3	16	42	92
S′	DL-alanine	0	0	1	4
S/sr	DL-alanine	0	3	10	12
S	Stm + DL-alanine		no growth		
S′	Stm + DL-alanine		no growth		
S/sr	Stm + DL-alanine	1	4	6	20

tomycin can be relieved considerably by pantothenic acid. In view of these findings with *E. coli*, coupled with the above cited evidence regarding α-alanine-pantothenic acid relationship in *B. abortus*, it does not appear surprising that streptomycin resistant *B. abortus* cells, which also might contain increased amounts of pantothenic acid, should prove to be more resistant to the effects of α-alanine.

DISCUSSION

The evidence obtained in the above studies appears to indicate that there is a relationship between the inhibitory effects of D- or DL-α-alanine upon smooth cells in growing *B. abortus* cultures, leading to increased population changes involving the establishment of more alanine resistant mutants and the synthesis or utilization of pantothenic acid. Increased levels of pantothenic acid, or its precursors β-alanine and pantoic acid, in the medium counteracted the α-alanine effects, whereas lowering of the intracellular or extracellular levels of pantothenate enhanced the inhibitory effects of α-alanine. In addition, other agents known to interfere with pantothenic acid synthesis have proved capable of producing the same effects as α-alanine, and a number of streptomycin resistant mutants, suspected of quantitatively altered pantothenic acid synthesis on the basis of studies by others, displayed increased alanine resistance.

The present studies fail to settle the actual site of such postulated interference but suggest the involvement of steps prior to the coupling of pantoic acid and β-alanine. This suggestion is supported by the findings that either β-alanine or pantoyl lactone can counteract the inhibitory effects of α-alanine and that salicylate, a known inhibitor of pantoate synthesis (Maas, 1952), can reproduce the effects of α-alanine. Another possible mechanism might involve the coupling of pantoic acid and α-alanine instead of β-alanine in the presence of inhibitory levels of α-alanine, leading to the production of a nonutilizable compound. However, the formation of such a compound has not been demonstrated, and if it were to occur, it would still require a clear demonstration that there are biological differences between the D- and L-forms of such a compound.

Since the inhibitory effects of α-alanine upon smooth cells lead to enhanced population changes, involving the establishment of more alanine resistant, usually nonsmooth mutants, the rate of pantothenic acid synthesis or the requirements for pantothenic acid might differ between smooth and nonsmooth types. Preliminary data on resting cells of *B. abortus*, strain 19 (Altenbern and Ginoza, 1954), suggest that smooth cells may be more dependent on the pantothenic acid which they synthesize, while nonsmooth cells, also capable of synthesizing pantothenic acid, can easily utilize added preformed pantothenate. Any agent interfering with pantothenic acid synthesis, therefore, would effect smooth cells more than nonsmooth cells. But these facts alone cannot explain all the observations made with growing cultures; further studies on requirements and synthesis of pantothenic acid by smooth and nonsmooth cells are needed in order to account fully for the differences in D-α-alanine resistance of smooth and nonsmooth types.

It is interesting to note that in the case of pantothenate deficient cells only pantothenic acid but not pantoic acid nor β-alanine can decrease the extent of population changes in normal and α-alanine supplemented cultures; whereas, any one of these three compounds can decrease population changes in cultures started with nondeficient cells. This might indicate that the end product, pantothenic acid, must be present in sufficient amounts to activate the enzyme coupling β-alanine and pantoic acid. However, the alternative possibility, that this difference between deficient and nondeficient cells is merely due to the fact that cells have been generally weakened by prior transfers through pantothenate-free media and thus are unable to utilize intermediates as efficiently as the final product, cannot be excluded on the basis of available data. In this connection it is also of interest that studies with *Neurospora* mutants (Wagner and Haddox, 1951) have indicated that the enzyme catalyzing the synthesis of pantothenic acid from β-alanine and pantoyl lactone is present under both *in vivo* and *in vitro* conditions but is inactive under certain *in vivo* conditions.

Some doubt has been cast on a direct interference by D-α-alanine with the synthesis of pantothenic acid by studies with resting cells of *B. abortus*, strain 19, in which such interference could not be demonstrated (Altenbern and Ginoza, 1954). Therefore, the simple relationships indicated with growing cells of *B. abortus*, strain 6232, might be misleading, or they might reflect

differences between strains used or the inability to duplicate with resting cells all of the events taking place in growing cultures.

Finally, this study again demonstrates that the extent of population changes in cultures of brucella depends not only upon metabolic events but also upon the prior history of cultivation of the cells employed (see Braun, 1952, for additional examples). In the present study population changes differed significantly in identical media, depending on whether the parent cells had grown previously in media with high or low pantothenate levels. This observation, in conjunction with the additional evidence cited above, also permits an answer to the initially raised question: Why are smooth cells previously grown in pantothenate containing media capable of fairly prolonged growth without inhibition after transfer into media containing a very large amount of D- or DL-α-alanine? The answer appears to be that the availability of intracellularly stored pantothenic acid or its precursors may serve to counteract any immediate inhibitory effects of D-α-alanine.

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SUMMARY

The inhibitory effects of D- or DL-α-alanine upon smooth cells of *Brucella abortus* lead to population changes involving the establishment of more alanine resistant, usually antigenically different mutants. These effects appear to involve an interference with pantothenic acid synthesis or utilization. This is indicated by observations on the effects of increased pantothenate levels in the media, the effects of partial depletion of intracellular pantothenic acid, and the activity of known inhibitors of pantothenic acid synthesis, including salicylate and of certain analogues of pantothenic acid. Additional indirect evidence is provided by the finding that many streptomycin resistant mutants simultaneously display increased resistance to the D-isomer of α-alanine.

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