STUDIES ON THE EFFECTS OF D-AMINO ACIDS ON BRUCELLA ABORTUS[1]

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The inhibition of growth of gram-positive and gram-negative bacteria by D-amino acids has been reported (Fox, Fling, and Bollenback, 1944; Fling and Fox, 1945; Kobayashi, Fling, and Fox, 1948). This paper reports the toxicity of the racemates and the unnatural isomers of some amino acids for strains of *Brucella abortus*.

EXPERIMENTAL METHODS

Broth (Albimi) containing 2 per cent "M" peptone, 0.1 per cent glucose, 0.2 per cent yeast autolysate, 0.5 per cent NaCl, and 0.01 per cent sodium bisulfite was the medium used. Four and one-half ml of medium were added to each tube, sterilized for 15 min at 15 lb pressure, and the tubes of media, after inoculation, were incubated at 37 C in air or under varying amounts of carbon dioxide.

Three strains of *B. abortus* were used: a strain which required added carbon dioxide, strain no. 1335; a relatively avirulent strain no. 19; and a highly virulent strain no. 2308. The latter two strains had no added carbon dioxide requirement. The inoculum was prepared by growing the desired strain in 10 ml of "Albimi" broth in air or under 10 per cent carbon dioxide at 37 C for 48 hours. After centrifuging the cells and resuspending them in sterile physiological saline, 0.1 ml of the saline suspension containing approximately 3×10^8 was used as inoculum for each tube of medium.

The desired amount of carbon dioxide was obtained by displacing the air with carbon dioxide in an anaerobic jar in which the inoculated tubes of broth were placed.

The essential amino acids (Rose, 1937) were made up separately in 0.1 M solutions, sterilized in the autoclave at 15 lb pressure for 15 min, and added aseptically to the broth in amounts to give the desired concentrations.

Growth was measured turbidimetrically with an Evelyn colorimeter using the 660 mμ filter. Five ml of water were added to the heat-killed broth cultures before they were read in the colorimeter. The colorimeter scale was set at 100 per cent transmission with the broth diluted with 5 ml of water. To determine the degree of inhibition of *B. abortus* by the amino acids, the control broth cultures were diluted with the diluted broth medium to 75, 50, 25, and 10 per cent of maximum growth and read in the Evelyn colorimeter. From these readings, a turbidity curve was plotted which was used as a standard of reference. The readings of the growth in the broth tubes containing the amino acids were then used to determine from this curve the amount of inhibition attributable to the amino acids.

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RESULTS

In table 1 the toxicity for 3 strains of *B. abortus* of the racemic forms of the indispensable amino acids is presented. There is some inhibition of growth of the 3 strains when grown under optimal conditions, but when the added carbon dioxide-requiring strain is grown under a suboptimal amount of added carbon dioxide, the inhibitory effect of the DL-amino acids is very pronounced. DL-Phenylalanine and DL-methionine are the most effective amino acid inhibitors tested, and DL-lysine is the least effective. The results of the initial experiments with DL-arginine were variable, but a new sample of the amino acid consistently shows toxic effects. Strain no. 1335, grown under 10 per cent added carbon dioxide, strain no. 19, and strain no. 2308 reached the amount of growth usually

TABLE 1

The effect of DL-*amino acids upon the growth of 3 strains of Brucella abortus*

AMINO ACID ADDED	μM PER ML OF MEDIUM	PER CENT OF INHIBITION OF GROWTH			
		Strain no. 19*	Strain no. 2308*	Strain no. 1335	
				Under 1 per cent added CO_2†	Under 10 per cent added CO_2*
None	—	0	0	0	0
DL-Threonine	10	18	20	92	15
DL-Isoleucine	10	15	13	92	13
DL-Tryptophane	saturated	15	17	92	17
DL-Lysine	10	6	6	80	10
DL-Methionine	10	34	36	100	30
DL-Phenylalanine	10	44	40	100	39
DL-Histidine	10	20	20	92	23
DL-Leucine	10	27	20	96	17
DL-Arginine	10	15	15	96	15
DL-Valine	10	21	27	100	25

* Killed and turbidity read after 4 days' growth.
† Killed and turbidity read after 9 days' growth.

produced in 4 days. When grown under 1 per cent added carbon dioxide, strain no. 1335 did not show the same amount of growth even after 8 to 9 days' incubation.

The inhibition of growth of the 3 strains of *B. abortus* by the racemate and the 2 optical isomers of some of the essential amino acids was tested, and the data are presented in table 2. The L-isomer of these amino acids is not inhibitory at the level tested. The inhibition of growth appears to be due entirely to the unnatural isomer. The degree of inhibition of growth of strain no. 1335 with the D-isomer, as with the racemic mixture, seems to be dependent upon the amount of carbon dioxide available to the bacteria. The amount of variation in inhibitory activity by the D-amino acids on strain no. 1335 grown under 10 per cent carbon dioxide in 4 separate experiments is presented in table 3. Newly prepared solutions of the amino acids were used in each of the experiments.

In figure 1 a typical series of curves is presented showing the degrees of inhibition of growth of strain no. 19 by various concentrations of the D-isomer of some amino acids. The curves for the L-isomers are not shown since there was no inhibition of growth by the natural isomer at 50 μM per ml of medium. The inhibi-

TABLE 2

The effect of stereospecificity of amino acids upon growth inhibition of 3 strains of Brucella abortus

AMINO ACID ADDED	μM PER ML OF MEDIUM	PER CENT OF INHIBITION OF GROWTH			
		Strain no. 19*	Strain no. 2308*	Strain no. 1335	
				Under 1 per cent added CO_2†	Under 10 per cent added CO_2*
None	—	0	0	0	0
D-Valine	5	29	25	100	25
L-Valine	5	0	0	0	0
DL-Valine	10	31	26	100	27
D-Leucine	5	21	19	92	20
L-Leucine	5	0	0	0	0
DL-Leucine	10	17	20	92	17
D-Histidine	5	23	20	88	21
L-Histidine	5	0	0	0	0
DL-Histidine	10	23	22	92	20
D-Methionine	5	34	36	100	34
L-Methionine	5	0	0	0	3
DL-Methionine	10	34	39	100	31
D-Phenylalanine	5	37	40	not done	42
L-Phenylalanine	5	0	0	0	0
DL-Phenylalanine	10	34	40	100	39

* Killed and turbidity read after 4 days' growth.
† Killed and turbidity read after 8 days' growth.

TABLE 3

Growth inhibition of Brucella abortus by D-amino acids in 4 different experiments

EXPERIMENT NO.	PER CENT OF INHIBITION		
	D-Valine	D-Histidine	D-Leucine
3-22	34	20	20
4-9	19	19	17
5-4	24	—	27
5-19	25	20	17

tion by the D-amino acids on an equimolar basis showed D-methionine > D-valine > D-leucine. Before the cultures were killed after 4 days' growth, plate counts were made of the number of viable bacteria in the media containing 50 μM per ml of the D-amino acids. At the time of inoculation each ml of medium had approximately 6×10^7 bacteria; after 4 days' contact with 50 μM per ml of

D-methionine, there were 56 bacteria per ml of medium. The media containing D-valine and D-leucine had 125 and 302 bacteria per ml of medium, respectively.

Strain no. 1335, when grown under 1 per cent carbon dioxide, showed almost complete inhibition by the D-amino acids. Under this low per cent of added carbon dioxide, the time required for growth in the control tube to be visually

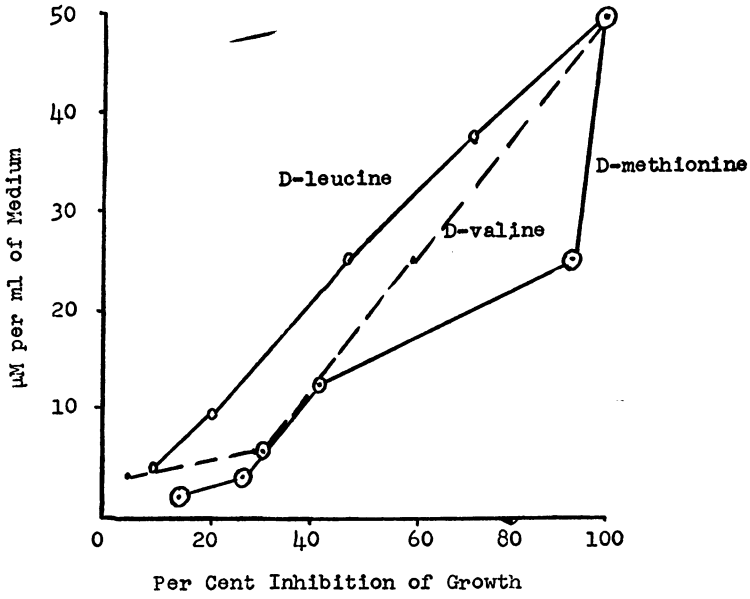

Figure 1. Inhibition of *Brucella abortus*, strain 19, by varying concentrations of 3 D-amino acids.

TABLE 4

Bactericidal effect of D-*valine upon Brucella abortus growing under 1 per cent added* CO_2

TIME OF TESTING	NUMBER OF VIABLE BACTERIA PER ML OF MEDIUM		
	Broth	Broth + L-valine 5 μM/ml of medium	Broth + D-valine 5 μM/ml of medium
At inoculation	5.6×10^7	5.6×10^7	5.6×10^7
After 1 day	2.5×10^7	2.9×10^7	1.6×10^6
After 2 days	3.3×10^7	3.5×10^7	2.2×10^3
After 3 days	7.9×10^7	7.0×10^7	1.0×10^3
After 4 days	visible growth	visible growth	no visible growth

apparent was from 3 to 4 days. Bacterial plate counts of strain no. 1335 growing in broth under 1 per cent added carbon dioxide were made for these first 3 days. Counts of the viable bacteria were made also from inoculated media to which L- and D-isomers of valine were added, and the results are shown in table 4. A bactericidal effect on strain no. 1335 is exhibited by the D-amino acid at 5 μM per ml of medium.

DISCUSSION

The inhibitory effect of D-leucine and D-valine upon the growth of *Lactobacillus arabinosus*, *Escherichia coli*, and *Staphylococcus aureus* and the effect of D-alanine upon the growth of *E. coli* have been reported (Fox, Fling, and Bollenback, 1944; Fling and Fox, 1945; Kobayashi, Fling, and Fox, 1948). In this work synthetic and complex media were used, and the molarity of the added D-amino acids which was found to be inhibitory was approximately that found to be inhibitory to the 3 strains of *B. abortus*. Dubos (1949) reported the toxicity of DL-serine and DL-alanine for virulent and avirulent strains of *Mycobacterium tuberculosis*. The inhibition reported may be due to the unnatural isomer. The avirulent strains of mycobacteria were much more resistant to DL-serine than were the virulent strains. With the brucellae no difference in toxicity was noted between the relatively avirulent strain no. 19 and the virulent strains nos. 2308 and 1335. With the mycobacteria and the brucellae there was a bactericidal and bacteriostatic effect by the addition of the 2 amino acids.

The inhibitory effect of amino acids on *B. abortus* grown in a synthetic medium has been reported (Schuhardt, Rode, and Oglesby, 1949), but no information was given on the isomeric configurations of the amino acids used. A study of the effect of stereospecificity upon the oxidation of glutamic acid, asparagine, and alanine by *B. abortus* indicated that the D-amino acid inhibited the oxidation of the L-isomer (Gerhardt, Levine, and Wilson, 1950).

The racemic mixtures of all the essential amino acids exhibit some toxicity to the 3 strains of *B. abortus*, though the inhibiting effect of DL-lysine is very slight; of the amino acids tested, the toxicity of the racemate was due entirely to the D-isomer.

It has been suggested (Kobayashi, Fling, and Fox, 1948) that the inhibition of growth of bacteria by D-amino acids may be due to the effect of the D-isomer upon the activity of the proteases or peptidases. The D-amino acids might interfere with the hydrolysis of the peptide bonds by the enzyme, or if this reaction is reversible, they may interfere with the formation of peptide bonds and thus inhibit bacterial growth by the blocking of protein synthesis. The inhibitory effect of D-phenylalanine, D-histidine, and D-isoleucine upon the action of crystalline carboxypeptidase (Elkins-Kaufman and Neurath, 1948) and the optical specificity required for the activity of leucine aminopeptidase (Smith and Polglase, 1949) add further evidence to this possible explanation.

The influence of the concentration of added carbon dioxide on the inhibition of the carbon dioxide-requiring strain by D-amino acids suggests that the added carbon dioxide is required in protein metabolism or synthesis in this bacterium. Some recent papers report results which might strengthen this hypothesis. The need for carbon dioxide by *Achromobacter fischeri* (Farghaly, 1950), grown in media washed free of that gas, could be replaced by the addition of some amino acids to the medium. Compounds involved in the Krebs cycle found by Ajl and Werkman (1948) to substitute for the carbon dioxide requirement of *E. coli* would not substitute for this requirement in the luminous bacterium. Marr and Wilson (1951) added $C^{14}O_2$ to carbon dioxide-requiring strains of *B. abortus* and

found that 80 per cent of the radioactivity of the cells was present in the protein fraction. Thorne and Gomez (1951) found that carbon dioxide was required for glutamyl polypeptide synthesis by virulent strains of *Bacillus anthracis*.

The possibility of using racemates of these amino acids in the therapy of brucellosis should be noted. A study of the combination of the beneficial effect of high protein diet upon antibody formation (Chien-Lung Yi, 1949) and the inhibition of the *Brucella* by the D-amino acids would be desirable. However, there is some toxicity to higher laboratory animals fed on high levels of D-amino acids; furthermore, these D-amino acids might not be retained effectively in the body of the animal. They might be either deaminated to the keto acid and re-aminated to the L-form, or they might be deaminated and the reaction product either excreted or further metabolized.

SUMMARY

The growth of 2 nonadded carbon dioxide-requiring and 1 added carbon dioxide-requiring strains of *Brucella abortus* in broth (Albimi) is inhibited by added D-amino acids at levels at which the L-forms do not exhibit such an effect; of the D-amino acids tested, the addition to the medium of D-phenylalanine and D-methionine caused the greatest amount of inhibition of growth. The inhibitory effect was general for the DL-form of all the essential amino acids, lysine being the least inhibitory.

The D-amino acids have a bactericidal effect upon *B. abortus* as well as a bacteriostatic effect.

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