THE TOXICITY OF CERTAIN AMINO ACIDS FOR BRUCELLAE

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In a previous paper (Schuhardt *et al.*, 1949) we suggested the possibility of certain oxidized amino acids being the antibrucella factor in toxic peptones. This possibility is in line with the numerous reported instances of the toxicity of amino acids for bacteria following the pioneer work of McLeod and his colleagues (Wyon and McLeod, 1923; Gordon and McLeod, 1926; McLeod, Wheatley, and Phelon, 1927) and that of Gladstone (1939). The latter report was doubly significant in that it introduced the principle of competitive neutralization of the antibacterial toxicity of one amino acid by other amino acids.

Several workers have reported the growth of *Brucella* spp. on chemically defined media containing amino acids (Koser, Breslove, and Dorfman, 1941; McCullough, Mills, Herbst, Roessler, and Brewer, 1947; Gerhardt and Wilson, 1948). The synthetic media used by the first two groups of workers contained 17 and 14 amino acids, respectively, and conceivably, therefore, might possess both toxic and neutralizing amino acids in their composition. The Gerhardt and Wilson medium contained DL-asparagine as the sole source of nitrogen.

McCullough and Dick (1943), in this laboratory, reported the growth of certain strains of all three *Brucella* species on an amino-acid-free medium containing inorganic ammonium salt as the sole source of nitrogen. None of these synthetic media will grow all strains of brucellae tested, and none of the authors claimed successful growth of freshly isolated, CO_2-requiring strains of *Brucella abortus*. The McCullough and Dick medium, although admittedly deficient for many strains of brucellae, seemed to offer a suitable base medium for testing the toxicity of various amino acids for strains of brucellae that would grow in this unsupplemented base medium.

EXPERIMENTAL RESULTS

The toxicity of casein amino acids for Brucella abortus 1257. Since the synthetic media of Koser *et al.* (1941) and McCullough *et al.* (1947) contained most of the amino acids of casein, and since casein digests are commonly advocated for *Brucella* culture media, we decided to do a preliminary test on the 19 amino acids found in casein combined in the relative concentrations in which they occur in natural casein. The casein amino acids (CAA) were then tested at concentrations of 0.5, 1.0, and 1.5 per cent casein equivalence in the basal medium of McCullough and Dick minus the ammonium salt. The pH of each medium was adjusted to 7.0, and each medium was distributed in 5-ml amounts to duplicate series of 10 tubes each and sterilized at 121 C for 20 minutes. The tubes were

inoculated with 0.1-ml amounts of 10-fold dilutions through 10^{-9} of a 48-hour culture of *Brucella abortus* 1257. The inoculated tubes were incubated at 37 C and examined for growth at 2, 3, and 5 days. Growth as evidenced by developing turbidity was arbitrarily designated 4+, 3+, 2+, 1+, ±, and −. Table 1 lists the results of this experiment.

These results indicate that at 0.5 per cent concentration our casein amino acids serve as an acceptable source of nitrogen with little or no evidence of toxicity for *B. abortus* 1257. When this concentration of amino acids is doubled or trebled, the medium acquires a toxicity very similar to that observed in toxic peptones in that only the largest inocula are able to attain visible growth.

TABLE 1

The toxicity of casein amino acids for Brucella abortus 1257 as measured by a graded inocula test*

INOCULA 0.1 ML.	CONCENTRATIONS OF CASEIN AMINO ACIDS								
	0.5%			1.0%			1.5%		
	Incubation period, days								
	2	3	5	2	3	5	2	3	5
Undil.	3+	3+	4+	1+	1+	2+	±	±	2+
10^{-1}	2+	3+	4+	−	±	1+	−	−	−
10^{-2}	2+	2+	4+	−	−	±	−	−	−
10^{-3}	1+	2+	4+	−	−	−	−	−	−
10^{-4}	±	1+	4+	−	−	−	−	−	−
10^{-5}	−	±	3+	−	−	−	−	−	−
10^{-6}	−	±	3+	−	−	−	−	−	−
10^{-7}	−	−	3+	−	−	−	−	−	−
10^{-8}	−	±	3+	−	−	−	−	−	−
10^{-9}	−	−	3+	−	−	−	−	−	−

* The 19 amino acids of casein combined in distilled water in the relative concentrations found in normal casein.

The only interpretation that we can visualize for these results is that one or more of the amino acids of our CAA is toxic for *B. abortus* 1257 at the concentration present in 1.0 and 1.5 per cent, and that this toxicity is not neutralized by comparable increased concentrations of the other amino acids above those in 0.5 per cent CAA. This observation necessitated the testing of the individual amino acids of this series for toxicity for this organism.

Toxicity of the individual casein amino acids for B. abortus 1257. The 19 amino acids that had been incorporated in the CAA were tested individually for toxicity to *B. abortus* 1257 at concentrations above and below that found in 1 per cent CAA. Tubes containing the various concentrations of the amino acid being tested, in 5 ml of McCullough-Dick medium, were inoculated with 0.1 ml each of a 10^{-3} dilution of a 48-hour broth culture of the test organism. The tubes were incubated at 37 C for 5 days and examined for growth in comparison with inoculated tubes of McCullough-Dick medium containing no amino acid.

Table 2 records the results of this experiment. A greater than (>) sign indicates that no inhibition of growth of the type indicated was observed at the amino acid concentration listed, which was the highest concentration tested. Where inhibition of growth was observed, the smallest concentrations giving partial and complete inhibition are recorded. These results indicate moderate toxicity of phenylalanine and methionine and marked toxicity of tryptophan and cystine for *B. abortus* 1257. As might be expected, some of the amino acids tended to stimulate rather than inhibit growth of this organism.

TABLE 2

Toxicity of individual casein amino acids for Brucella abortus 1257

AMINO ACID	QUANTITY PRESENT IN 1% AMINO ACID CASEIN,* μg PER ML	QUANTITY REQUIRED PARTIALLY TO INHIBIT GROWTH, μg PER ML	QUANTITY REQUIRED TO COMPLETELY INHIBIT GROWTH, μG PER ML
Glutamic acid	2,180	>2,000	>2,000
Proline	800	>1,000	>1,000
Histidine	760	>1,000	>1,000
Arginine	520	>1,000	>1,000
Lysine	260	>1,000	>1,000
Alanine	180	>1,000	>1,000
Glycine	40	1,000	>1,000
Serine	580	500	>1,000
Aspartic acid	410	500	>1,000
Hydroxyproline	20	500	>1,000
Threonine	390	250	>1,000
Tyrosine	650	4	>1,000
Valine	790	500	1,000
Leucine	970	500	1,000
Isoleucine		250	1,000
Methionine	360	6	500
Phenylalanine	390	4	500
Cystine	30	4	8
Tryptophan	220	<4	4

* The values for the amino acid content of casein were taken from R. J. Williams, Textbook of Biochemistry, 2d ed., D. Van Nostrand Co., Inc., New York, 1942.

Competitive neutralization of amino acid toxicity for B. abortus 1257. A series of experiments were conducted to determine which if any of the other amino acids of the CAA would reverse the inhibition of the toxic amino acids for *B. abortus* 1257. The technique employed was essentially the same as that given above except that varying concentrations of other amino acids were added to the tubes containing an inhibitory concentration of the toxic amino acid. When reversal was observed, tests were made to determine whether or not the reversal was competitive over a range of inhibitory concentrations of the toxic compound. A partial summarization of the results of these tests is given in table 3.

Tryptophan toxicity for *B. abortus* 1257 was competitively reversed by phenylalanine, tyrosine, and histidine over a 32-fold range of its individually toxic concentration. Also 400 μg per ml of tryptophan, when added to 1 per cent

casein hydrolyzate and to 1 per cent nontoxic tryptose, failed to render these media toxic for *B. abortus* 1257. Therefore we are convinced that tryptophan is not responsible for the toxicity of 1 per cent CAA nor for the toxicity of peptones for brucellae.

Methionine and phenylalanine toxicities for *B. abortus* 1257 were readily reversed by other amino acids at concentrations of the toxic pair well above those found in 1.0 per cent CAA. Also since they failed to toxify casein hydrolyzates and nontoxic tryptose, they too are eliminated from the probability of being the toxic factor in 1.0 per cent CAA and in toxic peptones.

TABLE 3

Neutralization of the toxicity of methionine, tryptophan, and cystine by other amino acids

INHIBITORY AMINO ACID, μG PER ML		NEUTRALIZING AMINO ACID, μG PER ML REQUIRED		GROWTH, 5 DAYS
Tryptophan	16	None		−
	32	Phenylalanine	10	+
	32	Tyrosine	40	+
	32	Histidine	80	+
	64	Phenylalanine	40	+
	64	Tyrosine	80	+
	64	Histidine	320	+
	128	Phenylalanine	80	+
	128	Tyrosine	160	+
	128	Histidine	640	+
Methionine	500	None		−
	1,000	Glutamic acid	10	+
	1,000	Alanine	1,000	+
	1,000	Lysine	1,000	+
Cystine	4	None		−
	4	Glutamic acid	8	+
	8	Glutamic acid	125	+
	16	Glutamic acid	1,000	+
	32	Glutamic acid	2,000	+
	64	Glutamic acid	2,500	−

Cystine toxicity for this organism, however, was reversed by none of the other 18 amino acids tested with the exception of glutamic acid. This reversal was competitive over a wide range, but in the vicinity of the concentrations of the two amino acids expected in 1.0 per cent casein digests, the glutamic acid neutralization of the cystine toxicity was borderline. Furthermore, the addition of cystine to 1.0 per cent casein hydrolyzate or to 1.0 per cent nontoxic tryptose tended to toxify these media for all but the largest inocula of *B. abortus* 1257.

Thus cystine could be the cause of the toxicity of 1.0 per cent CAA and could be the antibrucella factor in toxic tryptose. To be the latter, however, there would have to be either an excess of cystine, or an insufficiency of glutamic acid, or a counteraction of the cystine neutralizing effect of the glutamic acid by

other compounds present in the medium. That the latter might be a possibility is evidenced by the fact that 2,000 μg per ml of glutamic acid neutralized 32 μg per ml of cystine in the absence of other amino acids (table 3), whereas 2,180 μg per ml of glutamic acid failed to neutralize 30 μg per ml of cystine in the 1.0 per cent CAA experiment (table 1). The repetition of the 1.0 per cent CAA experiment with varying amounts of cystine, however, did not substantiate this concept (table 4). Probably differences in the number of organisms in different 48-hour cultures used in preparing the inocula and possibly other factors play some part in these borderline relationships. These interrelationships of cystine, glutamic acid, and other amino acids are being investigated further in an effort to determine their possible significance in toxic and nontoxic tryptose and other peptones.

TABLE 4

Toxicity of casein amino acids for B. abortus 1257 due to its cystine content

INOCULA	CYSTINE CONCENTRATION—μG PER ML					
	10	20	30	40	50	60
	Growth—5 days					
Undil.	4+	4+	4+	4+	4+	4+
10^{-1}	4+	4+	4+	4+	4+	4+
10^{-2}	4+	4+	4+	4+	−	2+
10^{-3}	4+	4+	4+	2+	−	−
10^{-4}	4+	4+	4+	−	−	−
10^{-5}	4+	4+	4+	−	−	−
10^{-6}	4+	4+	4+	−	−	−
10^{-7}	4+	4+	4+	−	−	−
10^{-8}	4+	3+	2+	−	−	−
10^{-9}	3+	3+	2+	−	−	−

Studies on the toxicity of cystine for brucellae. In an effort to determine whether or not the observed toxicity of our 1.0 and 1.5 per cent CAA was due to the cystine content, we repeated the casein amino acids toxicity experiment using all the other amino acids at 1.0 per cent CAA concentrations but varying the cystine concentration from 10 to 60 μg per ml. Table 4 gives the results of this experiment, which seems to leave little doubt that cystine was the cause of our previously observed toxicity of casein amino acids, although the lot of cystine used in this experiment was slightly less toxic for *B. abortus* 1257 than that used in the original experiment.

Having previously observed that neither the Koser *et al.* (1941) nor the McCullough *et al.* (1947) amino acid medium would support growth of small inocula of *B. abortus* 1257, we decided to determine whether or not the high cystine content (150 μg per ml in the former and 192 μg per ml in the latter) of these media was the cause of this failure. When the cystine content of these media was lowered to concentrations tolerated by *B. abortus* 1257, this organism gave excellent growth from small inocula in both media.

We next set up an experiment to determine the maximum cystine tolerance of 42 strains of *Brucella abortus*, 10 strains of *Brucella suis*, and 9 strains of *Brucella melitensis*. For this purpose we used 1 per cent tryptose broth as the base medium. A maximum cystine concentration of 512 μg per ml was attempted by dissolving the required amount of the amino acid in the minimum necessary HCl, adding this to the base medium, and immediately adjusting the pH to 7.0. No precipitate was observed, but we doubt that the preparation retained this concentration of dissolved cystine. Additional concentrations of cystine ranging from 256 to 2 μg per ml of base medium were included in the experiment. Each test culture was adjusted to a uniform density (approximately 1 billion cells per ml) by means of an Evelyn photoelectric turbidimeter. Each test preparation received 0.1 ml of a 10^{-3} dilution of the adjusted test culture. The cultures were incubated at 37 C for 5 days with or without increased CO_2 as required.

TABLE 5

Species differences in the brucellae and strain differences in the Brucella abortus species with respect to the inhibitory action of cystine

SPECIES	NUMBER OF STRAINS TESTED		MAXIMUM NONINHIBITORY LEVEL OF CYSTINE, μG PER ML					
			16	32	64	128	256	512*
			Number of strains growing					
B. suis	10		—	—	—	—	8	2
B. melitensis	9		—	—	3	—	6	—
B. abortus	42	9 strains, require CO_2	—	—	4	5	—	—
		7 strains, adapted	4	2	1	—	—	—
		26 strains:						
		13 parents, require CO_2	—	—	11	2	—	—
		13 mutants, adapted	1	2	9	1	—	—

* Put into solution in HCl, added to the medium, and the pH adjusted to 7.0 immediately. No precipitate noted, but it is doubtful if this amount remained in solution.

Table 5 summarizes the results of this experiment. All 10 strains of *B. suis* tested tolerated 256 μg per ml of cystine, and 2 of them grew in 512 μg per ml. This is an interesting contrast with the 42 strains of *B. abortus*, none of which tolerated 256 μg per ml of the cystine. This observation may prove to be a significant aid in the taxonomic differentiation of these two species, particularly since the acclimated strains of *B. abortus* seem to become less rather than more tolerant to the toxic effect of cystine. The 9 *B. melitensis* strains tested showed cystine tolerances ranging down to 64 and up to 256 μg per ml. Thus they seemed to occupy a position intermediate between *B. abortus* and *B. suis* in cystine tolerance.

An experiment was performed to determine whether cystine was brucellacidal or only brucellastatic. Flasks of McCullough and Dick medium were prepared containing 5 and 50 μg per ml of cystine. These and a control flask containing no

cystine were each inoculated with 0.1 ml of a 10^{-2} dilution of a 48-hour broth culture of *B. abortus* 1257 and incubated at 37 C. One-half-ml aliquots of the contents of these flasks were plated at intervals for 72 hours. The flasks containing the cystine showed progressive decrease in numbers of *B. abortus* colonies and were sterile in 24 hours, whereas the control flask soon reached and retained a status of confluent growth. Thus the cystine toxicity for *B. abortus*, like the previously described peptone toxicity, is definitely brucellacidal.

Comparative toxicity of cystine and cysteine for B. abortus 1257. In an experiment designed to test a number of compounds related to cystine for toxicity for *B. abortus* 1257, we encountered an interesting zone of inhibition when cysteine was tested. Growth of the organism occurred at concentrations of cysteine of 6± μg per ml and below, and 100 to 200 μg per ml and above. We were inclined to believe that this zone of inhibition was the consequence of the partial oxidation of the cysteine to cystine, which resulted in a three-zone effect. At low concentrations of added cysteine not enough cystine was produced to be toxic.

TABLE 6

The effect of varying both the cystine and the cysteine concentration on the growth of Brucella abortus 1257

CYSTINE CONCENTRATION, μG PER ML	CYSTEINE CONCENTRATION, μG PER ML									
	400	200	100	50	25	12.5	6.25	3.12	1.56	0
	Growth in 5 days									
100	+	+	+	−	−	−	−	−	−	−
3.12	+	+	+	−	−	−	−	−	−	−
1.56	+	+	+	−	−	−	−	−	−	+
0	+	+	+	−	−	−	−	+	+	+

At intermediate concentrations of added cysteine there was adequate oxidation to produce toxic concentrations of cystine and not enough cysteine left over to exert a reversing effect on the cystine toxicity. At higher concentrations, the unoxidized cysteine tended to reverse the toxicity of the oxidized toxic cystine.

An experiment was designed to test this hypothesis. Graded concentrations of both cystine and cysteine were added to tubes of McCullough and Dick medium and the tubes were inoculated with 0.1 ml of a 10^{-2} dilution of a 48-hour broth culture of *B. abortus* 1257 and incubated at 37 C for 5 days. Table 6 records the results of this experiment and confirms both the marked toxicity of cystine alone (3.12 μg per ml) and the zonal toxicity of cysteine alone. Also this experiment indicates that the toxicity of 100 μg per ml of added cystine is reversed by 100 or more μg per ml of added cysteine.

In a second effort to confirm our hypothesis relative to the zonal toxicity of cysteine for *B. abortus* 1257, we repeated the experiment recorded in table 6 except that we substituted the reducing compound sodium formaldehyde sulfoxylate for the cystine. Our reasoning for this substitution was that the sodium formaldehyde sulfoxylate in adequate concentration would suppress the oxida-

tion of the cysteine and thereby restrict or eliminate the zone of toxicity by restricting or eliminating the production of toxic concentrations of cystine. Table 7 records the results of this experiment, which confirm our reasoning both as to partial restriction and as to complete elimination of the zonal toxicity of cysteine.

TABLE 7

Neutralization of the zonal toxicity of cysteine by the addition of sodium formaldehyde sulfoxylate to the medium

CYSTEINE CONCENTRATION, μG PER ML	SODIUM FORMALDEHYDE SULFOXYLATE, μG PER ML			
	12.5	6.25	3.12	0
	Growth in 5 days			
100	+	+	+	+
50	+	−	−	−
25	+	+	−	−
12.5	+	+	−	−
6.25	+	+	+	−
3.12	+	+	+	+
0	+	+	+	+

DISCUSSION

With the demonstration that cystine toxicity for brucellae correlated the toxicity of certain lots of tryptose with regard to inoculum-size effects, neutralization by reducing agents, and brucellacidal activity, we had hoped that we would be able to state definitely that cystine is the toxic factor in these peptones. Many additional experiments have been performed to test this hypothesis. These include antibacterial spectra of cystine and toxic tryptose, tolerance adaptations to these substances, paper chromatography, and microbiological and chemical analyses of the toxic and nontoxic tryptose for cystine. Most of these experiments support the contention that cystine is or could be the toxic factor in toxic tryptose, but the microbiological and other analytic tests fail to support the cystine hypothesis. This failure may be more apparent than real because of the possibility of a deficiency of neutralizing agents in the toxic peptones. These possibilities are being investigated and the results will constitute a portion of another paper dealing with the nature of the antibrucella factor in toxic peptones.

The observation of cystine toxicity for certain strains of brucellae, as demonstrated in this report, has practical significance in the formulation of synthetic media for these organisms. However, since acclimated strains of *Brucella abortus* seem to tend to be less tolerant to cystine than CO_2-requiring strains, we are inclined to believe that some factor other than cystine content is involved in the failure of synthetic media to support the growth of freshly isolated strains of this organism. Another practical aspect of the observed cystine toxicity which needs further study is the possible taxonomic value in the differentiation of *Brucella suis* and *Brucella abortus*. Also the reversal of cystine (including oxidized

cysteine) toxicity for brucellae by sodium formaldehyde sulfoxylate and other reducing agents would seem to justify the incorporation of some such agent in media receiving small inocula of these organisms.

The theoretical implications of the zonal toxicity of cysteine for *Brucella abortus* are most intriguing. If a similar antibrucella toxicity-inducing, toxicity-reversing mechanism could be demonstrated *in vivo*, it might account for the exacerbations and recessions of activity of the organisms as observed in clinical brucellosis. This conceivably would involve quantitative variations in the activity of the oxidases and reductases within the tissues. Also variations in the normal resistance or susceptibility to infection might involve some such reversible antibacterial compounds.

SUMMARY

Tryptophan and cystine were found to be highly toxic to *Brucella abortus* 1257 in a medium containing no other amino acids. Two additional amino acids, methionine and phenylalanine, were slightly toxic to this organism, whereas none of the other 15 amino acids of the casein digest series proved toxic at concentrations well above those found in 1 to 2 per cent casein digests. Only the cystine toxicity for this organism showed a persistence in the presence of other amino acids, which might implicate it as the antibrucella factor of casein and other digest media.

Cystine toxicity for brucellae correlates in many respects the previously reported tryptose toxicity for these organisms. However, we are not yet sure whether the two are or are not identical.

Forty-two strains of *B. abortus* showed markedly less tolerance for cystine than did 10 strains of *Brucella suis*. Acclimated strains of *B. abortus* tended to be less tolerant than CO_2-requiring strains. Nine strains of *Brucella melitensis* were intermediate in their tolerance for cystine.

The cystine content of certain synthetic media was shown to be responsible for the failure to obtain growth of some strains of *B. abortus*.

Cysteine tended to give zonal inhibition of growth of *B. abortus* 1257. This zone was explained in terms of the balance between the tendency of cysteine to be oxidized to cystine and the toxicity-reversing effect of residual cysteine. Varying amounts of the reducing agent sodium formaldehyde sulfoxylate restricted and, with increasing amounts, eliminated the zonal toxicity of cysteine.

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