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STUDIES ON THE NITROGEN METABOLISM OF BACTERIUM TULARENSE

A dissertation submitted to the

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DOCTOR OF PHILOSOPHY

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by

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INTRODUCTION

With the exception of certain quantitative serological differences involving binding capacities of polysaccharides for antibody, of presumed changes in surface components as shown by a study of colonial variants, and of differing cultural and growth requirements there has been no success in determining the factors associated with virulence of Bacterium tularense. The present study has been undertaken in view of the known nutritional differences that exist between virulent and avirulent strains.

Since liquid cultures of virulent strains show a rising pH in contrast to the more stable pH of cultures of avirulent strains, initial studies were directed toward an investigation of the deaminase activity of this organism. Data from this study led to a detailed investigation of the glutamine and asparagine deamidases, citrulline ureidase, and serine dehydrase enzyme systems of both virulent and avirulent strains.

Previous investigations (1, 2) have shown that glutamic acid is required in unusually large amounts for the cultivation of B. tularense in synthetic medium. A study has been made regarding its function in transamination reactions. The role of several other amino acids in transamination was correlated with this study.

In addition, work has been done to investigate the synthesis of amino acids by the direct amination of keto acids.

MATERIALS AND METHODS

Virulent strains SCHU, SCHERM and HOLT and the avirulent 38 strain were used. Cultures of the virulent, stable, blue, "D" colony of both SCHU and SCHERM and the virulent, buff, "A" colony type of SCHU were also used. The variants were obtained by plating the parent strain on 2 per cent peptone cysteine plating medium (table 1), and incubating for 72 hours at 37 C. The plates were then examined under a dissecting microscope with oblique light. The colonial variants were "picked" with a straight needle and subcultured on routine dextrose cysteine blood agar (DCBA) medium. Stock cultures were maintained on DCBA slants at 4 C. All strains were titrated in mice for virulence and the LD_{50} values calculated according to the method of Reed and Muench. One-half ml of the appropriate dilution was inoculated I.P. into each of 10 mice. Four decimal dilutions were used. Plate counts were made concomitantly for each strain titrated. The LD_{50} data are presented in table 2.

Mass cultivation of the organism was accomplished with either a synthetic medium (table 3), a modified casein hydrolyzate medium, or various peptone broths. The cultures were shaken at either room temperature or at 37 C. Good growth was obtained at both temperatures. Colonial purity was determined by plating the terminal growth. The cells were harvested by centrifugation, washed twice with saline, and resuspended in either buffer or water, depending upon their destined use. The cell suspensions were preserved in the frozen state until

Table 1

PLATING MEDIUM

	Per Cent
Difco Peptone	2.0
NaCl	1.0
Glucose	1.0
Cysteine	0.1
Bacto Agar	1.5

pH 6.8 before autoclaving
Inoculum — 100 M/ml

Table 2

MOUSE VIRULENCE TITRATIONS OF *B. tularense* STRAINS

Strains	10^0 Billions of Bacteria/ml	LD_{50} Dilution	No. Bacteria/ml at LD_{50}
SCHU-D	5.57	$10^{-9.4}$	2.2
SCHERM	1.44	$10^{-8.7}$	3.0
HOLT	3.45	$10^{-9.4}$	1.3
38	Avirulent		

Mouse dose: 1/2 ml I.P.

Table 3

SYNTHETIC MEDIUM

	Mg/liter
l-tyrosine	160
l-methionine	160
l(+) histidine	160
l-arginine	240
l-aspartic acid	400
l-cysteine	400
l-leucine	400
dl-isoleucine	400
l-lysine	400
dl-serine	800
l-proline	800
dl-valine	800
dl-threonine	1200
l-glutamic acid	4800

NaCl	10.0 gm
Glucose	10.0 "
ATP, cryst.	0.1 "
Thiamine	50.0 µg
Biotin	50.0 "
"B" salts*	5.0 ml

pH adjusted to 6.8. 10 lbs. 10 min.

*$MgCl_2$	0.002	Molar
$CaCl_2$	0.002	"
$MnSO_4$	0.0000002	"
$FeSO_4$	0.0000002	"
KH_2PO_4	0.05	"

needed. Bacterial nitrogen contents were determined by the micro-Kjeldahl method of Kabat and Mayer (3).

Tenth molar phosphate buffers were used for all deaminase experiments. Two-tenths molar phosphate - NaOH was used as a buffer for the transaminase studies. The pH of all solutions was determined electrometrically with a Cambridge electronic pH meter.

Deaminase activity was measured by incubating the washed cell suspensions with the desired amount of amino acid and buffer in a 30 C water bath for 1 hour, or sometimes at other specified time durations. The reaction was stopped by the addition of 10 per cent trichloroacetic acid (TCA). The samples were then centrifuged and the supernatants were tested for ammonia nitrogen. Zero time samples and cell blanks were included for each test. Ammonia nitrogen determinations on the TCA supernatants were done either by direct nesslerization or by the micro-diffusion method of Conway and Byrne (4). In using the micro-diffusion, 1 ml of 0.2 N HCl was placed in the center well of the Conway unit. One ml of saturated aqueous Na_2CO_3 and 0.1 to 0.4 ml TCA supernatant were placed in the main compartment. Phenolphthalein was used as an indicator to insure alkalinity. The units were rimmed with anhydrous lanolin and covered with glass. The contents of the main compartment were then mixed by rotation and the unit allowed to stand at room temperature for 100 minutes. Five-tenths ml of the 0.2 N HCl was removed and tested for ammonia nitrogen by direct nesslerization. The Nessler's color was

measured with a Klett-Summerson photoelectric colorimeter, using a filter having a 400 to 465 mμ spectral transmission. A series of known standards of $(NH_4)_2SO_4$ was nesslerized by the same method. Appropriate calculations gave a Klett factor of 0.0553 in converting optical density to μg of nitrogen by this method. Table 4 presents the data from the Klett calibrations.

L-amino acids were used for all routine screening procedures, if available.

Partition chromatography was used to investigate the products other than ammonia formed during the incubation of cells and specified substrates. Unless indicated, 70 per cent ethyl alcohol was used as the solvent. Sheets or strips of Whatman's No. 1 filter paper were used for the chromatograms. All chromatograms were ascending. The solutions to be assayed were pipetted on spots one inch from the bottom of the paper in quantities of .005 to .02 ml. Capillary pipettes calibrated to deliver, 5, 10, and 20 lambda were used. If a larger quantity of material was desired, the spots were air-dried between successive applications. The sides of the filter paper sheets were tied together, thus forming a cylinder. The cylinders were then placed in a large flat petri dish containing the solvent. A bell jar was placed over the paper and petri dish, and the solvent allowed to ascend the filter paper for 15 to 17 hours. The front was lightly marked with pencil and the paper allowed to dry at room temperature. Other chromatograms were stapled to a glass rod and suspended over a tray containing the solvent. The paper was then lowered until the lower edge was immersed in the solvent. The

Table 4

SUMMARY OF CALIBRATION DATA

µg Nitrogen Added	Klett Reading	Klett Factor
1.0	17	.0588
1.0	20	.0500
1.0	20	.0500
1.0	16	.0625
1.0	18	.0556
1.0	20	.0500
5.0	95	.0563
5.0	94	.0532
5.0	95	.0563
5.0	80	.0625
5.0	81	.0617
5.0	82	.0610
5.0	91	.0549
5.0	95	.0526
5.0	90	.0556
10.0	188	.0532
10.0	194	.0516
10.0	212	.0472
10.0	164	.0610
10.0	165	.0606
10.0	173	.0578
10.0	188	.0532
10.0	190	.0526
10.0	200	.0500
20.0	415	.0482
20.0	425	.0471
20.0	365	.0548
20.0	351	.0570
20.0	337	.0593
20.0	350	.0571
25.0	463	.0540
25.0	445	.0562
25.0	440	.0568
25.0	445	.0562
25.0	455	.0549
25.0	460	.0543
25.0	435	.0575

(Continued)

Table 4 (Continued)

µg Nitrogen Added	Klett Reading	Klett Factor
25.0	440	.0568
25.0	438	.0571
25.0	442	.0566
25.0	442	.0566
25.0	450	.0556
25.0	461	.0542
25.0	450	.0556
25.0	460	.0543
25.0	448	.0558

Mean Klett factor = 2.5442/46 = 0.0553(09)

container was then covered and the solvent allowed to ascend the filter paper, as just described. Five-hundredths per cent ninhydrin (Pierce) in water-saturated butanol was sprayed on the air-dried chromatogram. The chromatogram was then placed in a 90 C oven for 10 minutes to develop the ninhydrin-positive areas. The quantitative determination of amino acids from the chromatograms was done according to the method of Housewright and Thorne (5). The tubes containing the filter paper squares and the ninhydrin reagent were heated in a boiling water bath for exactly 25 minutes instead of steaming in the autoclave. Small glass funnels containing a glass bead were placed in each tube to minimize evaporation. Amino acid standards were chromatogrammed with each series. The ninhydrin color was measured colorimetrically using the Klett-Summerson photoelectric colorimeter. A filter having a 500 to 570 mμ spectral transmission was used. Filter paper blanks were also done for each series.

Carbon dioxide was measured manometrically using the Warburg apparatus. The flasks and manometers were calibrated with mercury (6). The washed cell suspensions were placed in one side arm of the Warburg flasks and 6 M H_3PO_4, the acid tip, in the other side arm. The substrates and buffer were placed in the main compartment of the flasks. Two-tenths ml of 10 per cent KOH was placed in the center well for oxygen uptake determinations. A piece of fluted filter paper was placed in the KOH well. The gas phase was air. The flasks were shaken in a 30 C water bath for 1 hour. The endogenous respiration of the cell

suspension, without added substrate, was determined for each experiment. This value was subtracted from that obtained in the presence of specific substrate. Upon completion of the Warburg analysis, 10 per cent TCA was added and the flask contents centrifuged. The supernatant was tested for ammonia and other products, depending upon the substrate.

Ornithine was determined by the method of Stein and Moore (7). The TCA supernatants, containing 0.2 to 0.4 μ M ornithine, were added to Klett tubes calibrated to contain 10 ml. Two ml of glacial acetic acid and 1.0 ml of ninhydrin reagent were added to each tube. The tubes were covered with aluminum caps and heated in a 100 C water bath for exactly 60 minutes. After heating, the tubes were cooled, and the contents brought to 10.0 ml with glacial acetic acid. The color was read in the Klett-Summerson photoelectric cell using a filter with a 400 to 465 mμ spectral transmission. Ornithine standard solutions were done with each experiment. The ninhydrin reagent was prepared by adding 625 mg ninhydrin (Pierce) to 10.0 ml 6 M H_3PO_4 and diluting to 25.0 ml with glacial acetic acid. The reagent is stable for 1 week.

A satisfactory method for the determination of citrulline has not yet been found.

Transaminase activity was studied by incubating washed cell suspensions with known quantities of amino acid and keto acid. All substrates were prepared in 0.2 M PO_4-NaOH buffer, pH 7.3. The test solutions were diluted to volume

with the same phosphate buffer. The reaction mixtures were incubated aerobically in a water bath at 30 C for 14 to 18 hours. The reaction was stopped by immersion of the test tubes in an ice bath. The cells were removed by centrifugation at 34 F. The supernatant was used for chromatography and ammonia nitrogen determinations. The amino acids on the chromatograms were quantitated as previously described.

Pyruvic acid was quantitated by the method of Friedman and Haugen (8). The color developed with NaOH was measured by means of the Klett-Summerson photoelectric colorimeter. A filter having a 500 to 570 mμ spectral transmission was used. Standard pyruvate samples were done with each test. When the reaction mixtures contained semicarbazide, standard pyruvate samples were prepared containing the same amount of semicarbazide. Ten per cent TCA supernatants were used for analysis. All dilutions of standard and unknowns were made in 10 per cent TCA. Stock pyruvic acid standards were made up in 0.1 N H_2SO_4. The TCA was prepared fresh each week.

To study the direct amination of keto acids by B. tularense, the washed cell suspensions were mixed with known quantities of the specified keto acid and $(NH_4)_2SO_4$ in 0.2 M PO_4-NaOH buffer, pH 7.2. The reaction mixture was incubated aerobically for approximately 18 hours at 30 C. The amino acids formed by the reaction were quantitated from chromatograms as previously described.

Sonic lysates of cell suspensions were prepared by treating a water suspension

of washed cells in the Raytheon magneto-striction oscillator for 30 minutes, or longer if the volume was large. The plate and output voltages were set at from 90 to 110. The frequency was adjusted to the maximum pitch. The chamber was cooled with circulating tap water.

All glassware was cleaned with a concentrated H_2SO_4-dichromate solution, washed 8 to 10 times with tap water and rinsed 3 to 6 times with distilled water. The glassware was air-dried.

EXPERIMENTAL RESULTS

1. Preliminary Investigation: The preliminary investigation consisted of a screening of amino acids, amides, and other amino compounds for deaminase activity. The procedure used was to incubate washed, buffered cell suspensions with the specific substrate for 1 hour at 30 C. All tests were done aerobically. The reaction was stopped with 10 per cent TCA, and the TCA supernatants were tested for ammonia nitrogen by direct nesslerization. Of all the substrates tested, only glutamine, asparagine, serine and citrulline were deaminated to yield significant amounts of ammonia by the virulent SCHU strain.

A similar screening for deaminase activity was made with the avirulent 38 strain as the enzyme source. Marked deaminase activity was shown when either glutamine, asparagine, or serine was the substrate. Citrulline, however, was not attacked.

Since Schmidt (9) has stated that several amino acids interfere with the development of Nessler's color, a repeated screening for deaminase activity was carried out. In this instance, ammonia nitrogen determinations were determined by the Conway micro-diffusion technique (4). No significant differences were detected, and no new deaminases were found. Strain HOLT was used as the enzyme source.

The data obtained from these screening procedures are summarized in table 5. Glutamine, asparagine, and serine were degraded to ammonia by all strains.

Table 5

A SCREENING OF AMINO ACIDS AND OTHER SUBSTANCES FOR DEAMINASE ACTIVITY

Substrate	μ M NH_3 by Direct Nesslerization 5 μ-M Substrate		μ M NH_3 by Microdiffusion 20 μ M Substrate
	SCHU	38	SCHERM
a-alanine	0.2	0.4	0
β-alanine	0.1	0	—
amino acetic acid	0.1	0.2	—
l-aspartic acid	0.3	0.3	0
l-arginine	0	0.2	0
l-cysteine	0	0	0
l-glutamic acid	0	0.6	0.2
l-histidine	0	0	0
l-leucine	0.1	0.2	0
l-isoleucine	—	0.2	0
l-lysine	0	0.2	0
l-methionine	0	0.1	0
l-OH-proline	—	—	0
l-proline	0.1	0.2	0
l-phenylalanine	—	—	0
dl-serine	—	2.6	9.0
l-serine	2.6	—	—
d-serine	0.9	—	—
dl-threonine	—	0.6	0
l-threonine	0.9	—	—
d-threonine	0.2	—	—
l-tryptophane	0.1	0.2	0
l-tyrosine	0.1	0.1	0
dl-valine	0.1	0.1	0.5
l-asparagine	5.5	5.4	16.3
l-glutamine	3.9	2.4	16.5
histamine phosphate	0	—	—
glycinamide	0	0.2	—
urea	0	0	0
nicotinamide	—	0.4	—
dl-a-amino butyric acid	—	0.3	—
dl-citrulline	—	—	13.0
l-ornithine	—	—	0

pH 7.6 30 C 1 hr

SCHU - 0.4 mg Bact. N/ml
38 - 1.25 " " "
SCHERM - 1.3 " " "
— - Test not done.

Only the virulent strains attacked citrulline with the formation of ammonia.

Braunstein (10) has observed that deamination of some amino acids occurs through a complex enzyme system of transamination followed by deamination of the newly synthesized amino acid. Although we have never demonstrated significant deamination of either alanine, glutamic acid, or aspartic acid, tests were set up to screen amino acids for deamination in the presence of pyruvic acid (PU), alpha keto glutaric acid (KG) and oxalacetic acid (OA). Controls were run without keto acid. The tests were incubated at 30 C for 18 hours to detect any slow deaminase activity in either series. Prolonged incubation did not result in the deamination of amino acids other than those previously detected after incubating for 1 hour. The presence of keto acid in no way stimulated deamination. Since the results of all tests containing keto acid were negative for new deaminases, only the control series and the PU tests are recorded in table 6. The slight decrease in ammonia nitrogen in the presence of PU is probably due to the direct amination of PU.

A screening for deaminase activity was also carried out under anaerobic conditions. Thunberg tubes, evacuated with a water aspirator pump for 4 to 5 minutes, were used to establish anaerobic conditions. Strain SCHU was used as the enzyme source. Ammonia nitrogen was determined by direct nesslerization. No new deaminases were found under these conditions. The data are presented in table 7.

Table 6

A SCREENING OF AMINO ACIDS FOR B. tularense DEAMINASE ACTIVITY BY PROLONGED INCUBATION AND THE ADDITION OF PYRUVIC ACID

	μ M NH_3-N/18 hrs/30 C	
50 μ M Amino Acid	No Additions	50 μ M Pyruvate
None	6.5	2.7
l-histidine	1.6	0.1
l-glutamic acid	7.8	6.0
l-arginine HCl	0	0
l-cysteine	2.3	1.0
l-aspartic acid	9.6	7.5
l-serine	37.0	24.2
dl-citrulline	54.1	44.4
l-ornithine	8.2	6.6
l-threonine	12.2	5.1
glycine	5.1	8.4
l-leucine	3.5	7.5
l-proline	4.6	7.2
l-lysine	4.8	3.7
dl-methionine	4.0	7.4
l-phenylalanine	4.2	7.5
l-tryptophane	1.5	0.9
l-tyrosine	4.3	6.6
l-valine	4.4	7.9

Table 7

A SCREENING FOR DEAMINASE ACTIVITY

USING EVACUATED THUNBERG TUBES

Substrate	μ M/NH_3
l-isoleucine	0
l-leucine	0
l-histidine	0
l-tryptophane	0
dl-norvaline	0
dl-homocystine	2.1
dl-valine	0
l-alanine	0
l-proline	0
l-citrulline	48.1
l-serine	39.9
dl-homoserine	0.4
d -serine	4.7
l-ornithine • HCl	0.1
l-arginine	0
l-phenylalanine	0.2
d -threonine	0.4
l-threonine	4.9
l-cysteine • HCl	1.3
l-methionine	0.1
l-lysine • HCl	0.3
l-glutamic acid	1.6
l-aspartic acid	0.4
glycine	1.8
l-tyrosine	0

30 C 21 hr
40 μ M substrate
pH 7.4
Strain SCHU

2. The Deamidases: In view of the large amounts of ammonia formed from glutamine and asparagine on incubation with washed cell suspensions, a study has been made of some of the physical properties of these enzymes.

An experiment was designed to determine the optimum pH range of these deamidases. SCHU "A" cells were suspended in phosphate buffers of varying pH immediately after 2 washings with saline. The buffered cell suspensions were then frozen until used. The amides were freshly prepared in buffers of the same pH values. Deaminase activity was measured by the amount of ammonia nitrogen liberated after 60 minutes at 30 C.

The reaction mixtures at each pH value contained .005 M substrate and 0.8 mg bacterial nitrogen per ml. The data, presented in table 8 and figure 1, show optimum pH values of 7.8 to 8.1 for l-glutamine and 7.8 for l-asparagine.

To determine the effect of enzyme concentration upon the reaction, varying amounts of the buffered cell suspension (pH 7.8) were incubated with .005 M amide concentrations, pH 7.8, for 1 hour at 30 C. All mixtures were brought to constant volume with phosphate buffer, pH 7.8. Approximately 1 mg bacterial nitrogen/ml reaction mixture caused maximum deamination of both glutamine and asparagine. Table 9 presents the data for this experiment.

Table 10 presents the data from an investigation to determine the effect of substrate concentration on deamidation by strain SCHU.

A study of the enzyme-time relationship was also made. As indicated in

Table 8

THE EFFECT OF pH UPON THE DEAMINASE ACTIVITY OF B. tularense, STRAIN SCHU

μg NH_3-N/ml/hr

pH	.005 M l-glutamine	.005 M l-asparagine
5.0	13.2	3.6
5.6	25.0	15.6
6.3	37.8	26.4
7.1	47.4	24.2
7.8	51.2	29.2
8.1	53.1	25.8

0.8 mg Bact. N/ml
30 C

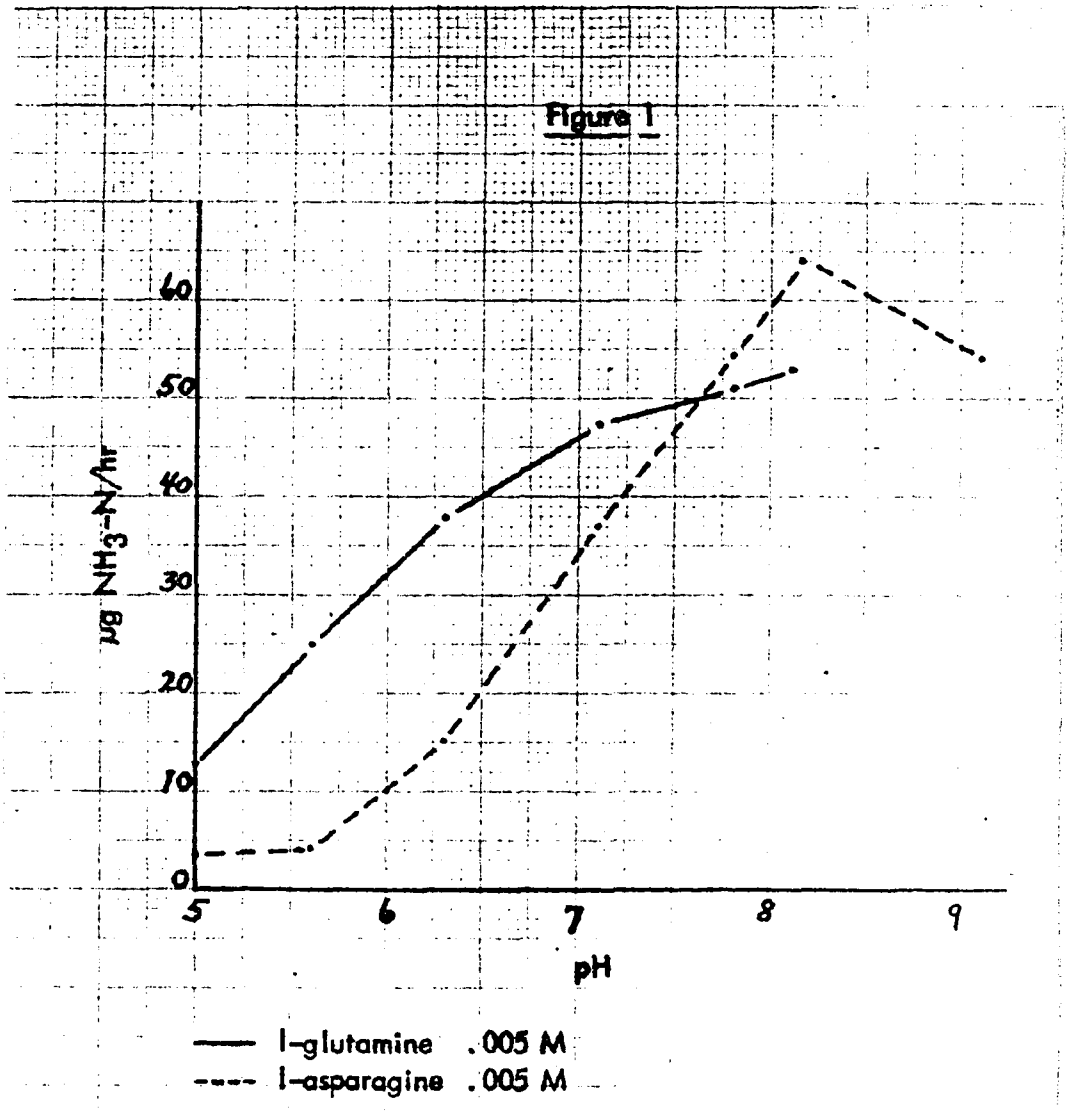


Table 9

THE EFFECT OF ENZYME CONCENTRATION ON THE DEAMINASE ACTIVITY OF SCHU

μg NH_3-N/ml/hr

Bact. N/ml mg	l-glutamine	l-asparagine
.1	19.6	18.2
.2	25.8	29.8
.9	54.6	69.8
1.8	51.1	77.0

pH 7.8
.005 M substrate
30 C

Table 10

THE EFFECT OF VARYING THE SUBSTRATE CONCENTRATION ON THE DEAMINASE ACTIVITY OF SCHU

Substrate concentration	μg NH_3-N/ml/hr	
	l-glutamine	l-asparagine
.0005 M	8.2	19.0
.001 M	17.6	25.0
.005 M	61.5	92.7
.01 M	109.8	155.8

0.9 mg Bact. N/ml
pH 7.8
30 C

table 11, deamination was complete at 60 minutes for both glutamine and asparagine. A graphic presentation of the data is recorded in figure 2.

To determine the heat stability of the deamidases, a preheated sonic lysate of strain HOLT was added to 25 μ M of amide in M/10 PO_4 buffer, pH 7.3. The test solution was then incubated in a 30 C water bath. After 1 hour, 10 per cent TCA was added, and the mixture centrifuged. Ammonia nitrogen determinations were done by direct nesslerization of the TCA supernatants. The data are presented in table 12. The glutaminase was found to be much more heat-labile than the asparaginase. Preheating the sonic lysate for 5 minutes at 45 C destroyed 52 per cent of the glutaminase activity. Asparagine deamidase activity was not destroyed by this temperature even after prolongation of heating to 1 hour. In order to destroy asparaginase activity, it was necessary to heat the enzyme for 60 minutes at 56 C (61 per cent inactivation). The temperature optimum for glutamine as determined by this analysis is 30 to 37 C, while that of asparagine is from 30 to 45.

The same sonic lysate of strain HOLT was used to determine the optical specificity of the deamidases. Incubation of 25 μ M of d-asparagine with the sonic lysate for 1 hour at 30 C, pH 7.3, resulted in the formation of only 0.6 μ M of ammonia nitrogen. L-asparagine, tested at the same time, yielded 22.7 μ M of ammonia nitrogen. The asparagine deamidase of B. tularense is, therefore, specific for the l-configuration of asparagine. We were unable to obtain a sample of d-glutamine for testing.

Table 11

TEMPORAL RELATIONSHIPS OF THE SCHU DEAMINASES

μg NH_3-N/ml

Time Min	l-glutamine	l-asparagine
5	17.1	13.8
10	25.7	22.2
15	33.7	33.5
30	47.7	49.1
60	68.9	76.2

0.8 mg Bact. N/ml
.005 M substrates
pH 7.8
30 C

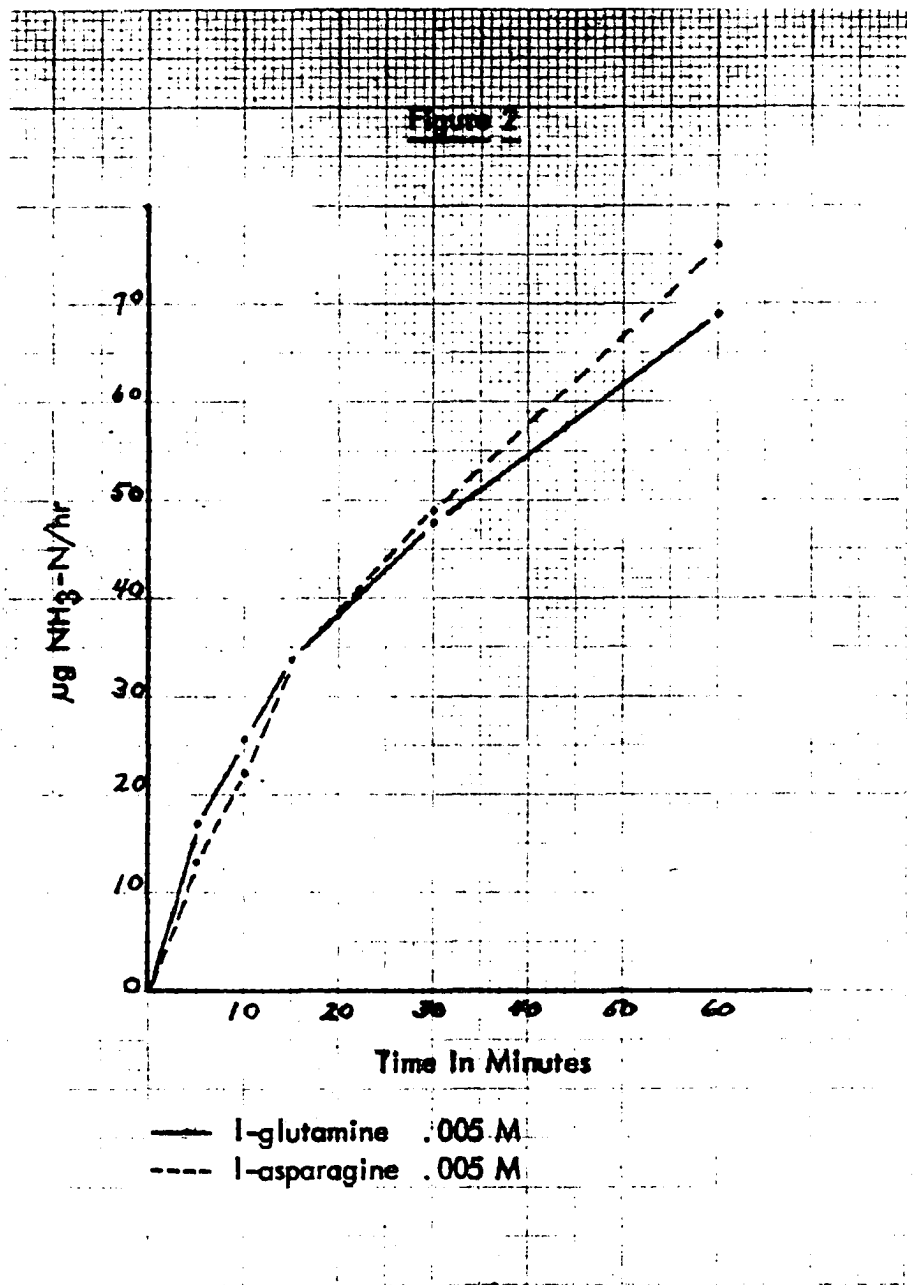


Table 12

THE EFFECT OF TEMPERATURE ON THE STABILITY OF THE DEAMIDASES OF *B. tularense*

Time of Heating Cell Suspension Prior to Testing		μ M NH_3-N/hr/30 C	
		25 μ M l-glutamine	25 μ M l-asparagine
C	Min		
30	60	26.0	22.7
37	5	24.3	23.2
	15	23.7	22.9
	30	23.1	23.1
	60	24.5	22.6
45	5	12.2	22.9
	15	5.7	23.4
	30	5.2	24.2
	60	5.2	22.6
56	5	5.2	21.6
	15	3.6	17.5
	30	3.9	15.0
	60	3.5	9.0

Competitive inhibition of enzymatic reactions by structurally similar compounds is a phenomenon of common occurrence. It was decided, therefore, to investigate the effect of l-glutamic acid and l-aspartic acid upon the deamidation of l-glutamine and l-asparagine, respectively, by strain SCHU. As anticipated, there was a marked inhibition of deamidation when glutamic and aspartic acids were present in reaction mixtures containing the corresponding amide. Neither glutamic acid nor aspartic acid is appreciably deaminated by B. tularense strains. Table 13 summarizes the data for this experiment.

The incubation of equimolar quantities of l-glutamine and l-asparagine in the same reaction mixture resulted in an ammonia nitrogen value equal to the sum of the ammonia liberated by each amide when incubated alone. Strain SCHU was the enzyme source. The data are presented in table 14. This additive effect, combined with the differences previously noted on the heat stability, presents convincing evidence for the existence of 2 separate enzymes in the deamidation of glutamine and asparagine.

The data presented in table 5 suggested that strain 38 was less efficient in deaminating l-glutamine than were the virulent strains. A test was set up to determine whether this difference was genuine and reproducible. Washed cell suspensions having approximately the same nitrogen content were incubated with .005 M glutamine, pH 7.8, for 1 hour at 30 C. It was again found that strain 38 did, indeed, deaminate glutamine to a lesser degree. The data are reported in

Table 13

THE INHIBITORY EFFECT OF GLUTAMIC AND ASPARTIC ACIDS

ON GLUTAMINASE AND ASPARAGINASE

l-glutamic acid added	μg NH_3-N/ml/hr from l-glutamine	l-aspartic acid added	μg NH_3-N/ml/hr from l-asparagine
0	64.0	0	75.9
.005 M	46.0	.005 M	67.6
.01 M	30.0	.01 M	42.4

0.8 mg Bact. N/ml
.005 M amides
pH 7.8
30 C

Table 14

SCHU GLUTAMINASE AND ASPARAGINASE

Substrate	Bacterial N/ml	µg NH_3-N/hr
.005 M l-glutamine	.238 mg	31.2
.005 M l-asparagine	.238 "	30.2
.005 M l-glutamine + .005 M l-asparagine	.238 "	60.2

table 15.

Since mammalian glutaminases have been grouped according to whether they are phosphate activated or not (11), an experiment was designed to determine whether the deamidases of B. tularense require phosphate for activity. Duplicate tests were set up in a veronal buffer, pH 7.4, prepared according to Michaelis (12), and a M/10 phosphate buffer, pH 7.4. Washed, frozen, strain SCHERM cells were incubated for 1 hour at 30 C with 10 μ M of l-glutamine and with 7.5 μ M of l-asparagine in each of these buffers. Ammonia nitrogen determinations on the TCA supernatants showed that phosphate was not essential to either deamidase. Table 16 presents the average of the data for this experiment.

To determine the products of the deamination of glutamine and asparagine, tests were set up in which buffered SCHERM cells (4.18 mg N/ml) were incubated with known quantities of glutamine and asparagine. Chromatograms were made of the TCA supernatants at zero time and after 60 minutes, using 70 per cent ethyl alcohol as the solvent. The 60-minute samples showed a disappearance of glutamine (Rf .52) and the presence of new ninhydrin-positive areas with an Rf value identical with that of glutamic acid (.72). The 60-minute asparagine sample also showed disappearance of asparagine (Rf .46) and the formation of a substance having an Rf value identical with that of aspartic acid (.58). The presence of glutamic acid and aspartic acid in these reaction mixtures was further confirmed by mixing the known amino acids with the test solution prior to

Table 15

COMPARISON OF THE SCHU AND 38 DEAMINASES

Substrate .005 M	μg NH_3-N/ml/hr	
	SCHU	38
l-glutamic acid	2.0	6.5
l-glutamine	61.8	22.1
l-aspartic acid	1.2	1.4
l-asparagine	68.8	69.7

SCHU - 0.8 mg N/ml final concentration
38 - 0.7 " " " "
pH 7.8
30 C

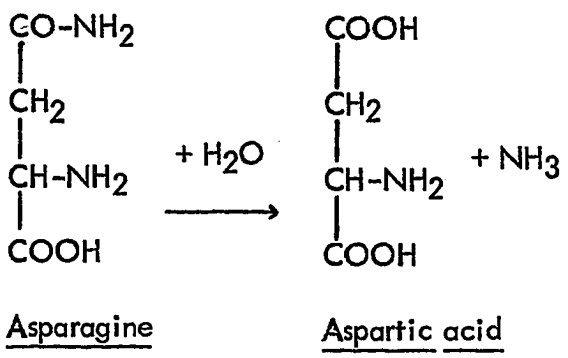
Table 16

THE EFFECT OF PHOSPHATE ON GLUTAMINASE AND ASPARAGINASE

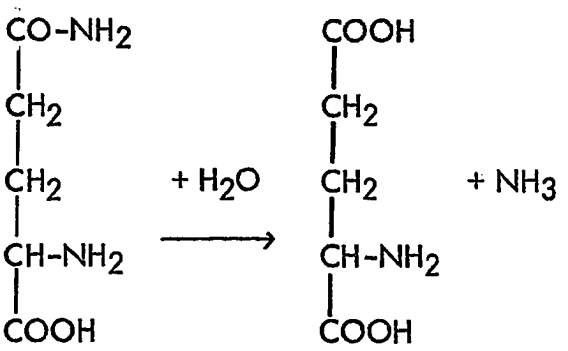
Substrate	μ M NH_3-N	
	Veronal Buffer	PO4 Buffer
glutamine 10μ M	6.25	6.35
asparagine 7.5μ M	4.40	4.70

Both buffers pH 7.4
30 C 1 hr
Strain SCHU

chromatographing. The glutamic and aspartic acid areas on the chromatograms were cut out and quantitated according to the method of Housewright and Thorne (5). Ammonia nitrogen determinations were done by direct nesslerization of the TCA supernatants. Repeated tests showed that nearly equimolar quantities of glutamic acid and ammonia were formed by the deamidation of glutamine. Asparagine was deamidated to yield equimolar amounts of aspartic acid and ammonia. The averages of data for 2 different tests are presented in table 17. The liberation of ammonia from asparagine and glutamine is therefore a deamidation and probably occurs as follows:



Asparagine — Aspartic acid



Glutamine — Glutamic acid

Table 17

THE PRODUCTS OF THE DEAMIDATION OF L-GLUTAMINE AND L-ASPARAGINE

Experiment No.	Amide	μ M NH_3	μ M Amino Acid
1	30 μ M glutamine	26.4	28.2 glutamic acid
	30 μ M asparagine	24.0	24.3 aspartic acid
2	5 μ M glutamine	4.5	4.8 glutamic acid
	5 μ M asparagine	4.1	4.1 aspartic acid

4.2 mg Bact. N added (SCHERM)
30 C 1 hr

3. Citrulline Ureidase: Schmidt, Logan, and Tytell (13) and Oginsky and Gehrig (14) have shown that the degradation of arginine to ornithine involves at least 2 enzymes. The first enzyme catalyzes the desimidation of arginine to citrulline and ammonia. The second enzyme catalyzes the breakdown of citrulline to ornithine, carbon dioxide, and ammonia. Slade and Doughty (15) have proposed the name, citrulline ureidase, to describe this latter enzyme. Schmidt, et al. were able to demonstrate attack on both arginine and citrulline by either washed or lyophilized cell suspensions of Cl. perfringens. Oginsky and Gehrig, however, were unable to demonstrate citrulline degradation using either washed cells or sonic lysates of washed cells of S. fecalis. Acetone-dried preparations of S. fecalis degraded both arginine and citrulline.

Reference to arginine dihydrolase activity has been made by other investigators (16 to 19), but citrulline was not considered to be an intermediate.

Both washed cell suspensions and sonic lysates of virulent strains of B. tularense possess enzymes which degrade citrulline to equimolar quantities of ornithine, ammonia, and carbon dioxide. Several studies have been made to determine some of the physical properties of this enzyme system.

There have been no reports on the heat stability of citrulline ureidase. The present study on heat stability was done by preheating aliquots of washed cell suspensions of strain HOLT for 30 minutes at 30, 37, 40, 45, and 59 C. Manometric studies were then done using the Warburg apparatus. Ten μ M l-citrulline in M/10

PO_4 buffer, pH 7.0, were placed in the main compartment of the Warburg flasks. The preheated cell suspension, containing 2.3 mg nitrogen, was placed in one side arm of the Warburg flask. After equilibration, the cells were tipped into the main compartment and the flasks were shaken at 30 C for 1 hour. Control flasks, without substrate, were included for each test. Ammonia nitrogen determinations were done on the flask contents at the conclusion of the manometric determinations. The data, presented in table 18, show complete inactivation of the enzyme after heating at 45 C for 30 minutes. Heating at 40 C for 30 minutes caused a 59 per cent decrease in activity.

Studies were made to determine the effect of pH upon enzyme activity. Phosphate buffers were used. Duplicate studies were made using 2 different virulent strains, and employing different methods of enzyme preparation. Washed, strain HOLT cells and a sonic lysate of washed strain SCHERM cells were used as the enzyme. Manometric studies were done at each pH level. The quantity of carbon dioxide and ammonia formed from 10 μ M of l-citrulline at 30 C for 1 hour was used as the criterion for pH effect. Ornithine was determined on some of the reaction mixtures. The method of Stein and Moore (7) was used. Proline was shown to be absent from the reaction mixture by partition chromatography. The data obtained from these tests show that highly efficient enzyme systems are present in both virulent strains and that both a sonic lysate and washed cells have good activity. The broad pH range of optimal activity using sonicized cells is probably

Table 18

THE EFFECT OF HEAT ON CITRULLINE UREIDASE

Preincubation Temperature C	μ M CO_2	μ M NH_3
30	8.6	10.1
37	7.0	9.9
40	3.61	4.6
45	0	0
59	0	0

10 μ M l-citrulline
2.3 mg Bact. N added
pH 7.0 30 C

a result of the increased nitrogen concentration of the enzyme. An optimal pH of 6.8 was found when using a lower bacterial nitrogen concentration. Data were also obtained from these tests showing the formation of approximately equimolar amounts of ornithine, carbon dioxide, and ammonia from citrulline. Tables 19 and 20 present the results of these studies.

Partition chromatography was also used to show that ornithine was a product of citrulline degradation.

The results of an enzyme-time study show that the reaction is 50 per cent completed at 15 minutes. Ten μ M of l-citrulline were incubated with 1.3 mg bacterial nitrogen at 30 C for this study. Both carbon dioxide and ammonia were determined at 15, 30, 60, and 120 minutes. The results are presented in table 21.

Additions of from 0.13 to 2.54 mg bacterial nitrogen to constant amounts (10 μ M) of l-citrulline at pH 7.2 show that approximately 1.0 mg bacterial nitrogen causes 50 per cent degradation of citrulline at 30 C in 1 hour. The data are presented in table 22 and figure 3. The formation of carbon dioxide was used as the index of activity for this experiment.

The addition of 5 μ M NaCN to reaction mixtures containing 10 μ M l-citrulline and 1.6 mg bacterial nitrogen inhibited completely the formation of carbon dioxide. The addition of 10 μ M l-ornithine to a reaction mixture had no effect on the formation of carbon dioxide and ammonia from citrulline.

Manometric studies, using the Warburg apparatus, were done to investigate

Table 19

pH CURVE FOR CITRULLINE UREIDASE USING WASHED STRAIN HOLT CELLS

pH	μ M CO_2	μ M NH_3
4.54	1.09	0.83
5.45	1.81	2.68
6.45	7.92	10.51
6.80	8.30	11.90
7.60	6.82	9.86
8.30	6.50	9.28

1.58 mg Bact. N added
10 μ M l-citrulline
30 C 1 hr

Table 20

pH CURVE OF CITRULLINE UREIDASE USING A SONIC LYSATE OF STRAIN SCHERM

pH	μ M CO_2	μ M NH_3	μ M Ornithine
4.55	1.82	1.79	—
5.40	3.93	4.6	—
6.10	9.59	9.58	9.59
6.6	9.53	9.00	9.52
7.0	9.30	9.14	9.30
7.6	9.44	—	9.62
8.3	8.27	9.95	8.75
9.0	8.61	9.05	8.73

2.5 mg Bact. N added
10 μ M l-citrulline
30 C 1 hr

Table 21

AN ENZYME-TIME STUDY OF CITRULLINE UREIDASE

Time Min	μ M CO_2	μ M NH_3
15	3.2	4.5
30	5.3	6.1
60	6.6	7.6
120	6.9	7.8

1.3 mg Bact. N
10μ M l-citrulline
pH 7.2 30 C

Table 22

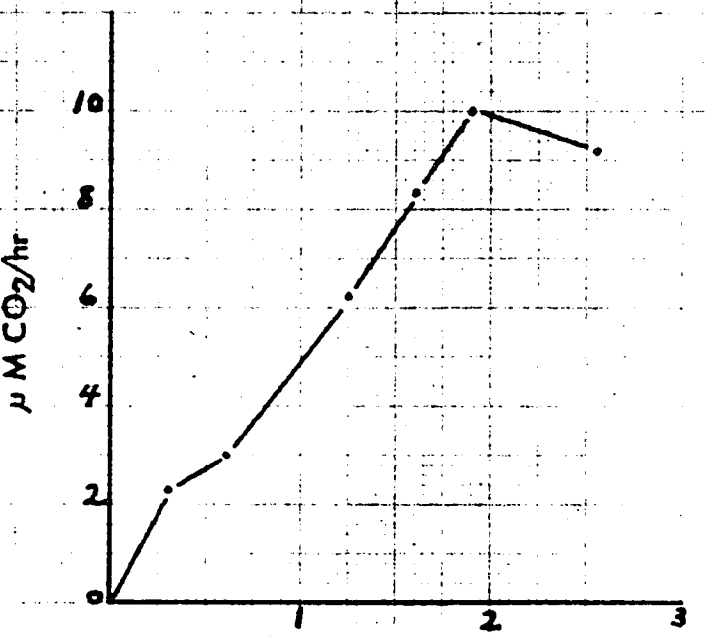
THE EFFECT OF BACTERIAL NITROGEN CONCENTRATION

ON CITRULLINE UREIDASE

Mg Bact. N Added	μ M CO_2
0.32	2.28
0.63	3.00
1.27	6.21
1.58	8.30
1.92	10.00
2.54	9.20

10 μ M l-citrulline
pH 7.2 30 C 1 hr
3.2 ml

Figure 3



10 μ M l-citrulline
pH 7.2, 30 C

the several commercial preparations of citrulline for use as substrate in citrulline ureidase experiments. Washed strain HOLT cells were used as the enzyme, and the tests were run for 1 hour at 30 C, pH 7.3. At the conclusion of the Warburg analysis, the samples were removed from the flasks, centrifuged, and ammonia nitrogen determinations made on the supernatants. L-arginine, l-canavanine, and dl-lanthionine were also used as substrates in this test. No differences were noted in the various preparations of citrulline used for dihydrolase activity. Dl-citrulline was degraded to the same extent as were the l-forms. Ten μ M dl-lanthionine yielded approximately 5 μ M carbon dioxide. Canavanine was degraded to carbon dioxide only slightly (2.6 μ M). The data for this experiment, presented in table 23, indicate that the citrulline ureidase of virulent strains of B. tularense is not optically specific.

Similar studies have been done using the avirulent 38 strain as the enzyme in testing for citrulline ureidase activity. All cultures tested have been negative for both carbon dioxide and ammonia formation from citrulline. This represents the first metabolic difference found between virulent and avirulent strains of B. tularense.

Repeated attempts to obtain arginine desimadase activity have met with failure. Both virulent and avirulent strains were used. Liquid cultures, both still and shaken, were incubated at both room temperature and 37 C, and in the presence of pyridoxal phosphate. The concentration of arginine in the medium

Table 23

CO_2 AND NH_3 STUDIES ON CITRULLINE, ARGININE, AND STRUCTURALLY-RELATED AMINO ACIDS

Substrate	μ M CO_2/hr	μ M NH_3-N/hr
No additions	0.4	0.8
10 μ M l-citrulline (Schwartz)	10.1	11.1
10 " " (GBI)	9.2	10.0
10 " " (Delta)	10.8	11.4
10 " dl-citrulline (NBC)	9.1	10.3
10 " l-arginine HCl	1.0	0.6*
10 " dl-lanthionine	5.6	1.0*
10 " l-canavanine	3.0	1.3*

* Conway nitrogen determination.

0.5 ml cells
10 μ M substrate
pH 7.3

has been increased to 1 gm per liter, and glycerol has been substituted for glucose. Cultures were grown from 18 to 72 hours. The cells harvested from these different cultural conditions and environments were tested for arginine desimadase activity both aerobically and anaerobically (evacuated Thunberg tubes) at pH ranges from 6.1 to 7.9. The following substances have been added, alone and in combination, to the reaction mixtures at the various pH levels under both aerobic and anaerobic conditions: Thiamine, pyridoxal phosphate, adenosine triphosphate, biotin, glucose, ure, Fe^{++}, MN^{++}, CO^{++}, glutathione, cysteine, and cetyltrimethylammonium bromide. No ammonia formation from arginine was detected under any of these conditions. The nitroprusside method of Weber (20) has also been used to determine whether there was disappearance of arginine after incubation with virulent strains. No disappearance of arginine was found.

The cell suspensions used to demonstrate citrulline ureidase activity have also been used to test for decarboxylation and deamination of both arginine and ornithine. Five-tenths μ M carbon dioxide was the largest quantity recorded in tests for ornithine decarboxylase activity. Carbon dioxide formation from arginine was never demonstrated. All tests made on arginine and ornithine were negative for ammonia formation.

Growth studies were done to determine whether either citrulline or ornithine could substitute for arginine in synthetic medium. The synthetic medium was made

up, omitting arginine, and distributed into flasks. Duplicate flasks contained either 20 μ M l-ornithine, 20 μ M l-citrulline, or 20 μ M l-arginine. Strain SCHERM was used as the inoculum. The flasks were shaken at 37 C. After 48 hours' incubation, growth was present only in the flasks containing arginine.

4. Serine Dehydrase: Various methods of cell preparations were tried to find one giving the greatest enzyme activity. Gale (21) has shown that a phosphate-glutathione (PO_4-GSH) buffer (table 24) "preserves the life" of the serine deaminase of E. coli. An experiment was designed to determine whether this is also true for SCHU serine deaminase. One-half of the cells from a 17-hour shaken culture of SCHU, having a terminal pH of 7.1, were washed twice with saline and resuspended in saline. The remaining cells were similarly treated using a phosphate-glutathione buffer at pH 7.8. Deaminase studies were performed on the saline-suspended organisms on the day of harvesting. Studies on the buffered cells were done 24 hours later. All reaction mixtures were buffered with M/10 PO_4, pH 7.8. The data for this experiment (table 25) show that the phosphate-glutathione buffer does "protect" the serine deaminase of strain SCHU.

Tests were also set up to determine the effect of freezing on serine deaminase activity. Ammonia nitrogen determinations were done at frequent time intervals on reaction mixtures containing a freshly prepared saline cell suspension, 50 μ M l-serine, and 0.2 M PO_4, pH 7.4. The remainder of the saline cell suspension was frozen for 18 hours, thawed, and a duplicate series of tests set up. A comparison of the data (table 26) from the 2 tests show greatly enhanced serine deaminase activity after freezing and thawing. Since freezing and thawing causes lysis of B. tularense cells, it seems likely that permeability is a factor in obtaining maximum serine dehydrase activity.

Table 24

COMPOSITION OF THE PO_4-GSH BUFFER

	Gm
glutathione	0.0030
K_2HPO_4	3.8838
KH_2PO_4	0.3403

Make up to 250 ml.
pH 7.8

Table 25

COMPARISON OF THE SERINE DEAMINASE ACTIVITY OF SALINE AND PO_4-GSH BUFFERED CELL SUSPENSIONS

Saline Cell Suspension		Buffered Cell Suspension	
Mg Bact. N/ml Final Conc.	μg NH_3-N/ml/30 min	Mg Bact. N/ml Final Conc.	μg NH_3-N/ml/30 min
.092	0.6	.097	6.2
.184	3.8	.194	12.5
.368	3.8	.354	19.3
.519	6.0	.561	19.6
.883	9.4	.930	24.1

.01 M dl-serine
37 C
pH 7.8

Table 26

THE EFFECT OF FREEZING AND THAWING

ON THE SERINE DEHYDRASE ACTIVITY OF STRAIN SCHU

Time		μ M NH_3	
hr	min	Freshly Prepared Saline Suspension	Frozen and Thawed Suspension
	5	2.5	17.7
	15	5.5	24.0
	30	8.2	27.4
	60	18.2	31.4
	120	33.3	—
18		40.6	50.2

50 μ M l-serine
0.5 ml cells 2.0 ml final volume
pH 7.4 30 C

The effect of bacterial nitrogen concentration on enzyme activity is demonstrated in table 25. The relationship of bacterial nitrogen to enzyme activity is linear up to 0.35 mg N/ml under the conditions of testing.

A comparison was also made between sonicized buffered cells and buffered cells. The serine deaminase of the sonic lysate showed a greater reaction velocity than the buffered cell suspension. However, if the time was prolonged (30 minutes) the buffered cells produced as much ammonia as the lysate. Table 27 presents the experimental data.

We have found that the deaminase activity of a PO_4-GSH buffered cell suspension decreases about 33 per cent when stored at 4 C for 4 days. However, if the buffered cell suspension is frozen immediately after preparation, the deaminase activity can be maintained for at least 1 month. Thawing of the buffered cell suspension produced a very mucoid material. A sonic lysate of buffered cells lost all serine deaminase activity when stored at 4 C for 2 weeks. Attempts to reactivate the enzyme with adenylic acid and biotin were unsuccessful.

Reaction mixtures were incubated at 24, 30, and 37 C to determine the optimal temperature for serine deamination studies. Maximal ammonia production occurred at 37 C. The data are presented in table 28.

The optimum pH of the serine dehydrase was found to be 7.6 for l-serine and 7.9 for d-serine (table 29 and figure 4). The conditions of testing were as follows: washed cells containing 2.3 mg bacterial nitrogen were added to buffers

Table 27

COMPARISON OF THE SERINE DEAMINASE ACTIVITY OF A SONIC LYSATE OF BUFFERED CELLS WITH THE BUFFERED CELL SUSPENSION

Mg Bact. N/ml Final Conc.	PO_4-GSH Buffered Cells		Sonic Lysate of Buffered Cells	
	µg N/15 min	µg N/30 min	µg N/15 min	µg N/30 min
.0376	3.0	6.6	2.6	6.2
.0752	5.0	12.6	6.6	13.2
.1881	11.6	29.4	27.0	39.8
.3761	19.6	41.0	49.0	58.2
.7522	37.6	57.8	60.4	61.0

.01 M dl-serine
37 C
pH 7.8

Table 28

THE EFFECT OF TEMPERATURE UPON THE SCHU SERINE DEAMINASE

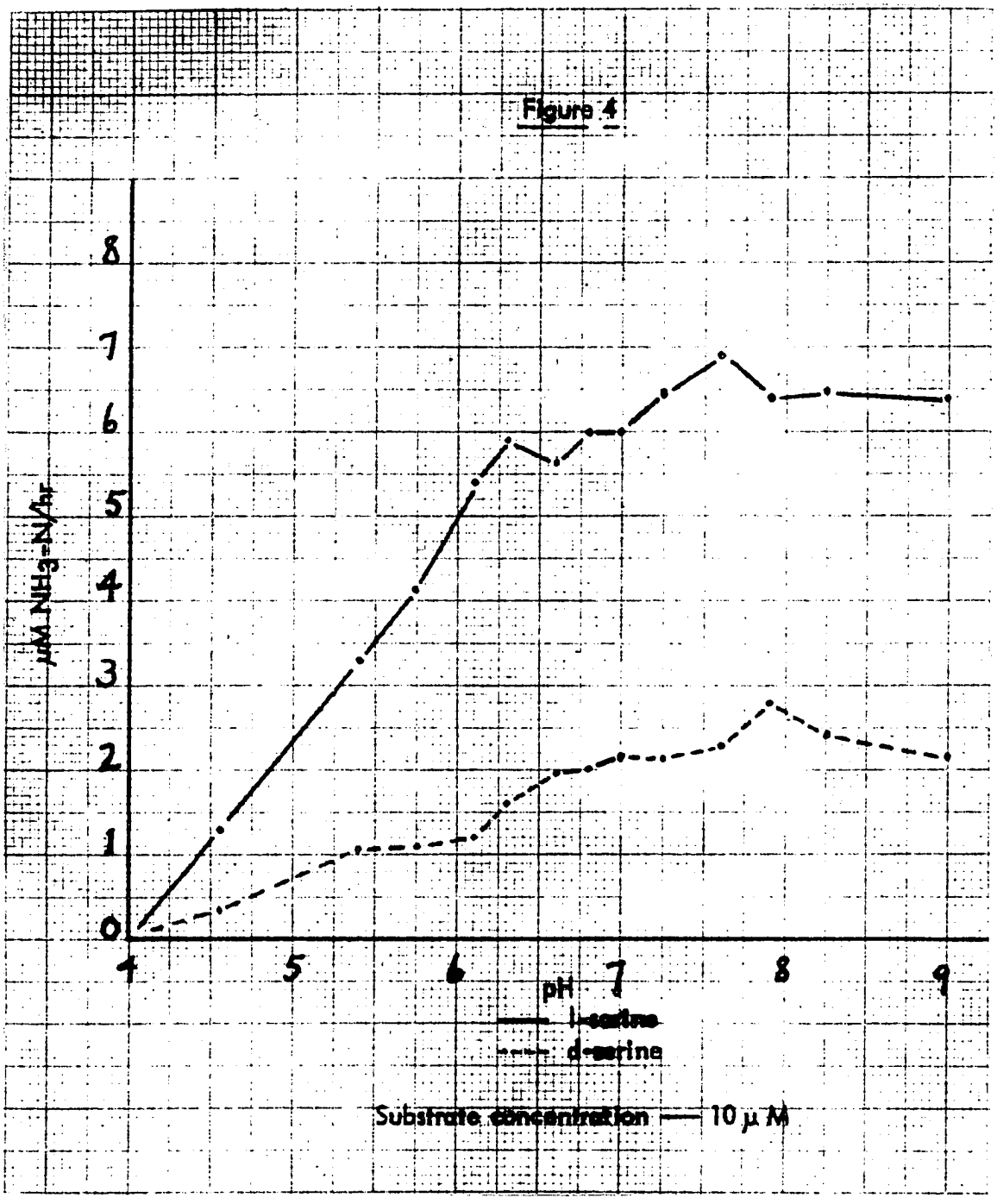
Temperature C	µg NH_3-N/ml/hr
24	24.9
30	27.8
37	31.3

0.8 mg Bact. N/ml reaction mixture
.005 M dl-serine
pH 7.8

Table 29

pH CURVES FOR D- AND L-SERINE

	μ M NH_3-N Produced	
pH	l-serine	d-serine
4.55	1.29	0.34
5.40	3.29	1.05
5.74	4.16	1.09
6.10	5.39	1.21
6.30	5.87	1.60
6.60	5.65	1.94
6.82	5.97	2.00
7.00	6.03	2.16
7.25	6.46	2.14
7.51	6.89	2.28
7.90	6.42	2.79
8.32	6.48	2.38
9.05	6.38	2.14



of various pH values containing 10 μ M of either d- or l-serine. The final volume was 2 ml. Maximum ammonia production in 1 hour at 30 C was used as the criterion for the optimum pH. D-serine was deaminated approximately two-thirds less than l-serine, and, as shown in table 30, does not competitively inhibit the l-serine dehydrase since the values for ammonia are additive.

Chromatograms of 18-hour incubation samples showed alanine to be a product of serine deamination. Quantitative determinations showed that the amino nitrogen of serine could be accounted for in ammonia, alanine, and residual serine. Table 31 presents the average of data from 2 tests. Further studies have been made in an attempt to quantitate the pyruvic acid which should be formed in this type of deamination. Pyruvate was quantitated according to the method of Friedman and Haugen (8), as previously described. Since all of the cell preparations tested slowly metabolized pyruvate, semicarbazide was added to the reaction mixture as a "trapping agent" for pyruvic acid. It was found that quantities of semicarbazide in excess of 25 μ M/ml inhibited serine dehydrase activity. It was also found that the addition of equimolar quantities of semicarbazide and serine was insufficient to completely trap the pyruvic acid formed. When semicarbazide was present in sufficient quantity, alanine was not formed. The maximum yield of pyruvate, quantitated under these conditions, was never greater than 79 per cent of the theoretical. The data for these studies are recorded in tables 32 and 33. Both virulent and avirulent strains gave similar results.

Table 30

THE ADDITIVE EFFECT OF D- AND L-SERINE DEHYDRASE

Substrate	µg NH_3-N/ml/hr
l-serine	17.4
d-serine	4.4
l-serine + d-serine	24.9

.005 M substrates
.24 mg Bact. N/ml Strain SCHU
pH 7.8 37 C

Table 31

MOLECULAR BALANCE SHEET OF SERINE DEAMINATION

L-serine	Strain	μ M NH_3	μ M Alanine	μ M Serine	μ M Total
50 μ M	HOLT	28.3	19.9	2.6	50.8
100 μ M	SCHU	51.5	11.5	38.5	101.5

pH 7.3
30 C 18 hr

Table 33

THE EFFECT OF THE ADDITION OF SEMICARBAZIDE ON QUANTITATIVE STUDIES OF L-SERINE DEHYDRASE

		μ M/ml						
Strain	Time	Semicarbazide Added	L-serine Added	PU	NH_3	Alanine	Residual Serine	Serine Used (by calc.)
	hr							
		250	25	0	0	0	25.0	0
38	18	25	25	11.8	17.8	4.2	4.6	20.4
		0	25	1.1	15.2	6.3	5.9	19.1
38	20	25	6.25	3.1	3.9	0	2.35	3.90

pH 7.4

The enzyme-time relationship (table 34) was nearly linear from one-half to 2 hours under the test conditions. One-half ml of a SCHU cell suspension was added to 50 μ M l-serine in M/5 PO_4 buffer, pH 7.4. The final volume was 2.0 ml. Duplicate tests were set up for each time interval. Ten per cent TCA was added at the designated time intervals. The supernatants were tested for ammonia nitrogen by the Conway micro-diffusion technique.

Both aerobic and anaerobic dehydration deamination of serine have been reported for bacteria. Chargaff and Sprinson (1943) (22 and 23) studied the serine and threonine deaminases of E. coli and found that pyruvate and butyrate, respectively, accumulated as the products of anaerobic deamination. Wood and Gunsalus (1949) (24) and Metzler and Snell (1952) (25) have demonstrated aerobic dehydration deamination of serine by E. coli. Comparative studies, using strain SCHU, show equally efficient deamination of serine to pyruvate and ammonia under both aerobic and anaerobic conditions, if the cells were cultivated in a casein hydrolyzate medium. Cultivation of the same strain in a peptone medium yielded cells which deaminated serine only under anaerobic conditions, and which did not metabolize pyruvate under anaerobic conditions. These findings are in marked contrast to the cells cultivated in casein hydrolyzate. Growth and terminal pH were comparable for the 2 media. The data are recorded in table 35.

Dl-homoserine, β-phenylserine, and O-methyl serine were not deaminated by virulent strains. Deaminase activity was tested for under both aerobic and

Table 34

THE ENZYME-TIME RELATIONSHIP OF THE SERINE DEHYDRASE OF STRAIN SCHU

Time		μ M NH_3
hr	min	
	5	2.5
	15	5.5
	30	8.2
	60	18.2
	120	33.3
18		40.6

50 μ M l-serine
0.5 ml cells
2.0 ml final volume
pH 7.4 30 C

Table 35

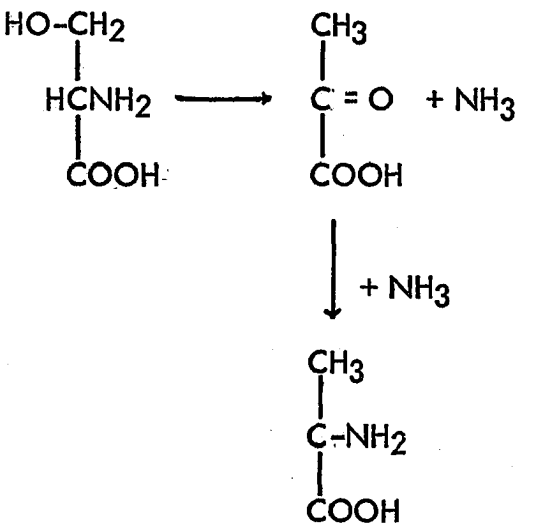
THE EFFECT OF CULTIVATION MEDIUM ON THE SERINE DEHYDRASE OF STRAIN SCHU

	Modified Casein Hydrolyzate		Peptone	
	50 μ M L-serine		40 μ M L-serine	
	μ M NH_3	μ M PU	μ M NH_3	μ M PU
Aerobic	43.2	14.2	0.3	1.8
Anaerobic	40.6	19.2	32.0	37.0

anaerobic conditions.

Acetone-dried cells of strain SCHU possessed no serine dehydrase activity.

In view of the evidence submitted on serine deamination, it appears that serine is degraded by dehydration deamination to pyruvic acid and ammonia, and that the alanine found as a product of long incubation periods is the result of direct amination of pyruvate as shown in the following equation:



The data in tables 31 and 32 support this statement. Additional evidence will be presented in the section on Transamination showing that direct amination of pyruvate occurs when NH_4Cl is added to a cell and buffer solution.

5. Transaminase Activity: In continuing our studies on the nitrogen metabolism of B. tularense, an investigation has been made of the transaminase activity of both virulent and avirulent strains. In addition, a determination of the "reductive amination" of keto acids was correlated with this investigation.

The technique used in determining transaminase activity has been described in the section on Materials and Methods. Strains SCHU-D, SCHERM, HOLT, and 38 were used. The bacteria were cultivated in both synthetic and casein hydrolyzate media. The washed cell suspensions were preserved in the frozen state. No significant decrease in activity was observed after storage of the cell suspensions for 2 weeks.

In discussing transaminase activity, the following symbols will be used in this paper:

Alanine	-AL
Aspartic acid	-AS
Glutamic acid	-GA
Alpha keto glutarate	-KG
Oxalacetic acid	-OA
Pyruvic acid	-PU

Several amino acids have been tested with PU, OA, and KG for transaminase activity using 2 virulent and 1 avirulent strain as the enzyme. The determinations were done at pH 7.4. A 0.2 M PO_4 buffer was used. The bacterial nitro-

gen concentrations were approximately the same for the 3 strains. One hundred μ M each of amino acid and keto acid were added to the reaction mixtures. All tests were adjusted to give a final volume of 2.5 ml, and were incubated aerobically at 30 C for 14 hours. Chromatograms were run on the supernatants. Table 36 presents the data for the positive findings. No transamination was observed with OA. Only alanine could transaminate with KG. The AL$\rightleftharpoons$ GA data will be presented later in this report. Excellent GA$\rightarrow$ AL and AS $\rightarrow$AL transaminases were present in all strains tested. Alanine was also formed in significant quantity from glycine, valine, leucine, phenylalanine, and methionine. Traces of alanine, not greater than the cell blanks, were formed from histidine, cysteine, lysine, tryptophan, arginine, ornithine, and proline. The data for citrulline and serine are probably not transamination. We have previously shown that alanine is a product of the deamination of serine. The addition of pyruvate to the serine deaminase system did not increase the quantity of alanine formed. Ammonia is readily formed from citrulline also. The alanine formed from citrulline and pyruvate is probably due primarily to the direct amination of pyruvic acid rather than to transamination. Direct amination of PU by the cells from NH_4Cl is demonstrated by the high yield of alanine. There was no amination of either KG or OA from NH_4Cl.

Similar transamination studies have been made using the amides, glutamine and asparagine. Strains HOLT and SCHERM were used. Chromatograms showed

Table 36

THE PYRUVATE TRANSAMINASE ACTIVITY OF VARIOUS STRAINS OF *B. tularense*

100 μ M	μ M Alanine Formed in 18 Hours		
	SCHERM 4.6 mg N/ml	SCHU-D 4.95 mg N/ml	38 4.47 mg N/ml
l-glutamic acid	64.6	55.0	42.8
l-aspartic acid	76.8	69.1	69.2
l-serine	19.2	29.9	9.5
glycine	9.9	4.9	1.2
l(+)valine	20.9	7.4	7.1
l-leucine	10.1	7.3	7.1
l-isoleucine	—	3.3	—
l-phenylalanine	6.4	7.4	8.5
methionine	6.1	5.2	4.5
l-citrulline	22.6	—	3.4
NH_4Cl	17.8	18.5	9.5
None	3.5	1.4	0.7

0.5 ml washed cell suspension
100 μ M each of amino acid and keto acid
pH 7.4 2.5 ml final volume
30 C 18 hr
— = Not done.

that transamination occurred between the amide and PU only. Ammonia nitrogen determinations on the reaction mixtures, showed a decreased ammonia formation from the amides in the presence of PU. OA and KG almost completely inhibited deamidation of both glutamine and asparagine. Quantitative studies were done to compare alanine formation from the amides and their corresponding amino acids. A decreased alanine formation was observed from the amides. The data are presented in table 37. Since the amides are so readily deamidated by B. tularense, the formation of alanine from reaction mixtures containing PU, amide, and cells, is, probably, primarily a transamination between the resultant amino acid (GA or AS) and PU. The increase in alanine over that observed for GA and AS may be due to the direct amination of PU from ammonia released by deamidation.

Several experiments have been done to determine the reaction velocity of the important transaminase systems of both virulent and avirulent cells. All tests were done in triplicate. Tables 38, 39, 40, and 41 present the averages of the data for AS⟶AL, GL⟶AL, AL⟶GA, and NH_4Cl⟶AL, respectively. The GL⟶AL reaction proceeds much more rapidly than the AS⟶AL with both virulent and avirulent cells. The AL⟶GL system is slower with both strains than the reverse reaction. Since the reaction proceeds well from each direction, equilibrium appears to be established at about 60 per cent to the right, i.e.,

$$GL \underset{40}{\overset{60}{\rightleftharpoons}} AL.$$

Table 37

A QUANTITATIVE DETERMINATION OF THE ALANINE FORMED BY

B. tularense TRANSAMINASES

Test 1

Substrate	μ M Alanine
glutamic acid + pyruvic acid	73
glutamine + pyruvic acid	77
aspartic acid + pyruvic acid	65
asparagine + pyruvic acid	72

100 μ M pyruvic acid
200 μ M amino acid or amide
pH 7.3 30 C 14 hr
Strain HOLT

Test 2

glutamic acid + pyruvic acid	32.6
glutamine + pyruvic acid	36.2
aspartic acid + pyruvic acid	38.4
asparagine + pyruvic acid	39.7

50 μ M each + pyruvic acid
pH 7.5 30 C 14 hr
Strain SCHERM

Table 38

SYNTHESIS OF ALANINE AND THE DISAPPEARANCE OF ASPARTIC ACID USING BOTH VIRULENT AND AVIRULENT STRAINS

Time	SCHU		38	
	μ M Alanine	μ M Aspartic Acid	μ M Alanine	μ M Aspartic Acid
hrs				
1/2	0.6	33.3	2.7	35.2
1	1.8	32.9	4.5	32.2
2	4.8	29.6	14.6	21.5
5	7.2	26.6	26.9	13.5
20	23.7	17.8	35.5	4.1

40 μ M each of aspartic acid and pyruvic acid
Bact. N — SCHU 1.4 mg
" " — 38 0.7 "

Table 39

SYNTHESIS OF ALANINE AND THE DISAPPEARANCE OF GLUTAMIC ACID USING BOTH VIRULENT AND AVIRULENT STRAINS

Time	SCHU		38	
	μ M Alanine	μ M Glutamic Acid	μ M Alanine	μ M Glutamic Acid
hr				
1/2	9.28	25.31	8.42	25.46
1	13.06	22.42	12.00	21.32
2	18.92	22.11	19.45	13.18
5	21.09	18.33	21.21	12.34
20	16.52	24.24	26.87	13.21

40 μ M each of glutamic acid and pyruvic acid
Bact. N — SCHU 1.4 mg
" " — 38 0.7 "

Table 40

SYNTHESIS OF GLUTAMIC ACID AND THE DISAPPEARANCE OF ALANINE USING BOTH VIRULENT AND AVIRULENT STRAINS

Time	SCHU		38	
	μ M Alanine	μ M Glutamic Acid	μ M Alanine	μ M Glutamic Acid
hr				
1/2	35.01	2.19	28.06	9.16
1	34.74	3.74	27.06	9.62
2	30.52	7.28	31.10	11.79
5	27.61	8.21	28.50	13.26
20	24.71	16.56	28.50	13.09

40 μ M each of alanine and α-keto glutarate
Bact. N — SCHU 1.4 mg
" " — 38 0.7 "

Table 41

SYNTHESIS OF ALANINE BY THE DIRECT AMINATION OF PYRUVATE

Time	μ M Alanine	
	SCHU	38
hr		
1/2	.48	1.90
1	.41	2.01
2	2.21	1.79
5	3.02	1.83
20	8.50	6.04

40 μ M each of NH_4Cl and pyruvate
Bact. N — SCHU 1.4 mg
" " — 38 0.7 "

Some differences were noted between virulent and avirulent strains in the AS⟶AL system. Although the reaction velocity is slow with both strains, the virulent SCHU strain was considerably slower than the avirulent strain 38. In addition, the reaction proceeded nearly to completion with strain 38, whereas it was only about 65 per cent complete with strain SCHU in the same time interval (20 hours).

Disappearance of glutamate and aspartate occurred at approximately the same rate as the synthesis of alanine.

The direct amination of pyruvate proceeded at a slow rate.

A study has also been made to disclose the effect of the bacterial nitrogen concentration on the synthesis of alanine and glutamic acid using both virulent and avirulent cells. Table 42 and figure 5 show the data for the virulent strain SCHU. Table 43 and figure 6 present the data for strain 38. All tests were done in triplicate, using a constant volume and pH. The reaction mixtures were incubated at 30 C for 18 hours. The data from this series of experiments show that, in terms of bacterial nitrogen concentration, the GL⟶AL system is the most efficient transaminase in both strains. A significant difference is apparent in the AS⟶AL transaminase in the 2 strains. Strain 38 not only transaminates to completion, compared to a maximum of 75 per cent for strain SCHU, but it does so with approximately one-half the bacterial nitrogen concentration necessary to yield optimum results for the virulent strain.

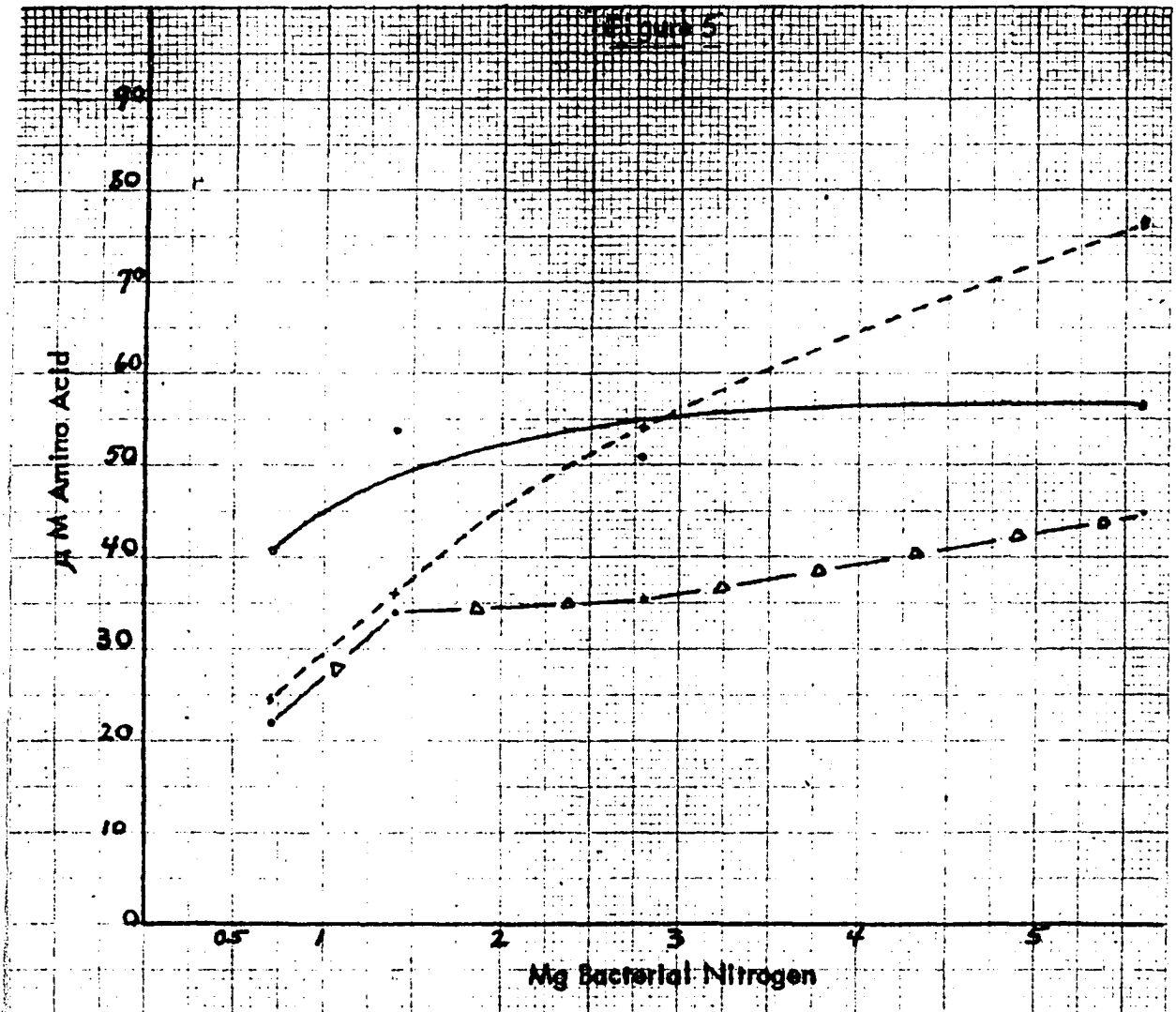
Table 42

THE EFFECT OF BACTERIAL NITROGEN CONCENTRATION ON THE SYNTHESIS OF ALANINE AND GLUTAMIC ACID BY STRAIN SCHU

Bact. Nitrogen	μ M Alanine		μ M Glutamic Acid
	Glutamic Acid + Pyruvic Acid	Aspartic Acid + Pyruvic Acid	Alanine + Keto Glutarate
mg			
0.699	40.6	24.3	21.8
1.398	53.8	36.1	34.0
2.796	50.4	54.2	35.6
5.592	56.4	75.8	44.7
6.990	52.8	76.6	39.5

100 μ M each of amino acids and keto acids
pH 7.4 30 C 18 hr
3.0 ml

Figure 5



100 μ M each of amino and keto acids, pH 7.4. Washed cells added as indicated. 3.0 ml final volume. Incubation 18 hours, 30 C.

——— alanine from glutamic-pyruvate

------- alanine from aspartic-pyruvate

—▲— glutamic acid from alanine-keto glutarate

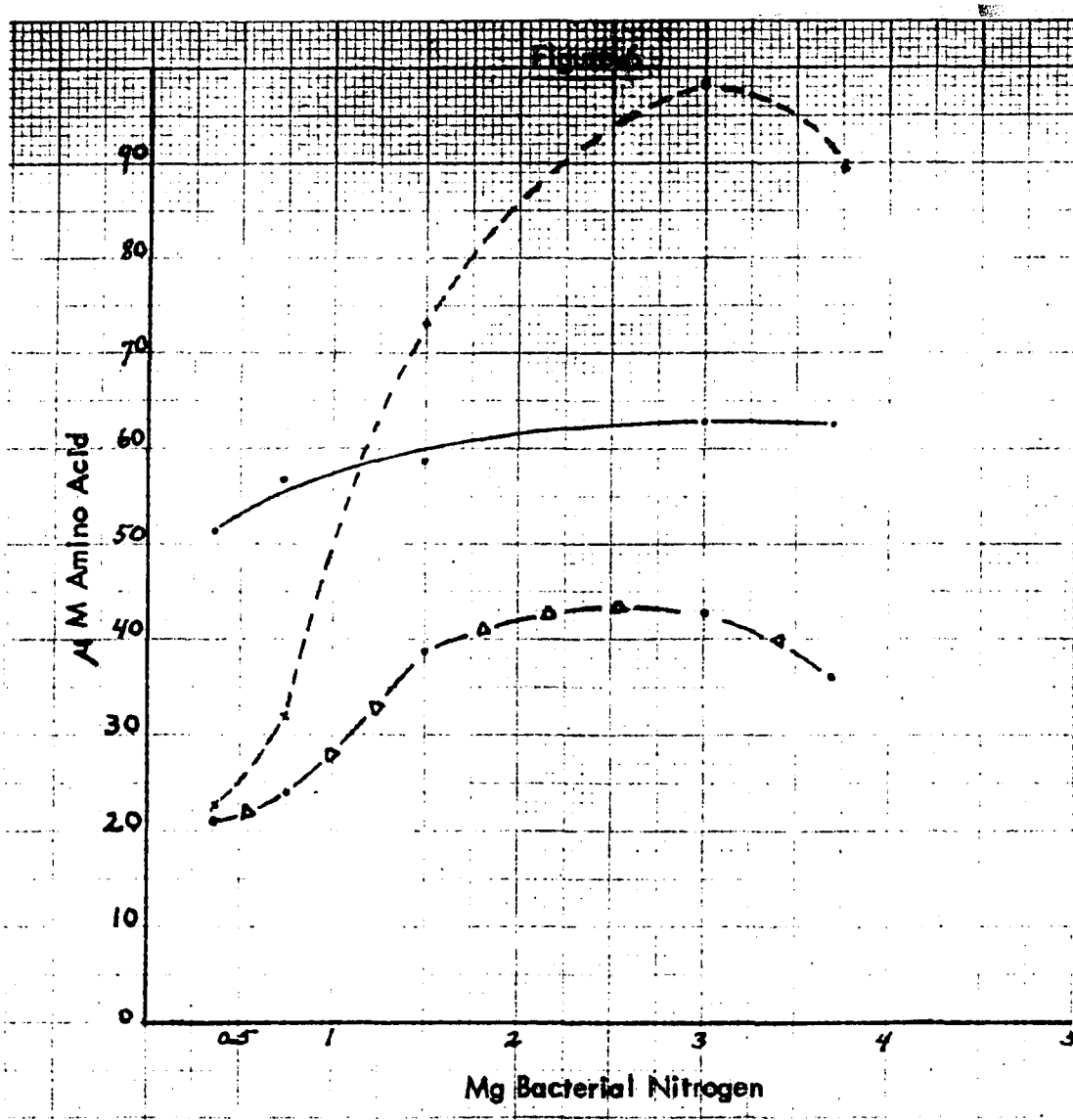
The effect of enzyme concentration on the transaminases of the virulent SCHU strain.

Table 43

THE EFFECT OF BACTERIAL NITROGEN CONCENTRATION ON THE SYNTHESIS OF ALANINE AND GLUTAMIC ACID BY STRAIN 38

Bact. Nitrogen	μ M Alanine		μ M Glutamic Acid
	Glutamic Acid + Pyruvic Acid	Aspartic Acid + Pyruvic Acid	Alanine + Keto Glutarate
mg			
0.369	51.5	22.8	21.3
0.738	56.8	31.9	23.9
1.476	58.7	73.1	38.8
2.952	62.9	98.6	42.8
3.690	62.4	89.4	35.1

100 μ M each of amino acids and keto acids
pH 7.4 30 C 18 hr
3.0 ml



100 μM each of amino and keto acids, pH 7.4. Washed cells added a
indicated. 3.0 ml final volume. Incubation 18 hours, 30 C.

——— alanine from glutamic-pyruvate

- - - - - - - alanine from aspartic-pyruvate

—Δ— glutamic acid from alanine-keto glutarate

The effect of enzyme concentration on the transaminases of the

avirulent 38 strain.

The effect of bacterial nitrogen on the AL⟶GA system was not strikingly different from those observed for GL⟶AL. No strain variation was observed.

A series of tests has been set up to determine the optimum pH for both the GA⟶AL and the AS⟶AL transaminases. Strain 38 was used. To determine the effect of pH, the cells, amino acids and keto acid were incubated in buffers of appropriate pH for 17 hours at 30 C. Constant volumes were maintained. The alanine synthesized at each pH level was quantitated from chromatograms. Table 44 presents the data for both systems. An optimum pH of 6.8 was found for both the GA⟶AL and the AS⟶AL transaminases.

In determining the effect of pH on the AS⟶AL transaminases, it was found that from pH 6.8 to 7.9 there remained only 0.1 to 1.6 μ M of aspartic acid in the various reaction mixtures. In this pH range, alanine synthesis had decreased, and chromatograms showed the presence of a new ninhydrin-positive area midway between aspartic acid and alanine. The product has not yet been identified.

Tests have also been carried out to determine whether the d form of glutamic acid and aspartic acid could transaminate with pyruvate. Strain 38 was used. The data (table 45) show that transamination does occur with the unnatural form, but to a very decreased extent. There was 50 per cent less transamination with d-glutamic acid and about an 85 per cent decrease with d-aspartic acid.

Table 44

pH CURVES FOR GA ⟶ AL AND AS ⟶ AL TRANSAMINASES

pH	μ M Alanine Synthesized from Aspartic Acid	μ M Alanine Synthesized from Glutamic Acid
6.0	16.8	7.5
6.4	21.1	11.6
6.6	22.8	11.6
6.8	29.0	12.4
7.0	27.0	11.6
7.2	25.4	11.8
7.4	19.9	11.0
7.6	19.9	—
7.9	20.4	—

0.5 ml strain 38, 30 μ M each amino acid and PU
3 ml final volume

Table 45

COMPARISON OF TRANSAMINASE ACTIVITY OF OPTICAL ISOMERS

	μ M Alanine Synthesized at pH 7.4
l-glutamic acid	11.0
d-glutamic acid	5.7
l-aspartic acid	19.9
d-aspartic acid	2.6

DISCUSSION

Both qualitative and quantitative enzymatic differences have been demonstrated between virulent and avirulent strains of B. tularense. Qualitatively, citrulline ureidase has not been demonstrated in the avirulent strain. Quantitatively, in terms of bacterial nitrogen concentration, strain 38 possesses less glutaminase and more aspartic-alanine transaminase than do virulent strains. Since the nutritional requirements of strain 38 are unknown, these enzymatic differences are of value in substantiating the nature of at least 1 biochemical mutation. Loss of virulence cannot be correlated with this particular enzyme deficiency until more avirulent strains have been tested.

The facility with which alanine is synthesized in both virulent and avirulent strains suggests that both transamination and the direct amination of pyruvic acid are important steps in the synthetic mechanism of B. tularense. Since direct amination occurs to a more limited extent, it seems probable that transamination represents the major pathway of alanine synthesis.

Wood and Gunsalus (24) obtained evidence suggesting that both serine and threonine are activated by a single enzyme. Using both resting cells and cell-free extracts of B. tularense, we have been unable to obtain data supporting this suggestion. Repeated trials have never shown more than minimum ammonia and pyruvate formation from threonine, in contrast to the complete degradation of serine.

SUMMARY

Evidence has been presented showing the presence of active glutamine and asparagine deamidases in both virulent and avirulent cell suspensions of B. tularense. Equimolar quantities of glutamic acid and ammonia were formed from L-glutamine. Asparagine was degraded to equimolar amounts of aspartic acid and ammonia. The pH optima for the 2 deamidases were essentially the same, 7.8 to 8.1 for glutaminase and 7.8 for asparaginase. The presence in reaction mixtures of .01 M glutamic acid or aspartic acid resulted in approximately 50 per cent inhibition of deamidation of .005 M concentrations of glutamine and asparagine, respectively. Only the l form of asparagine was attacked. Neither of the deamidases was found to be phosphate-activated. Although both deamidases were heat-labile, glutaminase was much more sensitive to temperature. Approximately 52 per cent loss of activity was found after heating the cells for 5 minutes at 45 C. Heating for 1 hour at 45 C did not destroy asparaginase activity. Heating for 1 hour at 56 C caused a 60 per cent loss of asparaginase activity. The ammonia formed from the incubation of both glutamine and asparagine in the same reaction mixture was equal to the sum of the ammonia liberated by each amide when incubated alone. This additive effect, combined with the differences observed on heat stability indicate that the deamidases are 2 different enzymes. On the basis of bacterial nitrogen concentration, avirulent cells contained less glutaminase than virulent cells. No differences were observed in asparaginase activity

between virulent and avirulent strains. No differences were observed between sonic lysates and washed cell preparations.

Virulent strains degraded citrulline to equimolar quantities of ammonia, carbon dioxide, and ornithine. The enzyme, citrulline ureidase, was not optically specific, possessed an optimum pH of 6.8 (based on ammonia and carbon dioxide production by 1.5 mg bacterial nitrogen at 30 C for 1 hour), and was heat-labile. Heating at 40 C for 30 minutes produced a 59 per cent decrease in activity. The enzyme was completely inactivated by heating at 45 C for 30 minutes. No differences were found between sonic lysates and washed cell suspensions. Cyanide inhibited enzyme activity. Strain 38 (avirulent) did not attack citrulline. Both virulent and avirulent strains failed to degrade arginine and ornithine.

Growth studies showed that neither citrulline nor ornithine could replace arginine in the cultivation of virulent strains in synthetic medium.

Both washed cells and sonic lysates of virulent and avirulent strains degraded serine to equimolar quantities of pyruvic acid and ammonia. On prolonged incubation periods (14 to 18 hours), significant quantities of alanine were found to accumulate as a product of serine dehydrase activity. The proposal has been made that alanine formation is the result of a second enzymatic reaction, the direct amination of pyruvate. Quantitative studies showed that serine nitrogen could be accounted for in alanine, ammonia, and residual serine. Since the cells slowly

metabolized pyruvic acid, a complete balance sheet study could not be carried out. In the presence of semicarbazide, however, alanine was not formed, thus substantiating the theory of direct amination of pyruvate.

Serine dehydrase activity occurred both aerobically and anaerobically. Both freezing and thawing and the presence of a phosphate-glutathione buffer significantly increased enzyme activity. No strain differences were observed. Although the enzyme attacked both d- and l-serine, d-serine was deaminated about two-thirds less than the l form. The pH optima were 7.6 for l-serine and 7.9 for d-serine. D-serine did not inhibit l-serine dehydrase activity. DL-homoserine, β-phenyl serine, and O-methyl serine were not attacked.

A wide variety of transaminases were found for B. tularense. Highly efficient glutamic acid ⟶ alanine, aspartic acid ⟶ alanine, and alanine ⟶ glutamic acid transaminases were present in both virulent and avirulent cells. Significant quantities of alanine were also formed from glycine, valine, leucine, phenylalanine, and methionine. Alanine was also formed when pyruvate was incubated with cells and either citrulline, glutamine, or asparagine. However, since these substrates are so readily degraded to ammonia and other products, the alanine formed in these instances is probably the result of direct amination of the pyruvic acid. With the exception of the AL⟶GA system, neither oxalacetic acid nor alpha keto glutaric acid could participate in transamination.[1]

An optimum pH of 6.8 was found for both the GL⟶AL and AS⟶AL systems. The d forms of both glutamic acid and aspartic acid participated only slightly in transamination with PU. The GA⇌AL system had an equilibrium established at about 60 per cent to the right. No reverse reaction was found for AS⟶AL. The GA⇌AL reaction velocity was more rapid than AS⟶AL. In terms of bacterial nitrogen concentration, the GA⟶AL enzyme system is also more efficient than the AS⟶AL. No strain differences were detected.

Some differences were observed between avirulent and virulent strains in the AS⟶AL system. Strain 38 apparently possesses more enzyme than virulent strains. Strain 38 not only transaminates 100 per cent to the right, compared to a maximum of 75 per cent for strain SCHU, but it does so with one-half the bacterial nitrogen concentration necessary for optimal results with virulent strains.

Direct amination of pyruvate has been reported for both virulent and avirulent strains. The reaction was slow, and no strain differences were noted. Direct amination of oxalacetate and keto glutarate was not observed.

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