AMINO ACID UTILIZATION BY REPRESENTATIVE STRAINS OF BRUCELLA

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ABSTRACT

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by Gladys Marie Thomas

The purpose of this study was to investigate the fate of amino acids removed from a minimal medium by representative strains of *Brucella*.

Amino acids and certain metabolites were detected as colored derivatives of an appropriate reagent after separation by one dimensional paper descending chromatography.

Variations in the uptake of amino acids were observed between species as well as within strains of species. In view of the variations obtained, a more extensive study was made with *Brucella abortus*, strain 19.

Extraction procedures employed did not detect the presence of intracellular acids, and the amino acid content of all hydrolysates appeared to be identical.

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By

Gladys Marie Thomas

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INTRODUCTION

Since the discovery of the Brucella organism, investigators have been studying the effects and seeking a control of the pathogenic manifestations of the organism in man and animals. Such investigations have been motivated by the great bearing which the genus Brucella have upon our domestic animal system (Hoyer, 1950). Many hours of research have gone into the development of media for producing maximum growth of these fastidious organisms.

Many observations of the metabolic activities of Brucella have been made from experiments on varied minimal media. Metabolic studies of single substrates by resting cells of brucellae have concerned themselves mainly with oxidative breakdown. These studies used the Warburg manometric technique and the Thunberg methylene blue reduction test.

The physiological needs of the bacterial cells determine the fate of the substrates utilized. The final destiny of the amino acids present in a medium may be preceded by several reactions. Bacterial cells are capable of internally concentrating small molecules, such as amino acids, and drawing from this reservoir to incorporate the simple substances into larger molecules. To incorporate the amino acids into peptides and protein they carry out such reactions as: (1) deamination, (2) decarboxylation, (3) transamination, and (4) peptide and protein synthesis.

Anabolically, amino acids from the external environment are absorbed into an internal pool for future protein synthesis.

The purpose of this study was to observe amino acid uptake from a minimal medium and the function of the amino acid in protein synthesis by resting cells of representative strains of Brucella. Acid hydrolysis and paper chromatography were used to study the amino acid extracts of cells and cell proteins.

LITERATURE REVIEW

Amino Acid Pool

For the production of macromolecules the building blocks must be transported within the cell. Many theories have been proposed for the mechanism of uptake: the permease theory, stoichiometric absorption site theory, and theories involving a carrier (Roberts and McQuillen, 1959). Roberts and McQuillen ask these questions concerning the biosynthesis of macromolecules: (1) What synthetic processes are interdependent, if any? (2) What areas within the cell are synthetic systems? (3) What determines the rate of assimilation of the basic molecules? With these questions in mind another phase of the internal concentration of amino acids must be considered. According to Gale (1951) the internal concentration of amino acids within gram positive organisms depends upon the species, external concentration of the nutrient and the phase of growth of the culture. The internal concentration is also related to the rate of cellular metabolism (Van Slyke and Meyer, 1913-14) and the rate of entry of the substances within the cell. The concentration gradient across the cell wall is a determining factor which varies with the species.

Gram positive organisms especially have been observed in many of the studies on amino acid assimilation. Gale (1953) has used gram positive organisms to study anabolic processes of amino acids and the effects of some antibiotics upon certain metabolic pathways. The organisms were divided into two groups using Taylor's technique of washed cell suspensions in determining the internal amounts of either glutamic acid or lysine present in both after washing (Taylor, 1947).

One of the groups maintained amino acids and the other did not. The gram positive cells contained measurable glutamic acid while the gram negative ones did not. Gram negative organisms may concentrate amino acids (Taylor, 1947), but the amount may be so small that if the cells are washed the intracellular amino acids may be lost to the external surroundings to meet the concentration barriers (Gale, 1953).

Gale (1951) gives examples of the entry of lysine and glutamic acid into the cell. Lysine passes across the wall by diffusion. The rate of entry of the amino acid is proportional to the external concentration. Lysine is not affected by temperature changes and will enter the cell even at O C. He has shown that glutamic acid will enter the cell only when exergonic metabolism, such as glycolysis, occurs simultaneously. Inhibitors, such as iodoactate, fluoride and cyanide, which poisons reactions of glycolysis also affect the concentration of glutamic acid. Extreme temperatures, O C, which slows down and stops glycolytic reactions causes the uptake of glutamic acid to be stopped.

Amino Acid Metabolism

The utilization of amino acids by Brucella has been a topic for discussion and observation. Previous studies in amino acid metabolism have centered around the Warburg manometric technique and the Thunberg reduction test.

Studies performed indicate the extent to which Brucella uses amino acids (Gerhardt et al., 1950a, b; Tucker, 1949; Kuwahara et al., 1951). They found that ammonium ions, such as ammonium sulfate, may serve as a nitrogen source for growth, amino acids employed

singly are good sources for nitrogen and for active deamination by resting cells (Gerhardt and Wilson, 1947 and 1948; Gerhardt et al., 1950b; Gerhardt, 1958). Gerhardt and Tucker observed that Brucella abortus, strain 19, aerobically grew luxuriantly with glutamic acid, asparagine, histidine, alanine, and arginine; this growth was determined photometrically. A high degree of specificity was demonstrated by the organism when structurally related compounds were employed; glutamine and asparatic acid were found to be poor replacements for glutamic acid and asparagine respectively. A comparison of the L and D isomers indicated that the natural, L isomers were used to a great extent, whereas the D isomers might or might not be metabolized.

Marr et al., (1953) showed that glutamic acid oxidation is one of the fastest reactions carried out by avirulent strains of Br. abortus. Glutamic acid may serve both as nitrogen and carbon source and is also important in the synthesis of other amino acids by transamination.

Cameron and Meyer, (1954), Mayer and Cameron, (1957) demonstrated the catabolic activities of Br. abortus upon amino acids by the Warburg technique. With this method no synthesis is detected; only the oxidative breakdown can be measured because of the pressure changes of the gases released.

Amino acid utilization may be hampered by the antagonism one amino acid produces toward another amino acid (Halvorson and Spiegelman, 1952; Gale, 1951). Such antagonistic amino acids are leucine, isoleucine and valine. If only one was added to a culture, growth occurred normally but the addition of the second or third caused inhibition of growth. This inhibition might be explained by the similarities in the structure of the amino acids (Gale, 1951). Breakdown products, such as the presence of elemental sulfur from cystine, have been shown to

inhibit some activities of Brucella; and the accumulation of metabolites, such as alanine, causes variants to appear in the culture (Gerhardt, 1958; Braun, 1950; Braun et al., 1951; Schuhardt et al., 1949).

Amination and Transamination

An observation which reaches farther than the measurments of the pressure changes resulting from release of the gas would be the fate of the end products of these catabolic processes. A positive correlation can be arrived at by stating that the amino acids may serve as an organic source of ammonia, which in turn is used as the primary source of nitrogen for synthetic processes (Gerhardt et al., 1950a).

Transaminases of Br. abortus, strain 19, were demonstrated under anaerobic conditions (Altenbern and Housewright, 1951, 1953). In transamination reactions there is an active transfer of the amino group from glutamate to pyruvate for the production of alanine. Transaminases apparently catalyze amino transfer from leucine, isoleucine, norleucine, and phenylalanine to pyruvate without proceeding through glutamic acid (Altenbern and Housewright, 1953).

Two methods of alanine production are transamination and direct amination or reductive amination (Altenbern and Housewright, 1951). The ammonium ions are used in the direct amination in alanine production. The exact method of incorporation is not known, but alanine is increased. This increase occurs when both L-asparagine and α-ketoglutaric acid and alanine are used as substrates; large amounts of glutamic acid, asparatic acid and alanine are formed. The reactions in this system are (1) hydrolysis of asparagine to aspartic acid, (2) transamination between asparate and α-ketoglutarate, and (3) transamination between glutamic acid and pyruvate (Altenbern and Housewright, 1951). Marr et al. (1953) using radioisotopes reported the oxidation of

glutamic acid in the presence of isotopic NH_3 indicated that both glutamic acid and free NH_4 ions were precursors for the amino group of alanine. Data showed that alanine contains more N^{15} than glutamic acid, but less than NH_4 ions. These results constituted evidence for the processes of transamination and direct amination. Altenbern and Housewright (1951) indicate that direct amination occurred only to a limited extent; however, the site and method of incorporation were unknown. Cameron and Meyer (1955) have investigated the utilization of CO_2 and NH_3 from urea breakdown in the synthesis of amino acids. In their studies it was found that Br. abortus, strain 19, fixed carbon dioxide to the carboxyl group of alanine. The synthesis and breakdown of urea provided pathways for the production of amino acids and allowed the cell to prevent its accumulation and toxic side effects.

Analysis of Amino Acids

An understanding of the chemical structure of protein and the components of its structure, amino acids, have depended upon hydrolysis. Investigators have demonstrated that amino acids can be obtained from protein by the following hydrolytic methods: (1) by acids, (2) by alkalies, and (3) by enzymes (Sahyun, 1944). The protein is treated with 5-20 times its weight of 3 N to 12 N acid or alkali at 100-120 C for 3 to 40 hr. (Meister, 1957).

Each of these methods has its advantages and disadvantages. Acid hydrolysis of protein is usually accompanied by destruction to tryptophan, and partial destruction of serine and threonine whereas alkaline hydrolysis offers some advantage over acid hydrolysis in as much as tryptophan is more stable under these conditions. However, extensive destruction of serine, thronine, cystine, cysteine, and arginine, and racemization of the amino acids occur with alkaline

hydrolysis (Meister, 1957). Hydrolysis by the enzymatic process is plagued by the necessity for careful control of pH and temperature while avoiding bacterial contamination. Since it is a slow process, it is not ideal for routine hydrolytic procedures (Meister, 1957).

Paper chromatography as practiced today was derived from the work of Consden, et al. (1944). Modified versions of Consden's technique have been made but the basic principles remain the same.

Paper chromatography is a technique for effecting the separation of chemically related compounds. Since many such compounds are colorless, the locations of these compounds are determined by the application of a color reagent to the dried chromatogram (Smith, 1960).

Several factors are important in the separation and migration of the solutes in paper chromatography. These include molecular weight of the solute, type of paper, size of chamber, temperature, length of time of the run. (Litwack, 1960; Smith, 1960; Brimley and Barrett, 1953). The results are reproducible provided that the factors mentioned remain constant. Paper chromatography permits quantitative as well as qualitative analysis of complex biological materials.

MATERIALS AND METHODS

Cultures

Representative strains of the suis, abortus, and melitensis species of Brucella were observed in these studies. The cells were maintained on tryptose agar (Difco) plates and examined periodically to confirm the typical characteristics of the smooth variant of the organisms. Examination of each culture was made by streaking a single colony over the surface of tryptose agar medium in petri plates so that dense and light areas of growth were present. The plates were incubated for four days at 37 C and observed using oblique illumination. Variants of Brucella may be identified by the different pastel hues of the colonies. The smooth variant exhibits a bluish green hue. The colonies were described as smooth according to shape, opacity, and color and consistency, the stability of the cells in a 1:1000 solution of acriflavine, and the absorption of an aqueous solution of crystal violet by the smooth colonies (Huddleson et al., 1952).

Aniline dyes, such as basic fuchsin and thionin, were used in the classification of the Brucella species. Br. abortus, strains 19, 1336, and 2308 grew only on the basic fuchsin agar plates; Brucella suis, strain 1856, grew only on the thionin agar plates and Brucella melitensis, strain 2480, grew on both basic fuchsin and thionin agar plates.

Cell Suspension

Test cultures were obtained by streaking a single colony to Brucella M agar (Albimi) slants and incubating for 48 hr. The cells

were removed from the slants with 0.85 per cent saline, spread on the surface of Brucella M agar medium in large bottles, and incubated for 48 hr. The resultant growth was harvested, washed, and suspended in M/15 phosphate buffer at pH 7.0 (Meyer and Cameron, 1959).

One ml of the washed cell suspension was removed to determine the cell concentration. The concentration of cells in the suspension was determined with the Libby Photonreflectometer from a prepared standard turbidity curve. The cell concentration was adjusted to 100×10^9 cells per ml in M/15 phosphate buffer.

Amino Acid Solutions

Solutions of amino acids were prepared in M/15 phosphate buffer in 0.01 M concentrations. In studies of mixtures of the amino or keto acids the dry solids were weighed and diluted to volume so that the concentration of each was 0.01 M. The solutions were autoclaved for 15 min. at 115 C.

A 20 per cent glucose solution was autoclaved separately from the amino acid solutions. After sterilization the glucose was aseptically diluted by addition to the amino acid solution to a final concentration of 1 per cent.

Brucella cells in solutions of amino acids sterilized by filtration were observed simultaneously with autoclaved solutions, but no difference in amino acid uptake was observed.

Reaction Mixture

The utilization of amino acids by resting cells was observed in the buffered solutions of amino acids. Two ml of the cell suspension (100×10^9 cells per ml) were added to 2 ml of an amino acid solution

in a sterile 50 ml erlenmeyer flask, which was plugged with cotton enclosed in gauze wrapping. The final concentration of the cells were 50 x 10^9 cells per ml and each amino acid was 0.005 M. In the test control the same number of cells was incubated in M/15 phosphate buffer only.

The reaction mixtures were incubated at 37 C on a push-pull type of shaker which operated at a rate of 55 strokes per minute. The time of incubation was for periods of 2 to 24 hr depending upon the rate of removal of the particular amino acid from the mixture.

Preparation of Samples for Paper Chromatography

Supernatant fluid from reaction mixtures

The reaction mixtures were removed from the incubator at the designated times, and a loopful of the suspensions were streaked on tryptose agar plates to detect the possible presence of contaminants. After the cells were removed by centrifugation, the supernatant fluids were retained for chromatographic analysis of possible amino acids and metabolic products. A schematic diagram of procedures illustrating the preparation of the fraction described as follows, is shown in Figure 1.

Cells from reaction mixtures

Efforts were made to determine the fate of the amino acids which were removed from the amino acid substrate solutions by the Brucella cells. The cells, washed three times in M/15 phosphate buffer, were treated by means which could damage the cell wall and membrane and cause leakage of the internal amino acid pool. The cell washings were concentrated to a 1 ml volume in a vacuum oven and examined chromatographically for trace amounts of the amino acids.

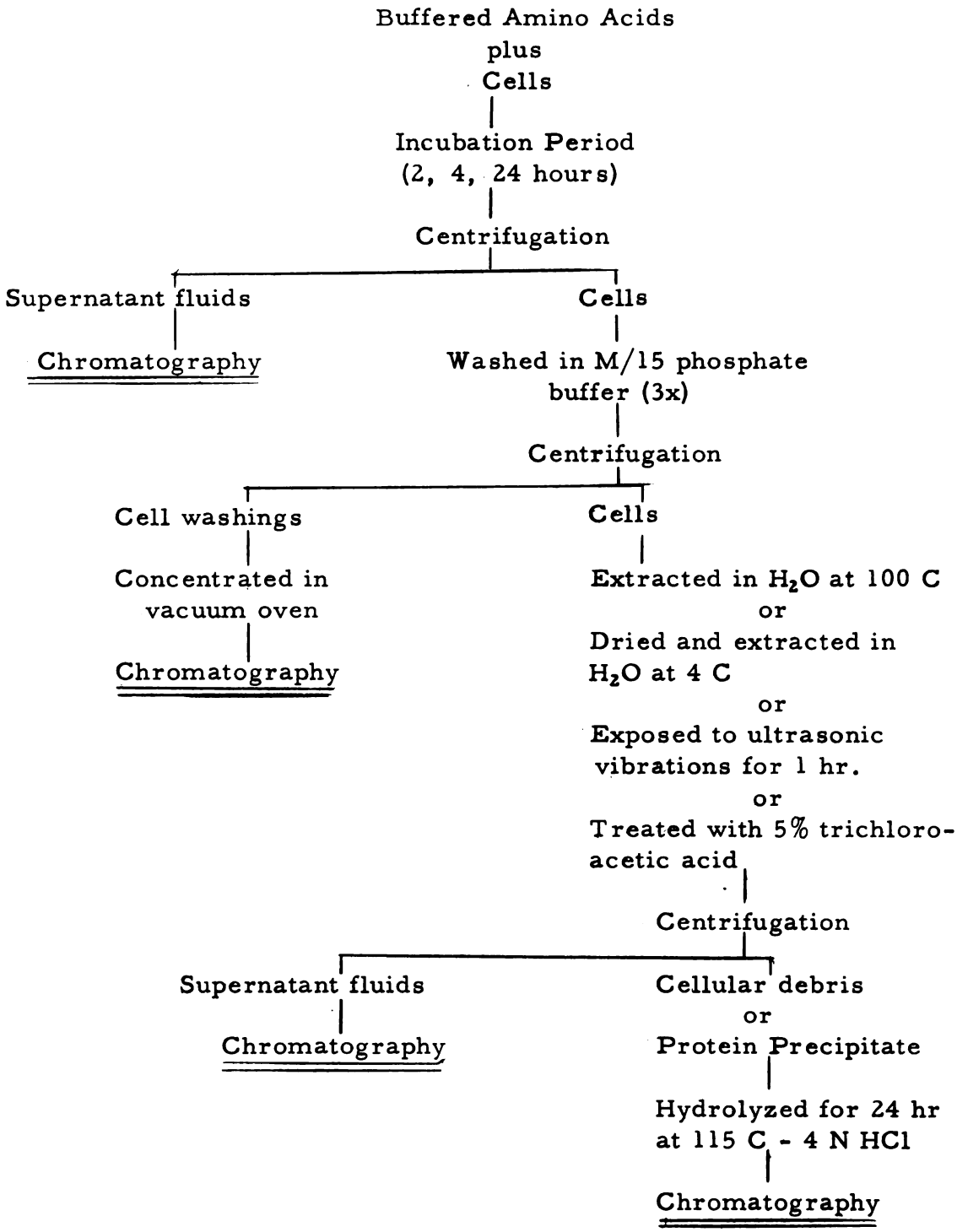

Figure 1. Flowsheet illustrating preparation of the several fractions for chromatographic analysis for amino acids.

Extraction of cells with water at 100 C. The washed cells were suspended in sterile distilled water and placed in a boiling water bath for 30 min. and cooled quickly (Speigelman et al., 1955).

Extraction of dried cells with water at 4 C. The wet cell mass was placed in a vacuum dessicator over "Dehydrite" and continuous pumping was maintained for 24 hr. The dried cells were then extracted for 20 min. at 4 C with sterile distilled water (Spiegelman et al., 1955).

Fractions of cells treated with ultrasonic vibrations. The cells were suspended in M/15 phosphate buffer and exposed to ultrasonic vibrations for 1 hr. The 25 ml sample was vibrated in a Raytheon Oscillator, model 3-102A, at a frequency in the order of 9 Kc. The principle of this technique is that magnetic material changes dimensions in a changing magnetic field. This effect is accomplished by attaching a sample cup to a diaphragm or a magnetic rod, wrapped in a coil of wire, which is connected to an electronic oscillator.

Extraction of cells with 5 per cent trichloroacetic acid. Protein was precipitated from the cells by treatment with 5 per cent trichloroacetic acid for 1/2 hr. at 37 C.

Following each of the above treatments of the cells, the materials were centrifuged to remove cell debris and all cell supernatant fluids were retained for chromatographic analysis.

Hydrolysates of cells. Washed cells from the reaction mixtures as well as the cellular debris and protein precipitates from trichloroacetic acid treated cells were hydrolyzed in 4 N hydrochloric acid at 115 C for 12 hr. The amino acids observed from the hydrolysis of whole cells would represent the amino acid pool and cellular protein whereas the hydrolysis of the trichloroacetic acid precipitable fraction

would represent cellular protein only. The hydrolysates were twice evaporated in a vacuum oven to dryness to remove the acid, and the residues were resuspended in sterile distilled water for chromatography.

Paper Chromatography

Amino acids and certain metabolites were detected with a color reagent after separation by one-dimensional paper descending chromatography. Technique followed was generally that of Consden et al. (1944).

Paper

Whatman No. 1 filter paper was used. This filter paper is graded as a medium rough and medium running paper (Brimley and Barrett, 1954; Smith, 1960). The strips were $5\frac{1}{2}$ inches by 17 inches and the solvent flowed with the grain of the paper.

Solvent

The isobutanol-acetic acid-water mixture is one of the commonly used solvents. It gives compact spots and is stable to temperature changes. The volume of isobutanol to acetic acid to water was at a ratio of 4:1:2 and the mixture was a single phase at normal room temperature.

Standard mixtures of amino acids

Amino acids were separated into two groups, A and B, which were individually resolved on the chromatogram to show the particular characteristics of each specific amino acid (Levy and Chung, 1953). The standards were used to aid in the identification of amino acids in

test solutions. Mixture A was prepared by dissolving 200 mM each of lysine, aspartic acid, glycine, threonine, proline, valine, tryptophan, phenylalanine and leucine in 2 ml of 1 N HCl and making up to 10 ml with 0.1 N HCl. Mixture B was prepared by dissolving 200 mM each of cystine, histidine, arginine, serine, glutamic acid, alanine, tyrosine, methionine and isoleucine in 2.4 ml of 1 N HCl and making up to 10 ml with 0.1 N HCl.

Preparation of paper strips and chambers

The papers were folded $\frac{1}{4}$ inch and $1\frac{1}{4}$ inches from the end. A starting line was penciled 1 inch from the last fold. The papers were then spotted on the starting line with the supernatant fluids, amino acid control solutions, and the standard mixtures of amino acids at points approximately 1 inch apart. Micropipettes were used to deliver 0.002 ml of the supernatant fluids and control solutions, and 0.001 ml of the standard mixtures of amino acids. The test spots were allowed to dry; then the paper strips were placed into the chambers which were Pyrex jars, 10 inches in diameter by 20 inches in height. These compartments contained stainless steel frames which hold a glass trough for the solvent. The folded ends of the strips were placed into the trough and held down by a glass rod. Thirty-five ml of the solvent were added to the trough after the papers were in position. A glass plate was sealed on the chamber with stopcock grease to prevent the evaporation of the solvent.

Temperature and time for chromatographic separation

The chromatographic chambers were placed in the 37 C incubator and were removed just before the solvent front reached the end of the paper (approximately 9 hr.).

In the earlier part of this study chromatograms were compared at room temperature and 37 C. At room temperature the spots after development were less compact, separation of amino acids was more variable than at 37 C. The normal variations in room temperature were factors in these results.

Color development on chromatogram

After the migration of the solvent, the papers were removed from the chambers; solvent fronts were marked and the papers dried in a hood with the aid of an exhaust fan for 2 to 3 hr.

The reagent used to develop the colors of the amino acids on the papers was 0.2 per cent ninhydrin containing 5 per cent 2, 4, 6-collidine (v/v) in ethanol. For the uniform application of the reagent the chromagrams were sprayed with a fine mist produced from a nozzle by air pressure. The papers were sprayed until thoroughly and evenly damp to obtain uniform color development of the spots.

The papers were again dried by the air exhaust fan for 5 min. The colors were developed in an oven at 105 C for 5 min.

The 2, 4, 6-collidine in the color reagent produced varieties of colors and hues of the individual amino acids. These characteristic colors and Rf's of individual amino acids were guides in the identification of amino acids. The concentration of the amino acids were estimated from the size and intensity of colors of the spots.

RESULTS

Utilization of Single Amino Acids

During the early part of this investigation a comparative study was made of the utilization of a limited number of single amino acids by strains of each of the three Brucella species.

Br. abortus, strain 19, Br. melitensis, strain 2480 and Br. suis, strain 1856 were observed for the individual utilization of seven amino acids (Table 1). After 24 hr. at 37 C, no evidence was demonstrated of uptake by any strain of four amino acids (glycine, L-isoleucine, L-leucine, and L-threonine). L-Lysine was removed by Br. suis only. The remaining two amino acids, L-alanine and L-glutamic acid, were removed by all of the strains tested. However, Br. suis utilized lesser amounts of these two amino acids than did the strains of Br. abortus or Br. melitensis.

Three strains of Br. abortus were used to study the removal of L-glutamic acid, L-histidine, and L-serine from the reaction mixtures (Table 2). Significant differences in utilization of two of these amino acids were noted. Each of the three strains utilized L-glutamic acid; strains 19 and 2308 utilized L-histidine whereas strain 1336 after 48 hr. removed none of this amino acid. A portion of L-serine was removed by 1336 and 2308, while strain 19 removed it completely from the reaction mixture.

In view of variations between species as well as within a species in the utilization of amino acids, a single strain of Br. abortus, strain 19, a strain of low virulence which is used in vaccines, was chosen for further extensive study. The amino acids which were removed by Br. abortus, strain 19, without the presence of a

Table 1. Amino Acid Utilization by Strains of Three Species of Brucella

Substrate*	Br. abortus strain 19	Br. melitensis strain 2480	Br. suis strain 1856
	Amount of Amino Acid Removed**		
	---------- Per cent ----------		
L-alanine	75	75	50
L-glutamic acid	100	100	75
glycine	0	0	0
L-isoleucine	0	0	0
L-leucine	0	0	0
L-lysine	0	0	100
L-threonine	0	0	0

*Amino acid substrate - 0.005 M in M/15 phosphate buffer, pH 7.

**Estimate (paper chromatogram) of amino acid removed by resting cells in 24 hr. at 37 C.

Table 2. Amino Acid Utilization by Strains of Brucella abortus

Substrate*	Br. abortus No. 19	Br. abortus No. 1336	Br. abortus No. 2308
	Amount of Amino Acid Removed**		
	----------Per cent----------		
L-glutamic acid	100	100	100
L-histidine·HCl	100	0	100
L-serine	100	50	75

*Amino acid substrate - 0.005 M in M/15 phosphate buffer, pH 7.

**Estimate (paper chromatogram) of amino acid removed by resting cells in 24 hr. at 37 C.

carbohydrate as an energy source were DL-asparagine, L-glutamic acid, L-glutamine; partial utilization occurred with L-alanine, L-histidine and L-serine (Table 3).

Multiple samples were used to determine the rates of removal of amino acids and to detect the accumulation of metabolites. L-Glutamic acid and L-glutamine were utilized at a very fast rate; their removal from the reaction mixtures occurred in 2 to 4 hr. No accumulation of metabolites was observed with the exception of aspartic acid which was detected after the removal of DL-asparagine.

Utilization of Single Amino Acids with Glucose

An energy source increases the uptake of an amino acid (Britten and McClure, 1962; Gale, 1953). In these experiments glucose was used to determine possible effects upon the amino acids which were partially removed within 24 hr. and several amino acids which were not removed (Table 4). L-Isoleucine, L-leucine, and L-lysine which were not removed from the reaction mixtures alone were not affected in the presence of glucose. L-Aspartic acid and glycine, not previously utilized, were completely removed in the presence of glucose. L-Alanine and L-serine which were partially utilized were completely removed whereas the uptake of L-histidine was not enhanced.

Effect of keto acids

Mixtures of the keto acids and amino acids were investigated to determine the effect of the presence of the keto acids upon the ability of the organism to concentrate an amino acid and possibly their role in protein synthesis. The keto acids were chosen in view that upon removal of the amino and keto acids from the medium transamination

Table 3. Utilization of Individual Amino Acids by Brucella abortus, strain 19.

Substrate*	Incubation (hr.)		
	2	4	24
	Amount of Amino Acid Removed**		
	----------Per cent----------		
L-alanine	0	50	75
L-arginine·HCl	0	0	0
DL-asparagine	50	75	100
L-aspartic acid	0	0	0
L-glutamic acid	100	100	100
L-glutamine	75	100	100
glycine	0	0	0
L-histidine·HCl	0	0	25
L-isoleucine	0	0	0
L-leucine	0	0	0
L-lysine·HCl	0	0	0
L-methionine	0	0	0
L-phenylalanine	0	0	0
L-proline	0	0	0
L-serine	0	0	25
L-threonine	0	0	0
L-tryptophan	0	0	0
L-valine	0	0	0

*Amino acid substrate-0.005 M in M/15 phosphate buffer, pH 7.

**Estimate (paper chromatogram) of amino acid removed by resting cells in 24 hr. at 37 C.

Table 4. The Effect of Glucose on the Utilization of Amino Acids by Brucella abortus, strain 19

Substrate*	Incubation (hr.) 2	4	24
	Amount of Amino Acid Removed**		
	----------Per cent----------		
L-alanine	0	50	75
L-alanine and glucose	0	50	100
L-aspartic acid	0	0	0
L-aspartic and glucose	50	100	100
glycine	0	0	0
glycine and glucose	0	50	100
L-histidine·HCl	0	0	25
L-histidine and glucose	0	0	25
L-isoleucine	0	0	0
L-isoleucine and glucose	0	0	0
L-leucine	0	0	0
L-leucine and glucose	0	0	0
L-lysine	0	0	0
L-lysine and glucose	0	0	0
L-serine	0	0	25
L-serine and glucose	0	25	100

*Glucose = 1%; amino acids = 0.005 M.

**Estimate (paper chromatogram) of amino acid removed by resting cells at 37 C.

reactions may occur and detection of metabolites in the medium would be possible (Altenbern and Housewright, 1951, 1953; Cameron and Meyer, 1954).

Mixtures of the keto and amino acids were: α-ketoglutaric acid and L-alanine; α-ketoglutaric acid and L-aspartic acid; α-ketoglutaric acid and DL-asparagine; oxaloacetic acid and L-glutamic acid; oxaloacetic acid and L-alanine; pyruvic acid and L-glutamic acid, and pyruvic acid and L-aspartic acid. Aspartic acid was the metabolite detected when DL-asparagine and α-ketoglutaric acid were placed in the medium and also when DL-asparagine was in the medium alone (Table 5). Other mixtures of the keto acids and amino acids did not show any detectable metabolites.

The keto acids enhanced the rate of removal of L-alanine (Table 6) and L-aspartic acid (Table 5). L-aspartic acid when observed singly was not removed from the medium and L-alanine was partially removed. Within 24 hr. L-alanine and L-aspartic acid were removed completely in the presence of the keto acids.

Mixtures of amino acids

Mixtures of amino acids were studied to determine if one amino acid enhanced the rate or removal of the others and would result in the effective concentration of amino acids and protein synthesis (Table 7).

The amino acid mixtures were: L-glutamic acid and L-alanine; L-glutamic acid and L-histidine monohydrochloride; L-arginine, L-glutamic acid, glycine, L-histidine monohydrochloride and L-serine. The rate of uptake of L-glutamic acid remained the same. The rate of uptake of amino acids which were partially removed (L-alanine, L-histidine monohydrochloride, and L-serine) was slightly enhanced. The amino acids which were not removed singly were not visibly affected.

Table 5. The Effect of Keto Acids on Utilization of Aspartic Acid and Asparagine by Brucella abortus, strain 19

Substrate*	Incubation (hr.) 2	4	24
	Amount of Amino Acid Removed**		
	----------Per cent----------		
L-aspartic acid	0	0	0
L-aspartic acid and α-keto-glutaric acid	0	50	100
L-aspartic acid and pyruvic	0	50	75
DL-asparagine	50	75	100
DL-asparagine and α-keto-glutaric acid	25***	50***	100***

*Keto and amino acids substrate = 0.005 M in M/15 phosphate buffer, pH 7.

**Estimate (paper chromatogram) of amino acid removed by resting cells at 37 C.

***Metabolite found in supernatant fluid was aspartic acid in a concentration comparable to the asparagine removed.

Table 6. The Effect of Keto Acids on the Utilization of Glutamic Acid and Alanine by Brucella abortus, strain 19

Substrate*	Incubation (hr.) 2	4	24
	Amount of Amino Acid Removed**		
	----------Per cent----------		
L-glutamic acid	100	100	100
L-glutamic acid and pyruvate	100	100	100
L-glutamic acid and oxaloacetic acid	100	100	100
L-alanine	0	50	75
L-alanine and oxaloacetic acid	0	50	100
L-alanine and α-keto-glutaric acid	0	50	100

*Keto and amino acid substrate = 0.005 M in M/15 phosphate buffer, pH 7.

**Estimate (paper chromatogram) of amino acid removed by resting cells at 37 C.

Table 7. Utilization of Individual Amino Acids from a Mixture by Brucella abortus, strain 19

Substrate*	Incubation (hr.) 2	4	24
	Amount of Amino Acid Removed**		
	----------Per cent----------		
(a) L-glutamic acid	100	100	100
L-histidine·HCl	0	25	50
(b) L-glutamic acid	100	100	100
L-alanine	25	75	100
(c) L-arginine·HCl	0	0	0
L-glutamic acid	100	100	100
glycine	0	0	0
L-histidine·HCl	0	25	50
L-serine	0	25	100

*Amino acid substrate = 0.005 M in M/15 phosphate buffer, pH 7.

**Estimate (paper chromatogram) of amino acid removed by resting cells at 37 C.

Trichloroacetic acid soluble fractions and cellular extracts

Soluble fractions from cells and cellular extracts were obtained to determine the fate of the amino acids. These materials were chromatogrammed to detect the presence of amino acids in an internal pool of the cell or to detect the incorporation of the amino acid into protein resulting in an increase of the test amino acid.

After incubation of the cells with the specific substrates, the cells were washed in M/15 phosphate buffer three times and treated with 5 per cent trichloroacetic acid to precipitate the protein.

The washings were tested chromatographically for the leakage of amino acids into the external environment; even when the washings were concentrated no amino acids were detected.

The trichloroacetic acid soluble fraction was maintained and examined chromatographically to determine the presence of an amino acid pool. Upon precipitation of the macromolecules the trichloroacetic acid should have caused damage to the cell wall and membrane, and leakage of the internal pool into the environment should occur if an amino acid pool was present (Britten and McClure, 1962). The trichloroacetic acid soluble portion was concentrated to detect traces of a pool. In all experiments conducted, all attempts to detect the pool failed.

Three other methods were used to obtain leakage from the cells by damaging the cell wall and membrane. These methods were: (1) boiling the cells and exposing these cells to low temperatures, (2) drying the cells over "Dehydrite" in a vacuum dessicator with continuous pumping and extracting the cells in the cold, and (3) utilization of sonic vibrations to cause damage to the cell wall and membrane which may result in leakage of the internal pool of amino acids.

All supernatant fluids obtained from the cells treated by these methods failed to have any measurable amino acid pool.

Trichloroacetic acid precipitable fraction and cellular debris

Following treatment of the cells with trichloroacetic acid and the methods cited above the precipitable protein and cellular debris were hydrolyzed and chromatograms were used to demonstrate a change in amino acids if protein synthesis had taken place. The test cells showed no qualitative and quantitative differences from the control cells. The amino acids found in the three species of Brucella were identical when compared chromatographically.

DISCUSSION

The uptake of amino acids by representative strains of three species of Brucella was determined by incubation of resting cells in an incomplete medium containing amino acids and the determination of the initial and final concentrations of the amino acids in the cells and solution.

Br. abortus, strain 19, Br. melitensis, strain 2480, and Br. suis, strain 1856 were compared in their utilization of L-alanine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-lysine and L-threonine. Br. suis, strain 1856, was slower in its removal of amino acids, L-alanine and L-glutamic acid, in comparison with other species. This was unexpected because in most metabolic and enzymatic activities, Br. suis is more active than Br. abortus and Br. melitensis. Br. suis is most active and contains the largest catalase and urease activity; Br. abortus is least active, while Br. melitensis falls between the two (Huddleson, 1943). In growth Br. suis cultures demonstrate colonies within 24 hr. while the other species require from 24 to 48 hr.

Br. suis differs significantly from Br. abortus and Br. melitensis in its ability to oxidize the substrates of the urea cycle (Meyer and Cameron, 1961). The difference in the rates of uptake of the amino acids may be dependent upon the pathways employed by the organisms in their synthetic processes. Br. abortus and Br. melitensis may use L-alanine directly in transamination reactions for synthesis of new compounds. In Br. suis the urea cycle may be a common pathway and through this cycle an interlocking occurs between it and the citric acid cycle which acts as a metabolic clearing house for any by-products (Cameron and Meyer, 1954).

A comparative study was made of the uptake of amino acids by Br. abortus, strain 19, 1336, and 2308. There was a difference in the rate of uptake; a difference occurred in the uptake of L-histidine and L-serine by strains 1336 and 2308. There was a rapid uptake of L-glutamic acid by all three strains of Br. abortus. The question arises as to whether glutamic acid may be used as a carbon source and energy source as well as a nitrogen source. This is expected in that upon deamination of glutamic acid, α-ketoglutaric acid is produced; this compound is active in the citric acid cycle. Upon deamination of L-alanine, pyruvic acid is produced; pyruvic acid is also a key component in the metabolism of carbohydrates and fats. Three strains were compared with only three amino acids, but evidence of differences in ability of uptake was observed within species.

In the studies of amino acid utilization of Br. abortus, strain 19, L-glutamic acid, its amide L-glutamine, DL-asparagine, and to a lesser extent L-alanine, L-histidine monohydrochloride and L-serine were removed from the original supernatant fluid. The utilization of the amides, L-glutamine and L-asparagine by Br. abortus, strain 19, may provide a device which permits ammonia to enter the cell and may also represent storage of the ammonia within the cell (Oginsky and Umbreit, 1955). The L-glutamic acid and DL-asparagine was completely removed in a 24 hr. period with the formation of aspartic acid. This was significant in that the amide group on the gamma carboxyl group was removed and may have been used in further metabolic processes. Aspartic acid which was not utilized alone was not utilized after the deamination of asparagine. Amino acids, such as glutamic acid, which were removed rapidly, showed no accumulation of metabolites after incubating for 24 hr. This is due to the vigorous oxidation by shaking the cultures.

The presence of glucose as an energy source enhanced the rate of removal of L-alanine, L-serine, glycine and L-aspartic acid, but did not result in the pool formation. The increase in the uptake of amino acids in the presence of glucose is caused by the exergonic metabolism which occurs.

In the mixtures of amino and keto acids, the rate of uptake of the amino acids was increased. Synthesis of other amino acids from the mixtures of these amino and keto acids: glutamate and pyruvate to alanine and α-ketoglutarate, glutamate and oxaloacetate to aspartate and α-ketoglutarate, aspartate and α-ketoglutarate to glutamate and oxaloacetate were not found. Reactions of aspartate and pyruvate to alanine and oxaloacetate, alanine and oxaloacetate to aspartate and pyruvate, and alanine and α-ketoglutarate to glutamate and pyruvate did not occur.

DL-Asparagine and α-ketoglutaric acid were selected to test under highly oxidative conditions in view of reports of transaminase reactions under anaerobic conditions. Altenbern and Housewright (1951) demonstrated alanine synthesis in a system containing L-asparagine and α-ketoglutaric acid. The reaction followed in this order: hydrolysis of asparagine to aspartic acid; transamination between aspartate and α-ketoglutarate to produce glutamic acid plus oxaloacetate; formation of pyruvate from oxaloacetate by presumably the loss of CO_2 and finally the transamination between glutamic acid and pyruvic acid. The process was demonstrated under anaerobic conditions. In this study the production of aspartic acid, the initial step in the line of reactions, was demonstrated. The failure to obtain an aspartic transaminase in Brucella originates from the vigorous oxidation procedure. The cultures were vigorously shaken during the incubation period; under such conditions the α-ketoglutarate would be removed by oxidation at a rate which would prevent its role in a transamination reaction

(Altenbern and Housewright, 1951). The vigorous oxidation may have affected the oxaloacetic and pyruvic acids in the same way.

Amino acid mixtures did not affect the uptake of those amino acids which were not removed when tested singly, but an increase in the removal of the partially utilized amino acids was observed. The enhancement of the uptake of the partially utilized amino acids may be due to the presence of glutamic acid. Glutamic acid is oxidized at a much faster rate than glucose, or the oxidizable amino acids, and it may be used as the sole source of carbon and energy in addition to being the nitrogen source in defined media (Hoyer, 1950). There was no accumulation of metabolites from these mixtures due to interaction between the amino acids present.

In the cell extracts, cellular debris and trichloroacetic acid precipitable fractions, no amino acid pool was found nor was there a quantitative or qualitative difference in the amino acids in hydrolysates of the test cells and control cells. After the removal of amino acids, if deamination occurs, the chromatographic method used would not detect the products. These products, keto acids and ammonia, may substitute for the amino acid pool (Kallio, 1955). The reassimilation of the compounds into needed products, such as amino acids and protein could possibly require a longer period of time and the amino acid pool or the increase in the amino acids which have been incorporated into protein would not be detected in the 24 hr. incubation period (Gerhardt et al., 1950a). If protein synthesis is represented by a breakdown of protein and reassimilation with the incorporation of the test amino acids, an increase in this amino acid would not be detected.

Amino acids play important roles in the interconversion of one amino acid to another. If the end products of the amino acid interconversions are utilizable by the organism and the enzymes for these

conversions are present in the cells, many nitrogenous compounds could be derived.

The main functions of amino acids involve their utilization for synthesis of new material and their breakdown to new compounds which are incorporated into new substances (Woods, 1948). The new material of the cell may be represented in the protein which is synthesized from the amino acids. The degradative products of an amino acid are the carbon chain which can be metabolized to fats and carbohydrates and the amino group which may be utilized in transamination reactions, direct amination, formation of excretory products, and formation of amides. These processes yield energy and nitrogen free intermediates. In this study the resting cells of Br. abortus, strain 19, 1336, 2308, Br. melitensis, strain 2480, and Br. suis, strain 1856 oxidized and deaminated the amino acids, which served as an organic source of ammonia and keto acids. The ammonia and keto acids in turn may serve as the primary source for synthetic reactions.

SUMMARY

Comparative studies were made in amino acid utilization by representative strains of three species of Brucella and Br. abortus, strains 19, 1336 and 2308.

Detection of amino acids was by paper chromatographic technique. The solvent contained isobutanol, acetic acid and water (5:1:2). The spray reagent, 0.2 per cent ninhydrin and 5 per cent 2,4,6-collidine in ethanol, was used in the development of the spots.

Seven amino acids were studied in amino acid uptake by Br. abortus, strain 19, Br. melitensis, strain 2480, and Br. suis, strain 1856. A difference in the amounts of the amino acid removed was observed in that L-alanine and L-glutamic acid were used by all three species in varying degrees of removal. L-Lysine was utilized by Br. suis, strain 1856 only.

Utilization of three amino acids by Br. abortus, strains 19, 1336, and 2308 was investigated. L-Glutamic acid was completely removed by all three strains; L-histidine was removed by strain 19 and 2308, but not by 1336, whereas L-serine was removed by strain 19 and partially removed by strains 1336 and 2308. The strains of a species also presented variations in uptake of the amino acids.

Studies of amino acid utilization by Br. abortus, strain 19, were conducted extensively. The amino acids removed from the medium by Br. abortus, strain 19, were L-glutamic acid, its amide L-glutamine, DL-asparagine and to a lesser extent L-alanine, L-histidine, and L-serine. The only metabolite observed in this study was aspartic acid upon the removal of DL-asparagine.

Glucose enhanced the rate of uptake of L-alanine, L-serine, glycine and aspartic acid but not L-histidine. L-Isoleucine, L-leucine and L-lysine, which were not removed from the reaction mixture containing the amino acid alone, were not noticeably decreased when in the presence of glucose.

The presence of keto acids enhanced the rate of uptake of the amino acids, but no metabolites were observed from the interaction between the compounds.

In mixtures of amino acids, no metabolites, amino acid pool nor protein formation was observed.

The presence of intracellular acids were not detected by extraction procedures.

The amino acid content of acid hydrolysates of cellular debris, trichloroacetic precipitates from cells, as well as whole cells, appeared to be qualitatively and quantatively identical.

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