Mechanism of ammoniation in Aerobacter aerogenes

Eric Beaumont Fowler
Iowa State College

Follow this and additional works at: https://lib.dr.iastate.edu/rtd
Part of the Biochemistry Commons, and the Microbiology Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/13159

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
NOTE TO USERS

This reproduction is the best copy available.

UMI®
MECHANISM OF AMMONIATION IN AEROBACTER AEROGENES

by

Eric Beaumont Fowler

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physiological Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College

1950
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>HISTORICAL</td>
<td>3</td>
</tr>
<tr>
<td>Survey of Significant Amino Acids</td>
<td>3</td>
</tr>
<tr>
<td>Ammoniation</td>
<td>9</td>
</tr>
<tr>
<td>Role of Intermediates of Carbohydrate Dissimilation</td>
<td>14</td>
</tr>
<tr>
<td>Significance of oxalacetate and carbon dioxide</td>
<td>14</td>
</tr>
<tr>
<td>Significance of α-ketoglutarate</td>
<td>17</td>
</tr>
<tr>
<td>Significance of pyruvate</td>
<td>19</td>
</tr>
<tr>
<td>Transamination</td>
<td>21</td>
</tr>
<tr>
<td>APPARATUS</td>
<td>25</td>
</tr>
<tr>
<td>Construction of Titrimeter-Dispenser</td>
<td>25</td>
</tr>
<tr>
<td>Results Obtained</td>
<td>31</td>
</tr>
<tr>
<td>METHODS</td>
<td>35</td>
</tr>
<tr>
<td>Preparation of Cells and Juices</td>
<td>35</td>
</tr>
<tr>
<td>Chemical Methods</td>
<td>36</td>
</tr>
<tr>
<td>Determination of residual ammonia</td>
<td>36</td>
</tr>
<tr>
<td>Determination of total α-keto acids</td>
<td>36</td>
</tr>
<tr>
<td>Determination of alanine</td>
<td>37</td>
</tr>
<tr>
<td>Synthesis of Compounds</td>
<td>37</td>
</tr>
<tr>
<td>Sodium bicarbonate containing C¹⁴</td>
<td>37</td>
</tr>
<tr>
<td>Pyruvate containing C¹⁴ in the carbonyl group</td>
<td>38</td>
</tr>
<tr>
<td>Reagents for removal of glycine and recovery of alanine</td>
<td>39</td>
</tr>
<tr>
<td>Microbiological Methods</td>
<td>39</td>
</tr>
<tr>
<td>Determination of alanine, glutamate and aspartate</td>
<td>39</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Recovery experiments</td>
<td>40</td>
</tr>
<tr>
<td>Determination of the isotopes C14 and N15</td>
<td>41</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>47</td>
</tr>
<tr>
<td>Ammoniation</td>
<td>47</td>
</tr>
<tr>
<td>Ammoniated products released during growth</td>
<td>47</td>
</tr>
<tr>
<td>Products from glucose</td>
<td>47</td>
</tr>
<tr>
<td>Products from α-keto acids</td>
<td>51</td>
</tr>
<tr>
<td>Ammoniated products from resting cells</td>
<td>57</td>
</tr>
<tr>
<td>Requirements of resting cells for ammoniation</td>
<td>61</td>
</tr>
<tr>
<td>Effect of anaerobic conditions on ammoniation</td>
<td>68</td>
</tr>
<tr>
<td>Requirements of dialyzed juices for ammoniation</td>
<td>74</td>
</tr>
<tr>
<td>Synthesis of glutamate</td>
<td>77</td>
</tr>
<tr>
<td>Synthesis of alanine and aspartate</td>
<td>77</td>
</tr>
<tr>
<td>Transamination</td>
<td>81</td>
</tr>
<tr>
<td>Carbon Dioxide and Pyruvate in Synthesis of Amino Acids</td>
<td>86</td>
</tr>
<tr>
<td>A mechanism for the synthesis of aspartate</td>
<td>87</td>
</tr>
<tr>
<td>Synthesis of alanine and α-ketoglutarate</td>
<td>90</td>
</tr>
<tr>
<td>Distribution of labeled nitrogen in synthesized amino acids</td>
<td>95</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>100</td>
</tr>
<tr>
<td>Ammoniation of α-Ketoglutarate</td>
<td>100</td>
</tr>
<tr>
<td>Possible Ammoniation of Oxalacetate</td>
<td>105</td>
</tr>
<tr>
<td>Role of Pyruvate in Ammoniation</td>
<td>108</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>110</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>112</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>126</td>
</tr>
</tbody>
</table>
INTRODUCTION

Interest in protein chemistry has grown extensively since the turn of the century. Perhaps the two approaches to an expanding field of science which have found greatest favor have been studies on various linkages in the protein molecule and a study of the ultimate degradation products of the proteins from various sources.

More recently investigations from the laboratories of Braunstein (1947), Kritzmann (1944-1948), Virtanen (1937-1949), Wiss (1948-1949), P. P. Cohen (1939-1947), Euler (1937-1940), Green (1945) and others have emphasized the relationship between amino-acid anabolism and carbohydrate dissimilation.

The position of α-keto acids is high in the scale of important compounds concerned with amino-acid synthesis. Considerable controversy exists concerning the assimilation of ammonium ion as regards biological systems. Perhaps only Euler has been able to point out an undisputed system for ammonium ion assimilation: α-ketoglutarate + ammonium ion → glutamate.

It seems reasonable to believe that such organisms as *Aerobacter aerogenes* may have other such systems since they grow and yield large amounts of protein on a diet of ammonia,
glucose and mineral salts. It also seems possible that an anaerobic and an aerobic system may exist separately and act independently for facultative organisms.

It was the purpose of this investigation to determine pathways of assimilation of ammonium ion through a study of the synthesis of alanine, aspartate and glutamate.
HISTORICAL

Two of the most fundamental reactions in biology involve the fixation of carbon as carbon dioxide and of nitrogen as ammonia. Elucidation of the mechanism of intermediary carbohydrate metabolism has aided in a study of the mechanism of carbon dioxide assimilation; recent studies using tracers, O\(^{18}\) and O\(^{16}\), have added much to our knowledge.

The picture is less clear in regard to ammoniation. Ammoniation and transamination reactions have not been completely separated, and since they are both so closely related to carbohydrate dissimilation, it is proposed to divide the historical background on the basis of ammoniation, significant products of carbohydrate dissimilation and transamination.

Survey of Significant Amino Acids

The role of alanine, aspartate and glutamate, important in transamination and ammoniation, will be reviewed.

Alanine was isolated from a protein hydrolyzate in 1875 by Schützenberger. It had been synthesized by Strecker twenty-five years previously and in this respect is unique. In 1888 Weyl found alanine in hydrolyzates of silk and the work of Fischer established its ubiquity. Because of its relationship to pyruvate, it has held special interest for the biochemist.
The alanine "residue" -CH$_2$CH$_2$NH$_2$CO$_2$H is found in thirteen of twenty-one amino acids listed by Sahyun (1944) as those occurring naturally. Alanine as such may not pass into or out of these amino acids. It is worthy of note, however, that Braunstein, et al., (1949) have observed the enzymatic hydrolysis of kynurenine to yield alanine. Reversal of the reaction would lead to the synthesis of tryptophan although Jackson and Jackson (1932) have claimed the reaction is not reversible. Wiss and Hatz (1949) reinvestigated the reaction in vivo and in vitro. They found that alanine was formed from tryptophan by rat liver, that anthranilic acid accumulates and that ammonia is not released. Krebs (1933) and Chargaff and Sprinson (1943) have shown that the oxidative deamination of serine, which is a substituted alanine "residue", yields pyruvate. Awapara (1950) noted an increase in the alanine content of rat liver when cysteine was injected intramuscularly. These and other investigations point to the importance of alanine as a possible precursor in the synthesis of other amino acids.

Frantz and coworkers (1947) studied the incorporation of carboxyl-labeled alanine into the protein of rat liver slices. They found a greater incorporation under aerobic than under anaerobic conditions and considered this relationship showed need for energy-rich phosphate bonds in protein synthesis. Snell and Guirard (1943) have shown that alanine
will substitute for pyridoxine in the nutrition of *Streptococcus lactis* and have suggested alanine as a precursor of pyridoxine. Nielsen (1944) has noted an antigrowth effect of alanine when yeast is fed on β-alanine in lieu of pantothenic acid.

Aspartic acid was isolated from asparagus juice by Vauquelin and Robiquet in 1806. Liebig reported the crystallization of aspartate and its correct empirical formula in 1838. Thirty years later Ritthausen isolated a mixture of aspartate and glutamate from hydrolyzed protein; later he was able to separate the two amino acids.

Virtanen and Laine (1939) suggested that aspartate is the first nitrogen-containing compound synthesized by bacterial cells in nitrogen fixation. Koser, Wright and Dorfman (1942) showed that aspartate could partly replace the biotin requirements of *Torula cremoris*. Stokes, Larsen and Gunnness (1947) and Potter and Elvehjem (1948) have reported the replacement of aspartic acid by biotin in the growth requirements of *Lactobacillus arabinosus*.

On enzymatic decarboxylation aspartate yields β-alanine. An enzyme has recently been described (Mardashev, *et al.*, 1949) which brings about "ω" decarboxylation. The product is α-alanine.

Glutamic acid like aspartic acid is a dicarboxylic, monoamino acid. It was first isolated and characterized by
Ritthausen in 1866. Since then the amino acid and its amide, glutamine, have been reported from numerous biological sources. Ryabinovskaya (1939) demonstrated a lowering of nerve excitability by sodium glutamate. In 1943 Sapirstein showed a protection against convulsions induced by ammonium chloride by the administration of sodium glutamate. Ammonium chloride is removed by glutamate or a product of glutamate dissimilation.

Gale and Taylor (1946) observed a decrease in the assimilation and concentration of internal glutamate when Micrococcus pyogenes var. aureus was subjected to low concentrations of penicillin. Gale and Mitchell (1947) noted a similar effect produced by triphenylmethane dyes. Studies on resistance by Gale and Rodwell (1949) led these workers to conclude that penicillin may prevent the synthesis of ribonucleic acids and thus prevent the assimilation of glutamate. Increased resistance was accompanied by an increase in the amount of penicillin necessary to prevent assimilation of glutamate. In 1947 Krumpitz and Werkman suggested that penicillin inhibited the synthesis of ribonucleic acids.

Polson (1948) reporting seventeen amino acids present in hydrolyzates of Escherichia coli found glutamate along with aspartate, serine, glycine and alanine present in higher concentration. Womack and Rose (1947) concluded that glutamate, proline and arginine were mutually interconvertible from results obtained in rat feeding experiments. Rose and
coworkers (1949) were able to show that both glutamate and aspartate were dispensable when a diet of nineteen other amino acids was fed but not dispensable when only ten amino acids were fed. Taylor (1947, 1949) demonstrated a lack of concentrated internal glutamate in gram-negative bacteria and an amount in gram-positive bacteria proportional to the external concentration.

On the other hand, Hartelius (1946) demonstrated an inhibition of growth by glutamate in several species of bacteria when β-alanine was present in low concentration. He concluded that β-alanine formed a peptide with glutamic acid and hence was not available for synthesis of pantothenic acid which is necessary for acetylation. However, glutamate was found to increase synthesis of acetylecholine in rat brain tissue by Nachmansohn, et al., (1943).

Glutamate utilization has been related to amylase production in Bacillus subtilis by Bhagwan (1948) and to the high concentration of d(-)glutamic acid in the capsular material of B. subtilis and B. anthracis by Hanby (1946) and Bovarnic (1942).

Ratner (1949) has restudied the Krebs-Henseleit urea cycle with partially purified enzymes and concluded that aspartate alone can act as an -NH₃ donor to form arginine from citrulline. Glutamate is of importance in the cycle through the production of aspartate by transamination.
Lipmann (1949) has discussed the mechanism of peptide bond synthesis and the incorporation of glutamate in the glutathione molecule. Waelsch and Rittenburg (1942) found both ammonium hydroxide containing $\text{N}^{15}$ and glutamate containing $\text{N}^{15}$ were incorporated in the livers of experimental animals. Both isotopically-labeled compounds were found in glutathione.

A relationship among glutamate, aspartate, serine and glycine was shown by Winnick, et al. (1948). Sixty per cent of the $\text{C}^{14}$ in methyl-labeled glycine was recovered in serine; small amounts of $\text{C}^{14}$ were found in glutamate and aspartate. Alanine did not contain $\text{C}^{14}$.

Nielsen and Johansen (1944) demonstrated the participation of glutamate in the synthesis of pantothenic acid from $\beta$-alanine. The latter arises through decarboxylation of aspartate. Cohen and coworkers (1949) and Minz and coworkers (1949) related glutamate to the synthesis of coenzyme A; they stated that of the amino acids only glutamate is active in coenzyme A synthesis.

The work of Dagley, et al. (1949) indicates that glutamate is an essential metabolite in reducing the lag period of cells when they are transferred to a new medium. Succinate has a similar effect in the case of \textit{A. aerogenes}. These workers believed that carbon dioxide fixation to succinate leads to the synthesis of glutamate. The synthesized
glutamate then stimulates the reproductive mechanism of the organism.

The three amino acids—alanine, aspartate and glutamate—are factors to be considered in numerous different reactions. The formation of such versatile compounds by reductive ammoniation would be important in the economy of the cell.

Ammoniation

One of the first suggested mechanisms of ammoniation was that of Virtanen and Laine (1937). The hypothesis proposed the formation of an oxime from hydroxylamine with oxal-acetic acid which was subsequently reduced to form aspartate. In a second paper (1938) Virtanen and Laine reported the synthesis of alanine from pyruvate by an enzyme system obtained from crushed peas. The amino group donor was aspartate.

In summarizing their results on nitrogen fixation Virtanen and Laine (1939) reported more than 90 per cent of the nitrogen excreted by root nodules to be amino nitrogen. One to two per cent oxime nitrogen was also found. L-aspartic acid formed the major part of the amino nitrogen when peas were harvested before flowering. Aspartate decreased with increasing age of the plants; the decrease was accompanied by an increase in β-alanine. The oxime appearing was characterized as oximinosuccinic acid. They
Theorized the synthesis of hydroxylamine from nitrogen and its nonenzymatic combination with oxalacetate to form the oxime. Enzymatic reduction of the oximinosuccinic acid was believed to yield aspartate.

Virtanen, et al., (1946) continued an investigation of the products excreted by root nodules. The nitrogen excreted by young plants was almost entirely in the form of \( \alpha \)-amino nitrogen; only a small amount of glutamate was present. Older plants excreted both glutamate and aspartate. The presence of glutamate suggested the reduction of hydroxylamine to ammonia followed by the formation of \( \alpha \)-imino-glutaric acid. No glutamate was found in excretions from young plants, although the workers thought it should be present.

Virtanen and Csaky (1948) suspended low-nitrogen yeast in solutions of oxime nitrogen, potassium nitrate and ammonium sulfate. These species of Torula grew on 200 p.p.m. hydroxylamine; oxime nitrogen was formed from potassium nitrate but not from ammonium sulfate. Normal Torula also formed oxime nitrogen.

Other observations on the reductive ammoniation of oxalacetate have been more indirect. Damodaran and Subramanian (1948) observed the presence of aspartase in germinating Phaseolus mingo within 24 hours and associated it with the early production of aspartic acid.
Jensen (1948) found that *Azotobacter indicum* grew as well on aspartate as in free nitrogen. He found hydrogenase in the reaction mixture when *Azotobacter* grew in the presence of free nitrogen. Hydrogenase was absent when aspartate acted as the source of nitrogen. The importance of a reduction in the synthesis of aspartate was indicated.

The laboratory of Euler studied coenzyme II of yeast, bacteria and higher plants. Adler, Hellström, Günther and Euler (1938) established the reversibility of the deamination of glutamate based on the following reaction which takes place non-enzymatically.

\[
\begin{array}{c}
\text{CO}_2\text{H} \\
\text{C}=\text{O} \\
\text{CH}_2 + \text{NH}_3 \\
\text{CH}_2 \\
\text{CO}_2\text{H}
\end{array}
\rightarrow
\begin{array}{c}
\text{CO}_2\text{H} \\
\text{C}=\text{NH} \\
\text{CH}_2 + \text{H}_2\text{O} \\
\text{CH}_2 \\
\text{CO}_2\text{H}
\end{array}
\]

They followed the disappearance of reduced coenzyme II spectrometrically when a suspension of *E. coli* was added to a solution containing reduced coenzyme II, ammonia and α-ketoglutarate.

Adler, *et al.*, (1938) were able to demonstrate a glutamate apoenzyme in *E. coli* or animal muscle which could be obtained in cell-free solution and which did not use coenzyme I. It was concluded that the series of reactions led to the fixation of ammonia when coenzyme II and a specific
apoenzyme were present in the cell, i. e., by reductive ammoniation. Adler, Günther and Everett (1938) observed a specificity of the apoenzyme for coenzyme II and pointed out the difference between this specificity and that of higher plants. Higher plants utilize coenzyme I. The dehydrogenation reaction was shown to be reversible and α-imino glutaric acid was identified as the primary product.

The overall reaction in this case of reductive ammoniation may be given:

\[
\begin{align*}
\text{CO}_2\text{H} & \quad \text{CO}_2\text{H} & \quad \text{CO}_2\text{H} \\
\text{C} = \text{O} & \quad \text{C} = \text{NH} & \quad \text{H-CN-NH}_2 \\
\text{CH}_2 + \text{NH}_3 & \rightarrow \text{CH}_2 + \text{H}_2\text{O} \xrightarrow{\text{H-Co II}} \text{CH}_2 + \text{O-Co II} \\
\text{CH}_2 & \quad \text{CH}_2 & \quad \text{CH}_2 \\
\text{CO}_2\text{H} & \quad \text{CO}_2\text{H} & \quad \text{CO}_2\text{H}
\end{align*}
\]

Euler, Adler, Günther and Elliot (1939) showed that isocitric dehydrogenase was present in yeast and higher plants and that it was specific for coenzyme II. The dehydrogenase is important in the synthesis of glutamate since oxalate succinate, hence α-ketoglutarate, arises from its action.

Gedrangolo (1943) observed an inhibition of oxygen uptake and a decrease in ammonia released into the medium when α-ketoglutarate was added to a reaction mixture containing l(-)amino acid and an enzyme preparation. The implication was that an ammoniation of α-ketoglutarate occurred followed by transamination thus removing oxidizable substrates.
The reductive ammoniation of pyruvate has been considered a mechanism for the assimilation of the ammonium ion. Kritzmann (1947) stated that reductive ammoniation of pyruvate could be demonstrated in tissue homogenates or cell-free extracts when such preparations were supplied with cozymase, a precursor of oxalacetate and "coenzyme of aspartic acid aminophorase". Green, Leloir and Nocito (1945) have criticized Kritzmann and suggested that the "coenzyme" used was in reality a small amount of a transaminase which converted oxalacetate to aspartate and this in turn transferred its amine group to pyruvate to form alanine.

Kritzmann (1944) showed a dependency of the synthesis of alanine on the presence of carbon dioxide and considered this relationship to involve the Wood-Werkman reaction in that the oxalacetate formed by carbon dioxide fixation could be transformed to aspartate by transamination. The aspartate was believed to transaminate with pyruvate to yield alanine. A ten-fold increase in amino nitrogen in the presence of pyruvate was attributed to carbon dioxide fixation. A specific method for the determination of aspartate and alanine might have shown that the amino nitrogen was present as aspartate and not as alanine.

The investigations of Wiss (1948) with rat liver homogenates indicated that pyruvate could be reductively ammoniated to alanine. However, Wiss took issue with Kritzmann on the
effect of carbon dioxide. His results showed the synthesis of alanine to be dependent upon an oxidative reaction which was inhibited by sodium cyanide and arsenic trioxide. Wiss could not find evidence for the presence of amino dicarboxylic acids as intermediates although he did not state that they took no part in the synthesis of alanine.

An interesting reaction was observed by Krueger (1950) who demonstrated the synthesis of glycine by ground pig liver in the presence of pyruvate and ammonium chloride. Glycine probably does not arise by reductive ammoniation.

Housewright and Thorne (1950) have shown that glutamate is synthesized from α-ketoglutarate and aspartate by B. anthracis; only L-aspartate was active, and only L-glutamate was formed. The transaminase enzyme was obtained in partially purified form by ammonium sulfate precipitation of cell-free extracts.

Role of Intermediates of Carbohydrate Dissimilation

Significance of oxalacetate and carbon dioxide

The simplest system for ammoniation would include ammonia and a carbon skeleton. A heterotrophic organism receives ammonium ion from the environment; the carbon skeleton would arise from the dissimilation of carbon compounds. The importance of intermediary carbohydrate metabolism is
evidenced in the requirements of the transamination reaction for dicarboxylic acids. Chibnall (1939) has expressed the relationship as follows:

... my reading of ... literature ... has impressed me with a firm belief that it is the group of substances we refer to as "organic acids"—which have ... been neglected ... because satisfactory methods of characterisation were not available—that occupy ... the key position, in the carbohydrate, protein and fat metabolism of plant cells.

Braunstein and Kritzmann (1939) and Kritzmann and Melik-Sarkisyan (1945a) indicated the importance of α-keto acids in the nitrogen economy of the cell. They added M/16,000 oxalacetate to enzyme preparations and found a catalysis of the transfer of -NH₂ groups to monocarboxylic acids. That an oxidative mechanism is important was shown by the catalytic effect of citrate, succinate, malate and fumarate under aerobic conditions. Monocarboxylic acids were probably not directly involved.

Krebs, et al., (1940) have shown that dissimilation of oxalacetate by animal tissue yields carbon dioxide, fumarate, malate, α-ketoglutarate, citrate, succinate and pyruvate. The dissimilation of oxalacetate alone would lead to the formation of precursors of alanine, aspartate and glutamate.

Wood, et al., (1941) isolated α-ketoglutarate containing C¹³ in only one carboxyl from the reaction pyruvate and C¹³O₂ in the presence of pigeon muscle. This observation supported
the theory of carbon dioxide fixation to pyruvate to yield oxalacetate. Krampitz, Wood and Werkman (1943) proved the above reaction by demonstrating an exchange of carbon dioxide in the β position of oxalacetate.

The role of oxalacetate and carbon dioxide fixation in amino acid synthesis was indicated by the work of Della and Wilson (1946). The injection of sodium bicarbonate containing C13 into rats resulted in fixation of C13 in the carboxyl of both aspartate and glutamate isolated from hydrolyzates of the muscle protein.

Ochoa, et al., (1948) have suggested a pathway of carbon dioxide fixation and reduction leading directly to malate; A.T.P. is not necessary, but T.P.N. is required by the enzyme. Malate might be important in reductive ammoniation.

The work of Lyman, et al., (1947) also indicates the importance of carbon dioxide in the synthesis of amino acids. In the absence of carbon dioxide, arginine, phenylalanine, tyrosine and pantothenic acid were essential to the nutrition of L. arabinosus. Carbon dioxide could be omitted only if pantothenic acid was present. The results indicate the occurrence of the series: pyruvate → oxalacetate → aspartate → β-alanine → pantothenate.

Koplanskii and Shmerling (1945) reported that succinate was as active as pyruvate in the synthesis of amino acids by liver and kidney slices and that carbon dioxide was not required for the synthesis of alanine. However, tissue slices
may retain sufficient carbon dioxide at physiological pH levels to give rise to questioning of their findings.

The work of Lwoff and Monod (1946) and Ajl and Werkman (1948, 1949) indicates fixation of carbon dioxide as necessary for growth. In the absence of carbon dioxide growth did not take place when pyruvate was added to the medium; however, the addition of oxalacetate resulted in extensive growth.

Das (1945) found that oxalacetate in the presence of ammonium sulfate initiated faster growth in "Bacterium coli anserogenes" than did \(\alpha\)-ketoglutarate, fumarate or pyruvate. Arsenite inhibited growth, but malonate and sodium fluoride had no appreciable effect.

Kritzmann and Melik-Sarkisyan (1948a) found large amounts of aspartate with some glutamate and a small amount of alanine when oxalacetate was used as a substrate by liver slices. The relative amounts of products were the same when pyruvate and sodium bicarbonate acted as substrate.

The conclusions of Ajl (1950) that a tricarboxylic acid cycle is not present in bacteria would invalidate many of the observations concerning \(\alpha\)-ketoglutarate and oxalacetate.

**Significance of \(\alpha\)-ketoglutarate**

The informative research of Euler, et al., (1937) and of Adler, et al., (1938) placed emphasis on \(\alpha\)-ketoglutarate as a
precursor of glutamate. The synthesis of α-ketoglutarate from citrate by pigeon muscle in the presence of arsenite was shown by Rossi and Ruffo (1941). Glutamate was also detected in the reaction.

A relationship existing between α-ketoglutarate and sodium bicarbonate was emphasized by Villano and d'Ambrosio (1947). In vivo experiments with man demonstrated an increase in urinary α-ketoglutarate when sodium bicarbonate was taken per os.

Krebs, Eggleston and Hems (1948) suggested that α-ketoglutarate could arise either anaerobically or aerobically since glutamate accumulated under either condition. They found more glutamate anaerobically than aerobically and observed an inhibition of the synthesis of glutamate by acetate and butyrate.

_E. subtilis_ was found to synthesize maximum amounts of glutamate from α-ketoglutarate by Konikova, _et al._ (1948). Yakobson, _et al._ (1949) reported an enzyme system present in a number of bacterial species which utilized α-ketoglutarate and pyruvate in the synthesis of amino nitrogen; glucose and cozymase were required in the reaction. Hunter and Leloir (1945) noted the need for α-ketoglutarate in the synthesis of citrate. The added α-keto acid had to be oxidized; this could take place anaerobically in the presence of an excess of oxalacetate the latter being reduced to malate. Hunter
(1949) in a further study suggested the need for phosphorylation in the reaction.

A mechanism for the origin of \(\alpha\)-ketoglutarate from isocitrate was observed by Ochoa (1945). Glucose-6-phosphate and T.P.N. were required in the reaction, and oxalsuccinate is an intermediate.

Morrison and Hinshelwood (1949) demonstrated the importance of \(\alpha\)-ketoglutarate in initiating reproduction in the cells of *A. aerogenes*. They believed a long lag phase was due to the lack of \(\alpha\)-ketoglutarate or its slow accumulation through retarded carbohydrate dissimilation.

Cohen-Bazire and Saissac (1947) reported an inhibition of oxidative deamination when \(\alpha\)-ketoglutarate was present as a substrate for *Clostridium sporogenes*.

**Significance of pyruvate**

Watt (1949) has reviewed extensively the literature relating to pyruvate. Pyruvate is often considered the hub of metabolism; i.e., it arises in glycolysis near the end of the classical Meyerhof scheme as phosphopyruvate. Its importance in amino acid synthesis lies in its relation to alanine. Phosphopyruvate may also arise through citrate dissimilation by muscle tissue in the presence of A.T.P. (Epstein, 1941). The importance of phosphorylation in pyruvate utilization was shown by Euler and Högberg (1940). Sodium
fluoride depressed the rate of utilization by yeast. Reiner (1947) reported that pyruvate neutralizes the sodium fluoride inhibition of yeast fermenting galactose.

Meister and Greenstein (1948) have demonstrated the release of pyruvate and acetate on enzymatic hydrolysis of 2,4-diketovaleric acid. A reversal of this reaction would lead to a \( C_4 \) + \( C_3 \) condensation. Such a condensation might be important in the formation of \( \alpha \)-ketoglutarate.

Lipmann (1939) suggested the separation of anaerobic and aerobic carbohydrate breakdown takes place at the point where pyruvate emerges from the Meyerhof scheme. The complete pattern of metabolism changes with the generation of pyruvate. This point has since become associated with the Krebs cycle and hence the production of other \( \alpha \)-keto acids important in ammoniation and transamination.

A metabolic relationship among pyruvate, citrate and \( \alpha \)-ketoglutarate was shown by the work of Simola and Krusius (1939). Sodium pyruvate fed to man resulted in a forty-fold increase in blood \( \alpha \)-ketoglutarate. The citrate level also rose after pyruvate consumption.

Simola and Alapeuso (1943) studied the metabolism of pyruvate by brain tissue. More pyruvate disappeared aerobically than anaerobically; 16-18 per cent of the amount disappearing could be accounted for as alanine in either case.
In 1947 Novelli and Lipmann reported an increased production of coenzyme A from added pantothenate when *L. arabinosus* was allowed to metabolize pyruvate. An acceleration in the rate of oxidation of pyruvate was reported by Tang and Hsueh (1943) when amino acid or ammonium chloride was added to the reaction mixture.

Anker (1948a) fed labeled pyruvate to rats and recovered, in the excreta, amines labeled in the acetyl group.

Transamination

Several excellent reviews have been written on transamination. Herbst (1944) reviewed the then known aspects of the reaction and compared model and enzymatic systems. The former system is irreversible by virtue of its loss of carbon dioxide and ammonia. Enzyme catalyzed systems are reversible and require a dicarboxylic amino acid or keto acid.

An important point made by Herbst is in regard to the poor quality of much of the work which had been done on transaminase systems. Crude analytical methods have led to much confusion and misunderstanding or misinterpretation of results. However, many necessary microanalytical methods were not available to early workers in the field.

Certain disagreements between the results obtained by Cohen and those obtained by Braunstein are discussed by
Cohen (1942). The $Q_{\text{(transaminase)}}$ values are listed for the glutamate $\rightarrow$ aspartate system as 1,600 and for the glutamate $\rightarrow$ alanine system as about 300. The equilibrium is in favor of aspartate in the former case; in the latter, the equilibrium constant is about one. An aspartate $\rightarrow$ alanine system is an artifact according to Cohen (1939).

An exhaustive review of the entire field of transamination is that of Braunstein's (1947) where the extensive work of Braunstein, et al., and the work of Kritmann is adequately presented.

The work of Snell (1945), Lichstein, Gunsalus and Umbreit (1945), Stokes and Gunness (1945) and Schlenk and Fischer (1947) has elucidated the structure of the coenzyme of glutamic transaminase. The coenzyme has been identified as pyridoxal. Lichstein, et al., (1945) indicate that it is phosphorylated.

Braunstein (1947) has indicated a lack of information on transamination in bacteria. The transamination reaction was studied by Cutinelli (1945); forty-three different species of bacteria were used. Many of the bacteria were pathogens. Pyruvate and glutamate were added to the reaction mixture and the disappearance of each used as an indication of transamination. Twenty-one species were considered to
utilize the transaminase system. Twelve species were reported which did not utilize pyruvate in any reaction.

More recently Konikova, et al., (1949) have demonstrated the synthesis of aspartate from malate, pyruvate from alanine and alanine from ammonia and pyruvate. Further, aspartate, glutamate and phenylalanine were synthesized from phenylpyruvate.

In 1948 Kritzmann and Samarian isolated a "narrowly specific enzyme" from pigeon liver which catalyzed the reaction aspartate $\rightarrow$ alanine in the presence of pyruvate. This is not in agreement with Cohen (1939).

A transamination observed in the Krebs-Henseleit urea cycle was studied by Krebs, Eggleston and Hems (1947) and Cohen and Hayana (1947). Glutamate and A.D.P. were both necessary in the operation of the cycle. Glutamate was shown to introduce $-\text{NH}_2$ at the citrulline-arginine step. Fahrlander, et al., (1948) demonstrated a suppression of the formation of arginine by malonate probably through the depression of the synthesis of $\alpha$-ketoglutarate.

Amidation has recently been studied by Elliott (1948), Speck (1949) and others. Glutamate, ammonium ion, A.T.P. and Mg$^{++}$ are required in the system. Oxygen and D.P.N. stimulate the synthesis and fluoride is inhibitory. The role and value of amidation in the economy of the cell are unknown. Amides may be involved in certain protein structures.
Recently Wood and Gunsalus (1950) reported partially purified transaminase systems specific for substrates other than pyruvate, oxalacetate and \( \alpha \)-ketoglutarate. Proof of the presence of many different transaminases could account for the formation of the different amino acids found in the cell.
APPARATUS

Filling of the numerous tubes required for the amino acid assays and titration of the samples was simplified by the use of a dual purpose titrimeter-dispenser.

Construction of Titrimeter-Dispenser

The apparatus comprises three separate units: (1) a device for measuring time; (2) a pressure regulator to maintain a constant flow of acid or base, and (3) two half-cells connected through a galvanometer to indicate the end point of titration.

The timing unit was constructed by mounting a 10-inch aluminum disk on the turntable of an electric record player (A) with brass escutcheon pins as rivets. A 1/4 inch diameter silver button (B) was soft soldered to the end of a 1/4 inch by 2 1/2 inch phosphorbronze strip. The silver acted as the contact point to the disk through a perforated mask of stencil paper which had twelve equally spaced perforations 3/4 inch in diameter. The mask was held in place by a lucite disk 3/16 inch thick and 6 1/2 inches in diameter and a brass knob which was pressed on to the center post of the turntable and held by friction. The twelve contacts per

\(^{1}\)Letters (A), (B), (a), (b) etc. refer to schematic wiring diagram, Figure 1.
Fig. 1. Schematic wiring diagram of titrimeter-dispenser. Refer to text for description.
revolution of the disk were recorded on a Veedor-Root magnetic counter (C). Thus, the end point of any titration was indicated by the total number of counts.

A single-pole, single-throw, two micro ampere Potter and Brumfield relay (D) was placed in the 110 volt circuit to activate the counter. The relay operated from a 45 volt "B" supply (G) and was activated by the contact point on the disk. The low amperage drain of the relay was necessary to cut arcing on the disk to a minimum.

Air line pressure was used to maintain a flow of base or acid into the assay tubes. Pressure was roughly controlled by a "Meco" dual gauge gas reducing valve and pressure regulator. The air was passed through sulfuric acid and 40 per cent sodium hydroxide to remove water and carbon dioxide. It was then passed through a column of "Ascarite" and a cotton plug filter before entering the reservoir used for base. A micro regulator was placed between the filter and the reservoir. This was constructed of glass in the form of a U tube; the inside diameter of the one arm was three times that of the other. The tube was half filled with mercury; a contact wire was placed in the smaller arm which was connected to a brass "T" by means of a copper tube and tygon tubing. The free end of the contact wire was passed along the bore of the tygon tubing and soldered to the copper tube. This arrangement completed a circuit from
the copper tube to the mercury in the larger arm. The vertical arm of the brass "T" was fitted with a carburetor check valve in such a way that the needle valve could drop out. The needle was held in place by an arm and spring. The arm was pulled downward by a solenoid when the rising mercury column made contact with a wire held at the desired level in the larger arm. The solenoid operated on a 3-volt battery. The orifice of the check valve was filled with soft solder through which a hole was drilled with a No. 60 drill to bleed off the air more slowly and prevent rapid and large changes in pressure in the system.

The remaining horizontal opening of the "T" was fitted with a second "T". One opening received the treated air, and the other opening supplied the reservoir. With such a valve, a pressure of ±1 mm of mercury could be maintained, and the pressure easily adjusted to the needs of the experimenter by changing the position of the contact wire in the open arm of the manometer.

The base used was approximately 0.02 N sodium hydroxide and was kept in a 20-liter pyrex bottle. The stopper was wired in place. Base was fed under pressure from the bottle through an all glass system to a solenoid valve (E) Guardian Electric No. 14, 110 volt AC. This was rebuilt in such a way that the plunger drew down a brass bar (a) against spring compression when the coil was activated. When not
activated, the brass bar (a) pressed against a second brass bar (b) so that a gum rubber tube passed between the two was pinched off. In this way the flow of base or acid could be regulated. A foot switch (H) was used to operate the solenoid valve and the counter, both being activated simultaneously. Thus the time of flow was recorded by the counter. The base fed into a glass capillary with a side exit orifice at about 45° to the horizontal axis of the capillary; the entering base aided in stirring the contents of the tubes. The capillary formed the center of the quinhydrone electrode.

Calomel and quinhydrone half-cells were used. The latter was made by wrapping a No. 26 B and S gauge platinum wire around the flared end of a 3/8 inch O.D. glass "T" tube which served as the entrance for base and carbon dioxide-free air. This arm was 9 1/2 inches long and reached to the bottom of the assay tubes. Bubbles of carbon dioxide-free air under pressure stirred the contents of the assay tubes.

The electrodes were connected by a bridge of saturated potassium chloride solution which fed dropwise slowly into the assay tube. This procedure maintained a clean surface at the open end of the bridge. It was found convenient to maintain a reservoir of saturated potassium chloride solution in a dropping funnel which fed into the calomel half-cell to replace solution passing out through the bridge.
The cells were connected to a Leeds and Northrup portable pointer type galvanometer, 1 μampere per scale division. The galvanometer was adjusted to read zero at a pH of 7.1. Assay tubes were titrated to this end point.

To titrate an assay tube, four drops of a saturated solution of quinhydrone were placed in the tube. This was then placed in such a way that the platinum electrode, stirring and base feed tube and salt bridge were immersed in the contents. The motor was started and the foot switch engaged. Base was fed into the assay tube until the galvanometer read zero. Passage through the zero point was rather rapid, and care had to be exercised not to overrun the end point.

The half-cells and counter are not required in a dispensing mechanism; otherwise the unit is the same as described above. Two changes were essential; the mask for the disk was changed to one having a sector removed so that contact was maintained through one-half of the revolution of the turntable. The counter was removed from its circuit and the magnetic valve placed in by means of a double-pole, double-throw switch (F).

The medium to be dispensed was placed in a suitable glass bottle. Pressure was maintained in the bottle from the line and the outlet connected to a gum rubber tube (I).

---

1See also Henderson, et al, 1948, J. Biol. Chem. 172, p. 31, for description of a similar electrometric device.
at the magnetic valve (E). Two-way glass stopcocks were used to shift pressure from one bottle to another or to change the liquid fed to the magnetic valve. A length of pressure tubing fitted with a glass tip served to dispense the medium into the assay tubes. The amount dispensed could be varied by changing the mask, the speed of the turntable or the orifice of the dispensing tip.

Results Obtained

Standard curves for aspartate and glutamate are shown in Figure 2. The activity of the assay organism and variation among batches of media will play a part in determining the shape of the curve. Data relating to the titration of 200 assay tubes are presented in Table 1. The rapidity of the titrations and narrow range of pH at the end point are of interest.

The weight of base delivered in grams as plotted against number of counts is shown in Figure 3. No attempt was made to calibrate the orifice to deliver a unit weight per count since such a ratio depends on the speed of the disk and the pressure of the system. Speed and pressure may be adjusted to need as long as they remain constant for any series. This is true since each series will be interpreted from its own standard curve.
Figure 2. Typical Standard Curves as Determined from Counts on the Titrimeter.
Table 1
Rate of Titration of Assay Tubes with Titrimeter-Dispenser

<table>
<thead>
<tr>
<th>No. of tubes</th>
<th>Av. count per tube</th>
<th>Total time$^1$ (min.)</th>
<th>Sec. per tube</th>
<th>Final pH (Av. of 20 tubes)</th>
<th>Final pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>104.5</td>
<td>120</td>
<td>36</td>
<td>7.09</td>
<td>7.04-7.12</td>
</tr>
</tbody>
</table>

$^1$Time included that for removal of tube caps and addition of quinhydrone.
Figure 3. Weight of Standard Base Determined by the Titrimeter-Dispenser Plotted against Time of Delivery (Counts).
METHODS

Preparation of Cells and Juices

Aerobacter aerogenes was grown in twelve-liter pyrex flasks with aeration. A liquid medium as described by Silverman and Werkman (1941) was used for growth of the organism. A one-inch aerating stone was sterilized separately and placed aseptically in the flask. Filtered pressure line air was used. Twelve-hour cells were generally used although cell ages varying from 8 to 24 hours were investigated.

Cells were harvested by means of a Sharples air-driven centrifuge, suspended in 0.05 N phosphate buffer at pH 7.0 and aerated at 37°C for 30 minutes. The cells were then washed twice with sterile saline and lyophilized or used for the production of juices. Cells treated in this manner had a low endogenous respiration.

Lyophilization was carried out by freezing in dry ice-acetone mixture and removal of water under vacuum. The lyophilized cells were stored at -20°C.

Juices were made by the glass grinding method of Wiggert, et al., (1940), or by treating the fresh cells in a Rathson sonic vibrator, Model S-102, at nine kilocycles. The particular treatment used in the preparation of juices will be referred to later.
The supernatant juice was removed from glass and/or cell debris by centrifugation at 10,000 R.P.M. in an International refrigerated centrifuge, Model PR-1. The juices were sharp frozen in flasks and stored at -20°C until needed. At the time of use the flasks were placed in ice cold water until the juice thawed.

Juices lost little activity over the two or three week period of storage and use. Cells remained active for a year or more although they lost some ability to assimilate ammonium ion after six to eight months. New cells were grown every two to three weeks to meet the need for juices.

Chemical Methods

Determination of residual ammonia

Ammonia was determined by a modified micro-Kjeldahl method as outlined by Pregl-Grant (1945). The samples were placed in a modified Parnas apparatus, 10 ml of 40 per cent sodium hydroxide were added and the sample steam distilled for seven minutes. The ammonia was trapped in two per cent boric acid and titrated with 0.005 N HCl.

Determination of total $\alpha$-keto acids

Residual pyruvate, oxalacetate and $\alpha$-ketoglutarate were determined manometrically by decarboxylation with $\text{CeSO}_4$ in
4 N H$_2$SO$_4$. The reactions were followed manometrically on the Barcroft-Warburg bath. One milliliter of sample containing less than 200 µl of pyruvate or other α-keto acid was added to 0.2 ml of concentrated H$_2$SO$_4$ in the Warburg flask. Eight-tenths milliliter of a solution of Ce(SO$_4$)$_2$ was added from the side arm after equilibration. The volume change was corrected for a blank and results recorded as µl Ce(SO$_4$)$_2$-CO$_2$. The method was adapted from that of Krebs and Johnson (1937) as used for pyruvate assay. Pyruvate, α-ketoglutarate and oxalacetate are decarboxylated quantitatively by cold ceric sulfate.

**Determination of alanine**

Initially alanine was determined chemically as outlined by Block and Bolling (1940). However, this procedure was found to be nonspecific and was replaced by microbiological assay.

**Synthesis of Compounds**

**Sodium bicarbonate containing C$^{14}$**

Sodium bicarbonate containing C$^{14}$ was synthesized from BaC$^{14}$O$_3$. The reaction was carried out in a two-armed vessel similar to that described by Sprinson and Rittenberg (1949). The BaC$^{14}$O$_3$ was weighed on a semi-micro balance and placed in
one arm. One to two milliliters of carbon dioxide-free water were added and the mixture frozen in dry ice and acetone. A small vial containing an excess of perchloric acid was placed on the frozen surface. The other arm contained sodium hydroxide equivalent to the theoretical yield of carbon dioxide. The two arms were placed in the female standard taper joints and a vacuum drawn on the system. Perchloric acid was then slowly mixed with the melting barium carbonate solution and evolution of gas controlled by cooling or heating. After completion of the reaction, the barium carbonate-acid solution was heated in boiling water for 15 minutes. The apparatus was allowed to stand at room temperature overnight to complete adsorption. The solution was colorless to phenolphthalein and assayed 98 per cent when treated with sulfuric acid and the evolved carbon dioxide measured manometrically.

**Pyruvate containing C¹⁴ in the carbonyl group**

Pyruvate was synthesized with labeled carbon in the carbonyl position using the method of Anker (1943b). Potassium acetate containing C¹⁴ in the carboxyl group was used as the starting compound. The over-all yield was 38 per cent based on the weight of potassium acetate used. The compound was stored as the pyruvamide and converted to pyruvic acid when needed. The pyruvamide was weighed in a pyrex test tube to which was added the equivalent amount of 0.1 N HCl. The
test tube was placed in a steam bath for two hours. Conversion was 98 to 100 per cent as determined by the method of Krebs and Johnson (1937).

Reagents for removal of glycine and recovery of alanine

Para-hydroxyazobenzene-p'-sulfonic acid and 5-nitronaphthalene-1-sulfonic acid were synthesized and used as outlined by Carter (1949). In experiments involving the recovery of specific amino acids, the latter compound was used to remove glycine from the reaction mixture; the former compound was used for the recovery of alanine. It was necessary to remove glycine first since glycine interferes in the recovery of alanine.

Microbiological Methods

Determination of alanine, glutamate and aspartate

The concentration of amino acids was determined by the assay method of Sauberlich and Baumann (1949). Leuconostoc citrovorum 8081 was used to determine glutamate and alanine quantitatively. Growth of L. citrovorum was erratic on the stock medium recommended by Sauberlich and Baumann. The addition of 100 ml of tomato juice per liter of medium resulted in more consistent growth and a more nearly typical standard curve. Aspartate was determined by microbiological assay using Leuconostoc mesenteroides P-60.
The amino acids, purines and pyrimidines required for the assay media were powdered in an agate mortar and stored in a desiccator. Vitamins were stored in solution under toluene. There was an occasional loss in the response of the assay organisms to low concentrations of amino acids. This was traced to the vitamin solution; a deactivation of riboflavin and pantothenic acid appeared to be responsible. The defect was corrected by making a fresh vitamin solution every week.

Glucose and salts were weighed out as needed. The complete assay medium could be stored for two weeks under toluene at 4°C without a change in response on the part of the assay organism as determined from the standard curve.

Assay tubes for the determination of an unknown and for the standard curves were run in duplicate. Data presented refer to the averages of the duplicates. Eighteen by 150 mm pyrex tubes containing a final volume of four milliliters of medium were used throughout. Aluminum caps were used on the tubes to facilitate handling.

Recovery experiments

A comparison was made between a chemical assay method for alanine and the microbiological assay method of Sauberlich and Baumann (1949) to determine the relative quantitative recoveries obtained by each method.
The data from recovery experiments on alanine are found in Table 2. Alanine alone was investigated since the methods for the chemical determination of micro amounts of glutamate and aspartate were found too time-consuming to render them of value when a large number of samples had to be assayed.

A cell hydrolyzate was used as the basal medium. The higher recoveries obtained by the method of Block and Bolling (1940) may be due to an aldehyde in the cell hydrolyzate since aldehydes interfere in this method. It was later shown that a volatile compound was present which gave a precipitate with 2,4-dinitrophenylhydrazine. The chemical method for the determination of alanine was discarded in favor of microbiological assay which was found to be more specific and less time consuming.

Determination of the Isotopes C\textsuperscript{14} and N\textsuperscript{15}

The presence of excess C\textsuperscript{14} was determined by the use of a "Q" gas flow counter Nuclear Model 2D and a Nuclear scaling unit. Chromatographed strips were surveyed with a Geiger-Mueller tube fitted with a brass double plate two millimeters below the window of the tube. A one-inch diameter circle of the paper strip was exposed to the mica window as the strip passed between the plates. The spots on the strip showing high counts were marked and removed for further study, e.g., microbiological assay and quantitative counting.
Table 2

Recovery of Alanine from Fortified Cell Hydrolyzates by Chemical and Microbiological Methods

<table>
<thead>
<tr>
<th>Alanine per ml cell hydrolyzate (mg)</th>
<th>Alanine added (mg)</th>
<th>Alanine recovered&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Alanine recovered&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>%</td>
<td>T</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10.8</td>
<td>108.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>26.0</td>
<td>104.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>47.0</td>
<td>104.4</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>14.0</td>
<td>107.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>30.0</td>
<td>107.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>47.0</td>
<td>97.9</td>
</tr>
</tbody>
</table>

<sup>1</sup>Chemical method of Block and Bolling.

<sup>2</sup>Microbiological assay of Sauberlich and Baumann.
Seventy millimeter kodak blue sensitive film was also used to locate the position of spots having excess C\textsuperscript{14}. The chromatographed strips were dried and secured to a drawing board by scotch tape. Film strips were placed over the full length of the chromatograms and secured in like manner. Time of exposure varied from one to seven days. The film strips were developed and matched with the respective chromatograms after the latter had been developed with ninhydrin to identify amino acid locations. The use of film for the location of excess C\textsuperscript{14} was not too successful due to the low activities obtained in the experiments.

A separation of the amino acids was necessary to determine the presence of excess C\textsuperscript{14}. The separations were accomplished by the use of chromatographic technics. An aliquot of the deproteinated reaction mixture was drawn into a one milliliter capillary pipette by reduced pressure and was then bled on to a sheet of Whatman No. 1 filter paper wrapped about the cylinder of a kymograph. The steel cylinder was replaced by a wooden cylinder three inches in diameter. A narrow band was obtained by drying the paper in a stream of air and the heat from an infra-red lamp as the drum rotated.

The sheet was cut into strips 18 inches long and two inches wide. The upper end of the strip was threaded on a nichrome wire B and S gauge No. 24. The lower end of the strip was threaded on a yoke of the same wire. The strips
were placed in 24-inch glass cylinders so that the lower one-
half inch hung in 80 per cent phenol.

The cylinders were placed in a water bath at 34°C. Transfer of the mobile phase was completed in 36 to 48 hours. Phenol has been suggested by Consden, et al, (1944) for amino acid separation on paper strip chromatograms. Under these conditions, the \( R_f \) values of alanine, aspartate and glutamate differ enough to give good separation.

The mass spectrometer was used to determine the presence of excess \( \text{N}^{15} \). The procedure used to effect a separation of the \( \text{N}^{15} \) containing amino acids is outlined in Figure 4. The reaction mixture was fortified with one-fourth mM of alanine and one-half mM each of aspartate and glutamate as indicated by (A) in the separation scheme. The addition of carrier was necessary to yield a sufficient sample of ammonium sulfate of the components (1), (2) and (3) to be able to determine the per cent excess \( \text{N}^{15} \) on the mass spectrometer. The ammonium chloride used in the reaction was obtained by conversion of ammonium nitrate containing 53.3 per cent excess \( \text{N}^{15} \) in the ammonium nitrogen.

Sodium hypobromite was used to oxidize the ammonium sulfate to gaseous nitrogen. The evolved gas was collected in evacuated sample bulbs containing adsorbents for water and carbon dioxide. Mass spectrometric analyses were made at five millimeters pressure.
(A) Reaction mixture fortified with alanine, aspartate and glutamate

Amberlite IR4B

- Basics and neutrals pass through
- Amberlite IR4B (Basics adsorbed)
- Treat neutrals with 5-nitronaphthalene-1-sulfonic acid
  (Glycine removed)
- Alanine in supernatant
  (Treat with p-hydroxy-azobenzene-p'-sulfonic acid)
- Filter

Filtrate (discard) Precipitate
(Alanine recovered by barium acetate treatment)
- Alanine in supernatant
  (1)
- Kjeldahl digestion to recover N\textsubscript{18}
- Kjeldahl digestion to recover N\textsubscript{18}

Glutamate and aspartate adsorbed
(Elute with 9 per cent HCl)
- Alanine and aspartate in eluate
  (pH adjusted to 2.4; autoclave 4 hours at 125\textdegree C; glutamate converted to pyrrolidone carboxylic acid)
- Extract with ethyl acetate
- Aspartate not extracted
  (2)
- Fyrrolidone into NaHCO\textsubscript{3} soln.
  (3)

Figure 4. Separation Procedure for the Recovery of Alanine, Aspartate and Glutamate.
Special methods applicable to a given experiment are further described in the experimental section to which they apply.

Results presented in all tables are corrected for appropriate blanks. Data obtained from control experiments have been given in some instances where a comparison may be desirable.
EXPERIMENTAL

Ammoniation

Ammoniated products released during growth

The problem of bacterial ammoniation was approached initially by attempting to determine whether alanine, glutamate and aspartate are to be found free in the growth liquors from a culture of *Aerobacter aerogenes*.

Products from glucose. The results of a series of growth experiments with glucose as a source of carbohydrate are set forth in Table 3. The flasks were prepared as outlined. *A. aerogenes*, I.S.C. No. 199, was grown for 16 hours in glucose broth, centrifuged, washed twice with sterile saline and resuspended. One drop of the suspension was used as an inoculum; the density of the inoculated control gave a reading of 2-4 on a Klett-Summerson photoelectric colorimeter with a No. 66 filter.

Turbidities were read to determine the amount of growth after 16 hours incubation. The cells were removed by the addition of sodium tungstate-sulfuric acid solution as outlined by Schurr, et al., (1950) followed by centrifugation. Assays to determine alanine, glutamate and aspartate, ammonium ion assimilation and residual α-keto acids were then conducted on the supernatant liquid.
Table 3

Synthesis of Amino Acids by *A. aerogenes* Growing in the Presence of Glucose and Glucose Plus Inhibitors

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative growth</th>
<th>μl CO₂</th>
<th>μM NH₃ uptake</th>
<th>Amino acid concentration in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A¹</td>
<td>An</td>
<td>A An</td>
<td>A An</td>
</tr>
<tr>
<td>None</td>
<td>95 23</td>
<td>57 3</td>
<td>44.5 38.6</td>
<td>10 22</td>
</tr>
<tr>
<td>0.003 M NaAsO₂</td>
<td>94 22</td>
<td>92 45</td>
<td>49.6 20.0</td>
<td>34 20</td>
</tr>
<tr>
<td>0.087 M NaF</td>
<td>9 35</td>
<td>16 35</td>
<td>27.3 31.2</td>
<td>8 20</td>
</tr>
<tr>
<td>0.087 M methadon-HCl</td>
<td>1 57</td>
<td>32 27</td>
<td>4.0 45.0</td>
<td>23 50</td>
</tr>
</tbody>
</table>

¹In each case "A" and "An" refer to aerobic and anaerobic conditions respectively.

Each flask contained 0.125% glucose, 0.15 M phosphate buffer, 0.02 M NH₄Cl and water to make total volume to 8.0 ml. Final pH = 6.8; time = 16 hours. Further additions are noted in the first column.

In all tables concentrations given are final, and a difference of two to three units in relative growth is considered nonsignificant.
Several observations presented in Table 3 are of interest. Glutamate accumulates in the medium to a greater extent than does aspartate or alanine; it is also formed in higher concentration aerobically than anaerobically. Taylor (1947) found that feeding glutamate does not result in its accumulation within the cells of *A. aerogenes*. The accumulated glutamate in the medium may be an excretion product. However, the precursor of glutamate, i.e., α-ketoglutarate since it is a source of energy can not easily be considered a waste product in actively growing cells. Later work will show the dependence of the synthesis of glutamate on enzymes which are easily removed from the cell. The enzymes are probably located at or near the surface of the cell; this relationship may explain the high rate of "excretion" of glutamate. α-Ketoglutarate is a product of aerobic dissimilation of carbohydrate; its greater production aerobically is reflected in a higher concentration of glutamate. It should be noted that the accumulation of alanine is favored by anaerobic conditions.

Sodium arsenite was used to retard the oxidative decarboxylation of α-ketoglutarate. Methadon hydrochloride has not been studied extensively but has been shown to retard pyruvate oxidation by an unknown mechanism. Sodium fluoride was used to inhibit phosphorylation in an attempt to determine its importance in ammoniation.
Sodium fluoride and methadon hydrochloride inhibit growth of \textit{A. aerogenes}; there are accompanying changes in the glutamate and aspartate concentration in the supernatant liquid. The effect of sodium fluoride suggests that the aerobic production of aspartate and glutamate from glucose is dependent on phosphorylation. Without phosphorylation there would be a lack of necessary precursors for the synthesis of alanine, aspartate and glutamate.

The difference in the action of methadon hydrochloride under aerobic and anaerobic conditions suggests there may be at least two different mechanisms associated with ammoniation. It is possible that $\beta$-carboxylation but not transamination is inhibited by methadon hydrochloride under anaerobic conditions since the inhibitor depresses the formation of aspartate but does not depress the synthesis of alanine as compared with the respective aerobic experiment. $\beta$-carboxylation of pyruvate yields oxalacetate necessary for the synthesis of aspartate. Under aerobic conditions aspartate does accumulate indicating that another pathway for ammoniation or the synthesis of oxalacetate is available to the cell. The change in action of methadon hydrochloride when \textit{A. aerogenes} was grown in its presence anaerobically resulted in the highest ammonium ion assimilation, the maximum amount of growth and the highest yield of alanine and aspartate. Since the anaerobic yield of $\alpha$-ketoglutarate is low, the results
may indicate carbon dioxide fixation and ammoniation of the resulting oxaloacetate to form aspartate or reductive ammoniation of pyruvate to form alanine. Ammonium ion could be assimilated by either mechanism.

In general the assimilation of ammonia is correlated with an increased concentration of amino acids in the medium. However, at no time did the decrease in ammonia quantitatively account for the increase in amino acids. The rather large amounts of ammonia released into the medium by resting cells and juices prevented an accounting. The accumulation of α-keto acids from glucose in the presence of inhibitors of oxidation is indicated by the volume of carbon dioxide released on the addition of ceric sulfate in assaying for residual α-keto acids. The use of either sodium arsenite or methadon hydrochloride should result in the accumulation of α-keto acids. Inhibition of phosphorylation would inhibit earlier reactions of glycolysis and lower the synthesis of α-keto acids. The results are in agreement with this conclusion.

Relative growth is correlated with the ammonium ion assimilation and with the production of glutamate in the absence of inhibitors.

**Products from α-keto acids.** The production of α-keto acids from glucose and the synthesis of alanine, aspartate and glutamate by growing cells suggested a series of experi-
ments in which the three \( \alpha \)-keto acids—pyruvate, oxalacetate and \( \alpha \)-ketoglutarate—acted as a source of carbon. These three acids are the respective precursors of alanine, aspartate and glutamate. The results are found in Table 4.

The assimilation of ammonium ion again correlates fairly well with growth. There is a difference in the response of the organism to pyruvate and \( \alpha \)-ketoglutarate in the presence of ammonium ion when conditions are changed from aerobic to anaerobic. It would appear that \( \alpha \)-ketoglutarate is more efficient in the initiation of growth aerobically than anaerobically, whereas this relationship is reversed in the case of pyruvate.

Pyruvate stimulates growth more than does \( \alpha \)-ketoglutarate. This might be a further indication of the importance of the fixation of carbon dioxide and ammoniation to yield aspartate and thus provide for the assimilation of ammonium ion.

Active decarboxylation by the organism precluded the use of oxalacetate in growth experiments.

To determine whether sodium arsenite or sodium fluoride would suppress ammoniation of \( \alpha \)-keto acids, the growth of \( A. \ aerogenes \) was studied in the presence of these inhibitors (Table 5). Pyruvate and \( \alpha \)-ketoglutarate were used separately and in combination as sources of carbon. The synthesis of glutamate is dependent upon the presence of \( \alpha \)-ketoglutarate; it is not inhibited by the concentrations of sodium fluoride.
Table 4

Growth of *A. aerogenes* in the Presence of α-Keto Acids

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative growth</th>
<th>( \mu \text{M NH}_4^+ ) uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A(^1)</td>
<td>An</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Pyruvate + α-ketoglutarate</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>( \text{NH}_4\text{Cl} )</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>( \text{NH}_4\text{Cl} + ) α-ketoglutarate</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>Pyruvate + ( \text{NH}_4\text{Cl} )</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>Pyruvate + ( \text{NH}_4\text{Cl} ) + α-ketoglutarate</td>
<td>42</td>
<td>64</td>
</tr>
</tbody>
</table>

\(^1\)In each case "A" and "An" refer to aerobic and anaerobic conditions respectively.

Each flask contained 0.15 M phosphate buffer, 0.033 M NaHCO\(_3\) and in addition 0.02 M NH\(_4\)Cl, 0.02 M pyruvate, 0.01 M α-ketoglutarate as noted and water to make total volume to 8.0 ml. Final pH = 6.8; time = 16 hours.
Table 5
Aerobic Growth of A. aerogenes in the Presence of α-keto Acids and α-keto Acids Plus Inhibitors

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative growth</th>
<th>NH₄⁺ μM uptake</th>
<th>Amino acid concentration in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaF</td>
<td>NaAsO₄</td>
<td>NaF</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>3</td>
<td>2</td>
<td>--</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>8</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>Pyruvate + α-ketoglutarate</td>
<td>13</td>
<td>6</td>
<td>--</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>4</td>
<td>0</td>
<td>-42.0</td>
</tr>
<tr>
<td>NH₄Cl + α-ketoglutarate</td>
<td>6</td>
<td>1</td>
<td>-41.5</td>
</tr>
<tr>
<td>NH₄Cl + pyruvate</td>
<td>24</td>
<td>3</td>
<td>-45.5</td>
</tr>
<tr>
<td>NH₄Cl + pyruvate + α-ketoglutarate</td>
<td>64</td>
<td>18</td>
<td>-47.1</td>
</tr>
<tr>
<td>Controls</td>
<td>125</td>
<td>102</td>
<td>-59.0</td>
</tr>
</tbody>
</table>

Each flask contained 0.15 M phosphate buffer and 0.033 M NaHCO₃ and in addition 0.02 M NH₄Cl, 0.02 M pyruvate, 0.01 M α-ketoglutarate, 0.0015 M NaAsO₄, 0.087 M NaF as noted and water to make total volume to 8.0 ml. Final pH = 6.8; time = 16 hours. Controls contain NH₄Cl, pyruvate and α-ketoglutarate but no inhibitor.
or sodium arsenite used. Smaller amounts of glutamate are found in the supernatant liquid when \( \alpha \)-ketoglutarate is used as a substrate than are found when glucose is the source of carbon (See Table 3).

The concentrations of sodium fluoride and sodium arsenite used partially inhibited the synthesis of alanine. Alanine formation appears to be independent of the presence of added pyruvate. This result suggests that added pyruvate is not readily available for ammoniation or transamination; a phosphorylated compound is important in the formation of alanine as shown by the sodium fluoride inhibition of alanine synthesis. Sodium fluoride may have an indirect influence on the production of a required precursor for the synthesis of alanine.

The synthesis of aspartate appears to differ from that of alanine in that it is dependent upon added pyruvate. It is very worthy of note that inhibited cells, where growth is held to one-half to one-hundredth of the control, synthesize large amounts of aspartate when pyruvate is present. \( \alpha \)-Ketoglutarate does not substitute for pyruvate under these conditions. The relationship of pyruvate to aspartate suggests a mechanism involving the Wood-Werkman reaction followed by or coupled with ammoniation or transamination. Further, the increased formation of aspartate by growing cells in the presence of sodium fluoride indicates that
phosphorylation is not too important to the intact cell for the synthesis of aspartate if pyruvate is supplied. Phosphorylation is important when glucose is supplied as was shown by a depression of the synthesis of aspartate in the presence of sodium fluoride.

Good growth was observed in the presence of a more nearly complete system containing ammonium chloride, pyruvate and α-ketoglutarate. The depression of aspartate synthesis by sodium fluoride in the presence of α-ketoglutarate under aerobic conditions is not explained by the available data.

Ajl and Werkman (1949) have reinvestigated the findings of Lwoff and Monod (1946) and have shown that oxalacetate and aspartate substitute for carbon dioxide in the initiation of growth of _Escherichia coli_. In view of the observed relationship between pyruvate and aspartate, one might interpret the observations of Ajl and Werkman as an aspect of the Wood-Werkman reaction perhaps closely allied to amino acid and protein synthesis. The oxalacetate formed by carbon dioxide fixation would be available for the synthesis of aspartate; growth could occur on the assimilation of ammonium ion. Virtanen (1939) has suggested the oxalacetate-aspartate system as a means of assimilation of ammonium ion. This would seem highly probable from the results obtained.
Ammoniated products from resting cells

Resting cells and juices were used to determine whether the synthesis of amino acids observed under conditions of retarded growth could be enhanced. A preliminary study of the effect of inhibitors on resting cells is reported in Table 6.

The inhibitory action of arsenite and methadon hydrochloride on oxidation of pyruvate is indicated by the increased R.Q. values. The concentrations of inhibitors used were considered sufficient to inhibit in part the oxidative dissimilation of the α-keto acids under observation; partial inhibition would leave more substrate available for ammoniation if the necessary enzyme were present.

An increase in the concentration of ammonium ion depresses the oxygen uptake until levels of 0.05 M are reached. At this level a decided stimulation in oxygen uptake is noted. Although the extent of stimulation of oxygen uptake at higher concentrations of ammonium ion varied with batches of cells, it was found present in all cell preparations examined. Its cause was not investigated. Similar stimulations have been noted by Lundsgaard (1943).

A time study on the disappearance of α-keto acids, ammonium ion and the appearance of amino acids indicated one and one-half hours as the most desirable length of time for the reaction. Although the time was not optimum for the
Table 6

Effect of Ammonium Ion Concentration and Inhibitors on Gas Exchange

<table>
<thead>
<tr>
<th>Additions</th>
<th>$\mu l$ O$_2$ uptake</th>
<th>$\mu l$ CO$_2$ evolution</th>
<th>$\mu l$ GS$_4$-CO$_2$</th>
<th>R.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03 M pyruvate) + 0.02 M NH$_4$Cl</td>
<td>549</td>
<td>-</td>
<td>545</td>
<td>-</td>
</tr>
<tr>
<td>0.033 M NaHCO$_3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+ 0.015 M NH$_4$Cl</td>
<td>546</td>
<td>-</td>
<td>296</td>
</tr>
<tr>
<td>-</td>
<td>+ 0.01 M NH$_4$Cl</td>
<td>590</td>
<td>-</td>
<td>435</td>
</tr>
<tr>
<td>-</td>
<td>+ 0.005 M NH$_4$Cl</td>
<td>604</td>
<td>-</td>
<td>635</td>
</tr>
<tr>
<td>-</td>
<td>+ 0.05 M NH$_4$Cl</td>
<td>1120</td>
<td>-</td>
<td>630</td>
</tr>
<tr>
<td>None</td>
<td>175</td>
<td>236</td>
<td>-</td>
<td>1.35</td>
</tr>
<tr>
<td>0.015 M pyruvate) + 0.02 M NH$_4$Cl</td>
<td>455</td>
<td>813</td>
<td>-</td>
<td>1.78</td>
</tr>
<tr>
<td>0.033 M NaHCO$_3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+ 0.015 M NH$_4$Cl</td>
<td>452</td>
<td>795</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+ (0.015 M NH$_4$Cl (0.0015 M NaAsO$_3$)</td>
<td>67</td>
<td>151</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+ (0.015 M NH$_4$Cl (0.087 M methadon·HCl)</td>
<td>35</td>
<td>310</td>
<td>-</td>
</tr>
</tbody>
</table>

Each Warburg flask contained 0.15 M phosphate buffer, 0.01 M α-ketoglutarate, and additions as noted, with water to make total volume to 2.3 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask.
Residual of α-keto acids remained. It was believed that data
and the oxidation of the substrate was at such a rate that a
since at that pH almost syntheses of α-keto acids was observed,
and a pH of 6.6 was chosen for the restant cell experiment.

Half-hour results are given in Figure 5.

Table 5. The reaction was allowed to proceed for one and one-
orthodox, pyruvate, a-keto glutarate system was detected.

The Meruque Islands each contributed an ammonium
amine, aspartate and glutamate by restant cells of A. t.

A study was made of the effect of pH on the syntheses of α-
with respect to amino acids, deacetylases and deaminases.

Br communities (1967) and others have suggested the importance of
that ammonium is considered a reversible reaction.

Factors which affect deamination should influence ammonium-

ammonium ion into the medium than did cells from 8-hour out-
several weeks. Cells from 24-hour cultures released more
the case with cells which have been in cold stored for
with periods of time in excess of one hour and is more often
not pronounced. An increase in ammonium ion has been noted
alteration by which to follow ammonium as has been pointed
the disappearance of ammonium ion is not a desirable
remained a desirable residual of α-keto acids.
Glutamate were oxygenated in moderate concentrations and there
syntheses of all three amino acids, alanine, aspartate, and

- 69 -
Figure 5. Effect of pH on the Synthesis of Amino Acids, Oxygen Consumption and Removal of α-Keto Acids by *A. aerogenes.*
There is a  

Table 7  

dependence on the presence of a-ketoacids, the data relative toable of a-ketoacids and the synthetase  

ected or the a-keto acid being studied. Aromatic  
carried out in buffer. Most of the reaction  

ferences were determined.  

the synthetase systems which had been shown to exist in  

differences in the substrates, the reductases of  

gain information on the mechanism of ammonium ion assimilation  

are necessary to separate these systems from each other and from an ammonium system in order to  

asparate and the synthetase  

Cohen (1932) has suggested at least two transamination sy.

reductases of resting cells for ammoniation  

investigation.  

metation of amino acids could not be directly related to this  

observed that the difference of a-keto acids and the for-  

need as evidence for transamination. However, it was later  

of amino acids which be valuable since such data have been  

relating the disappearance of a-keto acids and the synthetase  

- 61 -
### Table 7

Aerobic Synthesis of Amino Acids by Resting Cells of *A. aerogenes*

<table>
<thead>
<tr>
<th>Additions</th>
<th>Alanine (μg per ml)</th>
<th>Aspartate (μg per ml)</th>
<th>Glutamate (μg per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 M buffer + NH₄Cl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>0.0</td>
<td>248</td>
<td>10.3</td>
</tr>
<tr>
<td>-</td>
<td>8.2</td>
<td>341</td>
<td>25.7</td>
</tr>
<tr>
<td>+ (NH₄Cl + pyruvate)</td>
<td>59.7</td>
<td>0</td>
<td>28.8</td>
</tr>
<tr>
<td>+ (NH₄Cl + pyruvate + α-ketoglutarate)</td>
<td>46.4</td>
<td>372</td>
<td>0.0</td>
</tr>
<tr>
<td>0.15 M buffer + NH₄Cl</td>
<td>15.5</td>
<td>124</td>
<td>18.5</td>
</tr>
<tr>
<td>+ (NH₄Cl + pyruvate)</td>
<td>20.6</td>
<td>434</td>
<td>248</td>
</tr>
<tr>
<td>+ (NH₄Cl + pyruvate + α-ketoglutarate)</td>
<td>46.4</td>
<td>372</td>
<td>0.0</td>
</tr>
<tr>
<td>0.15 M buffer + NH₄Cl</td>
<td>15.5</td>
<td>0</td>
<td>56.6</td>
</tr>
<tr>
<td>+ (NH₄Cl + pyruvate)</td>
<td>37.0</td>
<td>0</td>
<td>123.6</td>
</tr>
<tr>
<td>+ (NH₄Cl + pyruvate + α-ketoglutarate)</td>
<td>128.7</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Each Warburg flask contained 0.02 M NH₄Cl, 0.02 M pyruvate, 0.01 M α-ketoglutarate as noted, and water to make total volume to 2.5 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask.
vente.

synthesize or a product of communication of cellular content.

that is a degradation product released in the absence of cell

In the absence of a-ketoalutanate, it is possible that

-a-ketoalutanate serves as an intermediate in the system

and of precursor to product as apparent as in the system

ketocutarate to the substrate. In no case is the relation-

glutamate is always found in the supernatant when a-

glutamate is always found in the cell.

Taylor (1947) observed that glutamic negative bacteria do not ac-

hylorotanes assessed for glutamate gave similar results.

the initial of the aspart method used. Lower registration of cell

amino acid interactivity present in low concentration to beyond

aminol and interactivity present in low concentration to beyond

amino acid interactivity present in low concentration to beyond

the larger inter-

however, that a higher concentration of cell hylorotanes was nec-

founded in the supernatant liquid. It should be pointed out,

concentration within the cell and the glutamate concentration

an inverse relationship exists between the glutamate

such upon transamination with glutamate as the amino donor.

action of pyruvate and a dependence of the synthesis of the

without a-ketoalutanate intermediates a lack of reduction apparent

seven fold increase over systems containing pyruvate but

nent liquid whenever a-ketoalutanate is added. The two to

marked increase in the amount of amine found in the super-

- 29 -
Oxalacetate appears to enhance aspartate production though the yield of aspartate from oxalacetate is lower than the yield of glutamate from α-ketoglutarate. Cohen (1942) stated that the equilibrium of the glutamate-aspartate transaminase system is in favor of aspartate. The observations in regard to the glutamate-aspartate ratio do not agree with the results of Cohen. However, Cohen was using a partially purified transaminase from muscle tissue. It is possible that the resting cells of *A. aerogenes* remove aspartate rapidly after its synthesis. Pyruvate appears to be as important as oxalacetate in the synthesis of aspartate.

The requirements of the ammoniating system were further studied in resting cells as shown in Table 8. Elliott (1948) has investigated the formation of glutamine from glutamate by cells of *Micrococcus pyogenes* var. *aureus*. He has reported the need for Mg++, A.T.P., a source of organic phosphate and cysteine in the reaction. It was reasoned that amidation and ammoniation might employ similar mechanisms. Through the omission of any one component from a complete system, its effect on the synthesis of amino acids was determined.

The observations may be summarized: (1) Absence of pyruvate stimulates production of glutamate but depresses production of aspartate and alanine. (2) The absence of α-ketoglutarate depresses the production of glutamate, al-
### Table 8
Components of the System Involving Synthesis of Alanine, Glutamate and Aspartate

<table>
<thead>
<tr>
<th>Omissions</th>
<th>Change in relative amounts of amino acids (γ per flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>-28</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>-45</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>-36</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>+41</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>-48</td>
</tr>
<tr>
<td>A.T.P.</td>
<td>+46</td>
</tr>
<tr>
<td>Glycerolphosphate</td>
<td>+20</td>
</tr>
<tr>
<td>Cysteine</td>
<td>-10</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>+91</td>
</tr>
<tr>
<td>No omissions</td>
<td></td>
</tr>
<tr>
<td>(Total in γ per flask)</td>
<td>350</td>
</tr>
</tbody>
</table>

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH₄Cl, 0.015 M pyruvate, 0.0015 M α-ketoglutarate, 0.0015 M oxalacetate, 0.045 M MgCl₂, 20 μM glycerolphosphate, 2.5 μM A.T.P., 0.025 M cysteine, 0.033 M NaHCO₃ and water to make total volume to 5.1 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask. Exceptions are noted in the first column.
anine and aspartate. The results suggest that ammoniation is via an α-ketoglutarate → glutamate mechanism followed by transamination to yield aspartate and alanine. Pyruvate and oxalacetate as amino acceptors would decrease the concentration of glutamate through active transamination.

(3) The absence of A.T.P. and Mg++ depresses the formation of aspartate but stimulates the synthesis of glutamate indicating the former is an energy requiring reaction. (4) The absence of oxalacetate stimulates the synthesis of aspartate. (5) The synthesis of alanine is less dependent on any of the metabolites used than is the synthesis of aspartate or glutamate.

A stimulation in the synthesis of glutamate and a depression in the formation of aspartate in the absence of Mg++ and A.T.P. indicated the possibility of glutamine formation and its action as a donor of the amide -NH₂. The result would be the formation of aspartate and alanine by transamidation.

Elliott (1948) showed that crystal violet and methionine sulfoxide act as inhibitors in the glutamate → glutamine system. Methionine sulfoxide and crystal violet were used to block the synthesis of glutamine and thus determine whether the system glutamine + oxalacetate → aspartate + glutamate might act as a pathway for the assimilation of ammonium ion. Results are given in Table 9. Cysteine was
Table 9
Effect of Glutamine on Synthesis of Alanine, Aspartate and Glutamate

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative amounts(^1) of amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>None</td>
<td>134</td>
</tr>
<tr>
<td>Glutamine</td>
<td>135</td>
</tr>
<tr>
<td>0.01 M methionine sulfoxide</td>
<td>150</td>
</tr>
<tr>
<td>0.01 M methionine sulfoxide + glutamine</td>
<td>139</td>
</tr>
<tr>
<td>0.0002 M crystal violet</td>
<td>16</td>
</tr>
<tr>
<td>0.0002 M crystal violet + glutamine</td>
<td>29</td>
</tr>
</tbody>
</table>

\(^1\)The amounts indicated are averages of counts as determined by the titrimeter.

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH\(_4\)Cl, 0.015 M pyruvate, 0.0015 M \(\alpha\)-ketoglutarate, 0.0015 M oxalacetate, 0.045 M MgCl\(_2\), 20 \(\mu\)M glycerolphosphate, 2.5 \(\mu\)M A.T.P., 0.033 M NaHCO\(_3\) and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask. Further additions are noted in the first column.
not added to these reaction mixtures since it reduces methionine sulfoxide to methionine.

There is no indication that transamination yields aspartate except for a slight relief of the crystal violet inhibition by glutamine. A similar investigation with cell-free juices gave comparable results although inhibition of the synthesis of amino acids was less marked in the presence of crystal violet. McCalla (1940) has shown that some dyes act at the surface of the cell. The action of these dyes might easily be lessened in the absence of a cell wall.

Effect of anaerobic conditions on ammoniation

Preliminary growth studies (See Table 3) had indicated the existence of anaerobic and aerobic ammoniating mechanisms. The question of a dual mechanism was further investigated with resting cells. Results are recorded in Table 10. The data confirm previous findings on the accumulation of more alanine and aspartate anaerobically than aerobically. The accumulation of glutamate anaerobically is slightly enhanced. The need of phosphorylation in the synthesis of alanine and aspartate is shown by a depression in the amount of these two amino acids formed in the presence of sodium fluoride; the synthesis of glutamate is only slightly affected by the same inhibitor. The depressing effect of oxalacetate on the synthesis of aspartate is to be noted again.
Table 10

Effect of Anaerobiosis on the Synthesis of Amino Acids

<table>
<thead>
<tr>
<th>Additions</th>
<th>CaSO₄-CO₂</th>
<th>Relative amounts of amino acids per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A⁴ An</td>
<td>A An</td>
</tr>
<tr>
<td>0.015 M pyruvate²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 0.087 M NaF</td>
<td>20  7</td>
<td>85  124</td>
</tr>
<tr>
<td>Complete system³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 0.087 M NaF</td>
<td>50  29</td>
<td>92  122</td>
</tr>
<tr>
<td>+ 0.0015 M NaAsO₄</td>
<td>62  37</td>
<td>81  93</td>
</tr>
<tr>
<td>+ 0.087 M methadon·HCl</td>
<td>123 107</td>
<td>116 131</td>
</tr>
<tr>
<td>Complete system except no oxalacetate</td>
<td>100 91</td>
<td>115 112</td>
</tr>
</tbody>
</table>

¹The amounts indicated are control corrected averages of counts as determined by the titrimeter.

²Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH₄Cl, 0.033 M NaHCO₃, pyruvate and NaF as indicated, and water to make volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask.

³Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH₄Cl, 0.015 M pyruvate, 0.0015 M α-ketoglutarate, 0.0015 M oxalacetate, 0.033 M NaHCO₃ and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask. Further additions are noted in the first column.

⁴In each case 'A' and 'An' refer to aerobic and anaerobic conditions respectively.
An unexplained effect of methadon hydrochloride on the synthesis of glutamate was observed. The formation of glutamate has been shown to be aerobically dependent; however, in the presence of methadon hydrochloride 23 per cent more glutamate accumulates anaerobically than aerobically. Anaerobic synthesis of aspartate is also at a high level in the presence of methadon hydrochloride. It is not possible to determine which is product and which is reactant without a specific inhibitor for the transamination reaction.

It is possible that anaerobic conditions yield higher concentrations of aspartate through a reversal of malate dehydrogenase. If malate is the compound ammoniated, one would expect malate to substitute for oxalacetate with a resultant increase in the yield of aspartate. Further, lactate might be ammoniated more readily than pyruvate.

The effect of malate on the accumulation of aspartate is recorded in Table 11. A slight decrease in the production of aspartate was noted when malate alone was the substrate; α-ketoglutarate and malate did not appreciably increase the yield of aspartate. Similar results were obtained for the synthesis of alanine from lactate. The effect of anaerobiosis was not found in the use of a reduced compound which could be more readily ammoniated.

Reduction of oxalacetate and analogous reduction of quinones suggested a possible explanation for the observed
Table 11
Replacement of Oxalacetate by Malate

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative amounts(^1) of amino acids per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>0.0015 M malate</td>
<td>145</td>
</tr>
<tr>
<td>0.0015 M α-ketoglutarate + 0.0015 M malate</td>
<td>136</td>
</tr>
<tr>
<td>0.0015 M oxalacetate</td>
<td>141</td>
</tr>
<tr>
<td>None</td>
<td>98</td>
</tr>
</tbody>
</table>

\(^1\)The amounts indicated are averages of counts as determined by the titrimeter.

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH\(_4\)Cl, 0.015 M pyruvate, 20 \(\mu\)M glycerolphosphate, 2.5 \(\mu\)M A.T.P., 0.025 M cysteine, 0.033 M NaHCO\(_3\), 0.045 M MgCl\(_2\) and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask.
depression in the synthesis of aspartate by oxalacetate.
Para and ortho quinone are listed by Braunstein (1947) as weak inhibitors of the glutamate + oxalacetate \[\rightarrow\] aspartate + \(\alpha\)-ketoglutarate reaction. The quinones may act as hydrogen acceptors being reduced to hydroquinone, and in like manner oxalacetate may serve as a hydrogen acceptor and be reduced to malate. The analogy suggested a possible competition on the part of oxalacetate for some reducing mechanism in the cell. If the reducing mechanism were required in the production of aspartate, oxalacetate could decrease the synthesis of aspartate. A reinvestigation of the data indicated that the rather erratic response of the cell to oxalacetate might be due to suboptimal amounts of cysteine.

The effect of cysteine on the synthesis of aspartate is recorded in Table 12. The results indicate an inverse effect of oxalacetate and cysteine. These results might also explain the anaerobic accumulation of aspartate and alanine. It is possible that high concentrations of cysteine result from anaerobic conditions. The activity of cysteine has been widely observed; it is generally assumed to act in a hydrogen transport system. Cystine would not replace cysteine.

The observed inhibition in the synthesis of aspartate by oxalacetate should not be interpreted to indicate that oxalacetate can not be ammoniated; the possibility of an enzyme active in ammoniation of oxalacetate must still be considered.
Table 12
Relief of Oxalacetate Inhibition of Aspartate Synthesis by Cysteine

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative amounts of amino acids per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>None</td>
<td>106</td>
</tr>
<tr>
<td>0.0016 M oxalacetate</td>
<td>117</td>
</tr>
<tr>
<td>0.025 M cysteine (no oxalacetate)</td>
<td>130</td>
</tr>
<tr>
<td>0.0032 M oxalacetate (no cysteine)</td>
<td>110</td>
</tr>
<tr>
<td>0.0032 M oxalacetate + 0.050 M cysteine</td>
<td>140</td>
</tr>
</tbody>
</table>

1The amounts indicated are averages of counts as determined by the titrimeter.

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH₄Cl, 0.015 M pyruvate, 0.045 M MgCl₂, 20 μM glycerolphosphate, 2.5 μM A.T.P., 0.033 M NaHCO₃ and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 40 mg of cells per flask. Further additions are noted in the first column.
Requirements of dialyzed juices for ammoniation

Cell-free juices were prepared and used to study the synthesis of amino acids in the absence of the formation of protein. Juices were dialyzed against distilled water at 4°C to gain more detailed information on the requirements of the systems synthesizing amino acids. Juices were prepared as follows.

<table>
<thead>
<tr>
<th>Juice</th>
<th>Sonic treatment (min.)</th>
<th>Centrifugation time (min.)</th>
<th>Speed (R.P.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1-1</td>
<td>10</td>
<td>10</td>
<td>10,000</td>
</tr>
<tr>
<td>S1-2</td>
<td>-</td>
<td>5</td>
<td>&quot;</td>
</tr>
<tr>
<td>S2-1</td>
<td>20</td>
<td>10</td>
<td>&quot;</td>
</tr>
<tr>
<td>S2-2</td>
<td>-</td>
<td>5</td>
<td>&quot;</td>
</tr>
<tr>
<td>G1-1</td>
<td>-</td>
<td>10</td>
<td>&quot;</td>
</tr>
<tr>
<td>G1-2</td>
<td>-</td>
<td>5</td>
<td>&quot;</td>
</tr>
<tr>
<td>S1N-1</td>
<td>10</td>
<td>10</td>
<td>&quot;</td>
</tr>
<tr>
<td>S1N-2</td>
<td>40</td>
<td>5</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Fifteen grams wet weight of cells were used for each preparation. "S" juices were made to a volume of 20 ml with distilled water and treated at nine kilocycles for the times indicated. The first juices obtained, at 10 minutes centrifugation, are labeled "-1" in each case. The residue was recovered and again made to a volume of 20 ml with distilled water and centrifuged for 5 minutes. The high nitrogen content associated with this fraction, Table 13, indicates the presence of particulate matter. These juices are the "-2" series. The first four juices were prepared by adding pow-
### Table 13

<table>
<thead>
<tr>
<th>Juice number</th>
<th>μM N₂ per ml</th>
<th>Type of system</th>
<th>μl O₂ uptake</th>
<th>μl CaSO₄-CO₂</th>
<th>μM NH₄⁺ uptake</th>
<th>Relative amounts¹ of amino acids per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁₋₁</td>
<td>10.0</td>
<td>Complete</td>
<td>+</td>
<td>78</td>
<td>- 5.1</td>
<td>249  62  106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>+</td>
<td>--</td>
<td>+ 2.9</td>
<td>243  71  55</td>
</tr>
<tr>
<td>S₁₋₂</td>
<td>20.8</td>
<td>Complete</td>
<td>4</td>
<td>129</td>
<td>-10.5</td>
<td>260  50  44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>+</td>
<td>--</td>
<td>+ 4.0</td>
<td>145  57  44</td>
</tr>
<tr>
<td>S₂₋₁</td>
<td>64.7</td>
<td>Complete</td>
<td>9</td>
<td>116</td>
<td>- 8.6</td>
<td>268  92  112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>--</td>
<td></td>
<td>+ 4.6</td>
<td>241  89  56</td>
</tr>
<tr>
<td>S₂₋₂</td>
<td>86.5</td>
<td>Complete</td>
<td>6</td>
<td>129</td>
<td>- 5.9</td>
<td>251  74  57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>--</td>
<td></td>
<td>+ 5.0</td>
<td>180  86  40</td>
</tr>
<tr>
<td>S₃₋₁</td>
<td>--</td>
<td>Complete</td>
<td>32</td>
<td>109</td>
<td>- 4.3</td>
<td>289  79  103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>5</td>
<td>--</td>
<td>+ 10.3</td>
<td>281  109  88</td>
</tr>
<tr>
<td>S₃₋₂</td>
<td>--</td>
<td>Complete</td>
<td>14</td>
<td>107</td>
<td>- 4.1</td>
<td>288  89  67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>--</td>
<td></td>
<td>+ 6.8</td>
<td>239  89  47</td>
</tr>
<tr>
<td>S₅₋₁</td>
<td>6.0</td>
<td>Complete</td>
<td>8</td>
<td>119</td>
<td>- 3.3</td>
<td>245  64  47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>28</td>
<td>--</td>
<td>+ 3.5</td>
<td>169  74  54</td>
</tr>
<tr>
<td>S₅₋₂</td>
<td>75.8</td>
<td>Complete</td>
<td>13</td>
<td>114</td>
<td>- 2.7</td>
<td>272  57  43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juice only</td>
<td>13</td>
<td>--</td>
<td>+ 5.4</td>
<td>134  59  58</td>
</tr>
</tbody>
</table>

¹The amounts indicated are averages of counts as determined by the titrimeter.

Each "Complete" Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH₄Cl, 0.015 M pyruvate, 0.0015 M α-ketoglutarate, 0.0015 M oxalacetate, 0.045 M MgCl₂, 20 μM glycerolphosphate, 2.5 μM A.T.P., 0.025 M cysteine, 0.033 M NaHCO₃ and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 0.50 ml of juice per flask. The "Juice only" flasks contained 0.15 M phosphate buffer.
dered glass at the rate of one gram per four grams wet weight of cells.

The "G" series of juices was prepared by grinding the cells with powdered glass (Wiggert, et al., 1940). Weights of cells and volumes of liquid were the same as used in the preparation of the "S" series of juices.

The "S\textsubscript{N}" series of juices was prepared by sonic treatment; powdered glass was not added to the cell suspension. The cell debris recovered after initial centrifugation was subjected to an additional 40 minutes sonic treatment. This fraction also contained particulate matter.

The characteristics of the prepared juices are recorded in Table 13. The \(\alpha\)-ketoglutarate-glutamate system is associated rather loosely with the cell and is removed by mild sonic treatment as noted with juices \(S_{1-1}\) and \(S_{2-1}\); it is less prominent in particulate matter from the disrupted cell, juices \(S_{1-2}\) and \(S_{2-2}\). The pyruvate-alanine system is more pronounced in juice \(S_{N-2}\) and is probably associated with particulate matter as seen from the high nitrogen content of the juice and the short time of centrifugation.

The oxalacetate-aspartate system is associated with the cell in a manner similar to the glutamate system. Elliott (1948) was able to remove a glutamine synthesizing system from \textit{M. pyogenes} var. \textit{aureus} by mild attrition, e.g., shaking with glow beads. Juices \(S_{1-1}\) and \(S_{2-1}\) were used to study the
synthesis of glutamate. Juice $S_{N-3}$ was employed in a study of the formation of alanine.

**Synthesis of glutamate.** To determine the requirements of any system, the juices were dialyzed for varying periods of time at 4° C in the cold box. The omission of a component indicated its effect on the synthesis of amino acids. Results of a typical experiment are reported in Table 14. Juice $S_{N-3}$ was dialyzed for 3 hours immediately before use.

Results were similar to those found when whole cells were used but differences were more marked. Dialysis reduces the endogenous ammonium nitrogen released by the juice in the dialyzed control to 22 per cent of that released in the undialyzed control, and the synthesis of glutamate is lowered 46 per cent. Ammonium ion assimilation does not correlate with the production of the amino acids by dialyzed juices. A.T.P., Mg++, sodium bicarbonate and cysteine are the more important components of the system. The effect of Mg++ is more noticeable in dialyzed juices and is the inverse of its action in whole cells; $\alpha$-ketoglutarate is the most important single component of the system. Since the dehydrogenases are probably not removed by dialysis, activation of the glutamic acid dehydrogenase system could readily take place.

**Synthesis of alanine and aspartate.** Results relating to juice $S_{N-3}$ and the synthesis of alanine and aspartate are found in Table 15. The juice was dialyzed for 7 hours at 4°C
<table>
<thead>
<tr>
<th>Omissions</th>
<th>O$_2$ uptake</th>
<th>NH$_4^+$ uptake</th>
<th>Relative amounts$^1$ of glutamate per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>All—not dialyzed</td>
<td>+</td>
<td>+11.7</td>
<td>100</td>
</tr>
<tr>
<td>All--dialyzed</td>
<td>0</td>
<td>+2.6</td>
<td>54</td>
</tr>
<tr>
<td>None</td>
<td>122</td>
<td>+3.8</td>
<td>169</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>97</td>
<td>-21.8</td>
<td>150</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>91</td>
<td>+5.5</td>
<td>134</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>87</td>
<td>-15.3</td>
<td>167</td>
</tr>
<tr>
<td>A.T.P.</td>
<td>91</td>
<td>-15.8</td>
<td>100</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>90</td>
<td>-2.5</td>
<td>112</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>--</td>
<td>+1.7</td>
<td>92</td>
</tr>
<tr>
<td>Cysteine</td>
<td>38</td>
<td>-18.5</td>
<td>73</td>
</tr>
<tr>
<td>α-Keto-glutarate</td>
<td>91</td>
<td>-21.7</td>
<td>60</td>
</tr>
</tbody>
</table>

$^1$The amounts indicated are averages of counts as determined by the titrimeter.

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH$_4$Cl, 0.015 M pyruvate, 0.0015 M α-ketoglutarate, 0.0015 M oxalacetate, 0.045 M MgCl$_2$, 20 μM glycerolphosphate, 2.5 μM A.T.P., 0.025 M cysteine, 0.033 M NaHCO$_3$ and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 0.50 ml of dialyzed juice per flask. Exceptions are noted in the first column.
Table 15
Some Requirements of a Dialyzed Juice S_{N-4} in the Synthesis of Alanine and Aspartate

<table>
<thead>
<tr>
<th>Omissions</th>
<th>μl O₂ uptake</th>
<th>μM NH₄⁺ uptake</th>
<th>Relative amounts of amino acids per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (juice only)</td>
<td>--</td>
<td>+ 2.7</td>
<td>65 Alanine 80 Aspartate</td>
</tr>
<tr>
<td>None</td>
<td>98</td>
<td>+ 3.0</td>
<td>152 Alanine 78 Aspartate</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>173</td>
<td>+ 2.7</td>
<td>115 Alanine 72 Aspartate</td>
</tr>
<tr>
<td>Glycerol phosphate</td>
<td>88</td>
<td>+ 2.8</td>
<td>107 Alanine 77 Aspartate</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>99</td>
<td>+ 5.1</td>
<td>118 Alanine 70 Aspartate</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>86</td>
<td>+ 3.3</td>
<td>134 Alanine 72 Aspartate</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>107</td>
<td>+ 1.2</td>
<td>139 Alanine 60 Aspartate</td>
</tr>
<tr>
<td>A.T.P.</td>
<td>84</td>
<td>+ 1.8</td>
<td>90 Alanine 67 Aspartate</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>77</td>
<td>+ 8.3</td>
<td>178 Alanine 52 Aspartate</td>
</tr>
<tr>
<td>Cysteine</td>
<td>50</td>
<td>0.0</td>
<td>131 Alanine 57 Aspartate</td>
</tr>
<tr>
<td>None + 0.0087 M NaF</td>
<td>90</td>
<td>+10.0</td>
<td>66 Alanine 49 Aspartate</td>
</tr>
<tr>
<td>None + 0.0087 M NaF + 0.045 M MnSO₄</td>
<td>29</td>
<td>+ 6.0</td>
<td>149 Alanine 60 Aspartate</td>
</tr>
</tbody>
</table>

1The amounts indicated are averages of counts as determined by the titrimeter.

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH₄Cl, 0.015 M pyruvate, 0.0015 M α-ketoglutarate, 0.0015 M oxalacetate, 0.045 M MgCl₂, 20 μM glycerol phosphate, 2.5 μM A.T.P., 0.025 M cysteine, 0.033 M NaHCO₃ and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 0.50 ml of dialyzed juice per flask. Exceptions and additions are noted in the first column.
and used immediately. A mild dependency of the synthesis of alanine on pyruvate is shown. The effects of glycerol-phosphate and A.T.P. indicate an energy requiring reaction. The conclusion is further supported by the observed relief of sodium fluoride inhibition by manganous sulfate. Similar effects have been discussed by Utter and Werkman (1942) in regard to carbohydrate dissimilation. The effect of sodium fluoride on Mg\(^{++}\) is probably associated with the production of phosphoeno1 pyruvate. Manganous ions can substitute for Mg\(^{++}\) in this reaction. If this reaction were blocked, "biological" pyruvate would not be available to the system. It is apparent that a phosphorylation is required for the synthesis of alanine and aspartate.

The absence of cysteine leads to a lowered yield of alanine. Oxygen uptake is always lowered by the absence of cysteine which further indicates its role in a hydrogen transport system.

The absence of sodium bicarbonate stimulates the synthesis of alanine and depresses the synthesis of aspartate. The formation of aspartate by cell-free juices is dependent on available pyruvate and carbon dioxide fixation.

The differences observed in the synthesis of aspartate are not as marked as are those mentioned for the synthesis of alanine. Inhibition by sodium fluoride is relieved by manganous ions. A direct effect of the absence of oxalacetate
on the synthesis of aspartate is noted after long dialysis. The effects of A.T.P., sodium bicarbonate and cysteine were noted in relation to the synthesis of alanine.

The formation of aspartate, alanine and glutamate by juices aerobically and anaerobically is similar to formation observed with whole cells (Table 16). In addition, the high aerobic and low anaerobic formation of endogenous glutamate supports the suggestion that a precursor of glutamate arises aerobically which can be more readily ammoniated. The opposite is true for precursors of alanine and aspartate which appear to be more readily ammoniated or transaminated anaerobically than aerobically.

Transamination

It is generally agreed there are as yet no inhibitors for the transamination reaction (Braunstein, 1947). Data on rates of reaction, equilibrium constants and direction of transamination can be obtained only by use of a highly purified enzyme system.

An attempt was made to determine the direction of reaction by removing α-ketoglutarate from a complete system and substituting glutamate or glutamine. The results are recorded in Table 17. The juice Sa-1 was dialyzed for 2 hours.
Table 16

Effect of Anaerobiosis on Synthesis of Amino Acids by Juices \(S_{a-1}\) and \(S_{N-2}\)

<table>
<thead>
<tr>
<th>Additions</th>
<th>Gas phase</th>
<th>(\mu l) Ce3O4 - CO2</th>
<th>Relative amounts of amino acids per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S_{a-1} + 0.0015 M)</td>
<td>air</td>
<td>19</td>
<td>Alanine 127</td>
</tr>
<tr>
<td>(a)-ketoglutarate</td>
<td>nitrogen</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>(S_{a-1}) only</td>
<td>air</td>
<td>22(^a)</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>nitrogen</td>
<td>23(^a)</td>
<td>110</td>
</tr>
<tr>
<td>(S_{N-2} + 0.015 M) pyruvate</td>
<td>air</td>
<td>52</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>nitrogen</td>
<td>45</td>
<td>144</td>
</tr>
<tr>
<td>(S_{N-2}) only</td>
<td>air</td>
<td>18(^a)</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>nitrogen</td>
<td>20(^a)</td>
<td>130</td>
</tr>
</tbody>
</table>

\(^a\)The amounts indicated are averages of counts as determined by the titrimeter.

\(^b\)Corrected for zero time concentrations.

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH4Cl, 0.045 M MgCl2, 20 \(\mu M\) glycerolphosphate, 2.5 \(\mu M\) A.T.P., 0.025 M cysteine, 0.035 M NaHCO3 and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 0.50 ml of juice per flask. Further additions are noted in the first column.
Table 17
Influence of Glutamate and Glutamine on the Synthesis of Amino Acids

<table>
<thead>
<tr>
<th>Additions</th>
<th>O₂ uptake</th>
<th>CO₂ uptake</th>
<th>NH₄⁺ uptake</th>
<th>Relative amounts of amino acids per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0015 M a-ketoglutarate present</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>173</td>
<td>64</td>
<td>+2.5</td>
<td>90</td>
</tr>
<tr>
<td>0.001 M glutamate</td>
<td>143</td>
<td>54</td>
<td>+3.6</td>
<td>230</td>
</tr>
<tr>
<td>0.001 M glutamine</td>
<td>133</td>
<td>47</td>
<td>+9.5</td>
<td>110</td>
</tr>
<tr>
<td>No a-ketoglutarate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>174</td>
<td>64</td>
<td>+7.2</td>
<td>190</td>
</tr>
<tr>
<td>0.001 M glutamate</td>
<td>155</td>
<td>63</td>
<td>+5.8</td>
<td>160</td>
</tr>
<tr>
<td>0.001 M glutamine</td>
<td>153</td>
<td>68</td>
<td>+7.2</td>
<td>150</td>
</tr>
</tbody>
</table>

The amounts indicated are averages of counts as determined by the titrimeter.

Each Warburg flask contained 0.15 M phosphate buffer, 0.02 M NH₄Cl, 0.015 M pyruvate, 0.0015 M oxalacetate, 0.045 M MgCl₂, 20 μM glycerolphosphat, 0.025 M cysteine, 0.033 M NaHCO₃ and water to make total volume to 3.1 ml. Final pH = 6.8; time = 1 1/2 hours; 0.50 ml of juice per flask. Further additions are noted in the first column.
There is a general reduction in the uptake of oxygen and a reduction in the residual α-keto acids which indicates removal of the latter by some mechanism other than oxidation. The disappearance of α-keto acids is reflected in increased synthesis of aspartate and alanine. It is apparent from the results of the first three experiments that the reaction is flowing toward the synthesis of aspartate and alanine.

In the absence of α-ketoglutarate there is a higher uptake of oxygen, a greater residual of α-keto acids and more extensive deamination. A reversal of the direction of synthesis becomes apparent with an increase in the amount of glutamate and a decrease in the amount of alanine and aspartate found. The results suggest the action of amino acid oxidases. The presence of transaminases, their reversal and a direction of reaction dependent on mass law and concentration of reactants and products is indicated. Since the reaction is reversible, ammoniation cannot be assigned to either α-ketoglutarate or oxalacetate without further information.

The accumulation of amino acids with time and under aerobic and anaerobic conditions was observed to determine whether the effect of α-ketoglutarate was due to transamination or ammoniation.

The ratios of glutamate/aspartate listed in Table 18 indicate the early accumulation of aspartate at a concentration
Table 18
Synthesis of Amino Acids as Related to Time and Oxygen

<table>
<thead>
<tr>
<th>Additions</th>
<th>Gas phase</th>
<th>Amounts(^1) of amino acids per flask (µg per ml)</th>
<th>Ratio of glutamate to aspartate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alanine</td>
<td>Aspartate</td>
</tr>
<tr>
<td>0.02 M pyruvate</td>
<td>air</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>nitrogen</td>
<td>80</td>
<td>140</td>
</tr>
<tr>
<td>0.01 M oxalacetate</td>
<td>air</td>
<td>56</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>nitrogen</td>
<td>76</td>
<td>110</td>
</tr>
<tr>
<td>0.01 M a-ketoglutarate</td>
<td>air</td>
<td>58</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>nitrogen</td>
<td>60</td>
<td>85</td>
</tr>
<tr>
<td>0.02 M pyruvate +</td>
<td>air</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>0.01 M oxalacetate +</td>
<td>nitrogen</td>
<td>83</td>
<td>128</td>
</tr>
<tr>
<td>0.01 M a-ketoglutarate</td>
<td>air</td>
<td>28</td>
<td>43</td>
</tr>
<tr>
<td>Control (a-keto acids omitted)</td>
<td>nitrogen</td>
<td>38</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^1\)The amounts indicated are averages of counts as determined by the titrimer.

\(^2,3\)In each case t\(^2\) = 10 minutes; t\(^3\) = 90 minutes.

Each flask contained 0.15 M phosphate buffer, 0.02 M NH\(_4\)Cl, 0.045 M MgCl\(_2\), 20 µM glycerolphosphate, 2.5 µM A.T.P., 0.025 M cysteine, 0.033 M NaHCO\(_3\) and water to make total volume to 3.1 ml. Final pH = 6.8; 0.50 ml dialyzed juice per flask. Further additions are noted in the first column.
higher than that of glutamate. This is especially true under anaerobic conditions where the "10 minute" aspartate is at three times the concentration of glutamate. The "10 minute" aspartate is about one and one-half times as great as the respective glutamate under aerobic conditions. The "90 minute" aspartate is only one-half that of the respective glutamate. The results obtained with oxalacetate were also observed in a general way with pyruvate.

"Ten minute" glutamate was found in excess of the respective aspartate only when α-ketoglutarate was used as a substrate. Such indirect evidence of a dual mechanism of ammoniation is of value in analyzing systems for which there is not a specific inhibitor. It is believed that the evidence is strongly in favor of ammoniation of aspartate. However, care should be exercised in the interpretation of results in view of the observations of Kritzmann, et al. (1945a), on catalysis of transamination and the work of Green, et al. (1945) on the high turnover rate of the partially purified glutamate → aspartate enzyme.

Carbon Dioxide and Pyruvate in Synthesis of Amino Acids

The work of Kritzmann (1944), Lwoff and Monod (1946) and Ajl and Werkman (1949) has shown the importance of carbon dioxide to growth and formation of amino acids.
Results presented here have also indicated a need for carbon
dioxide in the synthesis of aspartate.

A mechanism for the synthesis of aspartate

Sodium bicarbonate containing excess C\(^14\) was used to
determine the incorporation of carbon dioxide in amino acids.
The reaction was carried out in standard taper single side-
arm flasks having a volume of 50 ml. At the end of the re-
action time indicated the flasks were acidified, placed in a
water bath at 80°C and aerated with carbon dioxide free air.
Residual carbon dioxide was collected in 3 M base. The re-
action mixture was deproteinated, centrifuged and ether ex-
tracted to remove acids. The raffinate was reduced in volume
and an aliquot placed on a sheet of Whatman No. 1 filter
paper using the method previously outlined. The sheet was
cut into two-inch strips which were chromatographed using
a phenol-water solvent at 34°C. Transfer of the solvent was
complete in 36 to 48 hours. The strips were dried to remove
the phenol and surveyed with a Geiger-Mueller tube at a
distance of two millimeters from the window. The positions
of active spots were noted and the strips developed by
spraying with ninhydrin. Distances for R\(\text{f}\) values were
measured from the line of application to the front of the
developed spot and to the solvent front. Experimental R\(\text{f}\)
values were obtained from strips containing known amino
acids which were chromatographed in the same cylinders as the unknowns. Results are recorded in Table 19.

No attempt was made to obtain a balance of C\textsuperscript{14} in the various fractions obtained from the three experiments. About 12,250 counts were added in experiments 1 and 2 and about 6,000 counts in experiment 3. Approximately 97 per cent of the activity was recovered from the base in the center well of the reaction vessel. The ether extract contained a small fraction of activity indicating carbon dioxide fixation with the formation of ether extractable compounds. The small amount of active amino acid found high on the strip associated with an \( R_F \) value of .35-.88 is of interest but was not readily identified from the literature. The unknown would be in the region of methionine and arginine since the experimental \( R_F \) values were somewhat higher than those in the literature.

In elaborating the mechanism from pyruvate to aspartate, it would be important to determine whether oxalacetate might arise as the phosphoenol oxalacetate through carbon dioxide fixation to phosphoenol pyruvate. The phosphate ester might then be ammonolized giving rise to phosphoric acid and aspartate. Such a mechanism would explain the effect of A.T.P., Mg\textsuperscript{2+}, glycerolphosphate, pyruvate and sodium bicarbonate since these components would be required for the formation of oxalacetate.
Table 19
Appearance of C\textsuperscript{14} from NaHC\textsubscript{14}O\textsubscript{3} in Amino Acids

<table>
<thead>
<tr>
<th>Section number on strip</th>
<th>Activity (corrected)</th>
<th>(R_F) values</th>
<th>Possible amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Active spot</td>
<td>Literature\textsuperscript{1}</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(45 min.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>.85</td>
<td>--</td>
</tr>
<tr>
<td>16-18</td>
<td>33</td>
<td>.126</td>
<td>.12</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(45 min.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>.88</td>
<td>--</td>
</tr>
<tr>
<td>17-18</td>
<td>35\textsuperscript{a}</td>
<td>.131</td>
<td>.12</td>
</tr>
<tr>
<td>Expt. 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(30 min.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-11</td>
<td>12</td>
<td>.128</td>
<td>.12</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Consden, R., et al., 1944.

\textsuperscript{a}Green blue with ninhydrin.

\textsuperscript{a}In this case a separation of aspartate and glutamate was not clean-cut. This is often the case when the concentration of amino acid is high. Some of the activity may have been associated with glutamate.
The concentration of residual pyruvate was determined by the addition of reaction mixtures were harvested and deproteinized as usual. The concentration of residual pyruvate was synthesized with 0.1 M in the carbon-14 group.

Therapy

Adenosine triphosphatase was obtained through the use of CTR as a stimulus. The synthesis of substrate or substrate, or both, in the presence or absence of pyruvate accounted to a great extent in the absence of pyruvate not been adequately investigated. In several instances at least, the role of added pyruvate as a source of alanine has

Syntheses of alanine and acetocitrate

Pressed in the absence of carbon dioxide,

Extracted hence, the syntheses of substrate or substrate may also be de-

on acetocitrate is also dependent upon carbon dioxide

ammonium ion assimilation would be depressed. The formation of carbon dioxide, since with suboptimal amounts of acetocitrate

place in the presence of pyruvate and in the absence of
could be assimilated by the system. Growth would not take

were in reality operative mechanisms were ammonia

the syntheses of substrate made the observations of Aiz-

The importance of carbon dioxide fixation is the syn-
tion of ceric sulfate and a manometric measurement of the evolved carbon dioxide. Residual ammonium ion and concentrations of alanine, aspartate and glutamate were also determined on the supernatant liquid. The remainder of the reaction mixture was ether extracted for 72 hours, concentrated and placed on sheets of Whatman No. 1 filter paper for chromatographing. After transfer of the solvent, the two-inch strips were dried and backed with strips of honeycomb foundation. The honeycomb foundation acted as a planchet and was necessary to keep the flow counter from becoming contaminated. A sharp cork borer was used to remove a series of samples from the chromatographed strip. The circles were surveyed in a Nuclear Instrument Corporation "Q" gas counter; five 4-minute counts were averaged to obtain the count per circle.

The remaining half of each strip was sprayed with ninhydrin and the \( R_F \) values calculated from the position of the developed spots. These sections were then cut from the strips and their area determined by comparing their weights with the weight of a known area. A rough estimate was thus made of the activity of the chromatographed amino acid from the average count of the known area of the respective circles. Results of the calculations are presented in Table 20. The data are averages from two experiments.
Table 20

Estimated Recovery of C\textsuperscript{14} from Metabolized Carbonyl-labeled Pyruvate

<table>
<thead>
<tr>
<th>µM Pyruvate and counts added</th>
<th>µM Amino acid recovered</th>
<th>Activity in metabolic CO\textsubscript{2}</th>
<th>Activity in amino acid</th>
<th>Conversion of added pyruvate</th>
<th>Counts recovered in amino acid</th>
<th>Fractional counts converted per µM\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aerobic\textsuperscript{2}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µM--112 counts--29,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.05</td>
<td>430</td>
<td>1.14</td>
<td>1.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µM--112 counts--29,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.25</td>
<td>155</td>
<td>0.06</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1}This fraction = counts per µM amino acid recovered/ counts per µM pyruvate.

\textsuperscript{2}Unknown not transferred on chromatogram--98 counts, acid hydrolyzate of reaction juice--1,000 counts; observed under aerobic conditions only.
The juice used did not metabolize pyruvate in such a manner as to release the carbonyl carbon as carbon dioxide within the time limit of the experiment as seen from the zero activity of metabolic carbon dioxide. By far the greater amount of activity was recovered in the ether extract; it was not identified. The conversion of added pyruvate to alanine was slightly higher anaerobically than aerobically.

On the basis of counts per mole of pyruvate added and counts per mole of alanine recovered, about 43 per cent of the alanine was formed from the pyruvate which had been added when the reaction was carried on aerobically. The remaining 57 per cent would have arisen from some cellular activity such as the dissimilation of added glycerolphosphate or cellular constituents.

A similar calculation from the results of the anaerobic experiment indicates a 59 per cent conversion of added pyruvate to alanine. The results show that added pyruvate is a precursor of alanine but that it is not quantitatively converted to alanine.

Distribution of the pyruvate containing $C^{14}$ among the amino acids synthesized is recorded in Table 21. The two active sections on the chromatographed strip correspond to alanine and glutamate.

The most striking observation is the finding of an amino acid identified as glutamate containing high activity. The
Table 21

Distribution of $C^{14}$-labeled Pyruvate in Amino Acids

<table>
<thead>
<tr>
<th>Section number on strip</th>
<th>Activity (corrected)</th>
<th>$R_F$ values</th>
<th>Possible amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Active</td>
<td>Literature$^1$</td>
</tr>
<tr>
<td>Aerobic (45 min.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-12</td>
<td>36.4</td>
<td>.62</td>
<td>.57</td>
</tr>
<tr>
<td>4-5</td>
<td>18.2</td>
<td>.21</td>
<td>.19</td>
</tr>
<tr>
<td>Anaerobic (45 min.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-13</td>
<td>45.9</td>
<td>.61</td>
<td>.57</td>
</tr>
<tr>
<td>3-5</td>
<td>9.1</td>
<td>.22</td>
<td>.19</td>
</tr>
</tbody>
</table>

$^1$Consden, R., et al., 1944.
fractional counts converted per μM of glutamate formed under aerobic conditions, 1.65 (Table 20), indicates a condensation of at least two radioactive residues. This is not carbon dioxide fixation as seen from the lack of activity in the metabolic carbon dioxide. A C₅ + C₃ condensation could lead to glutamate through isocitrate and α-ketoglutarate. Pyruvate is important in the synthesis of glutamate and aspartate as well as alanine, and it may serve as the bridge between carbohydrate and protein metabolism.

It should be pointed out again that though the estimations are as exact as possible with available technics they leave much to be desired.

**Distribution of labeled nitrogen in synthesized amino acids**

The data presented in Table 18 showed the ratio aspartate/glutamate changing with time. The early formation of aspartate at a concentration higher than glutamate was interpreted as indicating preferential ammoniation of oxalacetate. Further evidence for oxalacetate ammoniation was obtained by determining the manner in which the concentration of labeled ammonium ion in aspartate and glutamate varied with time.

Equal volumes of juices S₁₈ and S₉ were used as the source of enzyme. The ammonium chloride used contained 53.3 per cent excess N¹⁵. The reaction was carried out in 150 ml
Warburg flasks. Two milliliters of juice were used; the final volume of reaction mixture was 8 ml. Components of the system were the same as those given in Table 13. After reaction, the solutions were harvested and deproteinated as previously outlined, and aliquots were removed for determination of residual α-keto acids, residual ammonium chloride and for amino acid assay.

An aliquot was fortified with 50 μM each of alanine, aspartate and glutamate to serve as carriers of the biosynthesized amino acids. The procedure outlined in Figure 4 (See page 45) was followed for the separation of the amino acids; the fractions were checked chromatographically for purity. Although the separations were good, recoveries based on Kjeldahl nitrogen were low, in some cases less than 50 per cent.

Results support the suggestion of ammoniation of oxalacetate by A. aerogenes (Table 22). The lower concentration of N18 in glutamate at 10 minutes as compared with the N18 concentration in the respective aspartate fraction indicates that transamination from cellular amino acids to α-keto-glutarate is competing with ammoniation. The results also indicate that aspartate is not the only compound which can serve as an amino donor in the synthesis of glutamate, since if aspartate were serving as the only amino donor, the N18 concentration in glutamate should be as high or higher than
<table>
<thead>
<tr>
<th>Time and gas phase</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu M )</td>
<td>Excess(^1) ( N^{15} )</td>
<td>Excess(^1) ( N^{15} )</td>
<td>Excess(^1) ( N^{15} )</td>
</tr>
<tr>
<td></td>
<td>amino acid formed</td>
<td>added as N(^{15}H_4)Cl</td>
<td>recovered from reaction mixture</td>
<td>in amino acid</td>
</tr>
<tr>
<td>10 min. (air)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>4.0</td>
<td>0.533</td>
<td>0.520</td>
<td>none</td>
</tr>
<tr>
<td>Aspartate</td>
<td>4.8</td>
<td>0.533</td>
<td>0.520</td>
<td>0.109</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.3</td>
<td>0.533</td>
<td>0.520</td>
<td>0.092</td>
</tr>
<tr>
<td>10 min. (nitrogen)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>4.3</td>
<td>0.533</td>
<td>0.525</td>
<td>none</td>
</tr>
<tr>
<td>Aspartate</td>
<td>5.3</td>
<td>0.533</td>
<td>0.525</td>
<td>0.142</td>
</tr>
<tr>
<td>Glutamate</td>
<td>3.0</td>
<td>0.533</td>
<td>0.525</td>
<td>0.080</td>
</tr>
<tr>
<td>90 min. (air)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>7.8</td>
<td>0.533</td>
<td>0.315</td>
<td>0.055</td>
</tr>
<tr>
<td>Aspartate</td>
<td>8.2</td>
<td>0.533</td>
<td>0.315</td>
<td>0.175</td>
</tr>
<tr>
<td>Glutamate</td>
<td>14.4</td>
<td>0.533</td>
<td>0.315</td>
<td>0.180</td>
</tr>
<tr>
<td>90 min. (nitrogen)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>14.4</td>
<td>0.533</td>
<td>0.415</td>
<td>lost</td>
</tr>
<tr>
<td>Aspartate</td>
<td>21.0</td>
<td>0.533</td>
<td>0.415</td>
<td>0.163</td>
</tr>
<tr>
<td>Glutamate</td>
<td>19.7</td>
<td>0.533</td>
<td>0.415</td>
<td>0.156</td>
</tr>
</tbody>
</table>

\(^1\)Normal \( N^{15} = 0.43 \); all figures report \( N^{15} \) in excess of normal.
that in aspartate. Other transaminases have been suggested by Wood and Gunsalus (1950).

A competition may also be present for oxalacetate in the case of synthesis of aspartate, but if present it is less marked than is the competition for α-ketoglutarate by cellular transaminases. The results are presented as evidence that aspartate is ammoniated for there could not be more N:\textsuperscript{15} present in aspartate than in glutamate if the only source of nitrogen for the formation of aspartate were from the glutamate molecule. However, a more complete knowledge of all reaction rates could change the interpretation of these results.

Higher concentration of N:\textsuperscript{15} aspartate anaerobically was observed and in view of the results presented here may indicate more active ammoniation of oxalacetate in the absence of a competing oxidative reaction. This conclusion is supported by the observed increase in aspartate when inhibitors of oxidation such as arsenite and methadon hydrochloride are used.

The excess N:\textsuperscript{15} in glutamate after 90 minutes appears to be reaching an equilibrium with that in aspartate. This result would be expected in a transamining system since transamination would eventually result in equal distribution of labeled nitrogen if either oxalacetate or α-ketoglutarate were ammoniated.
Ten minutes reaction in a system containing pyruvate and labeled ammonium chloride did not yield alanine with an N₁⁵ excess. There was an N₁⁵ excess in alanine after a 90 minute aerobic reaction. The similar anaerobic fraction was lost, and a definite conclusion can not be reached concerning reductive ammoniation of pyruvate; however, the 10 minute results indicate that reductive ammoniation of pyruvate does not occur in the described system.

The greater dilution of N₁⁵ in the 90 minute aerobic system may be accounted for by the action of oxidative deamination. Release of ammonia into the medium has been discussed. The results were obtained from one experiment.
The investigation has shown that choose is more effective and more direct than is expected. The Kohn theory predicts a Kohnite that leads to less acceptable results for the synthesis of benzine, and when a Kohnite is chosen as the substrate, the Kohnite is effective and leads to the synthesis of benzine.

The result of the examination is an example of the latter. The results of the examination are fundamental and reveal the mechanism of the cell. The mechanism of the cell is the case with certain nitrogen requirements of organisms. An examination of the literature reveals numerous references.
moniation and growth. The concentration of α-ketoglutarate arising from the glucose used in the growth experiments would be only 0.7 the concentration of α-ketoglutarate used in comparable growth experiments if each molecule of glucose were to yield a molecule of α-ketoglutarate. However, a six-fold increase in growth was observed when α-ketoglutarate was replaced by glucose as a substrate. Morrison and Hinshelwood (1949) concluded that carbohydrate yields a five-carbon fragment necessary for reproduction of the cell. Glucose also supplies energy-rich organic phosphate intermediates used in synthetic reactions. Phosphorylated pyridoxal is important in the reactions under consideration since it is the coenzyme of transamination (Lichstein, et al., 1945 and Schlenk and Fischer, 1947).

The importance of phosphorylation is indicated here by the observed depression of the synthesis of amino acids when glucose is the substrate and phosphorylation is inhibited by sodium fluoride.

Sodium arsenite and anaerobic conditions depress growth in a similar manner when α-ketoglutarate is the substrate; sodium fluoride is less inhibitory under these conditions. The differential effect of inhibitors indicates that when α-ketoglutarate is the substrate oxidation is more important in ammoniation and transamination than is phosphorylation, and it is highly probable that oxidation of α-ketoglutarate
is necessary to supply precursors for transamination or for other mechanisms of ammoniation. The effect of inhibitors was more pronounced in growth experiments than with resting cells.

A close relationship among α-ketoglutarate, oxalacetate and pyruvate is shown by the growth-stimulating affect of pyruvate under anaerobic conditions, i.e., pyruvate alone can substitute for the three α-keto acids which are necessary in ammoniation and transamination reactions (See Table 4). Oxalacetate may be formed by the Wood-Werkman reaction either anaerobically or in the presence of oxygen. It was shown that malate could replace oxalacetate in the synthesis of aspartate, alanine and glutamate. Hunter (1949) showed that malate and succinate are formed when α-ketoglutarate is oxidized anaerobically in the presence of oxalacetate. Thus a precursor of aspartate may be formed under aerobic conditions or in the absence of air. Stimulation to growth would result if ammoniation of oxalacetate occurred.

Another explanation for the growth-stimulating effect of pyruvate in the absence of oxygen may be offered. Ochoa (1945) demonstrated the anaerobic formation of α-keto-glutarate from isocitrate in the presence of glucose-6-phosphate. The scheme is shown in Figure 6. The possible condensation of "oxalacetate" and a C₃ fragment is supported
Figure 6. Possible Anaerobic Accumulation of α-Ketoglutarate and Glutamate.
by the observation that glutamate arising from carbonyl-
labeled pyruvate is at least doubly labeled.

The scheme is hypothetical, and it is not known whether it applies to \textit{A. aerogenes}. If it does, the increase in aspartate and alanine and decrease in glutamate under anaerobic conditions could not be interpreted entirely as ammoniation of oxalacetate since under these conditions, glutamate would accumulate in low concentrations and act as a catalyst in transamination.

Evidence for transamination was shown when glutamate or glutamine was added in lieu of \(\alpha\)-ketoglutarate. It was observed also that \(\alpha\)-ketoglutarate depressed oxygen uptake and the release of ammonium ion but enhanced the synthesis of alanine and aspartate. Transamination would likewise account for this observation. Cedrangoło (1943) observed a similar depression of oxygen uptake and ammonium ion release in experiments with tissue.

The requirements of resting cells for the synthesis of glutamate reflect the simplicity of the system. Of several metabolites usually involved in synthetic processes, only \(\alpha\)-ketoglutarate was required for appreciable synthesis of glutamate. Additional metabolites were required in the synthesis of alanine and aspartate. Long dialyzed juices required \(\alpha\)-ketoglutarate but required other factors also. It
is possible that the additional substituents were necessary
for the activation of coenzyme II or apoenzyme or both.

The enzyme system involved in reduction of a-imo-
glutamate is probably located near the surface of the cells
of A. aerogenes. Bradfield (1950) in a review of the liter-
ature on enzyme location within the cell gives no hint of a
possible location for enzymes acting in ammoniation. An ob-
servation in favor of surface location of the enzyme is the
rapid accumulation of glutamate in the medium when whole
cells are supplied with the cells under these conditions.
The enzyme is also easily removed by mild sonic treatment
and is only slightly active in particulate matter from the
cell after extended sonic treatment. Braunstein (1947) has
suggested a possible ammoniation of oxalacetate, or malate,
which locates secondary enzymes associated with the surface
of tissue cells.

Possible Ammoniation of Oxalacetate

Experiments to determine the differential accumulation
of glutamate and aspartate with time demonstrated a rapid
initial rate in the synthesis of oxalacetate, or aspartate. These results
suggested a possible ammoniation of oxalacetate, or malate,
with the fixation of labeled carbon dioxide in aspartate. The use
may be performed as a stepwise reaction. The scheme would yield apparatus by a stepwise mechanism. The transformation on the transporter of phospho-lactate to 
transporter, and results (1969) have shown a catalytic effect of am-
ion the need of phosphotransfer in the system of es-

Krittmann and Miller-Sarkisyan (1946) have

found the need of phosphotransfer in the system of es-

The requirements for Mg2+ and aM4+ in the sy-

theses of apparatus.

The substrate and co-substrate are readily utilized for the syn-

thesis of apparatus and co-substrate is not probable since

action of carbon dioxide and ammonium is not probable since

portant in the scheme shown in Figure 6. The simultaneous fix-

of the synthesizing oxalacetate. Oxalacetate would also be lam-

by the formation of carbon dioxide and ammonium

Krebs' intermediates. The greater extent of growth is a greater extent than a

show that the promotion of growth of A. aerogenes has

of pressure as a substrate for growth of A. aerogenes has
Such a scheme is speculative in the reactions studied; A.T.P. may only satisfy the coenzyme of transamination as suggested by Lichstein, et al. (1945). However, the Wood-Werkman reaction does require A.T.P. suggesting more than a single role for phosphorylation.

Ammoniation of oxalacetate is substantiated by the early accumulation of aspartate with a higher specific concentration of $^{15}N$ than that in the glutamate obtained from the same reaction. This is the strongest evidence offered in support of the hypothesis that assimilation of ammonium ion takes place through the ammoniation of oxalacetate as well as $\alpha$-ketoglutarate.

It should be pointed out, however, that proof of ammoniation of oxalacetate will depend on the use of specific inhibitors for transamination or a purified specific enzyme system; neither is available at present.
Role of Pyruvate in Ammoniation

Pyruvate was shown to be a more efficient substrate for the growth of A. aerogenes both anaerobically and aerobically than α-ketoglutarate. The response on the part of the cell may be explained by assuming that pyruvate can take part in a greater variety of reactions and interactions and thus yield precursors required by the cell. Pyruvate is known to undergo many reactions which have not been shown for α-ketoglutarate. Two active forms which have been shown to arise directly from pyruvate are phosphopyruvate (Lardy and Zeigler, 1945) and oxalacetate (Krampitz, Wood and Werkman, 1943).

A higher concentration of alanine was obtained in the presence of pyruvate, ammonium ion and enzyme under anaerobic conditions than under aerobic conditions. The alanine was not believed formed by reductive ammoniation since in each case some glutamate or aspartate could be demonstrated and transamination could have occurred. It was not possible to demonstrate N\textsuperscript{15} in alanine under anaerobic conditions which should have led to its incorporation if rapid reductive ammoniation had taken place. This is not in agreement with Kritzmann (1947) or Wiss (1948).

The addition of sodium arsenite or methadon hydrochloride or the presence of anaerobic conditions stimulated the syn-
thesis of alanine in a system containing pyruvate. More \( \alpha \) -keto acid is made available for transamination by inhibition of a competing oxidative reaction. Similar results were observed with muscle tissue (Agren, 1940) and bacteria (Cutinelli, 1947).
SUMMARY AND CONCLUSIONS

1. Whole cells and cell-free extracts of *Aerobacter aerogenes* possess an enzyme system which actively ammoniates \( \alpha \)-ketoglutarate. The enzyme was obtained in cell-free solution; its action was similar to that in resting cells or growing cells.

2. The concentration of glutamate formed by growing cells in the aerobic dissimilation of a carbon source is higher than that formed under anaerobic conditions. The same is not true for long dialyzed juices where more glutamate is formed anaerobically.

3. The enzyme(s) participating in the ammoniation of \( \alpha \)-ketoglutarate may be located near the surface of the cell.

4. Short time experiments employing \( N^{15} \) have shown aspartate as a possible product of ammoniation.

5. Alanine and aspartate are formed in high concentration as products of anaerobic dissimilation of a substrate.

6. Reductive ammoniation of pyruvate was not shown. Pyruvate is involved in the synthesis of aspartate because of its role in carbon dioxide fixation. Pyruvate is also significant in the formation of glutamate. A \( C_3 + C_3 \) condensation has been suggested to be followed by ammoniation of a resulting five-carbon compound.
7. Sodium fluoride inhibits ammoniation in dialyzed juices; the inhibition can be relieved by manganous sulfate. Sodium arsenite and methadon hydrochloride accentuate the formation of alanine and to a lesser degree that of aspartate and glutamate.
LITERATURE CITED

Adler, E., Günther, G. und Everett, J. E.

Adler, Erich, Hellström, Vidar, Günther, Gunnar und Eule, Hans

Agren, Gunnar

Ajl, Samuel J.

Ajl, Samuel J. and Werkman, C. H.

Ajl, Samuel J. and Werkman, C. H.

Anker, H. S.

Anker, H. S.

Awapara, Jorge

Bhagwan, S. Lulla
Chibnall, A. C.

Cohen, Georges N., Cohen-Bazire, Germaine and Minz, Bruno

Cohen, Philip P.

Cohen, Philip P.

Cohen, Philip P. and Hayana, Mika

Cohen-Bazire, Germaine and Saissac, Renée

Consden, R., Gordon, A. H. and Martin, A. J. P.

Cutinelli, C.

Cutinelli, C.

Dagley, S., Daws, E. A. and Morrison, G. A.
Damodaran, M. and Subramanian, S. S.  

Das, N. B.  

Delluva, Adelaide M. and Wilson, D. Wright  

Elliott, W. H.  

Epstein, S. F.  
1941. Synthesis of phosphopyruvic acid in muscle during oxidation of citric acid. Biochem. J. (Ukraine) 17, 139-143. (Original not seen; abstracted in Chemical Abstracts 36, 4561, 1942.)

Euler, H. v., Adler, E. und Eriksen, T. Steenhoff  

Euler, Hans v., Adler, Erich, Günther, Gunnar und Das, Nalin Bandhu  

Euler, Hans v., Adler, Erich, Günther, G. and Elliot, L.  
1939. Isocitric acid dehydrogenase and glutamic acid synthesis in higher plants and in yeast. Enzymologia 6, 337-341.

Euler, Hans v. and Högborg, Bertil  

Fahrländer, H., Favarger, P. and Leuthardt, F.  
Frantz, Ivan D. Jr., Lottfield, Robert B. and Miller, Warren W.

Gale, E. F. and Mitchell, P. D.

Gale, E. F. and Rodwell, A. W.

Gale, Ernest F. and Taylor, E. Shirley

Green, D. E., Leloir, Luis F. and Nocito, V.

Hanby, W. E. and Rydon, H. N.

Hartelius, Vagn

Herbst, Robert M.

Housewright, Riley D. and Thorne, Curtis B.

Hunter, F. Edmund
Hunter, F. Edmund and Leloir, Luis F.

Jackson, Richard W. and Jackson, W. T.

Jensen, H. L.

Konikova, A. S., Kritzmann, M. G. and Yakobson, L. M.

Konikova, A. S., Kritzmann, M. G., Yakobson, L. M. and Samarina, O. P.

Koplanskii, S. Ya. and Shmerling, Zh.

Koser, S. A., Wright, M. H. and Dorfman, A.

Krampitz, L. O. and Werkman, C. H.

Krebs, Hans Adolf

Krebs, H. A., Eggleston, L. V. and Hems, R.

Krebs, H. A., Eggleston, L. V. and Hems, R.


Krebs, H. A. and Johnson, W. A.

Kritzmann, M. G.
1944. The role of CO₂ in the formation of amino acids from pyruvic acid and NH₃ in liver slices. Biokhimija 9, 379-388. (Original not available; abstracted in Chemical Abstracts 39, 3315, 1945.)

Kritzmann, M. G.

Kritzmann, M. G. and Melik-Sarkisyan, S. S.

Kritzmann, M. G. and Melik-Sarkisyan, S. S.
Kritzmann, M. G. and Melik-Sarkisyan, S. S.

Kritzmann, M. G. and Samarina, O. P.

Krueger, R.

Lardy, Henry A. and Zeigler, John A.

Lichstein, Herman C., Gunsalus, I. C. and Umbreit, W. W.

Lipmann, Fritz

Lipmann, Fritz

Lundsgaard, Einar

Lwoff, André and Monod, Jacques

Lyman, Carl M., Moseley, Olive, Wood, Suzanne, Butler, Betty and Hale, Fred
(Original not available; abstracted in Chemical Abstracts 43, 5061, 1949.).

McCalla, T. M.

Meister, Alton and Greenstein, Jesse P.

Minz, Bruno, Cohen, Georges N. and Cohen-Bazire, Germaine

Morrison, G. A. and Hinshelwood, Sir Cyril

Nachmansohn, D., John, H. M. and Waelsch, Heinrich
1943. Effect of glutamic acid on the formation of acetylcholine. J. Biol. Chem. 150, 485-496.

Nielsen, Niels

Nielsen, Niels and Johansen, Gordon

Novelli, G. David and Lipmann, Fritz

Ochoa, Severo
Ochoa, Severo, Mehler, Alan H. and Kornberg, Arthur  
1948. Biosynthesis of dicarboxylic acids by CO₂ fixation.  
I. Isolation and properties of an enzyme from  
pigeon liver catalyzing the reversible oxidative  
decarboxylation of l-malic acid. J. Biol. Chem.  
174, 879-1000.

Polson, A.  
161, 351-352.

Potter, Richard L. and Elvehjem, C. A.  
1948. Biotin and the metabolism of Lactobacillus  
arabinosus. J. Biol. Chem. 172, 531-537.

Pregl, Fritz and Grant, Julius  

Ratner, Sarah  
1949. Mechanism of urea synthesis. Federation Proc. 8,  
603-609.

Reiner, John M.  
1947. Metabolic intermediates in adaptive fermentation  
of galactose by yeast. J. Gen. Physiol. 30, 355-  
365.

Rose, Wm. C., Oesterling, M. Jane and Womack, Madelyn  
1949. Comparative growth on diets containing ten and  
nineteen amino acids, with further observations on  
the role of glutamic and aspartic acids. J. Biol.  
Chem. 176, 753-762.

Rossi, A. e Ruffo, A.  
1941. Formazione di acido glutammico dall acido citrico  
nel muscolo. Boll. soc. ital. biol. sper. 16,  
652-654.

Ryabinovskaya, A. M.  
1939. Influence of sodium fluoride, sodium maleinate,  
glutamic acid and glyceraldehyde on muscular ac-  
tivity. Compt. rend. acad. sci. U. S. S. R. 23,  
953-961. (Original not available; abstracted in  
Chemical Abstracts 34, 1078, 1940.).

Sahyun, Melville  
Sapirstein, Milton R.

Sauberlich, H. E. and Baumann, C. A.

Schlenk, F. and Fisher, A.

Schurr, P. E., Thompson, H. T., Henderson, L. M. and Elvehjem, C. A.

Seits, I. F.
1949. Role of potassium and ammonium ions in the transfer of phosphate from phosphopyruvic acid to the adenylic system. Biokhimiya 14, 134-140. (Original not available; abstracted in Chemical Abstracts 43, 6281, 1949.).

Silverman, M. and Werkman, C. H.

Simola, P. E. und Alapeuso, HellM

Simola, P. E. und Krusius, F. -E.

Snell, Esmond E.

Snell, Esmond E. and Guirard, Beverley M.
Speck, John F.

Sprinson, David B. and Rittenberg, D.

Stokes, Jacob L. and Gunness, Marion

Stokes, J. L., Larsen, Alma and Gunness, Marion

Tang, P. S. and Haueh, T. Y.

Taylor, E. Shirley

Taylor, E. Shirley

Utter, M. F. and Werkman, C. H.

Villano, F. e d'Ambrosio, L.

Virtanen, A. I.
Virtanen, Artturi I. and Osaky, Tihamer Z.

Virtanen, Artturi I., Hakala, Maire and Järvinen, Helvi

Virtanen, Artturi I., Hilkka, Linkola, Hakala, Maire and Rautanen, Niilo

Virtanen, Artturi I. and Laine, T.

Virtanen, Artturi I. and Laine T.

Virtanen, Artturi I. and Laine T.

Waelsch, H. and Rittenburg, D.

Watt, Dean

Wiggert, W. P., Silverman, M., Utter, M. F. and Werkman, C. H.

Winnick, Theodore, Moring-Claesson, Ingrid and Greenberg, David M.


ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. C. H. Werkman for the opportunities, suggestions and assistance offered in the course of this investigation and to others for their cooperation and helpful suggestions.