March 7, 1974

Ms. Beverly J. Hungerford
Grant Administrator
National Aeronautics and Space Administration
Washington, D. C. 20456

Re: DHC-2 (B. J. Hungerford)
NASA No. NGR 26-003-023 completed 9-30-69
University of Missouri Research Fund Code No. 5814-2229

Dear Ms. Hungerford:

Your letter of January 31, 1974 to Mr. Rudy L. Koch, University of Missouri, Mgr., Research and Restricted Accounting, 118 University Hall, Columbia, Missouri 65201 has been referred to me.

A 15 page, Final Composite Progress Report has been already forwarded to Dr. Joe Saunders, NASA, on November 1, 1969. Enclosed is a copy for your records. If you wish additional copies, there are 20 or so copies on file in Dr. Joe Saunders' office, or you might duplicate this one if needed.

Page 14 of this report has a list of 10 publications from this NASA-supported research project. Since that was prepared in 1969, it is out of date. Therefore, I now enclose a revised bibliography that should be filed with the original report to Dr. Joe Saunders. Dr. Saunders has the reprints referred to therein (sent on May 5, 1970 and November 5, 1971).

I hope this Report meets your needs. If not, please advise me. We were certainly appreciative of the support for NASA for the two-year period during which this research grant was effective. If NASA sees a way in the future to continue this fundamental biochemical research which has also carry-over in bioregeneration-recycling, I hope you will so advise us. We have the laboratory expertise, procedural know-how, the intellectual resources and the literature knowledge to be able to continue this or closely related work.

Sincerely,

Robert L. Wixom, Ph.D.
Professor
Principal Investigator of earlier NASA Research Grant NGR-26-003-23

cc: Dr. Joseph F. Saunders
Mr. Rudy L. Koch
Mr. Willis E. Linneman
Dr. Benedict J. Campbell
Dr. Robert H. Schiffman
Project Title: Application of Nitrogen Metabolism in Autotrophic Bacteria to Chemosynthetic Bioregeneration in Space Missions.

Project Sponsor: National Aeronautics and Space Administration

Project Number: NASA No. NGR 26-003-23; University of Missouri, No. 5814-2229

Project Initiated: 10-1-67 (fiscal start)

Principal Investigator: Dr. Robert L. Wixom
    Associate Professor of Biochemistry and
    Research Associate, Space Sciences Research Center,
    School of Medicine
    University of Missouri
    Columbia, Missouri 65201

Period covered by earlier Final Composite Progress Report: 10-1-67 to 9-30-69; prepared on 10-20-69.

Date of this supplement prepared: 3-5-74

Scope of this summary: This Summary includes all the publications from the research supported by NASA and the Space Science Research Center, University of Missouri. Reprints of all except the last one have been previously forwarded to NASA.
List of Recent Publications Related to Grant


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Abstract

The chemolithotroph, *Hydrogenomonas eutropha*, is being considered as a life support, bioregenerative system. This project focuses on several metabolic functions that are related to the proposed nitrogen cycle between man and this microbe. Specifically this organism has the capability to utilize as the sole nitrogen source such urine components as urea and fifteen individual amino acids, but not nine other amino acids. The effectiveness of utilization was high for many amino acids. Several specific growth inhibitions were also observed.

The enzyme that catalyzes the incorporation of ammonia in the medium into amino acids has now been identified as a NADP-specific, L-glutamate dehydrogenase. This enzyme has a constitutive nature.

This organism can synthesize all of its amino acids from carbon dioxide and ammonia. Several of the enzymes that have been rigorously proven to participate in valine-isoleucine biosynthesis in the heterotroph, *Escherichia coli*, have now been found in cell-free extracts of *H. eutropha*, *H. facilis* and *H. ruhlandii*. Therefore with the background literature of multiple pathways of individual amino acid biosyntheses, our evidence to date is consistent with the *Hydrogenomonas* group having the same pathway of valine-isoleucine formation as the classical *E. coli*.
IA. Project Objectives

Since a key problem in prolonged human space flight is a continued source of food for the astronaut, recent NASA research has focused on investigation of unicellular organisms as a food-manufacturing, waste-conversion unit in a self-sustaining, life-support system. While others have examined the photosynthetic algal system, this project has centered on autotrophic bacteria and particularly on the utilization of nitrogen and amino acid-protein metabolism in these microbes. *Hydrogenomonas* would derive its energy for cellular synthesis by the oxidation of hydrogen from water electrolysis. It would use the nitrogen in urine and, perhaps also, feces for the biosynthesis of its amino acids and then its cell protein. After appropriate processing, these microbial cells would be utilized as a source of protein and other nutrients for human food. These metabolic steps would be necessary to complete and to balance the carbon, nitrogen, oxygen and hydrogen in the proposed closed ecological system.

This particular project focuses on the microbial part of the nitrogen cycle, namely the utilization of nitrogenous compounds found in urine and feces, the biosynthesis of the microbial amino acids, and amino acid-protein metabolism in these microbes. As stated in the original NASA grant application, the emphasis of these investigations continues to be on microbial growth, the enzymes related to these transformations, and their metabolic adaptation or control.

IB. Summary of Two Years Activities in Project

- Introduction - In this Final Progress Report, the experimental results in Section IBA are described in considerable detail with a portion of the data in Tables or Figures. Since Section IBB has been accepted for publication in Biochim. Biophys. Acta, the description here is very limited. The data in Section IBC and IBD is described in abstract form, since preparation of a full journal manuscript is under way.

The earlier, complete, 17-year literature search on *Hydrogenomonas* in Chemical Abstracts, Biological Abstracts, and STAR has 153 references, which are grouped by subject content. This bibliography has been invaluable as a guide to the past literature, for design of present experiments and deducing future research directions. This search has provided knowledge of a closed ecological system for prolonged human space travel - i.e. a balanced symbiotic relationship between man and a biological factory as a source of man's essential amino acids, carbohydrate, and lipids. Since other investigators have examined in some detail the oxidation of hydrogen, electron transport, CO₂ fixation, polymer formation, gas ratios, pilot plant projects, and various bioengineering questions on *Hydrogenomonas sp.*, our emphasis has been on its nitrogen metabolism. At the time of grant application, our area of research had not been studied by earlier investigators and represented fundamental studies on the proposed symbiotic nitrogen cycle between man and *Hydrogenomonas*. 
Some of the experimental work was supported by the Space Sciences Research Center, University of Missouri, which is hereafter abbreviated SSRC.

IIa. Utilization of Nitrogenous Compounds by Hydrogenomonas as Related to Life Support Systems

Project Report - The ability of Hydrogenomonas to grow on the nitrogenous substances found in human urine has been explored by testing these pure compounds singly and in pairs. To compensate for some biological variability, the following observations are derived from duplicate experiments with triplicate, 25 ml Erlenmeyer flasks. These flasks were incubated in stainless steel, anaerobic jars with a \( \text{H}_2:0_2:CO_2 (7:2:1) \) gas phase and equilibrated by rotary agitation in a \( 30^\circ \) water bath. The conditions of gas mixing, salts medium, and \( 30^\circ \) incubation with rotary agitation followed those of Repaske (J. Bacteriol 85, 418-422 (1962)).

Under these conditions, the L-isomers of aspartic acid, alanine, glutamic acid, glutamine, and asparagine and urea were readily utilized as the sole sources of nitrogen at 24 and 48 hrs. growth (Table I). The nitrogen of proline, leucine, glycine, phenylalanine, threonine, histidine, and serine was only slowly released during the first 24 hours of growth, but gave good growth at the 48 hour reading. Tryptophan, isoleucine, and valine gave intermediate results.

H. eutropha failed to grow significantly within 48 hours when hydroxyproline, arginine, lysine or methionine was the sole nitrogen source in the medium (Table I). These results are similar to those briefly reported recently by Fraser-Smith, E. C. B., Austin, M. A. and Reed, L. L. (J. Bacteriol. 97, 457-459 (1969)). De Cicco, B. T. and Stukus, P. E., reported last year that H. eutropha utilized histidine, tryptophan and alanine (J. Bacteriol. 95, 1469-1475 (1968)). Blake, E. E. in a 1966 M.S. thesis reported the utilization of L-glutamate, L-alanine and L-tyrosine (STAR, 21, N66-10956 (1966), abstract).

Since the methyl histidines are found in human urine in significant amounts, they were tested under the above conditions. L-1-Methyl histidine and L-3-methyl histidine did not support growth, unlike L-histidine which gave intermediate growth. Since arginine did not support growth, other members of the Krebs-Henseleit urea cycle were tested. L-Ornithine and L-citrulline were also found to be non-utilizable for growth. Taurine is found in human urine, and was found to support growth under our conditions.

Variation of the \( \text{NH}_4\text{Cl} \) concentration in the medium confirmed the finding that 0.019 M was the optimal concentration (Fig. 1). Seven nitrogenous compounds were selected to test for growth, each at 0.001, 0.002, 0.003, 0.005, 0.010 and the usual 0.019 M final nitrogen concentration in the medium (Fig. 2). The growth response curves at 48 hours were approximately the same for \( \text{NH}_4\text{Cl}, \text{L-alanine}, \text{L-glutamate}, \text{L-glutamine}, \) and urea; the curves for glycine and histidine were slightly lower. The growth at the 0.001 M nitrogen concentration was 17 to 32% that of the 0.019 M \( \text{NH}_4\text{Cl} \) control.
The next objective was to search for the possible interactions of the utilizable amino acids and the slowly metabolized amino acids. These interactions might be: inhibition of growth of the utilizable amino acid, facilitation of growth of the so-called slowly metabolized amino acid and other more complex responses. The nature of these interactions should be defined in view of the proposed use of *H. eutropha* in a closed cycle, bioregenerative system. More specifically, since the poorly utilized amino acids will accumulate in a growth media based on human urine over prolonged growth periods, the following series of experiments was performed (Table II). The eight readily utilized nitrogen sources were added to the Erlenmeyer flasks at the suboptimal concentration of 0.001 M of nitrogen in the test compound and the seven non-utilized amino acids at the usual concentration of 0.019 M. The growth data indicates that tryptophan or arginine, in combination with one of the utilizable nitrogen sources, always promoted growth to a greater extent than either alone and, in some cases, to a greater extent than the sum of each pair of individual figures. Presumably the catabolism of tryptophan or arginine in a mixture in the medium was greater under these conditions than when present from the initiation of growth as the sole nitrogenous source. Isoleucine or valine with the same combinations promoted growth to a greater extent than either alone, with the exception of combination with L-glutamate or L-glutamine respectively. Hydroxyproline and methionine, in addition to being metabolically inert, inhibited the microbe's utilization of nitrogen from almost all sources tested, except histidine and glutamine. Other more individualized results of both growth promotion or inhibition were noted for the other combinations of amino acids (Table II).

*H. eutropha* was also autotrophically grown in 1-l Erlenmeyer flasks with rotary agitation at 30° with a reproducible generation time of about 2.0 - 2.5 hours for NH₄Cl, urea, or alanine; the maximum turbidity was about the same for these tubes. Use of a 30° water - jacketed, tissue culture, spinner flask with the fastest possible magnetic stirring gave poor growth (7.0 hr generation time). At Fe concentration of 8 mg/l, FeSO₄, Fe(NH₄)₂(SO₄)₂, FeCl₃ or Fe(NH₄)SO₄ led to the same generation time and same maximum turbidity. Consequently subsequent experiments have continued to use Repaske's FeSO₄ at the above concentration.

To utilize these findings on a larger scale with multiple simultaneous comparisons and to harvest sufficient cells for enzyme experiments, a new incubation system was devised with 6 high speed magnetic stirrers underneath a plexiglass 30° water bath containing flasks linked to the gas distribution system. The new stirrers (max. speed - 1700 RPM) led to entrainment of gas bubbles in the liquid medium. The optimal conditions to achieve a reproducible time of 1.8 - 2.2 hours were use of a 1.5 inch magnetic bar rotating at about 1300 RPM and absence of the baffles in the flasks. These conditions were maintained for the following series of experiments for variation of gas phase, nitrogen source, and carbon source. Whenever air was substituted for the H₂O₂:CO₂ gas mixture for growth on alanine, glucose plus NH₄Cl, or glucose plus alanine, the generation time was prolonged and turbidity readings at 48 hours were lower. Alanine as the sole nitrogen source with the H₂O₂:CO₂
gas mixture for growth on alanine, glucose plus NH\textsubscript{4}Cl, or glucose plus alanine, the generation time was prolonged and turbidity readings at 48 hours were lowered. Alanine as the sole nitrogen source with the H\textsubscript{2}:O\textsubscript{2}:CO\textsubscript{2} gas phase gave the same generation time as NH\textsubscript{4}Cl, but lower maximum growth. Growth on alanine in air with its lowered CO\textsubscript{2} supply impaired growth by both criteria; but since positive growth occurred in these flasks, H. eutropha can degrade the carbon skeleton of alanine and reutilize the carbon for synthesis of other cellular components. Heterotrophic growth, achieved by addition of glucose, gave a similar growth response to chemolithotrophic growth with NH\textsubscript{4}Cl or alanine as the nitrogen source. These studies are also related to the efficient reutilization of urinary nitrogen through bacterial protein to complete the nitrogen cycle.

These results with exact concentrations and identities of amino acids can be reviewed in the light of information on the known composition of urinary nitrogen compounds. The \(\alpha\)-amino nitrogen concentration in normal human adult urine is 0.3-0.7 gm N per day. Within this nitrogen fraction are found the following amino acids (arranged in decreasing order of concentration): glycine, 71-416 mM x 10\textsuperscript{2} per 24 hours; taurine, 22-185; histidine, 13-137; 1-methyl-histidine, 13-93; glutamine and asparagine, 29-77; serine, 21-62; 3-methyl-histidine 18-52; alanine, 6-50, and variable lower concentrations of threonine, tyrosine, phenylalanine, isoleucine, leucine, cystine, lysine, glutamic acid, valine, aspartic acid, methionine, arginine and ornithine. Comparison of our H. eutropha growth data and this urinary composition indicates that 1- and 3- methyl-histidines, methionine, lysine and arginine were not utilized by H. eutropha (Table I). Lysine and methionine were poorly utilized in a mixed nitrogen source, and may be predicted to accumulate during continuous growth of H. eutropha on urine in a bioregenerative system.

In addition to these amino acids, other nitrogenous compounds are found in urine. A variety of project reports to NASA describe 
Hydrogenomonas strains that have been found to utilize urea, the main nitrogenous component of urine (see next Section IBb). Autotrophically-grown H. eutropha can utilize the nitrogen of uric acid, allantion, hippuric acid and urea, but not creatinine (Ammann, E. C. B., and Reed, L. J., Biochim. Biophys. Acta 141, 135-143 (1967). H. facilis utilizes thymine, cytosine, and uracil, whereas Hydrogenomonas H16 degraded only cytosine (Kaltwasser, H. and Kramer, J., Arch. Mikrobiol. 60, 172-181 (1968). All of the above studies and our investigations are related to the problem of efficient reutilization of urinary nitrogen through bacterial protein, human dietary protein, and repetition of the cycle.

**Personnel on Project** - Mr. R. S. Becker, Mr. K. S. Suh and Dr. R. L. Wixom.

**Degree of NASA Support** - Joint with SSRC research grant.

**Pertinent Publications Completed or in Preparation** - See II and 8.
## GROWTH OF H. EUTROPHA WITH SINGLE NITROGEN SOURCES

<table>
<thead>
<tr>
<th>Growth characteristic</th>
<th>Sole nitrogen source</th>
<th>Av. % growth of NH$_4$Cl control At 24 hrs.</th>
<th>At 48 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>130</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>126</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>118</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>112</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>110</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>100 (278*)</td>
<td>100 (547*)</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>33</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td><strong>Medium group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>33</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>30</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>30</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>27</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>22</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>17</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>13</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td><strong>Slow group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>17</td>
<td>40</td>
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</tr>
<tr>
<td>Isoleucine</td>
<td>9</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>3</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><strong>No growth group</strong></td>
<td>Hydroxyproline</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Arginine</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>3</td>
<td>2</td>
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</tr>
</tbody>
</table>
GROWTH OF H. EUTROPHA ON NH₄Cl

Growth at 48 hrs

Growth at 24 hrs

NH₄Cl CONC. IN MEDIUM (M)
UTILIZATION OF SINGLE N SOURCES BY H. EUTROPHA

% GROWTH OF NH₄Cl CONTROL

N CONCENTRATION IN MEDIUM (M)

- NH₄Cl
- Alanine
- Urea
- Histidine
- Glutamate
- Glycine
- Glutamine
INTERACTION OF TEST AMINO ACIDS WITH
UTILIZABLE NITROGEN SOURCES BY H. EUTROPHA

<table>
<thead>
<tr>
<th>Utilizable N source (0.001 M N)</th>
<th>None</th>
<th>Try</th>
<th>Arg</th>
<th>Ile</th>
<th>Val</th>
<th>Lys</th>
<th>Hyp</th>
<th>Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40</td>
<td>4</td>
<td>37</td>
<td>20</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>26</td>
<td>46</td>
<td>35</td>
<td>72</td>
<td>69</td>
<td>35</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>22</td>
<td>45</td>
<td>38</td>
<td>73</td>
<td>68</td>
<td>11</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>23</td>
<td>59</td>
<td>25</td>
<td>25</td>
<td>40</td>
<td>14</td>
<td>19</td>
<td>11</td>
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<tr>
<td>L-Glutamine</td>
<td>26</td>
<td>51</td>
<td>31</td>
<td>51</td>
<td>22</td>
<td>48</td>
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<td>20</td>
</tr>
<tr>
<td>Urea</td>
<td>37</td>
<td>104</td>
<td>53</td>
<td>108</td>
<td>74</td>
<td>6</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>Glycine</td>
<td>27</td>
<td>65</td>
<td>47</td>
<td>54</td>
<td>65</td>
<td>25</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>26</td>
<td>80</td>
<td>35</td>
<td>42</td>
<td>45</td>
<td>32</td>
<td>25</td>
<td>32</td>
</tr>
</tbody>
</table>
Enzymes in Nitrogen Metabolism in H. eutropha

Project Report - A review of the literature (our own continuing bibliography and that by Dr. J. Pulliam and F. Hong, G. Washington University Medical Center, Washington, D.C., NASA contract no. NSR-09-010-027, September, 1968) indicated that the enzymatic route of ammonia assimilation in Hydrogenomonas has not been demonstrated to date. Earlier investigators had shown that Hydrogenomonas extracts contained glutamate dehydrogenase activity, but in view of two other known microbial routes of ammonia assimilation, the mere presence of an enzyme does not prove the existence of a pathway. Since this area of our research has been written and accepted for publication, a photocopy of this manuscript for Biochim. Biophys. Acta is attached in lieu of description here.

For understanding metabolism, it is not sufficient to know solely that certain enzymes are present in a microbe; such knowledge must be coupled with both the biosynthesis and function of the enzyme under various conditions of medium, gas phase, growth cycle, continuous culture, and other environmental factors. To use more modern phrasing, the next research phase would be to elucidate the metabolic control mechanism(s) for the pertinent enzymes. Therefore, pending continued adequate research support, future research should emphasize the search for possible controls such as induction, repression, end product inhibition, allosteric effectors, subunit structure and other even more refined strategies for regulation of cellular events.

Our initial NASA grant application (prepared 9-1-66) raised a variety of questions and proposed experiments on the enzymatic utilization of urea. Shortly thereafter, the responsible enzyme in Hydrogenomonas H16 was identified as urease (König, C., Kaltwasser, H., and Schlegel, H. G., Arch. Mikrobiol. 53, 231-241 (1966)). Urease, found in autotrophically and heterotrophically grown cells, was induced by urea and repressed by ammonium salts. Urease underwent an oscillatory change during the growth cycle of Hydrogenomonas H16 (König, C., and Schlegel, H. G., Biochim. Biophys. Acta 152, 182-185 (1967). With awareness of these recent, careful studies, we have pursued other aspects in our original NASA grant proposal.

The enzyme responsible for utilization of uric acid, another urinary nitrogenous component, by Hydrogenomonas H16 has been demonstrated to be uricase (Kaltwasser, H., Arch. Mikrobiol. 60, 160-171 (1966). Uricase was found to be an inducible enzyme and found in a particulate fraction.

To return to our investigations, we have recently found glutaminase- and asparaginase activities in cell-free extracts of H. eutropha. Continued exploration of this finding and other enzymes in nitrogen metabolism will depend on an adequate source of funds.
Personnel on Project - Mr. A. A. Joseph, Mr. R. S. Becker and Dr. R. L. Wixom.

Degree of NASA Support - 100%.

Pertinent Publications Completed or in Preparation - See II-4, 7, and 8.

Ibc. Waline-Isoleucine Biosynthesis in Hydrogenomonas

Introduction - The pathways of the biosynthesis of amino acids in microorganisms have been deciphered in considerable detail for Escherichia coli and Neurospora crassa by mutant studies, radiotracer experiments and enzymatic investigations. Then other diverse microorganisms and plants were examined. After the initial reporting period of apparently identical pathways, it became apparent that there are two different pathways for the formation of lysine, glutamic acid and tryptophan in different microbial systems. The metabolic regulation of these biosynthetic pathways is even more heterogeneous. For instance, five different patterns of regulation of the formation of the aspartate family of amino acids are now known. The possibility of diversity in the biosynthetic routes in the chemolithotrophic bacterium has not been previously studied.

The pathway of valine-isoleucine formation involves four parallel biosynthetic steps by common enzymes, namely acetolactate synthetase, reductoisomerase, dihydroxyacid dehydratase, and an aminotransferase. The evidence for this pathway in E. coli and N. crassa has accumulated from the research of many investigators. Although the seven previous publications by this author have demonstrated this pathway in many other heterotrophs and many higher plants, there has been to date essentially no investigations other than the authors on amino acid biosynthesis in the chemolithotrophs.

Project Report - Research on the chemolithotrophic Hydrogenomonas proceeded by searching for the biosynthetic enzyme, dihydroxyacid dehydratase, E. C. 4.2.1.9. This enzyme is in the middle of the E. coli valine-isoleucine biosynthesis, and has a unique substrate. Log phase cells of heterotrophically grown H. facilis, heterotrophic and autotrophic, H. rublandii and H. eutropha were harvested, ruptured by ultrasonic oscillation and assayed for dihydroxyacid dehydratase. These extracts catalyzed the dehydration of DL-α, β-dihydroxyisovaleric acid and where tested, DL-α, β-dihydroxy-β-methyl-α-valeric acid to their corresponding keto acids. These compounds are known intermediates of respectively valine and isoleucine biosynthesis in Escherichia coli. The optimal pH of the dehydratase from autotrophic H. eutropha was pH 8.0-8.2. The enzymatic product was identified as α-ketoisovalerate by thin layer chromatography of the 2,4-dinitrophenylhydrazine derivatives on silica gel as compared with other known ketoacid hydrazones. This extract catalyzed the dehydration of DL-α,β-threo-dihydroxybutyrate at a faster rate than the erythro isomer, but did not alter 3 other dihydroxy acids. Serine dehydratase activity was found, but the dehydratases or desulphhydrases for threonine, homoserine, cysteine and homocysteine were absent.
Finding the above serine dehydratase activity led to experiments to differentiate these several activities. The addition of pyridoxal phosphate to the dehydratase assay system increased the rate of keto acid formation from L-serine, D-serine, and L-tartrate, but not from dihydroxyisovalerate as a substrate. Furthermore the addition of hydrazine or semicarbazide inhibited keto acid formation from L-serine, D-serine, and L-tartrate, but not from dihydroxyisovalerate as the substrate. Therefore these experiments demonstrate the existence of a dihydroxyacid dehydratase independent of other incidental dehydratases in the extract.

The reductoisomerase enzyme has also been demonstrated in H. eutropha extracts. Omission of the substrate, acetylactate, caused a loss of activity. Deletion of NADPH, or substitution of NADPH by NADH, led to a loss of activity. Omission of Mg$^{++}$ did not lead to appreciable decrease of activity in this crude system.

This enzymatic evidence is consistent with the presence of the E. coli route of valine-isoleucine biosynthesis in the chemolithotrophic bacteria Hydrogenomas eutropha.

Personnel on Project - Mr. A. A. Joseph and Dr. R. L. Wixom.

Degree of NASA Support - Shared with SSRC research grant.

Pertinent Publications Completed or in Preparation - See II - 1, 3, 4 and 9.

IBd. Metabolic Controls of Valine-Isoleucine Biosynthesis

Project Report - With the main emphasis on the previously described research areas, only shorter attention was given to this sub-research area, which was also described in the original research grant application. To introduce this subject, Cutinelli, et al. (Arkiv. Kemi 3, 315-320 (1951) found that C$^{14}$-acetate was incorporated into valine and other amino acids of the photoorganotroph, Rhodospirillum rubrum, in a different pattern from that previously obtained in Escherichia coli and Neurospora crassa. Thus, search for the presence of the valine-isoleucine biosynthetic enzymes in R. rubrum and Rhodopseudomonas spheroides, two members of the non-sulfur purple bacteria of the Athiromodaeae family was undertaken. Cell-free extracts of R. spheroides and R. rubrum, grown aerobically in the dark and anaerobically in the light, were found to catalyze the conversion of αβ-dihydroxyisovaleric acid to a keto acid. Dehydratase activity was present when cells were grown with glutamate-malate or acetate as carbon sources. Other characteristics of this dihydroxyacid dehydratase were described earlier (First NASA Semiannual Progress Report; Ref. 1 and 2). The activities of a valine-glutamate aminotransferase, reductoisomerase and acetohydroxyacid synthetase were also demonstrated several years ago and set the stage for the following new studies of one of several possible metabolic controls in the photosynthetic bacteria.

The first enzyme in the valine pathway is acetohydroxyacid synthetase; it was first studied in R. spheroides extracts in the summer of 1968 during the tenure of a medical student research fellowship. The required
components in its assay system were shown to be phosphate buffer, pH 7.4, pyruvate, thiamin pyrophosphate, Mg++, FAD, and extract. The product of the reaction was α-acetolactate, a known intermediate in the E. coli pathway of valine biosynthesis. After an initial lag, the enzyme activity was proportional to the time of reaction. Activity was also proportional to low pyruvate concentration; high concentrations caused substrate inhibition. The following characteristics were determined: optimal pH - 7.4, $K_m$ pyruvate - $4.5 \times 10^{-3}$M, $K_m$ TPP - $2.96 \times 10^{-5}$M, and $K_m$ Mg++ - $1.01 \times 10^{-3}$M. Addition of FAD at 5 uM enhanced the activity by 2.5 fold. The enzyme was sensitive to endproduct inhibition by valine in a competitive pattern according to a double reciprocal plot. Since isoleucine, leucine, alanine, phenylalanine, lysine, and glutamic acid were not inhibitory, the inhibition was specific for valine.

To summarize, the results of these experiments demonstrate that the E. coli pathway of valine biogenesis also occurs in the above photosynthetic bacteria, and that endproduct inhibition is at least one of the mechanisms to regulate valine biosynthesis in this photosynthetic bacterium.

**Personnel on Project** - Mr. R. J. Semeraro and Dr. R. L. Wixom.

**Degree of NASA Support** - Small amount of funds for consumable supplies - shared with SSRC research grant.

**Pertinent Publications Completed or in Preparation** - See II - 6, 9 and 10.
II. List of Recent Publications Related to Grant


2. Semeraro, R. J., and Wixom, R. L., The Pathway of Valine-Isoleucine Biosynthesis in Photosynthetic Bacteria. Trans. Mo. Acad. Sci. 1, 82 (1967); Abstract; reprints forwarded earlier to NASA and served as background for Hydrogenomonas research.

3. Joseph, A. A., and Wixom, R. L., The Biosynthesis of Valine and Isoleucine in Autotrophic Bacteria. Society for Experimental Biology and Medicine, Annual Meeting of the Missouri Section at the University of Missouri, Columbia, Mo., December 9, 1967; Abstract; reprints forwarded earlier to NASA.


III. Research Personnel Associated with NASA-SSRC grants

Dr. Robert L. Wixom, Principal Investigator of NASA grant, Associate Professor of Biochemistry, Research Associate in Space Sciences Research Center, University of Missouri, Columbia, Mo.

Dr. Augustine A. Joseph, Ph.D. degree completed December 19, 1968; thesis entitled, Amino Acid Metabolism of Propionibacteria and Hydrogenomonas; half-time Research Assistant on NASA grant as graduate student from 10-1-67 to 12-30-69 on subject areas IBb and Ibc; present position, Postdoctoral Research Fellow, Rockefeller University, New York, N. Y.

Mr. Russell J. Semeraro, Ph.D. candidate, degree expected February 1970; thesis entitled, Valine-Isoleucine Biosynthesis in Photosynthetic Bacteria; half-time Research Assistant on earlier SSRC project; a medical student since the fall of 1967; during the tenure of a summer 1968 medical student research fellowship, he completed his final thesis experiments, for which only small amounts of SSRC and NASA Consumable Supply funds were used for subject area IBa.

Mr. Richard S. Becker, Research Assistant on NASA grant from 2-1-68 to 8-31-69 on subject area IBa and Ibb, biochemistry - microbiology background.

Mr. Ki Surk Suh, new biochemistry graduate student, U. S. citizen; half-time Research Assistant on SSRC grant in 1968 on subject area IBa.