MAGNA CORPORATION

Research and Development Division

1001 SOUTH EAST STREET • ANAHEIM, CALIFORNIA

RESEARCH ON APPLIED BIOELECTROCHEMISTRY

Report Nr. 1

CONTRACT NASw-623

FIRST QUARTERLY PROGRESS REPORT
March 14 to June 30, 1963

Prepared for:
National Aeronautics and Space Administration
Washington, D. C.

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Object of the Research:

Utilization of human wastes as electrochemical fuels

Approved by:

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1.C PURPOSE

The purpose of work undertaken in this effort is to find environmental conditions and microbial life which are most favorable to bioelectrochemical utilization of human wastes in space vehicles. The principal function of this program is obtaining power from bioelectrochemical cells using human wastes. A secondary function of this program is conversion of the waste materials to chemicals that can be reused in maintenance of the space vehicle and its occupants.

This effort has been subdivided into three major tasks. The first, a literature search, and preliminary selection of organisms and enzymes, which are attractive (a) for production of electrochemically active chemicals from human wastes, and (b) for conversion of waste materials is completed. The experimental effort is divided between essentially biological functions and essentially electrochemical functions, into two tasks. The biological task is concerned with screening and characterization of microorganisms and enzymes with respect to conditions which predispose to their most effective conversion of wastes to electroactive chemicals and to otherwise useful chemicals. The electrochemical task has as its purpose the evaluation of biological electrodes with respect to optimizing and defining electrochemical parameters for effective utilization of the waste materials, primarily with respect to production of power. A further function of the electrochemical task is the preparation of biological electrodes having the biological material immobilized at the electrode surface.

Other programs which relate directly to the present work are being undertaken by Marquardt Corp. under Contract NASw-654 and Aeronutronic Corp. under Contract NASw-655. These programs are concerned with development and fundamental research in bioelectrochemistry of human wastes, respectively. Magna Corporation, under Contract DA 36-039 SC-90866, is performing research on biochemical fuel cells. Further, Magna has a contract with the Department of the Navy (NObs 84243) to study biochemically promoted power sources.

2.0 ABSTRACT

A literature search for microorganisms and enzymes attractive for (1) production of hydrogen and ammonia from urine and feces and (2) conversion of urine and feces to chemicals re-useable in a closed environment has been completed. The organisms and enzymes selected for screening and further study are the following:

(1) The urea bacteria for production of ammonia from urine.

(2) Escherichia coli, Aerobacter aerogenes, a Bacillus species, and a Clostridium species for hydrogen and ammonia production from feces and for proteolysis of feces.

(3) Cellulase and lipase for degradation of fecal cellulose and lipids, respectively.

(4) Urease for ammonia production from urine.

To attempt optimum utilization of feces and urine both as an electrochemical fuel and for conversion of re-useable chemicals is believed inconsistent. It has been decided tentatively to optimize primarily for utilization of these materials as electrochemical fuels.

Screening of the urea bacteria has been completed with a study of five organisms. The major criterion, anaerobic growth and hydrolysis of urea in urine, was met satisfactorily by only one organism, Bacillus pasteurii. This organism will be used in future work with urine.

Only limited effort has been expended in screening for fecal degradation. The high variability in human feces is the major reason. To attack this problem, a collection program has been initiated wherein volunteers consuming a simulated space diet are contributing feces and urine. These materials, when sufficient has been collected, will be composited and used as a source of consistent starting materials for the remainder of the program.

Electrochemical evaluation of urine has begun but results are inconclusive as yet.

3.0 CONFERENCES

June 10, 1963

A conference attended by personnel of Magna Corporation and Marquardt Corporation was held at Marquardt on June 10. The purpose of this conference was the discussion of (1) the two organizations' current efforts in bioelectrochemistry relating to Contracts NASw-623 and NASw-654, (2) coordination of the two efforts to avoid possibilities of duplication of effort, and (3) coordination of the two contractors' efforts in obtaining consistent starting materials (human feces and urine).

It was concluded that duplication of effort could most readily be avoided by Magna's efforts being directed primarily to pure cultures of microorganisms, or relatively well defined mixtures of organisms, and isolated enzymes, and by Marquardt's efforts being directed primarily, for the present, to indigenous organisms of feces. To maintain coordination, it was agreed to hold similar meetings at monthly intervals.

4.0 FACTUAL DATA

4.1 Introduction

Research on applied bioelectrochemistry as it relates to the use of the human wastes urine and feces has been initiated. A search of the literature, pertinent to the microbiology and enzymology of human feces and urine, and directed to the utilization of these materials as electrochemical fuels in space vehicles, was the first step in this research. The literature search is reported herein in its entirety.

Based on the literature search, microorganisms and enzymes were selected for study of their properties and requirements so as to lead to optimum utilization of urine and feces. The initial phases of this study are reported here in terms of biological evaluation of useful parameters and preliminary electrochemical evaluation.

4.2 Literature Search

It was found convenient to subdivide the literature search into a number of categories: (1) sewage digestion, (2) fecal components, (3) urine components, (4) enzymes, and (5) microbial production of ammonia and hydrogen. Preliminary evaluation of the literature revealed that very little information was available on biological conversion of human feces or urine per se, but that the most nearly related data existed in the literature on sewage digestion. In the absence of specific information on feces and urine, information was sought on components of these materials, to guide the search of the microbiological and enzymological literature. An early decision was made to consider only those enzymes which would provide relatively extensive breakdown of human wastes. Thus only hydrolytic enzymes were searched. Finally, in the interests of optimizing power production from urine and feces, the literature was searched for information on microbial production of hydrogen and ammonia, considered to be the most likely electro-active chemicals which can be obtained in significant quantities from urine and feces.

4.2.1 Microbiology

4.2.1.1 Sewage Digestion

Ingram[1] has adequately reviewed the microbiology and processes involved in conventional waste treatment. Rather than needlessly duplicate Ingram's effort, our literature search will describe the salient points concerning conventional waste treatment and concentrate more on the microbiology and degradation of human waste as it pertains to our particular interests.

Biological processes for waste disposal convert the organic components of the waste to cell materials which is then partially oxidized by the metabolic activities of the organisms. The products of this metabolic activity are inorganic oxidation products and a mass of microorganisms which is conventionally known as sludge. There are two basic types of biological processes for waste disposal, one aerobic (activated sludge) and the other anaerobic.

In general, there are two kinds of bacteria involved in aerobic waste disposal systems. The first group of bacteria utilizes the organic materials in waste and the second group feeds upon their metabolic by-products. This process requires good aeration of the waste and the formation of a floc which results from the growth of zoogleal organisms.[2] The organic materials adhere to the surfaces of the floc, which results in rapid degradation. Examining the microbiology of activated sludge, Butterfield[2] isolated a zoogleal bacterium and named it Zoogloa ramigera. Buck and Keefer[3] isolated a similar organism from activated sludge and reported that it produced significant quantities of ammonia from peptones. McKinney and Weichlein[4] isolated many floc-producing bacteria from activated sludge. They considered the organisms listed in Table I to be the most important in waste disposal. Russian workers[5] concerned with biochemical purification of industrial and domestic effluents have also studied the microbiological flora of activated sludge, and they have reported the isolation of many organisms cited by McKinney and Weichlein.[4]

Kaplovsky[6] has differentiated the anaerobic process into three basic stages:

1. Intensive acid production
2. Intensive digestion or liquification
3. Intensive digestion and gasification

TABLE I

Floc-Producing Bacteria Isolated from Activated Sludge

Zooglea ramigira

Escherichia coli

Escherichia freundii

Pseudomonas perluria

Pseudomonas ovalis

Alcaligenes faecalis

Alcaligenes metalcaligenes

Bacillus megaterium

Lactobacillus caseii

Neisseria catarrhalis

Aerobacter aerogenes

Flavobacterium solare

Flavobacterium breve

Micrococcus conglomeratas

Micrococcus varians

Achromobacter liquefaciens

The major gases produced are methane and carbon dioxide, with a little hydrogen sulfide and hydrogen. Up to 75% of the total volume of gas may be methane. It has generally been observed that obligate anaerobes do not form a major portion of the microorganisms involved in anaerobic sludge digestion but that facultative anaerobes predominate. The microorganisms reported to be in anaerobic sludge are presented in Table II.

One of the problems associated with standard waste disposal systems is the large amount of solids remaining after digestion. Leone[7] has reported on aerobic and anaerobic human waste treatment processes designed for space systems and has found that the activated sludge system stabilized in several hours with a 20% reduction in initial solids (3.7 gm/l). Subsequent processing in an aerobic digestor reduced total solids an additional 15%, but required a month to do so. Ingram[1] found anaerobic digestion of human wastes difficult to initiate and reported that poorly digested, foul smelling sludge resulted.

Kountz and Forney[8] advanced a theory concerning a two-phase activated sludge system that would result in complete oxidation. Phase I would consist of the assimilation of substrates and Phase II involved the endogenous oxidation of metabolic by-products. Their results indicated the accumulation of non-oxidizable sludge at the rate of 0.6% of the total weight per day.

Garret[9] reported on control of activated sludge growth by regulated overflow to a settling tank. The mathematics involved are very similar to that used in continuous culture of microorganisms, and he reported a more consistent growth rate of the floc. The use of continuous culture for aerobic waste disposal has been adapted to an algal growth unit by Golueke, Oswald and McGanky.[10] Their system used the air activated sludge process to digest raw sewage in order to provide the algae with nutrients such as carbon dioxide, while the algae produced oxygen for the growth of the bacteria. The waste used in this study was obtained from an individual who was on a roughage-free, low salt, and low fat diet. They found that after the addition of waste to the culture, the fecal odor disappeared in several hours and was replaced by a strong odor of ammonia.

TABLE II

Microorganisms Isolated from Anaerobic Sludge

Microorganism	Reference
Bacillus subtilis	Ruckhoft, Kallas and Edwards[37]
Bacillus endorhythmas	Buck, Keefer and Hatch[38]
Escherichia coli	Ruckhoft et al.[37]
Aerobacter aerogenes	Ruckhoft et al.[37]
Methane bacterium formicicum	Ingram[1]
Methanobacterium omelianski	Ingram[1]
Methanobacterium suboxidans	Ingram[1]
Streptococcus diploides	Buck, Keefer and Hatch[39]

After an additional few hours, an actinomyces odor developed; i.e., the odor of rich damp soil

Golueke and Oswald[10] reviewed the literature and reported that Chapman[11] was successful in stabilizing the waste of one man seeded with 1/8 cu. ft. of activated sludge culture using a highly aerated process. Bogen and Chapman,[12] in a later study, reported that human wastes contained a small amount of relatively stable or persistent organic matter amounting to 10 to 20% of the raw waste COD. His system consisted of a high concentration waste treatment process involving aerobic microorganisms.

Ingram[1] developed an aerospace-oriented human waste disposal system based upon the activated sludge process, and found the organisms most efficient if the dilution were similar to domestic sewage. In agreement with Moyer,[13] Ingram found the predominating gases liberated to be carbon dioxide and small amounts of ammonia.

Attractive features of the aerobic process include (1) the rapidity with which wastes are stabilized, (2) end-products which are generally non-odorous, and (3) almost complete degradation. The chief disadvantages with reference to a closed ecology, are the large consumption of oxygen and the production of carbon dioxide.

The chief advantage of an anaerobic process operating in a closed system is that oxygen is not required. Otherwise, this process is slower, is less efficient in reducing solids and produces more obnoxious and toxic by-products than an aerobic process. Also, most anaerobic digestors operate optimally at 55°C.

4.2.1.2 Human Fecal Components

Goldblith and Wick[14] reported that feces is generally known to contain 65 - 75% water, the remainder being indigestible food residues; i.e., cellulose, hexosans, pentosans and lignins, as well as small quantities of undigested and unadsorbed foodstuff, remains of mucosal cells, digestive fluids,

bacteria and bacterial by-products. Specific substances are indole, skatole hydrogen sulfide, methylmercaptan, urethane, hydrogen, carbon dioxide, ammonia, proteoses, peptones, peptides, amino acids. fats, minerals, trace elements and vitamins. The lipid content is primarily sterols with particular fatty acids consisting of palmitic, stearic, oleic, myristic, lauric, linoleic and isomers of oleic acid. The carbohydrate content is mainly cellulose and vegetable fibers, with very little reducing sugars. The protein content is largely bacterial, with some food residues and intestinal cells and enzymes. Cantarow and Schepartz[15] reported that feces contains the enzymes trypsin, lysozyme, maltase, lipase and nucleases. In addition to various peptides, Goldblith and Wick[14] reported the free amino acid content to consist of methionine, lysine, arginine, histidine, leucine, iso-leucine, valine, threonine, cystine, phenylalanine, tyrosine, and tryptophan.

The vitamin content is largely para-aminobenzoic acid, biotin, folic acid, pantothenic acid, pyridoxin, nicotinic acid, thiamine, and riboflavin; with small quantities of vitamins C, K, E and A. The mineral content is Na, K, Ca, Mg, Cl, P, and S with an average ash content of 3.1%. Bile pigments and metabolic products of the pigments are responsible for the color of feces and are mostly tetrapyrroles. Tischer, Tischer and Cook[16] have reported that these bile pigments did not appear to affect the growth of Escherichia coli and Pseudomonas fluorescens.

Many bacteria are present in human feces. Table III presents the fecal flora of man.

4.2.1.3 Urine Components

Urea is better characterized than feces. Detailed lists of urinary components have been reported,[17, 18, 19] but the major constituents in an average 24-hour output are water (1200 g), urea (30.0 g), chloride (as sodium chloride, 12.0 g), sodium (4.0 g), sulfur (as sulfur dioxide, 2.5 g), inorganic sulfates (as sulfur trioxide, 2.0 g), potassium (2.0 g), and creatine (1.2 g).

TABLE III

The Fecal Flora of Man

Microorganism	Reference
Bacteroides melaninogenicus	Zubrzycki and Spaulding[40]
Bactercides sp.	Smith and Crabb[41]
Escherichia coli	Zubrzycki and Spaulding[40]
	Smith and Crabb[41]
	Buthaux and Mossel[42]
Aerobacter aerogenes	Zubrzycki and Spaulding[40]
	Buthaux and Mossel[42]
Lactobacillus sp.	Zubrzycki and Spaulding[40]
	Smith and Crabb[41]
Streptococcus sp.	Zubrzycki and Spaulding[40]
	Buthaux and Mossel[42]
Streptococcus faecalis	Smith and Crabb[41]
Streptococcus faecum	Smith and Crabb[41]
Streptococcus liquefaciens	Smith and Crabb[41]
Diphtheroids	Zubrzycki and Spaulding[40]
Clostridium sp.	Zubrzycki and Spaulding[40]
Clostridium welchii	Smith and Crabb[41]
	Buthaux and Mossel[42]
	Collee, Knowlden and Hobbs[43]
Bacillus sp.	Zubrzycki and Spaulding[40]
Pseudomonas sp.	Zubrzycki and Spaulding[40]
Pseudomonas fluorescens	Brisou[44]
Proteus sp.	Zubrzycki and Spaulding[40]
Staphylococcus sp.	Zubrzycki and Spaulding[40]
Staphylococcus aureus	Smith and Crabb[41]
Yeast sp.	Zubrzycki and Spaulding[40]

4.2.1.4 Microbial Production of Ammonia and Hydrogen

It was pointed out earlier that aerobic disposal systems usually evolve carbon dioxide and ammonia while anaerobic systems evolve mainly carbon dioxide and methane. It would appear that most of the hydrogen produced is used for reductive reactions. In an attempt to determine the gases evolved from untreated raw human feces, Wheaton et al.[20] placed fecal samples into tin cans fitted with pressure gauges and gas sampling ports. Fecal samples produced from 3.5 to 6 mls of gas (at STP) per g within 7 days, under air or argon. Gas chromatographic analyses showed that the major constituents were carbon dioxide and ammonia with very little hydrogen and hydrogen sulfide. It would appear from the data that their results were very similar to that obtained in anaerobic sludge digestion processes These authors also reported a particularly interesting observation concerning two distinguishable types of fecal samples. The first type produced carbon dioxide primarily, with a little hydrogen; the second group produced carbon dioxide and methane.

Hermann[21] investigated the effects of adding nitrate to an algal sewage stabilization pond. He found that bacterial dentifrication led to an increase in ammonia production. The nitrate inhibited production of hydrogen sulfide and other odors, apparently by supplying an oxidizing agent. Hermann[21] learned that as much as 10,000 mg per liter of sodium nitrate was not toxic to the aerobic sewage-stabilizing organisms.

The Melpar Corp.[22] has evaluated a number of microorganisms for their ability to evolve hydrogen. Their study has shown that of the following bacteria: Escherichia coli, Aerobacter aerogenes, Aerobacter cloacae, Serratia kiliensis, Pseudomonas sp. 64A, E. coli produced the highest yield of hydrogen when grown on glucose or maltose. Aerobacter cloacae was next best when grown on galactose, arabinose or glucose. Aerobacter aerogenes produced a good yield of hydrogen from fructose, glucose, maltose, or lactose. Pseudomonas sp. 64A produced a fairly good yield when grown anaerobically on formate. This production of hydrogen by enteric organisms

grown on glucose is of interest due to the face that cellulose degradation results in glucose.

Gest[23] has reviewed various organisms capable of producing hydrogen and ammonia. Clostridium butylicum grown on pyruvate will evolve hydrogen and the reaction is apparently due to a phosphoroclastic split with the formation of acetyl phosphate and formate. The formate is then split to produce carbon dioxide and hydrogen. Cl. kluyverii produces hydrogen in the oxidation of acetaldehyde to acetate. Clostridial reactions also evolve ammonia; e.g., Cl. tetranomorphum attacks single amino acids, such as glutamate, with the production of ammonia, carbon dioxide, hydrogen and volatile acids. Cl. propionicum also attacks single amino acids, but produces carbon dioxide, ammonia and volatile acids, while hydrogen, though evolved, is used for the reduction of oxidized intermediates. Cl. sporogenes utilizes the Stickland reaction, in which one amino acid is oxidized and another is reduced, with the production of two organic acids and two molecules of ammonia. Stickland[24] reported that only specific compounds could act as hydrogen donors and as hydrogen acceptors. The donors were d-alanine, d-valine, pyruvate, d-leucine, l-phenylalanine, l-aspartate and d-glutamate. The acceptors were glycine, proline, and hydroxyproline. Stickland found that the best combinations for ammonia production by Cl. sporogenes were alanine and proline, valine and hydroxyproline, and leucine and glycine. He also reported that Cl. sporogenes would attack either serine or tyrosine alone, with the production of ammonia.

Nisman, Reynard and Cohen[25] studied the Stickland reaction in a number of bacteria, and showed that the following clostridia were physiologically similar to Cl. sporogenes: Cl. histolyticum, Cl. bifermentans, Cl. butyricum, Cl. acetobutylicum, Cl. flabelliferum, Cl. suprotoxicum and Cl. sordellii. They also found that the Stickland reaction did not occur with the following clostridia and facultative anaerobes: Cl. iodophilum, Cl. saccharobutyricum, Cl. welchii, Staphylococcus aureus, Proteus vulgaris, Klebseilla pneumoniae, and Escherichia coli.

Gest[23] also reported that the methane bacteria produce hydrogen during the formation of methane, but that the hydrogen is used to reduce carbon dioxide. If, however, Methanobacterium formicicum or M. vannielii are grown in an alkaline medium (above pH 8.6) carbon dioxide and hydrogen are produced as the major gases.

It was reported above that a number of bacterial species can produce hydrogen from the degradation products of cellulose, but these organisms generally cannot degrade cellulose itself. Khouvine[26] isolated an anaerobic cellulolytic bacterium, Bacillus cellulosae dissolvens, from human feces, herbivora, and soils. This organism produced hydrogen, carbon dioxide, organic acids, and ethanol from cellulose. Cowles and Rettger[27] reported the isolation of a cellulolytic anaerobe, Clostridium cellulosolvens, from horse feces. This organism fermented cellulose and produced gas, 75% of which was hydrogen, symbiotically with Aerobacter aerogenes, Escherichia coli, or Proteus vulgaris. These authors did not believe that Khouvine's culture was pure. Nagliski et al.[28] reported that Clostridium roseum fermented cryptostegia leaves rapidly with the production of a considerable amount of gas, and point out the interesting fact that the organism did not dissolve filter paper cellulose in tryptone broth. Hungate[29] discussed anaerobic, mesophilic, cellulolytic bacteria, and reported that although sewage sludge contained several species of cellulolytic bacteria, none were found in raw sludge. Fuller and Norman[30] reported that various aerobic, mesophilic soil bacteria were capable of hydrolyzing cellulose. They isolated and described Pseudomonas ephemerocyanea, Ps. lasia, Ps. erythra, Achromobacter picrum, and Bacillus aporrhoeus. Fuller and Norman[31] also performed various biochemical studies with the above organisms and found that each organism would utilize xylans and various hexosans. In a further study, Fuller and Norman[32] showed that lignin usually inhibited the fermentation of xylans and hexosans by all of the bacteria.

A small group of urea hydrolyzing soil bacteria have been termed "urea bacteria" by Alexander[33] because of their tolerance to high

levels of the compound, and their nutritional requirement for it. Bacillus pasteurii and Bacillus freudenreichii are representatives of spore formers, and Micrococcus ureae and Sarcina ureae are representatives of the coccus forms. Wiley and Stokes[34] have reported that B. pasteurii requires ammonia, formed by hydrolysis of urea, not only for growth, but also for development of the alkaline pH necessary for growth. Gibbons and Doetsch[35] reported that nearly all of the ureolytic bacteria are aerobic or facultative anaerobes, and that very few obligately anaerobic bacteria possess this capability. They reported the isolation and characterization of an obligately anaerobic, ureolytic bacterium, which has been classified as Lactobacillus bifidis var. ureolyticus.

Cooke and Keith[36] isolated Brevibacterium ammoniagenes from feces; this organism produced ammonia from urea both aerobically and anaerobically. Better growth occurred aerobically, however.

4.2.2 Enzymes

The literature was searched for information on those enzymes which could be expected to have relatively broad activity toward urine and feces. The use, for the purposes of this program, of enzymes which can act only on a minor component of human wastes would result in expenditure of considerable effort with little to show for it in terms either of power production from the wastes or degradation of the wastes. Accordingly, enzymes were considered from the general point of view that pretreatment of the wastes would facilitate bacterial action. Thus the saccharolytic enzymes cellulase, pectinesterase and polygalacturonase were considered. A proteinase, papain, was considered to the exclusion of other, more specific proteinases. Urease was considered because urea is the most predominant single component of human wastes. Finally, lipases were considered as a means of aiding in degradation of fecal lipids.

4.2.2.1 Cellulase

Cellulase (cell free) has been isolated from a large number of plants and animals. The list below are but a few representative samples.

Molluscs

Helix pomatia (snail)[45]

Teredo (shipworm)[46]

Protozoa

Endoplodium neglectum[47]

Arthropods

Termes obesus (teiminte)[48]

Plants

Malt[49]

Algae[50]

Fungi

Myrothecium verrucaria[51]

A. niger[52]

Bacteria

Rumen microorganisms[53]

Pseudomonas fluorescens[54]

The largest body of work concerning the purification of cellulase has been accomplished using the fungi as the source material. The reason for this lies in the fact that cellulase is an exoenzyme in most fungi and can be conveniently obtained from the medium by removing the cells and residual cellulose substrate. The fungus most commonly used is *Myrothecium verrucaria*. A procedure for obtaining crystalline cellulase from the fungus *Irpex lacteus* has been reported by Nisizawa.[55]

The ability of the enzyme cellulase to hydrolyze reprecipitated cellulose, soluble derivatives, or highly crystalline native celluloses varies widely between extracts from different sources. However, good evidence has been accumulated[56,57,58,59,60] that, as the crystallinity of the substrate decreases the rate of enzymatic hydrolysis increases.

Most plant cellulases have an optimum pH in the region of 5.0, animal cellulases in the range of 5.0 to 5.5, and bacterial cellulases between 5.8 and 7.0. Optimal pH values vary widely for fungal cellulase over a range from pH 3.0 to pH 8.0, however, most pH values are on the acid side, between pH 4.0 and pH 7.0.[61]

There is very little information regarding the temperature optimum of pure cellulase isolated from plant, animal and bacterial sources. In the case of the fungal cellulases where the more definitive work has been done, it has been shown (assay based on production of reduced sugar) that the maximum temperature varies from 40 to 70°.[62,63,64,65] The temperatures at which the fungal cellulases start to be inactivated vary from 40° to as high as 70°C. Below are a few examples.

Source	Temp.	Reference
Trichoderma koningi	70°C	66
Poria vaillanti	60-70°	63
Aspergillus niger	70°	67
M. verrucaria	50°	62

Work with fungal cellulase indicates that the enzyme is generally inhibited by mercury, silver, chromium, lead, and zinc salts, while manganese, cobalt, magnesium, and calcium with phosphate cause stimulation in certain cases.[61] The literature concerning the effect of certain oxidizing and reducing agents on the enzyme are conflicting, however: Permanganate and molybdate have been found to be stimulating; and bisulfite, dithionite and benzoquinone were inhibitory.[68]

Whitaker[69] reported that fungal cellulase from M. verrucaria was inhibited by iodoacetate and p-chloromecuribenzoate but that the inhibition could be reversed by the addition of glutathione, cysteine, sodium sulfide or potassium cyanide.

Reese, et. al.,[70] using cellulose isolated from various micro-organisms, found cellobiose to be generally inhibitory. There were two exceptions, and in these cases the presence of the cellobiose appeared to be stimulatory. These experiments were carried out at pH 5.0 using carboxymethyl cellulose as a substrate and a 2% solution of cellobiose.

The addition of certain proteins to preparations of cellulase from M. verrucaria[71] were shown to stimulate the cellulolytic activity.

There are many methods for the assay of cellulase, but most are based on one of the following procedures:

1. Increase in reducing sugar (colorimetric or volumetric).
2. Effect on viscosity of soluble cellulose derivatives.
3. Loss in weight of insoluble substrates.
4. Oxygen uptake during the enzymatic oxidation of glucose (glucose oxidase) produced by hydrolysis of the cellulose.
5. Decrease in mechanical properties of fibers or films.
6. Changes in bi-refringence of films.

4.2.2.2 Pectic Enzymes, Pectinesterase and Polygalacturonase

Pectic substrance is a group designation for colloidal polysaccharides which contain a large proportion of D-galacturonic acid and methanol and which are widely distributed in the plant kingdom. The pectin molecule has an α-1,4-glycosidic linkage between the pyranose rings of the methyl ester of the D-galacturonic monomer. The pyranose ring occurs mainly in the chair form, corresponding to the most stable configuration of D-galactose. The enzymes involved in the degradation of this pectic substance are pectinesterases and polygalacturonase.

Pectinesterase catalyzes the hydrolysis of the ester bonds of pectic substances to yield the pectic or pectinic acids and methanol.[72] Poly-galacturonase catalyzes the hydrolysis of glycosidic bonds between deesterified galacturonide residues.

4.2.2.2.1 Pectinesterase

Pectinesterase (PE) has been found to be widely distributed in plants, molds, and bacteria. However, those sources most often used and reportedly containing considerable quantities of PE are alfalfa,[72] tomatoes,[73] citrus fruit,[74] potato leaves, tobacco, elder,[75] and fungus.[76] In almost all cases the PE is usually fixed or adsorbed to the solid portions of the plant or fruit.

PE has been shown to be highly specific, saponifying only the methyl ester of pectic substances.[77] The PE from the skin of oranges has been reported to hydrolyze only the methyl ester groups adjacent to free carboxyl groups, the hydrolysis proceeding linearly along the pectin molecule as successive methoxy groups split off.[78]

The temperature stability of PE varies depending on the source from which it was isolated. Fungal PE is 50% inactivated in 0.1 M NaCl at pH 6.0 for 1 hour at 35° whereas, tomatoe PE at 70° and under otherwise similar conditions, was only 50% inactivated.[76] Orange PE was 50% inactivated at pH 7.5 in a borate-acetate extract in 30 minutes.[79]

The pH optimum for plant PE lies in the range of pH 7 to 8 at low salt concentrations. However, the fungal PE is markedly different in this respect and has a pH optimum in the range of pH 4.6 to 5.5.[80]

PE is relatively inert to chemical inhibition by such as cyanide, iodine, formaldehyde, etc.[76] Detergents and soaps are reported to be very effective inhibitors of PE.[76]

Cations have been found to increase by many fold the activity of PE in the pH range extending several pH units below neutrality. It has also been shown that divalent cations are more effective than monovalent cations. Because of this fact the explanation for the observed increase in activity can not be based on ionic strength. Although some theories have been put forth to explain this phenomena, none seem to completely fit all the facts.[76]

The activity of PE is assayed by measuring the increase of carboxyl groups at constant pH,[81] the increase of methanol,[73] or by measuring the evolution of carbon dioxide from an $NaHCO_3$ buffer using manometric methods.[82] To insure reliable results from any of these methods the cation content of the system must be known. The enzyme activity is expressed in milliequivalents of bonds hydrolyzed per minute per millimole of enzyme under established "standard" conditions. These "standard" conditions are, in general, the optimum conditions (pH, temp., etc.) and therefore vary slightly with the source of the enzyme.

4.2.2.2.2 Polygalacturonase

Polygalacturonase (PG) is most commonly found in the lower plants such as bacteria and fungi, but seldom in higher plants. In animals it is found only in the snails.[80] Isolation of PG often involves separation from PE by ion exchange treatment. Successful isolation of PG has been accomplished from the following organisms:

Organism	Method of Separation	Reference
Aspergillus niger	Partial inactivation, adsorption on Fuller's earth. Chromatography on Al_2O_3 and on filter paper.	83
Penicillium expansum	Precipitation with alcohol	84
Rhizopus tritici		

Most of the work done using purified preparations of PG indicates that PG is very specific.[85] Although PG is regarded as a hydrolase, it has not yet been clearly determined whether PG splits the glycosyl-oxygen bond or the aglycone-oxygen bond. Since no transferase action has yet been reported, it may be that a double displacement mechanism as proposed for many carbohydrases is operative.[77]

A classification of various PG's has been proposed based on their mode of action. There are three classes:[77]

PG I — Liquefying PG - splits the glycosidic linkages more or less at random, producing a rapid decrease in viscosity. This type acts best on pectins of a low degree of esterification

PG II — Brings about the hydrolysis of highly esterified pectins. The commercial preparation "Hydrolase" appears to be just such a PG.

PG III — Splits glycosidic linkages from one end of the pectin molecule - probably from the reducing end.

It has been observed that alkali salts increase the activity of PG as they do PE, however, there is not nearly the marked effect for PG. The effect follows the series:

$$L_i < Na < K < Rb < Cs$$ [86]

In contrast to the effect observed with PE, Mg^{++} caused 48% inhibition of PG.[87]

The pH optima for PG have been reported from 3 to 6. Although the optimum may vary depending on the source of the enzyme, pH 4.0 is most generally used.[80] PG's from bacteria and phycomycetes have a pH optimum near 7.0.[77] PG appears to be remarkably stable to acid; it is only 50 to 70% inactivated in 20 minutes at pH 0.6 and 23°C. PG also (from Aspergillus niger) appears to be rather thermostable.[88,89]

Assay procedure is based primarily on one of the following:

(a) Increase in reducing groups.[90]

(b) Decrease in viscosity.[91]

(c) Decrease in precipitability by calcium or nonpolar solvents.

(d) Decrease in optical rotation.

The assay procedure most commonly used is that based on the reducing group change. The results can be conveniently expressed in milli-equivalents of bonds hydrolyzed or millimoles of reducing group per minute under

standard assay conditions. Standard conditions consist of pH 4.0, 25°C and o.5% pectic acid.

4.2.2.3 Lipase

The enzyme lipase catalyzes the hydrolysis of oil and fats, i.e., triglycerides of long-chain fatty acids and can be thus differntiated from other esterases which hydrolyze simple esters of low molecular weight acids.

Isolations of the enzyme lipase have been done primarily with the pancreas of various animals, i.e., hog,[92,93] rats,[94] and bovine pancreas.[95] The isolation of lipase has also been reported from fungi,[96] yeast,[97] castor bean,[98] and various bacteria.[99,100]

Since it has been shown that lipase makes up 2.5% of the total protein of swine pancreas this has remained the primary source for this enzyme. A recent report for the isolation of pancreatic lipase (hog) is given by E. D. Wills.[101] This preparation was found to have maximum stability at pH 5.6, and was able to withstand storage in 5% NaCl at 5°C for 2-3 months without loss of activity. There is some indirect evidence that calcium ion is necessary for stability of the enzyme.[102] Hg and other heavy metals and also halogen ions (F > I > Br > Cl) seem to inhibit the enzyme.[103] However, it appears that -SH groups are not directly indispensable for the hydrolytic activity. Lipase is inactivated by surface-active compounds.[102]

Desnuelle[103] provides evidence that the lipase enzyme is more active when the substrate is emulsified (rather than in solution) and that the enzyme is active at the oil-water interface. He provides a series of curves showing that the initial enzyme rate varies with the interfacial area, and also reports Michaelis curves using partially soluble substrates (triacetin, methyl butyrate) which show a significant increase in lipase acrivity after the substrate has reached saturation and is in the form of an emulsion.

There is good evidence[104] that lipolysis occurs in three well-separated and consecutive steps and is due to the catalytic effect of one enzyme (lipase). The requirement for calcium ion does not in any way alter the course of the lipolysis but does promote the process by combining with the interfacial soaps.[105]

Desnuelle gives evidence that bile salts, such as taurocholate, increases the initial rate of hydrolysis by a factor of four at 37°C. He eliminates the possibility that the acceleration is due to an increase in interfacial area by insuring that it is already large enough to give maximal reaction rate before the addition of taurocholate. Since there are normally some bile salts present in fecal material one can anticipate some stimulation of the lipase activity when feces serves as the substrate. According to Goldblith and Wick,[106] feces contains 0.15% bile pigments. Optimum pH is about pH 7.0 for lower triglycerides; it is shifted to pH 8.8 for higher triglycerides.[107] Pancreatic lipase (although it varies with the state of purity of the enzyme, with buffers, with method of assay, etc.) has an optimum temperature of about 37°C for most substrates.[108]

Two methods have been used for assay. Continuous titration of fatty acids released during hydrolysis is one of these.[109] The other is measurement of carbon dioxide released from sodium bicarbonate by the fatty acids.[110]

4.2.2.4 Papain

Papain was chosen over such proteolytic enzymes as pepsin, chymotrypsin and trypsin for the following reasons: (1) the latter enzymes are not as heat stable, (2) they are more sensitive to low concentrations of urea, (3) their optimum pH values are at very acid levels (pH 2-3), (4) papain has shown to produce more extensive degradation of protein substrates than other proteases, and (5) crystalline papain is relatively easy to prepare and is commercially available.

Isolation is from the dried latex of the papaya plant. The dried extract is ground with sand in the presence of a dilute cysteine solution at pH 5.7. Inert material is removed from the clarified extract after the solution is brought to pH 9.0. The enzyme is precipitated first with ammonium sulfate and subsequently with sodium chloride Crystallization is achieved by allowing a solution of the enzyme at pH 6.5 to remain at 4°. Recrystallization can be accomplished from sodium chloride solution or from 70% ethanol by salting out with a lithium salt.

One of the most striking properties of papain is its rather high temperature stability: half-life, 56 min. at 75°C.[111] Resistance to elevated temperature is markedly pH - dependent, particularly below pH 4.0, where papain is rapidly and irreversibly inactivated.[112]

In the presence of protein substrates (as opposed to synthetic peptides) papain was found to be fully active after exposure to 9 M urea.[101] However, in the presence of synthetic substrates it was found that concentrations above 3M urea effected irreversible inactivation of papain.[113]

The enzyme is sensitive to sulfhydryl agents such as iodoacetate, hydrogen peroxide, heavy metals and p-mecuribenzoate; and is activated by H_2S, HCN, and other reducing agents.[114,115]

The broad specificity of papain was shown by Calvery,[116] who studied the hydrolysis of crystalline egg albumin by crude papain and found that after papain digestion, neither pepsin or trypsin could bring about further hydrolysis. The pH optimum for protein digestion was in the range pH 7.0 - 7.5.[117]

Papain may be conveniently assayed by measuring the rate of hydrolysis of a synthetic substrate such as benzoyl-L-argininamide (BAA). The assay is performed at pH 5 to 7 at 40° in the presence of appropriate activating agents.[118]

Papain can also be assayed with urea-denatured hemoglobin as substrate.[119,120] The reactions are performed at 39°C in the presence of

0.005 M cysteine and 0.001 M EDTA at pH 7 to 8.5. Aliquots of the reaction mixture are removed at appropriate intervals, treated with 5% trichloroacetic acid, and filtered. The filtrates are examined in a spectrophotometer at 280 mμ.

4.2.2.5 Urease

The presence of urease has been reported in numerous bacteria, animals and some plants. However, the two richest sources and those most frequently used for the isolation of the enzyme are jack bean meal[117] and the bacteria Bacillus pasteurii.[121] Jack bean contains 0.15% urease (on a dry weight basis) and Bacillus pasteurii 1.0%. Isolation from jack bean meal is fairly straightforward, an acetone extract giving impure crystalline material.[122] Isolation from Bacillus pasteurii is much more complex, involving six steps, including several sulfate fractionations, calcium phosphate gel treatment, and an acetone fractionation. Although the enzyme was not obtained in crystalline form, Kallio et al.[121] have reported activities of 150 to 190 units/mg compared to 130 units/mg for Sumner's jack bean urease.

The presence of sulfhydryl groups in the urease molecule has been amply confirmed.[123,124] The work in the last reference indicates that there are 23 -SH groups per molecule. Due to the presence of the -SH groups, urease is inhibited by typical -SH reagents such as the metal ions Ag^{+}, Hg^{++}, Cu^{++}, ferricyanide, p-mercuribenzoate, trivalent arsenicals, and furacin. Sodium and potassium ions inhibit, and phosphate ions activate, urease.[125]

Studies using tris-(hydroxymethyl)aminomethane sulfate (inert) as a buffer[126] show a pH optimum at 8.0. Wall and Laidler[127] indicate that at urea concentrations above 0.3 M the activity actually decreases. It has been postulated[127] that at high concentrations, urea may occupy that site on the urease surface normally occupied by water, and thereby inhibit the enzyme.

Extensive studies to determine the heat of activation of urease[128,129] indicate that thermal deactivation of the enzyme is significant at 40° - 50°C and that only 10% of original activity of the enzyme (at pH 7.0) remained after heating to 96° for six minutes.

By comparison, the bacterial urease isolated by Larson and Kallio[121] was very sensitive. They reported that their purest preparations (190 units/mg) were inactivated very rapidly on standing (even during refrigeration), by dialysis in the absence of reducing agents, by pH values lower than 5.2, and by organic solvents.

The enzyme is absolutely specific for hydrolysis of urea.[130,131]

A colorimetric assay using the Nessler reagent to determine the ammonia formed from the hydrolysis of the urea is convenient and simple.[117]

A titrimetric assay is reported[132] in which the ammonia formed is titrated with 0.1 M HCl.

4.3 Selection of Bacteria and Enzymes

The selection process has been guided by several features, the most important of which is the orientation toward using urine and feces as electrochemical fuels. Practical considerations point to conversion of urine and feces components to hydrogen and ammonia as the most likely route to optimizing power production. Optimum conversion of waste materials to re-usable chemicals, on the other hand, probably will require a different line of attack.

The first of these features has been given prominence as being most consistent with the objectives of the present effort. Therefore, organisms and enzymes were selected for further study on the basis of their predisposition to conversion of urine and feces components to hydrogen and ammonia.

As to enzymes, cellulase, lipase, and urease were selected for further study. Cellulase is of interest because (1) bacteria hydrolyze cellulose slowly and (2) the

glucose from cellulose is a good source of hydrogen by way of bacterial fermentation. Lipase is of interest primarily from the point of view of degradation of feces. Urease is a potent catalyst for hydrolysis of urea, which results in ammonia. Because proteolytic activity is common in bacteria, papain was rejected for further consideration. The pectic enzymes, pectinesterase and polygalacturonase, would be of limited value because of the small quantity of pectins in wastes.

Selection of bacteria for study was essentially on the basis of ammonia and hydrogen production and proteolytic activity. For ammonia production, the urea bacteria in general were selected for screening. It was planned to narrow this selection in preliminary screening for growth in urine and accompanying ureolytic activity. The organisms include Sarcina ureae, Micrococcus ureae, Lactobacillus bifidus var. ureolyticus, Brevibacterium ammoniagenes, and Bacillus pasteurii. For production of hydrogen, from glucose derived from hydrolysis of fecal cellulose, Escherichia coli and Aerobacter aerogenes were selected, both of which organisms are indigenous to feces. Proteolytic activity has been approached by way of selecting a Bacillus species and a Clostridium species. B. pasteurii is attractive for its proteolytic activity as well as its high ureolytic activity. B. coli is also capable of producing ammonia from fecal amino acids.

4.4 Bacterial Screening Program - Urine

Screening bacteria for growth and ureolysis in urine was done to select the most favorable organism(s). Both growth and ureolysis were considered of greatest interest under anaerobic conditions, because electrochemical utilization of the produced ammonia would be expected to proceed most favorably in the absence of oxygen. Further, it is desirable to minimize oxygen utilization.

Sarcina ureae - this organism grew and produced ammonia in aerobic but not in anaerobic cultures when urea broth and urine was used as media. Consequently, S. ureae was eliminated for further study.

Micrococcus ureae - the culture, received from a commercial culture collection, was contaminated. Attempts to purify the culture were unsuccessful; however, since the literature indicated that M. ureae, like S. ureae, is an obligate aerobe, the organism was rejected on this basis.

Lactobacillus bifidus var. ureolyticus - although the literature reports that this organism anaerobically hydrolyzes urea to ammonia, our culture failed to demonstrate ureolytic activity despite numerous subcultures in a medium containing urea. The investigator from whom the culture was obtained suspected that ureolytic ability was unstable and prolonged stock (five years) of the culture probably contributed to the loss of this characteristic. No other source of this organism is known.

Bacillus pasteurii - this organism demonstrated facultative growth and ammonia production in urea broth and urine. Somewhat better growth occurred aerobically in urea broth while anaerobic growth was better in urine. After two days anaerobic growth, 97% of the urea present in urine was utilized.

It was established early in the program that there was no difference in anaerobic growth of B. pasteurii when either freshly collected or frozen urine was used as the medium.

Bornside and Kallio[133] reported that the optimum pH for growth of B. pasteurii in a medium containing urea was pH 8.8. An experiment was performed which demonstrated that an initial pH of 9.0 supported optimal anaerobic growth in urine. The pH of ammonium carbonate, produced from urea hydrolysis, is approximately 9.2 and solutions containing this material are strongly buffered at that pH.

Wiley and Stokes[134] showed that B. pasteurii requires thiamine and certain amino acids for growth and that some strains also need biotin and nicotinic acid. Their cultures were grown in a synthetic medium. Our studies established that anaerobic growth was not enhanced by supplementing urine with these three vitamins, either alone or in combination. Either urine appears to contain sufficient amounts of these vitamins or our strain of B. pasteurii does not require them.

Brevibacterium ammoniagenes - this bacterium grew readily and produced ammonia aerobically and anaerobically in both urea broth and urine. Ureolytic activity was

was rapid (24-48 hours) in urea broth and aerobically in urine, but ammonia was not evident before six days anaerobic growth in urine. In the latter case, 46% of the urea present in the urine was utilized. The slow ureolytic activity under anaerobic conditions resulted in our dropping this organism.

4.5 Enzymatic Screening Program - Feces

Cellulase - the initial step in the study was to develop a colorimetric assay for cellulase and obtain a satisfactory calibration curve. The assay developed by Sumner[135] is based on the reduction of 3,5-dinitrosalicylic acid by the "reducing sugars" released during the hydrolysis of cellulose. The resultant reduction product imparts a characteristic brown color to the solution.

A preliminary assay of a crude cellulase preparation confirmed some cellulase activity but indicated the presence of contaminants capable of giving a reduced sugar reaction.

4.6 Electrochemical Evaluation of Urine

The feasibility of electrochemical energy conversion systems is governed by the thermodynamics of the reactants. The actual performance of a system depends on the electrode kinetics of the cell. For example, the actual steady potential of an electrode usually differs markedly from the potential calculated from thermodynamic considerations; the cell current is the algaebraic sum of the oxidation and reduction currents at each electrode. Since each current is a measure of the rate of a particular reaction, the cell current is the resultant of the behavior of perhaps several oxidation and reduction reactions, under the influence of the potential across the electrodes, and the ensuing polarization effects.

Some cells, which are seemingly simple, exhibit complex performances. The obviously complex systems involved in bioelectrochemistry may show the effects of several simultaneous oxidation-reduction reactions in addition to the ramifications from the various types of polarization.

In the first section of this study the electrochemical behavior of systems composed of urine and selected bacteria are observed. Bacillus pasteurii has been selected for its established ureolytic activity. Work with urine was initiated directly rather than work with simpler systems employing urea because this is being amply examined under another contract.[136] The study will be extended, and the conclusions derived will form the basis for the projected optimization of bioelectrochemical cells utilizing urine and feces. (The first data are for systems containing non-sterile urine with and without Bacillus pasteurii.) The open circuit potentials and potentials as a function of increasing current were measured. Since it is doubtful that the conditions of equilibrium and reversible reactions for thermodynamic oxidation potentials were obtained in these experiments, the open-circuit potential values imply only that no current was flowing during the measurement. The polarization curves permit the limiting current densities to be determined and allow some conclusions to be drawn regarding the reactions causing the currents.

The first data, in Figure 1, are on systems containing non-sterile urine with and without Bacillus pasteurii. Little difference between the anodic polarization curves for these two sets of data occurred at potentials more positive than 250 mV; the systems behaved nearly identically and the limiting current densities are the same. At potentials more negative than 250 mV, the curves are linear and apparently have different slopes. The non-equal slopes in the Tafel equation region signifies that different reactions are occurring. This conclusion will be tested in further measurements.

In the second group of data, shown in Figure 2, the effect of incubation of the urine with B. pasteurii is considered. Here the effect of an 18-hour incubation was measured. Again, it is difficult to distinguish differences in limiting current densities. However, that the same reaction is occurring is indicated by the essentially parallel linear sections of the curves. It is apparent that in this range the cell with the incubated B. pasteurii provides increased current for the same potential. For example, at 170 mV, the cell with incubated B. pasteurii carried 0.15 ma/cm^2, whereas only 0.01 ma/cm^2 was carried by the cell to which B. pasteurii had been

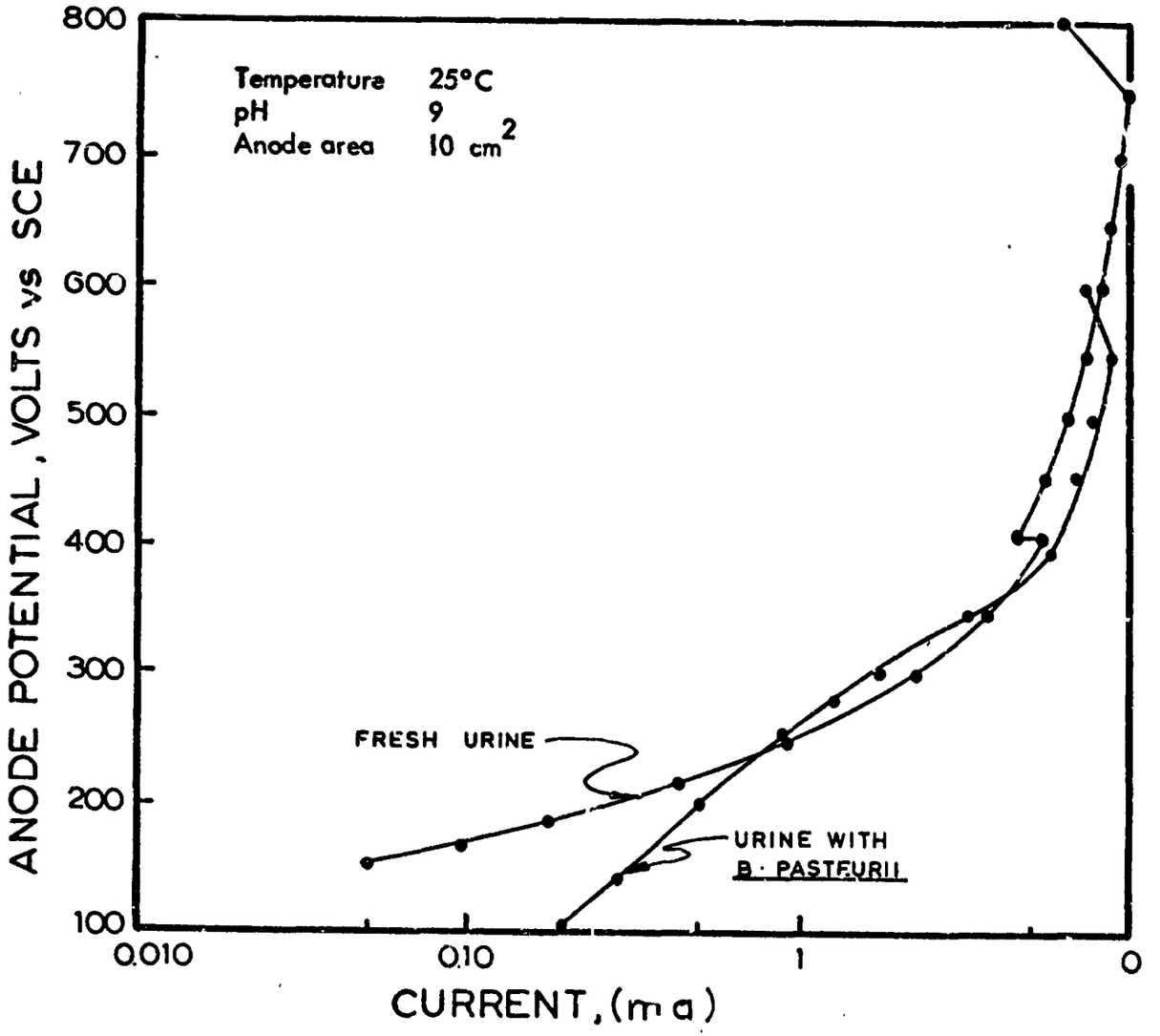

FIGURE I: Anodic Polarization of Platinum in Urine, Effect of Added B. pasteurii

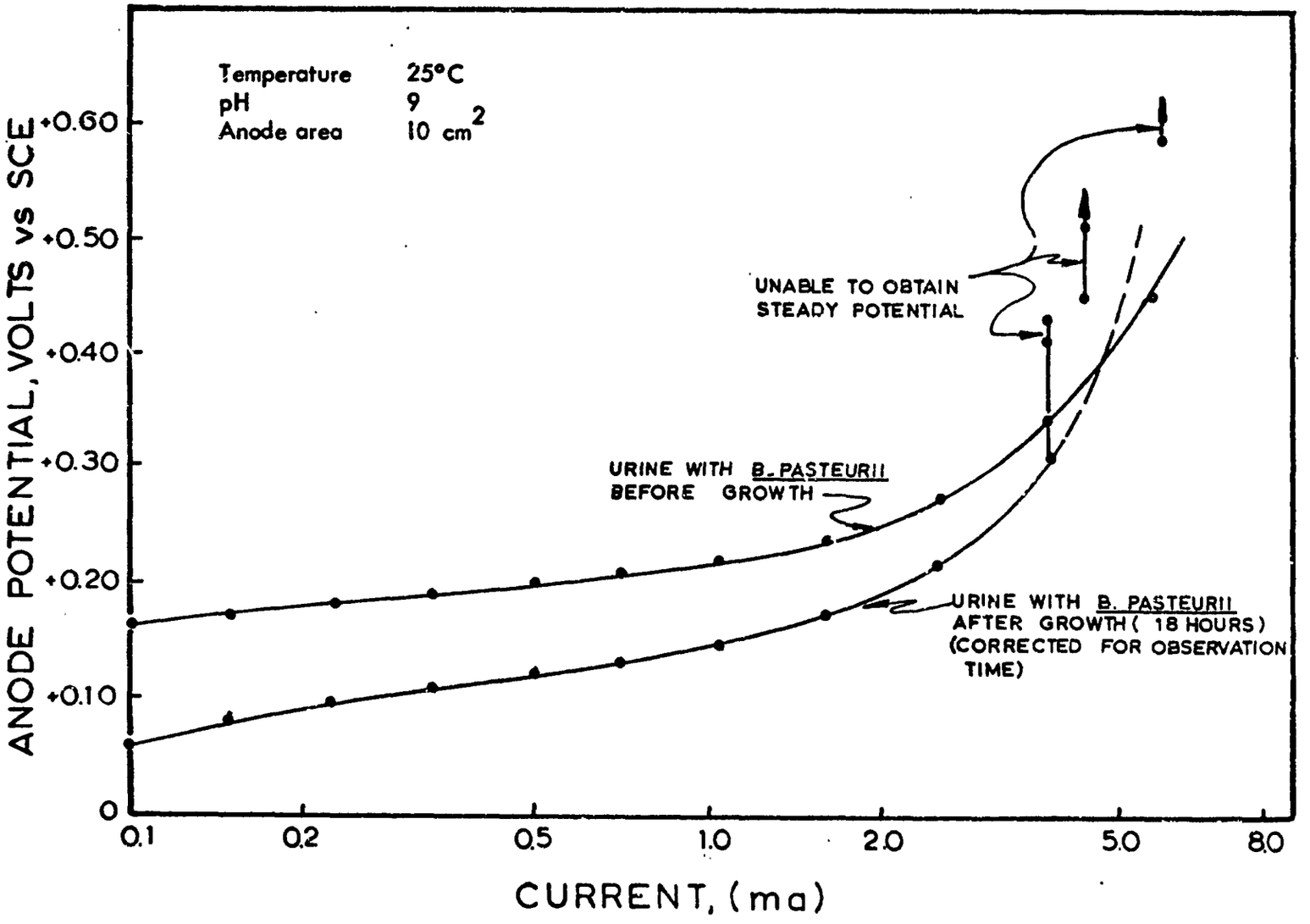

FIGURE 2: Anodic Polarization of Platinum in Urine, Effect of B. pasteurii growth

without incubation. The advantage of allowing time for the growth of B. pasteurii is apparent. This effect of bacteria population will be studied further.

4.7 Experimental

4.7.1 Collection of Feces and Urine

To obtain urine and feces, a collection program, with volunteers of Magna Corporation, Research and Development Division, has been initiated. The collection program is well under way. To date, 20 lbs. of feces and 75 liters of urine have been collected from our volunteers. To minimize individual variations in waste product composition and to reduce fecal cellulose, guidelines for food consumption have been provided. This approach is being taken to conform with several "space diets" currently under study elsewhere. Food abundant in cellulose, such as fresh fruits and vegatables, and foods which are difficult to digest, such as deep-fried foods, have been restricted or eliminated entirely from the diet. Reasonable precautions are being taken to insure "normal" samples. Volunteers to whom anti-microbial drugs are being administered are requested to immediately withdraw from the program. Each donor's urine is being examined for excess amounts of glucose and protein.

Urine is being stored at -20°C until the collection program is complete. The urine will then be thawed, composited and refrozen in suitable containers for use during the balance of the program. Feces is also being stored at -20°C during the collection period. The feces will be freeze-dried, composited and stored in suitable containers. Composited urine and feces will be analyzed for selected major chemical components such as cellulose, lipids, urea, etc.

Although we believe that freeze-drying will not significantly alter the properties of feces with respect to its ability to serve as a substrate

for bacteria, this will be determined expirically before the composite sample is subjected to this process.

Marquardt Corp. is initiating a similar program to complement our effort.

4.7.2 Sources of Culture Collection

1. Sarcina ureae - The National Collection of Industrial Bacteria, Aberdeen, Scotland (No. 8691).
2. Micrococcus ureae - The Institute for Fermentations, Osaka, Japan (No. 3767).
3. Lactobacillus bifidus var. ureolyticus - Dr. R. N. Doetsch, University of Maryland.
4. Brevibacterium ammoniagenes - The National Collection of Industrial Bacteria, Aberdeen, Scotland. (No. 8143)
5. Bacillus pasteurii - The American Type Culture Collection, Washington, D. C. (No. 11859).

4.7.3 Microbiological Experimental Details

4.7.3.1 Sarcina ureae. A 1% stock culture was inoculated into 100 ml of urea broth which consisted of Difco nutrient broth, 10 gm; Difco yeast extract, 5 gm; distilled water, 1 liter. After cooling, 100 ml of 20% urea (filter sterilized) was added. Final pH was 8.0. The inoculated medium was dispensed into a 250-ml screw cap flask and filled to capacity with urea broth (containing 0.1% ascorbic acid) similarly inoculated. The culture was incubated at 30°C for 24 hours. Turbidity was measured in a Beckman DU spectrophotometer using incident light of 660 - mμ wavelength. Growth (OD_{660} 0.95, 1/4 dilution) and ammonic production as evidenced by odor occurred only in the aerobic culture.

Urea broth agar plates were streaked with a culture of S. ureae and incubated aerobically at 30°C and anaerobically under hydrogen gas in an anaerobic jar for 24 hours. Again, growth was evident only aerobically.

The organism was inoculated into filter sterilized urine (Seitz) containing 0.1% ascorbic acid according to the procedures described above. Growth and ammonia were observed exclusively in the aerobic cultures.

4.7.3.2 Micrococcus ureae. The bacterium was received as an agar slant culture and inoculated into urea broth; subsequent growth was streaked on to urea broth agar plates to determine purity. Several types of colonies were observed. Isolated colonies were picked, inoculated into urea broth and growth was again streaked on to agar plates. Although this procedure was repeated several times, isolation of M. ureae was not achieved. In fact, even the original contaminated culture did not exhibit ureolytic activity.

4.7.3.3 Lactobacillus bifidus var. ureolyticus. The bacterium was subcultured repeatedly anaerobically at 37°C in the following medium based upon the recipe used by Gibbons and Doetsch:[137] Difco yeast extract, 3.0 g; Difco nutrient broth, 10.0 g; K_2HPO_4, 3.0 g; KH_2PO_4, 3.0 g; glucose, 5.0 g; Na_2CO_3, 3.0 g; resazurin, 0.001 g; sodium thioglycollate, 1.0 g; distilled water, 1 liter. 3.0 g urea (filter sterilized) was added to the cooled basal medium, final pH, 7.0 Excellent growth occurred within 24 hours but no ammonia was detected. Culturing in (1) same medium containing 20% urea, (2) urea broth, and (3) urine failed to stimulate ureolytic activity.

4.7.3.4 Bacillus pasteurii.

4.7.3.4.1 Relationship to Oxygen. Cultures were grown in urea broth as described earlier for S. ureae. After 24 hours incubation, the OD_{660} of the aerobic culture was 0.38 (1/4 dilution) compared to 0.12 (1/4 dilution) with anaerobic growth. The odor of ammonia was strongly evident in both cultures.

Urine growth studies were performed in a similar manner although optical density could not be measured due to the development of a heavy precipitate. Ammonia was detected in both anaerobic and aerobic cultures. Microscopic examination showed more cells in the anaerobic culture but this observation was not quantitated.

4.7.3.4.2 History of Urine. Anaerobic growth in fresh, refrigerated (four days), and thawed frozen urine from the same donor was compared. All urine was filter sterilized and 0.1% ascorbic acid was added. The pH was adjusted to 8.4 and a 1% inoculum of a urine-grown culture of B. pasteurii was used. Duplicate 30-ml screw cap test tubes were filled to capacity, and incubated at 30°C. Microscopic cell counts using a Petroff-Hauser bacteria counter were made initially and after 24 hours growth. The results are summarized in Table IV.

4.7.3.4.3 pH. Duplicate 30-ml screw cap test tubes were filled to capacity with filter sterilized urine (0.1% ascorbic acid) inoculated with 1% inoculum of an anaerobically urine-grown culture of B. pasteurii. The initial pH of duplicate cultures was adjusted to pH 7.5, 8.0, 8.5, 9.0 and 9.5 with NaOH and incubated at 30°C. Microscopic cell counts were made after 24, 48 and 72 hours growth. The results are reported in Table V.

4.7.3.4.4 Vitamins. The basal medium consisted of filter sterilized urine (0.1% ascorbic acid), pH 8.5. Nicotinic acid, thiamin hydrochloride and biotin were added to the basal medium to produce the following final concentration of the respective vitamins in urine: (1) nicotinic acid (0.5 μg/ml), (2) thiamine hydrochloride (0.5 μg/ml), (3) biotin (0.1 m μg/ml), (4) nicotinic acid (0.5 μg/ml), and biotin (0.1 m μg/ml), (5) nicotinic acid (0.5 μg/ml) and thiamine hydrochloride (0.5 μg/ml), (6) biotin (0.1 m μg/ml) and thiamine hydrochloride (0.5 μg/ml), (7) nicotinic acid (0.5 μg/ml),

TABLE IV

Effect of Fresh, Refrigerated, and Frozen Urine on the Anaerobic Growth of *B. pasteurii*

Age of Culture	History of Urine		
	Fresh	Refrigerated	Frozen
Inoculated urine	3.0×10^5	2.5×10^5	3.0×10^5
24 hours	1.5×10^7	1.5×10^7	1.0×10^7

thiamine hydrochloride (0.5 μg/ml) and biotin (0.1 mμg/ml). The control contained no added vitamins. Duplicate 30-ml screw cap test tubes were filled to capacity with the various media preparations, inoculated with a 1% inoculum of anaerobically urine-grown culture of B. pasteurii and incubated at 30°C. Microscopic cell counts were made after 24 and 48 hours growth. The results are summarized in Table VI. The nicotinic acid was obtained from Eastman Organic Chemicals, Rochester, New York; biotin from California Corporation for Biochemical Research, Los Angeles, California; thiamine hydrochloride from Hill Drugs, Anaheim, California.

4.7.3.4.5 Determination of Urea Hydrolysis. Filter sterilized urine (pH 8.5) containing 0.1% ascorbic acid, inoculated with a 1% inoculum of a anaerobic urine-grown culture of B. pasteurii, was dispersed into a 30-ml screw cap test tube and a 40-ml serum cap bottle. The cultures were incubated at 30°C for 48 hours. The utilization of urea was ascertained by comparing the concentration of urea present in uninoculated urine with that of the 48-hour culture. Urea was determined by the colorimetric method of Ormsby.[138] The results are reported in Table VII.

4.7.3.5 Brevibacterium ammoniagenes

Anaerobic and aerobic growth studies with urea broth and urine were performed in a manner similar to that described for S. ureae. The pH of urea broth and urine was adjusted to 7.4 and the cultures incubated at 30°C. Good growth and ammonia production was noted after 24 hours aerobic growth in urea broth but not until from 48 hours to five days under anaerobic conditions. After five days anaerobic growth in urea broth, the concentration of urea was reduced from 18.5 mg/ml to 11.65 mg/ml, a decrease of 37%.

TABLE V

Effect of pH on the Anaerobic Growth of B. pasteurii in Urine

Initial pH	7.5	8.0	8.5	9.0	9.5
Days after Inoculation					
1	7.3×10^{5}(1)	3.8×10^{6}	$2.7 \times 10^{}$	1.4×10^{7}	3.8×10^{6}
2	1.9×10^{7}	3.2×10^{7}	2.4×10^{7}	2.3×10^{7}	2.4×10^{7}
3	1.3×10^{7}	1.2×10^{7}	2.1×10^{7}	1.4×10^{7}	1.6×10^{7}
Final pH	9.5	9.5	9.5	9.5	9.6

(1) Average cell counts on duplicate samples

TABLE VI

The Effect of Vitamin-Enriched Urine on the Anaerobic Growth of B. pasteurii

Vitamin Added	None (control)	Nicotinic Acid	Thiamin hydrochloride	Biotin	Nicotinic Acid & Biotin	Biotin & Thiamin hydrochloride	Nicotinic Acid & Thiamin hydrochloride	Nicotinic Acid, Thiamin hydrochloride & Biotin
Days after Inoculation								
1	1.1×10^7(1)	2.1×10^7	1.6×10^7	1.8×10^7	1.6×10^7	2.2×10^7	1.4×10^7	1.7×10^7
2	2.4×10^7	1.3×10^7	1.9×10^7	2.0×10^7	1.7×10^7	1.6×10^7	2.0×10^7	1.6×10^7
Final pH	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5

(1) Average of cell counts from duplicate tubes.

TABLE VII

Utilization of Urea in Urine by B. pasteurii

Initial Urea in Urine		9.0 mg/ml
Final Urea in Urine		
(1)	Screw cap tube	0.243 [1] mg/ml
(2)	Serum bottle	0.238 [1] mg/ml

1. Mean of two determinations.

Ureolytic activity in aerobically grown urine was noted after 48 hours growth but not until after six days growth anaerobically. In the anaerobic urine culture the concentration of urea was reduced from 10.75 mg/ml to 5.8 mg/ml, a decrease of 46%.

4.7.4 Enzymatic Screening Program

Cellulase - A standard curve was prepared using glucose solutions containing weighed amounts of glucose from 0.05 mg/ml to 1.0 mg/ml. The data obtained gave a satisfactory linear relationship between optical density and glucose concentration in the range of 0.1 mg/ml to 1.0 mg/ml. However, at concentrations lower than 0.1 mg the absorption (at 540 mμ) of the developing compound (3,5-dinitrosalicylic acid) is greater than that of the brown reaction product.

A preliminary assay of the crude cellulase preparation (Nutritional Biochemical Corporation, Cleveland, Ohio) indicated that it possessed some cellulase activity (0.08 mg glucose/hr/mg dry at enzyme prep.) and a fair amount of reducing sugar contaminants. The zero time sample taken from the cellulose-cellulase reaction mixture (1 ml cellulase solution) gave a color equivalent to 3.0 mg of glucose. A check of both the cellulase and cellulose solutions clearly demonstrated that the reducing compounds were contained entirely in the cellulase preparation. A protein (Lowry[139] method) determination of the cellulase solution indicated that there was slightly less than 0.1 mg protein/ml. These preliminary studies show that the cellulase preparation is quite crude.

4.7.5 Electrochemical Experiments Details

The half-cells were 500 ml, five-neck flasks, separated by a cation membrane. The catholyte was 0.1 N KCl and the cathode was

platinized platinum. The anolyte was stirred with a Mag-mix and anaerobic conditions were maintained by a N_2 purge The temperature was not controlled. The potential was obtained with a standard calomel electrode placed in contact with an agar bridge immersed in the urine. A constant potential between SCE and anode (platinized platinum, 10 cm^2) was maintained with a potentiostat. No buffer or other solutions were used. The anode was positioned so that both faces were parallel to the current flow through the membrane. The agar bridge was positioned behind the anode (away from the membrane). An internal resistance at 175 ohms was found.

5.0 CONCLUSIONS

Based on the literature search, it appears that some inconsistency exists in trying to approach optimum utilization of urine and feces both as an electrochemical fuel and for conversion of these to chemicals reuseable in a closed environment. Thus it has been decided tentatively to follow predominantly the approach to optimum utilization of these materials as electrochemical fuels. Enzymes and bacteria were selected for study so as to lead primarily to production, from urine and feces, of ammonia and hydrogen.

In screening of five ureolytic bacteria, two were able to produce ammonia anaerobically in urine. Better than twice as much urea was hydrolyzed by B. pasteurii than by Brevibacterium ammoniagenes and in one-third the time (two days compared to six). As a result of this data, only B. pasteurii will be considered for further studies with urine.

Screening of bacteria and enzymes for conversion of feces to hydrogen and ammonia has not begun as yet.

Only preliminary electrochemical evaluation of urine with Bacillus pasteurii has been done and results are inconclusive as yet.

6.0 PROGRAM FOR NEXT INTERVAL

The screening program for ureolytic bacteria is complete; therefore, the major emphasis will be placed on feces degradation. Mixtures of urine and feces will also be evaluated as an approach to eliminating extensive separate studies. Selection of organisms will be based upon ability to function in the combined waste products. The addition of sewage bacteria and fecal microflora to urine/feces will be examined as an approach to establishing a suitable waste treatment process. Continuous culture parameters, including temperature, oxygen, pH and nutrition will be evaluated.

The screening program for appropriate enzymes will continue on the basis of chemical analysis of the feces/urine composite. The enzyme program will be integrated closely with the microbial program in a complementary effort to prevent duplication of efforts, except where comparative bacteria-enzyme studies are made, e.g., ureolytic bacteria and urease. Optimum activity for selected enzymes will be determined with respect to pH, electrolyte and substitute concentration and temperature.

Electrochemical measurements utilizing Bacillus pasteurii and urine will continue. Since it is anticipated that lyophilized feces will be available, suitable apparatus will be constructed and electrochemical measurements will be made with various media containing the feces.

7.0 REFERENCES

Three groups of references are given. Two of these groups are for literature cited in the literature search and the third is for references cited in the experimental work. Some duplication of references may be found as a result of this grouping. Because duplication is not severe, and in the interests of continuity of the separate literature searches, these duplications were allowed to remain.

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8.0 IDENTIFICATION OF KEY TECHNICAL PERSONNEL

Key technical personnel assigned during this quarter are as follows:

		Man-hours
J. H. Canfield	Head, Life Sciences (Project Leader)	115
J. J. Cavallo	Research Biochemist	98
B. H. Goldner	Senior Research Microbiologist	181
M. D. Lechiman	Research Microbiologist	200
R. Lutwack	Senior Research Chemist	108
C. Albright	Research Chemist	318