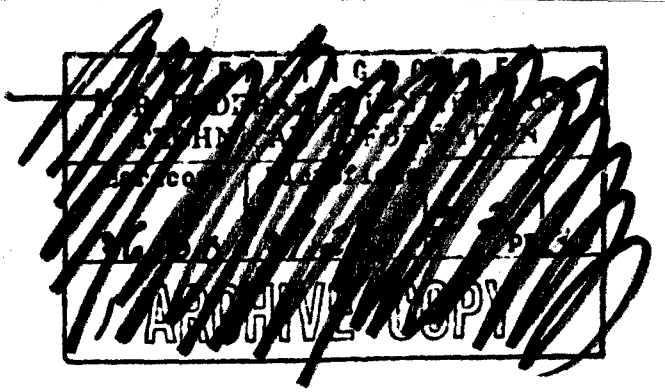
APL-TDR-64-70
PART III



MECHANISM OF MICROBIOLOGICAL CONTAMINATION OF JET FUEL AND DEVELOPMENT OF TECHNIQUES FOR DETECTION OF MICROBIOLOGICAL CONTAMINATION

Gordon C. Blanchard

Research Division, Melpar, Inc.

TECHNICAL DOCUMENTARY REPORT APL-TDR-64-70, PART III

September 1966

Air Force Aero Propulsion Laboratory
Research and Technology Division
Air Force Systems Command
Wright-Patterson Air Force Base, Ohio

MICROBIOLOGY

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FOREWORD

This is the final report prepared under Contract No. AF 33(657)-9186, Project No. 3048, Task No. 304801, "Mechanism of Microbiological Contamination of Jet Fuels and Development of Techniques for Detection of Microbiological Contamination," by Melpar, Inc., 7700 Arlington Boulevard, Falls Church, Virginia. This contract was initiated by the AF Aero Propulsion Laboratory, Research and Technology Division, Air Force Systems Command, Wright-Patterson Air Force Base. Mr. Jack Fultz was the Air Force Project Engineer.

This report was submitted by Dr. Gordon C. Blanchard in January 1966; it concerns work done from 1 March 1963 to 31 January 1966.

This technical report has been reviewed and is approved.

Arthur V. Churchill, Chief Fuels,
Lubrication and Hazards Branch,
Support Technology Division

ABSTRACT

This program was a portion of an Air Force effort to reduce or eliminate fuel system problems including the clogging of filters and fuel gages, destruction of sealants and coatings, and the corrosion of aircraft wing tanks. These problems were believed to be due to the growth of microorganisms in combination with other fuel contaminants.

The research effort in this program has been directed at developing rapid methods for detecting microorganisms in jet fuel water bottoms and at analyzing the chemical mechanisms by which microorganisms and other contaminants could cause operational difficulties in aircraft fuel systems. The mechanisms by which microorganisms produce emulsions, sludges, and fuel-soluble compounds and cause corrosion were studied.

Eight different methods for detecting microorganisms were evaluated including esterase, tetrazolium reduction, gas chromatography of lipids, CO_2 detection, O_2 utilization, radioactive silver binding, acridine orange staining, and fluorescein isothiocyanate staining. Only the last three methods had the sensitivity needed for detecting 10^3 to 10^4 microorganisms/ml of water bottom. The fluorescein isothiocyanate method was recommended for routine use because of its simplicity and minimum requirement for equipment and laboratory procedures.

The ability of fuel isolates to produce emulsions was demonstrated with jet fuel and pure hydrocarbons as substrates. The aldehyde and acid products of hydrocarbon oxidation, formed during the growth of the microorganisms, were believed to be responsible for some of the emulsions formed on jet fuel.

Fuel-oxidizing microorganisms were demonstrated to cause aluminum alloy corrosion by four different mechanisms: (1) alteration of the ionic composition of the growth medium, (2) production of corrosive compounds, (3) deposition on metal surfaces and establishing oxygen and metal concentration cells, and (4) production of electron mediators which transfer electrons from the metals to electron acceptors in the microorganisms.

During the first year of study, the growth medium was found to contain corrosion inhibitors and corrosion stimulators. Microorganisms were demonstrated to cause corrosion by removal of nitrate and phosphate as inhibitors. Nitrate was then tested for its ability to inhibit corrosion. The addition of 12 millimolar nitrate was found to inhibit the microbial corrosion of aluminum alloys. Aluminum alloy corrosion by natural water bottoms from fuel storage tanks was also inhibited by nitrate. Corrosion by the water bottoms was due principally to the additive ethylene glycol monomethyl ether.

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SECTION I

INTRODUCTION

This is the final report submitted in compliance with Contract No. AF 33(657)-9186 covering the work performed during the period 1 March 1963 to 31 January 1966. When this study began, the Air Force was experiencing severe fuel contamination problems at several Air Force bases resulting in the plugging of filters, fuel gauge malfunctions and pitting corrosion in aircraft wing tanks. The exact cause of these problems was not known at the time, but were believed to be caused by microorganisms in combination with other contaminants, such as water, iron rust, surface-active material, and extraneous materials.

The objectives of the studies performed under this contract were to: (1) determine the mechanism by which microorganisms in combination with other fuel contaminants degrade jet fuel and cause corrosion and the formation of emulsions, sludges, and fuel-soluble products; (2) develop techniques for the rapid detection of microorganisms in fuel water bottoms.

The first year of this study was devoted to determining the nature of fuel contaminants. Alterations in the microbial ecology of fuel/water systems were followed and the findings related to chemical changes in those systems. Knowledge was obtained also on how to efficiently grow organisms isolated from fuel, and experimental work was accomplished on methods for detecting microorganisms in fuel systems.

During the last 2 years the hydrocarbon-oxidizing microorganisms isolated from jet fuel-water bottoms and from aircraft wing tanks were shown to have the ability to produce several different metabolic products which caused emulsion formation, sludge formation, and corrosion. Some of the products were soluble in fuel, others were soluble in the water phase, and still others were insoluble in both phases and concentrated at the interface or on the bottom.

The mechanism by which the microbes produced each of these products was revealed by studying their production from purified hydrocarbons and glucose and by fractionation, purification, and identification studies. The ability of fuel isolates to grow on various purified hydrocarbons was tested. These studies revealed that some of the olefins, hexene, heptene, octene, and xylene were lethal to the microorganisms. These studies were extended to the Warburg respirometer where the effec of the olefins on respiration of resting cells and cell-free extracts were determined. The effects of the lethal olefins on dehydrogenases were also determined.

When these studies began, there were no theories which would explain the mechanism by which fuel-oxidizing microorganisms caused aluminum alloy corrosion. After examining most of the applicable corrosion literature and performing several unsuccessful experiments, the corrosive process was

examined theoretically, and four possible mechanisms by which microorganisms could cause corrosion were hypothesized. The hypotheses were that microorganisms cause corrosion by (1) altering the relative concentration of biologically essential ions in the growth medium, (2) producing corrosive materials from the oxidation and transformation of hydrocarbon substrates and inorganic medium components, (3) depositing on metal surfaces and establishing oxygen and metal concentration cells, and (4) producing substances which react directly with metal surfaces and transfer electrons from the metal surface to some terminal acceptor in the microorganism. During the past 2 years, research has been accomplished to show the importance of each of these hypotheses in the corrosive process.

Research on the ability of a natural water bottom to support growth of fuel isolates and to cause corrosion was also accomplished. The water bottom was fractionated by Sephadex chromatography, and the corrosivity of each fraction was determined. The components responsible for corrosion were also identified.

The ability of nitrate to control corrosion caused by microorganisms and water bottoms was studied and its potential application as an additive for aircraft wing tanks was determined.

Eight different methods for detection of microorganisms in fuel water bottoms were explored. The sensitivity of each method was examined first, and the most hopeful methods were then tested for speed and interference from water bottom components. The method that appears to be best suited for routine use with the minimum of equipment and laboratory procedures is described in Section IV, "Recommendations."

The laboratory media and general procedures used throughout this study are described in the appendix.

SECTION II

SUMMARY

This research program has been concerned with (A) determining the mechanisms by which microorganisms cause (1) aluminum alloy corrosion, (2) hydrocarbon oxidation; the formation of (3) emulsions, (4) sludges, and (5) fuel soluble compounds during their growth on JP-4 jet fuel; and (B) the development of techniques for the rapid detection of microorganisms in jet fuel.

A. Mechanism Studies

1. Corrosion

The corrosion studies have been confined to the four hypotheses by which microorganisms could cause corrosion.[1] The first hypothesis proposed that microorganisms cause corrosion by altering the relative concentration of biologically-essential ions in the growth medium. The results of this effort showed that when bacteria removed phosphate and nitrate from a growth-supporting medium the medium becomes more corrosive to aluminum. It was concluded that the greater corrosivity resulted from increasing the proportion of iron, calcium, and chloride present in the medium. Examination of the corrosive process by weight loss measurements showed that the growth of microorganisms for 30 and 90 days modified Bushnell-Haas/JP-4 fuel media, containing between 0 and 0.8 millimolar KNO_3 as the only nitrogen source for growth, caused significantly greater corrosion of aluminum alloys 7075 and 2024 than was observed in controls. All nitrate concentrations inhibited corrosion by the media alone. Microorganisms failed to cause corrosion even after 90 days when the nitrate concentration was increased to 12 millimolar KNO_3. The rate at which microorganisms utilize this from the growth medium was followed to show the importance of nitrate in inhibiting corrosion. The results showed that the nitrate concentration of the medium increased from 12 millimolar to 0.1 millimolar within the first 20 days of growth, and remained constant thereafter. The nitrite concentration increased from 0 to 2 millimolar in the same 20 days, and remained constant thereafter. It was postulated that the inhibition of microbial corrosion observed even after 90 days incubation was due to the nitrite concentration rather than nitrate, since the nitrate concentration of the media (0.1 millimolar) was too low to inhibit corrosion by other components of the medium.

The ability of both nitrite and nitrate to inhibit the corrosion caused by corrosion $CaCl_2$ solutions was tested. The results showed that nitrite at 10 times the concentration of $CaCl_2$ caused no inhibition of $CaCl_2$ corrosion of 7075 and 2024 alloys. Nitrate at equimolar and 10 times concentration completely inhibited corrosion of these alloys. Therefore nitrite is not a corrosion inhibitor for these alloys and the

lack of corrosion by the 90-day cultures had to be due to the presence of some other inhibitor formed by the organisms.

The second hypothesis was that microorganisms can produce corrosive materials from the oxidation and transformation of hydrocarbon substrates and inorganic medium components. The noncorrosive 90-day 12 millimolar KNO_3 growth medium was fractionated and tested for corrosion. The medium was found to contain corrosive anionic components and cationic components upon fractionation by ion exchange chromatography. These corrosive components were demonstrated by Sephadex chromatography to be large molecules with molecular weights greater than 5000. The lack of corrosion by microorganisms after 90 days of incubation in the 12 millimolar KNO_3 medium was believed to be due to the production of corrosion-inhibiting products which inhibit corrosion by the high molecular weight corrosion materials produced by the microorganisms.

The presence of corrosive microbial products was also demonstrated in low nitrate modified BH media (0.4 and 0.6 millimolar KNO_3) after growth of microorganisms for 30 days. These products, in contrast to those formed after 90 days incubation, had low molecular weights (approx. 500) and could be easily separated on the G-25 Sephadex column. Thus, it appears that microorganisms do produce corrosive products during their growth on jet fuel, but the products formed after 30 days on low nitrate media are different from those obtained after 90 days of incubation on high nitrate media.

Research was accomplished on the third hypothesis: microorganisms cause aluminum alloy corrosion by depositing on metal surfaces and establishing oxygen and metal concentration cells. These studies showed that fungi are capable of accumulating very large amounts of metals on or within the mycelial mass and that this mass when deposited on the surface of aluminum alloys causes corrosion pits. Sulfate-reducing bacteria were also shown to cause severe pits in 7075 aluminum, but not in 2024 under the conditions of the experiment. The pits in this case were believed to be caused by the presence of hydrogen sulfide in the medium after growth of the bacteria.

The fourth hypothesis proposed that microorganisms produce substances which react directly with metal surfaces and transfer electrons from the metal surface to some terminal acceptor in the microorganism. The ability of the corrosive products isolated from the previously described studies to act as electron mediators has not been tested. An attempt was made with electrochemical half cells to measure the oxidation of aluminum by microorganisms. These studies were unsuccessful because of the lack of sensitivity and reproducibility of the methods. However, evidence to support the theory was obtained indirectly when electron mediators were added to the cultures. Methylene blue addition caused the microorganisms to deposit on the aluminum surface. The turbidity of the growth medium decreased as the microorganisms were deposited on the

alloys, and corrosion pits occurred under the deposits after 5 days. If microorganisms produce compounds similar to methylene blue, then they would be expected to cause corrosion. However, tests for this type of compound were not performed in this study.

2. Hydrocarbon Oxidation

The ability of microorganisms to oxidize hydrocarbons is a prerequisite to their growth in jet fuel-water systems and to the ultimate problems they can cause. Studies on hydrocarbon oxidation were begun by testing saturated and unsaturated paraffins from C_6 to C_{16} for their ability to support the growth of jet fuel isolates. Early in these studies it was realized that the microorganisms would grow on jet fuel and other hydrocarbons regardless of how the culture was maintained. That is, the enzymes involved in hydrocarbon oxidation were constitutive; good growth occurred on the hydrocarbons even though the cultures were maintained for a year or so on rich TGY media. This observation was confirmed by Warburg studies where oxidation of hydrocarbons was as good with TGY cells as with jet fuel cells.

Early in the studies C_6 to C_9 olefins were observed to be lethal to fuel cultures regardless of the substrate on which they were growing. A screening study was performed with all the jet fuel cultures, and hexene was found to be lethal to all the cultures; heptene, octene, and nonene were lethal for some cultures, but not for others. The concentration of hexene required to maintain sterility is between 2.5 and 5% in the fuel. The effect of the C_6 to C_9 olefins were then extended to Warburg studies where they were found to inhibit hydrocarbon oxidation by resting cells. Furthermore, it was shown that one of the mechanisms by which the C_6 - C_9 olefins inhibit respiration is by inhibition of the dehydrogenase enzymes. The ability of cell-free extracts to reduce DPN in the presence of jet fuel, glucose, octane, and ethanol was demonstrated, and the olefins inhibited DPN reduction in each case. Purified alcohol dehydrogenase was also inhibited when it was incubated with the olefins. No information is available yet to explain why the C_6 to C_9 unsaturated molecules are so potent while the saturated molecules either have no effect or serve as good growth substrates.

3. Emulsions

The ability of microorganisms to produce emulsions during their growth in jet fuel-water systems is due to the composition of the bacteria themselves and to metabolic products. The growth of one culture, strain 101, emulsified all the fuel placed over the inoculated medium. In a typical experiment 100 ml of Bushnell-Haas medium was overlaid with 1000 ml of fuel. After 2 days, the entire fuel layer was emulsified into a stable emulsion, which did not break upon standing. The high lipid content of the fuel isolates accounts for a portion of the emulsion; however, if these cells are removed by centrifugation and

filtration, soluble products still remain which will cause emulsions. Clarified culture media was fractionated, and weakly acidic emulsion-forming components were isolated and characterized.

The mechanism of emulsion formation by microorganisms was investigated by growing the cells on purified hydrocarbons and by testing products which are likely to be produced during their growth. Nonane, nonene, decane, decene, dodecene, tetradecane, and tetradecene all served as growth media for the microbial production of emulsions. The products formed first during hydrocarbon oxidation are likely to be alcohols, aldehydes, and acids. The ability of saturated hydrocarbons with chain lengths varying from C_8 to C_{10} and the corresponding saturated aldehydes, alcohols, and acids were tested in the absence of microbial growth. The results show that emulsions were formed from some of the acids and aldehydes, but not from the hydrocarbons and alcohols. The emulsions formed by the pure reagents are as stable as those formed by microorganisms and have the same appearance. Thus it appears that the immediate hydrocarbon oxidation products may be responsible for the emulsions observed with microorganisms growing on jet fuel.

4. Fuel-Soluble Products

A yellow fuel-soluble substance was produced on media containing jet fuel and nitrate; it was not produced on media containing ammonium ion as the sole nitrogen source or on pure hydrocarbons. The substance was produced in large amounts just prior to emulsion formation and remained in the fuel at acidic pH but entered the aqueous phase under alkaline conditions. The pH of the functional group which controlled the color of the compound was at 10.8. This compound did not cause corrosion and did not appear to be related to any problem.

5. Sludge

Sludge refers to a dense aqueous and fuel-insoluble product which is produced on all media containing nitrate and JP-4 fuel. This material, which contained all the insoluble components of the growth medium, including old cells, was insoluble in all the organic solvents tested and was solubilized only by H_2SO_4. Although this material did cause aluminum alloy corrosion, it was not studied extensively because of its complexity.

B. Detection Techniques

The purpose of this study was to develop a procedure which could be used for the early detection of any microorganism growth in a fuel system. When this study began the sensitivity limitations on the detector were not as great as they presently are. This change occurred because the Air Force now adds the bactericidal anti-icing additive, EGME, to its fuels and because better housecleaning practices have been initiated.

Implementing these changes, the number of organisms present in the fuel water bottoms has decreased from $10^6 - 10^7$ /ml to around 10^3/ml. The detection of 10^3 cells/ml places severe restrictions on the microbial detector; it places it in the sensitivity range required by most Biological Warfare Detectors, which by necessity are expensive and complex.

Eight different detection methods were evaluated for sensitivity, specificity, and lack of interference from materials in water bottoms. The results obtained with these methods are summarized in Table 1. Only three methods: acridine orange stain, fluorescein isothiocyanate stain, and radioactive silver stain possess sensitivity to detect 10^3 to 10^4 bacteria. Of the three methods, the acridine orange method is the only one which has the potential to lead to instrumentation that would be simple, reliable, reasonably inexpensive, and logistically desirable.

Acridine orange is a peculiar stain because it will form a complex with bacteria in water solutions that can be dissociated with ethanol. Thus, if one could find a filter which does not bind the acridine orange, one could run the entire detection reaction on a filter. The steps would be: (1) concentrate organisms from the water bottom onto the filter, (2) wash, (3) stain with acridine orange, (4) wash to remove excess stain, (5) dissociate with ethanol, and (6) measure fluorescence of the acridine orange which was bound to the organisms. The only problem area is to find a suitable filter for the reaction. This, however, does not appear to be an insurmountable problem. The studies of filters did not a appear to be warranted until information showing the sensitivity of the method had been developed. This data was obtained by using centrifugation techniques and Sephadex chromatography for separating free dye from stained bacteria. Neither of these methods is suitable for instrumentation because they are time consuming and introduce unnecessary losses of cells. However, they were suitable for developing information concerning the sensitivity of the detection approach. Further studies are required to find suitable filters for carrying out the entire reaction on the filter including collection, processing, and detection.

The radioactive silver method possesses the required sensitivity but has the disadvantage that it would be hazardous for routine field use.

The fluorescein isothiocyanate (FITC) method would be essentially the same as the acridine orange method except the method of detection would be different. FITC does not dissociate, thus detection would have to be done by some expensive and complex microscopic scanning system. The procedure would be as follows: (1) concentrate organisms from the water bottom onto the filter, (2) wash, (3) stain with FITC, (4) wash, and (5) count the stained cells on the filter. This procedure suffers the same limitations as the acridine orange method; namely, most of the filters react and bind with the dye thus making it impossible to distinguish the stained organisms on the filter. If this problem can be overcome, then instrumentation of the method will be feasible.

TABLE 1.

SUMMARY EVALUATION OF SENSITIVITY, SPECIFICITY AND LIMITATIONS OF DETECTION TECHNIQUES FOR JET FUEL MICROORGANISMS

Method*	Sensitivity	Specificity	Comment
Esterase	10^7 cells	Viable cells only	Enzyme activity was not proportional to cell number on jet fuel media.
Tetrazolium reduction	10^6-10^7 cells	Viable cells only	
Gas chromatography of lipids	10^7-10^8 cells	Both viable, nonviable cells	Lipid content varied with age of cells and growth medium. Lipid content low on jet fuel medium. Subject to interference by lipids in natural water bottom.
CO_2 detection	10^8-10^{10} cells	Viable cells only	Requires 8 to 24 hours for detection. Indicating soda lime column wets easily.
Oxygen utilization	10^8-10^{10} cells	Viable cells only	Requires 40 hours for detection in Fernback flasks, closed system.
Radioactive silver binding	(calculated) 5×10^3 cells	Both live and dead cells	Requires trained personnel for controlling and containing radioactivity.
Acridine orange stain	5×10^5 in 3ml 1×10^4 in 0.5 ml 1×10^3 visual count	Both live and dead cells	
Fluorescein isothiocyanate	5×10^5 in 3 ml 1×10^4 in 0.5 ml 1×10^3 visual count	Both live and dead cells	

*The procedures used in the above assays are described in Section III of this report.

SECTION III

EXPERIMENTAL WORK

A. Ecology and Nutritional Requirements of Jet Fuel Microorganisms.

1. Biological Changes

An ecology study was performed to establish (1) a basis for understanding major ecological phenomena occurring in fuel-water systems, (2) a basis for understanding interrelationships of the environment and biochemical activities of microorganisms as related to corrosion, and (3) clues that can be expanded into methods for preventing deteriorative activity. The ecology study established that major changes in a fuel-water system occurred during the first month of microbial growth.

Organisms used in this study were pure culture fuel isolates and mixed cultures of organisms found in water bottoms of petroleum tanks. Water bottom cultures were maintained on Medium 1 (See Appendix) for a period of 6 weeks. During this interval samples of the growing culture were periodically examined on semiselective media for changes in the relative proportions of microbial types present.

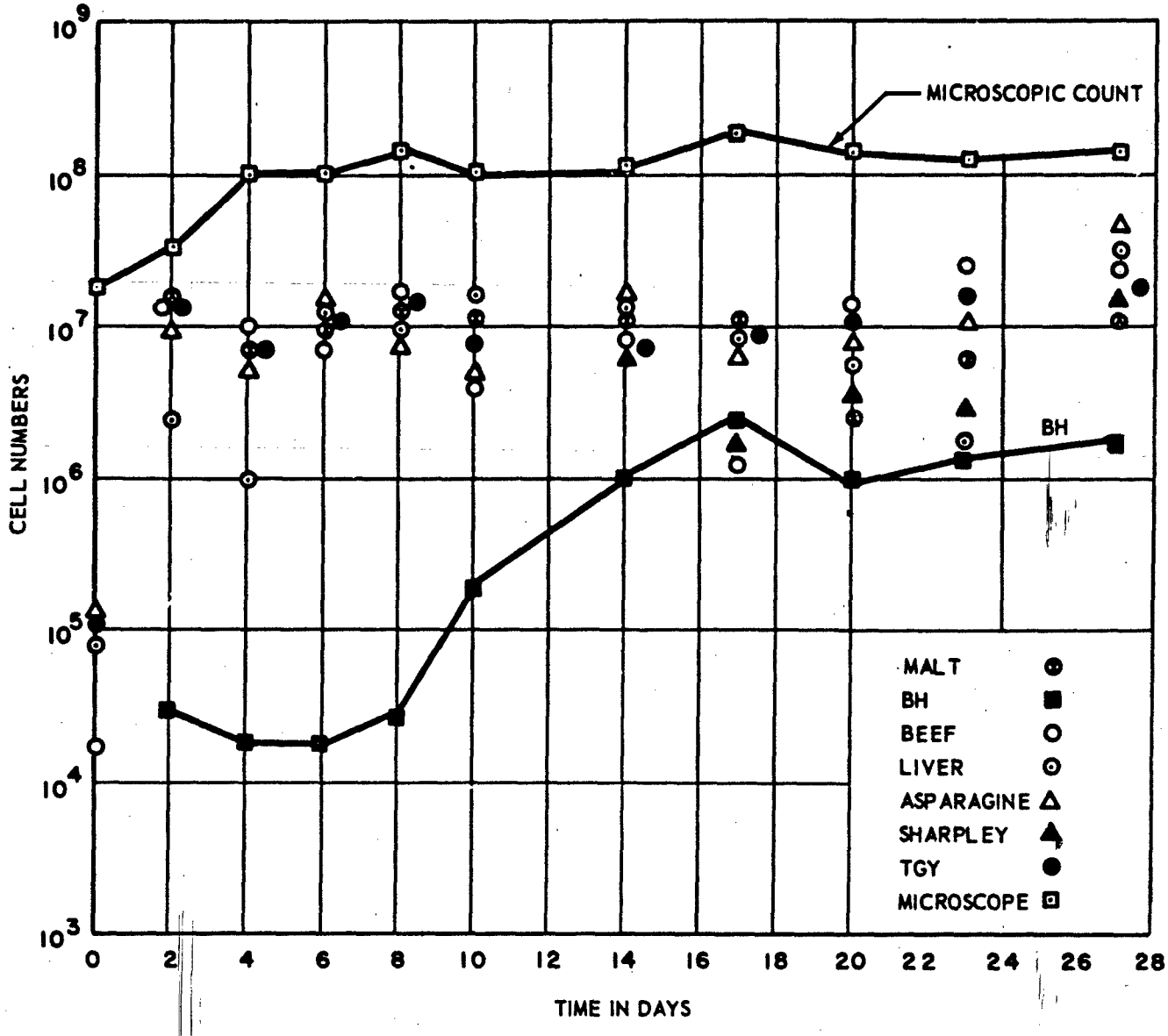
Sharpley's medium[2] was used for the isolation of anaerobic autotrophs, and aerobic autotrophs were screened using beef extract, Silverman's 9K medium[4] and thiosulfate medium;[5] the latter two media were selective. Anaerobic heterotropic organisms were isolated in deep liver medium,[6] and aerobic heterotrophs were grown on tryptone glucose yeast extract.[6] Molds and actinomyces were isolated, respectively, on malt extract agar and asparagine dextrose agar.[6] (See Appendix for medium compositions.)

The changes in number and types of microorganisms were followed for 57 days. The flora predominating in the medium during the first 28 days is shown in Figure 1. Thus, after 28 days, essentially the entire microbial population was composed of cells capable of oxidizing fuel in the presence of NH_4NO_3 and a few metal ions. The flora predominating in each agar after 2 months of growth are shown in Table 2.

2. Nutritional Studies*

Studies concerning the microbiological attack of fuels have been hindered by the lack of a medium that would provide good microbial growth of hydrocarbon-oxidizing microorganisms in a short time in the laboratory. The design of such a medium was undertaken as described below.

*Sharpley Laboratories, Inc., Fredericksburg, Va.



LEGEND:

THE ECOLOGY FLASKS CONTAINED 500ml MEDIUM, [1] 290ml JP-4 FUEL, 2 STRIPS OF 7075 AND 2024 ALUMINUM AND 3ml OF WASHED CELL SUSPENSION. THE MIXED CELL SUSPENSION WAS OBTAINED AFTER 10 DAILY TRANSFERS OF A WATER BOTTOM INOCULUM IN MEDIUM 1. DUPLICATE INOCULATED AND UNINOCULATED FLASKS WERE INCUBATED IN AIR UNDER STATIC CONDITIONS AT 28°C. SAMPLES WERE REMOVED DAILY FOR MICROSCOPIC COUNTS AND VIABLE COUNTS. THE COMPOSITIONS OF PLATING MEDIA ARE DESCRIBED IN THE APPENDIX.

Figure 1. Numbers of Microorganisms Isolated from Ecology Flasks

TABLE 2

NUMBERS AND MORPHOLOGY OF MICROORGANISMS
ISOLATED FROM WATER BOTTOM

Agar	Count	Predominant Flora
TGY	3.2×10^5	gram (-) rod, small round glisteni colony
Deep liver	6.5×10^6	gram (-) rod, green large spreadin colony
Sharpley	6×10^6	small round, raised, gram (-) rod, white colony
Beef extract	1.3×10^5	gram (-) rod, white smooth raised glistening colony
Malt	1.4×10^5	gram (-) rod, small yellow punctat raised opalescent colony
Asparagine dextrose agar	2.2×10^5	gram (-) rod, white raised trans-lucent colony
Thiobacillus plus TSA overlay	3.5×10^5	gram (-) rod, round raised white colony
BH Agar	3.6×10^4	gram (-) rod, round regular trans-lucent colony
9K Silverman*		growth after 7 days

*No attempt was made to quantitate the number occurring in the water bottom.

The experimental approach used was to first examine a number of media and other substrates described in the literature. Next, the substrates were examined using statistical techniques that would indicate the specific ingredients influencing growth. These data only indicate whether a given component was effective or ineffective at a high or low concentration. After the important ingredients were determined in this fashion, the optimum concentrations were found by factorial designs and experimental media compounded on these bases. The optimum physical conditions for cultures were then determined using the medium providing best growth in the previous tests.

A statistical technique was used in this work, since the very large numbers of variables in the media could not be analyzed by simple methods.

a. Screening of Known Media and Other Substrates

Nine different substrates were screened for ability to support growth of a Pseudomonas sp. (culture 179) and a Hormodendrum sp. (culture 181) when fuel was used as the sole carbon source. As shown in Table 3, Medium 1 provided the best growth of Pseudomonas; but Leadbetter's medium gave superior growth of the Hormondendrum. These two media were used as a starting point for the development of an improved medium.

b. Statistical Study of Leadbetter, Bushnell-Haas, and Other Media

A fractional factorial statistical design, as outlined by Davies,[10] was used to study the components of all of the media investigated during this study. The medium finally recommended was compounded on the basis of these data. Table 4 shows the detailed analysis of Leadbetter's medium using Hormodendrum as a test organism and Bushnell-Haas medium using Pseudomonas as a test microcrganism. These data were then statistically analyzed to determine which ingredient significantly increased growth. Further designs of this nature were used to separate factors that could not be fitted into a single analysis. The trace elements in factor E of Table 4 are an example. Using designs of this type the following media components appeared to accelerate growth of the test organisms as follows:

Pseudomonas	Hormodendrum
High concentration of buffer	Low buffer concentration
Magnesium sulfate	Sodium nitrate
Nitrogen source	Ferrous sulfate
	Potassium chloride
	Calcium chloride

After the constituents of the media had been indicated by the statistical studies, the concentrations were determined by simple factorial designs that modified only one ingredient.

TABLE 3

MICROBIAL GROWTH ON VARIOUS SUBSTRATES USING FUEL AS A CARBON SOURCE

Substrate	Nitrogen Source	Pseudomonas Mean O.D. (c)	Hormodendrum Mean mg mycelia (d)
Bushnell-Haas[1]	b	0.310	25.7
Stone-Fenske[7]	b	0.043	6.9
Sea water	a	0.052	14.7
15% Sea water	a	0.100	17.7
Water bottom	a	0.115	14.0
Tap water	a	0.101	3.2
Distilled water	a	0.066	0.3
Artificial sea water[8]	a,b	0.0	0.0
Leadbetter's[9]	b	0.075	36.0

(a) 1.0 g/liter ammonium nitrate added as a nitrogen source

(b) As referenced.

(c) 14 days static incubation at 25°C

(d) 22 days static incubation at 25°C

TABLE 4

FRACTIONAL FACTORIAL DESIGN FOR SIX OR SEVEN FACTORS

Leadbetter's Medium with Hormodendrum Using 7 Factors (A-G)
Bushnell-Haas Medium with Pseudomonas Using 6 Factors (A-F)

	FACTORS							Pseudomonas (2)	Hormodendrum (1)
Bottle	A	B	C	D	E	F	G	Mean OD	Mean mg mycelia
y1	-	-	-	-	-	-	-	0.088	28.4
y2	-	+	+	-	-	-	+	0.048	41.4
y3	+	-	-	-	+	-	+	0.035	26.6
y4	+	+	+	-	+	-	-	0.118	23.6
y5	-	-	-	+	-	+	+	0.067	13.4
y6	-	+	+	+	-	+	-	0.280	31.4
y7	+	-	-	+	+	+	-	0.063	74.7
y8	+	+	+	+	+	+	+	0.358	135.0
y9	+	+	-	+	-	-	+	0.111	19.3
y10	+	-	+	+	-	-	-	0.322	65.3
y11	-	+	-	+	+	-	-	0.113	14.4
y12	-	-	+	+	+	-	+	0.125	14.3
y13	+	+	-	-	-	+	-	0.116	24.4
y14	+	-	+	-	-	+	+	0.273	27.2
y15	-	+	-	-	+	+	+	0.085	23.3
y16	-	-	+	-	+	+	-	0.147	45.9
Control									36.0

Notes:

+ = high concentration
- = low concentration
(1) 22 days static incubation at 28°C
(2) 14 days static incubation at 28°C
(3) Factors A-F in Pseudomonas study represent $MgSO_4 \cdot 7H_2O$, $CaCl_2$, KH_2PO_4+K_2HPO_4, NH_4NO_3 and $FeCl_3$, respectively. A high concentration of 2X Normal BH and a low concentration of (0.1X) Normal BH were used.
(4) Factors A-G in Hormodendrum study are as follows on the next page:

TABLE 4 (Continued)

FRACTIONAL FACTORIAL DESIGN FOR SIX OR SEVEN FACTORS

Factor	Substance	High (grams/l)	Low (grams/l)
A	$NaNO_3$	4.0	0.2
B	$MgSO_4 \cdot 7H_2O$	2.0	0.4
C	$FeSO_4 \cdot 7H_2O$	2.0	0.1
D	Na_2HPO_4	0.42	0.021
	NaH_2PO_4	0.18	0.009

Factor	Substance	High (grams/l)	Low (grams/l)
E	$CuSO_4 \cdot 5H_2O$	1×10^{-5}	5×10^{-7}
	H_3BO_3	2×10^{-5}	1×10^{-6}
	$MnSO_4 \cdot 5H_2O$	2×10^{-5}	1×10^{-6}
	$ZnSO_4 \cdot 7H_2O$	1.4×10^{-4}	7×10^{-6}
	MoO_3	2×10^{-5}	1×10^{-6}
F	KCl	8×10^{-2}	4×10^{-3}
G	$CaCl_2$	3×10^{-2}	1.5×10^{-3}

c. Effect of Particulate Material and Iron

One of the factors previously associated with growth of organisms in fuel-water environments was the presence of rust. Preliminary laboratory studies indicated that better growth occurred in the presence of metallic iron than in its absence. The data in Table 5 were obtained to determine whether particulate material, metallic iron, or dissolved iron was responsible for the accelerated growth.

These data indicate that, of the substances tested, metallic iron is responsible for growth stimulation with Pseudomonas. With Hormodendrum the presence of particulate material appeared to greatly accelerate growth.

d. Effect of Trace Elements

Although a medium composed of the mineral salts found in Bushnell-Haas, with addition of iron as discussed previously, was shown to give the most rapid growth of the microorganisms under study in preliminary investigations, it was felt that certain trace elements might enhance growth. A concentration of 10 ppm of Cu^{++}, B^{---}, Mn^{++}, Zn^{++}, F^{-}, Sr^{++}, and Fe^{+++} was added to Bushnell-Haas using a fractional factorial statistical design. The only factor to enhance growth was copper. A separate factorial design was set up as shown in Table 6.

e. Summary of Important Medium Constituents

The following factors were found to be most responsible for increasing growth. The composition of the final recommended media can be found in the Appendix.

1. Pseudomonas:

Phosphate Buffer: The laboratory data with different buffers indicate that growth stimulation provided by a high concentration of potassium phosphates (mono and dibasic) is due primarily to the phosphate ion rather than pH stabilization. (See Table 7.)

Copper: The addition of copper as cupric sulfate increases the final yield of bacteria but also the lag time; i.e., with copper present, greater growth finally will be obtained. Faster initial growth is obtained without copper. (See Table 6.)

Nitrogen Source: Ten different inorganic nitrogen compounds were tested as nitrogen sources as shown in Table 8. Ammonium nitrate, 1:1 ratio of mono and dibasic ammonium phosphate, and ammonium sulfate when used on an equivalent nitrogen basis gave about the same level of growth at 100 ppm nitrogen.

TABLE 5

GROWTH OF PSEUDOMONAS AND HORMODENDRUM IN SUBSTRATES CONTAINING IRON OR PARTICULATE MATERIAL

Substance	Concentrations (grams/l.)	Growth Bushnell-Haas	
		Pseudomonas (a) (Mean O.D.)	Hormodendrum (b) Mean (c) mg mycelia*
Ferric chloride	0.1	0.149	2.8
Ferric chloride	0.005	0.118	4.3
Iron powder	0.2	0.328	71.0
Iron powder	0.010	0.305	8.4
Sand	0.2	0.067	65.7
Sand	0.010	0.062	8.3
Talc	0.1	0.137	91.7
Talc	0.010	0.128	12.6
Control	——	0.113	3.4

(a) Static incubation for 14 days at 28°C

(b) Static incubation for 22 days at 28°C

(c) Average of 3 determinations

TABLE 6

GROWTH OF PSEUDOMONAS SPECIES* IN COPPER DILUTION SERIES STUDY

$CuSO_4 \cdot 5H_2O$ (PPM)	Optical Density at 550 mμ		
	2 Days Mean	7 Days Mean	14 Days Mean
1	0.17	0.27	0.16
3	0.136	0.30	0.105
5	0.08	0.39	0.34
7	0.11	0.311	0.10
10	0.068	0.262	0.27
12	0.059	0.31	0.08
15	0.055	0.278	0.195
20	0.04	0.322	0.412
25	0.027	0.26	0.26
30	0.019	0.322	0.48
35	0.022	0.19	0.14
40	0.015	0.24	0.228
0	0.33	0.18	0.320
Bushnell-Haas	0.17	0.32	0.085

*Incubated 14 days under static conditions.

TABLE 7

GROWTH OF PSEUDOMONAS SPECIES IN PHOSPHATE AND TRIS BUFFER SERIES

Phosphate Buffer (a) (Concentration g/50 ml)	Optical Density at 550 mμ (Mean)
0.8024g KH_2PO_4 + 0.800 g K_2HPO_4	0.147
0.4012g KH_2PO_4 + 0.400 g K_2HPO_4	0.270
0.2006g KH_2PO_4 + 0.200 g K_2HPO_4	0.355
0.1003g KH_2PO_4 + 0.100 g K_2HPO_4	0.420
0.05 KH_2PO_4 + 0.05 K_2HPO_4	0.255
0.025 KH_2PO_4 + 0.025 K_2HPO_4	0.200
0.0125 KH_2PO_4 + 0.0125 K_2HPO_4	0.115
0.006 KH_2PO_4 + 0.006 K_2HPO_4	0.053
0.003 KH_2PO_4 + 0.003 K_2HPO_4	0.012
Control 0	0.002

Tris Buffer	Tris Salt (mg/50 ml)	Optical Density at 550 mμ (Mean)
1	1.56	0.0025 (b)
2	0.78	0.0007
3	0.39	0.005
4	0.195	0.00
5	0.097	0.001
6	0.048	0.003
7	0.024	0.00

(a) All indicated buffers replaced the phosphate buffer in normal Bushnell-Hass medium.

(b) Phosphate allowed growth when added to this series.

TABLE 8

GROWTH OF PSEUDOMONAS SPECIES IN VARIOUS NITROGEN SOURCES

Chemicals Used as Nitrogen Sources

N= PPM	1	2	3	4	5	6	7	8	9	10
48 hours										
0	0.120	0.120	0.120	0.051	0.085	0.121	0.087	0.084	0.084	0.123
1	0.169	0.166	0.113	0.066	0.067	0.152	0.137	0.137	0.056	0.086
10	0.285	0.248	0.265	0.070	0.054	0.203	0.097	0.057	0.030	0.147
100	0.206	0.053	0.241	0.032	0.012	0.0095	0.128*	0.442*	0.057	0.060
1000	0.018	0.025	0.035	0.010	0.037*	0.018	0.013	0.020*		0.148*
Control	0.036		0.098			0.172	0.116			
7 days										
0	0.091	0.014	0.034	0.022	0.052	0.077	0.067	0.018	0.039	0.036
1	0.116	0.078	0.107	0.034	0.079	0.099	0.107	0.114	0.116	0.089
10	0.640	0.301	0.257	0.308	0.149	0.291	0.117	0.036	0.108	0.360
100	0.302	0.122	0.362	0.432	0.017*	0.005	0.182	0.055*	0.027	0.143
1000	0.022	0.012	0.007	0.009	0.026*	0.0015	0.0015	0.006*		0.179
Control	0.024		0.013			0.108	0.017			
14 days										
0	0.029	0.015	0.020	0.016	0.017	0.029	0.032	0.011	0.0075	0.026
1	0.027	0.031	0.020	0.018	0.068	0.107	0.064	0.069	0.0305	0.060
10	0.459	0.533	0.465	0.098	0.122	0.217	0.126	0.412	0.093	0.306
100	0.266	0.310	0.295	0.401	0.036*	0.003	0.106	0.011	0.017	0.035
1000	0.026	0.027	0.008	0.004	0.025*	0.0025	0.005	0.008		0.200*
Control	0.021		0.097			0.031	0.020			

1 - NH_4NO_3
2 - $NH_4H_2PO_4$
$(NH_4)_2HPO_4$
3 - $(NH_4)_2SO_4$
4 - KNO_3
5 - KNO_2
6 - NH_4HCO_3
7 - NH_4HSO_4
8 - $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
9 - $FeNH_4(SO_4)_2 \cdot 12H_2O$
10 - $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$

* Where indicated, color development or chemical solids partially nullifies the turbidometric readings.

Sulfate Ion: The anion rather than the cation was found important for optimum growth. Either magnesium or sodium sulfate may be used as a SO_4^{--} source, but the former is preferable at this time.

(2) Hormodendrum:

Phosphate Buffer: A low phosphate buffer concentration provides the best growth. The optimum is about 150 ppm of PO_4^{---}. It is not certain whether the phosphate system exercises its effect on the fungus by buffering effect or phosphate metabolism.

Talc: Inert material provides better growth of Hormodendrum. The concentration providing best growth is at the high range; i.e., 2.0-4.0 grams per liter of sand, talc, or powdered iron. This is in distinct contrast to Pseudomonas where only metallic iron stimulated growth to a great extent.

Calcium Chloride: Preliminary data indicated that calcium chloride stimulated the growth of Hormodendrum. A confirming series gave poor growth but confirmed the use of calcium chloride at low levels.

Sodium Nitrate, Ferrous Sulfate, and Potassium Chloride: Preliminary statistical data indicated good growth of Hormodendrum using these salts. The optimum concentrations have not been defined, and the medium recommendation is based on the high-low statistical range. Refer to the Appendix for complete composition of recommended Hormodendrum and Pseudomonas medium.

f. Influence of Physical Parameters on Growth of Pseudomonas in Pseudomonas Medium

The influence of pH, temperature, agitation, and oil-water ratio on the growth of bacteria in Pseudomonas medium was investigated. The results are shown in Table 9. Based on optical density measurements, agitation increases growth of Pseudomonas by at least 10-fold; optimum pH is about 7.6; optimum temperature is about 28°C, and the higher the oil-water ratio the greater the turbidity.

The growth reported in the text and tabular material is much lower in many cases than the growth actually obtained. The Pseudomonas used as a test culture characteristically grows in clumps in this medium when used in static culture. The optical density readings are consequently quite low, since a thorough dispersion of the cells was not attempted prior to measuring the OD. We recommend that the medium be agitated. When grown in this fashion, optical density readings of 2.0 and above are not unusual after 3 days of incubation at 28°C.

TABLE 9

GROWTH IN PSEUDOMONAS TEST MEDIUM. STATIC VS. AGITATED CULTURES

Agitation	3 days (Mean O.D.)	7 days (Mean O.D.)	14 days (Mean O.D.)
Static	0.220	0.083(b)	0.204(b)
Agitated(a)	1.207	1.683	1.880
pH (agitated)			
5.0	0.070	0.057	0.082
6.0	0.131	0.064	0.137
7.0	0.416	0.402	0.418
8.0	0.169	0.169	0.097
9.0	6.170	0.239	0.267
Temperature, C° (not agitated)			
28°	0.253	0.254	0.496
37°	0.247	0.175	0.104
42°	0.111	0.301	0.161
Oil-water ratio (agitated)			
380 ml JP-4: 20 ml Pseudomonas medium	0.170	0.334	0.690
20 ml JP-4: 380 ml Pseudomonas medium	0.171	0.262	0.328
10 ml JP-4: 50 ml Pseudomonas medium	0.272	0.239	0.348

(a) 180 rpm, rotary shaker
(b) Settling and clumping of bacteria
(c) Except where indicated incubation at 28°C.

B. Mechanisms of Aluminum Corrosion by Microorganisms

1. Introduction

Attempts to demonstrate aluminum corrosion in Medium 1 under controlled conditions in the early phases of this research were unsuccessful. The absence of corrosion was puzzling for two reasons. First, it was anticipated that the alloys tested would be corroded simply as a result of exposure to salts of the sterile growth medium. Second, it was anticipated that the presence of microorganisms would enhance the corrosivity of the medium. Because the microbial corrosion of aluminum alloy was not easily demonstrated in this system, efforts were directed to determining the importance of the hydrogenase theory in corrosion by jet-fuel microorganisms.

When this study began, the hydrogenase theory was the only theory to explain microbial corrosion. This theory postulated that the anaerobic sulfate-reducing bacteria, Desulfovibrio desulfuricans, caused ferrous metal corrosion by their ability to bring about cathodic depolarization through the oxidation of hydrogen gas absorbed on the metal surface and by their ability to reduce elemental sulfur to hydrogen sulfide[11, 12].

The jet fuel microorganisms, however, would not grow anaerobically with jet fuel as the sole carbon source. Since some bacteria, the nitrogen fixing organisms, grown aerobically could synthesize hydrogenase[13] and since a putative relationship had been considered between the capacities of bacteria to oxidize hydrogen and hydrocarbons,[14, 15] studies were done to determine if a correlation between this enzyme and corrosion of aluminum could be demonstrated.

a. Preliminary Hydrogenase Assay

The hydrogenase assay was conducted by measuring methylene blue reduction manometrically.[16] Duplicate test and endogenous control flasks were run with each organism assayed. Test flasks contained 8μ moles methylene blue, 0.0625 M phosphate buffer pH 7.3, 0.2 ml KOH and convoluted filter paper in the center well, and 0.3 ml of culture in the side arm. Endogenous metabolism was determined by substituting distilled water for methylene blue. The final volume in each case was 3.2 ml. All flasks were incubated at 30° for 30 minutes under an atmosphere of hydrogen. After incubation, the flasks were tilted carefully to mix the organism and flask contents. Initial readings were made at zero time; subsequent readings were taken every 30 minutes.

Eight cultures isolated from jet fuel were used in this study. These cultures were transferred to medium and incubated at 30° C for 26 days. After incubation, the cells were collected by centrifugation and washed twice with 0.06 M phosphate buffer, pH 7.3. Five ml of buffer were added

to the packed cells, and the number of organisms per ml were determined microscopically.

The stock culture flasks contained sterile aluminum strips. Visual observations of aluminum alloy corrosion were made.

The hydrogenase content of the eight microorganisms growing on BH fuel media was compared with the corrosion of aluminum. The results of this study are shown in Table 10. Compared with the positive hydrogenase control (Escherichia coli grown on hydrogenase-inducing medium), very little enzyme activity was observed in any of the cultures. There were two possible explanations for this result; (1) the growth conditions were not anaerobic thus the hydrogenase enzyme was not induced,[17] (2) the growth medium did not contain the proper nitrogen source for synthesis of the enzyme. Pakes and Jollyman[18] found that nitrate inhibited the hydrogenase system of the colon-aerogenes group. Billen[19] found that hydrogenase synthesis was markedly suppressed by 10-20 mg ammonium nitrate/100 ml of medium. The BH medium contains 100 mg ammonium nitrate/100 ml medium.

b. Screening of 86 Strains of Fuel Isolates for Hydrogenase Enzyme

Preliminary screening studies to speed up the Warbury hydrogenase assay were initiated with 10 strains of fuel isolates. The criterion for the presence of the hydrogenase enzyme was gas production.

Durham fermentation tubes were prepared by inverting micro tubes (0.5 x 15 cm) in a larger tube (1.5 x 15 cm). Each organism was tested against three different media: TGY broth, Medium 1, and Medium 2. The TGY medium is known to induce hydrogenase.[16] One set of each medium was incubated aerobically at 30° C, and a duplicate set was incubated anaerobically. Observations for gas production were made after 24 and 48 hours of growth.

Results of preliminary screening studies are presented in Table 11. Four organisms produced gas in rich medium, but none formed gas in the fuel media. The lack of gas in the anaerobic fuel media was probably due to lack of growth. In the aerobic fuel media, the lack of gas was probably due to inability of the fuel isolates to synthesize hydrogenase aerobically. Screening of the remainder of Melpar stock cultures (86 in all) yielded only one gas-producing organism.

c. Quantitative Determination of Hydrogenase

The quantitative hydrogenase assay was conducted on the five positive hydrogenase cultures by measuring methylene blue reduction manometrically.[16] The test conditions were identical with those stated above with the exception that readings were made at zero time, 5 minutes, 10 minutes, and 15 minutes, with a final reading at 30 minutes. The

TABLE 10

PRELIMINARY COMPARISONS OF HYDROGENASE ACTIVITY WITH CORROSION*

Organism Control**	μl H_2 Evolved x 10^{-9}/30 Min/cell	Corrosion
(E. coli)	330	
	421	
1	2.9	±
	2.7	
3	0.10	±
	0.10	
7	1.2	+
	0.7	
11	0.0	
	0.09	—
13	10.1	±
	0.0	
18	0.46	+
	0.01	
20	0.0	+
	0.0	
31	12.4	
	0.0	—

* Corrosion. No visible pits were observed; blackening of aluminum was called positive.

** Control was grown aerobically on TGY to induce a hydrogenase activity. This organism was obtained from American type culture collection No. 9637 and was not an isolate from fuel.

TABLE 11

PRELIMINARY SCREENING FOR HYDROGENASE ENZYME*

Culture No.	TGY		BH-Fuel		BH-Fuel Minus Nitrate	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
87	-	-				
88	+	+	-	-	-	-
89	+	+	-	-	-	-
90	+	+	-	-	-	-
91	-	-	-	-	-	-
92	+	+	-	-	-	-
93	-	-	-	-	-	-
94	-	-	-	-	-	-
95	-	-	-	-	-	-
96	-	-	-	-	-	-

*(+) indicates gas production
(-) indicated no gas production

cultures were transferred to TGY broth, a rich hydrogenase-inducing medium, and incubated anaerobically at 30°C for 18 hours. After incubation, the cells were collected by centrifugation and washed twice with 0.06 M phosphate buffer, pH/7.3. Three milliliters of buffer were added to the packed cells, and the number of organisms per milliliter was determined microscopically. The hydrogenase activity of the five cultures was compared with Escherichea coli (also grown on TGY broth). Results in Table 12 show that cultures 88, 92, and 28 possess good enzyme content, while cultures 89 and 90 are essentially devoid of enzyme.

d. Effect of Nitrate Ion on Hydrogenase Activity

Since nitrate ion has been reported as an inhibitor of the hydrogenase enzyme system in the colon-aerogenes bacteria 18 and 19, the effect of this ion on hydrogenase activity of fuel isolates was investigated. Three media, i.e., TGY plus 2 grams of ammonium nitrate per liter and TGY plus 3 grams of ammonium nitrate per liter, were prepared, and 100-ml quantities were dispensed in 250-ml flasks. The desired culture plus a positive E. coli control culture was then grown anaerobically for 24 hours. Cells were then harvested and quantitative determination of hydrogenase activity was determined by the Warburg Technique 16.

The results presented in Table 13 show that all three cultures possess hydrogenase enzyme activity when grown in TGY medium indicating that the cultures are capable of utilizing molecular hydrogen. However, nitrate, 2g/liter (twice that employed in the BH medium), suppressed enzyme formation. With three times the concentration of nitrate used in Medium 1, no hydrogenase enzyme was formed. From this investigation it is apparent that nitrate suppresses the hydrogenase synthesis in fuel organisms.

The lack of growth of jet fuel microorganisms on jet fuel under anaerobic conditions and the absence of hydrogenase in microorganisms grown aerobically on jet fuel suggested that the microbial corrosion of aluminum alloys was accomplished by mechanisms other than those operative in the corrosion of ferrous metals.

e. Corrosion Inhibition by Nitrate Ion

Early studies showed that microorganisms caused no corrosion in Medium 1. Medium 9 was substituted for Medium 1 and good corrosion occurred (Table 14). In this study, however, only a few controls were examined. To verify this observation, the stock cultures were tested for their corrosiveness. In this experiment, 32 controls were run. Screening of 98 stock cultures, believed to be involved in the corrosion process, was accomplished by growing organisms in the nitrate-free medium (Medium 9) in the presence of alloys 7075 and 2024.

TABLE 12

QUANTITATIVE DETERMINATION OF HYDROGENASE ACTIVITY

Organism	μl H_2 utilized x 10^{-9}/30 min/cell
E. coli	30.6
	38.7
	34.8
88	26
	17
89	8.7
	4.2
90	0
	0
92	46.5
	44.7
28	40.9
	39.2

TABLE 13

QUANTITATIVE DETERMINATION OF THE EFFECT OF NITRATE ON HYDROGENASE ACTIVITY

Culture	Ammonium Nitrate (grams/100 mls in TGY growth medium)	μl H_2 utilized/hr $x10^{-8}$/cell
E. coli	0	50.2
88	0	57.7
88	0	43.9
88	0.2	6.59
88	0.2	0
88	0.3	0
88	0.3	
E. coli	0	42.5
89	0	7.8
89	0	8.4
89	0.2	0
89	0.2	0
89	0.3	0
89	0.3	0
90	0	47.6
90	0	38.1
90	0.2	1.14
90	0.2	2.83
90	0.3	0
90	0.3	0

Assay conditions: 0.3 mls culture in side arm of Warburg vessel; 0.2 ml 20% KOH plus convoluted filter paper in center well. 8 μ moles methylene blue and 0.625M phosphate buffer, pH 7.3. Final volume 3.2, temperature 30° C.

TABLE 14

LONG-TERM CORROSION STUDIES IN MEDIUM 1, MEDIUM

Culture	Medium 1						Medium 9	
	Aerobic			Anaerobic			Aerobic	
	7075-2024		Final pH	7075-2024		Final pH	7075-2024	Final pH
87	+	+	6.6	+	+	6.5	+ +	4.6
88	-	-	4.1	-	-	4.0	+ +	4.9
89	-	-	3.9	-	-	4.4	+ +	5.8
90	-	-	3.6	-	-	4.6	+ +	6.8
91	-	-	4.1	-	-	4.5	+ +	5.1
92	-	-	4.0	-	-	4.6	+ +	5.3
93	-	-	4.7	-	-	4.8	+ +	4.9
94	-	-	4.4	-	-	6.3	+ +	4.8
95	-	-	6.6	-	-	7.7	+ +	7.8
96	-	-	4.3	-	-	5.7	+ +	5.0
Control	-	-	6.5	-	-	7.1	- -	6.5

* + indicates corrosion pits on aluminum in the water layer
  - indicates no corrosion pits on aluminum in the water layer

Conditions: 100 ml media in 250 Erlenmeyer flasks; 5 ml inoculum; 10 ml JP-4 fuel ove
Control flasks contained no bacteria.

A

14

DIUM 1, MEDIUM 9, AND TGY MEDIUM

Medium 9				TGY MEDIUM			
obic		Anaerobic		Aerobic		Anaerobic	
Final pH	7075-2024		Final pH	7075-2024	Final pH	7075-2024	
4.6	+	+		-	-	8.6	-	-
4.9	-	-		-	-	8.8	-	-
5.8	+	+	4.5	-	-	8.7	-	-
6.8	+	-		-	-	8.9	-	-
5.1	+	+		-	-	8.8	-	-
5.3	+	-	5.8	-	-	8.8	-	-
4.9	+	-		-	-	8.8	-	-
4.8	-	-	6.2	-	-	8.8	-	-
7.8	-	+	6.9	-	-	9.0	-	-
5.0	+	+	5.5	-	-	8.8	-	-
6.5	-	-	7.1	-	-	8.7	-	-

JP-4 fuel overlay; 7075 and 2024 alloy flask - Incubation at 30°C for 6 weeks.

B

Observations of test and control flask were made after 4 weeks of incubation at 30° C. As was expected, 28 out of 32 control strips of the 7075 alloy were corroded, but all 98 strips of this alloy placed in growing cultures were corroded. On alloy 2024 corrosion occurred on 72 of the 98 strips placed in growing cultures, and 6 of the 32 control strips of this alloy were corroded.

Although these experiments did not definitely demonstrate the role of microbes in the corrosion process, they did show that with the 2024 alloy more corrosion occurred with organisms than in controls.

f. Four Mechanisms of Aluminum Corrosion

These observations posed the question of how microorganisms without hydrogenase growing in a nitrate-containing medium cause aluminum corrosion. In the course of these investigations, four mechanisms were tentatively proposed to account for the corrosion of aluminum alloys associated with aerobic growth of microorganisms. First, microbial growth or metabolism effects changes in the proportion of biologically-essential ions in a medium, thus causing the medium to become more corrosive. Second, microbial metabolism results in the production of compounds which react with the protective oxide films or gas absorbed to metal surfaces and thereby stimulates corrosion. Third, microorganisms produce substances which react directly with the metallic surfaces and transfer electrons from it to some terminal acceptor. Fourth, microorganisms effect large changes in the electro-chemical properties of very confined areas through microcolony formation, and such microbial deposits restrict the diffusion of gases and metabolic products and result in corrosion by oxygen cell or concentration cell formation.

2. Corrosion Due to Alteration in Ionic Composition of the Medium

a. Corrosion Caused by the Components of Bushnell-Haas Medium [1]

The mineral constitutents of Medium 1 were altered systematically, and the effect of this alteration was related to bacterial growth and to aluminum corrosion. To evaluate the contribution of microbial growth to aluminum corrosion, it was first necessary to determine the ability of the individual ions of the growth medium to cause corrosion in the absence of inhibitors such as nitrate and in the absence of microorganisms.

Tests for corrosion were made by placing 7075 and 2024 alloys in a 250-ml Erlenmeyer flask which contained 100 ml of mineral media and 10 ml of JP-4 fuel. Both alloys were 0.635 cm wide and 10.16 cm long. The 7075 alloy was 0.081 cm thick and the 2024 alloy was 0.027 cm thick. Growth took place on a rotary shaker at 30° C.

Aluminum alloy coupons were cleaned before testing by soaking them in methanol, wiping the strips with paper towels, and immersing them in 5% NaOH at 50°-60° C for 1-2 minutes. The bars were then washed thoroughly in running tap water and immediately immersed in a solution of 1:1 nitric acid for ½ minute. The bars were again washed in running water; finally, they were dipped in acetone or methanol, blotted dry, and stored in a desiccator.

Tests for corrosion were essentially qualitative and consisted of visual and microscopic examination of the alloys after various periods of exposure in microbial cultures. Medium 1 was thought to be corrosive to aluminum because it contained ferric ions which at the pH of the medium were largely in the form of ferric hydroxide. Ferric hydroxide or rust was known to corrode aluminum alloys. The medium also contained calcium, and this ion was corrosive to these alloys.

Tests were made to determine the concentrations at which ferric hydroxide and calcium sulfate corrode alloys 2024 and 7075. Table 15 shows the corrosion of these alloys after 48 hours at pH 7 by various quantities of ferric hydroxide suspended in 100 ml of distilled water. These determinations show that the quantity of ferric hydroxide present in Medium 1 will corrode the two alloys in the absence of the corrosive calcium ion and the other ions of the medium.

TABLE 15

ALUMINUM CORROSION CAUSED BY $Fe(CH)_3$

Moles $Fe(OH)_3$	Alloy 7075	Alloy 2024
$8x10^{-6}$	-	-
$8x10^{-5}$	-	-
$8x10^{-4}$	+	+
$8x10^{-3}$	++	++
$8x10^{-2}$	+++	+++

The corrosion of aluminum alloys by various concentrations of calcium sulfate in 48 hours at pH 7 is shown in Table 16. The concentration of calcium which is used in the Medium 1 produces severe corrosion in a short period of time in the absence of the other ions of the medium.

Similar tests were made with magnesium sulfate at 10^{-4} concentration, but this metal did not corrode aluminum. The corrosion of these alloys in aqueous solutions with only nitrate or phosphate was studied. Neither

nitrate nor phosphate, at 10^{-1} M and pH 7, caused aluminum corrosion. Phosphate is known to be a metal passivator, and nitrite, but not nitrate, has been shown to passivate some metals.[20] The absence of corrosion of aluminum coupons submerged in Medium 1 was thought to result from a possible interaction of corrosive and noncorrosive ions.

TABLE 16

ALUMINUM CORROSION CAUSED BY $CaSO_4$

Moles $CaSO_4$	Alloy 7075	Alloy 2024
8×10^{-6}	-	-
8×10^{-5}	-	-
8×10^{-4}	+	+
8×10^{-3}	++	++
8×10^{-2}	+++	+++

b. Corrosion Inhibition by Components of Bushnell-Haas Medium

The ability of nitrate and phosphate to act as inhibitors of the corrosion produced by calcium ion and ferric hydroxide was explored. Both anions were found to prevent corrosion caused by ferric hydroxide and calcium ion at the relative concentrations used in the growth media. Table 17 shows the inhibition of corrosion as a function of nitrate concentration in solutions which contain 10^{-4} moles of ferric hydroxide per liter. Table 18 shows the ability of nitrate to inhibit corrosion caused by 10^{-4} moles of calcium sulfate per liter. Tables 19 and 20 show the phosphate inhibition of the corrosion caused respectively by ferric hydroxide and calcium sulfate. All observations were made at the end of 48 hours.

These observations reveal that aluminum corrosion is stimulated by some biologically essential ions and inhibited by other biologically-essential ions. Therefore the question was raised concerning the type of growth medium which would be appropriate for a study of microbial corrosion of aluminum. It was obvious that a medium containing too little nitrate or phosphate would permit iron and calcium to stimulate aluminum corrosion independent of microbial action. But media high in nitrate or phosphate would prevent the occurrence of the corrosion which would perhaps be mediated directly by metabolic products or by the action of microbial enzymes.

The realization that certain biologically-essential ions were inhibitors and other ions stimulants of corrosion led to a new concept of microbial corrosion. Thus, it was conjectured that microorganisms remove phosphate and nitrate more rapidly than calcium or iron from the medium in which they grow. By means of this selective and differential utilization of ions, microorganisms make the medium in which they grow progressively more corrosive. This notion is consistent with the relative quantities of calcium and iron and nitrogen and phosphorous found in the microbial cell. The metals are present in trace amounts in microorganisms and often function as cofactors in enzyme-catalyzed reactions, but the elements of nitrogen and phosphorous exist as part of the macrostructure of the cell in their function as components of protein and nucleic acids.

c. Corrosion Due to Microbial Alteration of Components of Bushnell-Haas Medium

In the present study, this concept of the microbial corrosion of aluminum alloys was partially tested. Uninoculated and inoculated media were each replicated 15 times. The inoculum consisted of fuel-grown pseudomonads, which had been washed three times in 50 volumes of water or pure cultures of pseudomonads isolated from jet fuel systems. The results obtained with individual strains of bacteria and with mixed culture were essentially the same in these studies. Growth took place again at 30°C on a rotary shaker.

TABLE 17

NITRATE INHIBITION OF ALUMINUM CORROSION CAUSED BY 8×10^{-4} M $Fe(OH)_3$

Moles KNO_3	Alloy 7075	Alloy 2024
0	+++	+++
1.2×10^{-6}	+++	+++
1.2×10^{-5}	++	++
1.2×10^{-4}	+	+
1.2×10^{-3}	-	-
1.2×10^{-2}	-	-

TABLE 18

NITRATE INHIBITION OF ALUMINUM CORROSION CAUSED BY 8×10^{-4} M $CaSO_4$

Moles KNO_3	Alloy 7075	Alloy 2024
0	+++	+++
8×10^{-6}	+++	+++
8×10^{-5}	++	++
8×10^{-4}	+	+
8×10^{-3}	-	-
8×10^{-2}	-	-

TABLE 19

PHOSPHATE INHIBITION OF ALUMINUM CORROSION CAUSED BY 8×10^{-4} M $Fe(OH)_3$

Moles K_2HPO_4	Alloy 7075	Alloy 2024
0	+++	+++
8×10^{-6}	+++	+++
8×10^{-5}	++	++
8×10^{-4}	+	+
8×10^{-3}	-	-
8×10^{-2}	-	-

TABLE 20

PHOSPHATE INHIBITION OF ALUMINUM CORROSION CAUSED BY 8×10^{-4} M $CaSO_4$

Moles K_2HPO_4	Alloy 7075	Alloy 2024
0	+++	+++
8×10^{-6}	+++	+++
8×10^{-5}	++	++
8×10^{-4}	+	+
8×10^{-3}	-	-
8×10^{-2}	-	-

The extent of corrosion and the time of its occurrence is recorded in Figure 2. After 30 days incubation, the alloy 7075 was corroded only in inoculated media and in media which contained no nitrate or in media with low concentrations of nitrate, 0.02 g KNO_3 per liter This alloy was not corroded in inoculated media containing high nitrate concentrations, even though cell growth was abundant. In uninoculated controls, corrosion occurred in media without nitrate but corrosion did not occur in the presence of nitrate even after 97 days at the lowest nitrate concentration studied.

The microbial corrosion of the aluminum alloy 7075 was progressive and, with the passage of time and the growth and metabolism of microorganisms, corrosion occurred even in cultures which contained initially 1.2 g of KNO_3 per liter.

The presence of corrosion in both uninoculated and inoculated media without added nitrate was attributed to the actions of the corrosive ions of the Bushnell-Haas medium, the activities of which were somewhat diminished by the presence of phosphate.

The extent of corrosion initially was inversely proportional to the nitrate concentrations of inoculated media, but, after long periods of time, all inoculated media contained corroded coupons of alloy 7075. However, terminal corrosion was most profound in these cultures which initially contained 1.2 g KNO_3 per liter, and which were the last to corrode. An explanation of this terminal result perhaps lies in the discontinuous physiological response of the fuel isolates studied to increasing concentrations of nitrate. At 0.08 g KNO_3 per liter, the culture media was yellow after a 97-day growth, but at 1.2 g KNO_3 per liter, a compound(s) is formed which is insoluble in water and in jet fuel. The material is black and it is suspended in the aqueous phase of cultures which are shaken. This "sludge" adheres to aluminum surfaces during growth and possibly contributes to the corrosivity of the growth medium at high nitrate concentrations.

The pattern of microbial corrosion in relation to nitrate concentration depended also on the alloy tested. Figure 3 shows the corrosion of alloy 2024 in media with and without nitrate added. Again nitrate inhibited the corrosion of this alloy brought about by the calcium and iron of the growth medium. However, in inoculated media containing no nitrate or high concentrations of nitrate, corrosion differed significantly from sterile controls following 97 days of incubation. In media containing added nitrate, corrosion of aluminum alloy 2024 took place only in the presence of microbial growth. In general the 2024 alloy appeared to be more resistant to corrosion than the 7075 alloy, although its response to ions of the growth medium which were corrosion inhibitors and corrosion stimulants was essentially the same as the 7075 alloy.

E4532

0.0 KNO_3

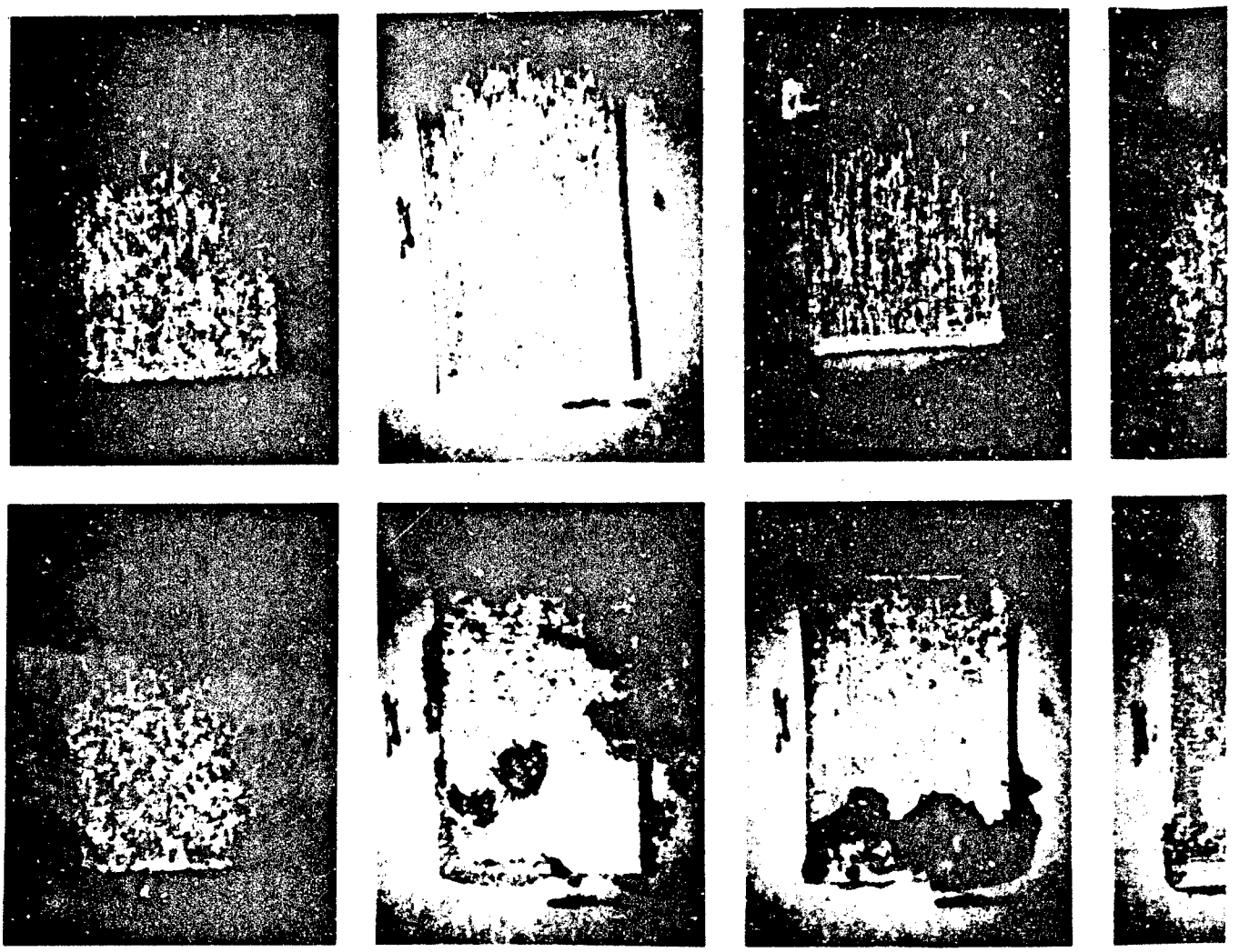
10

0.02 $\frac{g}{l}$ KNO_3

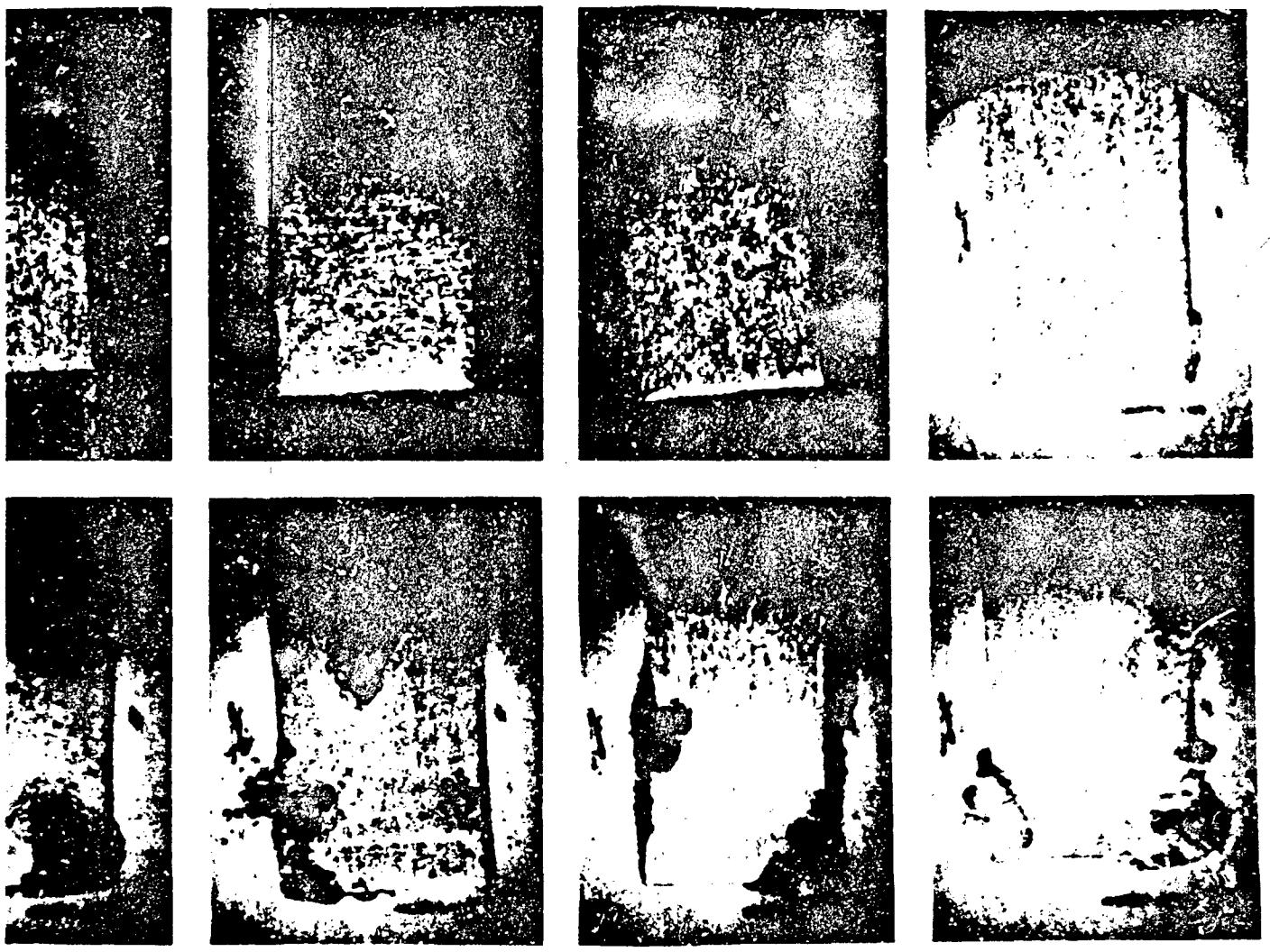
3

0.04 $\frac{g}{l}$ N

11

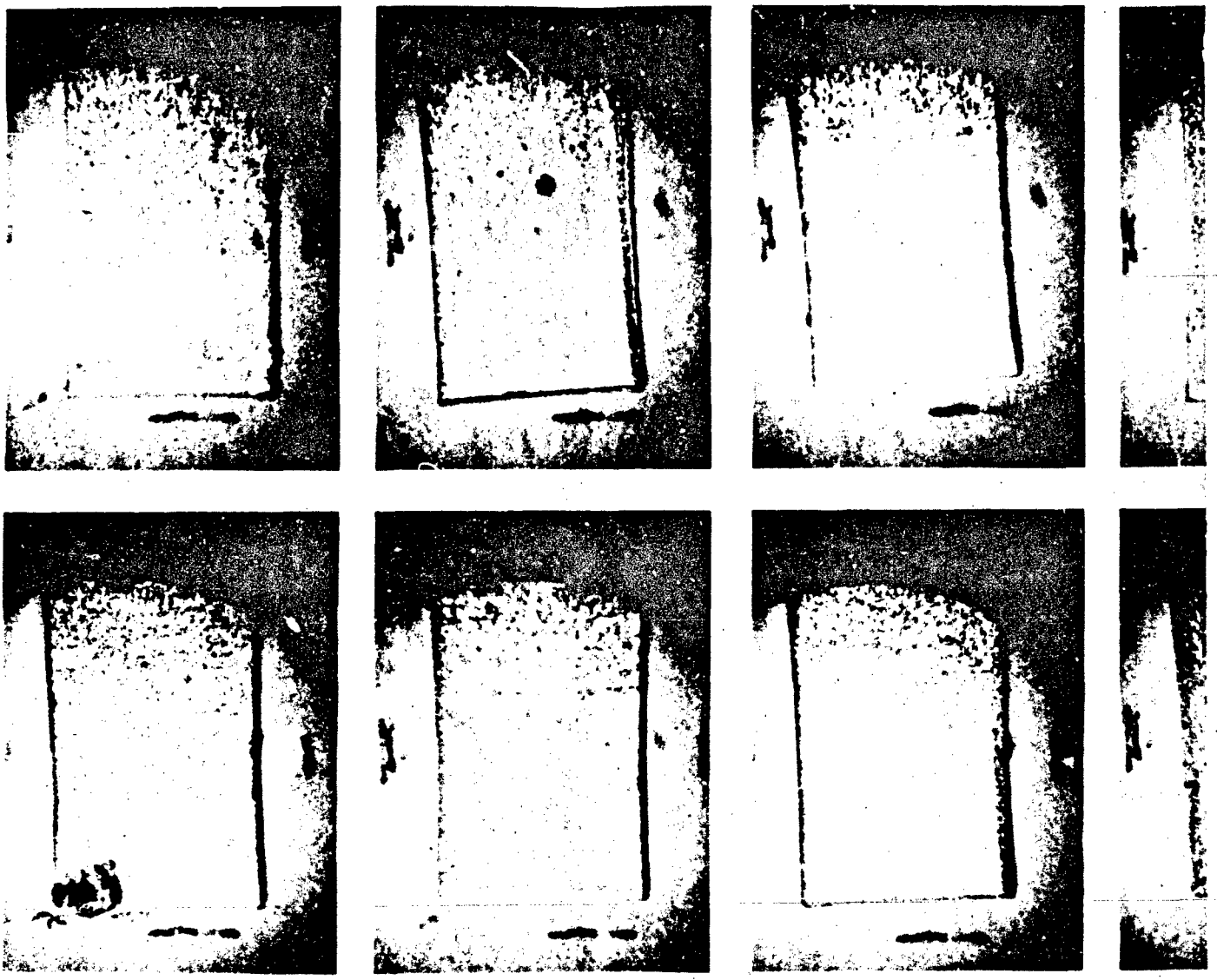


A



FLASKS WERE INOCULATED WITH 5 ml OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN MEDIUM 1, HARVESTED BY CENTRIFUGATION, AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C ON A NEW BRUNSWICK SHAKER. THE COUPONS FROM LEFT TO RIGHT WERE IN MEDIUM 10, 3, 11, 4, 5, 6 AND 9 RESPECTIVELY. ALL MEDIA WERE OVERLAID WITH 50 ml JP-4 FUEL. THE STRIPS SHOWN ON THE TOP ROW ARE FROM UNINOCULATED FLASKS AND THOSE ON THE BOTTOM ROW ARE FROM INOCULATED FLASKS. INCUBATION WAS FOR 97 DAYS.

Figure 2. Corrosion of 7075 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source

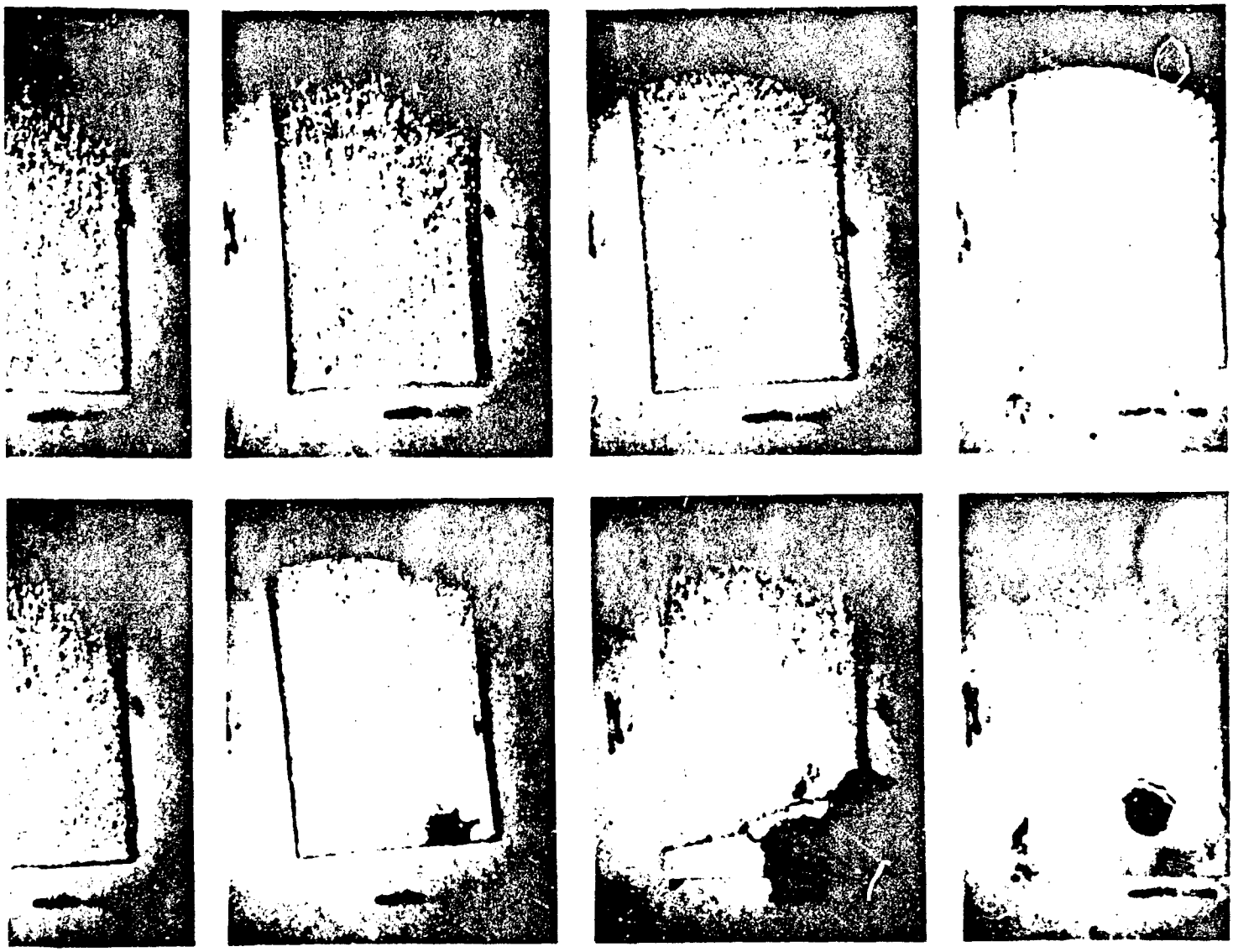


LEGEND

CONDITIONS WERE THE SAME AS THOSE DESCRIBED IN THE LEGEND OF FIGURE 2.

Figure 3. Corrosion of 2024 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source

A



B

d. Effect of Nitrate Concentration on Microbial Corrosion -- Corrosion Determined Quantitatively

Heretofore corrosion was determined by visual observations and these studies showed that the time required for microbes to make a non-corrosive medium corrosive depends on the nature and concentration of ions in the medium; nitrate is an especially effective corrosion inhibitor. It was demonstrated that the growth of microorganisms caused corrosion of 2024 and 7075 aluminum alloy coupons after 20 days when grown on Medium 3, versus 86 days needed to produce corrosion when medium 6 was used. The objective of the present studies was to repeat these studies with media which would be more favorable for demonstrating corrosion and to quantitate corrosion by measuring weight loss from the coupons.

To determine the effect of nitrate on corrosion and to minimize the inhibition effect of phosphate on corrosion, the media were modified to contain less phosphate. Ten flasks of each medium (Medium 12-17) were prepared. To five flasks of each medium, washed cells of culture 96 and culture 101 were added to give an initial concentration of 5.0×10^6 cells per ml of culture 96 and 5.4×10^6 cells per ml of culture 101 in each flask. The remaining five flasks of each medium were used as uninoculated controls. Coupons of aluminum alloys 7075 and 2024 were added to each flask, and all of the flasks were overlayed with JP-4 fuel.

Aluminum alloys 7075 and 2024 were cut into coupons (4 by 1/4 by 1/16 inches). The coupons were coded at one end with a diamond point, cleaned, and weighed (tare) before testing. The coupons were cleaned before test by washing with acetone, wiping with paper towels, and immersing in 50% HNO_3 for 1 minute. Next, the coupons were rinsed with flowing tap water, rinsed with distilled water, dipped in acetone, blotted dry on a lintless towel, and stored in a desiccator until weighed (tare).

The procedure of Robertson [21] was used for cleaning the alloy coupons after test; the coupons were heated for 10 minutes at 70°-80°C in the cleaning solution (20g $K_2Cr_2O_7$, 28 ml 85% H_3PO_4 sp. gr. 1.7, distilled water to 1000 ml), brushed clean of loose debris under running tap water, rinsed in distilled water, dried as described above, and reweighed. Weight was calculated from differences in weight obtained before and after test.

The data points after 30 days of incubation (see Figure 4) are means of triplicate determinations. The results show that 7075 is more resistant to corrosion by the growth medium and by microorganisms than is alloy 2024. The presence of microorganisms in the growth medium caused greater corrosion of both alloys. Nitrate inhibited the corrosion of alloy 2024, but had little or no effect on the corrosion of alloy 7075. Corrosion of alloy 2024 occurred in the control medium (no microorganisms) at 0, 0.2, and 0.4 millimolar KNO_3, but not at 0.6 millimolar KNO_3 or above. In the inoculated medium, corrosion of alloy 2024 occurred between 0 and 0.8 millimolar KNO_3, but not at 12 millimolar KNO_3.

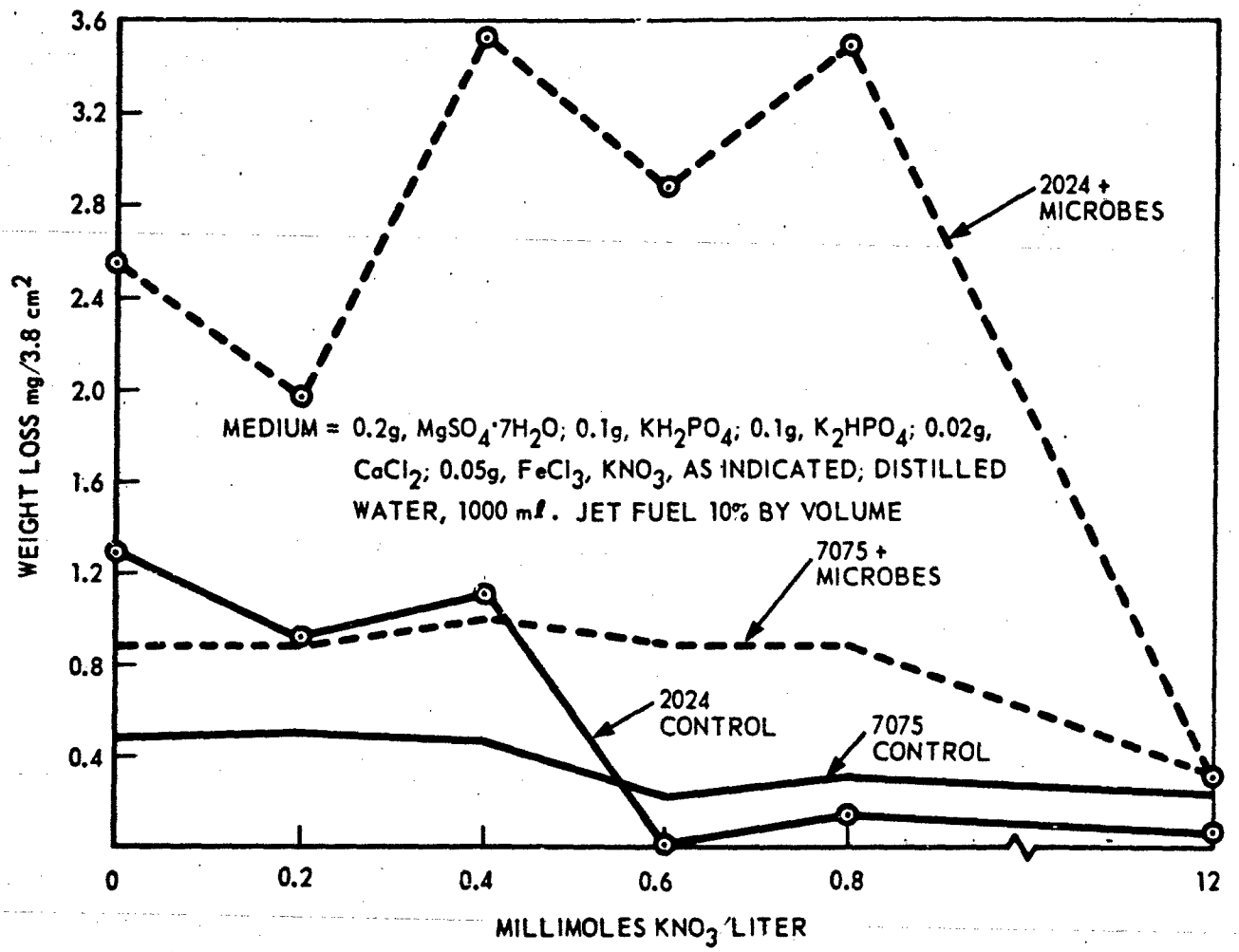


Figure 4. Effect of Nitrate on Microbial Corrosion of 7075 and 2024 Aluminum Alloy after 30 Days.

A statistical analysis was performed on the weight loss of alloy 2024 from data obtained between 0 and 0.8 millimolar KNO_3. The results of this analysis show a "t value" of 2.71, with 28 degrees of freedom. The probability of a larger "t value" is about 0.01. This means that there is a significant difference, at the 99% confidence limit, between inoculated and sterile media. In other words, if one inoculates this modified BH medium containing between 0 and 0.8 millimolar KNO_3 with the mixed culture and allows it to incubate for 30 days, one could expect more corrosion in the inoculated samples than in the control samples 99% of the time.

The data in Figure 4 shows that high concentrations of nitrate, 12 millimolar KNO_3, protected both alloys from corrosion. This agrees with our previous data[22] and with Uhlig and Gilman[23], who found that $NaNO_3$ completely inhibited pitting of 18-8 stainless steel in 1 - 15% $FeCl_3$ solutions. Corrosion in the latter system occurred in a very acidic environment (pH 1.0 to 2.0), whereas corrosion by microorganisms occurred in a neutral environment (pH 7.0-8.0). Thus the ability of nitrate to passivate metals appears to be a generalized phenomenon independent of the pH of the corrosive medium. The mechanism by which the passivity is induced is not known.

Previously, visual observations indicated that microorganisms caused corrosion in the high nitrate medium (Medium 6) if the coupons were allowed to remain in the medium for 3 months.[22] This experiment was repeated using the same conditions as in Figure 4 except there were 90 days of incubation for determining whether the corrosion occurs in the high nitrate media (12 millimolar). The data in Figure 5 show that nitrate concentrations between 0.2 and 0.8 millimolar protect the alloys from corrosion in the absence of microorganisms, but fail to protect the alloy in the presence of microorganisms. The results between 0.2 and 0.8 millimolar KNO_3 were analyzed statistically by the "t test", and it was found that highly significant differences at the 0.1% confidence limit exist between the corrosion observed in the controls and corrosion observed with microorganisms.

At these high nitrate concentrations (12 millimolar) no differences in weight loss were observed between the inoculated and the control media or between 30 days and 90 days of incubation. The lack of corrosion by microorganisms in the high nitrate media is in disagreement with our previous data[22] where microbes caused corrosion in 90 days (Figures 2 and 3). These differences may be due to the use of two different mixed cultures or to the different methods of assessing corrosion. The present experiments used a mixed culture containing two pseudomonads, strains 96 and 101. The previous experiments used a mixed culture containing strain 101, Cladosporium resinae, Aspergillus niger, and Desulfovibrio desulfuricans; D. desulfuricans did not grow in the aerobic system and the fungi grew poorly. The present method of measuring corrosion was by weight loss, whereas, previously, corrosion was assessed by visual observation of pitting and/or blackening.

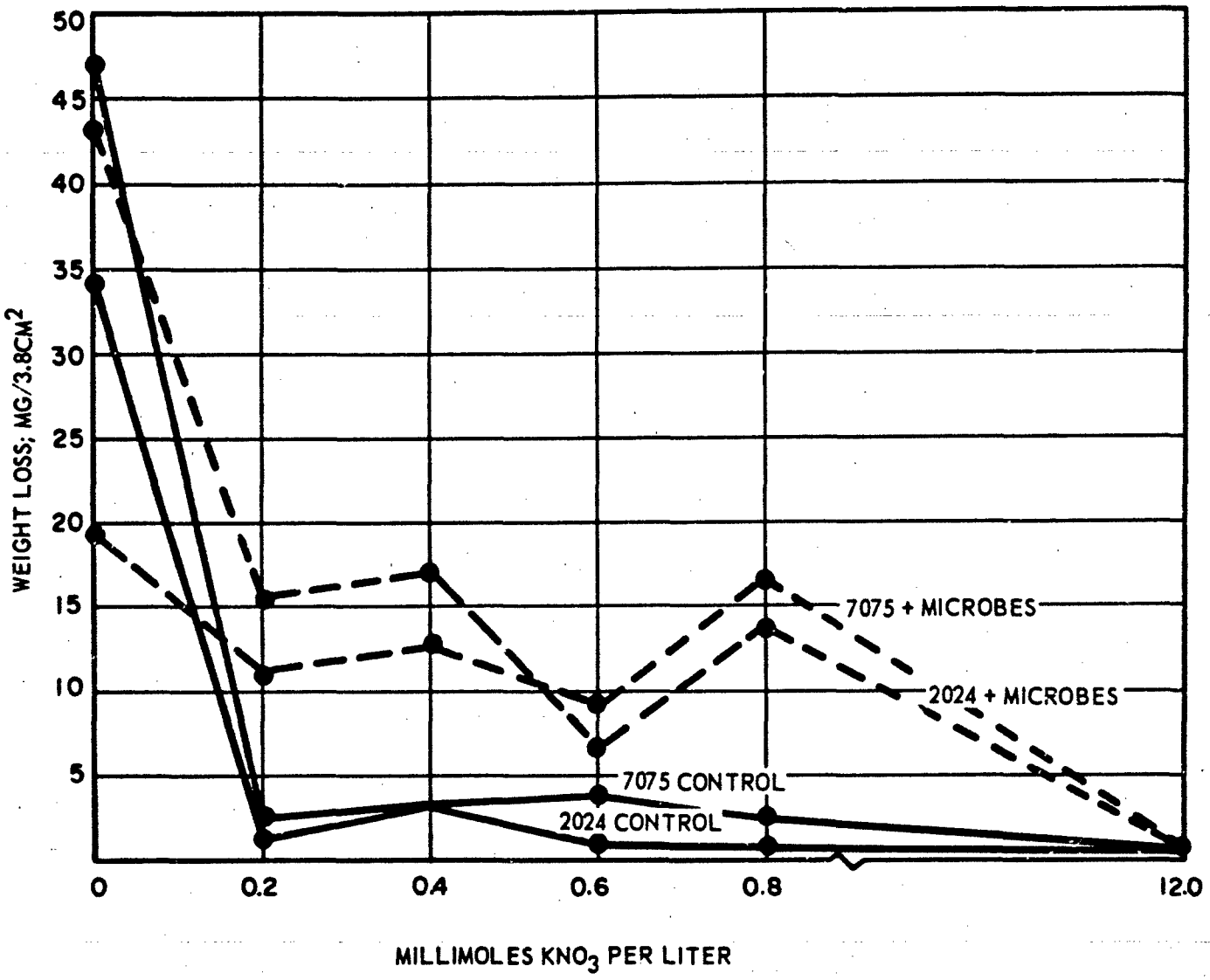


Figure 5. Effect of Nitrate on Microbial Corrosion of 7075 and 2024 Aluminum Alloy after 90 Days

e. Quantitative Comparison of Nitrate and Nitrite Corrosion Inhibition

Experiments were performed to determine whether strains 96 and 101 would reduce nitrate to nitrite and to determine the effectiveness of nitrate and nitrite as inhibitors of aluminum alloy corrosion. The concentration of nitrite was of interest because it is the first intermediate formed by the microorganisms in nitrate reduction,[24] and because it has been shown to inhibit stainless steel corrosion by $FeCl_3$.[23]

During the first 19 days of incubation the microorganisms changed the nitrate concentration from 12 millimolar to 0.1 millimolar and the nitrite concentration from 0 to 2 millimolar (Table 21). The remaining nitrate was probably converted into ammonia and to nitrogen-containing materials in the microbial cells. Since the microorganisms decreased the concentration of nitrate to 0.1 millimolar, it was postulated that the lack of corrosion during the 90 days was due either to nitrite inhibition of corrosion or to the production of products which inhibit corrosion.[25]

Nitrate and nitrite were tested as corrosion inhibitors of $CaCl_2$ corrosion. The alloys were immersed in 100 ml of mixture of $CaCl_2$ and KNO_3 or $CaCl_2$ and KNO_2 for 4 days at 28°C on a gyratory shaker. The weight losses observed are shown in Figures 6 and 7.

Three different concentrations of $CaCl_2$ were studied at four different ratios of chloride ion to KNO_3 or chloride ion to KNO_2; at 0.2, 2.0, 20, and infinitive ratios, the weight loss data in Figure 6 show that nitrate causes a variety of responses in a corrosive system. At a high molar ratio of Cl/KNO_3, 20, nitrate stimulates corrosion, whereas at lower molar ratios, 2 and 0.2, nitrate inhibits $CaCl_2$ corrosion of the alloys. These effects were not observed with KNO_3 (Figure 7). In fact, no inhibition was observed even at a molar ratio of 0.2. This proves that nitrite is not a corrosion inhibitor for these aluminum alloys. At a Cl/KNO_2 molar ratio of 2, nitrite stimulated corrosion of alloy 7075. This effect was observed initially, and the experiment was repeated with 10 coupons in 5 separate flasks. The weight loss observed was 82.2± 27 mg/3.8 cm^2 (mean ± maximum deviation). Thus, in contrast to the results observed with KNO_3 which inhibits corrosion of both steel and aluminum, KNO_2 appears to be an effective inhibitor only against steel corrosion. Although both nitrate and nitrite are good inhibitors of steel, it is not possible from the data presented herein or from the literature data cited to determine which inhibitor is the more effective inhibitor of steel corrosion.

It is obvious from the weight-loss data that nitrite does not inhibit aluminum alloy corrosion and that the inhibition of corrosion observed in the microbial experiment between 20 and 90 days must have been due to the presence of some microbially-produced corrosion inhibitors.

TABLE 21

NITRATE AND NITRITE CONCENTRATIONS IN MEDIUM 6 SUPPORTING MIXED CULTURE

Time (Days)	Nitrate (moles/liter)	Nitrite (moles/liter)
0	120.0×10^{-4}	0
19	0.949×10^{-4}	20.7×10^{-4}
43	1.19×10^{-4}	19.4×10^{-4}
89	0.791×10^{-4}	20.7×10^{-4}

Inoculm: Mixed culture - Cladosporium resinae (99). Aspergillus niger (100) Pseudomonas aeruginosa (101). Desulfovibrio desulfuricans (102), grown on medium 1. 48 hours, washed 3x, then transferred to medium 6.

Nitrate determined by method of Skujins[26].
Nitrite determined by method of Pappernhagen and Mellon[27].

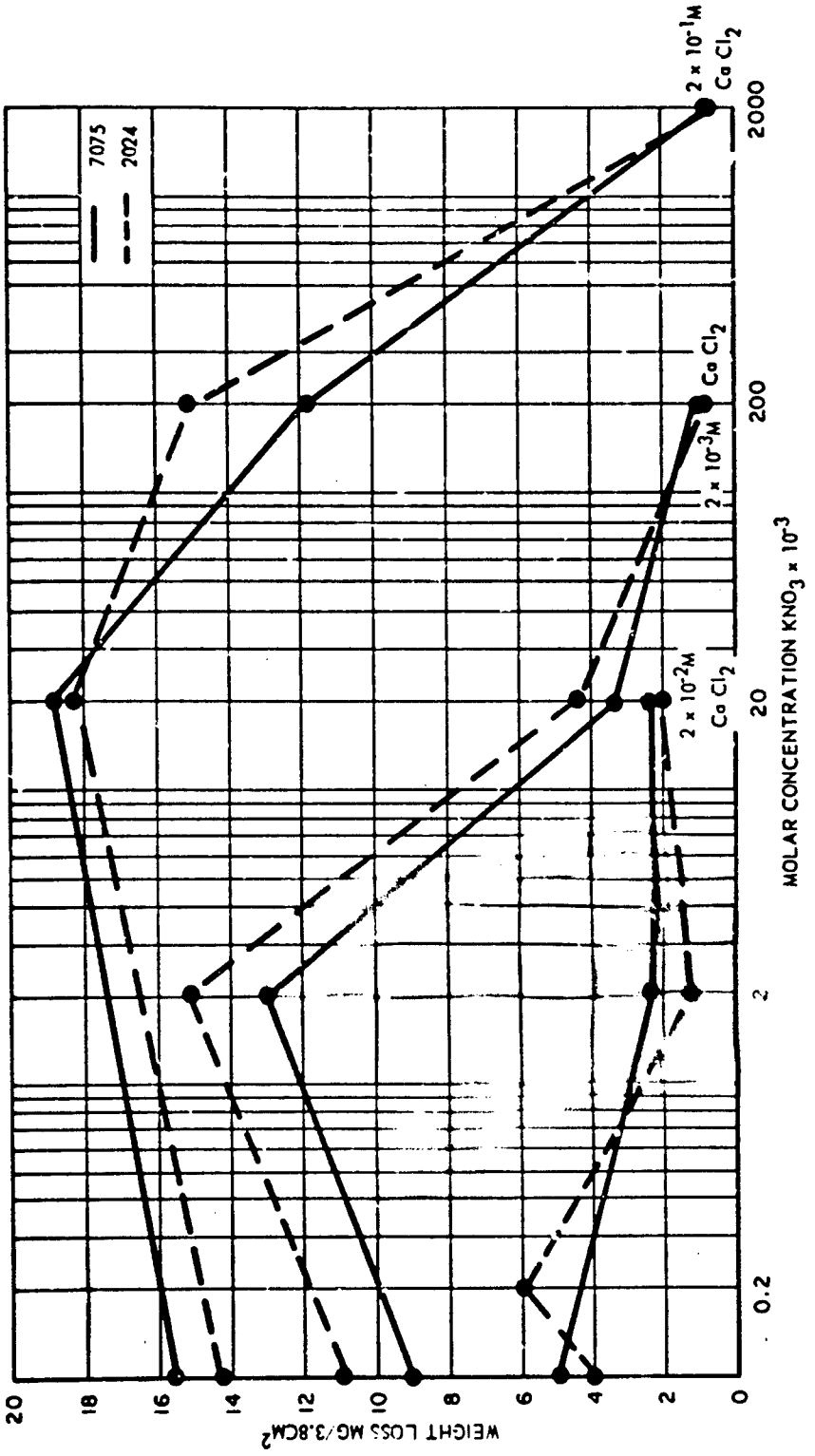


Figure 6. Effect of Nitrate on $CaCl_2$ Corrosion of 7075 and 2024 Aluminum Alloys

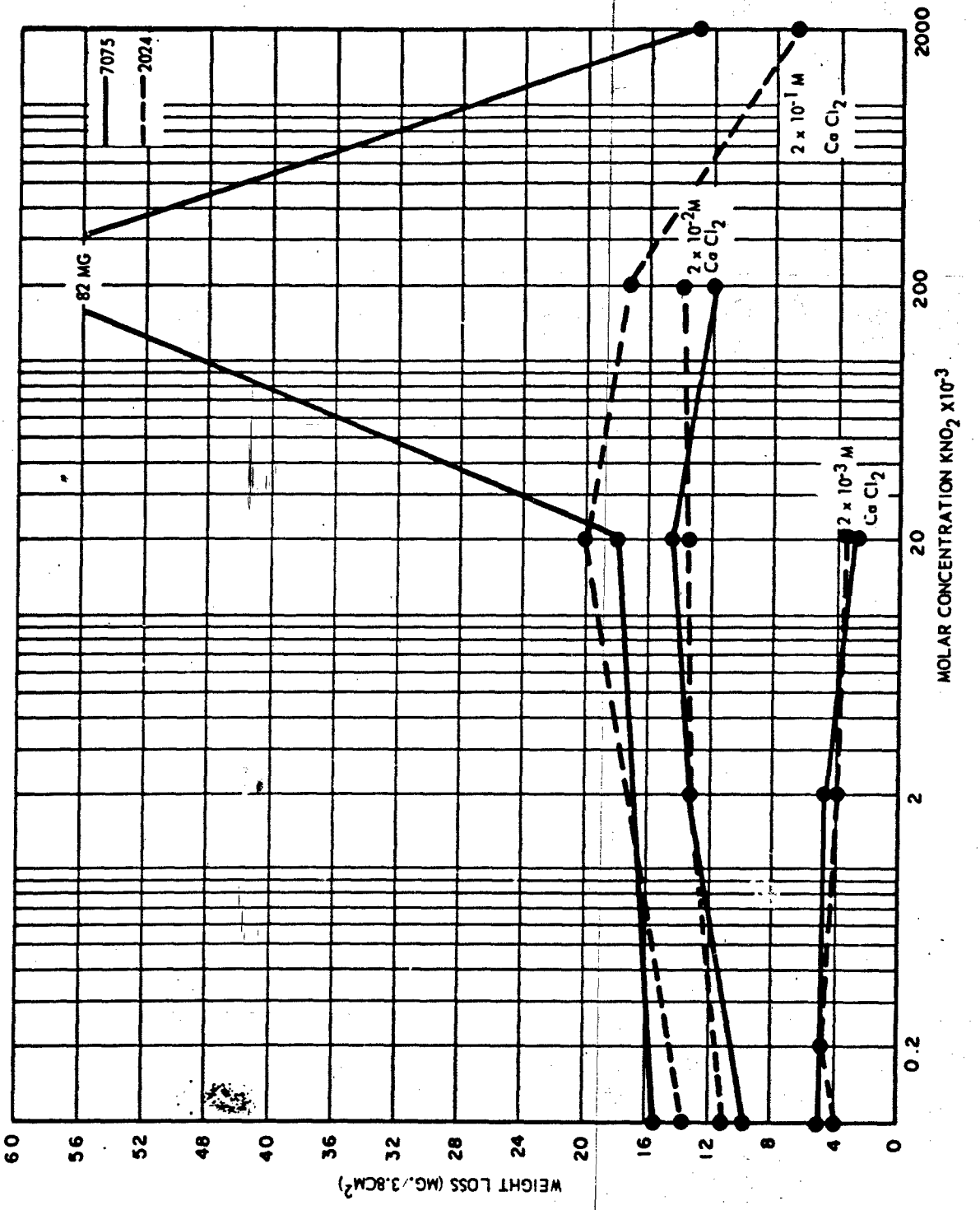


Figure 7. Effect of Nitrite on $CaCl_2$ Corrosion of 7075 and 2024 Aluminum Alloys

3. Production of Compounds which React with Metal Surface and Corrode

a. Characterization of Corrosive and Corrosion-Inhibiting Components in the Aqueous Phase of 90-Day Cultures

The purpose of this study was to determine which microorganisms caused no corrosion in high nitrate media after 90 days of incubation even though most of the nitrate had been utilized after 20 days of incubation. The approach used was to isolate the corrosive fractions from 90-day growth media and to determine whether other fractions of the growth media inhibit corrosion by these fractions. Also differences in location of corrosive fractions and corrosion inhibitors of the media were also of interest.

Both pure strains and mixed cultures produce dark, corrosive pigments, which are soluble in the aqueous phase. The production of these brown-black pigments depends on the composition of the media and on time. They are produced only on media which contain both nitrate and JP-4. Color increases with nitrate concentration. Uninoculated controls are colorless. Both strains 96 and 101 produce pigments in 15 days on Medium 1 and in 5 days on Medium 6. Apparently the ammonium ions are metabolized before the nitrate ions, thus delaying pigment production. Six liters of the corrosive aqueous phase produced by strain 101 after 90 days growth on medium 6 were fractionated as shown in Figure 8. The concentrated filtrate was fractionated by ion exchange chromatography and by Sephadex chromatography.

Dowex-1 and Dowex-50 ion exchange resins* were packed in glass columns, 2 cm x 20 cm, and generated to the OH^- and H^+ form, respectively. The Dowex-1 resin was brought to its analytical form (OH^-) by flushing it with 0.1 N NaOH until the presence of Cl^- ion was not detectable by the silver chloride precipitation test.[28] The column was then washed with flowing distilled water until the pH was neutral. The Dow-50 resin was purchased in the H^+ form and was washed extensively with about 10 volumes of distilled water allowed to flow through. Approximately 75 ml of concentrate was placed on the column and eluted with distilled water. The eluate, approximately 250 ml, was collected and tested for corrosivity.

G-25 and G-10 Sephadex gels were allowed to swell in distilled water overnight at room temperature. The water was decanted to remove the fines. A glass column, 1.5 cm x 87 cm, was used to hold this cross-linked dextran gel. After allowing the column to pack in flowing distilled water, filter paper was placed on top of the gel, a 3-ml sample was applied, and distilled water allowed to flow through. All effluant liquid including the void volume was collected until after the last colored fraction passed through the column.

Aluminum alloys, 7075 T-6 and 2024 used in the study, were cut into coupons 4 by 1/4 by 1/16 inches. The coupons were coded at one end with a

*Dowex resins were purchased from Calbiochem., 4921 Cordell Ave., Bethesda, Maryland. Sephadex was purchased from Pharmacia Fine Chemicals, Inc., 50 Fifth Ave., New York.17 N.Y.

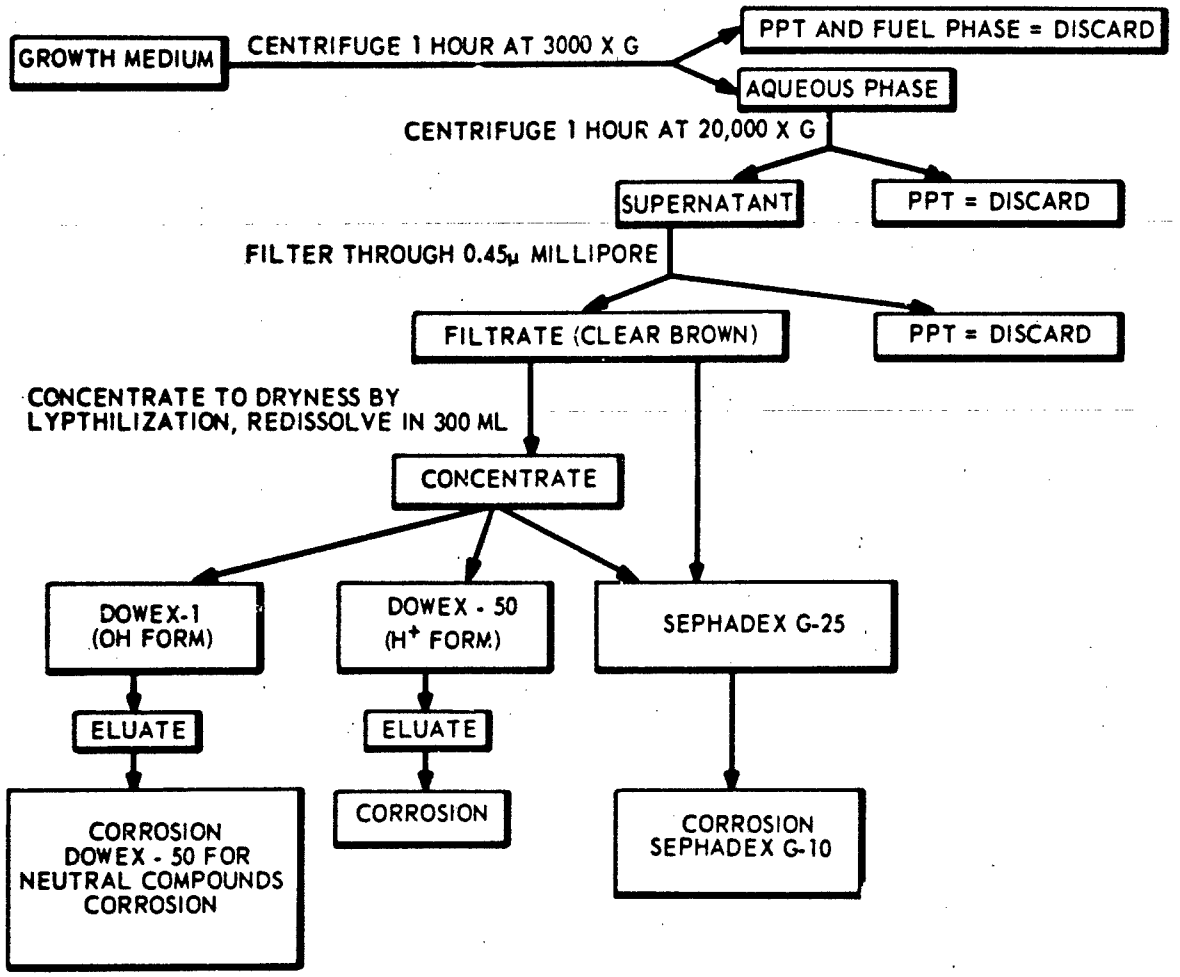


Figure 8. Scheme for Extraction, Concentration, and Fractionation of the Aqueous Phase

diamond point, cleaned, and weighed (tare) before testing. The alloys were cleaned before test by washing with acetone, wiping with paper towels, and immersing in 50% HNO_3 for 1 minute. Next, the coupons were rinsed with flowing tap water, rinsed with distilled water, dipped in acetone, blotted dry on a lintless towel, and stored in a desiccator until weighed (tare).

The procedure used by Robertson[21] was used for cleaning the alloy coupons after test; coupons were heated for 10 minutes at 70°-80°C in the cleaning solution (20g $K_2CR_2O_7$; 28 ml 85% H_3PO_4 sp gr; 1.7, distilled water to 1000 ml) rinsed, dried as described above, and reweighed. Weight loss was calculated from differences in weight obtained before and after test.

Separation of the concentrate into colored bands by Sephadex molecular-sieve-type chromatography was investigated. Three ml of concentrate was analyzed by this method. Eight colored bands were observed on the G-25 Sephadex column. This gel separates compounds with molecular weights ranging from 100 to 5000.[29] The last four bands on the G-25 apparently had a molecular weight of less than 700, since they were resolved on the G-10 gel. The corrosivity of each of these bands was tested. The weight losses observed with the concentrate, Dow-1 eluate, Dow-50 eluate, and Sephadex fractions are shown in Table 22.

The objective of these experiments was to separate and characterize the corrosive compounds from the growth media, thus no attempt was made to obtain a weight loss balance in the fractionation. The original 90-day sample used in the fractionation study was noncorrosive. The concentrate caused appreciable corrosion of both alloys; 7075 showed about twice as much weight loss as the 2024. Both anionic and cationic compounds are produced by the microorganisms and both fractions cause aluminum alloy corrosion. The eluate from the Dow-1, which contains cations, OH^- ions, and neutral compounds, caused much more corrosion of alloy 7075 than alloy 2024; 61.3 mg compared with 5.7 mg weight loss. The eluate from the Dow-50 column which contains anions, H^+ ions, and neutral compounds, like the unfractionated concentrate, caused about twice as much weight loss from 7075 alloy as from 2024 alloy. Very little weight loss was observed with the distilled water controls and sterile medium controls, which were processed the same as the inoculated samples. Neutral compounds, if they existed, did not cause corrosion of either alloy.

Very little corrosion was caused by the Sephadex fractions. The reason for this effect was not known, but it was postulated that corrosion would be observed if larger samples of corrosive media were used and if fractions of equal volume were collected and tested. The experiment was repeated using the previously described conditions except for the following changes. A large column (5 cm x 65 cm) was used, 300 ml of clear brown filtrate were applied to the column, and 80-ml fractions were collected until it appeared that all materials were removed from the column. The results obtained show that the first seven fractions contained the corrosive components of the medium (Figure 9). No corrosion was observed in any of the fractions containing the pigmented materials. This data indicated that old cultures

TABLE 22

CORROSIVITY OF FRACTIONS FROM 90-DAY CULTURE OF 101

	Milliliters	7075*	2024*
Original Culture		9.4	0.3
Concentrate		62.9	33.9
Sterile Medium Concentrate Control		0.1	0.1
DOW-1-Distilled Water		3.5	0.2
DOW-1-Sterile Medium Conc.		3.4	2.9
DOW-1-Concentrate		61.3	5.7
DOW-50-Distilled Water		0.1	0.1
DOW-50-Sterile Medium Conc.		2.5	2.2
DOW-50-Concentrate		35.7	17.6
DOW-1-Concentrate-DOW-50		0.1	0.1
Sephadex Fractions			
1. Void Volume	48	0.6	0.5
2. Light Brown	24	0.4	0.3
3. Yellow	38	0.5	0.1
4. Pale Amber	6	0.8	0.4
5. Brown	5	0	0
6. Deep Brown	3	0.1	0.4
7. Purple	5	0.3	0.4
8. Yellow-Pink	4	0.3	0.3
9. Yellow	13	1.5	0.3
10. Water	10	0.4	0.4

*Weight loss in mg/3.8cm^2 after 8 days exposure at room temperature

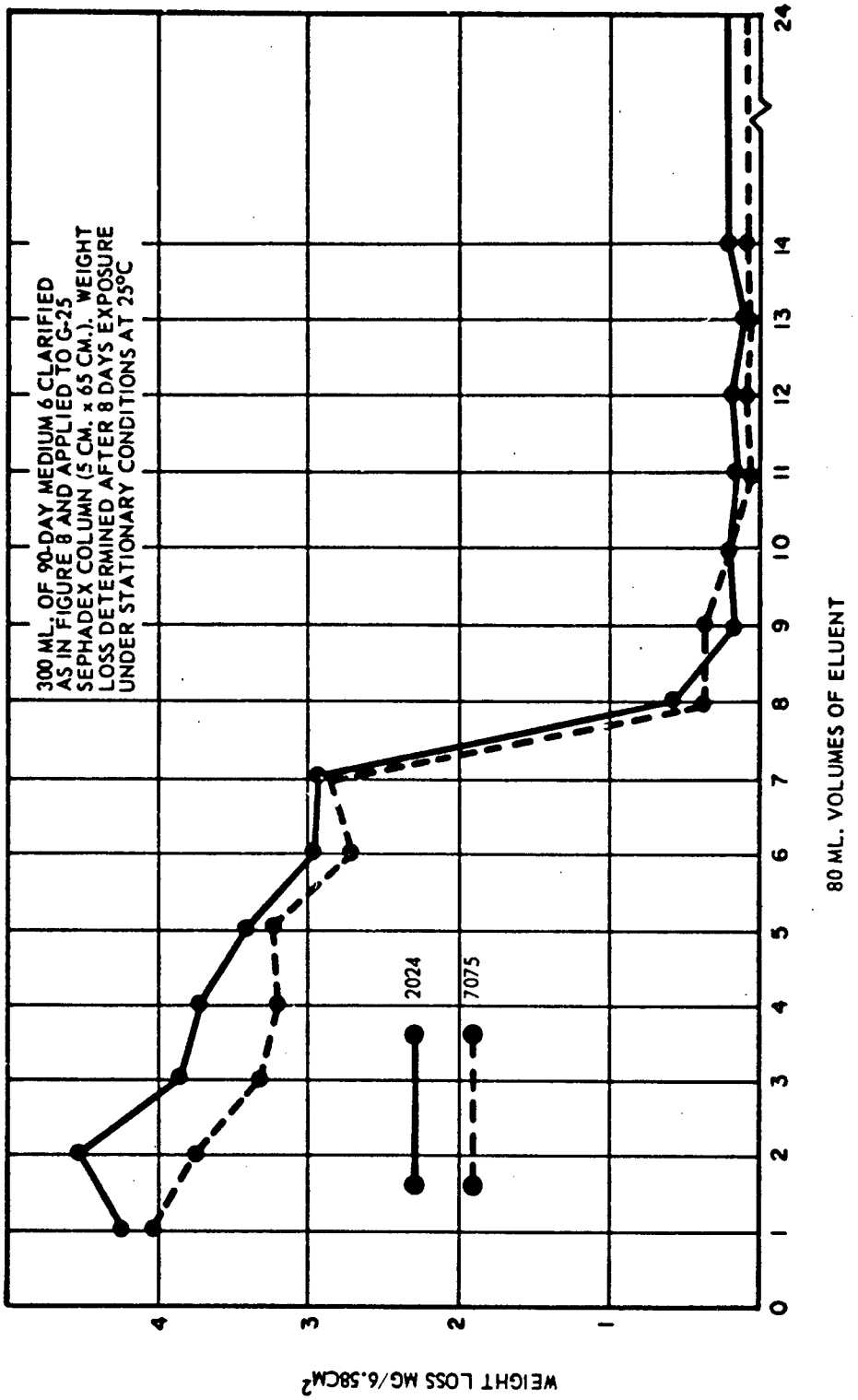


Figure 9. Corrosion by Sephadex Fractions of 90-Day Media After Growth of Culture 101

could cause corrosion by producing large molecular weight components, molecular weight 5000 and above in the medium. The composition of these large molecular weight materials is presently unknown.

b. Characterization of Corrosion and Corrosion-Inhibiting Components in the Aqueous Phase of 30-Day Cultures

After demonstrating that microorganisms do produce corrosive compounds during the 90-day incubation in high nitrate media even though the media itself is noncorrosive, it was questioned whether the high m.w. compounds were responsible for the corrosion caused by 30-day cultures growing on low nitrate media. To determine this, flasks of BH media modified with 0.4 and 0.6 millimoles KNO_3 per liter were inoculated with a 10% culture, overlayed with sterile jet fuel, and incubated on a gyratory shaker for 30 days at 28°C.

After 30 days of growth, the cultures were centrifuged and fractionated by procedures outlined in Figure 8. Aliquots of these clarified 30-day-old cultures were tested for aluminum corrosion with coupons of alloys 2024 and 7075.

The growth media were pooled and passed through a G-25 fine Sephadex column 5 x 65 cm followed by a distilled water wash. Twenty 80-ml fractions were collected, and each fraction was tested for corrosive activity with preweighed aluminum coupons of alloys 2024 and 7075. (See Figure 10.)

From previous experiments with 90-day cultures, it was determined that the compounds responsible for corrosion were of high molecular weight and came off the Sephadex column in the first seven 80-ml fractions. Corrosion by 30-day cultures is due to low molecular weight compounds, which are in the last 10 fractions.

c. Corrosion by Biologically-Produced Compounds

One of the hypotheses for microbial corrosion was that microorganisms could produce a variety of organic compounds that would react with aluminum alloys and cause corrosion. Because natural water bottoms in fuel storage tanks and aircraft wing tanks could contain a variety of organic materials collected from air, dust, and soil, it was postulated that many of these materials would also cause corrosion. To test this hypothesis, a variety of compounds from the laboratory shelf including morin, a chelating agent for aluminum ions, were examined. The data in Table 23 show that citrate, lactate, hydroxyl amine hydrochloride, and morin caused good corrosion, whereas the other acids, proteins, amino acids, fatty acids, and sugars produced essentially no corrosion. The large amount of corrosion by hydroxylamine hydrochloride was unexpected and cannot be explained on the basis of chloride content. The chloride ion concentration in the present experiment is about 0.143 M, and the corrosion observed after 15 days was about 225 µg weight loss. In an earlier experiment (Figures 5 and 6), 0.4 M chloride ion in the form of $CaCl_2$ caused only 15 mg weight loss in 4 days. This indicates

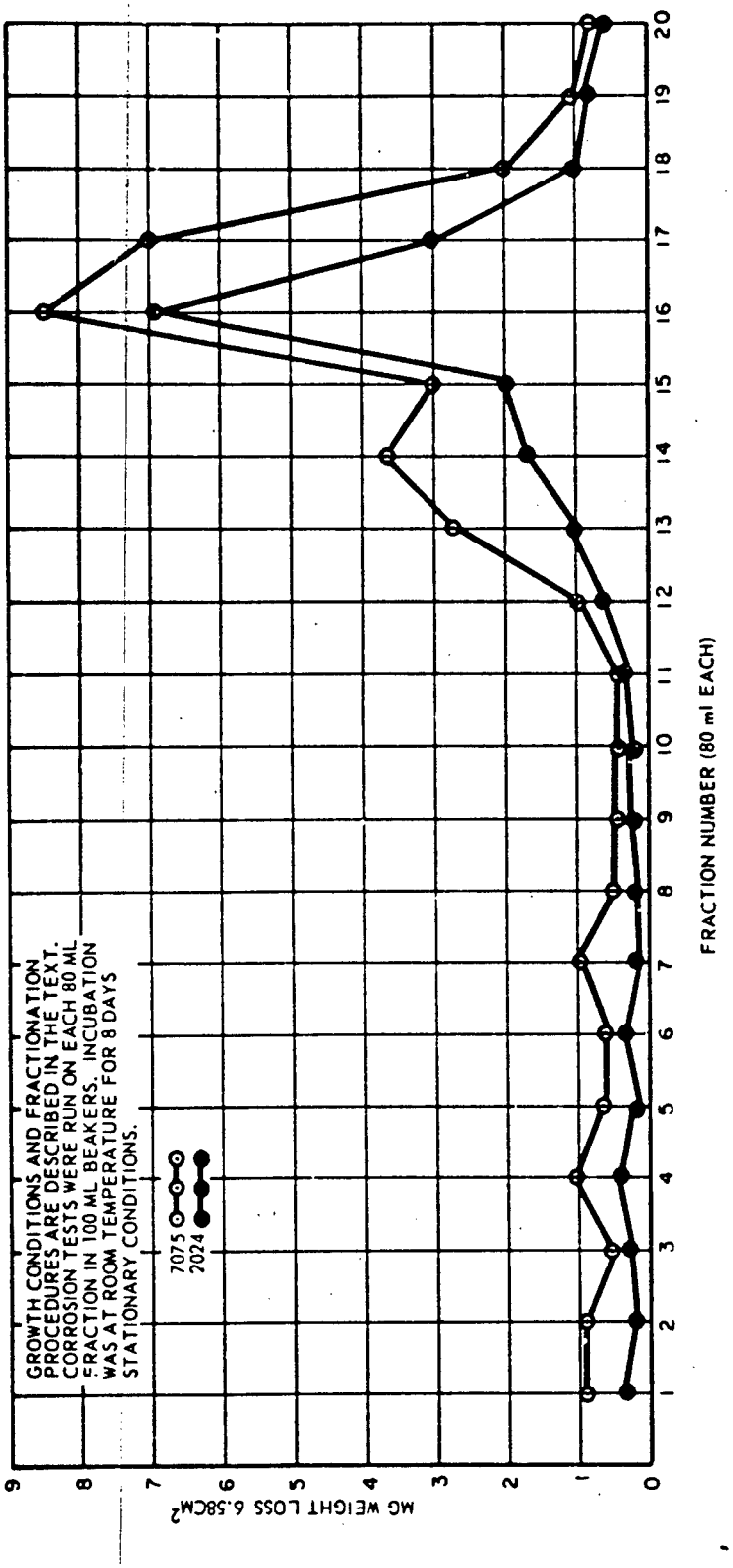


Figure 10. Corrosion by Sephadex Fractions of 30 Day Media after Growth of Culture 101

TABLE 23

WEIGHT LOSS CAUSED BY 1% SUSPENSIONS OR SOLUTIONS OF VARIOUS COMPOUNDS AFTER 15 DAYS INCUBATION AT 28°C

SOLUTION	pH		Weight Loss (mg/3.8 cm²)	
	Before	After	2024	7075
Hydroxylamine hydrochloride	3.3	5.5	205.35	257.37
Sodium citrate	8.0	9.3	23.73	41.08
Morin		4.9	7.85	10.51
Calcium lactate	6.5	7.2	4.73	4.94
Salicylous acid		4.8	0.83	2.56
Citric acid		2.3	1.19	1.72
Papain		7.0	0.62	1.31
Heptanoic acid		3.3	0.89	0.98
Octanoic acid		3.5	0.58	0.95
Blood fibrin		8.3	0.80	0.70
Hexanoic acid		3.0	0.73	0.78
Valeric (pentanoic) acid		2.9	0.71	0.77
Casein hydrolysate		8.6	0.75	0.57
l-leucine		—	0.42	0.75
Nonanoic acid		3.6	0.45	0.56
Hemoglobin		8.6	0.44	0.56
Decanoic acid		4.1	0.49	0.54
Undecanoic acid		4.5	0.50	0.51
l-tyresine		6.8	0.40	0.43
dl-asparagine		7.3	0.34	0.38
l-cystine		5.8	0.36	0.19
l-arabinose		5.2	0.35	0.27
Casein		8.4	0.16	0.33
Dehecahoic acid		4.6	0.30	0.15
Cellobiose		5.8	0.25	0.21

that hydroxylamine is highly active in corrosion and may function in corrosion in systems coupled with nitrate reduction or nitrogen fixation. The ability of the corrosive compounds to cause corrosion may be pH dependent; e.g., citric acid caused much less corrosion than sodium citrate. This effect may also have been due to the lack of solubility of citric acid in the water.

The theory that, because one has an acidic or alkaline environment or a high-protein media, one would have a corrosive environment is not supported by the data presented here. Instead, the results show that corrosive materials have a very definite structure and property which set them apart from noncorrosive materials.

4. Production of Oxidation -- Reduction Products

The hypothesis was investigated that microorganisms would cause corrosion if mediators of electicn transfer were present in the medium.

In this study methylene blue was used as a model electron transfer mediator. This dye can react both with the electron transport system of the cell and with molecular oxygen.

Cultures of fuel isolates were prepared in Medium 2. Culture media were made to 8-, 40- and 80-millimolar methylene blue hydrochloride. The media were inoculated with fuel isolate strain 101, and coupons of aluminum alloys 2024 and 7075 were submerged in the aqueous phase of each culture. Controls contained the same concentrations of methylene blue and aluminum coupons, but they were not inoculated with microorganisms.

The aluminum coupons were examined for corrosion 5 days after inoculation. The cell concentration changed from 5×10^8 cells per ml to 3.2×10^9 cells per ml during this time. The dye affects the aluminum surface very little in the absence of microbial growth, but when this electron mediator and microorganisms are included in the same culture, the organisms adhere to the surface of the aluminum. It was observed that pitting corrosion had occurred beneath the absorbed organisms or debris. It is believed that the effect of methylene blue was predominantly on the metabolic activity of the microorganisms rather than on the surface of the aluminum coupon. These results emphasize again the necessity of carefully evaluating the environment and medium in which microorganisms cause corrosion.

5. Concentration Cell Corrosion*

It was theorized that if microorganisms were deposited on metal surfaces they could cause aluminum alloy corrosion: (1) by producing an oxygen gradient and thus a difference in potential between the center and the edge of the deposit; (2) by accumulating metals from the medium and thus forming a galvanic cell when it comes in contact with the aluminum; and (3) by acting as cathodic depolarizing agents because of hydrogenase activity or because of metabolic products, i.e., H_2S, formed at local sites; or (4) by removing metals from the alloys and concentrating them in the microbial deposit.

The studies described below attempted to show the importance of the various concentration cells in corrosion by microorganisms.

a. Accumulation of Metals by Fungi and Their Relationship to Galvanic Corrosion of Aluminum

Fungi growing in a fuel-water system will form slime mats which consist of fungus mycelium, dirt, and other debris. Depending on what is available in the system, fungi may accumulate metals which are bound to the mycelium extra or intracellularly. If the metal bound by the mycelium is more noble in the electromotive series than aluminum, then the metal-containing mycelium can form a galvanic cell when it comes into contact with an aluminum surface. In such a system the aluminum surface functions as an anode which corrodes, while the metal-containing mycelium functions as the cathode.

In this work it was of interest to gain information as to which metals, more noble than aluminum, are accumulated by microorganisms. The binding of metals by fungal mycelium is defined as that metal which cannot be removed from the mycelium by repeated washing with distilled water. The choice of metals used in this work was restricted to copper, iron, and zinc. First, these metals are more noble than aluminum and, second, these metals might be reasonably expected to be present in fuel-processing and handling systems.

The general procedure was to prepare Fernbach flasks which contained 500 ml of sterilized media with and without various mineral salts. The flasks, following inoculation with fungi, were placed on a rotary shaker (160 strokes per minute) and incubated from 4 to 17 days at a temperature of 28°C. Following incubation, the contents of each flask were transferred to 100-ml centrifuge tubes and centrifuged at 1500 rpm for 30 minutes. After the supernatent liquid was removed, 50 ml of deionized water was added to the Aspergillus cultures. The same quantity of ethyl alcohol was added to the cultures of Pullularia and Hormodendrum harvests. In the latter case, ethyl alcohol was required for adequate filtration of the harvest. The contents of the tube were filtered through a Millipore filter holder using No. 4 Whatman filter paper. The filter cake of mycelium was

*This work was performed by Sharpley Laboratories, Inc., Fredricksburg, Va.

washed three times with 50-ml quantities of water or alcohol. The mycelial harvest was then dried overnight in an oven at 80°C and stored in a desiccator until used. Small amounts of the dried mycelium were placed on cleaned aluminum coupons (7075). The mycelium was rewetted by adding a drop of distilled water to the mycelium. The cleaned coupons with wet mycelium containing varying concentrations of metals on the surface of the coupons were placed in petri dishes containing filter paper. The paper was moistened, and a wide rubber band was placed around the petri dish cover to provide a moisture-tight container which will maintain a relative humidity of 95 to 100 percent for about 30 days.

Aluminum coupons with copper-containing mycelium of Aspergillus niger, zinc-containing mycelium from both Hormodendrum resinae and Pullularia pullulans were placed not only in petri dishes but also in a desiccator from which the air was evacuated to provide an atmosphere of reduced oxygen content. Water was added to the bottom of the desiccator to provide uniform moisture.

The media used in this work were potato dextrose broth (P.D.B.), 1.0 percent peptone or mixtures thereof.

The inoculum consisting of Hormodendrum resinae SL-181, Pullularia pullulans SL-272, and Aspergillus niger SL-23 were grown on malt agar slants. The spores and mycelium from two malt agar slants were harvested by adding distilled water containing 0.1 percent Tween 80 and gently scraping the mold growth. The water-spore mixture was diluted to 100 ml. Two ml of the spore suspension was used for inoculum.

A ferric chloride ($FeCl_3.6H_2O$) solution was prepared which contained 2.42 gms/100 ml. This amount of salt provides a 0.5 percent concentration of iron.

A copper sulfate ($CuSO_4.5H_2O$) solution was prepared which contained 1.96 gms/100 ml. This amount of salt provides a 0.5 percent concentration of copper ion.

A zinc sulfate ($ZnSO_4.7H_2O$) solution was prepared which contained 10.97 gm/100 ml. This amount of salt provides a 5.0 percent concentration of zinc ion. These salt solutions were sterilized by filtration through a 0.45-μ membrane filter.

Aside from the control flasks which did not receive salt solution, copper sulfate and ferric chloride were added in 1- and 5-ml amounts up to 500 ml of media to provide a concentration of 0.0, 10.0 and 50 ppm. Zinc sulfate was added in a similar fashion to the media to provide a concentration of 0.0, 100.0 and 500.0 ppm.

Weighed portions of dry mycelium varying from 0.06 to 0.2 gm were digested in Kjeldahl flasks containing 5 ml of acid. A digestion mixture of four parts concentrated sulfuric acid to one part concentrated nitric acid was found to be satisfactory. The digestion proceeded until the

solution had cleared. This normally required 1 to 3 hours. Following digestion, the excess acidity was neutralized by the addition of sodium hydroxide. The neutralized digestion solution was added to a 100-ml volumetric flask along with the acid rinse water from the Kjeldahl flasks. Kjeldahl flasks containing zinc were adjusted to pH 7.0 to 8.0, while those containing iron and copper were adjusted to pH 4.0 to 5.0. Reagent controls consisted of Kjeldahl flasks containing all materials except the dry mycelium.

b. Analysis of the Copper Content of Mycelium and Corrosion of Aluminum Coupons (7075) in Contact with Copper-Containing Mycelium

The method of Hach[30] was employed for the determination of copper. This method employs cuprethol as a chelating agent and the copper chelate is measured colorimetrically using a B and L spectrophotometer at 435 μ. Isoamyl alcohol was used to extract the copper chelate.

The amount of copper bound by Aspergillus niger is presented in Table 24. The small amount of copper bound by Aspergillus niger, growing in a medium containing 50.3 ppm of copper, was unexpected.

A total of 18 aluminum coupons with mycelium containing varying concentrations of copper, each concentration being replicated three times, were distributed equally into petri dishes and the air-evacuated desiccator. At the end of a 60-day storage period, examination of the coupons revealed little or no pitting corrosion, regardless of whether the coupons had been stored aerobically or anaerobically. The state of the water in the mycelium may be important from a biocolloid standpoint because preliminary results indicate that the wet copper-containing mycelium (never dried) did cause corrosion of aluminum coupons (7075).

TABLE 24

ACCUMULATION OF COPPER BY ASPERGILLUS NIGER

Concentration of Copper in Media (ppm)	Copper (ppm) Per Gram of Dry Weight of Mycelium
0.3*	3.0
10.3	31.0
50.3	4.0

*Copper present in potato dextrose broth.
Reagent control - 0.6 ppm.

c. Analysis of the Iron Content of Mycelium and Corrosion of Aluminum Coupons (7075) in Contact with Iron-Containing Mycelium

The method of Hach[30] was employed for the determination of iron. This method employs 1, 10-phenanthroline as the chelating agent, and the iron chelate is measured colorimetrically using a B and L spectrophotometer at 510 μ.

The amount of iron bound by Hormodendrum and Pullularia is presented in Table 25.

TABLE 25

ACCUMULATION OF IRON BY FUNGI

Concentration of Iron in Media (ppm)	Iron (ppm) Per Gram of Dry Weight of Mycelium and/or Spores	
	Hormodendrum	Pullularia
0.3*	0.0	12.0
10.3	2.0	70.0
50.3	297.0	952.0

*Amount of iron present in 1.0% peptone medium.
Reagent control - 0.0 ppm iron.

Pullularia bound more iron than Hormodendrum. The maximum amount of iron bound by the fungus mycelium increases as the available iron present in the growth medium increases.

A total of 18 aluminum (7075) coupons with mycelium of each species containing varying concentrations of iron, each concentration being replicated three times, were placed in petri dishes and stored for 60 days. The amount of pitting corrosion observed on the coupons is presented in Table 26. It appears that in general more pitting corrosion of aluminum coupons occurs as the concentration of iron bound by the fungus mycelium increases.

Because these coupons in contact with iron-containing mycelium were stored under atmospheric conditions, the pitting corrosion which occurred may reflect an oxygen concentration cell system as well as galvanic corrosion.

d. Analysis of the Zinc Content of Mycelium and Corrosion of Aluminum Coupons (7075) in Contact with Zinc-Containing Mycelium

The method as outlined in "The Manual on Industrial Water and Industrial Waste Water"[31] was employed for the determination of zinc. This method employs 2-carboxy-2'-hydroxy-5'sulfo-formazylbenzene (zincon) as the chelating agent, and the colored zinc chelate is measured using a B and L spectrophotometer at 620 μ.

Hormodendrum and Pullularia grew poorly in the 1.0 percent peptone, and it was necessary to add potato dextrose broth to initiate and promote the growth of these fungi. The addition of P.D.B. changed the concentration of zinc in the medium to approximately those concentrations listed in Table 27. The amount of zinc bound by Hormodendrum and Pullularia is presented in Table 27.

The maximum amount of zinc that can be tolerated by Hormodendrum is less than 418 ppm; however, the maximum tolerance of Pullularia to zinc was not determined.

A total of 18 aluminum (7075) coupons with the mycelium of Pullularia and 12 coupons with the mycelium of Hormodendrum containing varying concentrations of zinc, each concentration being replicated three times, were distributed equally into petri dishes and an air-evacuated desiccator. The coupons were held in storage for 60 days at room temperature (20°C).

Examination of the coupons at the end of the storage period revealed little or no corrosion regardless of whether the coupons were stored aerobically or anaerobically. In view of the fact that zinc is immediately below aluminum in the electromotive series, the results are not surprising. One would not expect as much galvanic activity between aluminum and zinc as compared to aluminum and copper or iron.

e. Corrosion of Aluminum Coupons (7075) in Contact with Copper-Containing Mycelium Mixed with Azotobacter vinelandii Cells

Galvanic corrosion of aluminum coupons in contact with metal-containing mycelium must be demonstrated in the absence of oxygen to eliminate the possibility of oxygen concentration cell corrosion occurring simultaneously. In previous experiments when coupons and mycelium were stored anaerobically, the lack of coupon corrosion may have been related to the polarization of the cathodic metal-containing mycelium. Hence, some depolarizing agent which does not contain oxygen is needed for the galvanic system to operate.

Azotobacter vinelandii with known hydrogenase activity was grown in Burk's medium [32] and harvested. The Azotobacter harvest was stored in the refrigerator in the form of a viscous water mixture.

TABLE 26

VISUAL GRADING OF PITTING CORROSION OF ALUMINUM COUPONS IN CONTACT WITH MYCELIUM CONTAINING VARIOUS AMOUNTS OF IRON

Concentration of Iron Per Gram of Dry Weight Mycelium (ppm)	Fungal Mass on Coupon	Visual Grading of the Average Corrosion Based on Triplicate Exposure of Aluminum Coupons to Metal Containing Fungi
0.0	Hormodendrum	0.0
2.0	Hormodendrum	0.7
297.0	Hormodendrum	2.0
12.0	Pullularia	2.7
70.0	Pullularia	2.0
952.0	Pullularia	3.7

System of grading:

0.0 - No visible pitting corrosion
1.0
2.0
3.0
4.0 - Severe pitting corrosion

The numerical ratings of the triplicate exposures were averaged together which accounts for the reporting of fractions of one unit number.

TABLE 27

ACCUMULATION OF ZINC BY FUNGI

Concentration of Zinc in Media (ppm)	Zinc (ppm) Per Gram of Dry Weight of Mycelium and/or Spores	
	Hormodendrum	Pullularia
0.0	12.0	10.0
83.0	70.0	37.0
418.0	No growth	946.0

Reagent control - less than 0.5 ppm
Medium consisted of 500 ml 1.0% peptone and 100 ml potato dextrose broth

A total of 21 aluminum (7075) coupons with wet Aspergillus niger mycelium containing varying concentrations of copper with and without Azotobacter cells were stored in an air-evacuated desiccator for 60 days at room temperature. Four to 5 drops of Azotobacter cells were mixed with the selected portions of mycelium in hopes that Azotobacter with its hydrogenase activity would function as a depolarizer under the anaerobic conditions of the experiment.

The amount of pitting corrosion observed on the coupons is presented in Table 28. In general, there was not much pitting corrosion observed on any of the coupons. Those coupons in contact with mycelium mixed with Azotobacter appeared to be more corroded than coupons without the benefits of the added Azotobacter cells.

It should be pointed out that the pitting corrosion which occurred probably reflects galvanic corrosion and not oxygen concentration cell corrosion.

f. Final Experiment to Determine Whether Metal-Containing Mycelium Can Cause Galvanic Corrosion of Aluminum

In this work the experimental design incorporated all of the factors gained from previous work as well as new ideas. The principal factors are as follows:

(1) Metal to be Bound by Fungus: Copper was used because there is the greatest amount of difference between copper and aluminum in the electromotive series and is one of the principal metals found in a jet fuel.

(2) Condition of Mycelium in Contact with the Aluminum Coupon: Metal-containing mycelium which had never been dehydrated was used. Past work revealed that initially wet mycelium tended to dry out during the course of the experiment, and where there is reduced moisture electrolytic activity is reduced. Therefore in this work, 20 to 25 gm of wet mycelium were added to 125-ml beakers, which permitted the addition of water as required. Second, the large amount of mycelium provided a large cathode in relation to the aluminum coupons and also permitted analysis of the mycelium for change in its aluminum content when the experiment was terminated.

(3) Aluminum Coupons (7075): It was realized that in past work no provision for evaluating galvanic corrosion on a weight-loss basis had been made. Hence, in this work, all coupons were weighed before and after exposure to determine weight loss as well as judgment by visual examination. All coupons were cleaned as described previously. Three numbered coupons were placed in each of eight beakers representing the factorial combinations.

TABLE 28

VISUAL GRADING OF PITTING CORROSION OF ALUMINUM COUPONS IN CONTACT WITH MYCELIUM CONTAINING VARIOUS AMOUNTS OF COPPER WITH AND WITHOUT AZOTOBACTER

Concentration of Copper Per Gram of Dry Weight Mycelium (ppm)	Aspergillus niger Mucelium on Coupon		Visual Grading of Avg. Corrosion Based on Triplicate Exposure of Al to Metal-Containing Fungi
	With Azotobacter	Without Azotobacter	
1.54		+	0.3
4.54		+	0.3
9.99		+	0.6
1.54	+		1.0
4.54	+		1.6
9.99	+		0.3

Systems of grading:

0.0 - No visible pitting corrosion
1.0
2.0
3.0
4.0 - Severe pitting corrosion

The numerical ratings of the triplicate exposures were averaged together which accounts for the reporting of fractions of one unit number.

(4) Addition of a Sensitizing Agent: Aluminum metal rapidly becomes passive when exposed to water or atmospheric oxygen. If, however, there are agents present in the environment to remove all or a portion of the aluminum oxide film from the surface of the metal, then the underlying metal becomes sensitive to electrolytic action. According to commercial brochure information, gluconic or citric acid will remove the alumium oxide film and not the underlying metal. In this work, sufficient gluconic acid was added to each of eight beakers to provide a concentration of 1000 ppm.

(5) Grounding the System: Past experience indicated the need for grounding all the coupons. Grounding the coupons was accomplished by preparing an aluminum screen with slots cut into the screen. The screen was superimposed over the coupons contained in the beakers and each coupon was fitted into a slot in the wire screen to provide electrical contact. The entire wire screen was grounded to aluminum foil which extended from the inside of the desiccator across the lip to the outside. The foil on the outside of the desiccator was then grounded in a conventional manner. A photograph of the grounded and sealed desiccator is presented in Figure 11. The arrangement of the coupons in the fungal mass is illustrated in Figure 12.

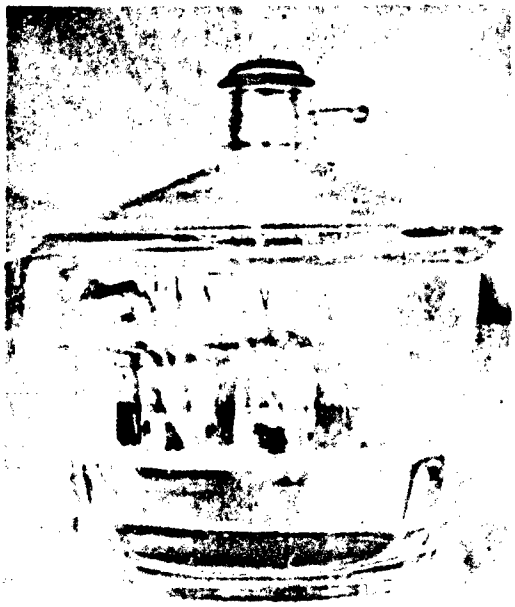


Figure 11. Grounded and Evacuated Myceleal Masses in Contact with Aluminum Coupons

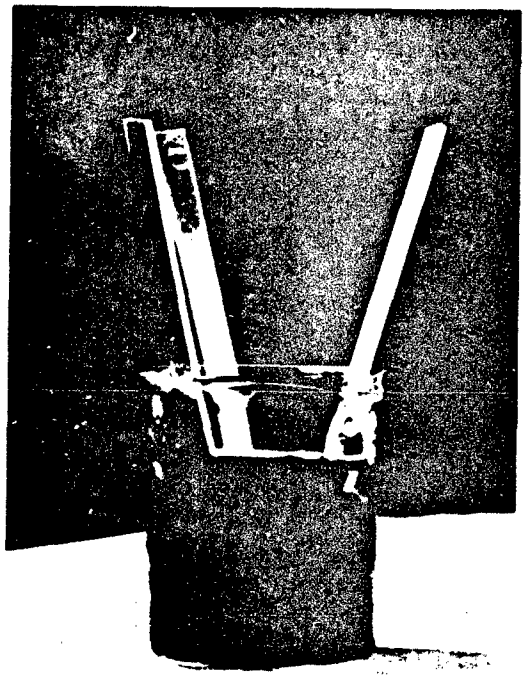


Figure 12. Arrangement of Coupons in Fungal Mass for Corrosion Experiments

In this experiment, Aspergillus niger was grown in P.D.B.-containing flasks with varying concentrations of copper and harvested in the same manner described previously. To each of six 125-ml beakers, 20 to 24 gm of copper-containing mycelium were added along with 1 ml of gluconic acid. Approximately 0.5 ml of Azotobacter cells were added to the appropriate beakers. Sufficient distilled water was added to each beaker to make the total weight of each beaker 25 gms. Beakers 7 and 8 represented controls. Beaker No. 7 contained 1 ml of gluconic acid, 0.5 ml of Azotobacter, and sufficient water to make a total of 25 gm. Beaker No. 8 contained 1 ml of gluconic acid and 24 ml of water. To each beaker, three cleaned and weighed aluminum coupons (7075) were inserted. All of the beakers and their contents were placed in the desiccator, which was evacuated of air. The design of the experiment is listed in Table 29. The coupons were under test for approximately 60 days.

Visual examination of the coupons after storage was not very fruitful. As viewed with the unaided eye, there appeared to be little or no corrosion. When viewed microscopically, corrosion was more evident. There was evidence of small pits developing in many of the coupons. Figure 13 is a low-power photomicrograph of a corroded area and Figure 14 is a higher power photomicrograph of the same area. Note the "rotten" appearance of the metal.

The coupon weight loss data is listed in Table 30. The coupon weight loss was determined as follows:

$$\text{Coupon weight loss} = \frac{\text{True weight loss (coupon weight loss-coupon cleaning weight loss)}}{\text{Original weight of coupon}}$$

The weight is also expressed in terms of mils per year (MPY). The MPY date was determined by using the method suggested by NACE and API.[33]

The presence of mycelium and bacteria appears to protect the aluminum coupon against the corrosive nature of gluconic acid. However, it can be seen that as the copper content of the fungal mycelium increases, so does the corrosion rate as judged by coupon weight loss. These data would tend to confirm the mechanism of galvanic corrosion by metal-containing mycelium. The role of Azotobacter with its hydrogenase system is obscure. Azotobacter and gluconic acid together caused more corrosion by themselves than when added to mycelium which contained more copper than the gluconic acid-Azotobacter combination. In addition, the presence of Azotobacter appeared to increase the corrosion of coupons when in contact with mycelium of low copper content. In those cases where the copper content of the mycelium was higher than the control mycelium (low copper), the presence of Azotobacter appeared to reduce the corrosion rate.

In industrial operations 5 MPY is often quoted as the maximum amount of corrosion that can be tolerated. This figure represents

TABLE 29

DESIGN OF FINAL EXPERIMENT TO DETERMINE WHETHER METAL-CONTAINING MYCELIUM CAN CAUSE GALVANIC CORROSION OF ALUMINUM 7075

Beaker No.	Gluconic Acid (ppm)	Azotobacter Vinelandii	Asp. Niger Mycelium	Conc. of Copper in Growth Medium	Coupon No.
1	1000		+	0.3 ppm	1 2 3
2	1000	+	+	0.3 ppm	4 5 6
3	1000		+	10.0 ppm	7 8 9
4	1000	+	+	10.0 ppm	10 11 12
5	1000		+	50.0 ppm	13 14 15
6	1000	+	+	50.0 ppm	16 17 18
7	1000	+			19 20 21
8	1000				22 23 24

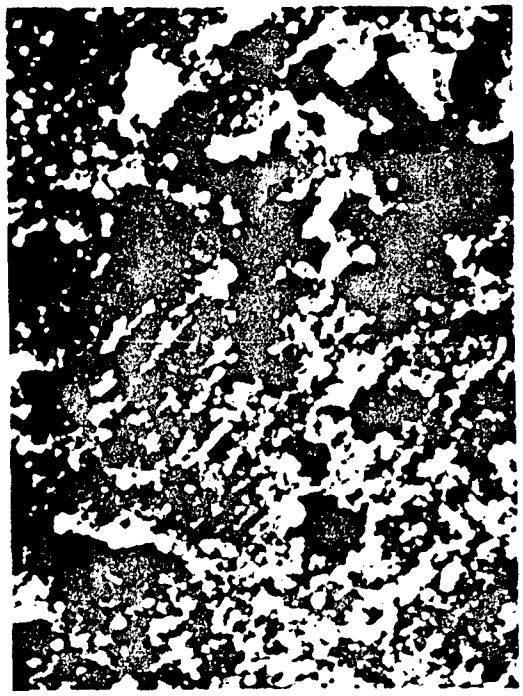


Figure 13. Corrosion from Copper-Containing Fungal Mass, Low-Power Photomicrograph

Figure 14. Corrosion from Copper-Containing Fungal Mass, High-Power Photomicrograph

TABLE 30

WEIGHT LOSS OF 7075 ALUMINUM COUPONS AFTER 60 DAYS STORAGE IN WET MYCELIUM WITH AND WITHOUT COPPER, AZOTOBACTER, AND GLUCONIC ACID

Beaker No.	Coupon No.	Percentage Weight Loss	Average Percentage Weight of Group of Coupons in One Beaker	M.P.Y.	Average M.P.Y.
1	1	0.113		0.8	
	2	0.082	0.090	0.6	0.66
	3	0.077		0.6	
	4				
2	5	0.127	0.146	0.9	1.05
	6	0.165		1.2	
	7	0.257		1.9	
3	8	0.210	0.210	1.5	1.53
	9	0.165		1.2	
	10	0.212		1.5	
4	11	0.214	0.200	1.5	1.4
	12	0.174		1.2	
	13	0.252		1.8	
5	14	0.353	0.283	2.6	2.06
	15	0.245		1.8	
	16	0.276		2.0	
6	17	0.229	0.246	1.7	1.90
	18	0.234		2.0	
	19	0.169		1.2	
7	20	0.159	0.163	1.1	1.16
	21	0.162		1.2	
	22	0.257		2.0	
8	23	0.171	0.223	1.4	1.83
	24	0.243		2.1	

generalized corrosion losses and not pitting corrosion. The data obtained in this work would indicate that corrosion from galvanic action associated with microorganisms is within the normal acceptable corrosion limits of industry if expressed on an MPY basis. It should be pointed out that the data submitted was obtained in the absence of oxygen concentration cell corrosion. However, MPY losses are not an accurate nor realistic way to express damage caused by pitting corrosion. As shown in the attached photomicrograph, localized damage could be quite severe in spite of insignificant overall loss of metal.

The copper content of the Aspergillus mycelium is presented in Table 31. Earlier checks by spectrographic analysis of fungal ash samples indicated little or no agreement between the colorimetric method for copper analysis and spectrographic methods. Spectrographic analysis indicated that the fungal ash contained a fair number of metals in Group II of qualitative analytical schemes, which would interfere with the accuracy of the cuprethol method of analysis. Therefore, in the present work, considerable effort was made to separate copper from the other interfering metals present in the fungal ash. While the two different analytical methods provide data in the same order of magnitude, the degree of agreement between the two methods is not as good as expected.

The mycelium that had been in contact with aluminum coupons was ashed and analyzed spectrographically to compare the metal content with the same mycelium prior to contact with aluminum coupons. A comparison of metal content of mycelium from beaker No. 6 is listed as a typical example in Table 32. The results were not as expected. Aluminum 7075 is an alloy containing small amounts of silicon, iron, copper, manganese, magnesium, chromium, zinc and titanium. The increase of metal content of the mycelium after contact with the aluminum coupons consisted principally of those metals higher than copper in the electromotive series. Much more magnesium was removed from the coupons than aluminum. Surprisingly enough there was more copper present in the mycelium after contact with the aluminum coupons. If the analytical data is relatively accurate, then the mycelium may be functioning as an ion exchange system as well as the cathode of a galvanic cell.

g. Concentration Cell Corrosion of Aluminum by Anaerobic Bacteria

The occurrence of sulfate-reducing bacteria in sludges removed from corroded aircraft was reported in the late 1940's and early 1950's. Since that time there have been comparatively few such reports. The reason is not known. It is generally accepted that sulfate-reducing bacteria establish concentration cells that corrode ferrous metals, but similar data is not specifically available for nonferrous metals. In view of the absence of information concerning the corrosion of aluminum by sulfate-reducing bacteria, and the possible occurrence of Desulfovibrio in aircraft sludge, an investigation of the anaerobic corrosion of aluminum was initiated.

TABLE 31

ACCUMULATION OF COPPER BY ASPERGILLUS NIGER

Concentration of Copper in Medium (ppm)	Percentage of Copper in Fungal Ash		Copper(ppm)per Gram of Dry Weight of Mycelium	
	Spectrographic	Colorimetric	Spectrographic	Colori-metric
0.3	0.01	0.01	3.3	1.9
10.3	0.38	0.69	144.0	260.0
50.3	2.0	3.13	626.0	509.0

TABLE 32

THE METAL CONTENT OF ASPERGILLUS NIGER MYCELIUM BEFORE AND AFTER CONTACT WITH 7075 ALUMINUM COUPONS AS DETERMINED BY SPECTROGRAPHIC ANALYSIS

Metal	Percentage of Metal in Fungal Ash Residues	
	Before Contact with Aluminum Coupons	After Contact with Aluminum Coupons
Aluminum	0.012	0.70
Magnesium	1.1	2.2
Iron	0.50	0.74
Manganese	0.012	0.77
Copper	2.0	3.4

Two groups of anaerobic bacteria were chosen, since both have been reported as corroding ferrous metals. The action of Desulfovibrio has been well documented in the literature and was an obvious choice. Species of clostridia, specifically Clostridium nigrificans, also have been implicated in the corrosion of steel and were chosen as the second group.

Two-phase agar systems were used in all of the work. One phase was an appropriate nutrient substrate for the specific microorganism. The second phase was plain nonnutrient agar containing 0.25 percent morin (2',3, 4'5, 7-pentahydroxyflavone). Morin is a sensitive fluorometric test for aluminum under ultraviolet radiation, and it was postulated that the migration of the aluminum ions could be followed by this technique. The second purpose of the morin was to provide a neutral-to-slightly-oxidizing pole opposing the strongly reducing side produced by hydrogen sulfide.

Two-phase systems were used in petri dishes and incubated anaerobically. The results obtained were confusing, and this approach was abandoned in favor of techniques using sealed bottles and test tubes. The small volume of agar from test tubes was found difficult to use in some analytical determinations and bottles of 6 oz. size were used for most of the work.

A good deal of effort was expended on analytical determinations of metals in the substrate in an effort to provide quantitative corrosion data. Colorimetric techniques were extensively used, but emission spectrographic analyses were found more useful. These determinations were used for comparison with visual corrosion estimates and were particularly intended to determine whether portions of the alloying constituents in aluminum were selectively removed.

Difficulty was encountered cultivating a strain of Desulfovibrio that would grow on a salt-free medium. The bulk of the research done in this country, and all of the work in this laboratory, has been with sulfate-reducing bacteria isolated from salt-containing waters such as marine and estuarine mud or oil field brines. European reports frequently discuss a fresh water sulfate-reducing bacterium and as a matter of record, the taxonomic literature states that the type species, Desulfovibrio desulfuricans, grows best in fresh water media as does D. rubentschikii. The salt-requiring species is thus D. aestuarii. Whatever the taxonomic confusion, we were unable to obtain growth in salt-free media of any of a number of cultures labeled D. desulfuricans; two of the cultures were of European origin and supposed to be type strains.

Next, a number of primary isolations from stream mud, swamps and other soils were made. Sulfate-reducing bacteria were isolated but these did not live on salt-free media. A large sample of estuarine mud from the Chesapeake Bay finally yielded a strain of Desulfovibrio that would grow, though slowly, in a conventional sulfate reducer medium using lactate as a carbon source, and with no sodium chloride in the medium. The species is unknown, in view of the current taxonomy, but the organism is a typical sulfate-reducing vibrio that grows reasonably well without sodium chloride. Previous experiments with salt-containing media corroded 7075 aluminum in spite of oxygen exclusion, and it was felt that chlorides must be absent from the substrate for meaningful results.

No problem was encountered with the clostridia. An extensive collection of clostridia is available in our stock culture collection and the required species were obtained from this source.

Corrosion of 7075 Aluminum by Clostridia: The following bacteria were used in the corrosion experiment:

Clostridium leutoputrescens, SL-3:
Produces hydrogen sulfide in protein media.

Clostridium nigrificans, SL-9:
Implicated in oil field corrosion. This is the organism once erroneously classified as a spore-forming, sulfate-reducing vibrio; i.e., "Sporovibrio." Produces large amounts of hydrogen sulfide.

Clostridium sporogenes, SL-17:
Produces a foul odor but little hydrogen sulfide.

Clostridium aerofoetidum, SL-4:
Produces a foul odor but little hydrogen sulfide.

A two-phase medium was used in screw-capped test tubes. The bottom portion consisted of liver-veal agar inoculated with one of the clostridia and the top of the tube contained 0.25 morin in nonnutrient agar. An aluminum coupon extended across both phases.

After 60 days of incubation, there was distinct discoloration at the top of the coupon in the morin agar. This did not occur on the uninoculated controls, and various species of bacteria produced different amounts of discoloration. However, fluorescence under UV was not observed; thus the presumptive test for aluminum was negative.

The coupons were removed and cleaned. The observations are described in Table 33. "Top" of the coupon is that portion in the morin agar; "bottom" is that portion where the bacteria were grown. In general the corrosion was more pronounced at the top of the coupon, but was not particularly severe. With one exception, the corrosion appeared to be general corrosion but limited to small areas. We are not certain that this is pitting corrosion. The difference between a "shallow pit" and "small circumscribed areas of general corrosion" appears to be primarily a semantic difference. The pits obtained on these coupons are certainly not the deep pits obtained in later work.

The photomicrograph in Figure 15 is typical of the minor corrosion obtained.

h. Corrosion of 7075 and 2024 Aluminum by Sulfate-Reducing Bacteria

A number of different approaches were used to demonstrate corrosion by sulfate reducers. Petri dishes were poured with morin plus agar on one side and inoculated sulfate-reducing agar on the other side. Strips of 7075 aluminum were laid across the two phases of agar. The theory was that the very reducing environment on the sulfate reducer side would allow the strip to polarize and aluminum ions would migrate into the indicating morin agar. This works well when a small EMF is imposed, but it failed in the experiment. Presumably, sulfides formed on the aluminum causing positive and negative areas that confounded the theory. Chemical analyses

TABLE 33

CORRISION OF 7075 ALUMINUM BY CLOSTRIDIA

Control (uninoculated)

No corrosion observed

Cl. aerofoetidum

1. Small areas of general corrosion on both ends
2. General shallow corrosion on top
3. General corrosion at both ends, mostly bottom
4. Slight corrosion at top. Edge corrosion?

Cl. sporogenes

1. General corrosion at top
2. General corrosion at top
3. Slight general corrosion at top
4. General corrosion and shallow pits at top

Cl. nigrificans

1 -4 All the general corrosion at top with shallow pits.

Cl. lentoputrescens

1. Small pit at top
2. General corrosion at top
3. General corrosion at top, slight and general
4. Small pits at top. Little general corrosion

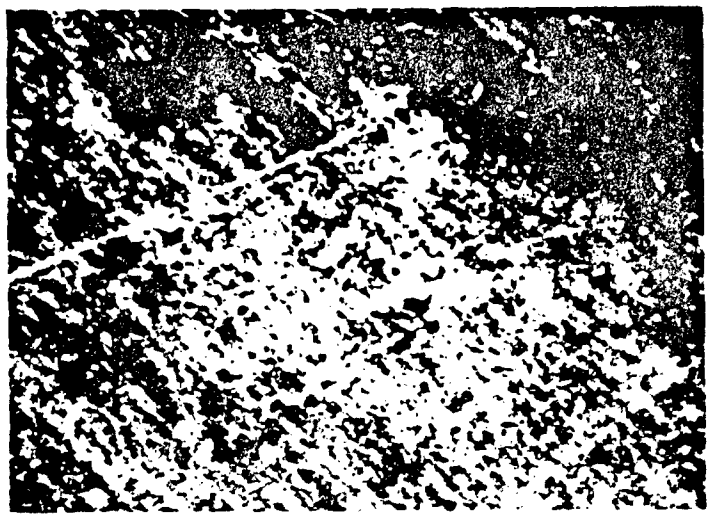


Figure 15. Corrosion of 7075 Aluminum by Clostridia. Total Corroded Area is about 1.1 x 2.0 mm

showed less than 0.1 ppm of aluminum in the morin side and about 1-2 ppm aluminum on the hydrogen sulfide side. This approach was abandoned since its reproducibility was in doubt.

Comparatively large 6-ounce prescription bottles using the two-phase agar system with a 1/4 x 4 inch coupon had been satisfactory in some preliminary experiments. One can be quite certain of oxygen exclusion; the sample is sufficiently large for analytical determinations and the corrosion process is somewhat visible. Test tube cultures were also set up in the same manner and showed about 4x the aluminum loss on the hydrogen sulfide side.

Several series of these bottles containing salt-free, sulfate-reducing agar, inoculated with the strain of bacteria previously described, were prepared with either 7075 or 2024 series aluminum. The appearance is illustrated in Figure 16. When good growth was established with abundant hydrogen sulfide, deep pits were produced on 7075 aluminum coupon. No attack was observed on 2024 series aluminum. The pits were present only on the inoculated end of the coupon. A series of these experiments were prepared for analytical work and yielded the data in the following tables and photographs.

The pits formed were very deep and sharp-sided: typical microbial corrosion pits as observed in the field. Figures 17 and 18 indicate the appearance and size of the pits.

Analytical work on the substrate was intended to demonstrate whether segregated metals had been removed from the aluminum. The results are presented in Tables 34 and 35. There is no observable correlation between the severe pit corrosion and analyses for metals. These findings simply substantiate previous observations that chemical analyses and weight losses are not significant when evaluating pit corrosion. Pit corrosion causes severe damage by the removal of small amounts of metal; these cannot be distinguished from the "background" of minor general corrosion.

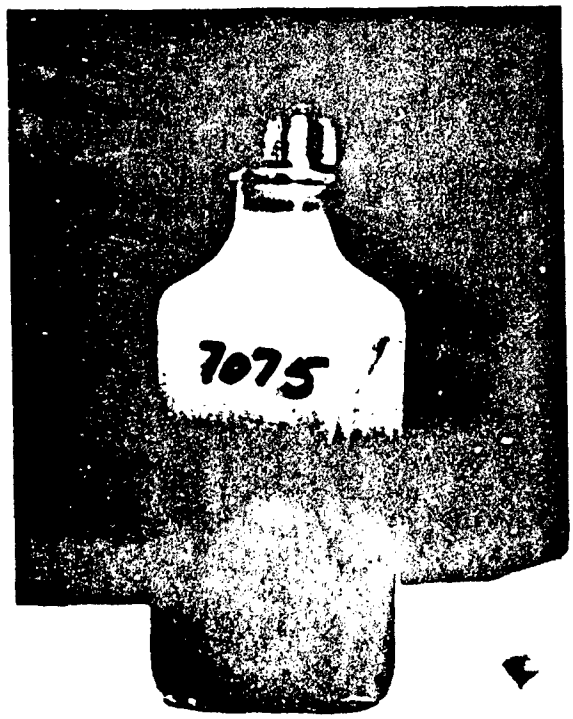


Figure 16. Technique used for Corrosion by Sulfate-Reducing Bacteria

Figure 17. Corrosion Pit on 7075 Aluminum in Contact with Sulfate-Reducing Bacteria

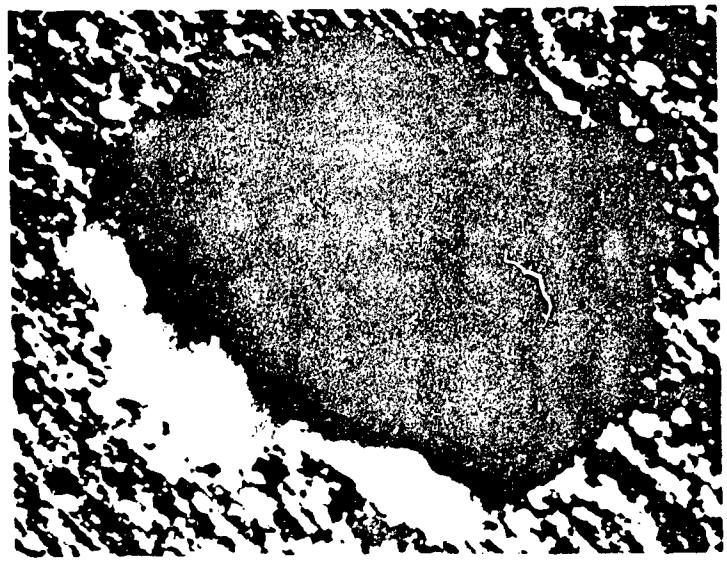


Figure 18. Corrosion Pit on 7075 Aluminum in Contact with Sulfate-Reducing Bacteria

TABLE 34

CORROSION BY SULFATE-REDUCING BACTERIA

No.	Coupon-alloy	Corrosion	Medium	Wet wt.	Dry wt.	Wt.ashed	Ash wt.
1	31-7075	None (2)	Morin-agar (1)	77.33	1.12	0.7761	0.0934
2	31-7075	Deep pits	Salt-free DS (3)	94.15	1.33	0.9268	0.1213
3	30-7075	None	Morin-agar	93.76	1.49	1.1958	0.1377
4	30-7075	Deep pits	Salt-free DS	95.66	1.21	0.7329	0.0924
5	5-2024	None	Morin-agar	104.66	1.43	1.0173	0.1553
6	5-2024	None	Salt-free DS	74.37	1.12	0.7156	0.1028
7	6-2024	None	Morin-agar	67.47	1.02	0.8136	0.1134
8	6-2024	None	Salt-free DS (4)	104.95	1.44	1.1368	0.1744

(1) 0.25% morin in 2.0% plain agar.

(2) There is some uniform weight loss on all coupons. "None" refers to deep pits and other severe penetrating corrosion. See photomicrographs.

(3)

Sodium lactate	4.0	ml
Yeast extract	1.0	g
Ascorbic acid	0.1	g
Magnesium sulfate	0.2	g
Potassium phosphate, dibasic	0.1	g
Ferrous ammonium sulfate	0.1	g
Agar	3.0	g
Water, q.v.	1000	ml

(4) No growth

TABLE 35

EMISSION SPECTROGRAPHIC ANALYSES* OF SUBSTRATES LISTED IN TABLE 34

	1	2	3	4	5	6	7	8
Sodium	22.	25	25.	25.	26.	21.	26.	21.
Potassium	4.6	4.7	4.8	7.0	6.4	5.2	4.7	5.7
Aluminum	0.23	0.16	0.14	0.17	0.064	0.48	0.098	0.92
Calcium	0.41	0.42	0.37	0.26	0.36	1.3	0.38	0.77
Silicon	1.6	1.8	2.9	1.8	1.5	5.7	2.1	5.9
Iron	0.56	0.47	0.39	0.33	0.29	0.38	0.43	0.46
Phosphorus	5.7	1.7	trace	trace	trace	trace	trace	trace
Magnesium	0.82	1.0	1.4	0.34	0.38	3.0	0.72	1.5
Boron	0.10	0.21	0.21	0.073	0.041	0.13	0.091	0.054
Manganese	0.0035	0.0057	0.0044	0.0040	trace	0.0062	0.0030	0.0027
Chromium	0.0035	0.0016	0.0023	0.0061	0.0012	0.0036	0.0032	0.0045
Lead	0.0095	0.0065	0.0052	0.0073	0.0049	0.0058	trace	0.0061
Molybdenum	trace	trace	0.0055	0.0025	nil	trace	0.0024	nil
Tin	trace	trace	0.010	0.0035	nil	trace	trace	nil
Vanadium	0.0021	0.00091	0.00082	0.0016	0.00093	trace	trace	0.0019
Lithium	0.010	0.017	0.010	0.016	0.035	0.028	0.011	trace
Copper	0.023	0.021	0.032	0.026	0.019	0.021	0.021	0.024
Silver	trace	trace	0.00035	trace	trace	trace	trace	nil
Titanium	0.0075	0.0030	0.0031	0.0067	0.0023	0.011	0.0030	0.049
Nickel	0.0022	0.0022	0.0023	0.0027	0.0013	0.0014	0.0014	0.0018
Cobalt	0.0021	0.0025	0.0030	0.0046	trace	trace	0.0098	0.0098
Strontium	0.016	0.011	0.013	0.010	0.018	0.015	0.013	0.020
Zirconium	nil	nil	nil	nil	nil	0.0014	nil	0.0020
Other elements	nil	nil	nil	nil	nil	nil	nil	nil

* Percent of sulfate ash.

C. Mechanism of Aluminum Corrosion by Natural JP-4 Fuel-Water Bottom

Microbial contamination of fuel can cause aluminum corrosion as a result of oxidizing and transforming hydrocarbon substrates to water-soluble corrosive materials which collect in the water bottom of fuel storage tanks. Sources other than microbial contamination also contribute to the corrosivity of the water bottoms. Samples of water bottom from JP-4 fuel storage tanks were obtained from Andrews Air Force Base, Maryland. This water was sterile at the time of sampling and would not support the growth of strains 96 and 101. This effect was expected because the fuel contained a biocidal anti-icing compound, ethylene glycol monomethyl ether. The 7075 abd 2024 aluminum alloys were cut into coupons, weighed, and cleaned before and after test as described previously. (See Section B, 2, d).

1. Effect of Nitrate Concentration and pH on Corrosivity of Fuel-Water Bottom

Aliquots of 100 ml of water bottom were added to 250 ml Erlenmeyer flasks and overlayed with 20 ml of JP-4 fuel. Ten flasks contained unaltered water (no nitrate added and no pH adjustment); five flasks which were adjusted to pH 7.0 with KOH and had no nitrate added; five flasks contained one concentration of KNO_3 (0.2, 0.4, 0.6, 0.8, and 12 millimoles per liter) and no pH adjustment; and five flasks were glass-distilled water controls. After 30 days, the test coupons were removed from five of the unaltered water samples and from three of each of the other samples. The remaining five unaltered water samples and two of each of the other samples were retained for 90 days. Incubation was at 28°C and the flasks were undisturbed. The weight loss data in Figure 19 show that nitrate concentrations between 0 and 0.8 millimolar KNO_3 caused no appreciable reduction in corrosion. High concentrations of KNO_3 (12 millimolar) however, did inhibit corrosion of both alloys in 30 days and 90 days.

The water bottom was neutralized with KOH to determine if neutral conditions would alter its corrosivity. Incubation was for 30 days and 90 days at 28°C. The results obtained are shown in Table 36. These data were analyzed statistically by the "t test" at the 5% confidence limit, and it was found that at pH 4.9 significant differences exist in the weight losses observed in 2024 and 7075 at 30 days but not at 90 days. Also significant differences exist in corrosion of 2024 at pH 4.9 and 7.0 at 30 days with alloy 2024. Other workers indicate that less corrosion occurs in neutral solutions than in acid solutions.[35] Therefore, the effect observed here may be due to increased potassium ion concentration rather than pH, since after 90 days 2024 and 7075 corroded at about the same extent regardless of pH.

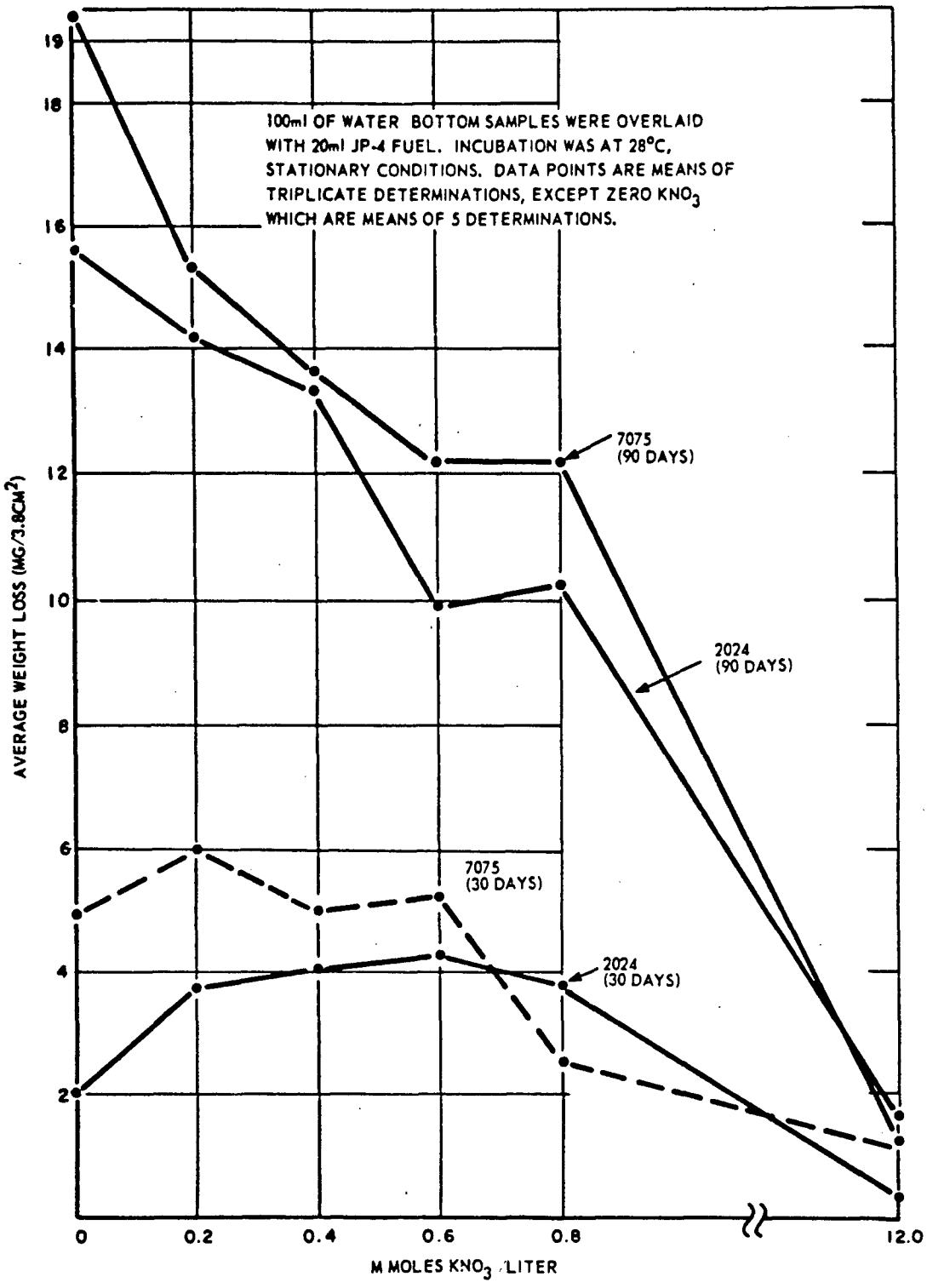


Figure 19. Effect of Nitrate on Fuel Water Bottom Corrosion of 7075 and 2024 Aluminum Alloy. Corrosion after 30 Days and 90 Days

TABLE 36

EFFECT ON pH ON CORROSION OF 2024 and 7075 ALLOYS BY WATER BOTTOM

Coupon No.	Weight Loss (mg/3.8 cm^2) 2024	7075	Reaction time (days)	pH*
62	2.28	4.88	30	4.9
66	1.91	3.92	30	4.9
67	1.66	4.31	30	4.9
68	2.81	6.36	30	4.9
69	1.96	5.36	30	4.9
72	3.93	4.40	30	7.0
73	—**	3.75	30	7.0
74	4.01	5.14	30	7.0
61	9.59	22.88	90	4.9
63	15.53	16.95	90	4.9
64	19.26	20.29	90	4.9
65	16.72	20.92	90	4.9
70	16.86	16.36	90	4.9
71	10.82	12.80	90	7.0
75	8.36	10.01	90	7.0

* Dilute KOH used for adjustment of pH

** flask broken during incubation

2. Fractionation of a Natural Water Bottom

The corrosive activity of water bottom material can be adequately assayed through the use of a column of Pharmacia's G-25 fine Sephadex. This column chromatographic technique (gel filtration) allows for a good separation by molecular weight of all components in a clarified water bottom sample.

Clarification of crude jet fuel water bottom material was accomplished by filtration with Gelman Instrument Company's 10-micron, pore-size glass filters. After clarification of the water bottom sample two aliquots were removed, one for application on a Sephadex column and the other for qualitative and quantitative chemical analysis. Three hundred milliliters of this clarified material were then applied to a 5 x 65 cm G-26 fine Sephadex column, and washed through with distilled water. Twenty 80-ml fractions were collected and each fraction was tested for aluminum corrosion with coupons of alloy 2024 and 7075.

Data on aluminum alloy weight loss by corrosion of fractionated water bottom material is in Table 37. This weight loss information revealed two items of interest: one, that the compound responsible for aluminum corrosion was of small molecular weight (50-100), and two, that there may be some corrosion-inhibiting material present, possibly of higher molecular weight. The latter statement is based on the observation that non-fractionated water bottom material is not as corrosive as fractionated material.

3. Chemical Analysis of Corrosive Fractions from a Jet Fuel-Water Bottom Fractionation

Because fractions 10 through 13 were corrosive an immediate attempt was made to identify those compound(s) responsible. The first identification scheme devised employed two general qualitative reactions. The first was Molische's alpha-naphthol-sulfuric acid test, and the second test was that of controlled Periodic Acid Oxidation.[36]

The alpha-naphthol-sulfuric acid test is a general test for the presence of a carbohydrate radical (CHO), and the Periodic Acid Oxidation reaction is specific for 1, 2-glycols, a-hydroxy aldehydes, and 1, 2-diketones. Aliquots from fractions 10 through 13 gave good strong positive reactions with both of these tests.

Because these two positive reactions indicated a water-soluble glycol-type compound and because this natural jet fuel-water bottom was suspected of containing, as an additive, ethylene glycol monomethyl ether, a modification of the Maloprade reaction, was used to determine titrimetrically the percent ethylene glycol present. This reaction was developed by Scribner.[37] This procedure is for glycerine.

Since no dichromate was suspected, the following procedure was used:

(1) Add by pipet 1.0 ml of sample to a clean, dry 250-ml Erylenmeyer flask. If greater accuracy is desired and sufficient sample is available for all analyses, a 10-ml aliquot of a 10-ml sample diluted to 100 ml in a volumetric flask may be employed. Place the equivalent amount of water in two flasks and carry these through the procedure as blanks.

(2) Add by pipet 10.0 ml of 0.5 M periodic acid to each flask.

(3) Allow the flasks to stand for 1 hour; swirl the flasks occasionally to mix well.

(4) Add sufficient water to the flask to bring the volume to about 30 ml.

(5) Add 2 gm of potassium iodine crystals. Swirl to dissolve.

(6) Add 5 ml of 6 N sulfuric acid. Swirl to mix well.

(7) Titrate the evolved iodine with standardized 0.1 N sodium thiosulfate to a pale yellow color. Add "Paragon" or starch indicator; swirl to disperse well. Carry on the tritration carefully until a blur or grey starch-iodine color disappears.

The volume of thiosulfate required for the blank is usually 36 to 38 ml. The volume of thiosulfate required to titrate the glycerol must be equal to or greater than 80% of the volume required for the blanks. If it is less than this amount, the glycerol is present in quantities greater than 1.78% (w/v) and the analysis must be re-run using larger volume of periodic acid. If the net titration with thiosulfate (volume required for blank less volume required for sample) is less than 0.50 ml, the glycerol concentration in the samples is less than 0.12% (w/v). If greater accuracy is required below this concentration, the analysis must be re-run using 5 ml of sample and either 10 ml or 5 ml of periodic acid.

(8) Calculations:

ml thiosulfate (blank-ml thiosulfate sample)

= net ml thiosulfate

$$\frac{(\text{net ml thiosulfate})\ (\text{normally thiosulfate})\ (2.302)}{\text{ml sample}}$$

= % w/v glycerol

If an aliquot of a diluted sample has been taken, the dilution factor must be taken in consideration in the calculation.

Solutions containing 1%, 17%, and 20% of Fischer reagent grade glycerol and Phillips Fuel Additive EGME were used as standards for the procedures. The glycol content in the three Sephadex fractions was 14%. The nonfractionated but clarified jet fuel-water bottom material had a glycol content of 17%.

Therefore, since corrosion was observed only by the fractions containing the glycol, it appears that the additive is the responsible agent in the corrosion observed by water bottoms. The previous data with nitrate, however, indicates that addition of nitrate at 12 millimoles/liter of water bottom is sufficient to inhibit corrosion by the additive.

TABLE 37

WEIGHT LOSS OBSERVED IN 2024 AND 7075 COUPONS EXPOSED TO WATER BOTTOM FRACTIONS SEPARATED ON SEPHADEX G-25 GEL*

Fraction No.		Weight Loss, mg/6.58 cm^2	
		Alloy 2024	Alloy 7075
Unfractionated sample		1.7	2.5
order of decreasing molecular weights ↓ 1	High molecular weight components	0.3	1.0
2		0.3	0.7
3		1.1	1.8
4		1.0	1.1
5		0.8	0.8
6		0.3	0.6
7		0.9	0.6
8		3.8	0.9
9		-	5.3
10	50 to 100 molecular weights	11.6	16.1
11		---	16.9
12		12.3	13.9
13		13.8	---
14	Low molecular weight components	1.8	3.3
15		---	6.3
16		5.4	4.5
17		1.5	2.3
18			
19		(less than 1 mg weight loss)	
20			

--- indicates NO COUPON

* Corrosion tests were run on each 80-ml fraction in 100 ml beakers. Incubation was at room temperature for 8 days, stationary conditions.

D. Mechanism of Hydrocarbon Oxidation by Jet Fuel Microorganisms

The growth of microorganisms on jet fuel depends on the oxidation of hydrocarbons. Although many species of taxonomically unrelated microorganisms have been isolated from the water bottoms of jet fuel tanks, most of these species share the ability to utilize hydrocarbons. The oxidation of hydrocarbons by jet fuel contaminants is of importance from at least two standpoints: (1) it provides energy for the uptake and transformation of corrosion inhibitors such as nitrate and phosphate, and (2) it results in the growth of cells and production of organic fuel contaminants which clog filters and react with aluminum alloy surfaces.

1. Growth on Jet Fuel and Hydrocarbons

During the screening of 98 stock cultures for corrosion in the early part of this study, it was found that these cultures would grow on salt medium with JP-4 fuel as the only source of carbon. To characterize these organisms and their metabolic products, it was necessary to determine their capacity to grow on hydrocarbons of different chain length and structure. Table 38 shows the responses of microorganisms in the presence of the particular alkanes and olefins used in growth media.

Pentane, hexane, and heptane did not support growth and did not kill the fuel isolate tested. The organisms are not killed by 1-pentene and are killed only slowly by 2-pentene. However, hexene, heptene and octene unsaturated at the 1 or 2 position kill these fuel isolated readily. But the lethal effects of these short-chain olefins ends with nonene; 1-decene and 1-dodecene support growth to about the same extent as octane, nonane, decane, and dodecane. Population densities change on these saturated hydrocarbons from 10^6 cells per ml to about 10^8 cells per ml in 48 hours.

A study was also made of the growth of strain 101 on media with purified hydrocarbon overlays containing either ammonium chloride or potassium nitrate instead of ammonium nitrate. The results of this study are shown in Table 39 and Table 40.

The lethal effect of 1-hexene, 1-heptene, and 1-octene was not influenced by the source of nitrogen in the salt medium. However, 1-nonene was lethal to strain 101 only when the source of nitrogen was ammonium nitrate; this olefin supported growth on both ammonium chloride and potassium nitrate; nonane supported growth with ammonium chloride and ammonium nitrate, but not with potassium nitrate.

The toxicity of short-chain olefins is not confined to organisms that grow on fuel. Figure 20 shows that E. coli is killed more rapidly by 1-heptene than by jet fuel. E. coli is distinguished from the pseudomonads isolated from fuel by its sensitivity to jet fuel, but both organisms are sensitive to the lethal properties of 1-heptene.

TABLE 38

GROWTH OF STRAIN 101 ON JP-4 AND 33 PURIFIED HYDROCARBONS

Hydrocarbon	6-day Incubation cells/ml	Final pH	Hydrocarbon	6-day Incubation cells/ml	Final pH
JP-4	1.4×10^8	4.2	Hexadecane	4×10^8	6.5
Pentane	5.8×10^6	6.3	1-Hexadecene	1.6×10^8	6.7
2,2,4-Trimethyl-pentane	1×10^7	6.6	Mesitylene	3.4×10^4	6.8
1-Pentene	6.9×10^6	6.3	1,2,4-Trimethyl-benzene	9×10^4	6.9
2-Pentene	2.1×10^6	6.9			
Hexane	1.5×10^5	6.3	O-Xylene	0	7.0
1-Hexene	0	6.2	m-Xylene	0	7.0
2-Hexene	0	7.1	p-Xylene	0	7.0
Heptane	2.8×10^5	6.3	Cymene	1×10^3	7.0
1-Heptene	0	6.2	Methylcyclo-pentane	1.4×10^6	6.8
Octane	4×10^8	6.2	2,3-Dimethyl-butane	7.1×10^6	6.9
1-Octene		6.9			
Nonane	1.4×10^6	6.4	Methylcyclo-hexane	1.7×10^6	6.7
1-Nonene	5×10^6	6.2	2,2,5-Trimethyl-hexane	7×10^6	6.9
Decane	1×10^9	4.6			
1-Decene	1.6×10^8	4.5			
Undecane	8.7×10^7	4.2			
1-Undecene	1×10^8	6.3			
Dodecane	2.4×10^8	4.2			
1-Dodecene	8.6×10^8	6.3			
Tridecane	1.7×10^6	6.3			
Tetradecane	4.8×10^8	4.4			
1-Tetradecene	3.9×10^8	4.2			

Inoculum: Medium-2 grown cells were washed three times with distilled water; initial concentration 10^6 cells per ml; initial pH, 7.0; incubation at 28°C in gyratory shaker in Medium 7.

TABLE 39

GROWTH OF STRAIN 101 ON MEDIUM 7

Hydrocarbon	6-day Plate Count per ml	10-day Plate Count per ml	13-day Plate Count per ml	Final pH
Jet Fuel	1.4×10^8	7×10^8	3.7×10^8	3.2
Pentane	5.8×10^6	6×10^6	5.8×10^6	6.3
1-Pentene	6.9×10^6	1.2×10^7	1.1×10^7	6.3
Hexane	1.5×10^5	3×10^5	2.3×10^6	6.3
1-Hexene	0	0	0	6.2
Heptane	2.8×10^5	2.4×10^5	2.1×10^5	6.3
1-Heptene	0	0	0	6.2
Octane	4×10^8	2.6×10^8	1.5×10^8	6.2
1-Octene	0	0	0	6.3
Nonane	1.4×10^6	1.6×10^6	1.2×10^6	6.4
1-Nonene	5×10^6	7.9×10^7	2.1×10^7	6.2
Decane	$1.x 10^9$	1.3×10^9	7.1×10^7	4.6
1-Decene	1.6×10^8	1×10^8	$3. \times 10^8$	4.5
Undecane	8.7×10^7	2.7×10^8	2.6×10^8	4.2
1-Undecene	1×10^8	2.3×10^8	1×10^8	6.3
Dodecane	2.4×10^8	2.9×10^8	3×10^8	4.2
1-Dodecene	8.6×10^8	1.2×10^9	9.3×10^8	6.3
Tridecane	1.7×10^6	1.9×10^6	3.9×10^6	6.3
Tetradecane	4.8×10^8	6.1×10^8	4.3×10^8	4.4
Tetradecene	3.9×10^8	2.5×10^8	5.5×10^8	3.2

Inoculum: TGY-grown cells washed three times with distilled water; initial concentration, 1×10^7 cells/ml; temperature, 25°C; on New Brunswick shaker; Medium 7.

TABLE 40

GROWTH OF STRAIN 101 ON MEDIUM 8

Hydrocarbon	6-day Plate Count per ml	10-day Plate Count per ml	13-day Plate Count per ml	Final pH
Jet Fuel	2×10^8	2.7×10^8	1.4×10^8	6.7
Pentane	1.1×10^6	2×10^5	3.4×10^5	7.0
Pentene	2.9×10^6	2.3×10^5	1.3×10^5	7.0
Hexane	1.2×10^5	6.3×10^3	1.2×10^4	7.2
1-Hexene	0	0	0	7.1
Heptane	1.8×10^5	1.1×10^5	4.8×10^4	7.15
1-Heptene	0	0	0	7.0
Octane	6.9×10^7	4.4×10^7	1.3×10^8	7.3
1-Octene	0	0	0	7.1
Nonane	7.2×10^5	1.7×10^5	2×10^4	7.2
1-Nonene	1.1×10^8	3.4×10^8	4.3×10^8	6.9
Decane	1.5×10^8	5.6×10^8	2.8×10^8	6.9
1-Decene	6.2×10^4	1.3×10^7	2.3×10^8	6.8
Undecane	2×10^6	2.4×10^3	1.3×10^5	6.8
1-Undecene	1.5×10^{10}	7.4×10^9	2.7×10^9	7.0
Dodecane	9×10^6	8.6×10^6	2.1×10^7	7.2
1-Dodecene	1.2×10^8	3.2×10^8	4.2×10^8	7.1
Tridecane	1.1×10^6	5.8×10^5	2.8×10^5	7.1
Tetradecane	1.8×10^6	9.5×10^6	2.4×10^7	7.2
Tetradecene	3.4×10^8	1×10^9	8.7×10^8	7.2

Inoculum TGY-grown cells washed three times with distilled water; initial concentration, 1×10^7 cells/ml; temperature, 25°C; on New Brunswick shaker Medium 8.

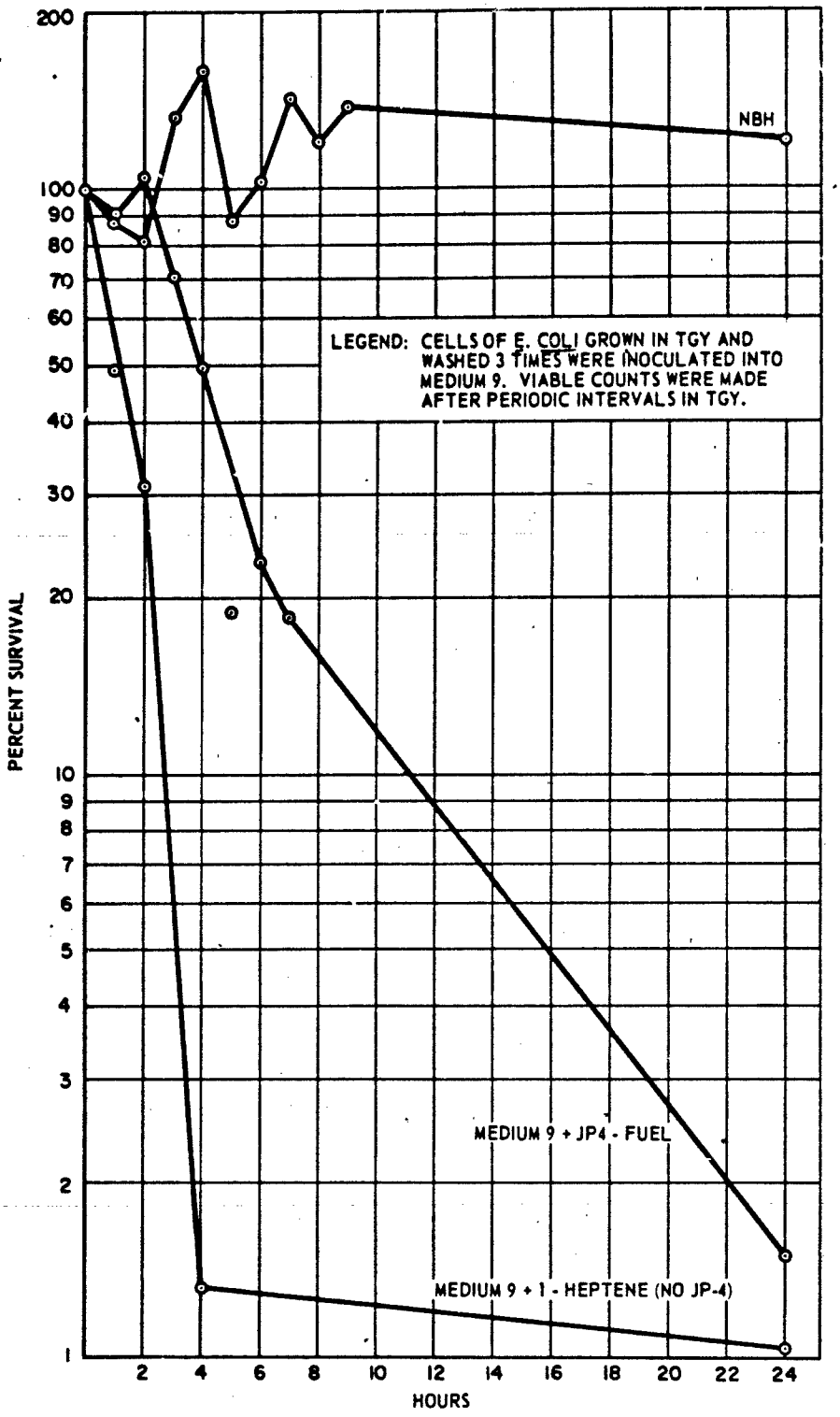


Figure 20. Effect of 1-Heptene on the Viability of E. coli

2. Lethal Effect of 1-Hexene, 1-Heptene, and 1-Octene on Growth of Different Strains of Fuel-Oxidizing Organisms on Medium 8 and on TGY Medium

The lethal effect of 1-hexene, 1-heptene and 1-octene was believed to be potentially useful as a means of controlling bacteria in fuel-water bottoms. However, before suggesting this method, it was necessary to determine whether the olefins were lethal to other fuel-oxidizing strains.

The results in Table 41 show that all the strains tested were killed by 1-heptene in 4 hours except strain 28 which was not killed in 4 hours but in 24 hours. However, only strains 96 and 101 were killed by 1-octene; the other strains grew on this olefin. In one case, strain 87, there was more growth with 1-octene than with TP-4 fuel.

The presence of organic material generally reduced the effectiveness of inhibitors. TGY broth contains glucose and various proteins which are of a similar chemical nature as exist in bacterial protoplasm. The effectiveness of the olefin inhibitors would be expected to be reduced because of competition of the TGY protein with the bacterial components in reacting with the olefins.

Flasks of TGY broth were overlayed with 15 ml of olefin and inoculated with washed cells of the strains of fuel-oxidizing organisms which were tested above. The results of this study are shown in Table 42. Only 1-hexene was lethal to all the strains.

3. Effect of 1-Heptene Concentration on Survival of Strain 101

The objective of this study was to determine the effect of incubation time and 1-heptene concentration in the 20 ml overlay of jet fuel on survival of strain 101 on medium 6 after 4 days. The cells were harvested and washed three times by centrifugation in sterile distilled water and suspended in 18-ml of sterile distilled water. A standard plate count showed the concentration of this stock suspension to be 2.8×10^9 cells per ml.

TABLE 41

COMPARATIVE EFFECTS OF 1-OLEFINS ON THE GROWTH OF DIFFERENT STRAINS OF FUEL-OXIDIZING ORGANISMS ON MEDIUM 8

Strain 23	JP-4 Fuel	1-Hexene	1-Heptene	1-Octene	No overlay
0-hr	9×10^6	9×10^6	9×10^6	9×10^6	9×10^6
4-hrs	1.8×10^7	0	0	2.9×10^6	1.6×10^7
24-hrs	3.5×10^7	0	0	2.3×10^6	3.2×10^7
48-hrs	1.7×10^9	0	0	6.1×10^6	1.4×10^7
5-days	6.9×10^8	0	0	1.7×10^7	2×10^7
9-days	9.7×10^8	0	0	3.6×10^7	9.3×10^6
Strain 86					
0-hr	5.3×10^7	5.3×10^7	5.3×10^7	5.3×10^7	5.3×10^7
4-hrs	5×10^8	0	0	2.2×10^4	3×10^7
24-hrs	3.3×10^8	0	0	3.4×10^4	5.7×10^7
48-hrs	4.9×10^9	0	0	5.8×10^7	2.5×10^7
3-days	1.3×10^9	0	0	3.7×10^8	2.1×10^7
6-days	4.1×10^9	0	0	3.8×10^9	2.4×10^7
7-days	4×10^9	0	0	1×10^8	3.4×10^7
Strain 2					
0-hr	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6
4-hrs	9×10^6	0	0	2.5×10^6	9.1×10^6
24-hrs	2.1×10^8	0	0	9.9×10^8	1.9×10^7
48-hrs	5.2×10^9	0	0	3.4×10^9	1×10^7
5-days	1×10^8	0	0	7×10^8	1.2×10^7
9-days	9.4×10^8	0	0	1.9×10^8	8.7×10^6
Strain 28					
0-hr	7.7×10^6	7.7×10^6	7.7×10^6	7.7×10^6	7.7×10^6
4-hrs	2.3×10^7	5.4×10^5	0	6.7×10^7	6.6×10^7
24-hrs	1×10^9	0	0	1.3×10^9	4×10^7
48-hrs	2.6×10^{10}	0	0	2.9×10^9	1.2×10^7
5-days	1.2×10^{10}	0	0	6.7×10^8	1.8×10^7

(Continued on next page)

TABLE 41 (Continued)

Strain 87	JP-4 Fuel	1-Hexene	1-Heptene	1-Octene	No Overlay
0-hr	1.9×10^5	1.9×10^5	1.9×10^5	1.9×10^5	1.9×10^5
4-hrs	2.7×10^5	0	0	2.1×10^5	7.9×10^6
24-hrs	5.9×10^7	0	0	1.3×10^6	1.5×10^7
48-hrs	6.8×10^7	0	0	2.9×10^6	1.5×10^7
5-days	6.3×10^8	0	0	1.1×10^9	7.3×10^6
Strain 15					
0-hr	4.2×10^7	4.2×10^7	4.2×10^7	4.2×10^7	4.2×10^7
4-hrs	2.3×10^8	0	1×10^3	1.3×10^7	5.9×10^7
24-hrs	2.9×10^8	0	0	2.1×10^7	1×10^7
48-hrs	7.9×10^8	0	0	4.3×10^9	4.2×10^7
3-days	8.6×10^9	0	0	1.6×10^9	3.1×10^7
6-days	2.6×10^9	0	0	1×10^9	3.5×10^7
7-days	3.2×10^9	0	0	2×10^9	3×10^7
Strain 96					
0-hr	1.3×10^7	1.3×10^7	1.3×10^7	1.3×10^7	1.3×10^7
72-hrs	4.2×10^8	0	0	0	2.6×10^7
6-days	1.1×10^9	0	0	0	1.1×10^7
Strain 101					
0-hr	1.1×10^7	1.1×10^7	1.1×10^7	1.1×10^7	1.1×10^7
4-hrs	3.2×10^7	0	0	0	2.1×10^7
24-hrs	4.8×10^7	0	0	0	3.2×10^7
48-hrs	8.1×10^7	0	0	0	1.9×10^7
72-hrs	5.6×10^8	0	0	0	2.3×10^7
5-days	9.6×10^8	0	0	0	1.6×10^7

Cells were washed three times in distilled water before incubation at 28°C on a rotary shaker; plate counts are expressed in cell count per ml. Medium 8 with purified hydrocarbons or jet fuel were used in all studies except one control (no overlay).

TABLE 42

COMPARATIVE EFFECT OF 1-OLEFINS ON THE GROWTH OF DIFFERENT STRAINS OF FUEL-OXIDIZING ORGANISMS ON TGY MEDIUM

Strain 23	1-Hexene	1-Heptene	1-Octene	TGY Control
0 - Time	1.1×10^8	1.1×10^8	1.1×10^8	1.1×10^8
5 - hrs.	1×10^6	4.4×10^7	1×10^8	7.8×10^8
24 - hrs.	0	3.7×10^6	1.6×10^9	2×10^{10}
48 - hrs.	0	8×10^7	1.7×10^9	1×10^{10}
3 - days	0	3.9×10^7	7.7×10^8	3.5×10^{10}
4 - days	0	2.6×10^8	7.1×10^8	1.7×10^{11}
7 - days	0	7×10^8	3.8×10^9	1.1×10^{11}
Strain 86				
0 - Time	7.9×10^7	7.9×10^7	7.9×10^7	7.9×10^7
5 - hrs.	2×10^1	2.8×10^3	5.2×10^6	2.1×10^8
24 - hrs.	0	2.7×10^5	1×10^8	4.2×10^{10}
48 - hrs.	0	2.9×10^7	2.4×10^8	2.6×10^{10}
3 - days	0	5.2×10^6	2×10^8	2.2×10^{10}
4 - days	0	4.6×10^6	7.3×10^8	3.4×10^{10}
7 - days	0	1.1×10^7	2.5×10^8	1.1×10^{10}
Strain 2				
0 - Time	3.1×10^7	3.1×10^7	3.1×10^7	3.1×10^7
5 - hrs.	5×10^1	4.1×10^5	1.1×10^7	7.3×10^7
24 - hrs.	0	1.9×10^7	4.7×10^6	9.1×10^{10}
48 - hrs.	0	1.7×10^9	6.7×10^7	2.3×10^{10}
3 - days	0	5.4×10^8	2.1×10^8	2×10^{10}
4 - days	0	7×10^9	5.7×10^8	9.2×10^9
7 - days	0	9.2×10^7	2.3×10^8	2.9×10^9
Strain 7				
0 - Time	3.3×10^4	3.3×10^4	3.3×10^4	3.3×10^4
4 - hrs.	0	0	$2.x 10^4$	3.9×10^4
24 - hrs.	0	0	3.8×10^4	2.2×10^6
48 - hrs.	0	0	1.2×10^6	8.4×10^6
4 - days	0	0	1.6×10^8	6.8×10^8

(Continued on next page)

(TABLE 42 CONT.)

Strain 15	1-Hexene	1-Heptene	1-Octene	TGY Control
0 - Time	3.3×10^7	3.3×10^7	3.3×10^7	3.3×10^7
5 - hrs.	1.8×10^7	3×10^7	4.8×10^7	8.9×10^7
24 - hrs.	4.6×10^2	2.5×10^6	6.8×10^8	7.3×10^9
48 - hrs.	0	7.8×10^7	2.8×10^9	7.9×10^9
3 - days	0	8.4×10^7	2.6×10^8	1.2×10^{10}
4 - days	0	3×10^8	1.5×10^9	1.3×10^{10}
7 - days	0	8.4×10^8	1.7×10^9	1.4×10^{10}
Strain 28				
0 - Time	7.7×10^6	7.7×10^6	7.7×10^6	7.7×10^6
4 - hrs.	0	0	2×10^7	1×10^8
24 - hrs.	0	0	3.1×10^8	4.1×10^9
48 - hrs.	0	0	1×10^9	2.7×10^9
5 - days	0	0	1.2×10^9	5.8×10^9
7 - days	0	0	1.3×10^8	1.6×10^9
Strain 87				
0 - Time	1.9×10^5	1.9×10^5	1.9×10^5	1.9×10^5
4 - hrs.	0	0	3.1×10^5	5.6×10^6
24 - hrs.	0	0	2.1×10^7	2.4×10^8
48 - hrs.	0	0	3.4×10^8	
5 - days	0	0	5.5×10^9	5.1×10^9
7 - days	0	0	2.1×10^9	5.1×10^{10}
Strain 101				
0 - Time	4.2×10^6	4.2×10^6	4.2×10^6	4.2×10^6
6 - hrs.	0	0	0	1.3×10^7
24 - hrs.	0	0	0	1.2×10^9
48 - hrs.	0	0	0	1.8×10^9
22 - days	0	0	0	1.2×10^8
Strain 96				
0 - Time	6.7×10^6	6.7×10^6	6.7×10^6	6.7×10^6
6 - hrs.	0	0	0	1.6×10^7
24 - hrs.	0	0	0	2.1×10^9
48 - hrs.	0	0	0	3.8×10^9
22 - days	0	0	0	1.5×10^8

Note: Cells were washed three times in distilled water before inoculation; 15 ml of olefin overlayed 100 ml of TGY broth (BBL) except the control flasks which had no overlay; incubation was at 28°C on a rotary shaker; plate counts are expressed in cell count per ml.

Eleven flasks of Medium 6 were overlaid with 20 ml of sterile JP-4 fuel and 1-heptene, in varying percentages. The media were as follows:

% 1-Heptene	Volume Heptene	Volume JP-4
25%	5 ml	15 ml
12.5%	2.5 ml	17.5 ml
5%	1 ml	19 ml
2.5%	0.5 ml	19.5 ml
2%	0.4 ml	19.6 ml
1.5%	0.3 ml	19.7 ml
1%	0.2 ml	19.8 ml
0.5%	0.1 ml	19.9 ml
0.05%	0.01 ml	19.99 ml
0.0%	0.0 ml	20 ml

The eleventh flask had no overlay and served as a control.

One ml of the stock 101 suspension was added to each flask giving an initial concentration of 2.8×10^7 organisms per ml. The flasks were then covered with two layers of polyethylene over the cotton plug to decrease losses of 1-heptene by evaporation. All flasks were incubated at 28°C with shaking. Samples were taken for viable count on TGY agar after 5, 24, 48, and 120 hours of incubation.

The results in Figure 21 show that complete kill was achieved with 5% heptene in JP-4 overlay. The aqueous phase contained 100 ml, which is 5 times that of the JP-4 overlay. In this case 7.1 millimoles of 1-heptene resulted in complete kill of 101 organism in 100 ml of Medium 6.

A study was performed to determine whether an absolute concentration (7.1 millimoles) or an absolute percentage (5%) of 1-heptene was required to express its lethal effect. Five Fernback flasks each containing 100 ml of Medium 6, were overlayed with 1000 ml of JP-4 fuel. One ml of 1-heptene was added to two of the flasks, and 5 ml of 1-heptene was added to two flasks. The fifth flask served as a control. All five flasks were inoculated with washed strain 101 cells to give an initial concentration of 3×10^7 cells/ml. Samples were withdrawn periodically and plate counts were made. The results of this study are shown in Table 43.

The conclusion of this study is that the percentage of olefin in the fuel is more important than the concentration of olefin. Although 7.1 millimole (5% 1-heptene in JP-4 fuel) was previously lethal to strain 101, this study showed that as much as five times this concentration (35.5 millimole) was not lethal when it was overlayed as 0.5% 1-heptene in JP-4 fuel.

Therefore, for the short-chain olefins to be effective as a lethal agent for microorganisms in fuel-water bottoms, they would have to be added to fuel to about 5% concentration.

Figure 21. Effect of Incubation Time and 1-Heptene Concentration in the 20-ml Overlay of JP-4 Fuel on the Survival of Strain 101 in Medium 6

TABLE 43

EFFECT OF 1-HEPTENE ON GROWTH ON JP-4 FUEL BY STRAIN 101

1-Heptene added	4 hour Plate count	24 hour Plate count	6 days Plate count	29 days Plate count
1.0 ml (7.1 mmoles)	2×10^7	1.8×10^7	3×10^9	2.3×10^9
1.0 ml (7.1 mmoles)	3×10^7	1.3×10^7	1×10^9	2.5×10^9
5.0 ml (35.5 mmoles)	1×10^7	5×10^6	1×10^9	5.5×10^8
5.0 ml (35.5 mmoles)	1.3×10^7	9×10^6	5.8×10^8	2.1×10^9
0 ml	3×10^7	9.3×10^7	2×10^9	1.4×10^9

100 ml of Bushnell-Haas medium (containing 1.2 g/l of KNO_3 instead of NH_4NO_3) overlayed with 1000 ml of JP-4 fuel. Initial concentration in each, 3×10^7 cells/ml. Cells from 7 day culture (BH with JP-4 fuel overlay) washed three times with sterile distilled water. Incubated at 28°C on rotary shaker. Plate counts are expressed in cell counts per ml.

4. Oxidation of Jet Fuel, Saturated and Unsaturated Hydrocarbons, and Glucose by Fuel Isolate and Inhibition of Whole-cell Respiration by Lethal Olefins

Hydrocarbons affect the growth of fuel isolates in different ways: (1) viability is not altered and the organism does not grow, (2) the organisms are killed, and (3) the hydrocarbon supports growth. The short-chain olefins, 1-heptene, 1-hexene, and 1-octene, were lethal to strains 96 and 101, both in the presence of salt medium and in the presence of TGY broth. To determine if the respiratory response of strain 101 to hydrocarbons would show the same pattern as the growth response, hydrocarbon oxidation was determined by oxygen uptake studies in the Warburg respirometers.

Cells of strain 101 were grown on TGY broth or on Medium 2. Cells on Medium 2 were incubated for 4 days at 28°C. The TGY cells were grown for 18 hours at 37°C. The cells were harvested by centrifugation (13,000 rpm for 10 minutes), washed three times with 0.01 M phosphate buffer (pH 6.5), and finally suspended in 0.1 M phosphate buffer (pH 6.5). Suspensions of organisms added to Warburg vessels were diluted to the same O. D. at 540 mu. These suspensions contained approximately 9×10^9 cells/ml. A thermobarometer was set up for each determination.

Wicks soaked with 0.2 ml 20% KOH were used in all Warburg flasks to absorb CO_2. The thermobarometers contained buffer and substrate. All reaction mixtures contained 0.5 ml cell suspension (10^9 cells/ml). The following quantities of substrates were used: 1.0 ml 1% glucose, 0.5 ml JP-4 fuel, or 0.5 ml octane. Five-tenth milliliter aliquots of desired olefins were used. The reaction mixture volumes were adjusted to a total volume of 3.2 ml with 0.1 M phosphate buffer, PH 6.5. The flasks were placed on the Warburg shaker and brought to 25°C during a 15-minute equilibration period before initiation of the reaction by addition of substrate from the sidearm. Endogenous respiration is indicated in each figure.

a. Oxidation of Pure Hydrocarbons by Cells Grown on Jet Fuel Medium

Figure 22 shows the oxidation of the C_5 through C_{10} alkanes and alkenes by these cells. A parallel is noted when oxidation of these hydrocarbons by strain 101 as determined by oxygen uptake is compared with the growth response (see Table 41). The outstanding parallel is that the three olefins, 1-hexene, 1-heptene and 1-octene, which were lethal to this organism, also failed to show oxygen uptake when used as substrates.

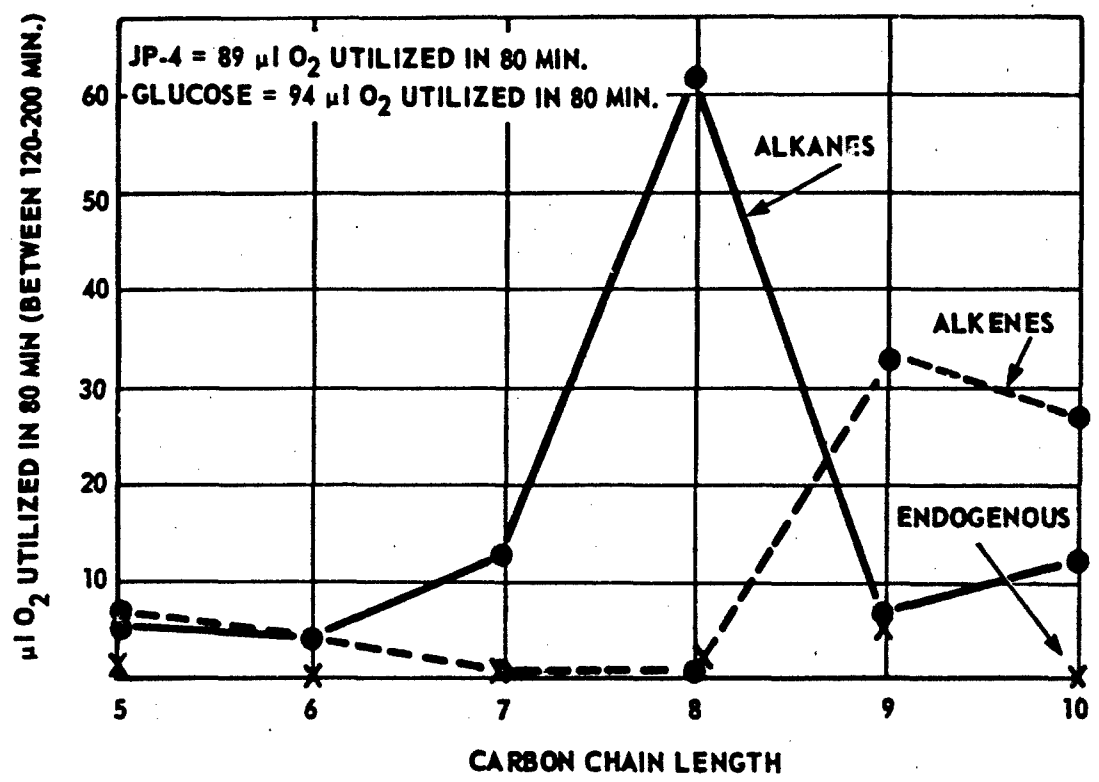


Figure 22. Oxidation of N-Alkanes and N-Alkenes by Washed Cells of Strain 101 Grown on Medium 2

b. Oxidation of Pure Hydrocarbons by Cells Grown on TGY Broth Medium

Jet fuel-water bottoms could contain organic material as well as inorganic salts. The origin of the organic material could be lysed micro organisms or outside contamination. TGY broth medium contains proteinaceous material, such as amino acids and peptides, as well as glucose. Cells adapted to growth on this medium, could show a different respiratory pattern with hydrocarbon substrates than cells grown on Medium 2.

Several aspects of the respiratory pattern of TGY cells differ from fuel-grown cells (Figure 23). The TGY-grown cells appear to be adapted to octane and decane, whereas fuel-grown cells oxidize only octane. Decane is readily oxidized by TGY cells, but not by fuel-grown cells. Also, jet fuel oxidation is lower with TGY-grown cells.

Difficulty was encountered in measuring oxygen uptake of the C_5 hydrocarbons because of the high volatility of these compounds. The C_6 - C_{10} alkenes of TGY-grown cells and C_6 - C_8 alkenses of fuel-grown cells inhibited endogenous uptake. With fuel-grown cells, nonene and decene were oxidized. These differences in respiratory activities between fuel-grown and TGY-grown cells suggested that the growth substrates regulate the ability of cells to oxidize specific hydrocarbons.

c. Oxidation of Hydrocarbon - JP-4 Fuel Mixture by Cells Grown on Jet Fuel Medium

In this study mixtures of pure hydrocarbons and JP-4 fuel were used as substrates to determine whether the added hydrocarbon was more readily oxidized than the fuel hydrocarbons.

The results in Figure 24 show that most of the hydrocarbons inhibited respiration, compared with oxidation of JP-4 fuel. One exception in Figure 24 was octane, which stimulated respiration. In all, alkenes inhibited more than alkanes, and C_6 - C_8 and decene were very effective inhibitors of respiration.

d. Oxidation of Hydrocarbon - JP-4 Fuel Mixture by Cells Grown on TGY Broth Medium.

With TGY-growth cells, the added alkane or alkene rather than the JP-4 fuel appears to regulate respiration (Figure 25). No differences were observed between respiration with mixtures of added hydrocarbon and jet fuel than with the pure hydrocarbon alone (see Figure 23). Again, as noted above, the exceptionally high rate of oxygen uptake of 1-pentene

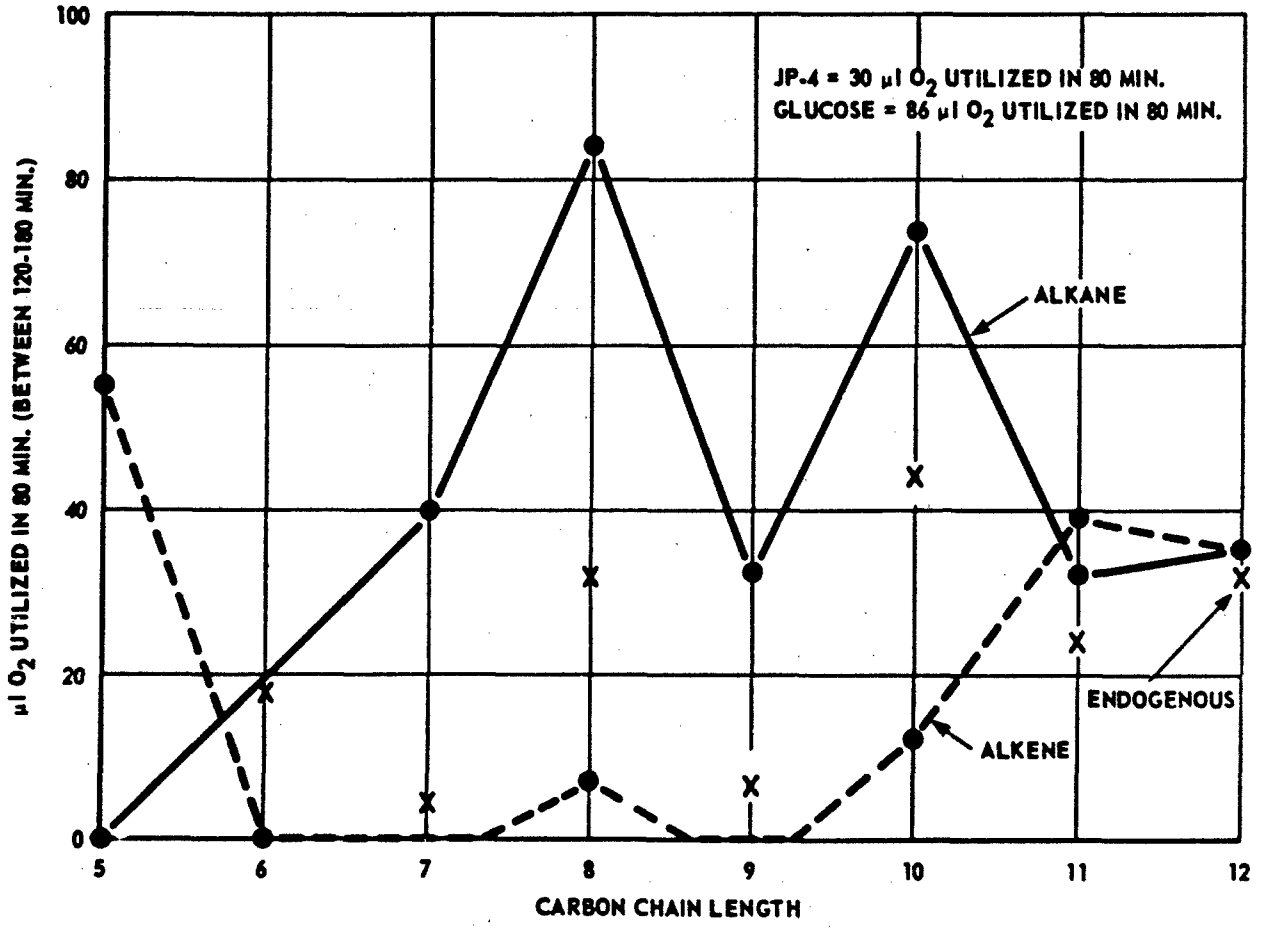


Figure 23. Oxidation of N-Alkanes and N-Alkenes by Washed Cells of Strain 101 Grown on TGY Medium

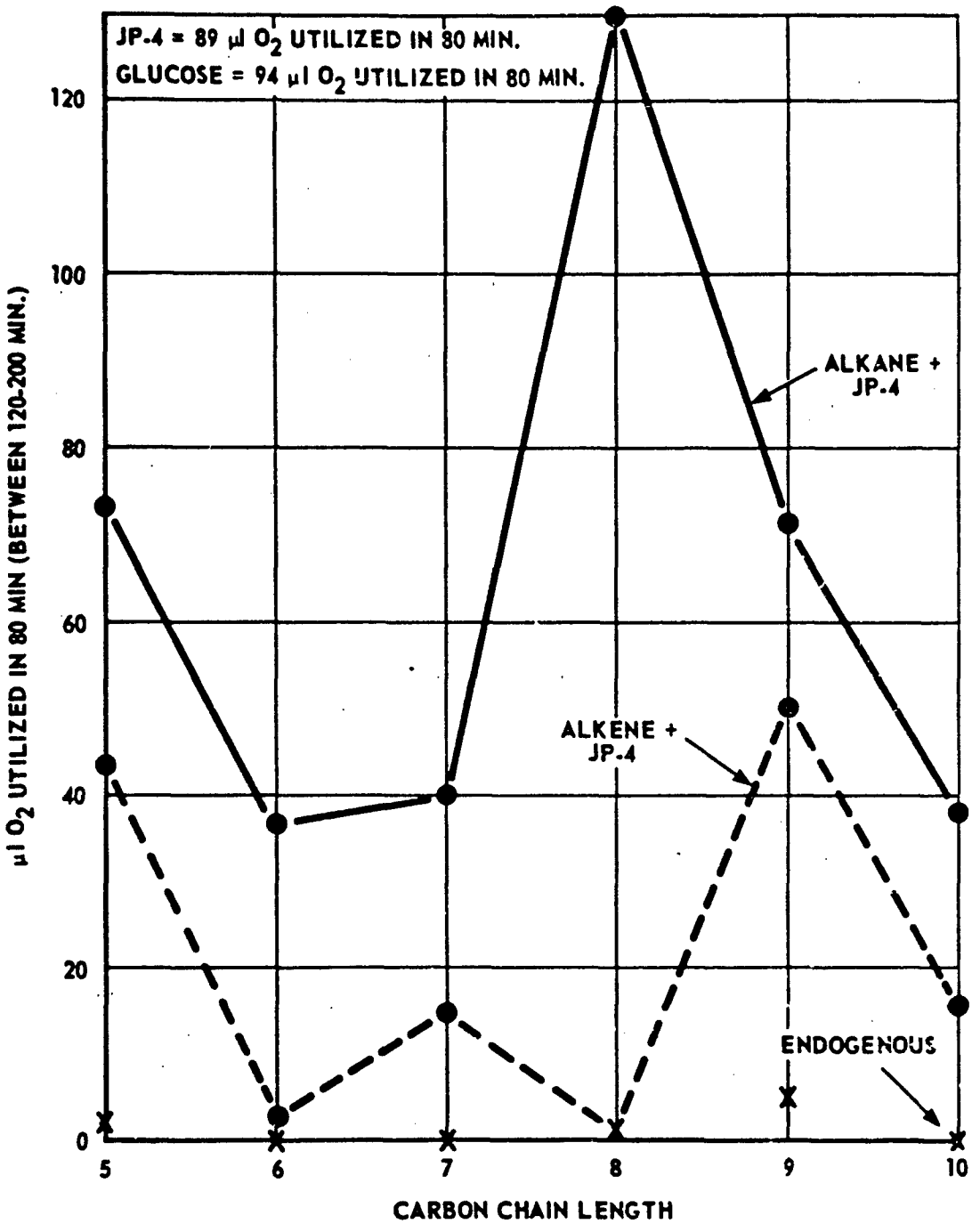


Figure 24. Oxidation of N-Alkanes and N-Alkenes in the Presence of JP-4 Fuel by Washed Cells of Strain 101 Grown on Medium 2

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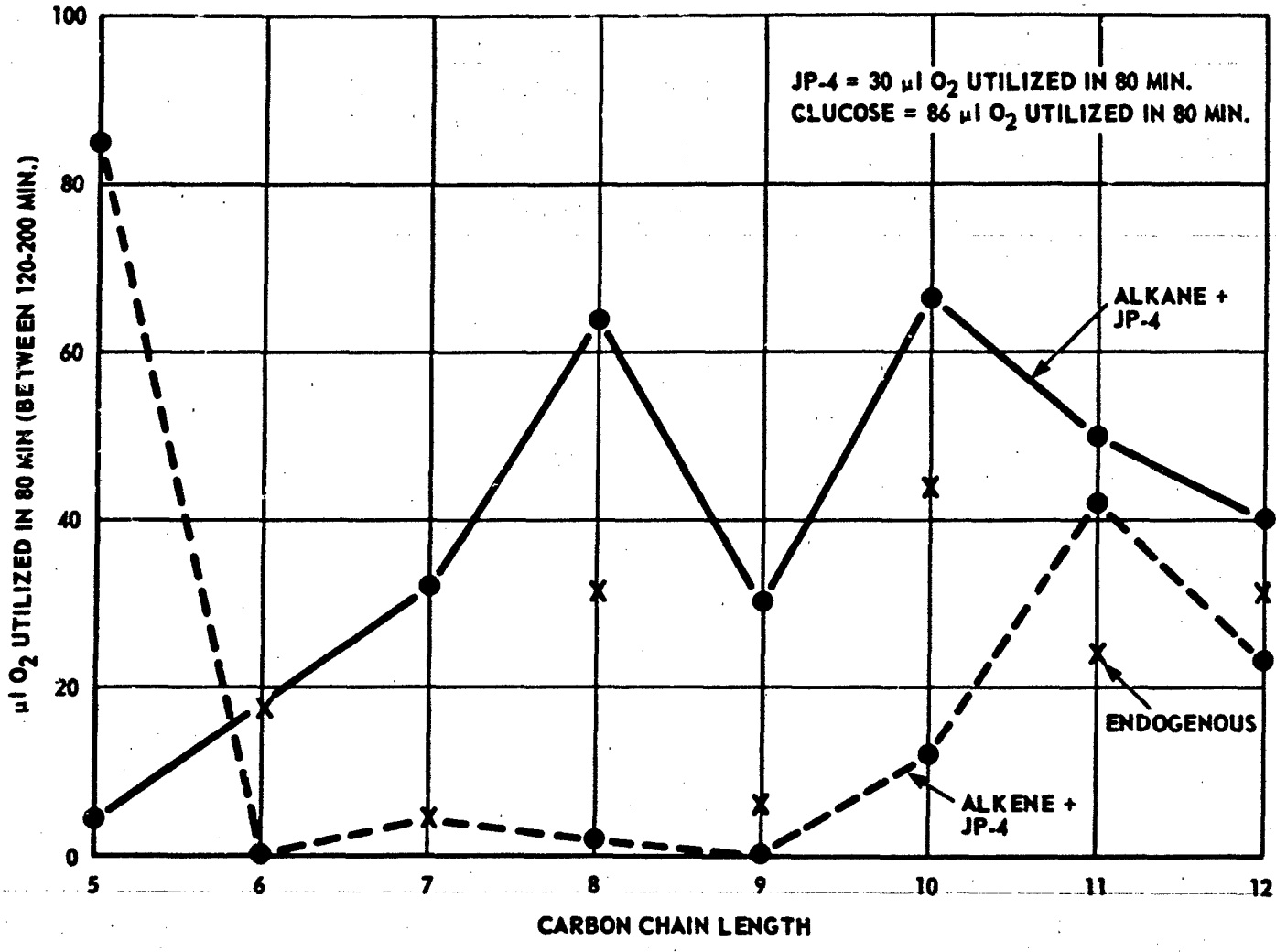


Figure 25. Oxidation of N-Alkanes and N-Alkenes in the Presence of JP-4 Fuel by Washed Cells of Strain 101 Grown on TGY Medium

was probably an attribute of the volatility of this hydrocarbon rather than due to true oxygen uptake. The C_6 - C_{10} alkenes in the presence of jet fuel, as in the absence of jet fuel, were not oxidized and inhibited endogenous respiration.

e. Oxidation of Hydrocarbon-Glucose Mixture by Cells Grown on Jet Fuel Medium.

Since the addition of jet fuel to pure hydrocarbons had essentially no effect on oxygen uptake of the hydrocarbons, the question arose: What effect would the addition of an organic compound have? Glucose was chosen, since it serves as a readily available source of energy for microorganisms.

The effect of the mixed substrate on the respiration of strain 101 fuel isolate is shown in Figure 26. Here, it is observed that C_5 - C_8 and C_{10} alkenes inhibit the oxidation of glucose, The C_5, C_6, and C_{10} alkanes also somewhat inhibited the oxidation of glucose; but octane, nonane, and 1-nonene stimulated respiration of glucose.

f. Oxidation of Hydrocarbon-Glucose Mixture by Cells Grown on TGY Broth Medium

With TGY-grown cells, except for hexane and C_6 - C_8 olefins, the added hydrocarbon did not appear to have any effect on glucose oxidation (Figure 27). TGY-grown cells are distinguished from fuel-grown cells by the susceptibility of the respiratory system of the latter to be inhibited by decane.

It was surprising that not much difference existed between TGY-grown cells and fuel-grown cells in regard to their ability to oxidize various hydrocarbons. Robinson[38] and Thijsse[39] and others have indicated that alkanes induced the formation of enzymes that were involved in the oxidation of alcohols, aldehydes, and fatty acids. Robinson also found that there was no oxidation of alkane if Pseudomonas was not grown on alkane. However, the organisms used in this study are obviously different. A unique character of these organisms is that they do not remain viable longer than 5 days on TGY medium, whereas they survive for years in Medium 1. The reason for the extreme sensitivity of these Pseudomonas to the TGY medium is not known.

g. Effect of 2-Hexene on the Oxidation of Fuel and Glucose

The three lethal short-chain hydrocarbons, 1-hexene, 1-heptene and 1-octene, were not oxidized by the cells and also inhibited the oxidation of jet fuel and glucose. That the site of unsaturation of these olefins was on the first carbon atom prompted the study to determine if the site of unsaturation was the determining factor.

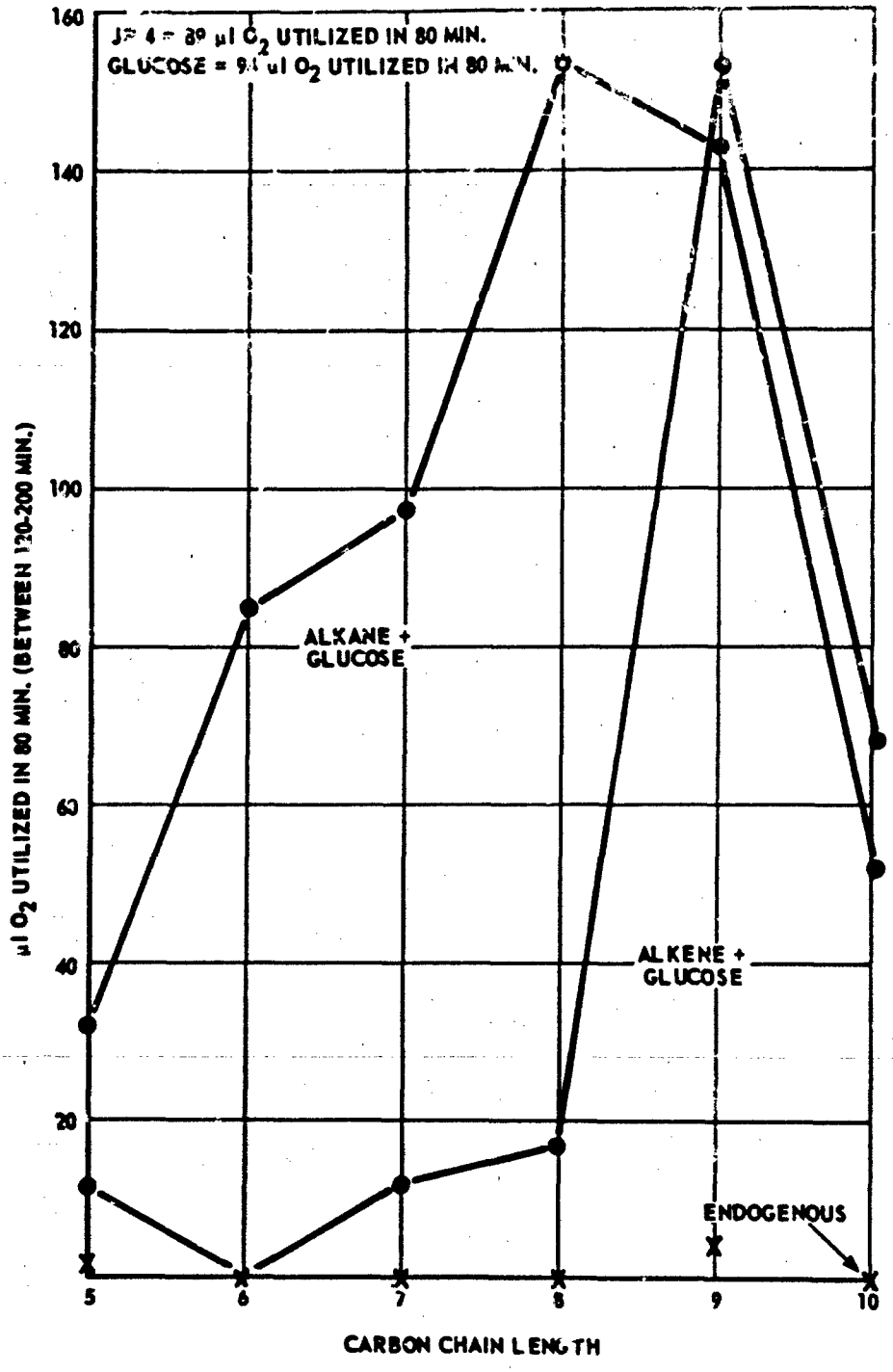


Figure 26. Oxidation of N-Alkanes and N-Alkenes in the Presence of Glucose by Washed Cells on Strain 101 Grown on Medium 2

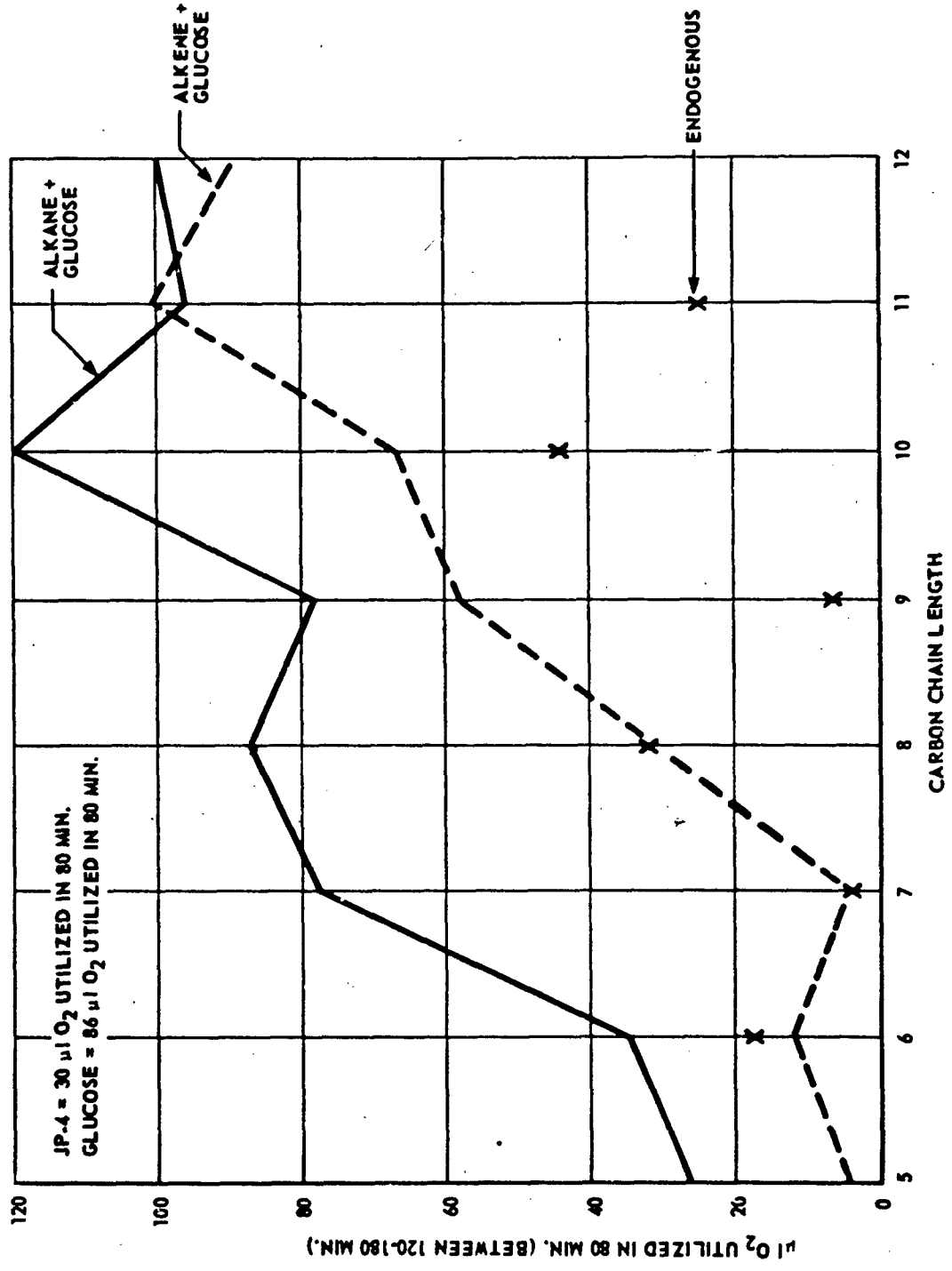


Figure 27. Oxidation of N-Alkanes and N-Alkenes in the Presence of Glucose by Washed Cells of Strain 101 Grown on TGY Medium

A typical profound effect of a lethal olefin with the unsaturation, other than on the first carbon atom, is shown in Figure 28. The inhibition of glucose (not shown) assumed essentially the same course as that shown for jet fuel oxidation. Thus the site of unsaturation of these lethal olefins does not determine their lethality.

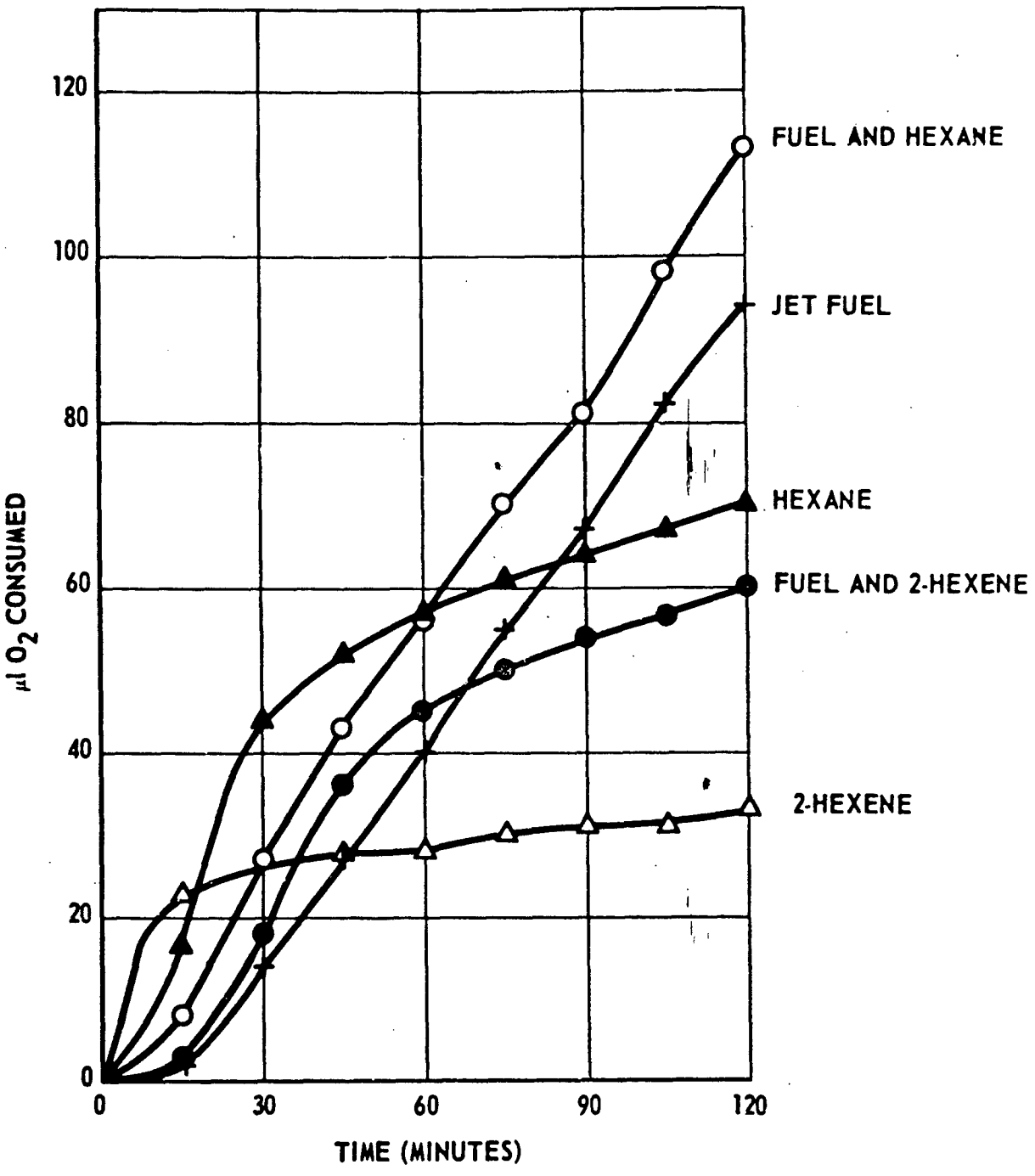
h. Effect of P-Xylene on the Oxidation of Fuel and Glucose

The parallel of lethality and respiration inhibition of the straight short-chain hydrocarbons was correlated with chain length and structure. The unsaturated ring structure, p-xylene, was demonstrated to be lethal to fuel isolates (see Table 38). Data shown in Figure 29 indicates that respiration of fuel isolates on both jet fuel and glucose is also profoundly affected by unsaturated ring structures such as p-xylene.

5. Oxidation of Jet Fuel, Hydrocarbons, and Glucose by Cell-Free Extracts and Inhibition by Lethal Olefins

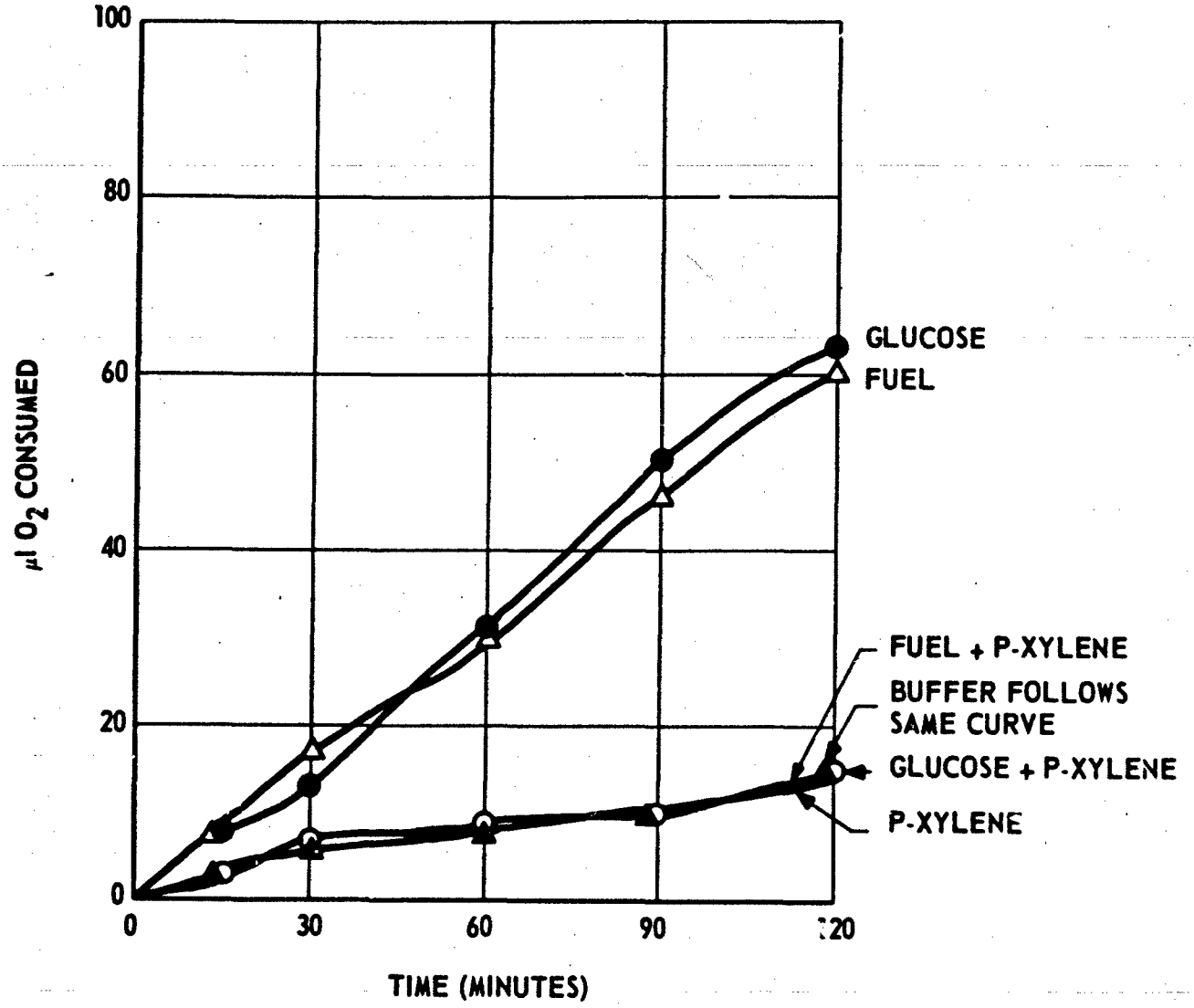
Examination of the growth responses and whole-cell respiration of jet fuel organisms in the presence of pure hydrocarbons showed a variety of responses depending on the chain length and saturation of the hydrocarbon molecule. The C_6 - C_9 olefins killed the organisms and also inhibited respiration by whole cells regardless of substrates used. Because these olefins were potential agents for controlling organisms in water bottoms, it was important to determine the mechanism by which the olefins caused the lethal effect. The first question asked was: do the olefins cause their effect by reaction with the cell wall or cell membrane or do they react with intracellular enzymes or coenzymes? Attempts to answer this question were made by using cell-free extracts of fuel isolates prepared by sonication.

Strain 101 was grown on TGY medium for 4-5 days at 37°C, washed 3 times with 0.05 M phosphate buffer pH 7.2, and suspended in 0.1 M phosphate buffer pH 7.2. The total volume was 40 ml, and the cell concentration was 1×10^{11} cells/ml. The cell suspension was placed in a glass-water jacketed sonication chamber and 1.2 mm glass beads were added to 1/4 the volume. The jacket was submerged in a salt-ice bath on a magnetic stirrer. The disruption of the cells was induced by the use of a Branson Sonifier. The 40 ml suspension was exposed to a sound field of approximately 75 acoustical watts for various time intervals. The temperature was maintained below 80°C. After sonication for 28 minutes, the count decreased from 1×10^{11} cells/ml to 1×10^{10} cells/ml, which indicates that 90% of the cells were broken. These homogenates were centrifuged for 20 minutes at 15,000 rpm rotor SS-34, in a Servall centrifuge. The cell-free extracts containing 6 mg protein/ml were kept frozen until used. Oxidative activity was demonstrated in the extracts using the Warburg respirometer.



LEGEND: FLASK CONTENTS: 1.0 ML CELLS (1.4 X 10^{10}/ML); 1.0 ML 10^{-2}M PHOSPHATE BUFFER pH 7.1; 0.5 ML FUEL OR GLUCOSE; 0.5 ML HEXANE OR 2-HEXENE OR BUFFER; CENTER WELL 0.2 ML 25% KOH. REACTION RUN AT 37°C.

Figure 28. The Effect of Hexene on the Oxidation of Jet Fuel Culture 101



LEGEND: FLASK CONTENTS: 1.0 ML CELLS (9.2×10^9/ML); 0.5 ML GLUCOSE OR FUEL; 1.0 ML 10^{-2} M PHOSPHATE BUFFER pH 7.1; 0.5 ML P-XYLENE OR BUFFER; CENTER WELL 0.2 ML 25% KOH. REACTION RUN AT 37°C.

Figure 29. The Effect of P-Xylene on the Oxidation of JP-4 Fuel and Glucose by Culture 101

Wicks soaked with 0.2 ml 20% KOH were used in all flasks to absorb CO_2. The thermobarometers contained buffer and substrate. All reaction mixtures contained substrate (appropriate quantities), 0.2 ml of 0.012 M DCPI, 0.5 ml olefin, and 0.5 ml of cell-free extract. The following quantities of substrates were used: 1.0 ml 1% glucose, 0.5 ml JP-4 fuel, and 0.5 ml alkane or alkene. In tests where olefins were used as respiration inhibitors of glucose, jet fuel, or alkane oxidation, they were added in a 0.1 ml aliquot. The reaction mixture volumes were adjusted to a total volume of 3.2 ml with 0.1 M phosphate buffer, pH 7.2. The Warburg flasks were allowed to equilibrate for 10 minutes at 25°C before mixing the cell-free extract (in the side arm) with the reaction mixture components. Oxygen uptake was followed for a period of 240 minutes. Good respiratory activity was observed with these extracts. After correcting for endogenous respiration and thermobarometer, oxygen uptake was as follows: glucose, 500 µl; octane, 150 µl; and JP-4 fuel, 70 µl.

a. The Effect of C_6 - C_9 Olefins on Fuel Oxidation

The ability of C_6 - C_9 olefins to inhibit respiration by cell-free extracts on JP-4 fuel is shown in Figures 30 and 31. Hexene was the most effective inhibitor of JP-4 oxidation, but nonene also showed good activity. Heptene and octene were less effective inhibitors. This differs from the effect observed with whole cells grown on TGY where the C_6 - C_9 olefins inhibited respiration completely (see Figure 25). It is interesting to note that as in the case with whole cells, olefins not only inhibit oxidation of jet fuel but also inhibit endogenous respiration.

b. The Effect of C_6 - C_{11} Alkenes and Octane on Glucose Oxidation

The effect of olefins on the oxidation of glucose by cell-free extracts was about the same as with jet fuel except that oxygen uptake with glucose was about twice that observed with JP-4 fuel (Figures 32, 33, and 34).

Decene also inhibited respiration, but undecene had little or no effect. (Figure 35).

In general, the effects observed with the olefins mixed with glucose were the same as those observed with whole cells. Thus, good correlation exists between response of extracts and response of whole cells exposed to the "lethal" olefins.

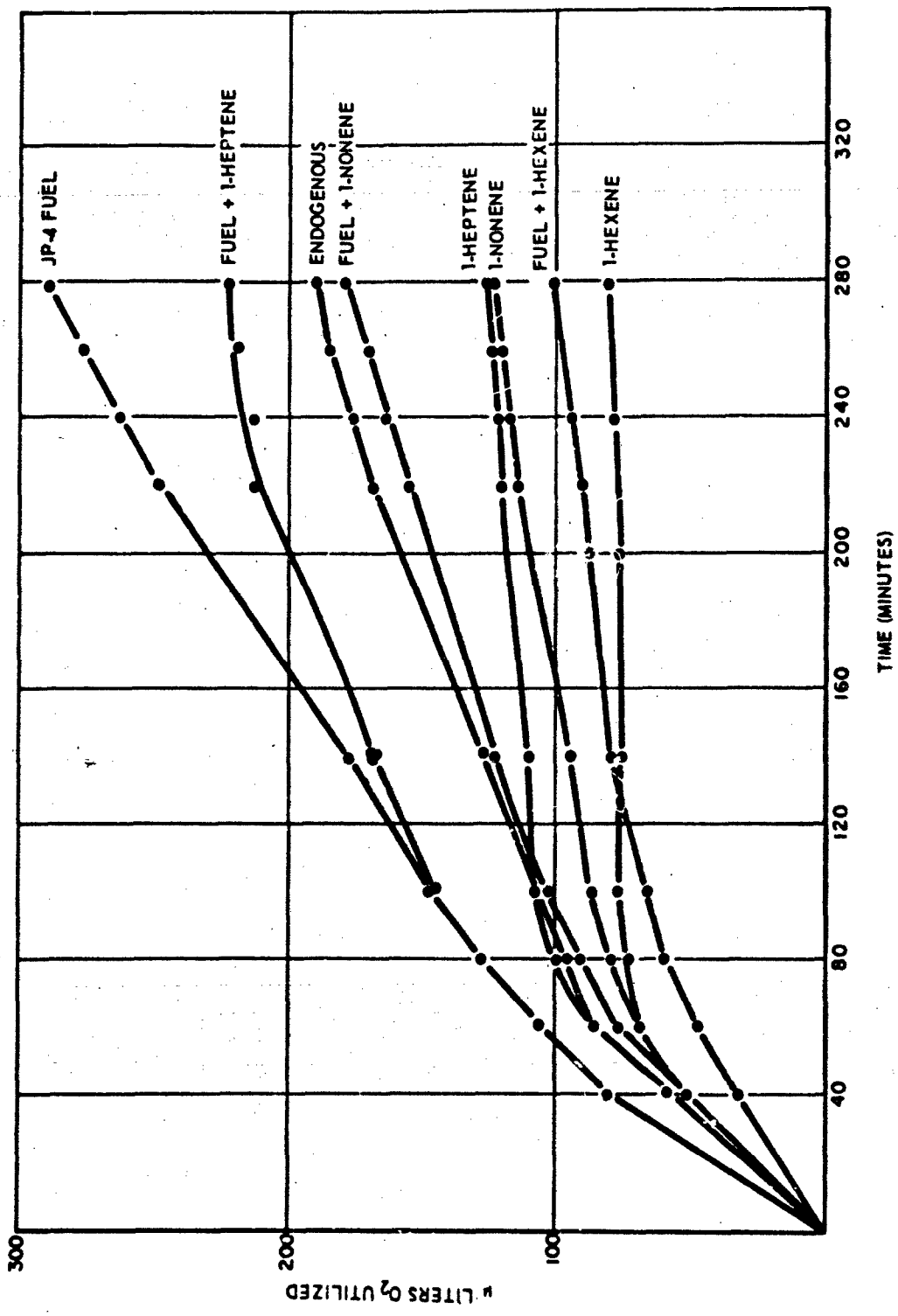


Figure 30. Effect of 1-Hexene, 1-Heptene and 1-Nonene on JP-4 Fuel Oxidation by Cell-Free Extracts of Strain 101

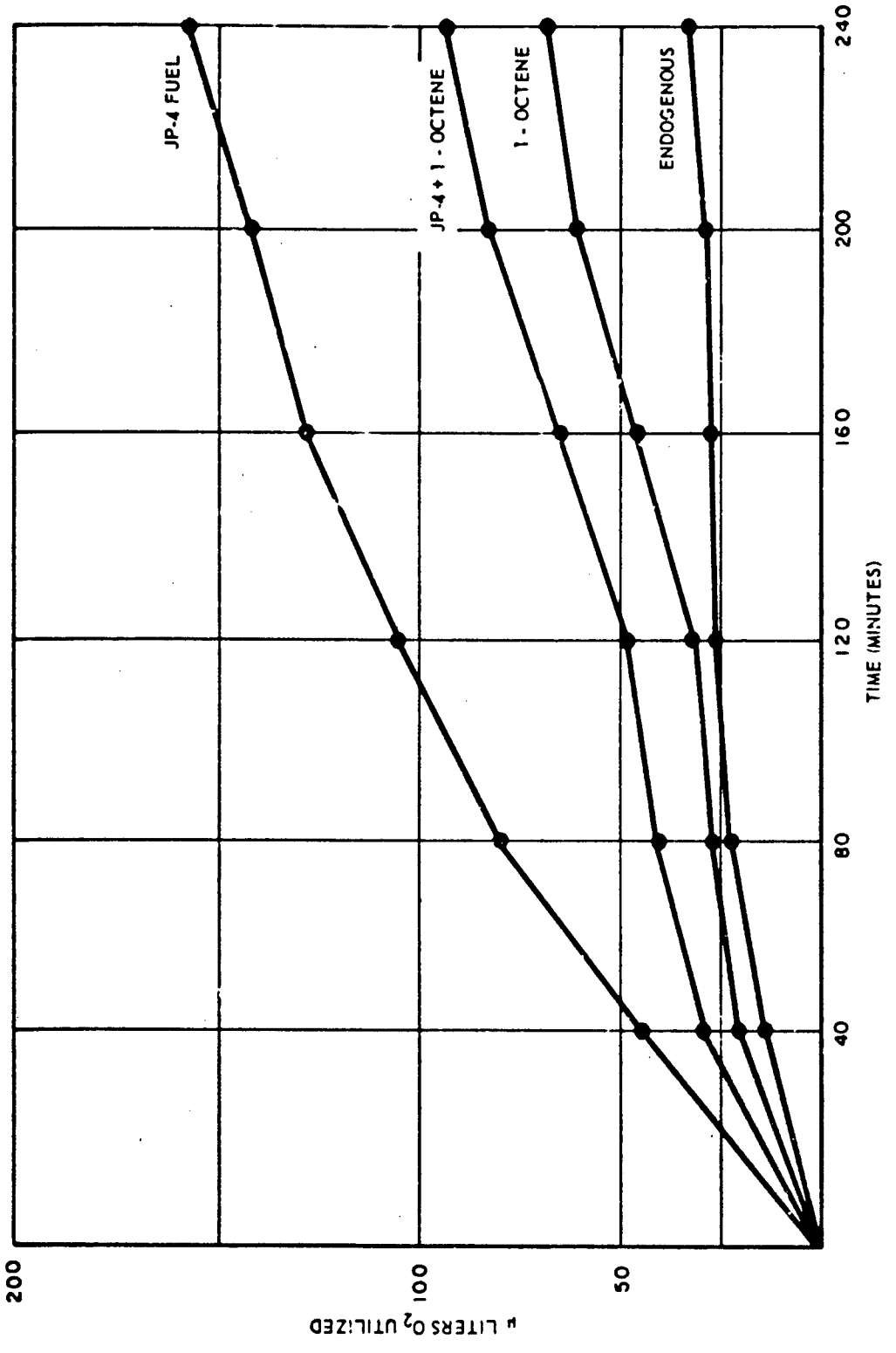


Figure 31. Effect of 1-Octene on JP-4 Fuel Oxidation by Cell-Free Extracts of Strain 101

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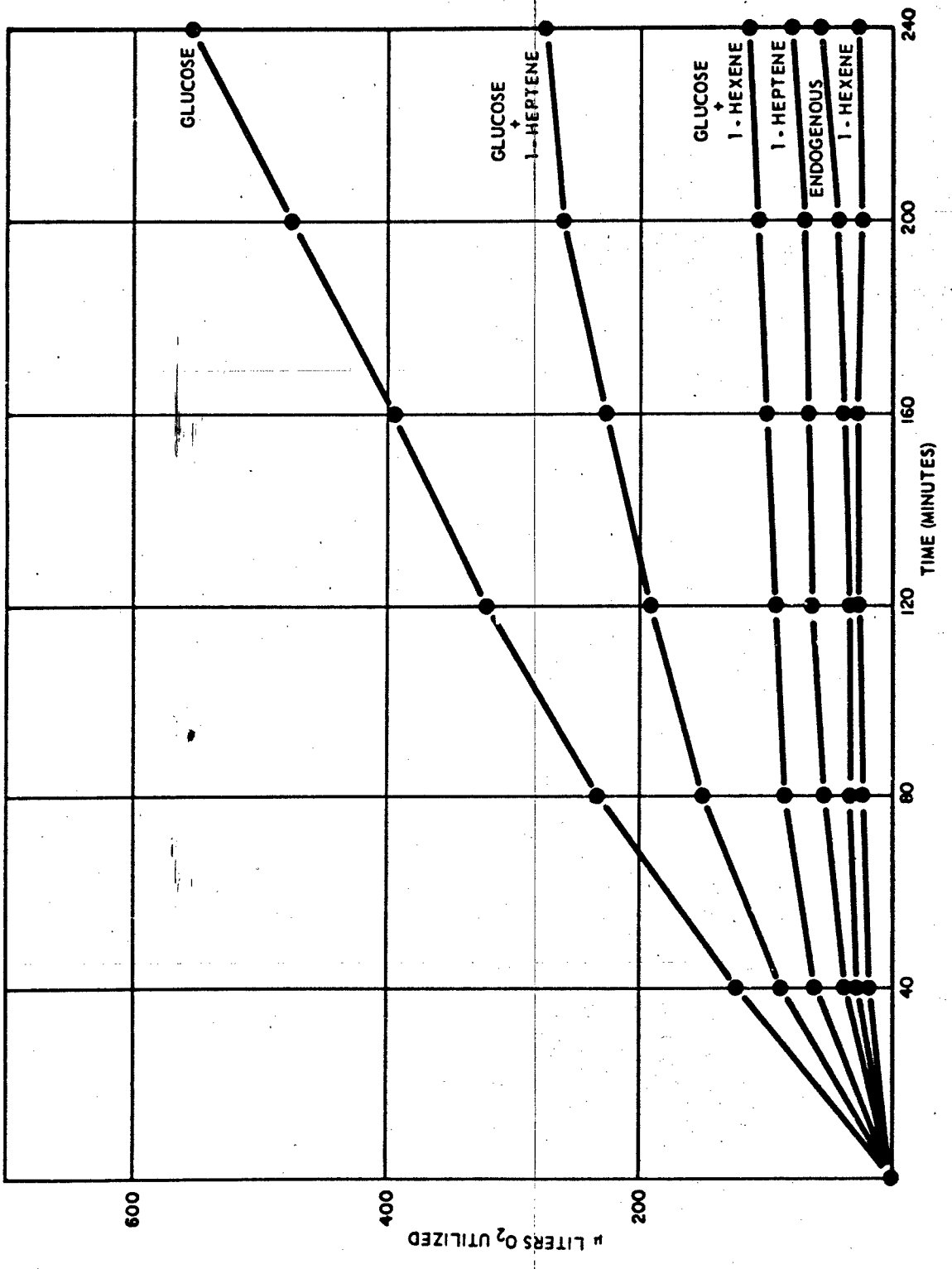


Figure 32. Effect of 1-Hexene and 1-Heptene on Glucose Oxidation by Cell-Free Extracts of Strain 101

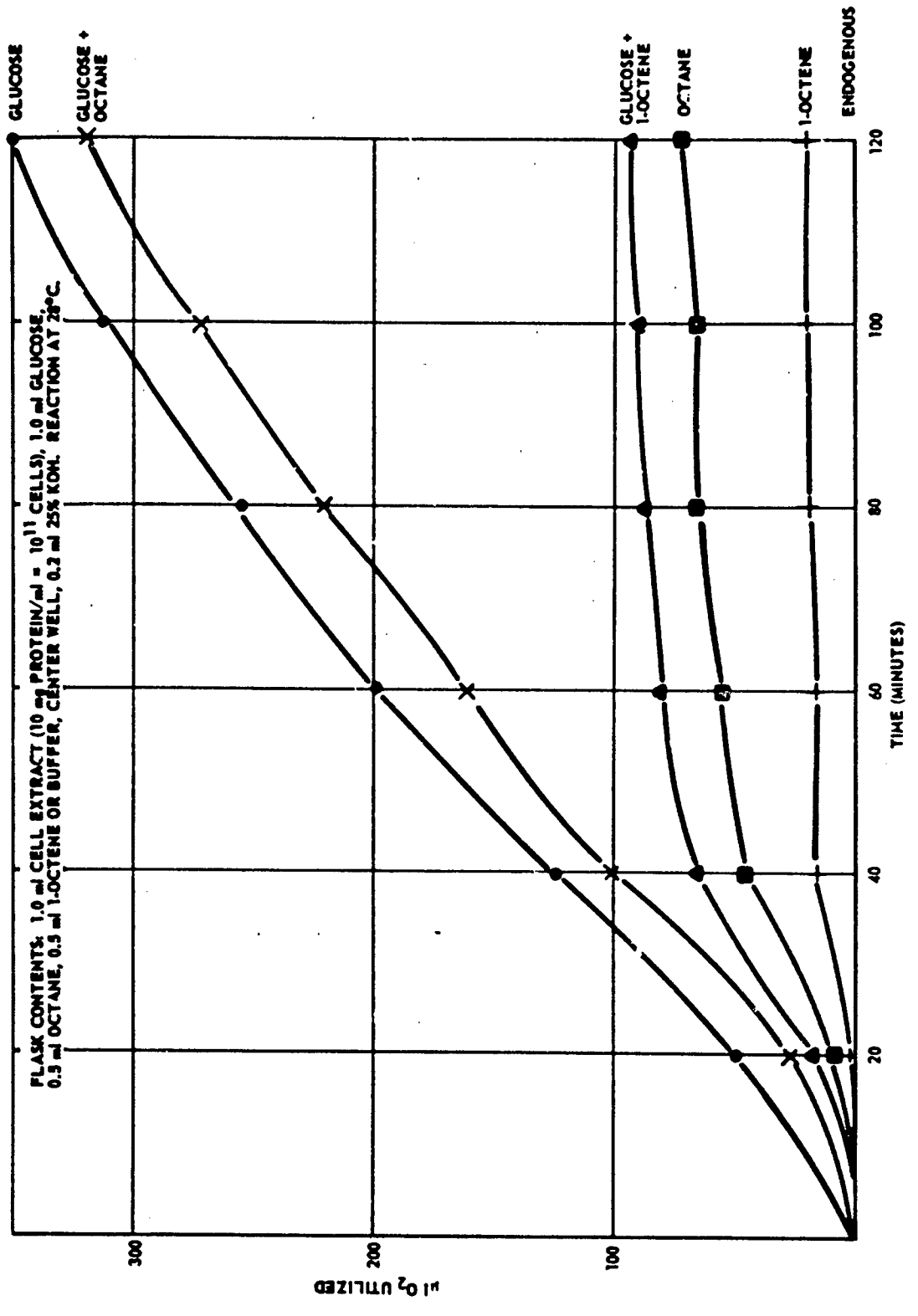


Figure 33. The Effect of Octane and 1-Octene on the Oxidation of Glucose by Cell-Free Extracts of Strain 101

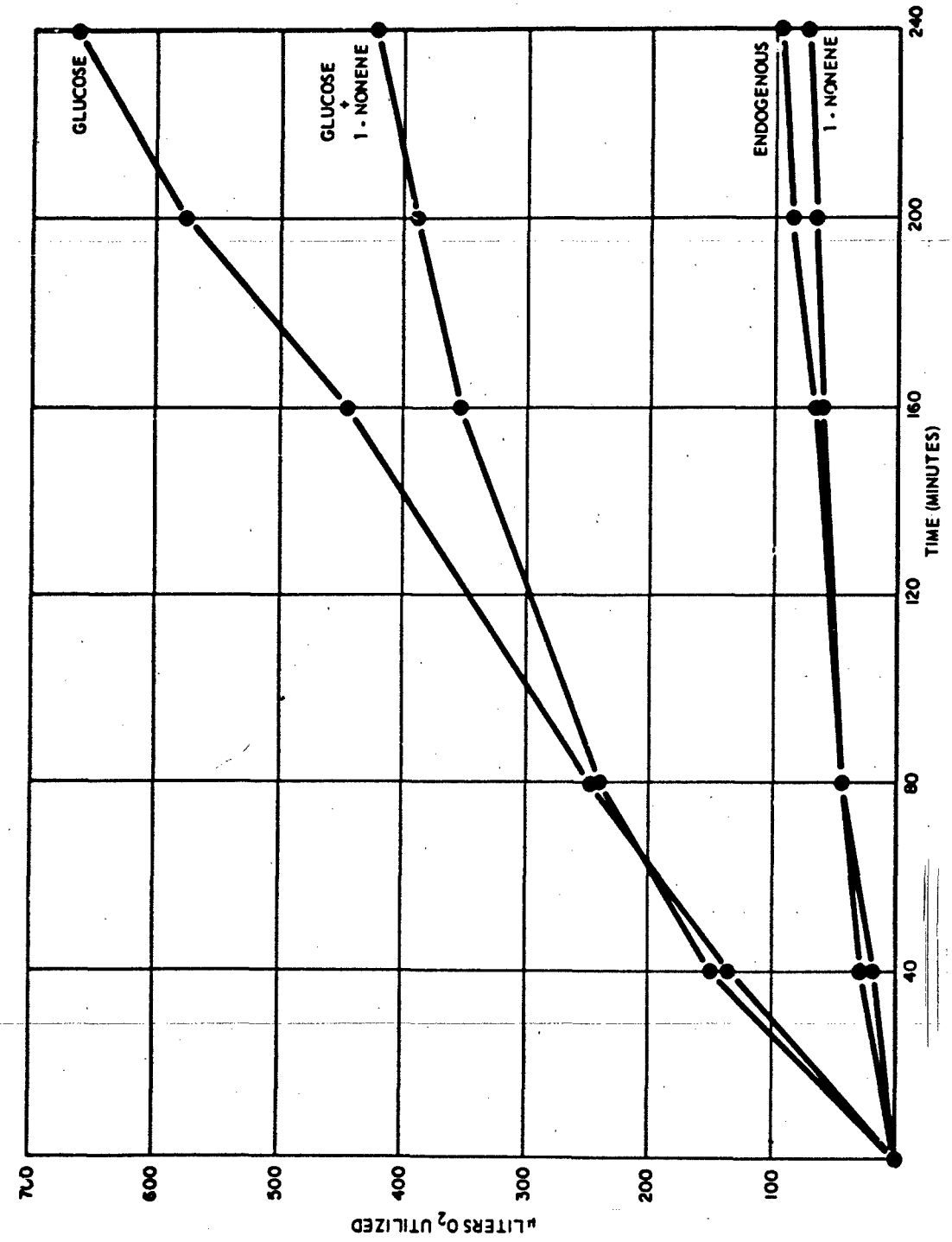


Figure 34. Effect of 1-Nonene on Glucose Oxidation by Cell-Free Extracts of Strain 101

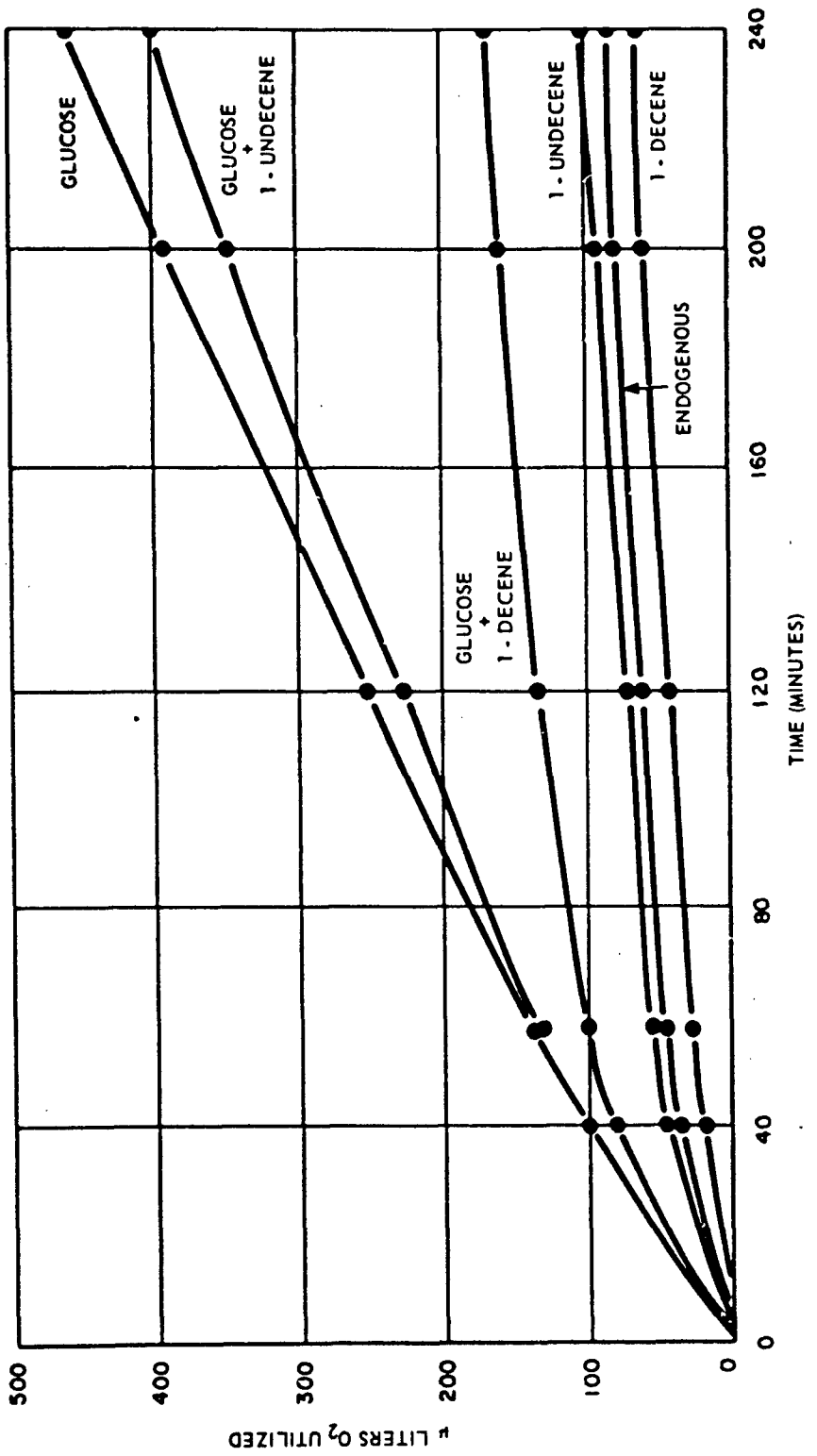


Figure 35. Effect of 1-Decene and 1-Undecene on Glucose Oxidation by Cell-Free Extracts of Strain 101

c. The Effect of C_6 - C_{11} Olefins on Octane Oxidation

The effect of olefins on the oxidation of octane was investigated because octane was readily oxidized by whole cells (Figures 22 and 23) and cell-free extracts. It was also believed that the study of olefin inhibition of the oxidation of an alkane would lead to a better understanding of the mechanism of olefin inhibition and the mechanism of hydrocarbon oxidation by the jet fuel microorganisms. The results in Figure 36, 37, and 38 show that hexene markedly inhibits respiration, while heptene, octene, decene, and undecene cause much less inhibition.

6. Dehydrogenase Activity of Cell-Free Extracts and Purified Enzymes and Inhibition by Lethal Olefins

The inhibition of respiration by C_6 - C_9 olefins with glucose and jet fuel as substrates could be due to inhibition of enzymes (dehydrogenases and oxidases) directly involved in the oxidation of the hydrocarbon molecules or to inhibition of some intermediate step in oxidation or to inhibition of terminal oxidation.

Dehydrogenase activity was followed by measuring the increase in concentration of reduced DPN at 340 mu. The reaction mixture and conditions used are described in Figures 39 thru 43. Jet fuel readily serves as substrate for DPN reduction, and hexene inhibits this reduction (Figure 39.) Greater inhibition is observed if cell-free extract and hexene are incubated together before assay. Similar results were observed with octane (Figure 40). Also the ability of hexene to inhibit octane dehydrogenase appears to be dependent upon hexene concentration in the reaction mixture; inhibition was nearly doubled by increasing the volume of hexene in the 3.0-ml reaction mixture from 0.1 to 0.5 ml.

Hexene causes marked inhibition of DPN reduction with glucose as substrate (Figure 41). Also reaction with dehydrogenases was rapid, since 2 hours of incubation of cell-free extract and hexene caused no increase in olefin inhibition. The reasons for the more rapid reaction and greater inhibition by olefins of dehydrogenases, involved in sugar metabolism, are not known.

Lethal olefins also markedly inhibited alcohol degydrogenase in cell-free extracts (Figure 42). In this case heptene was used as the inhibitor, and nearly complete inhibition was obtained. The reason for the 6-minute lag before DPN reduction was believed to be due to the DPNH oxidase activity of the cell-free extract. To confirm this, the DPNH oxidase of the cell-free extract was tested. The rate of decrease in absorption at 340 mu as DPNH was oxidized was measured. The reaction mixture contained 0.1 ml octane, 0.1 ml DPNH (0.002 M), 2 - 7 ml phosphate buffer (0.03 M, pH 7.4) and 0.1 ml enzyme. Change in absorption is recorded every 30 seconds for 3 minutes. When this was performed with undiluted cell-free extract, the activity was too great to read: the DPNH was completely

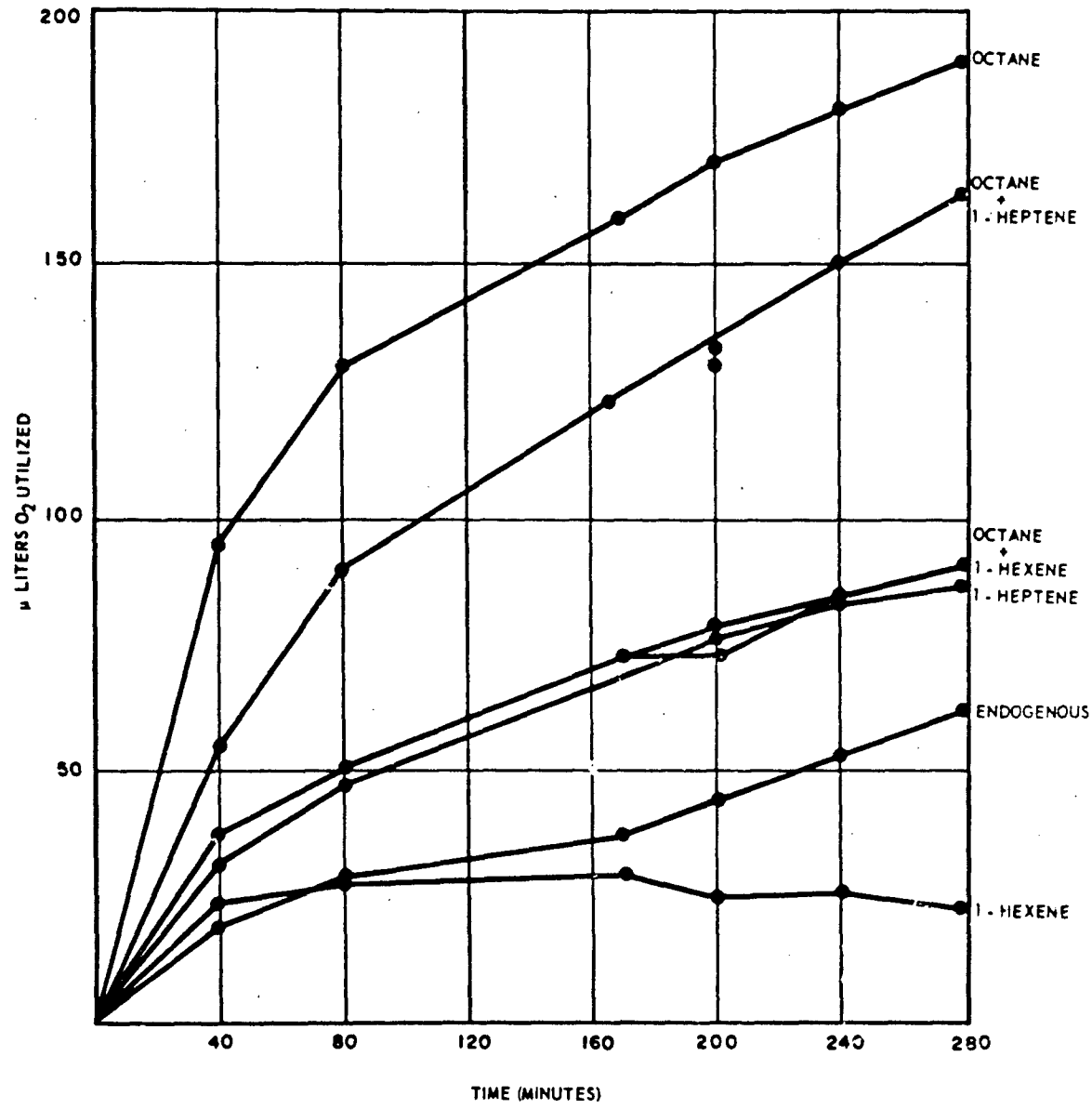


Figure 36. Effect of 1-Hexene and 1-Heptene on Octane Oxidation by Cell-Free Extracts of Strain 101

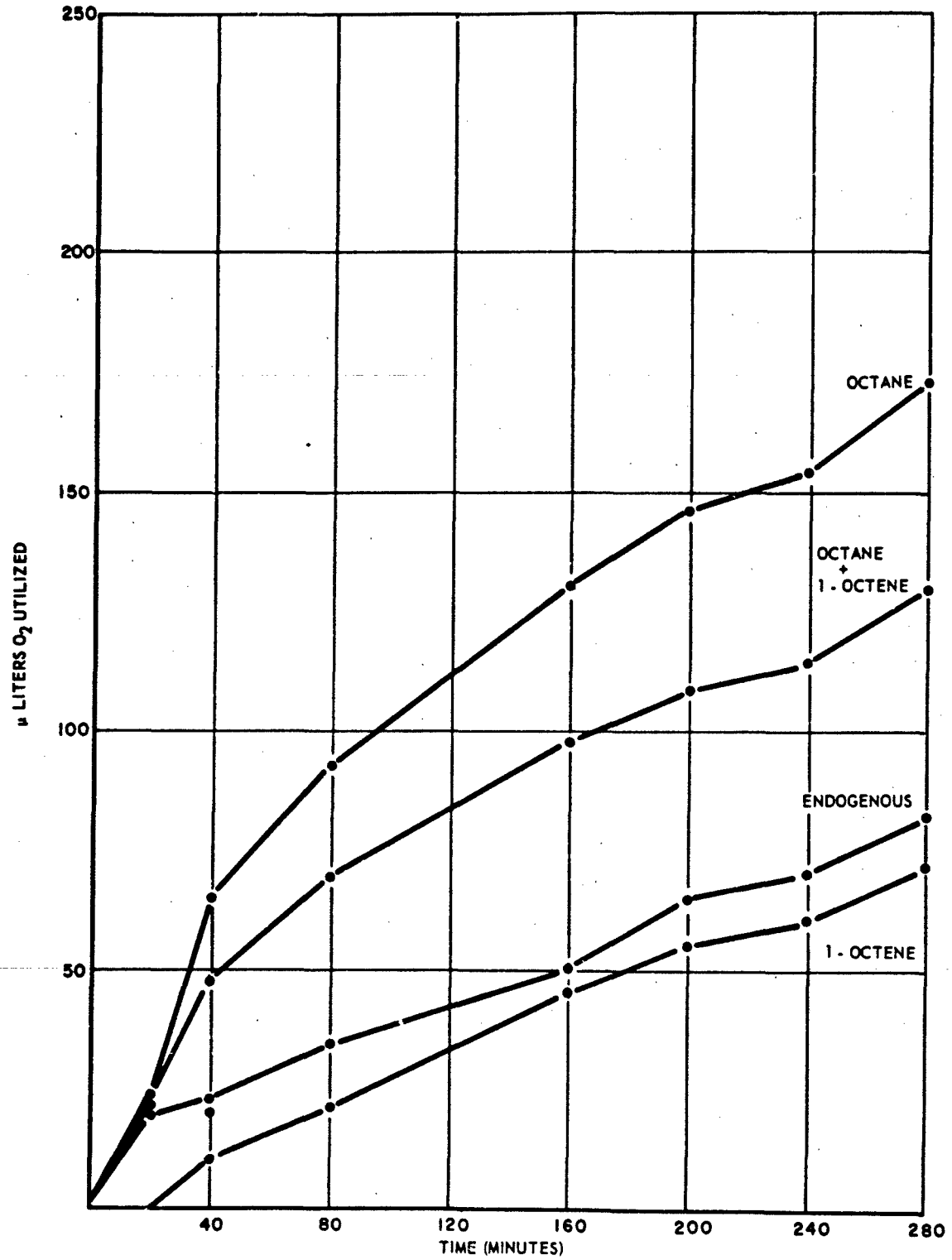


Figure 37. Effect of 1-Octene on Octane Oxidation by Cell-Free Extracts of Strain 101

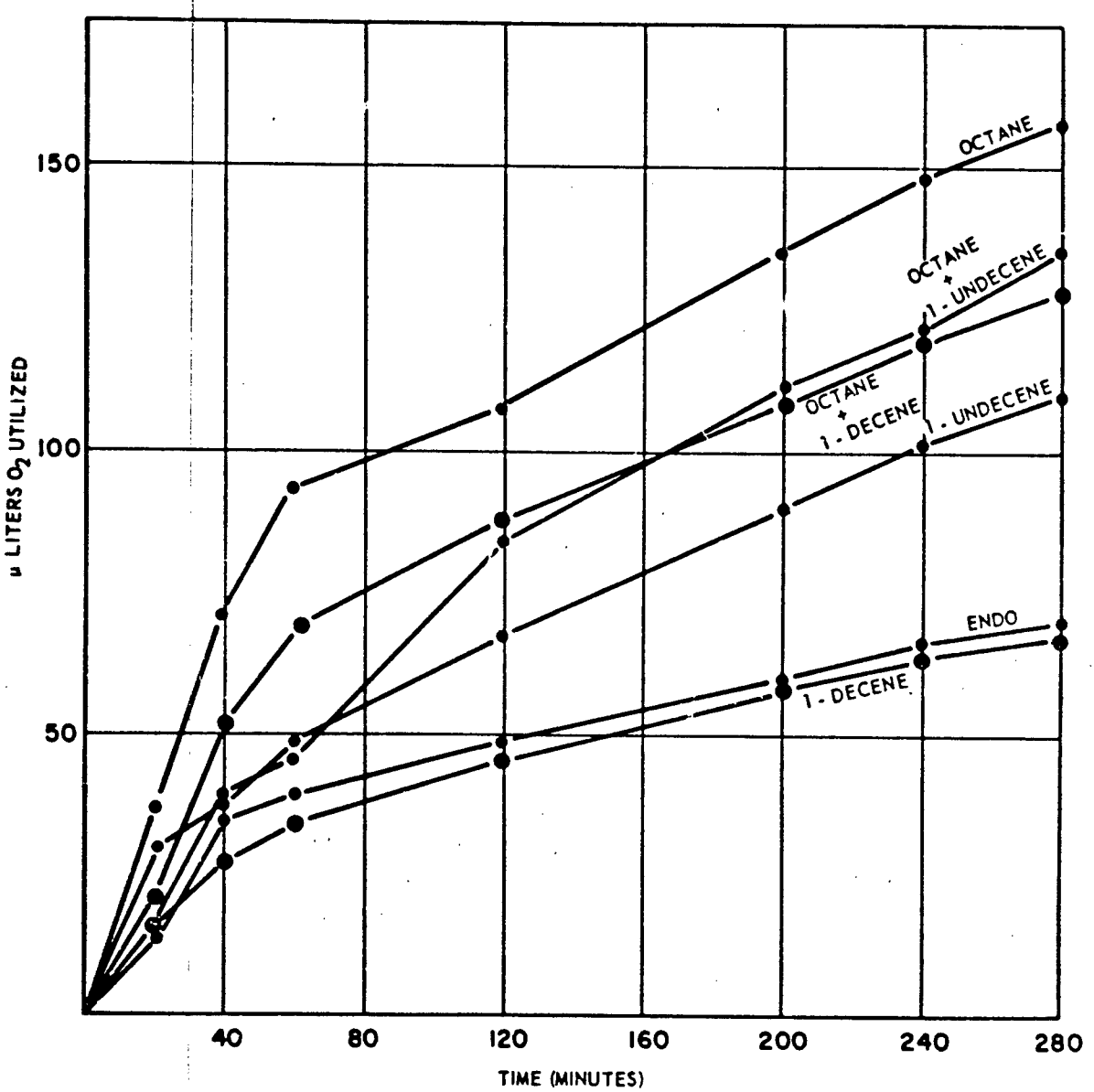


Figure 38. Effect of 1-Decene and 1-Undecene on Octane Oxidation by Cell-Free Extracts of Strain 101

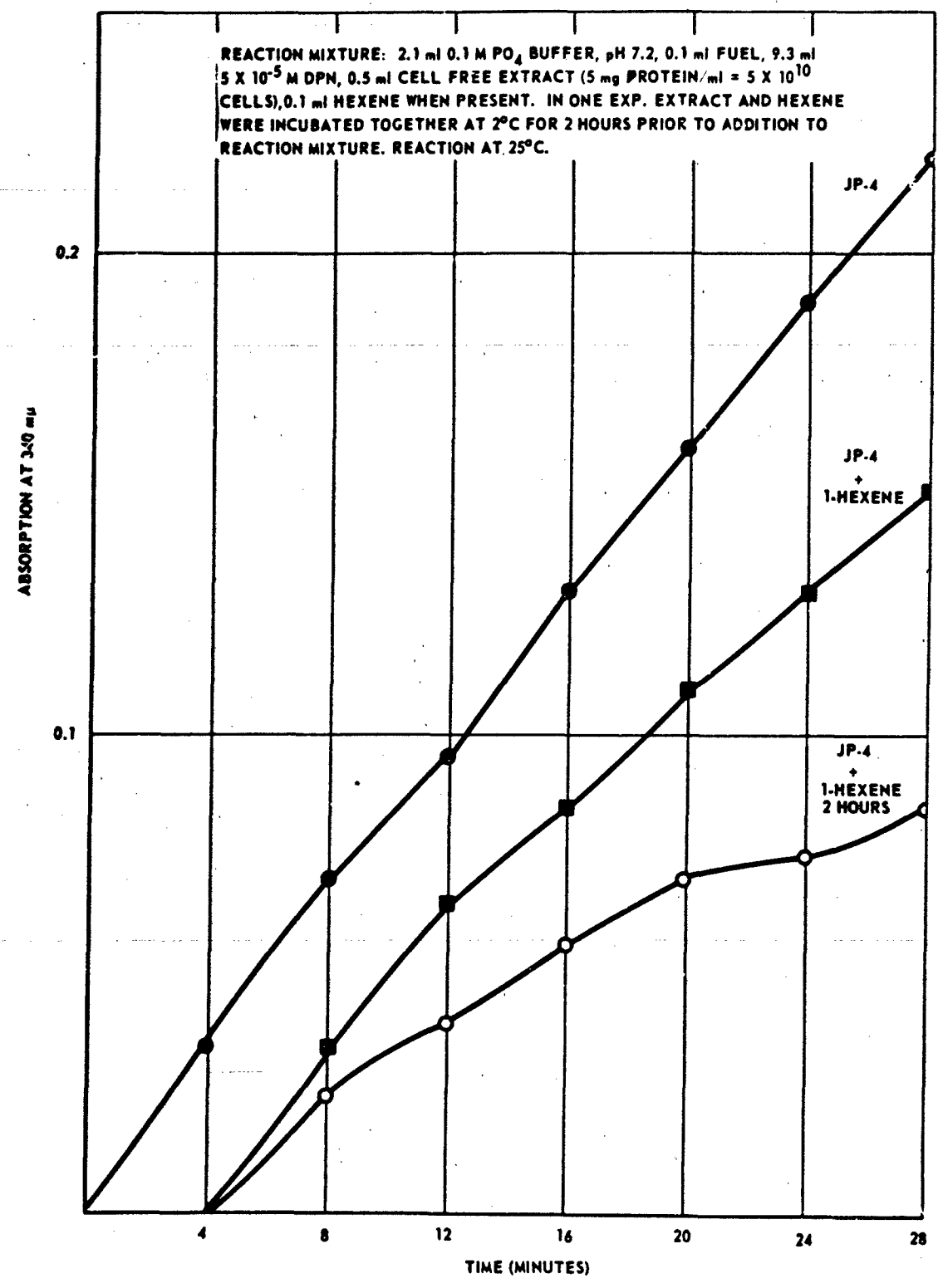


Figure 39. The Effect of 1-Hexene on DPN Reduction by Cell-Free Extracts of Strain 101

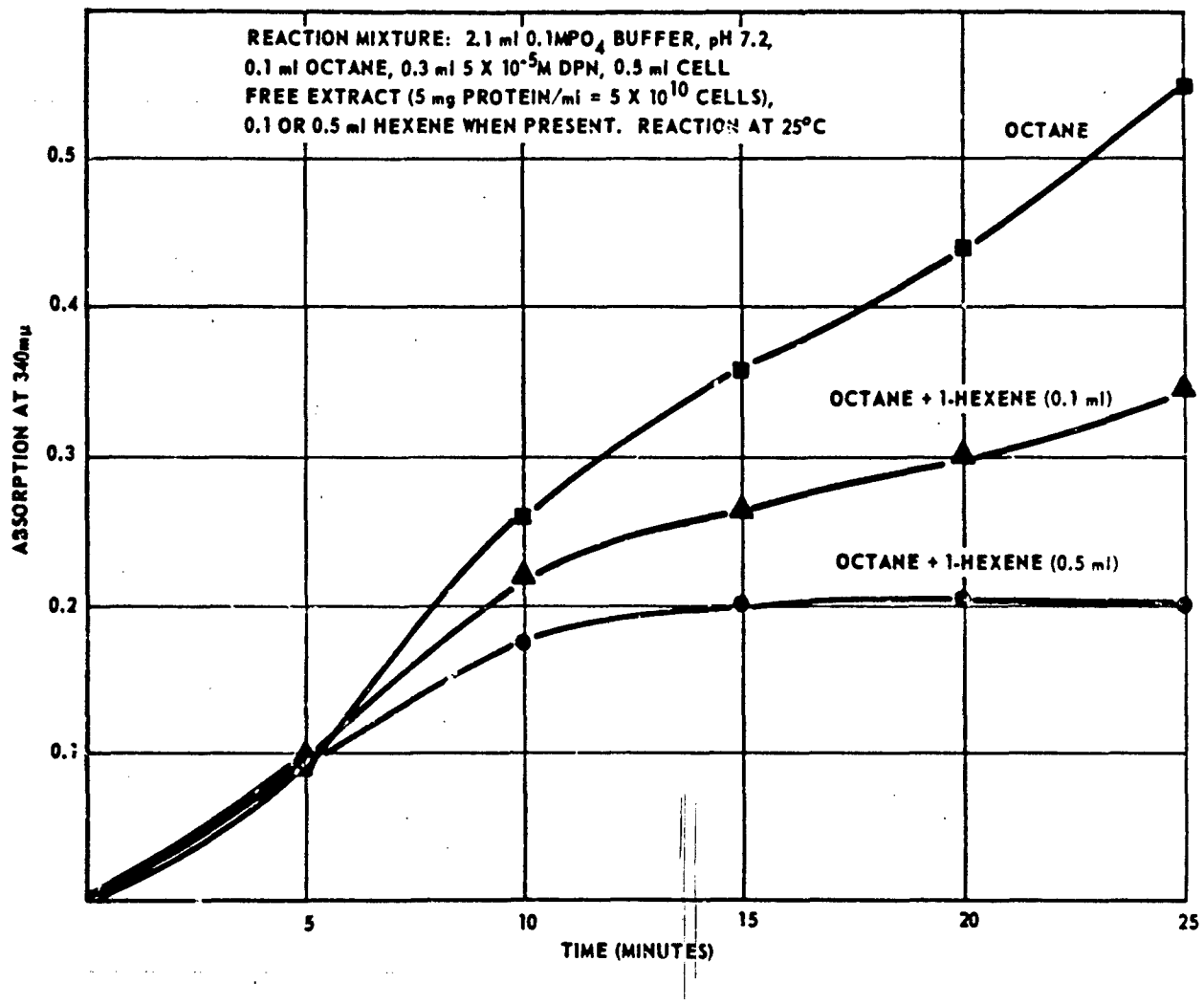


Figure 40. The Effect of 1-Hexene on DPN Reduction by Cell-Free Extracts of Strain 101 Using Octane as Substrate

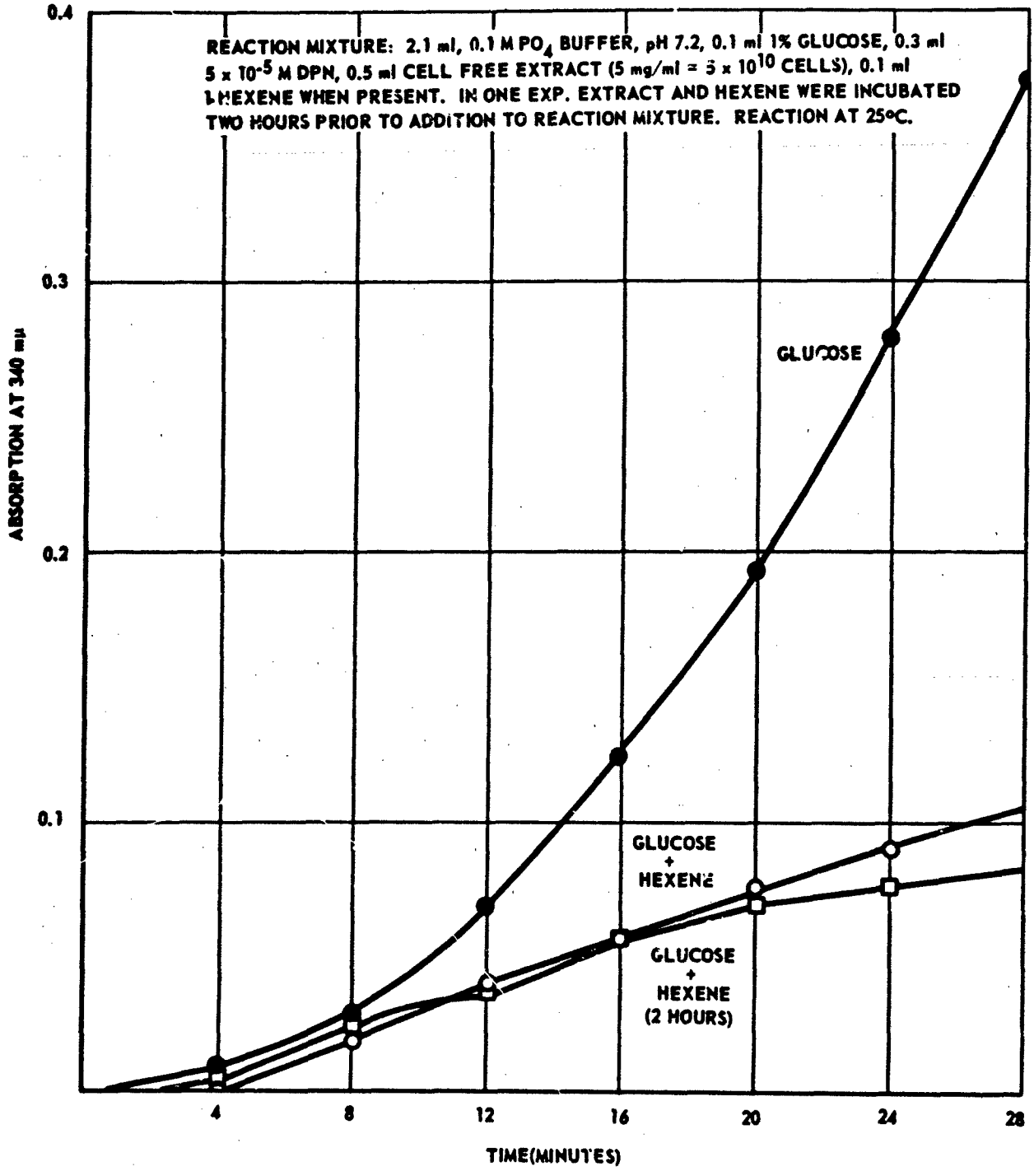


Figure 41. The Effect of 1-Hexene on DPN Reduction by Cell-Free Extracts of Strain 101 Using Glucose as Substrate

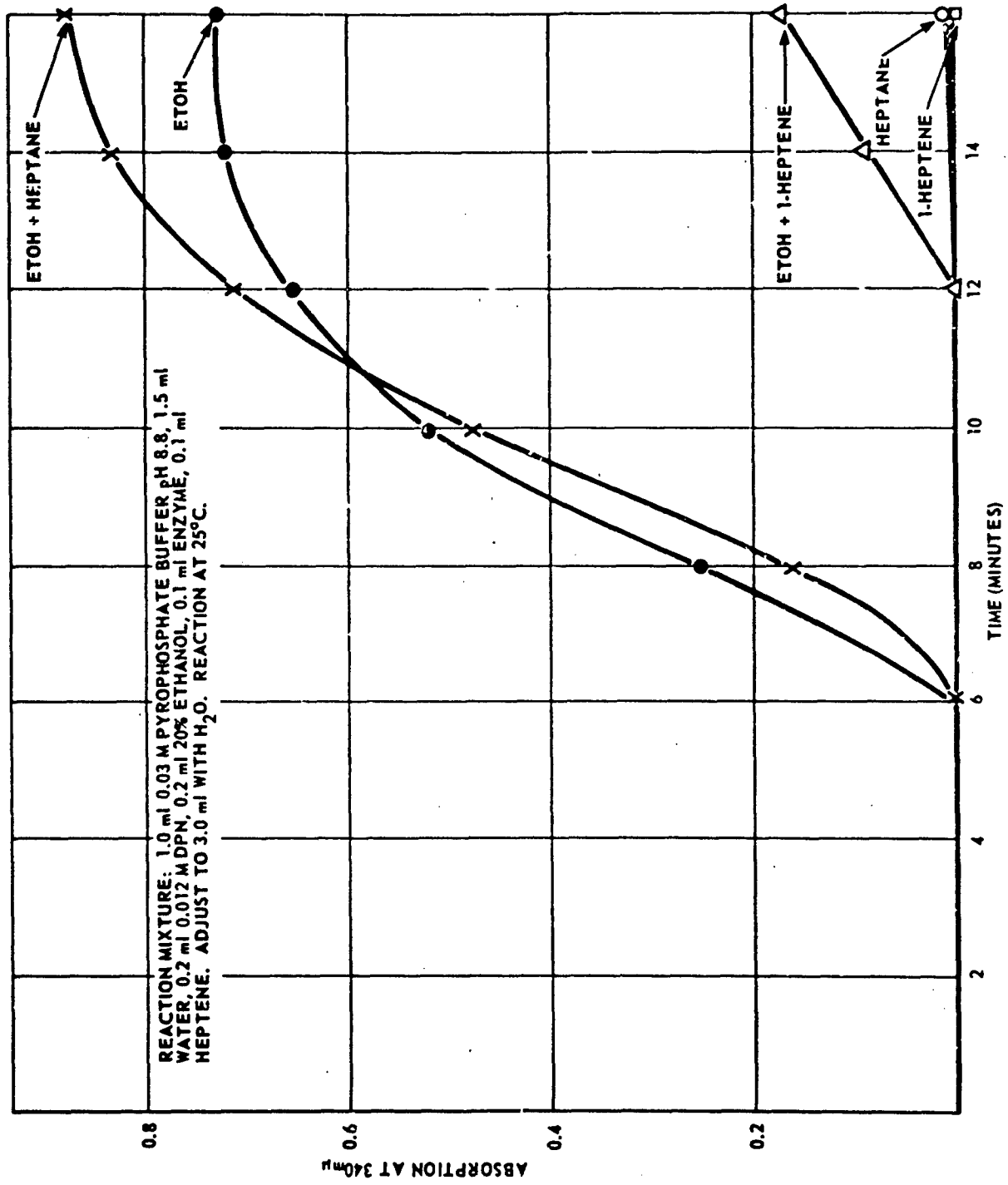


Figure 42. Effect of Heptane and 1-Heptene on DPN Reduction by Cell-Free Extracts of Strain 10[illegible]

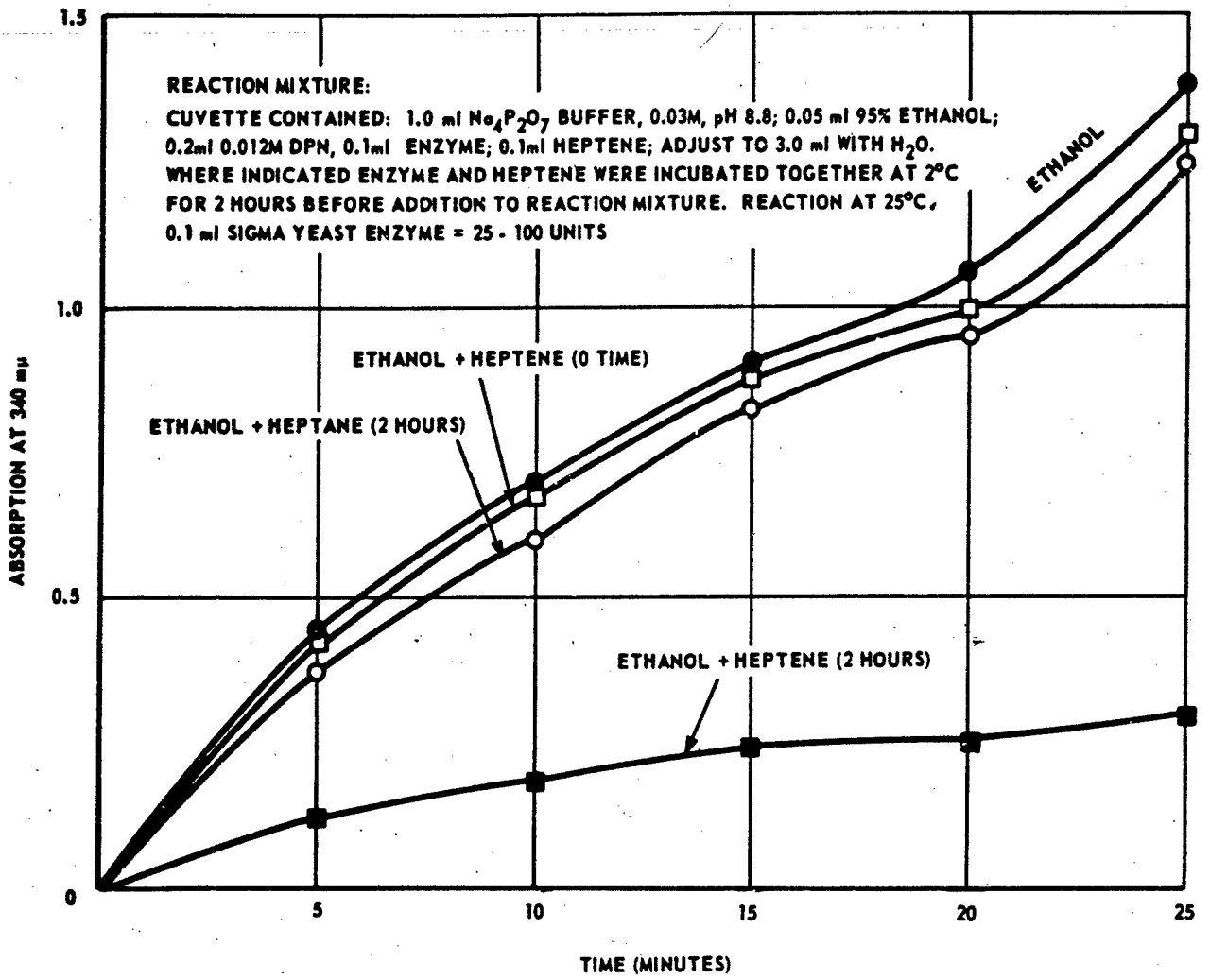


Figure 43. Effect of 1-Heptene on Alcohol Dehydrogenase (Purified)

oxidized within the first 30 seconds. The extract had to be diluted by 100 fold before the rate could be followed. Therefore, the highly active DPNH oxidase in the cell-free extract, is apparently responsible for the lag observed in Figure 42.

The next step was to determine the effect of heptene on purified yeast alcohol dehydrogenase. In this case heptene did not inhibit alcohol dehydrogenase when added at zero time, but did inhibit when the enzyme and heptene were incubated together for 2 hours prior to addition of substrate (Figure 43). Thus it appears that the inhibition of dehydrogenases by C_6 - C_9 olefins is one of the reasons for their lethal effect and for their inhibition of respiration. However, because the C_6 - C_9 olefins also inhibit endogenous respiration, it is questionable that the dehydrogenase sites are the only sites involved; inhibition may be occurring at other sites in the terminal oxidation pathway.

E. Jet Fuel Contamination by Metabolic Products Formed by Microorganisms

1. Emulsions

Both pure strains and mixed cultures emulsify jet fuel media containing nitrate or ammonium. The time required for emulsification depends both on the medium and on the inoculum. On Medium 2, strain 96 emulsifies within 48 hours, while strain 101 emulsifies after a longer period of growth. Time required for emulsification of Medium 2 provides presumptive evidence for distinguishing strain 96 from strain 101. On Medium 6, both strains begin to emulsify within 48 hours. Initially, both strains are distributed homogeneously in the aqueous phase. After 24 hours, some of the organisms concentrate at the interface and begin to form an emulsion there. Within 48 hours, the emulsified bacterial mass progressively penetrates the fuel phase but remains near the interface. When 48-hour cultures on Medium 6 are centrifuged at 29,000 x g for 30 minutes, four fractions are recovered: JP-4, floated cells at the interfaces, aqueous phase, and a pellet containing sedimented cells. Although the proportion of floated cells increases with the age of the culture, the floated and sedimented cells have essentially the same viability and respiratory activity.

Investigations have been conducted on the effect of centrifugation on the emulsions produced by 7-day cultures of strain 101 grown on Medium 6. A sample of the emulsion was centrifuged at 50,000 x g for 15 minutes; four fractions were recovered as above. The top three fractions were removed by pipette, the pellet was washed by centrifugation with distilled water in a clinical centrifuge, and the wash removed with a pipette. Fresh Medium 6 was added to the washed cells, and the suspension was stirred vigorously, shaken, and pipetted back and forth several times to break the pellet. A soapy emulsion was formed almost immediately at the interface. Upon recentrifugation at 50,000 x g for 15 minutes, most of the cells were at the interface, and the pellet was very small.

Forty-eight-hour cultures of strain 96, grown on Medium 2 and centrifuged at 29,000 x g to separate, floated from sedimented cells. The sedimented and floated cells were separately dried, then extracted with chloroform-methanol (1:1); the extracts were dried and redissolved in n-hexane. These extracts, probably lipid, were transesterified with 10% HCl in methanol at 100°C for 3 hours. After cooling, 2,2-dimethoxypropane was added to the scavenge water, and the resulting extract was analyzed in a Beckman GC-2 hydrogen flame gas chromatograph. The lipids of floated and sedimented cells were qualitatively similar: methyl esters having equivalent chain lengths of 16 to 24. Quantitatively, however, floated and sedimented cells showed significant differences. Floated cells contained 34% lipid by weight, whereas sedimented cells contained only 5.4% lipid. Lipid content was determined by evaporating the chloroform-methanol extract to dryness; the weight of the dry residue, divided by the weight of the dried cells, gave the percent lipid.

a. Fractionation of 90-Day Growth Media and Characterization of the Fractions

When Medium 6 is inoculated with strain 101, the organism oxidizes the jet fuel and produces a number of compounds; some are corrosive, some may inhibit corrosion, and some emulsify the JP-4 fuel.

A number of attempts have been made to separate or fractionate the materials responsible for aluminum corrosion and emulsifying activity by chemical means, ion exchange chromatography, paper chromatography, and gel filtration.

Gel filtration has proved to be the best technique for learning about these compounds because this process sorts out a complex mixture by gross molecular weights without introducing any extraneous factors, such as precipitated salts or acid and base hydrolysates.

Using Sephadex G-25-fine from Pharmacia Co. as the gel and 3-month-old clarified cultures, known to be corrosive or to emulsify jet fuel, as the sample material to be passed through that gel, two facts were immediately discovered: (1) the highly pigmented material had nothing to do with aluminum corrosion but did contain the emulsion forming compound(s) and (2) the compound(s) responsible for aluminum corrosion were excluded from the G-25-fine Sephadex and were colorless.

An effort was made to identify the emulsifying compound(s) by solubility classification tests and qualitative organic reactions.

The brown compounds in Sephadex fractions responsible for emulsifying JP-4 fuel are assumed to have low molecular weights because they are the last compounds to be eluted before the medium salts. These compounds are alkali soluble and acid insoluble, but formed good emulsions at either pH extreme. If these brown fractions were acidified with HCl, a deep brown flocculant precipitate formed which was collected by centrifugation. This precipitate was then washed with distilled water and tested for emulsifying activity. The precipitate was insoluble in ether and 10% $NaHCO_3$ and was therefore according to the classification scheme of Shriner and Fuson.[36]

The precipitate was an A_2 compound--a weak acid. Some qualitative organic reactions for weak acid -A_2 compounds were run on this precipitate. The following is a list of the positive reactions which the precipitate gave:

(1) Bromine water--A test for unsaturated compounds. The precipitate in this reaction chased away the bromine color without any evolution of hydrogen bromide indicating some degree of unsaturation.

(2) Molisch's Naphthol--The precipitate gives a positive reaction with this test indicating the presence of a carbohydrate radical.

(3) Periodic acid test--The precipitate was positive with this test and indicates a 1,2-glycol, α-hydroxy aldehyde or ketone, 1-2-diketone, or a α-hydroxy acid group present in this compound(s).

All other reactions suggested by the above reference for weak acid compounds were not definitely positive or negative, which indicates that interfering functional groups were co-precipitated with the emulsion-forming compound.

Further solubility studies indicate that the emulsion-forming precipitate was soluble in methyl alcohol.

Upon heating and evaporating the methanol in hopes of crystalizing the acid-insoluble brown precipitate, it was discovered that a new light brown or white precipitate formed. When this precipitate was collected, the methanol was still dark brown, yet nothing else came out of the solution. This methanol insoluble compound was tested for emulsifying activity with jet fuel. The compound gave a good positive reaction. This precipitate was then washed with ethyl ether, water, and then ethyl alcohol, none of which dissolves this compound.

Attempts are presently being made to identify this compound by infrared spectrophotometry.

b. Emulsion Formation by Strain 101 on Purified Hyrdocarbons

The objective of these experiments were to demonstrate that pure hydrocarbons could be metabolized by strain 101 to yield emulsion-forming compounds.

The results in Table 44 show that in 10 days a very good emulsion was produced on 1-hexene, nonane, 1-nonene, 1-decene, 1-undecene, tetradecane, and JP-4 fuel. A fair emulsion was produced on dodecane. It is interesting to note that good emulsion is produced on 1-hexene yet this olefin is lethal to strain 101 in 5 hours. Also, there was no emulsion produced on octane, decane, 1-dodecene, undecane, tridecane and 1-tetradecene, yet these hydrocarbons support growth of strain 101. It is particularly interesting that there is a selectivity by the organisms for media which support growth and emulsion formation.

An experiment was performed to determine if these 10-day-old, purified hydrocarbon, overlay cultures contained substances which would cause emulsion of JP-4 fuel. From each of the cultures listed in Table 44, 5 ml of medium-cell suspension and 1 ml of the hydrocarbon overlay were withdrawn, and 5 ml of JP-4 fuel were added. Control tubes were included which contained sterile media, hydrocarbon, and JP-4 fuel. All the tubes were shaken vigorously, and observation for emulsion of the JP-4 fuel was made. There was no emulsion of the JP-4 fuel in any of the control tubes. Four of the cultures, nonane, 1-nonene, decane, and tetradecane, exhibited considerably more emulsion of the JP-4 fuel than the others, but the nature of the emulsion was not the same for all of these hydrocarbons. The emulsion produced by nonane was a soap-suds-like small bubble emulsion in contrast to the medium to large-size-bubble emulsion produced by organisms growing on decane, nonane, and tetradecane.

TABLE 44

EMULSION FORMATION BY STRAIN 101
ON PURIFIED HYDROCARBONS

Hydrocarbon	Emulsion of Hydrocarbon (millimeters)	Emulsion of JP-4 Fuel* (millimeters)
Pentane	0	0
1-Pentene	0	0
Hexane	0	0
1-Hexene	5	0
Heptane	0.5	0
1-Heptene	0	0
Octane	0	0
1-Octene	0	0
Nonane	5	9
1-Nonene	5	15
Decane	0	6
1-Decene	4	2
Dodecane	2	0
1-Dodecene	0	2
Undecane	0	0
1-Undecene	4	0
Tridecane	0	0
Tetradecane	4	6
1-Tetradecene	0	2
JP-4 Fuel	5	7

Medium 7 was overlayed with 15 ml of hydrocarbon; Inoculum: 4-day old culture 101 cells washed three times with sterile H_2O, initial concentration of each, 2.5×10^8 cells per ml; incubated for 10 days at 28°C on rotary shaker. Controls of sterile medium with hydrocarbon overlay showed no emulsion. Five ml of culture and 1 ml of overlay was withdrawn, introduced into 150 x 18 mm screw cap tubes and shaken vigorously.

*Five ml of JP-4 was added to 5 ml of culture and 1 ml of hydrocarbon after 10 days incubation, and this mixture was vigorously shaken.

As a comparison, a 4-month-old culture of strain 101 with JP-4 fuel overlay was tested in the same manner as the cultures above, and the entire amount of added JP-4 fuel was converted into a soap-suds-like, small-bubble emulsion of semisolid rigidity, which resisted loss of shape even upon inverting the tube containing it.

The long incubation period is not required for emulsion formation, however. Figure 44 shows the response observed when 100 ml of Medium 1 was overlaid with 1000 ml of jet fuel after 3 days growth on a gyratory shaker in the presence of growing cells of strain 101. The entire 1000 ml of fuel was completely emulsified.

That these particular hydrocarbons, nonane, 1-nonene, decane, and tetradecane, should be metabolized to a product which causes emulsion of JP-4 fuel could be important in elucidating the mechanism of fuel oxidation by microbes and the formation of compounds causing emulsion of fuel.

c. Emulsion of JP-4 Fuel by Hydrocarbons, Alcohols, Aldehydes, and Acids

The growth of microorganisms on fuel depends on the oxidation of hydrocarbons. Some of the products of oxidation cause emulsions. The objective of this study was to determine if in the absence of microorganisms any of the potential oxidation products of hydrocarbon oxidation would cause emulsion of JP-4 fuel.

The saturated hydrocarbons and their homologous aldehydes, alcohols, and acids used in this study are listed in Table 45. Several were not tested because they were unavailable. Five ml of JP-4 fuel and 5 ml of sterile Medium 7 were introduced into a 150 x 18 mm screw cap and 0.05 ml (or 0.05 g in the case of solids) of the test compound was added. This mixture was vigorously shaken for about 1 minute. The tubes were then permitted to remain undisturbed at room temperature for 24 hours after which observation of emulsion was made. Percentage of emulsion in the 5 ml of fuel was recorded. The nature of the emulsion in all cases was a soap-suds-like emulsion.

The results shown in Table 45 indicate that none of the hydrocarbons tested caused any emulsion of fuel, but the aldehydes and acids do; C_8-C_{10} and C_{14} acids were particularly good emulsion formers.

2. A Yellow Fuel-Soluble Microbial Product

A yellow fuel-soluble substance is produced by both pure strains and by mixed inocula cultivated on media containing both nitrate and JP-4. This substance is not observed on media containing ammonia as the only nitrogen source or on purified hydrocarbons. This substance is produced within the first 2 days of incubation; its color is most prominent at the onset of emulsion formation. On continued growth, the substance vanishes from the fuel phase.

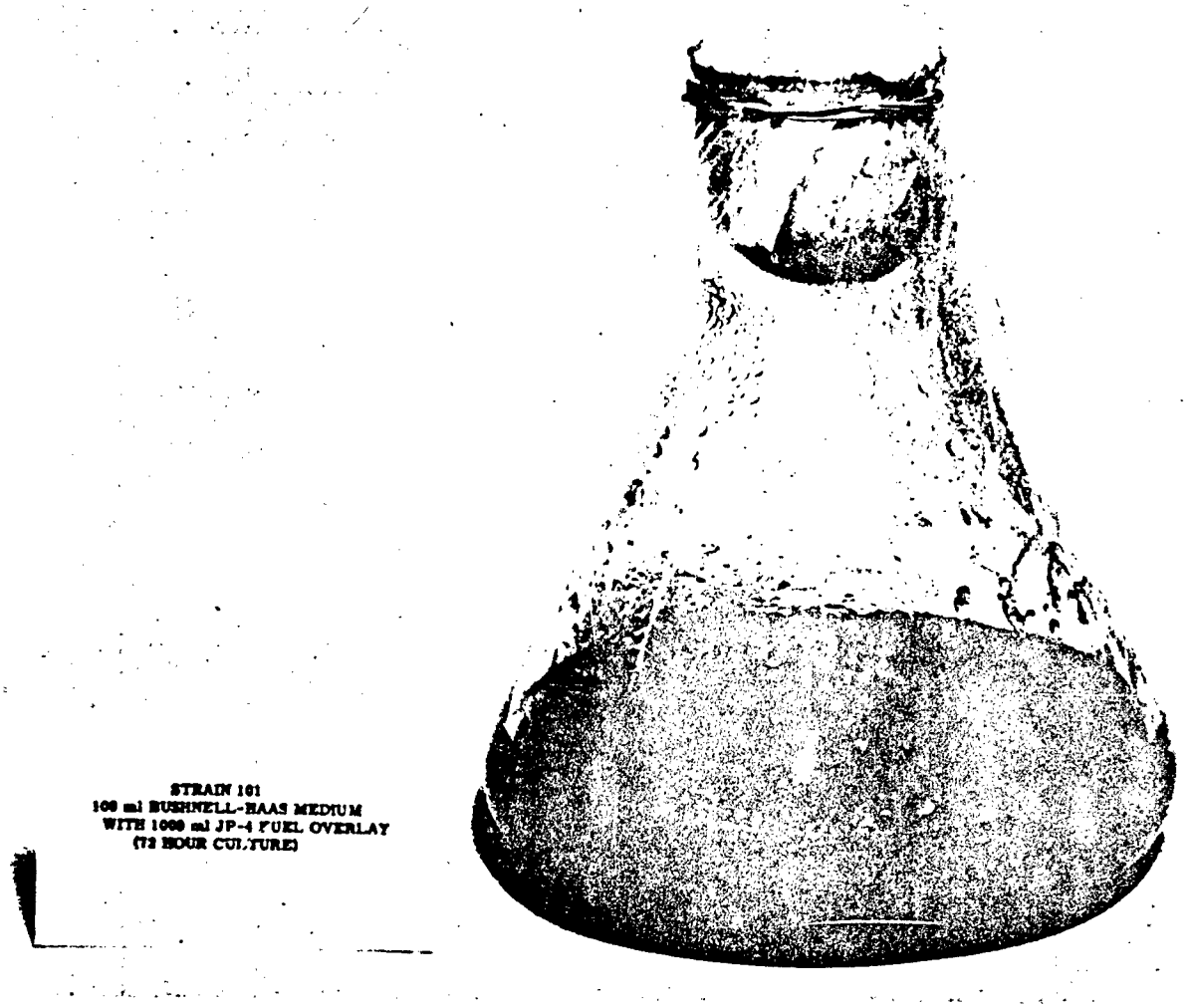


Figure 44. Emulsion Formation by Strain 101 on Medium 1

TABLE 45

EMULSION FORMATION IN JP-4 FUEL BY SATURATED HYDROCARBONS AND THEIR HOMOLOGS, ALDEHYDES, ALCOHOLS, AND ACIDS

Carbon Chain Length	Percent of Emulsified JP-4 Fuel			
	Hydrocarbons	Aldehyde	Alcohol	Acid
C_5	0	*	*	1
C_6	0	*	0	1
C_7	0	*	0	3
C_8	0	20	0	4
C_9	0	20	0	30
C_{10}	0	2	0	60
C_{11}	0	2	0	40
C_{12}	0	3	0	5
C_{13}	*	*	*	3
C_{14}	0	*	0	15
C_{15}	*	*	*	3
C_{16}	*	*	1	1
C_{17}	*	*	*	1
C_{18}	*	*	1	2

To five ml of JP-4 fuel and 5 ml of Medium 7 in 150 x 18 mm screw cap tubes, 0.05 ml (or 0.05 g) of the test compound was added, vigorously shaken, and percent emulsion of JP-4 fuel determined after 24 hours of undisturbed settling at 24°C.

*Not done.

The yellow substance produced on Medium 6 by strain 101 remains in the fuel phase at acidic pH, but enters the aqueous phase as alkalinity is increased; it is easily extracted into the aqueous phase by adjusting the pH to 11.5. The intensity of the yellow substance diminishes with increasing hydrogen ion concentration; the substance behaves as though it has a weak acidic group which exercises an inductive effect on a chromophore. A spectrophotometric titration was made using a mixed buffer containing 0.1 M Tris buffer and NH_4OH; pH was adjusted with HCl. Figure 45 shows this determination; the clearly defined inflection point at pH 11.0 sets the apparent pK of the functional group(s) which control color change. It is probable that this group also controls the water solubility of the substance. Figure 46 shows the UV absorption spectra of this substance at pH 6.8 and 11.5. The existence of only two isosbestic points at these extremes of pH suggests that this substance is a single molecular species, which is deprotonated at alkaline pH. These determinations show that this substance produced by bacteria can contaminate the fuel phase at hydrogen ion concentrations permitting microbial growth. This contaminant, unlike emulsions, is not confined to the interface but dissolves in the fuel and undoubtedly alters its properties.

3. Sludge

A dense, aqueous- and fuel-insoluble product (sludge) is produced by both pure strains and by mixed cultures on all media which contain both nitrate and JP-4. Sludge is not produced on media which contain only ammonium as the nitrogen source, nor on media which contain purified hydrocarbons, regardless of the nitrogen source. The sludge is an extremely soft gum, not soluble in hexane, ether, ethanol, acetone, dioxane, alkali, and dilute acid. The sludge is soluble in concentrated H_2SO_4.

To test the corrosivity of sludge, strains 96 and 101 were grown for 86 days on Media 3, 4, 5, and 6. The sludge was collected by decanting the culture fluids, washed three times by centrifugation with 100 volumes of distilled water at each washing, and violently agitated for 15 minutes between washings to equilibrate entrapped ions with the wash fluid. The sludge was then suspended in 25 ml of distilled H_2O, adjusted to pH 7.0, and dispensed into duplicate tubes, each containing a 10-ml suspension. The alloy coupons were introduced into these tubes and incubated for 48 hours on a gyratory shaker. Figure 47 shows the corrosion patterns and the relationship between extent of corrosion and nitrate concentration. Medium 3 produced the least amount of sludge, but this sludge caused more corrosion in 48 hours than sludges from media containing higher nitrate concentrations.

An attempt was made to acetylate the microbial sludge by refluxing with acetic anhydride. This treatment resulted in the partial dissolution of the sludge in the acetylating agent. The dissolution of this material may have resulted from the masking of charged groups by acetylation.

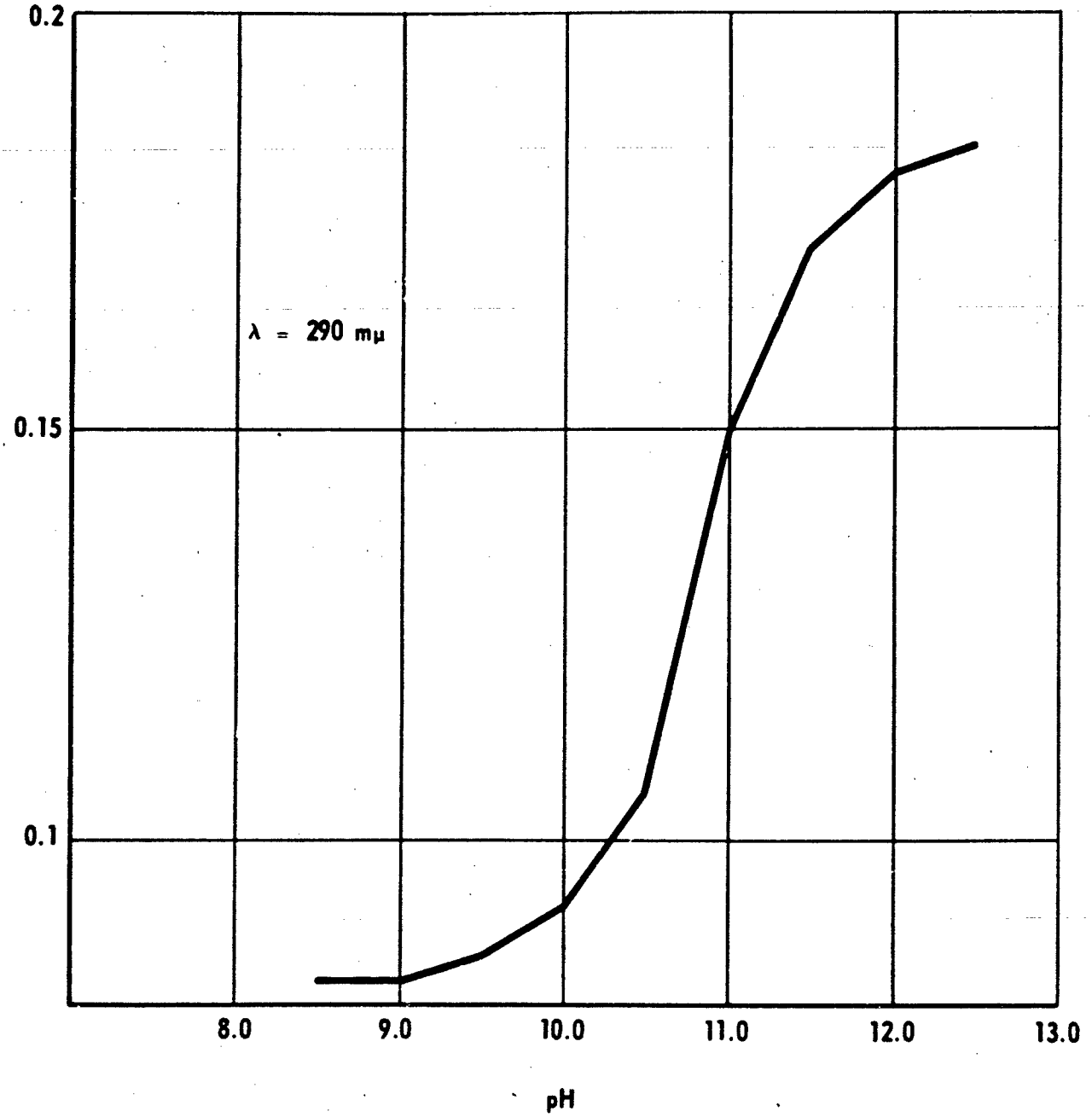


Figure 45. Spectrophotometric Titration of Fuel-Soluble Yellow Substance

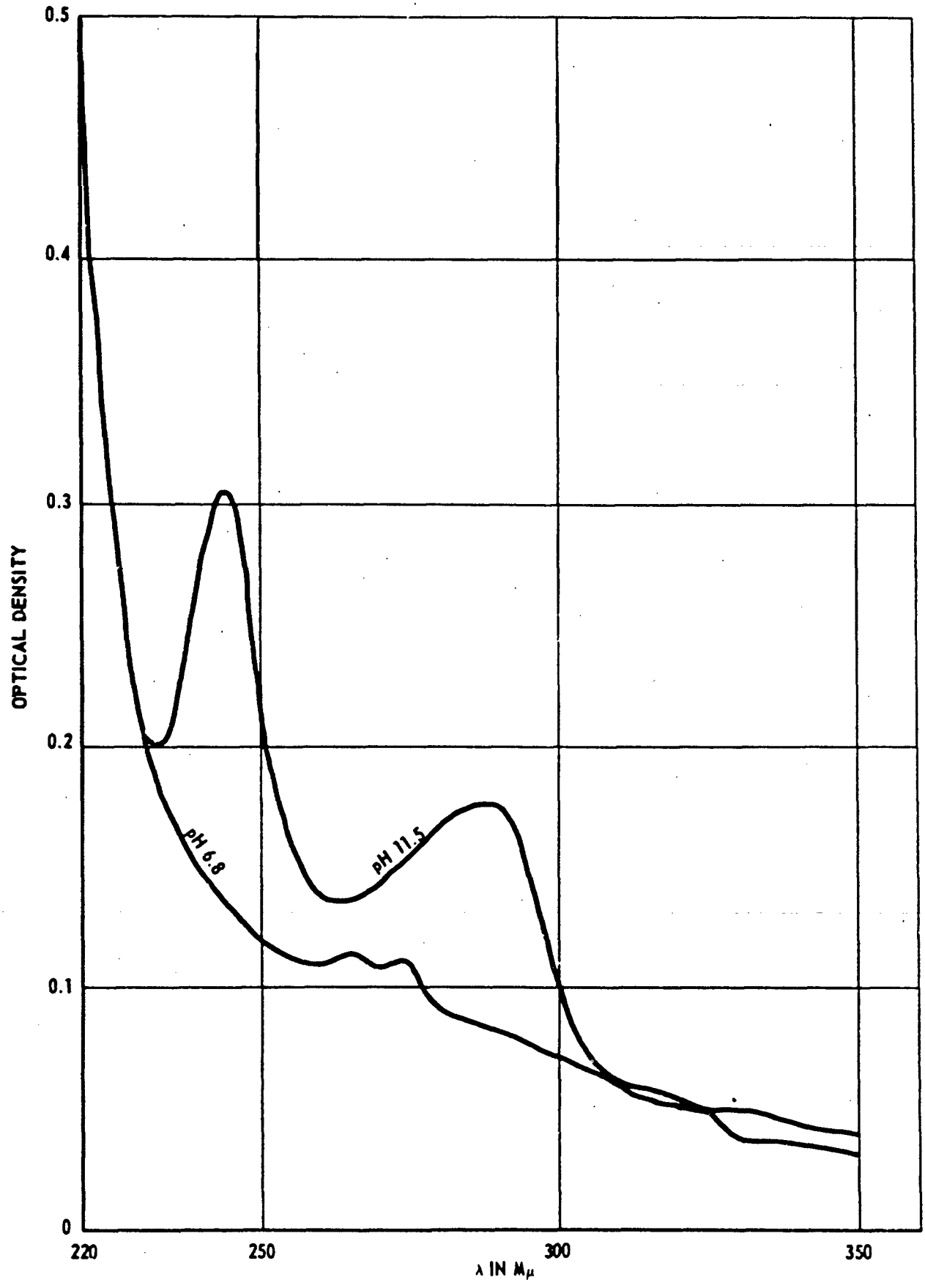
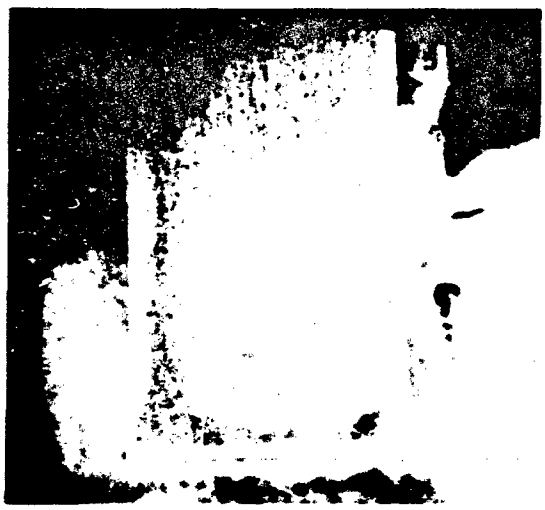


Figure 46. Absorption Spectra of Yellow Fuel-Soluble Substances

MEDIUM 6
1.2 g/l KNO_3

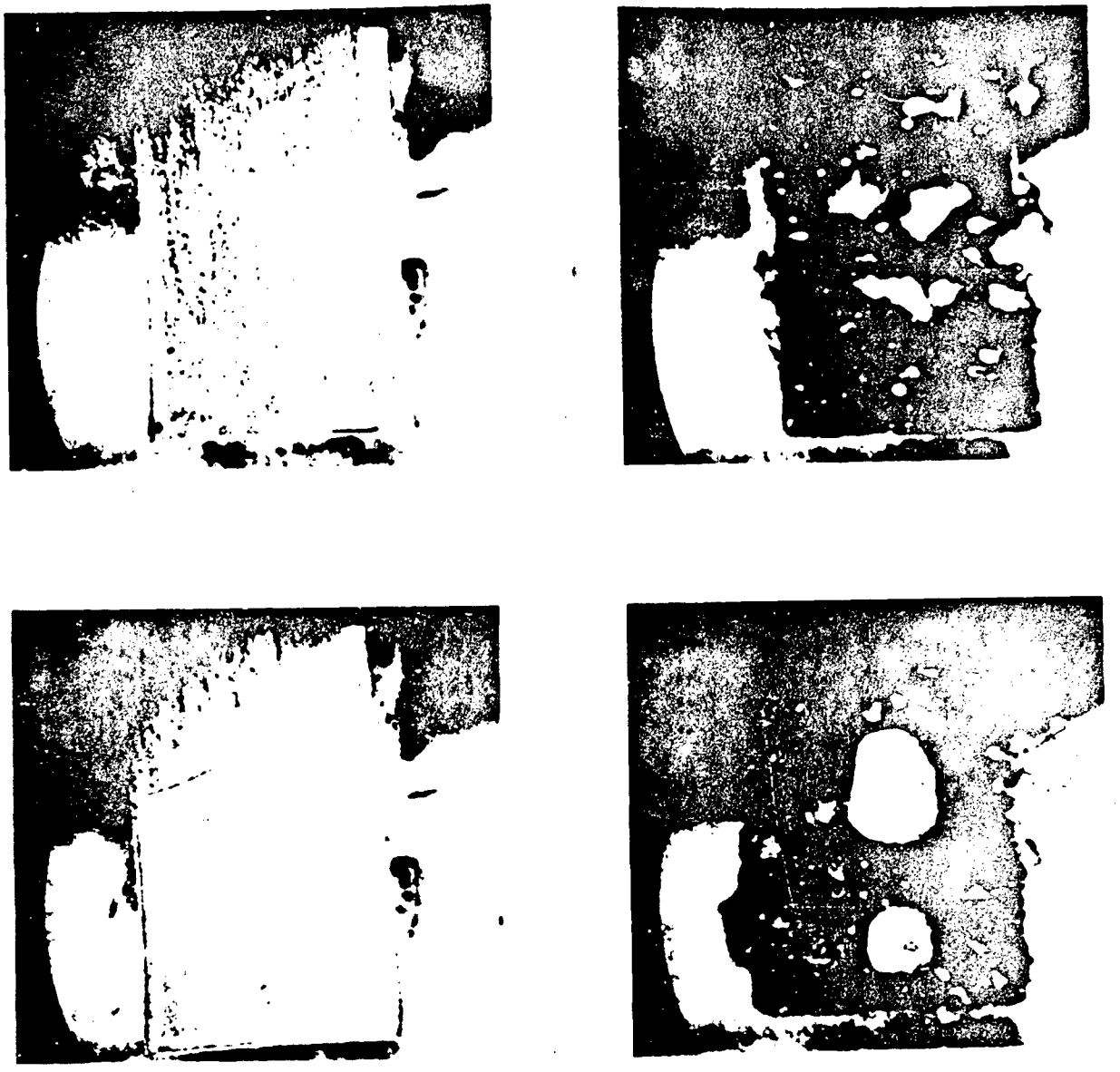
MEDIUM 5
0.08 g/l KNO_3



A

MEDIUM 4
0.06 g / KNO_3

MEDIUM 3
0.02 g / KNO_3



LEGEND

CULTIVATION: STRAIN 101 INCUBATED 86 DAYS IN 1 LITER OF MEDIUM 3,4, 5 AND 6.
SLUDGE: WASHED WITH DISTILLED H_2O DURING 3 CENTRIFUGATIONS, DILUTED TO 25 ML pH ADJUSTED TO 7.0
COUPONS - 2024 AND 7075 ALLOYS ADDED TO DUPLICATE TUBES CONTAINING 10 ML SLUDGE SOLUTION.
FROM LEFT TO RIGHT, SLUDGE COLLECTED FROM MEDIUM 6, 5, 4 AND 3. ALLOY 2024 AT TOP, ALLOY 7075 AT BOTTOM (48 HOUR EXPOSURE).

Figure 47. Corrosivity of Bacterial Sludge: Dependence on Concentration of KNO_3 in Initial Medium

B

The acetylated material, or that material treated with acetic anhydride, was dried and dissolved in methanol with BF_3 as a catalyst. The mixture was refluxed to permit the formation of methyl esters. Following this treatment, the sludge residue in methanol-BF_3 was dissolved in benzene-ethyl ether. To this organic solution, water was added, and a large quantity of brown material was extracted into the water phase with precipitate formation. The organic phase was separated and washed; it was then analyzed for long-chain methyl esters. The results of that analysis are shown in Table 46.

The large proportion of long-chain acids present was of interest. The identity of the fuel components that act as substrates for the biosynthesis of C_{22} acids appears to be of particular importance because of the emulsifying properties of long-chain fatty acids. The appearance of fatty acids in this transacetylated material leaves open the status of the long-chain acids in the native bacterial sludge. If the long-chain acids contained more than one functional group, they could act as ligands and concentrate mineral constituents of the medium which cause corrosion. These ligands with their bound metals would agglomerate at the bottom of fuel-water systems and exercise a corrosive effect over a confined area.

A portion of the bacterial sludge was further analyzed. It was dialyzed for 24 hours and subjected to C, H, and N analyses. (Table 47.) Another portion (0.104 g) was extracted with 10% HCl-methanol for 48 hours. The resulting material was separated into a soluble fraction and an insoluble fraction by centrifugation. CHN analyses were performed on these fractions. The weight of the methanol-insoluble fraction actually was greater than indicated because much of it formed a residue on the centrifuge tubes. This residue was not dialyzed out because the residual material in the dialyzed sample was much greater than that present in the extracted sample. The C, H, N, and O values were adjusted to eliminate the effect of this residue.

The methanol-insoluble portion contained much more nitrogen than the methanol-soluble portion, with the dialyzed portion having an intermediate value. This was to be expected, since the methanol-soluble portion contained, primarily, the lipid portion. The protein concentration was obtained by assuming that normal proteins were responsible for the nitrogen concentration. The residual C, H, and O values were obtained by subtracting the CHO contributions of the protein from the total CHO concentrations. The oxygen value probably has a large error.

These results appear to indicate that the protein lipid and carbohydrate concentrations are approximately 50, 20, and 30% respectively. The presence of nitrogen in this water-insoluble, fuel-insoluble sludge suggests the presence of nitrated hydrocarbons, microbially produced, which contribute to the corrosivity of fuel-water bottoms.

TABLE 46

FATTY ACID CONTENT OF MICROBIAL SLUDGE FRACTIONS

Retention Time (minutes)	Peak Height (inches)	Probable Fatty Acid Ester (Chain length: Unsaturations)	Percent of Total Material Analyzed
13.34	0.47	14:1	0.09
13.86	0.80	14:0	0.16
14.42	0.25		0.05
17.84	16.88	16.0	3.48
18.98	14.00		2.89
19.44	20.00	17.0	4.12
21.38	2.95	18.1	0.61
21.80	1.02	18.0	0.21
23.24	6.76	19.0	1.39
26.02	0.47	21.1	0.09
26.36	1.73	21.0	0.36
28.50	419.20 (5.24 x 80)	22.0	86.45
32.74	0.38		0.08
Total height	484.91		

TABLE 47

A CARBON, HYDROGEN, NITROGEN, AND OXYGEN ANALYSIS OF MICROBIAL SLUDGE

	Methanol Soluble	Methanol Insoluble	Dialyzed
Dry weight	0.0322 g	0.0187 g	0.060 g
% C		45.7	25.9
% H		7.8	6.5
% N	4.8	11.3	4.7
% Residue		4.4	39.4
Adj. C		48.0	42.8
H		8.2	10.7
N	4.8	11.9	7.8
O		31.9	38.8
Protein	30.0	74.4	48.8
Residual C		9.4	17.4
H		3.4	7.5
O		15.8	28.0

F. Detection of Jet Fuel Microorganisms

The objective of this study was to develop a simple detection method which could be employed in the field for early detection of microorganisms in jet fuel-water bottoms.

During this study, eight different detection methods were evaluated for their sensitivity, specificity, and lack of interference from background materials in water bottom. The results of this study are summarized in Table 1. Only three of the methods, acridine orange stain, fluorescein isothiocyanate stain, and radioactive silver stain, had a potential sensitivity to 10^3 - 10^4 bacteria. Studies on these three methods are described in detail below, whereas studies on the other methods are only summarized.

1. Radioactive Silver

Previous studies have shown that bacteria bind considerable amounts of silver whether living or dead; therefore, the detection of bound silver would give a quantitative measure of the total number of organisms present in a system. This method was tested by reacting 10^8 cells of bacteria with radioactive Ag^{110}, 0.5 μc of Ag^{110} per ml in 0.01 M $AgNO_3$ for one hour, washing the cells to remove the free Ag^{110} and measuring the bacterial residue in a proportional counter. The results in Table 48 show that 0.4% of the silver was bound by the bacteria, and this amount is approximately 1% of the bacterial weight. Using the above concentration of Ag^{110}, and assuming that there is no background contribution other than the normal background count of 20 CPM, 10^7 cells would give a detectable signal of 40 CPM.

TABLE 48

DETERMINATION OF Ag^{110} BINDING TO SERRATIA MARCESCENS

Detector	Sample CPM	CPM of Ag^{110} Reacted	% Ag^{110} Bound to Cells
Scintillation Counter	5458	1,268,000	0.41
Proportional Counter	438	109,000	0.4
Proportional Counter	353	109,000	0.32
Proportional Counter	413	109,000	0.38

Procedure: Serratia marcescens were grown for 18 hours in TGY medium at 37°C, washed three times with distilled water by centrifugation and diluted to a concentration of 4.2×10^8 cells/ml. The reaction mixture contained 1 ml of bacterial suspension, and 1 ml of a solution containing 0.5 microcuries of Ag^{110} in 0.1 M $AgNO_3$. Reaction was for 1 hour. The cells were centrifuged and washed with distilled H_2O until no radioactivity was detected in the supernatent.

However, if the radioactive silver solution is not diluted with unlabeled silver, the sensitivity of the method can be greatly increased. If a solution of concentration of 1 curie/gram of Ag^{110} were used and 1% of the silver bound, 5×10^3 cells would give an easily detectable signal above background.

The use of this high specific activity Ag^{110}, however, would make rigid safety measures a must for any instrument designed to use this approach. The instrument would have to be completely automated and shielded to be safe for use by nontechnical personnel.

In an automated process, it would be possible to concentrate the cells in a complex suspension, such as jet fuel-water bottom, by filtering a large volume onto Millipore tape,* reacting with Ag^{110}, washing the tape, and counting. To test this procedure, the staining reaction was attempted on a millipore filter. It was discovered that the filter control bound Ag^{110}, resulting in a large background. Several attempts were made to lower the background. The lowest background was obtained by soaking a millipore filter overnight in $Al(NO_3)_3$ solution. The background was reduced to 150 CPM above normal background of 30 CPM. However, if this background level, 150 CPM, can be reproducibly maintained, it should be possible to detect 1×10^4 bacteria. It is anticipated that further work with this system will reduce the background even lower.

Studies with this method were discontinued because it appeared that this principle would present logistic problems involving personnel safety in the routine use of an instrument developed to utilize high specific activity Ag^{110}.

2. Acridine Orange Stain

A general technique using Acridine Orange (A.O.) was studied. This dye is a peculiar type of stain because it will bind to bacteria in water and dissociate from bacteria in ethanol. It was postulated that a reliable process for this reaction would make it possible to use the present esterase instrument (soluble product instrument) for detecting both live and dead microorganisms from any source.

The original objective of this study was to determine the feasibility of performing the entire collection, processing, and detection reactions on a bacterial filter. The steps in the process would be: (1) collect the microorganisms from water bottom by filtration, (2) apply the acridine orange stain to the filter and permit it to react with microorganisms (3) wash the filter to remove excess dye from the filter, (4) dissociate the dye from the microorganisms with ethanol, and (5) measure the fluorescence of the eluted dye.

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* Millipore Filler Corporation, Bedford, Mass.

The first studies carried out with this process showed that Millipore filters bound acridine orange about as well as the microorganisms, and this dye could not be washed out to leave only stained organisms. Therefore, it became obvious that alternate methods would have to be used for determining quantitatively the quantities of dye bound to bacteria. Since the sensitivities and volumes of acridine orange solution needed by present instruments are known, knowledge of the moles of dye bound by the microorganisms can be used to determine the feasibility of the approach for detecting 10^3 - 10^4 bacteria. Therefore, the following steps were studied in an attempt to obtain the moles of dye bound to the microorganisms:

(1) Reaction of A.O. and bacteria
(2) Separation of free A.O. from the stained bacteria (A.O.-bacteria complex)
(3) Dissociation of the A.O.-bacteria complex to free bacteria and free dye
(4) Separation of free dye in ethanol from the free bacteria. This was believed to be needed, since the bacteria interfered with measurement of fluorescence of free A.O.
(5) Detection of A.O.

No problems were found in obtaining a good staining reaction. The following conditions resulted in a good stain:

(1) One ml suspension of bacteria in water or water bottom
(2) 2.0 ml A.O., 1.44×10^{-4} M in 10% ethanol
(3) Incubation for 15 minutes at room temperature.

Four different methods: (1) millipore filtration, (2) centrifugation, (3) dialysis, and (4) Sephadex chromatography were studied for the separation of free A.O. from the stained bacteria. Millipore filtration was not useful, since the filter also bound dye. The best technique for removal of the unreacted dye from stained cells was separation on a Sephadex gel column. The results in Table 49 show that Sephadex and centrifugation yield cells which have about the same amount of stain. After removing free dye, the stained bacteria were collected by centrifugation or Millipore filtration, then used for the dissociation study.

TABLE 49

COMPARISON OF METHODS FOR REMOVAL OF EXCESS ACRIDINE ORANGE

Purification Method	Cell Concentration	m moles/2 ml of A.O. Bound in Sample	m moles of A.O. Bound Per Cell
Sephadex	5.5×10^{7}	1.6×10^{-6}	2.9×10^{-14}
Dialysis	2.8×10^{8}	3.8×10^{-7}	1.4×10^{-15}
Centrifugation	8.5×10^{7}	2.4×10^{-6}	2.8×10^{-14}

Method Used to Remove Excess Dye from Sample:

(a) Sephadex: Sample containing stained bacteria and free dye was eluted with acetate buffer 0.01 M, pH 4.5 through a G-25 coarse grade column, 20 cm x 1.3 cm.

(b) Dialysis: Sample was diluted to 10 ml with distilled water, placed in a dialysis bag, and dialysed against tap water overnight.

(c) Centrifugation: Sample was centrifuged, then resuspended in 2 ml distilled water and washed three times.

Procedure: After sample purification, each sample was measured by suspending the bacteria in 2 ml of ethanol for 5 minutes, removing the cells by centrifugation, and measuring the fluorescence of the supernatent containing the free dye.

A study was performed to determine the percentage of cells bound by Sephadex G-25 under the conditions of elution described above. The results in Table 50 show that better than half the bacteria are recovered. Since this is not a large loss considering the simplicity and speed of the step, it was decided that this was the preferred method for dye removal until a better one is found.

TABLE 50

LOSS OF LABELED EXCHERICHIA COLI
DUE TO BINDING ON G-25 SEPHADEX

Number of Cells Placed on Column	Number of Cells Removed from Column	% of Cells Recovered from Column
3.9×10^8	2.0×10^8	51
1.4×10^9	7.8×10^8	56
4.1×10^8	2.1×10^8	51
4.1×10^8	2.8×10^8	69
3.5×10^8	2.7×10^8	75
3.5×10^8	1.7×10^8	49
8.8×10^8	2.8×10^8	32
8.8×10^8	6.5×10^8	74

Escherichia coli were grown on a gyratory shaker at 37°C for 18 hours on TGY medium. The cells were harvested by centrifugation and washed 3 times with distilled water by centrifugation. The packed cell paste was suspended in water at the above number/ml. One ml of suspension was applied to the column in each case. Cell counts were determined by standard dilution plate count techniques using TGY agar and more microscopic counts.

The dissociation reaction was studied by (a) dissociation of the A.O.-bacterial complex by addition of two ml of ethanol to the cells for 5 minutes, then (b) removal of the cells by centrifugation. The bound A.O. was dissociated into the ethanol by this procedure, and the fluorescence was measured in a 3-ml curette on an Aminco-Bowman spectrophotofluorimeter. The lower limit of sensitivity with the centrifugation method was 5×10^5 cells. If the reaction is performed by the same procedure as used for Melpar's present soluble product instrument, then the entire reaction would be carried out in 0.05 ml and detection of 1×10^4 bacteria would be possible. The Aminco-Bowman was used for comparative purposes to determine whether the dissociation reaction could be carried out on a filter. This process could be implemented into a detection system if the A.O.-bacterial suspension could be filtered, and the dissociation then carried out on the filter with the ethanol being filtered through and measured. However, most of the membrane filters which retain bacteria also bound acridine orange. Several filters

were tested for their capacity to bind A.O. The results are shown in Table 51. The Gelman Versapor filter bound the lowest percentage of dye, 10%. Attempts are presently being made to carry out the entire collection, staining, washing, and dissociation reactions on this filter.

A study was performed with A.O.-stained bacteria to determine if stained bacteria can be quantitatively measured directly in the Aminco-Bowman spectrophotofluorimeter. The results are shown in Figure 48. The standard curve for A.O. is shown in Figure 49.

When the signal obtained by the dissociation procedure is compared with a direct measurement of the A.O.-cell suspension, the lower detection limit of the suspension is approximately one log higher (5×10^5 cells) than the dissociation procedure. Thus, it appears that the dissociation reaction rather than the direct measurement of the A.O.-labeled cell suspension is the most sensitive.

TABLE 51

COMPARISON OF BINDING OF ACRIDINE ORANGE BY VARIOUS FILTERS

Type of Filter	A.O. Concentration	Reading of A.O. Before Passing through Filter	Reading of A.O. After Passing through Filter	% A.O. Not Bound
HAWG Millipore Filter	1.44×10^{-5} M	0.98	0.0123	1.3
	1.44×10^{-6} M	0.12	0.00015	0.1
Gelman VM Filter	1.44×10^{-6} M	0.12	0.0189	16
	1.44×10^{-6} M	0.12	0.0201	17
Flotronics Silver Filter	1.44×10^{-6} M	0.102	0.043	43
Versapor Filter	1.44×10^{-6} M	0.102	0.090	88

Procedure: Two ml of the appropriate A.O. solution were filtered slowly through each filter, collected, and measured on the Aminco-Bowman spectrophotofluorimeter. The A.O. solutions were also measured before being filtered. All readings were converted to an arbitrary scale of fluorescent units.

Excitation = 490 mu
Emission = 525 mu

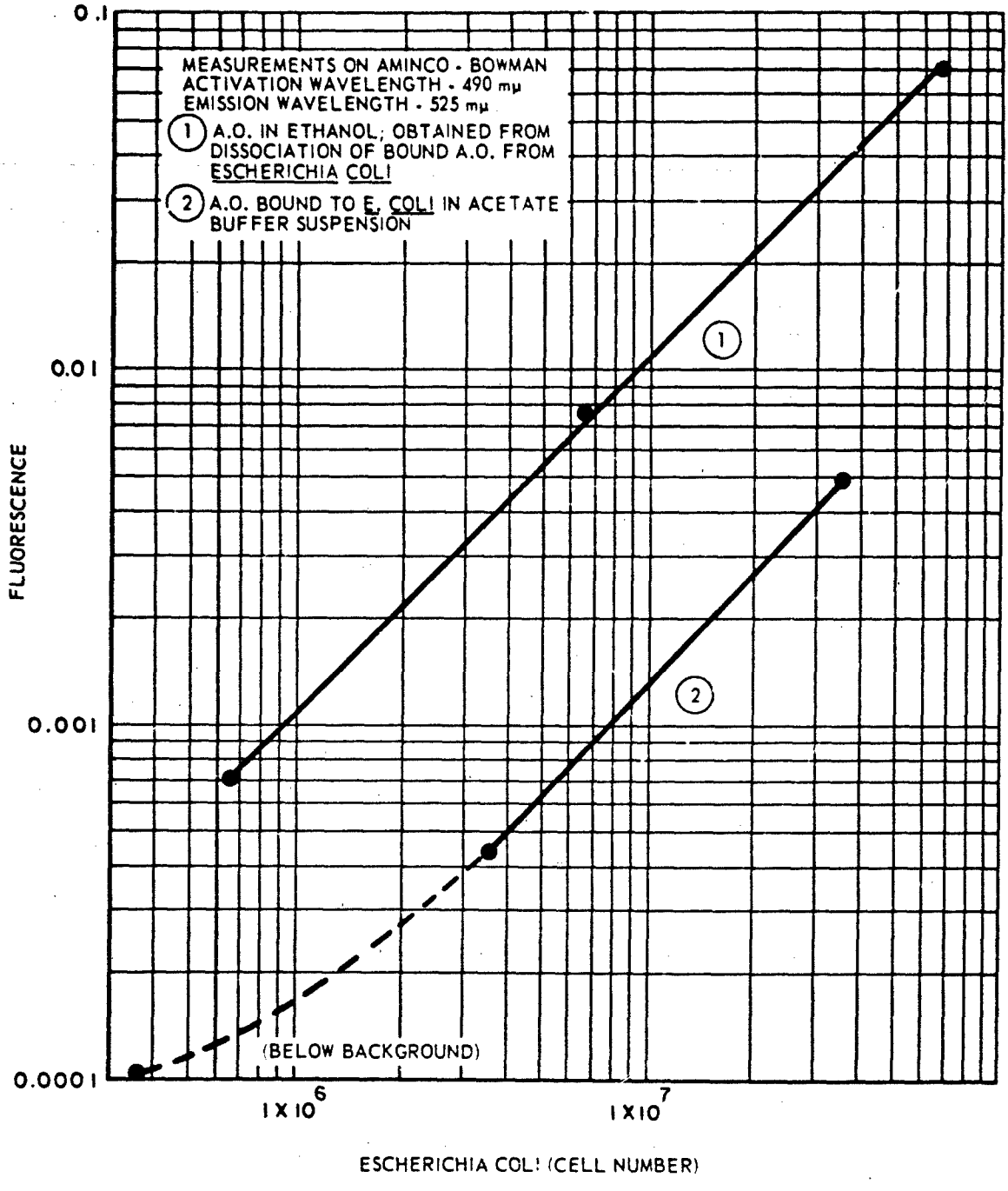

Figure 48. Comparison of Acridine Orange Procedures. Dissociation Versus Direct Reading

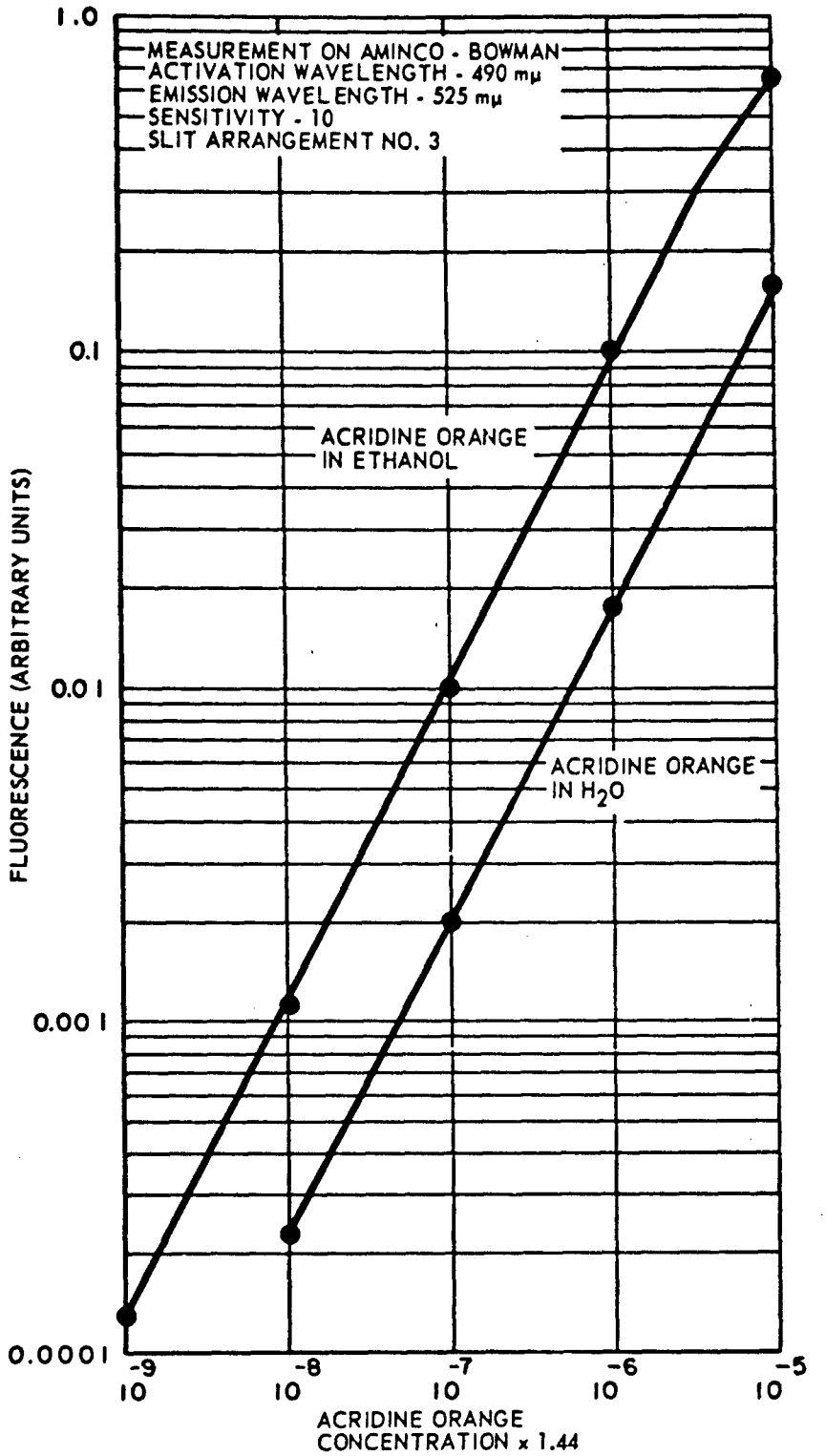

Figure 49. Acridine Orange Calibration

3. Fluorescein Isothiocyanate Stain

Labeling bacteria with FITC was attempted by a procedure similar to the one described above for direct measurement of A.O. One ml of bacterial suspension, Escherichia coli, was reacted for 15 minutes with 2 ml of a solution of FITC containing 1 mg/ml FITC in 50% acetone - 50% of 0.05 M carbonate-bicarbonate buffer, pH 9.6. The sample was then eluted through a Sephadex G-25 coarse grade column with 0.05 M carbonate-bicarbonate buffer, pH 9.6. The FITC-Labeled cell suspension was then measured directly on the Aminco-Bowman for fluorescence at 490 mu activation wavelength and 525 mu emission wavelength. The results are shown in Figure 50. The lower limit of detection is approximately 5×10^5 cells. The suspension was also observed under a fluorescent microscope. The bacteria were brightly stained. To determine the relative intensity of the stain, these cells were compared under incident light with streptococcal cells stained with FITC-labeled antibody, and were judged to be of equal intensity.

The FITC-stained bacteria obtained from the Sephadex treatment were also filtered onto a Millipore filter and counted directly under the microscope. The total filtering area of a 25-mm Millipore filter is approximately 314 mm. The size of one block on the gridded filter is 3 mm; therefore, the area is 9 mm^2. The diameter of the microscope field at 500X is approximately 300μ. Therefore, to scan the width of one block would require approximately 10 fields, and if one were to filter 1000 organisms over the area of 314 mm^2, then 30 organisms would populate each block. Thus, if one counts 10 different microscope fields, he should count about 30 bacteria.

This method was tested and it was found that counting 10 fields did result in the expected count. Thus, the sensitivity of direct eyeball counts is about 10^3 bacteria on the Millipore filter. Attempts are presently being made to determine the minimum time required to perform the entire process staining, Sephadex separation, and Millipore filtration and counting.

A second procedure to run the FITC reaction on a filter is described in Section IV, "Recommended Procedure for Early Detection of Microorganisms in Fuel-Water Bottoms."

4. Esterase

Other studies at Melpar have demonstrated that all bacteria, fungi, spores, and yeast examined to date have enzymes which hydrolyze fluorescein diacetate.

The first studies were to determine the esterase activity of the jet fuel microorganisms. The stock cultures were grown for 1 week on Medium 1 at 30°C on a gyratory shaker. The cell number/ml of growth media was

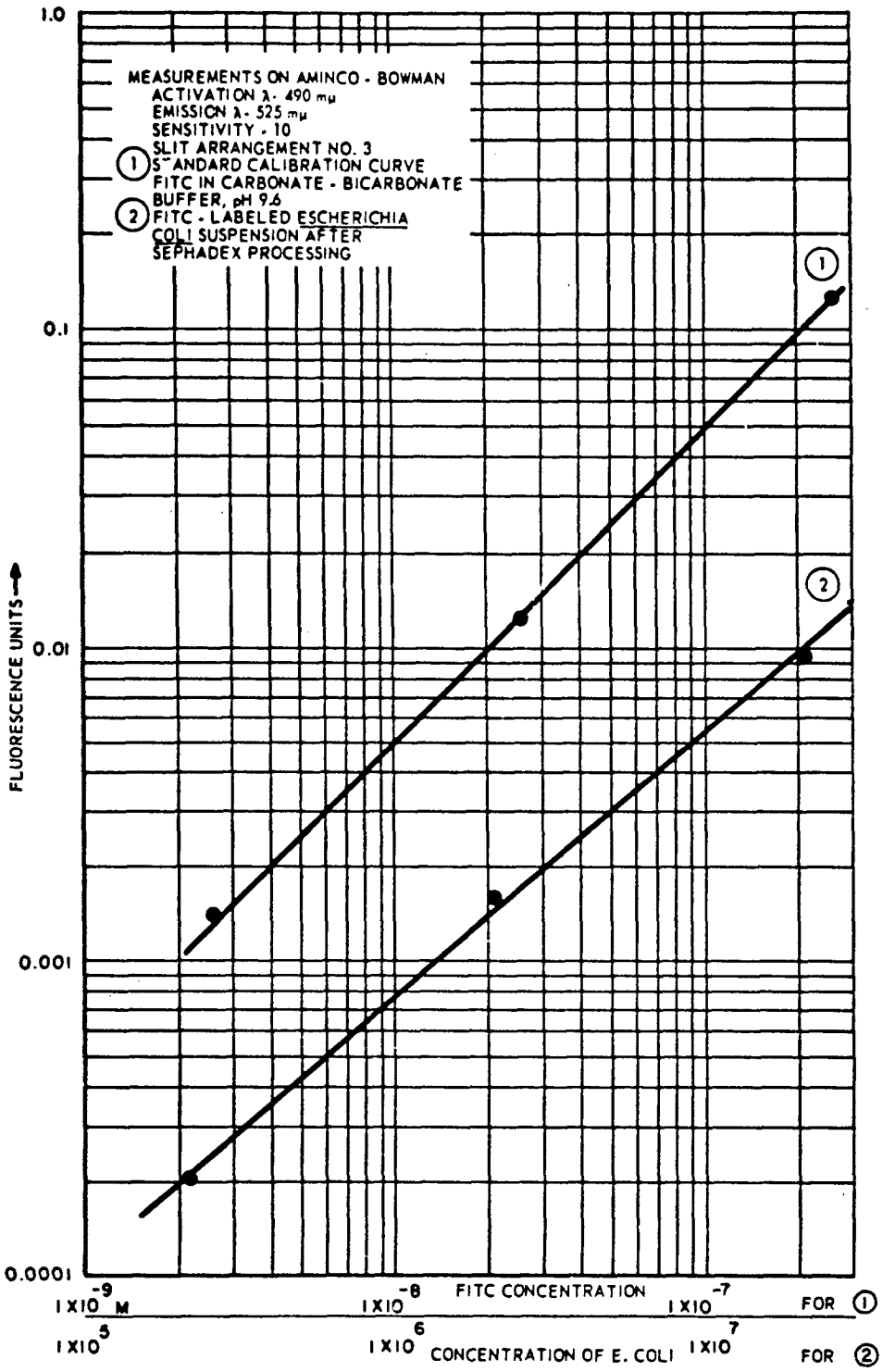

Figure 50. Fluorescence of FITC and FITC Labelled Escherichia coli

determined by microscopic counts. The reaction mixture contained 1.0 ml of bacterial suspension after 1 week's growth, 25 μ moles of 0.5 M phosphate buffer, pH 7.0, 0.1 μ moles fluorescein diacetate, and water to 3.0 ml. The reactions were initiated by addition of substrate, and readings were made at 5, 10, and 20 minutes. Three controls were run with each group of test organisms: (1) reaction mixture containing JP-4 fuel in the place of test organisms, (2) reaction mixture containing Medium 1 in place of test organisms, and (3) reaction mixture containing distilled water in place of organisms. The enzyme activity of 33 fuel isolates and a sample of the aqueous layer from a kerosene storage tank, Hotchkiss Fuel Oil Company, Fredericksburg, Va., is shown in Table 52.

A variety of growth responses and enzyme activities were observed and enzyme activity did not appear to be a function of cell number. This observation was confirmed in later studies with stain 5, 14, and mixed water-bottom cultures, grown on Medium 1 and TGY medium. In this experiment, the esterase activity of the cultures was proportional to cell number when the cells were grown on a rich TGY medium, but this relationship did not exist when the cultures were grown on jet-fuel medium. The reasons for these differences are not known; but it did appear to limit the usefulness of this approach for detection.

5. Tetrazolium Reduction

This technique depends upon the reduction of a tetrazolium salt to a deeply colored formazan by the microorganisms. Several tetrazolium salts were investigated including MTT (3, 4, 5 dimethylthiazolyl-1,2), 2, 5 diphenyl tetrazolium bromide, NTV (neotetrazolium violet), and NTC (neotetrazolium chloride).

To evaluate these compounds with respect to their usefulness as detectors of microbial systems, it was desirable to determine their ability to be extracted by jet fuel. Preliminary studies showed that NTV formazan was most easily extracted, and MTT formazan was next most easily extracted. But subsequent experiments indicated that NTV was not readily reducible by microbial dehydrogenases when jet fuel was used as an oxidizable substrate.

Fuel-grown organisms were then assayed for their ability to reduce tetrazolium salts in the presence of various dehydrogenase substrates. The substrates tested were lactate, glyceraldehyde-3-phosphate, and serccinate. Reduction of MTT in the presence of the three substrates was higher than in their absence. These data indicated that MTT is readily reduced in the presence of suitable substrates and that it is a more suitable dye than the others for use with jet-fuel microorganisms.

TABLE 52

ESTERASE ACTIVITY OF JET FUEL ISOLATED

Organism	Cell Count per ml	AMINCO-BOWMAN UNITS X 0.001			Temperature
		5'	10'	20'	
8	4.2×10^7	3	6	12	27°C
15	2.6×10^8	10	25	64	27°C
24	1.0×10^8	3	6	12	27°C
31	3.5×10^8	7	19	43	27°C
27	7.5×10^7	1	21	59	27°C
28	1.1×10^8	15	27	54	28°C
12	1×10^7	9	9	15	28°C
9	7.5×10^7	6	12	21	28°C
20	5.7×10^7	9	18	33	28°C
6	1.5×10^7	9	12	24	28°C
5	1.05×10^8	15	42	99	28°C
17	4.25×10^7	3	6	12	26°C
23	9.5×10^8	3	9	15	26°C
7	1×10^8	3	72	147	26°C
25	8.2×10^7	3	33	57	26°C
29	9.5×10^6	1	9	15	26°C
19	3.0×10^7	3	39	75	26°C
32	3.5×10^7	8	17	32	26°C
10	4.0×10^7	5	14	29	26°C
26	2.65×10^7	17	38	94	27°C
4	2.2×10^8	20	44	89	27°C
30	5×10^6	8	17	32	27°C
2	3.4×10^7	5	11	23	27°C
33	1×10^8	14	32	83	27°C
11	3.25×10^7	2	8	20	27°C
18	3.25×10^7	5	11	32	27°C
16	1.5×10^8	5	8	14	27°C
21	5.8×10^7	2	5	5	27°C
14	1.2×10^8	20	56	137	27°C
3	2.5×10^6	1	2	2	27°C
1	5.2×10^7	5	20	59	27°C
13	1.25×10^7	1	5	5	27°C
Serratia Marcescens	1×10^9	9	21	60	27°C
Kerosene-H_2O Bottom	8.2×10^7	160	410	670	28°C

A standard curve was obtained by the following procudure. A stock solution of MTT, 0.5 mg/ml, was used. The appropriate dilution of MTT was added to 1 ml of sterile Medium 1 (salts) and 4 ml of jet fuel JP-4, and a small amount of solid sodium hydrosulfite was added to reduce the MTT to its formazan, a violet precipitate. The aqueous reaction mixture was overlayed with 4 ml of JP-4 fuel and stirred on a Vortex stirrer. The formazan was completely extracted into the jet fuel layer. An extraction blank without MTT was subjected to the same procedure, and the jet fuel layer was used as a blank. All MTT dilutions were analyzed for absorption against this blank using a Zeiss Spectrophotometer at 570 mu. The results obtained are shown in Figure 51.

A week-old culture of a Pseudomanas strain 96, grown in Medium 1, was tested for its ability to reduce MTT. The reaction mixture consisted of 0.5 ml bacteria, 1.0 ml 0.1 M Tris buffer at pH 9.0 0.5 ml 1% lactate as substrate, and 0.4 ml MTT 0.5 mg/ml. MTF formed was extracted with 4 ml of JP-4, and O.D. readings were made at 570 mu. The results shown in Table 53 indicate that a detection limit for jet fuel organisms could presently be set at 10^7 cells, since in all cases the O.D. obtained at 10^7 corresponds to a visually detectable color. However, the fact that cell concentration and O.D. do not correlate in a linear manner in every sample suggests that some MTT is reduced by components of the dead cells present.

Several experiments have been run with Bacillus globigii (B.g.) under conditions similar to those above. The B.g. cultures were grown 18 hours in TGY broth, washed twice, and resuspended with distilled water. The reaction time was 50 minutes at room temperature. Experiments 3 and 4 were run by filtering one ml of B.g. through a 0.45μ pore size Millipore filter. The B.g. were retained on the filter, and the reaction mixture was placed over the filter in a test tube to react. The results in Table 54 show that the limit of detectable reduction of MTT by B.g. was approximately 1×10^7 cells. With the freshly grown B.g. cultures, there appears to be a linear correlation of cells concentration to O.D.

The above results prompted studies to determine the effect of the variables in the system and to optimize each so that a system of maximum response might be obtained. The enzyme system utilizing lactate had previously been investigated using MTT; thus, the following cyclic reaction system was adopted for detection of the diphosphorpyridine nucleotide (DPN) present in bacteria.

$$\text{Lactate} + \text{DPN (from bacteria)} \xrightleftharpoons{\text{LDH}} \text{Pyruvate} + \text{DPNH}$$

$$\text{DPNH} + \text{MTT} \xrightleftharpoons{\text{Diaphorase}} \text{DPN} + \text{MTT (violet color)}$$

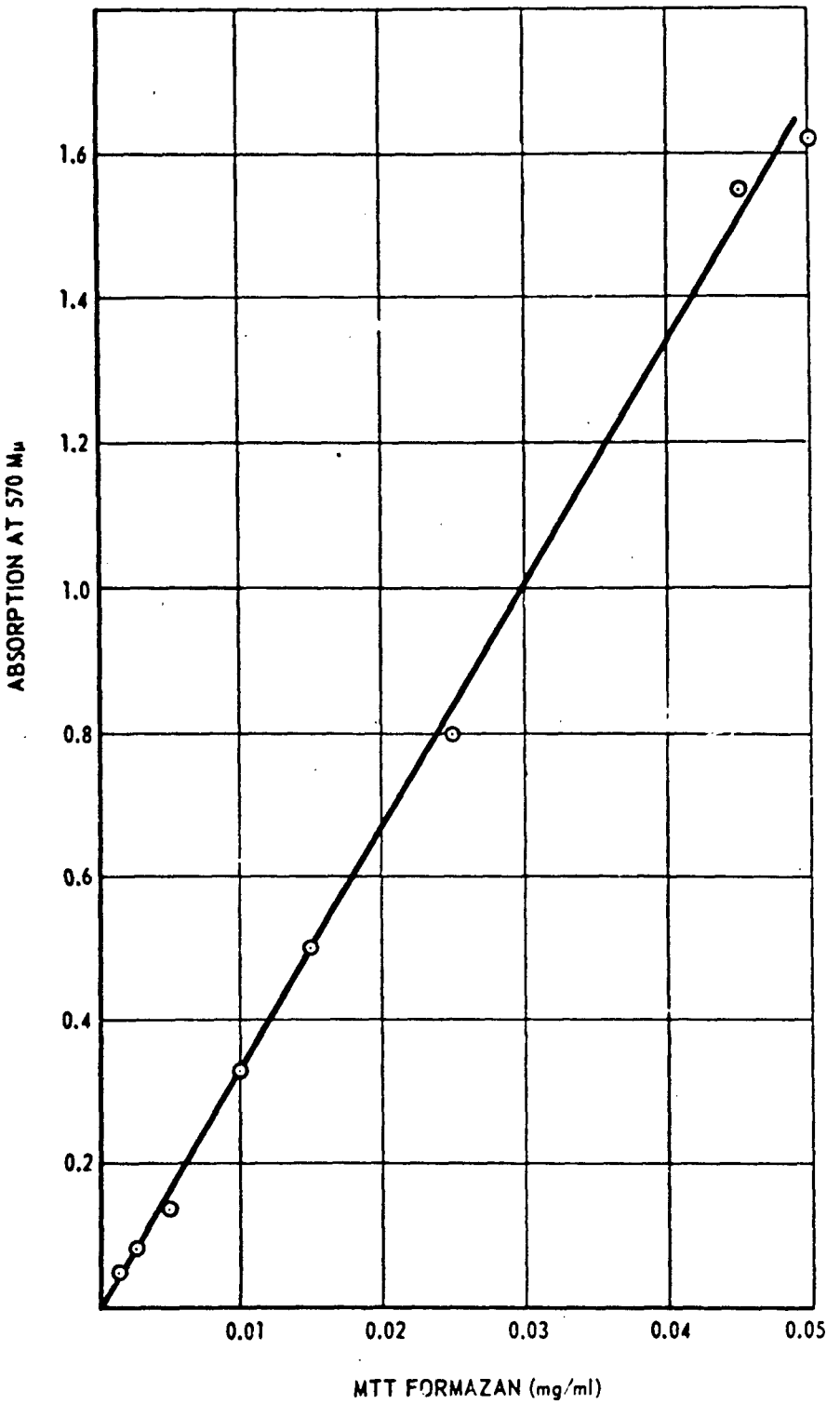

Figure 51. Standard Calibration Curve of MTT Formazan

TABLE 53

MTT TETRAZOLIUM REDUCTION BY JET FUEL MICROORGANISMS

Culture	Age	Bacterial cells/ml	Absorption at 570 mμ	Reaction Time in Minutes
27	2 wks	1.5×10^6	0.103	50
96	20 days	1.2×10^7	0.145	63
		1.2×10^6	0.165	68
27	3 wks	2.4×10^6	0.038	60
		2.4×10^5	0.020	60
		2.4×10^4	0.010	60
96	10 days	Approx. 10^7	0.105	60
		10^6	0.025	60
		10^5	0.000	60
96	1 mo	Approx. 5×10^7	0.296	60
		5×10^6	0.020	60
		5×10^5	0.000	60

Reaction Mixture:

0.5 ml bacteria in H_2O
1.0 ml in 0.7 M Tris buffer, pH 9.0
0.5 ml of 1% lactate
0.4 ml MTT, 0.5 mg/ml

TABLE 54

MTT TETRAZOLIUM REDUCTION BY BACILLUS GLOBIGII

Expt. No.	Bacterial No. cells/ml	Substrate	Absorption at 570 mμ
1	3.6×10^7	Lactate	0.36
2	2.5×10^6	Lactate	0.266
3	8.3×10^8	Succinate	0.33
	8.3×10^8	Succinate	0.024
4	5×10^8	Lactate	0.52
	5×10^7	Lactate	0.05

Reaction Mixture:

0.5 bacteria in H_2O
1.0 ml of 1% lactate in 0.7 M Tris buffer, pH 9.0.

TABLE 55

EFFECT OF ADDITION OF LACTIC DEHYDROGENASE (LDH) AND DIAPHORASE ENZYMES TO MTT REDUCTION BY MICROORGANISMS

Culture	O.D. of MTF at 570 mμ No. Enzymes	Enzymes *	% Increase due to Enzymes
BG	0.046	0.070	50
15	0.037	0.050	45
H_2O Bottom Extract (Pseudomanas)	0.043	0.065	50

Reaction Mixture:

0.5 ml Bacteria
1.0 ml of 0.2 M Lactate in 0.7 M Tris buffer, pH 9
0.4 ml MTT, concentration 0.5 mg/ml

* 0.07 mg LDH and 0.21 mg Diaphorase added to each ml of lactate solution.

The reaction mixtures consisted of 0.5 ml bacteria in H_2O, 1.1 ml of 0.2 M lactate in 0.7 M Tris buffer, PH 9.0, containing 0.07 mg lactic dehydrogenase (LDH) and 0.21 mg diaphorase, and 0.4 ml of MMT, 0.5 mg/ml. Incubation was for 30 minutes at room temperature. This reaction mixture was compared with the reduction obtained from the same mixture without the enqymes using whole cells of B. globigii. The MTF formed was extracted with 4 ml JP-4, and the absorption of the formazan was measured at 570 mu. The results in Table 55 show that the addition of enzymes increased MTT reduction by 50%.

Several bacteria were tested with the cyclic enzyme system in an attempt to correlate MTT reduction to cell concentration. The results in Table 56 indicate that the effective amount of DPN reacting varies with the bacterial species and possibly with the growth history of the organism.

Several methods of lysis of the bacteria were tested in an attempt to free the DPN for reaction. The results in Table 57 show that none of the methods tested (heat, heat and acid, sonication, and toluene) increased tetrazolium reduction. Lysozyme treatment followed by sodium lauryl sulfate inhibited reduction under the one condition tested.

The conclusion from most of the studies with the coupled enzyme approach is that cell breakage does not appreciably increase MTT reduction. This could be interpreted to mean that DPN is not released in a utilizable form by the cell-breaking techniques or that most of the free DPN in the whole cells are available to the cyclic enzyme system.

The tests run with Millipore filters indicate that extraction of the formazan from Millipore filters is less complete than extraction from aqueous solution. The cyclic enzyme reaction was next set up to test the feasibility of using the MTT reduction on a Millipore filter as a spot test. The reaction was run on filtered bacteria by adding the reaction mixtures in aliquots to the Millipore filters. A visible violet color developed after 1 hour on filters containing 10^8 cells and higher. Fewer cells than this gave no response. Therefore, the spot test technique was found to be one order of magnitude more sensitive than the corresponding solution test.

TABLE 56

MTT REDUCTION BY VARIOUS MICROORGANISMS

Culture	Age	Concentration Total Cells	Absorption at 570 mμ
Bacillus globigii	1 day	3.8×10^7	0.13
		3.8×10^6	0.062
		3.8×10^5	0.0
		3.8×10^4	0.0
15	1 mo	1.6×10^8	0.057
		1.6×10^7	0.007
22	1 mo	4.5×10^6	0.018
		4.5×10^6	0.00
H_2O Extraction from Fuel-Water Bottom (Isolated Pseudomonas)	1 mo	1.9×10^8	0.07
		1.9×10^7	0.007

Reaction Mixture:

0.5 ml bacteria in H_2O
1.0 ml of 0.2 M lactate, 0.07 mg LDH, and 0.21 mg Diaphorase in 0.7 M Tris buffer, pH 9.0
0.4 ml MTT, 0.5 mg/ml

TABLE 57

EFFECT OF LYSIS TREATMENTS OF ORGANISMS ON MTT REDUCTION

).	Culture	Lysis Treatment	O.D. at 570 mμ
1	B.g.	None (Control for 2-7)	0.064
2	B.g.	102 toluene added, shaken 60 seconds	0.02
3	B.g.	102 toluene added, shaken 5 minutes	0.02
4	B.g.	0.1 ml of acetic acid, 100°C for 60 seconds	0.03
5	B.g.	0.1 ml of 0.1 N acetic acid, 100°C for 5 minutes	0.02
6	B.g.	100°C for 60 seconds	0.00
7	B.g.	100°C for 5 minutes	0.01
8	B.g.	None (Control for 9)	0.266
9	B.g.	Sonication for 2 minutes	0.00
0	B.g.	None (Control for 12)	0.07
1	515	None (Control for 15)	0.050
2	S22	None (Control for 16)	0.018
3	H_2O Bottom Extract	None (Control for 17)	0.065
4	B.g.	Solid Lysozyne Added	In all cases no MTT reduction was observed. On addition of JP-4 and shaking, an emulsion was formed.
5	S10	Shaken for 1 minute, 0.1 ml sodium laurylsulfate added for 1 minute, then MTT reaction mixture added.	
6	S22		
7	H_2O Bottom Extract		

te: 1 ml of 0.2 M lactate and 0.07 mg LDH, 0.2 mg diaphorase dissolved in 0.7 M Tris buffer, pH 9.0, plus 0.4 ml MTT, 0.5 mg/ml were added for MTT reduction.

To determine the effect of growth time on the MTT reduction, a series of daily tests were run on jet-fuel Culture 101. The culture was grown on Medium 2. Samples were harvested and washed each day then resuspended in distilled H_2O. The cells were reacted each day with the above-mentioned cyclic enzyme reaction mixture for 30 minutes. The results are shown in Table 58. There appears to be no correlation between cell concentration and O.D. However, in all cases, the 10-fold dilution of stock 101 gave a higher O.D. than would be expected if one assumed a linear relationship. This observation points to the likelihood that not all MTF formed is being extracted by the JP-4. This hypothesis was tested by running 30-minute reactions, extracting formazan with 4 ml of JP-4, then making a second extraction with 4 ml JP-4. The O.D. readings for these extractions are listed in Table 59.

It appears that, in the presence of organisms, the extraction efficiency of the JP-4 decreases when larger amounts of formazan are produced in the reaction.

It has been observed in the literature that tetrazolium reduction may be, in some cases, increased under anaerobic conditions. MTT reduction by B. globigii and Culture 101 was tested under aerobic conditions and under anaerobic conditions. Anaerobic conditions were produced by bubbling the reaction mixture with nitrogen. The results in Table 60 show that MTT reduction under anaerobic conditions was approximately one-half that observed under aerobic conditions.

TABLE 58

EFFECT OF GROWTH TIME OF STRAIN 101 ON REDUCTION OF MTT

Age of Culture	Cell Concentration	O.D. at 570 mμ	Conc. MTF in mg/ml
1 day	1.75×10^9	0.77	0.023
	1.75×10^8	0.14	0.004
2 days	3.5×10^9	0.47	0.014
	3.5×10^8	0.068	0.002
3 days	0.5×10^8	0.378	0.011
	0.5×10^7	0.0	0.0
4 days	3.2×10^9	1.035	0.031
	3.2×10^8	0.158	0.004

Reaction Mixture:

0.5 ml bacteria in H_2O
1.0 ml 0.2 M lactate, 0.07 mg diaphorase in 0.7 M Tris buffer, pH 9.0
0.4 ml MTT, 0.5 mg/ml

TABLE 59

EFFICIENCY OF MTF EXTRACTION BY JP-4 FUEL

Organism	Extraction 1	Extraction 2	% MTF in Extraction 1
B.g.	0.09	0.04	70
101	0.77	0.64	55

Reaction Mixture:

0.5 ml bacteria in H_2O
1.0 ml of 0.2 M lactate, 0.07 mg LDH, 0.2/mg diaphorase in 0.7 M Tris buffer, pH 9.0
0.4 ml MTT, 0.5 mg/ml

TABLE 60

EFFECT OF ANAEROBIC CONDITIONS ON MTT REDUCTION

Culture	O.D. Aerobic Reaction	O.D. Anaeorbic Reaction
B.g.	0.10	0.04
101	0.255	0.14

Reaction Mixture:

0.5 ml bacteria in H_2O
1.0 ml of 0.2 M lactate in 0.7 M Tris buffer, pH 9.0
0.4 ml MTT, 0.5 mg/ml

6. Gas Chromatography of Lipids

The fatty acid composition of bacteria was analyzed because of the preponderance of lipid material in most bacteria, the ease with which the fatty acids may be analyzed, and the great sensitivity achieved in gas chromatographic analysis of these acids.

The procedure used in evaluating this approach for sensitivity for detecting jet-fuel microorganisms was as follows: (1) centrifugation and drying of the microorganisms, (2) methylation of a portion of the bacteria, and (3) analysis of an aliquot of the solution for fatty acid methyl esters by means of gas chromatography.

a. Procedures

Gas Chromatographic Equipment: A Barber-Coleman Model 61-C chromatograph equipped with a tritium foil argon diode detector was used for all fatty-acid analyses. A 25% coating of diethylene glycol succinate was evaporated onto 80/100 mesh Chromosorb W. This packing was filled into a 10-foot x 1/16-inch ID aluminum column and used for all analyses. Column temperature was maintained at 170°C and the detector temperature was maintained at 220°C. Argon carrier gas was used at a flow-rate of 25 ml per minute through the column. An additional purge of 25 ml of argon per minute was used in the detector. A sensitivity setting of 100 and an attenuation of X64 were used as the standard sensitivity (referred to as 1X in the report), since the noise level was slightly less than 1% at this setting when the detector was operated at 800 volts.

Fatty Acid Compositional Analyses: Fatty acid methyl ester samples were injected with a 10 µl capacity Hamilton syringe. Most of the solvent was vented to the atmosphere for 30 to 40 seconds before the analysis was begun. This venting procedure allowed much larger samples to be introduced without disrupting the column or detector. The injection part was then rapidly heated to 250°C in order to vaporize the fatty acid methyl esters. The time at which the injection port reached 150°C was arbitrarily chosen as time zero, and all peaks were measured from this point. Like all gas chromatographic columns, each compound was eluted at a certain specific time with the most volatile compounds being eluted first. In addition, mono-olefins, and diolefins were retained longer than their saturated normal fatty acid homologs.

A known prepared standard fatty acid methyl ester sample consisted of the following fatty acids made up to a concentration of 2% with methanol; approximately 0.2 of each was included:

C_8	Caprylate	C_{18}	Stearate
C_{10}	Caprate	$C_{18:1}$	Oleate
C_{12}	Laurate	$C_{18:2}$	Linoleate
C_{14}	Myristrate	$C_{18:3}$	Linolenate
C_{16}	Palmitate	C_{20}	Arachidate

and was used to calibrate the detector response and column retention characteristics. A straight line relationship was obtained when the fatty acid carbon content was plotted against the logarithm of the retention time. This plot was used to assign equivalent carbon numbers to unknown bacterial peaks for identification purposes.

Methylation Procedures: All lipid samples were transesterified with methanol before being analyzed for fatty-acid composition. Boron trichloride was used as the catalyst for earlier work.

Briefly, this method consists of refluxing the bacteria sample with methanol in the presence of BCl_3. The resulting fatty-acid methyl esters are dissolved in ethyl ether and water washed. This method was found to work quite well for large samples (over 100 mg), but the many handling procedures caused serious losses when small samples were used.

All bacteria samples analyzed during this period were methylated by a new procedure, which required a minimum number of transfers. This method consisted of weighing the bacteria sample, usually 0.1 to 1.0 mg., into a 1/8 inch O.D. by 5 inch glass tube closed at one end. Fifty microliters of 10% HCl in methanol were added, and the tube was sealed. It was then heated for 3 hours in boiling water and opened. A mixture of free fatty acids, methyl esters, and nonlipid material was expected at this time. The relative amounts of methyl esters and free fatty acids depends on the quantity of water originally present. Fifty microliters of chloroform and a known aliquot, usually 1 to 5 microliters were analyzed. In most samples, it was found helpful to add an equal quantity of water to the chloroform solution. This helped to reduce the amount of more volatile, nonlipid components eluting at the beginning of a chromatogram.

b. Fatty Acid Composition of Various Bacteria

The fatty acid composition of a number of bacteria samples was determined (Table 61). The identity of the fatty acids was inferred from the retention time of the fatty acid peaks when compared to known fatty acid retention times. The first number refers to the total carbon chain length, and the second number refers to the number of unsaturated bonds.

TABLE 61

FATTY ACID COMPOSITION OF VARIOUS BACTERIA

	Bacteria, Melpar Stock Culture Number		
Fatty Acid	0-8	0-9	0-10
11:0			0.1
11:1			1.4
12:0	2.3	13.8	5.5
12:1	2.2		
13:0	7.7	1.6	
13:1	0.9		2.7
14:0	1.3	1.0	1.4
14:1	3.6	1.1	
15:0	3.5	0.2	0.4
15:1	2.9		
16:0	21.7	28.5	31.6
16:1	0.6	13.9	8.1
17:0	2.3	8.4	0.8
17:1	1.6	0.3	
18:0	2.6	9.0	7.5
18:1	31.8	22.9	40.2
19:0	9.4		
20:0	5.4		
Wt. (mg.)	1.5	13.6	4.9
% F.A.	2.9	3.0	2.3

TABLE 61 (Continued)

Bacteria, Melpar Stock Culture Number

Fatty Acid	0-12	0-13	0-14	0-15	0-1	0-4	0-16	0-17
11:0		0.5			13.1		2.7	
11:1	2.3	0.9					0.9	
12:0	0.6	7.6		2.6			0.1	0.6
12:1	15.7		9.2	0.9			0.3	12.0
13:0	0.3	1.0	4.9	4.1	0.6		2.8	
13:1	0.2	5.3	10.2	0.6			1.3	0.6
14:0	3.2	3.4	0.8	0.4	2.6		0.2	4.3
14:1	1.5	1.0	0.2	2.1	1.0	7.4	10.9	
15:0	1.2	0.7	4.2	2.6	1.4	2.0	2.5	1.1
15:1	0.3	0.8	0.9	14.1	0.1	0.5	5.5	0.6
16:0	19.1	21.7	20.7	27.6	22.2	30.0	31.1	23.4
16:1	18.8	7.9	2.9	12.8	15.9	3.0	8.8	6.3
17:0	5.9	0.5	2.0	0.9	5.6	0.7	2.7	5.7
17:1	0.7	3.6	1.5	1.3		5.0	11.8	
18:0	11.4	8.5	1.2	1.1	2.8	3.0	3.4	4.3
18:1	18.5	15.9	23.5	27.2	30.7	23.6	13.1	40.2
18:2		2.7						
19:0		4.1	2.4			8.8	1.6	
19:1		2.0	11.6		0.7	5.5		0.6
20:0		11.6	2.3			3.7		
20:1			1.1		2.3			
21:0						6.6		
21:1					1.0			
Wt. (mg.)	5.8	0.4	3.5	2.9	2.3	7.1	2.3	4.7
% F.A.	1.4	2.9	7.1	3.0	6.5	3.0	6.3	1.1

TABLE 61 (Continued)

Bacteria, Melpar Stock Culture Number

Fatty Acid	0-19	0-21	0-25	0-26	0-28
12:0	10.2	4.0	0.6		
12:1	18.2			3.3	
13:0		10.7			
13:1	3.1			1.1	
14:0	9.8	22.1	4.8	1.7	1.7
14:1	0.2			2.9	
15:0	0.5	2.6		4.9	4.5
15:1	2.0	4.0		0.1	
16:0	15.6	22.8	39.5	22.7	34.7
16:1	10.5	15.2	9.2	20.7	9.4
17:0	5.6	4.9	7.9	6.3	2.8
17:1	2.7	2.6	1.1		2.1
18:0	2.7	2.0	1.1	3.0	0.4
18:1	8.6	2.3	29.3	33.2	41.6
19:0	2.1	0.6			
19:1	0.7		6.4		2.7
20:0	3.0	3.6			
20:1	1.1	0.6			
21:0	0.8	0.8			
21:1	2.8	1.1			
Wt. (mg.)	4.4	3.6	22.1	5.4	16.4
% F.A.	3.8		3.0		

TABLE 61 (Continued)

Bacteria, Melpar Stock Culture Number

Fatty Acid	0-24	0-31	0-34
12:1		3.1	6.5
14:0		3.6	2.4
14:1	0.2		1.8
15:0	2.4	0.1	
15:1			1.1
16:0	16.1	36.0	29.3
16:1	12.5	10.8	19.7
17:0		3.3	5.7
18:0	8.9	8.4	6.9
18:1	19.5	34.5	26.6
19:1	0.7		
21:1	4.2		
24:0	35.5		
Wt. (mg.)	6.6	9.5	2.8
% F.A.	3.5	2.5	1.4

7. Carbon Dioxide Indicating Flasks

George[40] previously described a method of detecting growth of microorganisms by measuring the CO_2 evolved by microorganisms during growth in fuel media. The evolved CO_2 was absorbed in Indicating Soda Lime (Mallinckrodt Chemical Works, St. Louis) in small columns inserted in closed systems.

No attempt was made by George[40] to quantitate the technique. Thus, its use for detection of microbial growth in jet fuel would require standardization. Three flasks were set up, each containing a known amount of Na_2CO_3. Inserted in the top of each was a small column (1.5 mm X 100 mm) packed with indicating soda lime. The entire system was sealed, leaving only a small opening in the top, covered by a rubber septum by which HCl could be added to liberate carbonic acid.

Carbon dioxide of HCl was generated by adding 2×10^3 μ moles, 1×10^4 μ moles and 2×10^4 μ moles, respectively, to flasks containing Na_2CO_3, 1×10^3 μ moles, 5×10^3 μ moles, and 1×10^4 μ moles.

The flasks were opened after 24 hours, and the columns removed. The color change was measured in millimeters and plotted against CO_2 concentration.

The standard curve shown in Figure 52 demonstrates that the color change in the indicator in 24 hours is proportional to the CO_2 concentration. It would appear from these results that the technique might be considered as a means for detecting microbial growth in fuel.

When the columns were placed approximately 50 mm over a week old mixed culture in 500 ml of Medium 1, a definite change of 2 mm was observed in 24 hours. The Fernback flasks were tightly stoppered and contained 1.5×10^8 cells/ml. The 2-mm color change would correspond to approximately 6×10^2 μ moles of CO_2. Difficulties were encountered with wetting of the columns after 2 dyas. The reasons for this difficulty were not investigated because other detection techniques appeared to be better.

8. O_2 Utilization

Several methods were employed to test for oxygen utilization of organisms grown on fuel. Most successful measurements were made with a commercial polarographic oxygen analyzer, manufactured by Beckman Instruments, Inc. The analyzer was calibrated for oxygen content of water using a Winkler titration.[41] Scale readings of the oxygen analyzer were expressed following calibration in parts per million of O_2.

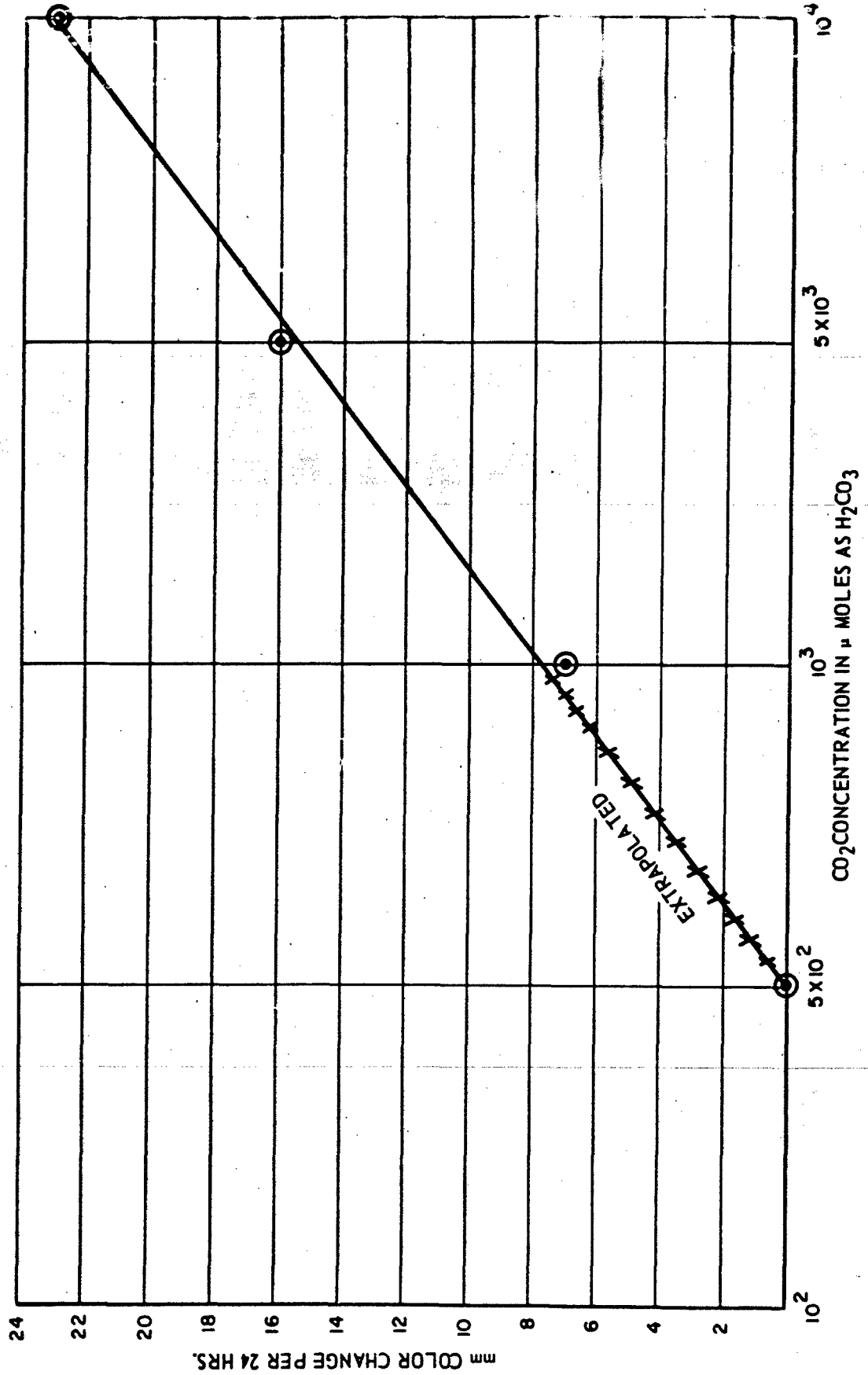

Figure 52. MM Color Change vs. CO_2 Concentration
Conditions: Carbon dioxide was generated by adding $2 \times 10^3 \mu$ moles, $1 \times 10^4 \mu$ moles, and $2 \times 10^4 \mu$ moles of HCL respectively to flasks containing Na_2CO_3; $1 \times 10^3 \mu$ moles, 5×10^3 moles, and $1 \times 10^4 \mu$ moles, A column (1.5 mm x 100 mm) packed with indicating soda lime was inserted through a cork stopper into the flasks, the system closed, and all flasks incubated at 30°C.

The oxygen sensor unit was inserted in 500 ml of Medium 2 overlaid with 500 ml of JP-4 fuel. The water-fuel solution was equilibrated 24 hours at 30°C; approximately 50 ml of water-bottom inoculum were added, and the system was sealed past the sensor with a cork stopper. A magnetic stirrer maintained continuous flow of the sample. The system was constantly monitored by use of a recorder during each of the four experiments carried out (Figure 53).

Run 1 was conducted by placing the oxygen sensor in the fuel layer of the BH without nitrate medium, and the flask was plugged with cotton so that the amount of air entering the system was not restricted. In Run 2 the sensor was placed in the water layer of the medium, and the system was closed but not sealed, thus allowing restricted air to reach the sample. The third and fourth Runs were closed completely, with 25 ml of inoculum for Run 3 and 50 ml of inoculum for Run 4. The sensor in Runs 3 and 4 was in the water layer.

The results in Figure 53 show that no changes in the oxygen concentration of the fuel layer occurred during growth of the water-bottom inoculum (Run 1). Changes in oxygen concentration did occur, however, in the water layer under only partially closed conditions (Run 2). In completely closed systems, the rate of oxygen depletion in the medium was a function of the inoculum size. With 50 ml of water-bottom inoculum, about half as much time (theoretically 40 hours) was required for depletion as was required for a 25-ml inoculum.

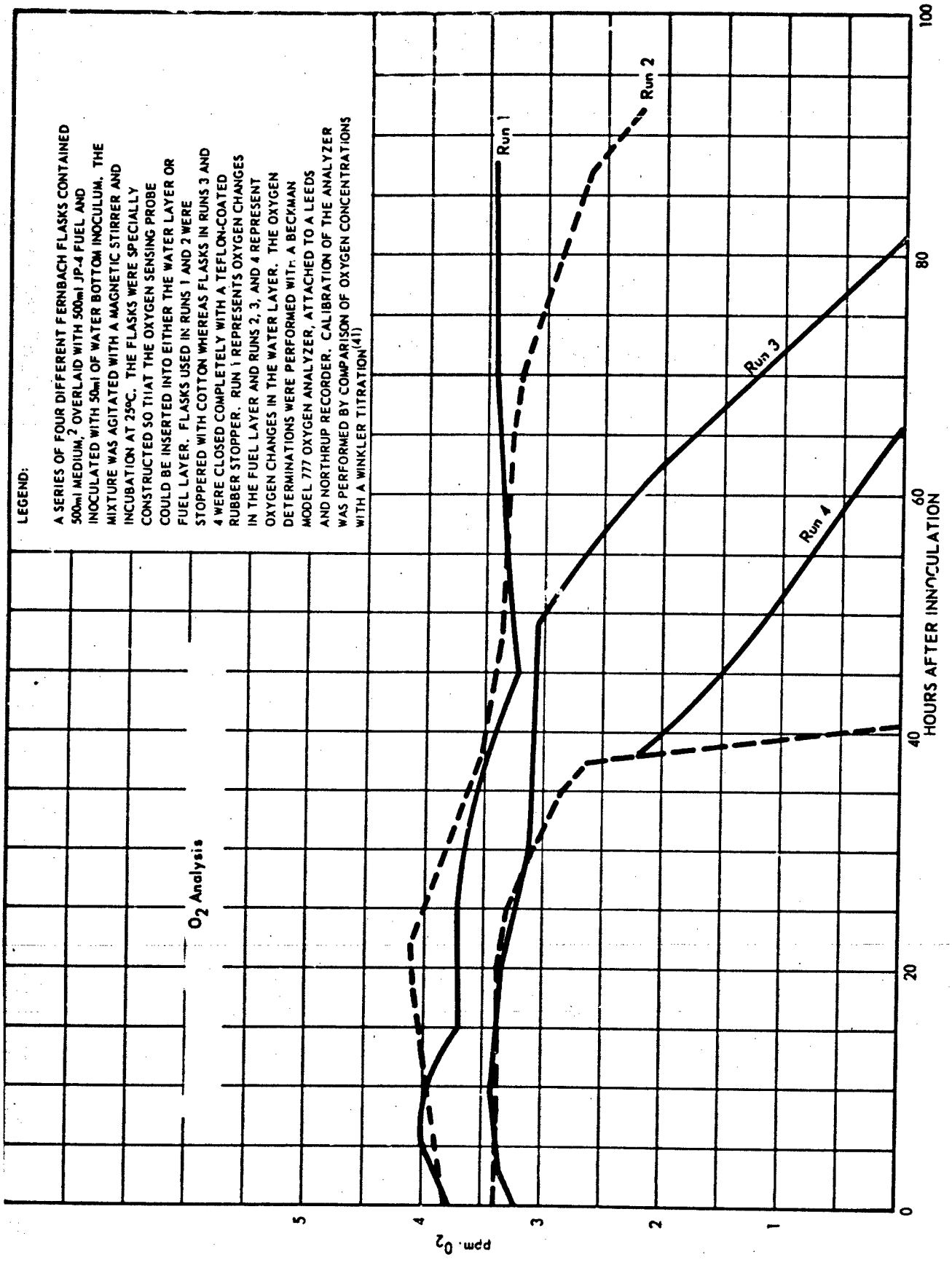

Figure 53. Changes in Oxygen Content during Growth of Water-Bottom Culture

SECTION IV

CONCLUSIONS

The first conclusion that can be drawn from this study is that microorganisms in JP-4 jet fuel-water systems, in the absence of anti-icing additive ethylene glycol monomethyl ether (EGME), can oxidize jet fuel hydrocarbons and produce microbial cells (growth), emulsions, sludges, fuel-soluble substances and conditions, and compounds which cause the corrosion of aluminum alloys. The mechansims by which microorganisms produced these conditons and compounds during their growth on jet fuel were defined and elucidated. These mechanism studies were particularly rewarding, since they revealed that short-chain olefins, C_6 to C_9, particularly hexene, and xylene were lethal to the jet fuel cultures when present at 5% concentration in the fuel. Although this method of controlling organisms in fuel-water bottoms is not presently needed, it would be a convenient and a compatible additive for the jet fuel systems if it were ever needed.

There is the possibility that microorganisms which will utilize the present additive, EGME, will arise eventually in the fuel systems by natural mutation or by artificially induced mutation. It is not inconceivable that mutant microorganisms could develop to utilize these rather simple molecules especially since microbes have adapted to much worse conditions (e.g., growth in sulfuric acid at pH 1, growth in the hot sulfur springs, dependence of some microorganisms on antibiotics for growth) and since it is already known that Gluconobacter melanogenus,[42] Acetobacter ascendens, and Acetobacter aceti[43] grow on ethylene glycol. Kausbal and Walker[44] have reported that A. ascendans grows on a synthetic medium with ethylene glycol as sole carbon source with the formation of a thick pellicle, which most likely contains cellulose.

Very little work has been done with ethylene glycol monomethyl ether. However, it was shown that Gluconobacter suboxygdans oxidizes the compound.[45] Therefore, the present conclusion is that a concentrated research effort on the screening of acetic acid bacteria and on artifical induction of mutations in these microbes would lead to strains that readily utilize these substrates in the presence of fuel. This research effort obviously would be worthwhile by the enemy, for it has the potential of incapacitating the defense of this country if these organisms were introduced into the air and eventually found their way into fuel storage tanks and aircraft. Thus, the alternate method of controlling microbial growth in fuels would be useful under these conditions. Also, research studies should be performed on the mechanism of oxidation of EGME, since inhibition of this reaction by specific metabolic inhibitors could then be used to control the growth of EGME-oxidizing microorganisms should they develop.

Another important finding in this study concerned the mechanism of corrosion by microorganisms and methods for preventing corrosion. In this

study it was found that 12 millimolar concentration of nitrate protected aluminum alloys from corrosion, not only by microorganisms but also by natural water bottom from Andrews Air Force Base. The natural water bottom was corrosive to both iron and aluminum alloys. Upon analysis the water bottom was found to contain 17% EGME, and Sephadex fractionation showed corrosion only in the fractions containing the additive. Therefore, it is possible that the corrosion that occurs occasionally in aircraft wing tanks is due to the additive itself or to other inducers of corrosion such as micro-rust particles carried-over from storage tanks into wing tanks. Although the present approach is to coat the wing tanks, a better, cheaper, and easier solution, which would result in less weight, might be the addition of simple inorganic additives, e.g., nitrate to fuel storage tanks and aircraft wing tanks. A study of the best corrosion inhibitor for fuel storage tanks would have to be done, since the effect of nitrate on iron corrosion has not been determined. It is anticipated, however, that nitrite or possibly nitrate would easily control the rust in iron tanks.

Except for methods of controlling growth of fuel microorganisms, no methods for preventing emulsion formation or sludge formation were found. Although some of the oxidation products of hydrocarbons that result in emulsion were identified, the total composition of fuel was not tested because of its complexity. Therefore, the present solution for controlling the production of these microbial products appears to be inhibition of growth.

A variety of detection methods for microorganisms were explored and almost any one of them would have been suitable for detecting microorganisms in fuel-water bottoms when the contract began. However, since that time the problem has become much more difficult, since the numbers of organisms present in the water bottoms has decreased from around 10^6 to 10^8 cells/ml to 10^3 to 10^4 cells/ml by the addition of the anti-icing additive, EGME, and better housekeeping techniques. Therefore, only three methods were found which show potential for development into instrumental approaches for monitoring the microbial concentration of water bottoms.

Although the recommended rapid detection method proposed herein is based on visual counting of stained organisms on a filter, this is not the method best suited for simple instrumentation. The best method for instrumentation utilized filters for collecting and concentrating the microorganisms and for processing; however, detection is based upon a dissociation reaction for separating dye from the stained organisms. The present data indicates that the use of an acridine orange staining approach can be instrumented into a simple rugged instrument that will automatically determine microbial cell concentration by a nonscanning technique.

SECTION V

RECOMMENDATIONS

A. Future Work

(1) Continue the detection studies with the objective of developing a microbial detector which will operate automatically using a microbial filter for collection and concentration of the organisms present in water bottom samples and for processing. Detection will be by the method of least cost providing it will detect 10^4 microorganisms in 10 minutes and not show any background interference.

(2) Initiate a study to determine if the addition of nitrate to fuel storage tanks and to aircraft wing tanks protects them from corrosion by the anti-icing additive and other corrosive substances in the fuel. This program should first determine if KNO_3 is a more effective inhibitor of steel corrosion than KNO_2. The most inhibitory compound of the two should be tested in the fuel storage tanks.

B. Rapid Detection of Bacteria Technique

The following is a recommended technique for the rapid detection of bacteria using simple procedures and routine laboratory equipment:

(1) Filter water-bottom sample (50 ml) through a 25-mm glass fiber filter, Type A, purchased from Gelman Instrument Company, Ann Arbor, Michigan. This filter removes the large particulate materials and allows bacteria to pass through.

(2) Filter microorganisms through 0.45-micron, 25-mm HAWG Millipore filter, purchased from Millipore Filter Corporation, Bedford, Mass.

(3) Add 1 ml of fluorescein isothiocyanate (FITC), 0.2 mg/ml in carbonate buffer pH 9.0, to the filter and allow to react for 1 minute. The fluorescein isothiocyanate was purchased from Baltimore Biological Laboratories, Baltimore, Md.

(4) Draw the FITC through the filter and wash the filter with a 10-ml soltuion containing two parts of 0.01 N KOH and one part butanol. This step removes most of the color from the filter but leaves the bacteria brightly stained.

(5) Dry and mount the filter on the slide.

(6) Clarify the filter with oil and scan and count the fluorescent particles in 10 fields.

It should be possible to carry out the entire procedure in 25 to 30 minutes. The entire procedure would include:

(1) Pre-filtration of 10 ml of water-bottom sample -- 1 minute.

(2) Filtration through a 0.45-micron filter -- 1 minute.

(3) Staining with FITC -- either 1 minute or 5 minutes.

(4) Wash with butanol-KOH -- 5 minutes.

(5) Dry and mount filter on slide -- 10 minutes.

(6) Count organisms in 10 fields -- 5 minutes.

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APPENDIX:

Laboratory Media

TABLE 62

COMPOSITION OF MEDIA USED FOR GROWTH OF HYDROCARBON-OXIDIZING MICROORGANISMS

Salts Solution, A	Salts Solution, B
0.2g $MgSO_4 * H_2O$	0.2g $MgSO_4$
1.0g KH_2PO_4	0.1g KH_2PO_4
1.0g K_2HPO_4	0.1g K_2HPO_4
0.02g $CaCl_2$	0.02g $CaCl_2$
0.05g $FeCl_3$	0.05g $FeCl_3$
1000 ml distilled water	1000 ml distilled water

Hydrocarbon overlay was 10% by volume. Salts solutions were sterilized by autoclaving. Hydrocarbons were sterilized by Millipore filtration.

(Continued on the next page)

TABLE 62 (continued)

Medium	Salt Solution	Nitrogen Source	(grams per liter)	Carbon Source
1*	A	NH_4NO_3	1.0	Jet fuel JP-4
2	A	NH_4Cl	1.0	Jet fuel JP-4
3	A	KNO_3	0.02	Jet fuel JP-4
4	A	KNO_3	0.06	Jet fuel JP-4
5	A	KNO_3	0.08	Jet fuel JP-4
6	A	KNO_3	1.2	Jet fuel JP-4
7	A	NH_4CL	0.67	Purified hydrocarbons (see Table 3)
8	A	KNO_3	1.0	Purified hydrocarbon
9	A	$(NH_4)_2 SO_4$	1.C	Jet fuel JP-4
10	A	None	0	Jet fuel JP-4
11	A	KNO_3	0.04	Jet fuel JP-4
12	B	None	0	Jet fuel JP-4
13	B	KNO_3	0.02	Jet fuel JP-4
14	B	KNO_3	0.04	Jet fuel JP-4
15	B	KNO_3	0.06	Jet fuel JP-4
16	B	KNO_3	0.08	Jet fuel JP-4
17	B	KNO_3	1.2	Jet fuel JP-4

*Bushnell and Haas (1941) [1]

ARTIFICIAL SEA WATER[8]

Stock Solution No. 1

Dissolve the indicated amounts of the following salts in the distilled water and dilute to a total volume of 7.0 liters:

$MgCl_2 \cdot 6H_2O$	3,889.0 grams
$CaCl_2$(anhydrous)	405.6 grams
$SrCl_2 \cdot 6H_2O$	14.8 grams

Stock Solution No. 2

Dissolve the indicated amounts of the following salts in distilled water and dilute to a total volume of 7.0 liters:

KCl	486.2 grams
NaHCO3	140.7 grams
KSr	70.4 grams
H_3SO_3	19.0 grams
NaF	2.1 grams

Preparation:

Dissolve 245.34 g of NaCl and 40.94 g of anhydrous Na_2SO_4 in 8 to 9 liters of distilled water. Add slowly with vigorous stirring 200 ml of stock solution No. 1, then 100 ml of stock solution No. 2. Dilute to 10.0 liters. Adjust the pH to 7.2 with 0.1N NaOH solution.

STONE-FENSKE SALT SOLUTION[7]

$CaCo_3$	5.0 grams
NH_4NO_3	1.5 grams
$Na_2HPO_4 \cdot 7H_2O$	1.0 grams
KH_2PO_4	0.5 grams
$MgSO_4 \cdot 7H_2O$	0.5 grams
$MaCl_2 \cdot 4H_2O$	0.2 grams
Distilled water	1000.0 ml

LEADBETTER'S SALT SOLUTION[9]

$NaNO_3$	2.0	grams
$MgSO_4 \cdot 7H_2O$	0.2	grams
$FeSO_4 \cdot 7H_2O$	1.0	grams
Na_2HPO_4	0.21	grams
$Na\ H_2PO_4$	0.09	grams
Cu (as $CuSO_4 \cdot 5H_2O$)	5.	micrograms
B (as H_3BO_3)	10.	micrograms
Mn (as $MnSO_4 \cdot 5H_2O$)	10.	micrograms
Zn (as $ZnSO_4 \cdot 7H_2O$)	70.	micrograms
Mo (as MoO_3)	10.	micrograms
KCl	0.04	grams
$CaCl_2$	0.015	grams
Distilled water		

POTATO DEXTROSE BROTH

Potato dextrose broth consists of the water infusion of 200 grams of potatoes boiled until soft:

20 grams glucose
0.5g Na_2HPO_4
Distilled water to 1 liter

MALT EXTRACT AGAR[6]

Distilled H_2O	1000	ml
Malt Extract	20.0	grams
Peptone	1.0	grams
Dextrose	20.0	grams
Agar	20.0	grams

pH 5.0 - 5.5

*9K SILVERMAN[4]

$(NH_4)_2SO_4$	3.0	grams
KCl	0.1	grams
K_2HPO_4	0.5	grams
$MgSO_4 \cdot 7H_2O$	0.5	grams
$Ca(NO_3)_2$	0.015	grams
$FeSO_4 \cdot 7H_2O$	44.2	grams
$10NH_2SO_4$	0.1	ml

pH 3.0 - 3.6

SHARPLEY'S MEDIUM[2]

Sodium Lactate	4.0	grams
Yeast Extract	1.0	grams
Absorbic Acid	0.1	grams
$MgSO_4 \cdot 7H_2O$	0.2	grams
$Fe(SO_4)_2(NH_4)_2 6H_2O$	0.1	grams
NaCl	10.0	grams
Agar	20.0	grams
Distilled H_2O	1000	ml

pH 7.0 - 7.5

BEEF EXTRACT AGAR[3]

Beef Extract	0.5	grams
Agar	20.0	grams
Tap H_2O	1000	ml

pH 7.0 - 7.2

*Agar plates of this medium were not prepared since hydrolysis of the agar occurs during sterilization when pH of the medium is below 5.

TGY AGAR[6]

Tryptone	5.0 grams
Yeast Extract	5.0 grams
Glucose	1.0 grams
K_2HPO_4	1.0 grams
Agar	20.0 grams
Tap H_2O	1000 ml

pH 7.0

(TGY broth contained 5% glucose and no agar)

DEEP LIVER MEDIUM[6]

Liver Extract	100 ml
Yeast Extract	5.0 grams
Tryptone	10.0 grams
K_2HPO_4	2.0 grams
Glucose	5.0 grams
Agar	20.0 grams

pH 7.4

ASPARAGINE DEXTROSE[6]

Tap H_2O	1000 ml
Asparagine	0.5 grams
K_2HPO	0.5 grams
Beef Extract	2.0 grams
Agar	17 grams
Dextrose	1.0 grams

pH 6.8 - 7.0

THIOBACILLUS[5]

(a) K_2HPO_4	2.0 grams
$CaCl_2$	0.1 gram
$MgSO_4$	0.1 gram
$MnSO_4$	trace
$FeSO_4$	trace
Tap H_2O	900 ml
Agar	15 grams

pH 7.8

Sterilize in Separate Flasks:

(b) $Na_2S_2O_3$	10 grams
Tap H_2O	50 ml
(c) $(NH_4)_2SO_4$	0.1 gram
Tap H_2O	50 ml

At time of inoculating add 5 ml aseptically of each (b) and (c) to (a).

RECOMMENDED MEDIA FOR CULTIVATION OF PSEUDOMONAS AND HORMODENDRUM

Based on the data, the recommended media for cultivation of Pseudomonas and Hormodendrum using JP-4 as the sole carbon source are set forth below:

Pseudomonas Medium		Hormondendrum Medium	
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.4 g	Sodium nitrate ($NaNO_3$)	4.0 g
Calcium chloride ($CaCl_2$)	0.02 g	Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	2.0 g
Potassium phosphate, monobasic (KH_2PO_4)	2.0 g	Potassium chlcride (KCl)	0.080 g
Potassium phosphate, dibasic (K_2HPO_4)	2.0 g	Manganese sulfate ($MnSO_4 \cdot H_2O$)	0.030 g
Ammonium nitrate (NH_4NO_3)*	0.286 g	Calcium chloride ($CaCl_2$)	0.040 g
Copper sulfate ($CuSO_4 \cdot 5H_2O$)	0.027 g	Potassium phosphate, monobasic (KH_2PO_4)	0.120 g
Iron powder	4.0 g	Potassium phosphate, dibasic (K_2HPO_4)	0.120 g
Distilled water	1000 ml	Talc	2.0 g
Adjust to pH 6.8-7.0		Distilled water	1000 ml
		pH 6.3	

*The use of nitrate is sometimes considered undesirable, particularly for corrosion studies. The ammonium nitrate in the above medium may be substituted by the following nitrogen sources but at some sacrifice in growth:

Ammonium phosphate, 1:1 ratio mono-dibasic	0.599 g
or	
Ammonium sulfate	0.471 g

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Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)	2a. REPORT SECURITY CLASSIFICATION
Melpar, Inc. 7700 Arlington Boulevard Falls Church, Virginia	UNCLASSIFIED 2b. GROUP

3. REPORT TITLE

MECHANISM OF MICROBIOLOGICAL CONTAMINATION OF JET FUEL AND DEVELOPMENT OF TECHNIQUES FOR DETECTION OF MICROBIOLOGICAL CONTAMINATION.

4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*

Final Report for work done 1 March 1963 to 31 January 1966

5. AUTHOR(S) *(Last name, first name, initial)*

Blanchard, Gordon C.

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
January 1966	214	45

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
AF 33(657)-9186 b. PROJECT NO. 3048 Task No. 304801 c.	4019 9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)* APD-TDR-64-70, PART III

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	AF Aero Propulsion Laboratory, Research and Technology Division, AF Systems Command, Wright-Patterson AF Base, Ohio

13. ABSTRACT This program was a portion of an Air Force effort to reduce or eliminate fuel system problems including the clogging of filters and fuel gages, destruction of sealants and coatings, and the corrosion of aircraft wing tanks. These problems were believed to be due to the growth of microorganisms in combination with other fuel contaminants. The research effort in this program has been directed at developing rapid methods for detecting microorganisms in jet fuel water bottoms and at analyzing the chemical mechanisms by which microorganisms and other contaminants could cause operational difficulties in aircraft fuel systems. The mechanisms by which microorganisms produce emulsions, sludges, and fuel-soluble compounds and cause corrosion were studied. Eight different methods for detecting microorganisms were evaluated including esterase, tetrazolium reduction, gas chromatography of lipids, CO_2 detection, O_2 utilization, radioactive silver binding, acridine orange staining, and fluorescein isothiocyanate staining. Only the last three methods had the sensitivity needed for detecting 10^3 to 10^4 microorganisms/ml of water bottom. The fluorescein isothiocyanate method was recommended for routine use because of its simplicity and minimum requirement for equipment and laboratory procedures. The ability of fuel isolates to produce emulsions was demonstrated, with jet fuel and pure hydrocarbons as substrates. The aldehyde and acid products of hydrocarbon oxidation, formed during the growth of the microorganisms, were believed to be responsible for some of the emulsions formed on jet fuel. Fuel oxidizing microorganisms were demonstrated to cause aluminum alloy corrosion

(continued on next page)

DD FORM 1 JAN 64 1473

UNCLASSIFIED

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Microbial Contamination of Jet Fuel	Detection of Fuel Microorganisms					
Microbial Corrosion Mechanisms	Hydrocarbon oxidation					
Emulsion Formation by Microorganisms	Fuel Soluble Compounds and Sludges					

by four different mechanisms: (1) alteration of the ionic composition of the growth medium, (2) production of corrosive compounds, (3) deposition on metal surfaces and establishing oxygen and metal concentration cells, and (4) production of electron mediators which transfer electrons from the metals to electron acceptors in the microorganisms. During the first year of study, the growth medium was found to contain corrosion inhibitors and corrosion stimulators. Microorganisms were demonstrated to cause corrosion by removal of nitrate and phosphate as inhibitors. Nitrate was then tested for its ability to inhibit corrosion. The addition of 12 millimolar nitrate was found to inhibit the microbial corrosion of aluminum alloys. Aluminum alloy corrosion by natural water bottoms from fuel storage tanks was also inhibited by nitrate. Corrosion by the water bottoms was due principally to the additive, ethylene glycol monomethyl ether.

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GPO 886-551