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APL-TDR-64-95

Microbial Contamination of Air Force Petroleum Products

TECHNICAL DOCUMENTARY REPORT APL-TDR-64-95

March 1965

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

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Project 3048, Task 304801

(Prepared under Contract MIPR-33-657-3-RD-123 by United States Army Biological Laboratories, Fort Detrick, Frederick, Maryland; Charles E. Wilkes, Warren P. Iverson, Ralph R. Cockey, Howard M. Hodge, Authors)

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FOREWORD

This report was prepared by the U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland, and describes work performed at the request of the U.S. Air Force under Air Force Systems Command, Research and Technology Division MIPR 33-657-3-RD-123, entitled "Study of Microbiological Contamination." This work was initiated under Project 3048, "Aviation Fuels," Task 304801, "Hydrocarbon Fuels," and covers the period of March 1963 to March 1964, inclusive. The MIPR was administered under the direction of AF Aero Propulsion Laboratory, Research and Technology Division, Wright-Patterson Air Force Base, with Mr. A.V. Churchill of the Fuels and Lubricants Branch as project monitor; he was later succeeded by Mr. Jack R. Fultz as project coordinator.

Previous work requested by the Commander in Chief, SAC, in a letter dated 12 June 1961 and continued from July 1962 through March 1963 under Research and Technology Division MIPR 33-657-2-RD-264 was published as RTD-TDR-63-4119 in January 1964.

ABSTRACT

A study was undertaken to develop information on the control of microbial growth in jet fuel storage tanks and aircraft wing tanks. One year after the addition of anti-icing additive to the fuel, six Air Force installations were selected and surveyed periodically. The survey revealed sterile or nearly sterile fuel systems at the Air Force bases, and a similar condition was observed at other installations where the fuel contained the additive.

The biocidal properties of ethylene glycol monomethyl ether and other mono ethers were evaluated. The monomethyl ether was found to be superior to ethyl and butyl mono ethers when tested under realistic conditions.

Of several compounds evaluated for use as potential or standby fuel-soluble biocides, arsenosobenzene at a concentration of three ppm in the fuel was effective when tested under realistic conditions.

Fifty-four cultures of Pseudomonas were partially characterized. Pathogenic strains of Pseudomonas grew poorly or not at all on JP-4 as a sole carbon source. Three strains of Desulfovibrio desulfuricans grew well and could be enumerated on the surface of trypticase soy agar plates when incubated under a hydrogen atmosphere. Desulfovibrio desulfuricans, Cladosporium spp., and Pseudomonas aeruginosa were isolated from an aluminum tubercle and a corrosion pit. The action of Desulfovibrio was also studied in an oxygen concentration cell.

This technical documentary report has been reviewed and is approved.

Marc P. Dunnam

MARC P. DUNNAM, Chief
Technical Support Division
AF Aero Propulsion Laboratory

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I. INTRODUCTION

Before the advent of the wet-wing aircraft and more sophisticated fuels, sporadic incidents of microbiological contamination of fuels had occurred. In 1960 and 1961, the USAF became alarmed at the increase in incidents such as fuel probe failures, fuel control malfunctions, clogged drains, and wing-tank corrosion. In June 1961, the Strategic Air Command requested the U.S. Army Biological Laboratories to cooperate in obtaining practical information that would solve their problems. An early survey of Air Force bases revealed the necessity of improved housekeeping coupled with higher fuel standards. The areas of most concern were the bulk storage facilities and the fuel handling systems.

Early in 1962, the USAF issued a Military Interdepartmental Purchase Request to the U.S. Army Biological Laboratories at Fort Detrick to develop information pertaining to microbiological growth and its effect on fuels and other military products. This program also proposed the development of water-soluble and fuel-soluble additives for the prevention and control of microbiological growth. The work was initiated because even a condition of practical cleanliness does not preclude the presence of water and foreign materials in aircraft fuel and bulk storage tanks. In the laboratory, early work with ethylene glycol monomethyl ether (EGME) had indicated its potential as an effective biostatic agent. After the Air Force incorporated EGME as an anti-icing additive in their jet fuels, successive surveys of Air Force bases confirmed our laboratory results. This work was reported in RTD-TDR-63-4119[1] as part of our MIPR contract.

Repeated attempts in the laboratory to adapt microorganisms to high concentrations of EGME have been unsuccessful, and recent surveys of Air Force bases have not shown any indication of their adaptation to additive fuel under field conditions. However, such a possibility still exists, so that it is desirable to have other effective candidate additives in readiness.

In these studies, an effort has been made to develop information that would be useful under practical operating conditions. Biocides have been evaluated using high fuel-to-water ratios, and relatively long-term corrosion tests were conducted with simulated aluminum wing tanks in which microbial adhesion to susceptible topcoatings was enhanced by providing large horizontal surfaces.

Manuscript submitted to Air Force by authors 1 July 1964 as an RTD Technical Documentary Report.

A general and detailed description of the work designed by Research and Technology Division under MIPR-33-657-3-RD-123 is given below.

A. GENERAL DESCRIPTION

A general program for developing information pertaining to microbiological growths and their effects on fuels and other widely used Air Force products will be conducted by the Army Biological Laboratories. This includes the development of water-soluble additives and fuel-soluble additives to control and prevent microbiological growths.

B. DETAILED DESCRIPTION

A survey of selected Air Force base aircraft and bulk storage facilities will be conducted periodically to determine the amount of microbial contamination present and its side effects, such as wing-tank corrosion. Samples from other areas will also be evaluated upon request.

Fuel-soluble biocides will be evaluated to determine their potential value to the Air Force as additional or standby additives. Candidate compounds will be evaluated upon recommendation from industry, government, or defense agencies. Ethers of ethylene glycol will be compared as potential biocidal additives. Coating materials that show some biocidal properties will also be tested.

Identification studies of microbiological isolates from fuel samples will be continued.

Methods to determine the effects of microbial growth on corrosion will be studied. The cultivation and assessment of sulfate-reducing bacteria and their role in corrosion will be evaluated.

II. MICROBIOLOGICAL CONTAMINATION OF JET FUEL IN AIRCRAFT AND BULK STORAGE FACILITIES

A SURVEY OF AIR FORCE BASE AIRCRAFT AND BULK STORAGE SAMPLES OF JET FUEL

Six Air Force bases were selected for a survey that started 1 July 1963 to ascertain the level of microbial fuel contamination approximately one year after the addition of the AF anti-icing additive to the JP-4 fuel systems. Two groups of samples spaced approximately one month apart were received from each base. Where possible, the same aircraft and storage tanks were sampled each time. The samples were taken by base personnel and shipped to Fort Detrick for microbiological assay.

It was requested that each sample contain fuel and water (where available) in approximately a 1:1 ratio. Sixty-three samples were received of which 29 contained fuel and water and 34 contained only fuel. The water bottoms were plated on trypticase soy agar (TSA) and Sabouraud's dextrose agar. One-tenth ml of fuel was plated in triplicate directly on both agar media. One-half, 1.0, 5.0, or 10.0 ml of fuel from each sample was added to screw-capped test tubes that contained ten ml of sterile trypticase soy broth. The tubes were shaken vigorously and placed in racks. After one week at room temperature, the plates and tubes were examined for microbial growth. The descriptive data and the results of the investigations of samples of JP-4 and water from aircraft and storage tanks are shown in Tables 1 and 2. No contamination was found during this survey. The additive content in all water samples was well above the inhibitory level for biological growth. There was no indication of the development of additive-resistant organisms in the field samples.

B. MILITARY AIR TRANSPORT SERVICE

In February 1963, a periodic sampling program at 90-day intervals was established with the 1254th Air Transport Wing at Andrews Air Force Base. The samples, which were taken by Air Force personnel, were immediately transported to Fort Detrick and were examined for viable microbiological contamination. Because the original 30 samples received from Andrews AFB contained no water bottom, 0.1-ml quantities of fuel were plated on trypticase soy broth agar (TSBA) and rose bengal agar (RBA). A portion of the fuel was also used to overlay sterile Bushnell-Haas (B-H) mineral salts solution. The results of the assay are shown in Table 3.

TABLE 1. AIR FORCE BASE AIRCRAFT SUMP SAMPLES

AFB	Aircraft Tail	Tank	Biological Contamination		Per Cent Additive in Water
			Fuel	Water	
Amarillo	60685	L. Outboard	0	0	31
	60688	Tip Tank	0	0	32
	60688	R. Outboard	0	0	32
	60627	L. Outboard	0	0	26.5
	60692	L. Outboard	0	-[a]	-
	60692	X[b]	0	-	-
	60688	X	0	-	-
	60627	X	0	0	28
	60664	X	0	0	28
	60685	X	0	0	28
KI Sawyer	60313	Center Wing	0	-	-
	60043	Mid. Body	0	-	-
	60313	Center Wing	0	-	-
Bergstrom	090	R. Outboard	0	0	21.5
	598	Forward Body	0	-	-
	090	X	0	-	-
Castle	366	X	0	-	-
	160	L. Outboard	0	-	-
	368	X	0	-	-
	749	L. Outboard	0	0	31
	368	L. Outboard	0	-	-
	366	L. Outboard	0	0	25
	160	L. Outboard	0	0	29.5
	749	L. Outboard	0	0	28
Blytheville	10231	X	0	-	-
	10292	R. Main	0	-	-
	279	X	0	-	-
	267	X	0	-	-
	295	X	0	-	-
	286	X	0	-	-
	76514	X	0	-	-
	80242	X	0	-	-
	275	X	0	0	25
	10272	X	0	0	25
	10267	X	0	-	-
	10286	X	0	-	-
	76514	X	0	-	-
	10272	X	0	-	-
	10275	X	0	-	-
	10281	X	0	-	-
	10295	X	0	-	-
	10292	X	0	-	-
	10279	X	0	-	-
Travis	Aircraft samples were not received				

a. - indicates no water present in the sample received.
b. X indicates tank not identified.

TABLE 2. AIR FORCE BASE BULK STORAGE SAMPLES

AFB	Tank	Volumes Represented		Biological Contamination		Per Cent Additive
		Fuel, gal	Water, gal	Fuel	Water	in Water
Amarillo	26	1,137,537	0	0	-b/	-
	26	1,127,000	0	0	-	-
K. I. Sawyer	5	718,381	68	0	0	38
	4 Pmp. Hse. 2	20,654	0	0	-	-
	3	758,979	68	0	0	37.5
	4 Pmp. Hse. 2	47,308	0	0	-	-
Bergstrom	C-1	473,825	-a/	0	0	25
	B-14	664,189	-	0	0	25
	C-1	297,862	0	0	0	23.5
	B-14	812,131	0	0	0	23.5
Castle	1-H	1,515,550	302	0	0	10.3
	1-H	1,380,900	602	0	0	23.5
Plattsburgh	1	384,878	-	0	-	-
	1	802,733	715	0	0	10.5
Travis	25C	-	-	0	0	19.5
	26C	-	-	0	0	19.5
	3A	-	-	0	0	15
	25C	215,616	663	0	0	26.5
	26C	457,097	1,257	0	0	26.5
	3A	408,046	350	0	0	19.5

a. Information not received from AFB.
b. Water not available; no determination of additive.

TABLE 3. MICROBIOLOGICAL ASSAY OF FUEL SAMPLES FROM 1254th ATW, ANDREWS AFB

Date	Aircraft	A/C No.	Tank	Fuel	Contamination	
					Plates	B-H
27 Feb 63	VC-140B	61-2489	#4	JP-4	0	0
	VC-140B	62-4197	Right	JP-4	0	Fungi
	VC-140B	62-4197	Left	JP-4	0	Fungi
	VC-140B	61-2491	L. Aux.	JP-4	0	Fungi
	VC-140B	61-2491	R. Aux.	JP-4	0	Fungi
	VC-140B	61-2493	R. Aux.	JP-4	0	0
	VC-140B	61-2493	L. Aux.	JP-4	0	0
	VC-137C	62-6000	#2	JP-4	0	0
	VC-137C	62-6000	#3	JP-4	0	Fungi
	VC-137A	58-6972	#2	JP-4	0	Fungi
	VC-137A	58-6972	#3	JP-4	0	0
8 Aug 63	VC-137C	62-6000	#3	JP-4	0	0
	VC-137C	62-6000	Center	JP-4	0	0
	VC-140	61-2491	#2	JP-4	0	0
	VC-140	61-2491	#4	JP-4	0	0
	VC-140B	61-2490	#3	JP-4	0	0
	VC-140B	61-2490	#2	JP-4	0	0
	C-118	53-3299	#3 Main	115/145	0	0
	C-118	53-3299	#2 Main	115/145	0	0
	VC-140	63-7023	Engine #1	7808D	0	0
18 Oct 63	VC-118	53-3240	#2 Main	115/145	0	0
	VC-118	53-3240	#3 Main	115/145	1 Bact.	0
	VC-137C	62-6000	Center	JP-4	0	0
	VC-137C	62-6000	#2 Main	JP-4	0	0
	VC-140B	61-2493	#1 Main	JP-4	1 Bact.	0
	VC-140B	61-2493	#2 Main	JP-4	1 Bact.	0
	C-140B	62-4197	L. Aux.	JP-4	1 Fungi	0
	C-140B	62-4197	R. Aux.	JP-4	1 Fungi	Fungi
	C-140B	62-4199	L. Aux.	JP-4	0	0
	C-140B	62-4199	R. Aux.	JP-4	0	0

contamination is often less than 10 viable microorganisms per ml of fuel and cannot be detected by direct plating of small samples of 0.1 ml. Growth in the B-H solution could result from one viable microorganism and does not indicate the degree of contamination. In view of these shortcomings, an alternative technique was used for the 18 samples received in March and April 1964. The entire fuel sample (no sample contained water) was passed through a Millipore filter (HA Type, 0.45-micron pore size). The membrane was rinsed free of fuel with a sterile 0.1 per cent solution of Triton X-100 followed by sterile B-H solution. One half of the membrane was plated on the TSBA and one half on RBA plates. The plates were examined after 5 days and the number of bacteria and fungi were recorded. The results of these examinations are shown in Table 4.

MISCELLANEOUS SAMPLES

1. Pan American Airways

Pan American Airways furnished a collection of 65 JP-4 samples taken at locations throughout the world. Each pint sample was filtered through a membrane filter. The filters were rinsed free of fuel, and the samples were divided in half and incubated on plates of TSBA and B-H saline agar. The B-H plates each received 4 ml of JP-4 as an energy source and were examined at 5 and 10 days for growth. The B-H agar plates were all negative. Thirty-one of the TSBA plates contained from 1 to 4 contaminants. The majority of the contaminants were not of the type usually associated with fuel. Since none of the cultures could utilize JP-4 as an energy source, the very low levels of contamination present were apparently introduced by atmospheric dust.

2. Castle Air Force Base - Samples of 25 February 1963

A fuel and water sample containing a slimy interface was recovered from a filter separator. The interface material contained no viable organisms as determined by direct streaking on agar plates. The fibrous material at the interface was not soluble in concentrated sulfuric acid, which indicates that it was not cellulose or of fungal origin. Samples of aircraft fuel obtained from a furan-coated tank were sterile.

3. Ramey Air Force Base

A water bottom sample taken from a diesel bulk storage tank on 25 February 1963 contained 3×10^5 cells per ml when plated aerobically, and showed the presence of Desulfovibrio when cultured anaerobically.

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TABLE 4. MICROBIOLOGICAL ASSAY OF FUEL SAMPLES FROM 1254th ATW, ANDREWS AFB WITH MEMBRANE FILTER

Date	Aircraft	A/C No.	Tank	Fuel, ml	Contamination	
					Bacteria	Fungi
28 Jan 64	VC-137C	62-6000	#1 Main	JP-4 400-500	0	1
	VC-137C	62-6000	#4 Main	JP-4	1	1
	VC-137B	58-6970	#2 Main	JP-4	0	0
	VC-137B	58-6970	#3 Main	JP-4	0	0
	VC-140B	61-2489	#2 Main	JP-4	11	2
	VC-140B	61-2489	#3 Main	JP-4	2	0
	VC-140B	61-2490	#1 Main	JP-4	5	0
	VC-140B	61-2490	#4 Main	JP-4	1	0
	C-140B	62-4199	#1 Main	JP-4	1	0
	C-140B	62-4199	#4 Main	JP-4	4	0
21 Apr 64	VC-137C	62-6000	#1 Main	JP-5 425	7	3
	VC-137C	62-6000	#4 Main	JP-5 475	4	4
	C-140B	62-4200	#1 Main	JP-4 375	1	8
	C-140B	62-4200	#3 Main	JP-4 340	3	1
	C-140B	62-4201	#1 Main	JP-4 450	9	2
	C-140B	62-4201	#4 Main	JP-4 430	5	0
	VC-140B	61-2492	#2 Main	JP-4 450	1	2
	VC-140B	61-2492	#3 Main	JP-4 485	27	3

4. Lockheed, Marietta, Georgia - JP-5 Samples

Two fuel and water samples from Australian C-130 aircraft (No. 3644 and 3633) were received from Lockheed, Marietta.* These were heavily contaminated with a fungus, possibly Cladosporium. Lockheed also submitted a sample from a B-47 (No. 2209) that was sterile.

5. RCAF Samples - JP-5

Four fuel and water samples were received from RCAF C-130 aircraft. The water was plated on TSBA and inoculated into API medium for sulfate-reducing bacteria. The samples contained from 10^5 to 10^6 aerobic bacteria per ml of water and no anaerobic organisms.

* Lockheed Georgia Co., Marietta, Georgia.

III. EVALUATION OF FUEL-SOLUBLE BIOCIDES

A. TESTS ON ARSENOSOBENZENE, PYROGALLOL, 2-NITRO-RESORCINOL, β-NITRO-STYRENE, AND DODECYLBENZYLTHIOURONIUM HYDROCHLORIDE

Two chemicals were recommended to us as potential biocides. Arsenosobenzene was recommended by industry, and pyrogallol through a contact at Fort Monmouth, N.J. Pyrogallol was recommended at a level of 1 to 2 ppm in fuel on the basis of a Swedish publication.[2] Arsenosobenzene has several favorable properties for use as a fuel biocide: it can be obtained in pure or technical grades, is effective at low ppm level in aqueous solutions, decomposes at temperatures above the melting point, is volatile with steam, stable in alkaline or acidic solutions, has a low molecular weight, and has a partition coefficient of 17:1 in a water/oil system.

In preliminary tests, the two chemicals were screened using 1, 2, 4, and 8 ppm in nonadditive JP-4 fuel. The test system employed was one gallon of fuel containing 40 mg of ferrous oxide and 38.0 ml of a microbiologically contaminated B-H medium as an underlay, to provide a 100:1 ratio of fuel to water. A control using untreated fuel was run in conjunction with the biocide-containing fuels. The test systems were assayed weekly. Pyrogallol failed to show any inhibitory properties at the levels tested, but the arsenosobenzene was effective at all levels tested. The arsenosobenzene systems became sterile within one week. To determine whether an inhibitory concentration of this biocide remained in the fuel after a single contact with an aqueous phase, the fuel overlays (Searsport JP-4) used in the above experiment were removed from the test containers and again tested with one per cent by volume of Bushnell-Haas medium that had been inoculated with one per cent of a mixed culture of Pseudomonas aeruginosa, Cladosporium resinae, and Candida lipolytica. Viable cell counts after 4, 7, and 14 days are shown in Table 5. Note that 8 ppm arsenosobenzene in the original fuel phase was sufficient to inhibit biological growth in the second exposure of the fuel to a nutrient-containing aqueous system using a 100:1 ratio of fuel to water.

At this point, several experiments were set up to determine the concentration of arsenosobenzene that would be effective for a more realistic ratio of 1000:1. After several confusing weeks of work we found that the JP-4 being used (Searsport, nonadditive, referee, standard fuel) was inhibitory by itself when used at a 1000:1 ratio. The remaining stocks of Searsport JP-4 on hand were then tested for ability to support growth at a 1000:1 ratio of fuel to water. A previously tested, hand-blended, nonadditive JP-4 received from Gulf was used as a control. The results are shown in Table 6.

TABLE 5. MICROBIAL INHIBITION OF ARSENOSOBENZENE AFTER TRANSFER TO A FRESH UNDERLAY

Concentration of Arsenosobenzene in Fuel Phase Originally Used, ppm	Viable Cell Count of Aqueous Phase at Indicated Days			
	0	4	7	14
1	89.0×10^6	25.2×10^8	6.2×10^8	20.5×10^8
2	89.0×10^6	21.2×10^8	4.5×10^8	32.8×10^8
4	89.0×10^6	41.6×10^8	5.1×10^8	32.3×10^6
8	89.0×10^6	0	0	0
0 (Control)	89.0×10^6	18.7×10^8	19.9×10^8	40.6×10^8

TABLE 6. TEST OF SEARSPORT JP-4 FOR INHERENT INHIBITION

JP-4 Barrel No.	Viable Cell Count of Aqueous Phase at Indicated Days					
	Tested in Metal Can			Tested in Glass Container		
	0	5	14	0	5	14
1 (Searsport)	26×10^6	2200	0	26×10^6	3900	0
2 (Searsport)	26×10^6	22.0×10^6	18×10^6	26×10^6	2.0×10^6	4×10^6
3 (Searsport)	26×10^6	0.9×10^6	12×10^6	26×10^6	1.2×10^6	12×10^6
4 (Searsport)	26×10^6	1.0×10^6	8×10^6	26×10^6	0.4×10^6	3.2×10^6
5 (Searsport)	26×10^6	3400	3×10^6	26×10^6	2200	0.7×10^6
6 (Gulf control)	26×10^6	200.0×10^6	340×10^6	26×10^6	310×10^6	220×10^6

This test indicated that (i) the Searsport JP-4 (Barrel 1) currently being used was inhibitory, and (ii) that the ability of this fuel to support microbiological growth varied from barrel to barrel and in all cases was less than that of a JP-4 known to be free of inhibitory materials. Since the Searsport JP-4 was received from the Air Force in barrels that had been previously used to transport a JP-4 rust inhibitor, an experiment was set up to determine the biocidal concentration of that additive (see Section III, B).

For final evaluation of fuel-soluble biocides on a laboratory scale, an attempt was made to simulate realistic conditions by: (i) using a 1000:1 ratio of fuel to water, (ii) adding 40 mg of ferrous oxide to each gallon of fuel to be certain that the biocide under test would not be inactivated by rust in a storage tank bottom, (iii) using the mixed culture of organisms recommended by the Society for Industrial Microbiology Committee for Method Standardization of Biocide Testing (see Section VII) as including the most frequently found organisms in fuel systems, and (iv) employing two water-contact steps because under field conditions the fuel is in contact with water in a bulk storage tank where a portion of the biocide partitions to the aqueous phase, and is then transferred to a ready tank and/or an aircraft wing tank where further aqueous extraction of the biocide occurs. To each gallon of fuel containing the biocide under test, 3.8 ml of B-H medium previously inoculated with one per cent by volume of a mixed culture of P. aeruginosa, C. resinae, and C. lipolytica were added. The test samples were held at room temperature for three weeks and examined for viable cells by plating 0.1-ml quantities of the aqueous layers on TSA plates. After three weeks, the fuel overlays were decanted and 3500 ml of the once-extracted fuel were again placed in contact with 3.5 ml of fresh B-H and inoculum. Viable cell assays were performed at two and three weeks on the first contact and at two and four weeks for the second contact.

In the first test, a previously tested, noninhibitory JP-4 from Ramey AFB was used as the fuel stock. Various concentrations of 2-nitroresorcinol, β-nitro-styrene, and arsenosobenzene were tested (Table 7).

In the second test, the nonadditive CIE* fuel was used to evaluate arsenosobenzene and dodecylbenzylthiouronium hydrochloride. The latter compound has been found to be an effective biocide in injection flood waters in concentrations less than 10 ppm (Table 8).

---

* CIE = compression ignition turbine engine fuel.

TABLE 7. BIOCIDAL ACTION OF SELECTED CHEMICALS IN RAMEY AFB JP-4

Biocide	Concentration in Fuel, ppm	Viable Cells Present in Aqueous Layer after Indicated Holding Time			
		First Contact		Second Contact	
		2 Weeks	3 Weeks	2 Weeks	4 Weeks
2-nitro-resorcinol	50	+	+	+	+
	25	+	+	+	+
	10	+	+	+	+
β-nitro-styrene	50	-	-	+	+
	25	-	-	+	+
	10	+	+	+	+
Arsenosobenzene	2	-	-	-	-
	1	-	-	-	-
	1/2	-	-	+	+
	1/4	-	-	+	+
	1/8	+	+	+	+
Control[a/]	0	+	+	+	+

a. Aqueous phase contained 10×10^6 cells/ml at the start of the experiment.

TABLE 8. BIOCIDAL ACTION OF SELECTED CHEMICALS IN CIE FUEL

Biocide	Concentration in Fuel, ppm	Viable Cells Present in Aqueous Layer after Indicated Holding Time			
		First Contact		Second Contact	
		2 Weeks	3 Weeks	2 Weeks	4 Weeks
Arsenosobenzene	4	-	-	-	-
	2	-	-	+	+
	1	-	-	+	+
	1/2	+	-	+	+
	1/4	+	+	X[a/]	X
Dodecylbenzyl-thiouronium Hydrochloride	20	+	+	X	X
	15	+	+	X	X
	10	+	+	X	X
	5	+	+	X	X
	2	+	+	X	X
Control[b/]	0	+	+	+	+

a. Not tested.
b. Aqueous phase contained 18×10^6 cells/ml at the start of the experiment.

B. RUST INHIBITOR

DuPont No. 2 rust inhibitor was added to Gulf hand-blended JP-4 and tested in a 1000:1 ratio of fuel to water with the same inoculum as described in Section III, A. The results (Table 9) indicate that this additive was completely inhibitory at 500 ppm and had a detectable effect on microbiological growth when present at much lower concentrations in the fuel.

TABLE 9. EFFECT OF A RUST INHIBITOR ON MICROBIOLOGICAL GROWTH

Concentration of Anti-Rust Additive Added to JP-4, ppm	Viable Cell Count of Aqueous Phase after Indicated Days		
	0	5	14
0 (Control)	$26x10^5$	$20.0x10^7$	$38.0x10^7$
10	$26x10^5$	$16.2x10^7$	$19.4x10^7$
50	$26x10^5$	$14.0x10^7$	$4.0x10^7$
100	$26x10^5$	$1.5x10^7$	$0.4x10^7$
500	$26x10^5$	0	0
1000	$26x10^5$	0	0

C. ATTEMPT TO DEVELOP A FLORA RESISTANT TO BIOCIDAL ACTION OF ARSENOSOBENZENE IN FUELS

Arsenosobenzene has shown promise as a potential standby or emergency fuel biocide when used in fuel in a low ppm range. It was of interest to determine whether a resistant flora would develop rapidly in a system containing such relatively small quantities of toxic material. A mixed culture inoculum was used in an attempt to develop a flora capable of growing in B-H medium overlaid with CIE fuel containing arsenosobenzene as a biocide. A composite of water bottoms (1 ml) obtained from previous tests that included bacteria and fungi resistant to arsenosobenzene at 0.125 to 0.5 ppm in fuel when used at a 1000:1 ratio of fuel to water and one gram of

a composite mixture of twelve soil samples were added to 100 ml of B-H medium. This inoculated medium was used as the underlay in test systems using CIE fuel containing 0 to 5 ppm arsenosobenzene and a 1000:1 ratio of fuel to water. The results of this test are shown in Table 10.

TABLE 10. RESULTS OF EXPOSING MIXED CULTURES TO FUEL CONTAINING ARSENOSOBENZENE AT CONCENTRATIONS OF 1 TO 5 PPM

Container No.	Arsenosobenzene in Fuel Phase,[a] ppm	Viability[b] of Cells in Underlay at Indicated Days			
		0	10	20	30
1	1	+	+	+	+[c]
2	2	+	+	+	+[c]
3	3	+	-	-	-
4	4	+	-	-	-
5	5	+	-	-	-
6	0 (Control)	+	+	+	+[c]

a. Fuel also contained 40 mg/gallon iron rust.
b. Viability determined by streaking 0.1 ml of underlay on TSA plates.
c. Visible fungal development.

After ten days, 1 ml each of the water bottoms of containers numbered 1 and 2 (Table 10) were added to 100 ml of B-H medium and this medium again was overlaid with CIE fuel containing varied quantities of arsenosobenzene at a 1000:1 ratio of fuel to water. Results obtained after a 20-day incubation period are shown in Table 11.

TABLE 11. RESULTS OF SECOND EXPOSURE TO ARSENOSOBENZENE

Container No.	Arsenosobenzene in Fuel,[a] ppm	Viability[b] of Cells in Underlay at Indicated Days		
		0	10	20
1	1	+	+	+[c]
2	2	+	+	-
3	3	+	-	-
4	4	+	-	-
5	5	+	-	-
6	0 (Control)	+	+	+[c]

a. Fuel also contained 40 mg iron rust per gallon.
b. Viability determined by streaking 0.1 ml of underlay on TSA plates.
c. Fungi developed on TSA plates.

The test samples containing arsenosobenzene in fuel were held for a total of 60 days. There was no development of resistance during this holding period to quantities of arsenosobenzene above 2 ppm in the fuel.

D. NITRO-SUBSTITUTED BENZONITRILES

Two compounds, 2-nitrobenzonitrile (NBN) and 2,4-dinitrobenzonitrile (DNBN), previously screened and recommended by a research laboratory as microbial inhibitors for hydrocarbon fuels, were evaluated. The test compounds were dissolved in nonadditive JP-4 in concentrations that ranged from 1 to 100 ppm and were underlaid with B-H mineral salts solution containg a one per cent inoculum of a mixed culture of *P. aeruginosa*, *C. resinae*, and *C. lipolytica*. The ratio of fuel to water was 1000:1. The aqueous underlays, which originally contained 1.9×10^6 cells per ml, were plated after 7 and 14 days. Viable cells were present at all biocide levels. After 5 weeks, obvious visible growth had developed in all of the test samples. These biocides were therefore ineffective in concentrations through 100 ppm in the fuel.

E. TEST OF FURAN COATING FOR BIOSTATIC ACTION

1. Coupon Testing

Mild steel coupons (½ inch by 4½ inches) were dip-coated with furan primer followed by a gray, a black, and a gray furan coating.* Coupons were air-dried for 24 and 48 hours and then placed in 100 ml of B-H medium, inoculated with mixed contaminants, and overlaid with 50 ml of Searsport fuel. Under these conditions, which resulted in a 3-square-inch exposure of furan to 100 ml of aqueous medium, there was no reduction in biological growth during a three-week observation period. Data are tabulated in Table 12.

TABLE 12. EXPOSURE OF FURAN-COATED COUPONS

	Coating Exposed	Bacterial Count at Indicated Days, cells/ml	
		0	21
1	None (Control)	2.0×10^6	40×10^6
2	Furan, Air-Dried, 24 hours	2.0×10^6	24×10^6
3	Furan, Air-Dried, 48 hours	2.0×10^6	26×10^6

2. Simulated Tank Tests

Two small simulated aluminum wing tanks were coated with furan at the factory. On receipt, one tank was held at room temperature and exposed to air for a week. The other tank was treated in the following sequence: subjected to a continuous wash with running tap water for four days, drained, filled with nonadditive JP-4 and held for two days, and air-dried for one day. A third tank that was used as a control had an aged polyurethane topcoating. To supply a nutrient water bottom and a mixed-culture challenge inoculum, one ml of contaminated water bottom from a diesel bulk storage tank at Ramey AFB was added to 500 ml of B-H medium. One hundred

* MIL-C-4556B, an approved coating for bulk storage fuel tanks.

ml of this nutrient and inoculum was overlaid with 200 ml of Searsport non-additive referee fuel in each of the test systems. A glass jar with the same ratio of nutrient to JP-4 was used as a control. Five exposures were run concurrently with each of the test tanks. Each tank was sampled weekly for a 2- or 3-week period, then emptied and prepared for the next exposure. Since the results of the exposures could not be predicted, additional observations were made in the first two exposures.

The results of the initial tank exposure are shown in Table 13. The underlay in the water- and fuel-extracted furan tank continued to exert a slow biocidal action even when removed from the top-coated tank and transferred to a glass container, which became sterile after 31 days.

The results of the second exposure are shown in Table 14. The air-washed furan coating, which showed some inhibition in the initial exposure, was no longer biostatic. After a one- to two-week holding period, the underlay in the tank coated with furan, which had been water- and fuel-washed, had extracted a biocidal material from the coating and the system became sterile within three weeks.

The results of the third exposure are shown in Table 15. All systems showed growth within three days. After one week, the underlay in the water- and fuel-washed furan tank approached sterility and finally became sterile by the second week.

The results of the fourth exposure are shown in Table 16. The water- and fuel-washed furan tank, while still slowly bactericidal, permitted growth of fungi at the fuel/water interface. The change in appearance of each system can be followed by noting the visual observations at the end of each table.

The results of the fifth exposure are shown in Table 17. The water- and fuel-washed furan tank lost its biocidal properties and the water/fuel interface was covered with a fungal mat.

The coupon and simulated wing tank tests uncovered two important factors. The biocidal or bacteriostatic properties of furan coating is related to curing time and curing conditions and to the area of coating exposed to the water underlay. Biocidal or bacteriostatic properties were not observed in the coupon test when only three square inches of coating was exposed to 100 ml of water bottom. The biocidal and bacteriostatic properties were observed in the simulated wing tanks when 27 square inches of coating were exposed to the 100 ml of nutrient and water underlay. Evidence of failure became noticeable after the fourth exposure of the simulated wing tank. The loss of bactericidal and bacteriostatic material from the coating is rapid during exposure to air. The loss of this property is slower when the fresh coating is submerged in liquids. The importance of the area of coating exposed to an underlay of nutrient and water became apparent when the biocidal action of a furan-coated drum was lost after seven weeks' exposure.

TABLE 13. MICROBIOLOGICAL GROWTH IN JP-4 AND B-H MEDIUM IN CONTACT WITH TOPCOATING MATERIALS AND AFTER REMOVAL TO GLASS; INITIAL EXPOSURE

Test	Surface Exposed	Viable Cell Count/ml after Indicated Days						
		In Contact with Topcoating Material						After Removal to Glass Container
		0	3	5	7	14	24	7
1	Polyurethane	9.6×10^{4}	1.5×10^{7}	4.0×10^{8}	1.9×10^{8}	5.5×10^{7}	1.2×10^{6} a/	11×10^{4} a/
2	Furan-Coated; Air Washed	9.6×10^{4}	1.3×10^{5}	3.5×10^{6}	5.8×10^{6}	16.9×10^{6}	0.5×10^{6} b/	28×10^{4} b/
3	Furan-Coated; Water- and Fuel-Washed	9.6×10^{4}	10.9×10^{4}	6.5×10^{4}	4.0×10^{4}	2.0×10^{3}	5.0×10^{2} b/	0
4	Glass (control)	9.6×10^{4}	1.4×10^{8}	2.4×10^{8}	3.0×10^{8}	4.0×10^{8}	0.3×10^{6} a/	20×10^{4} a/

a. Aqueous underlay and interface showed heavy contamination.
b. Aqueous underlay and interface were clear.

TABLE 14. MICROBIOLOGICAL GROWTH IN JP-4 AND B-H MEDIUM IN CONTACT WITH TOPCOATING MATERIALS; SECOND EXPOSURE

Test	Surface Exposed	Viable Cell Count/ml after Indicated Days					Visual Observation
		0	3	7	14	21	
1	Polyurethane	28.5×10^4	22.5×10^6	150×10^6	10×10^4	2.3×10^6 a/	Interface had a lacy growth
2	Furan-Coated; Air-Washed	28.5×10^4	1.3×10^4	10.0×10^6	1.2×10^6	-b/	Interface had a heavy fungal mat
3	Furan-Coated; Water- and Fuel-Washed	28.5×10^4	0.26×10^6	2.1×10^6	<100	0	Interface and aqueous layer clear
4	Glass (control)	28.5×10^4	0.71×10^6	$>10^8$	40×10^6	32×10^6 b/	Interface had a heavy fungal mat

a. A pure culture of Candida lipolytica was present.
b. Heavy fungal mat - impossible to obtain an accurate count.

TABLE 15. MICROBIOLOGICAL GROWTH IN JP-4 AND B-H MEDIUM IN CONTACT WITH TOPCOATING MATERIALS; THIRD EXPOSURE

Test	Coating Exposed	Viable Cell Count/ml after Indicated Days				Visual Observation
		0	7	14	21	
1	Polyurethane	2.5×10^6	17.0×10^7	2.3×10^7	2×10^7	Cloudy underlay
2	Furan-Coated; Air-Washed	2.5×10^6	8.8×10^6	6.0×10^6	4×10^6	Heavy fungal mat and cloudy underlay
3	Furan-Coated; Water- and Fuel Washed	2.5×10^6	<200	0	0	Cloudy underlay
4	Glass (Control)	2.5×10^6	4.8×10^7	12×10^7	6×10^7	Cloudy underlay

TABLE 16. MICROBIOLOGICAL GROWTH IN JP-4 AND B-H MEDIUM IN CONTACT WITH TOPCOATING MATERIALS; FOURTH EXPOSURE

Test	Coating Exposed	Viable Cell Count/ml in Underlay after Indicated Days				Visual Observation at Fourteen Days
		0	3	7	14	
1	Polyurethane	4.6×10^5	2.1×10^7	8.6×10^6	6.4×10^7	Cloudy underlay
2	Furan-Coated; Air-Washed	4.6×10^5	9.0×10^7	3.2×10^7	4.0×10^4	Heavy fungal mat, 95% coverage of interface
3	Furan-Coated; Water- and Fuel-Washed	4.6×10^5	1.5×10^7	0 bacteria 15 fungi	0	Fungal mat forming 10% coverage of interface
4	Glass (Control)	4.6×10^5	2.2×10^8	1.6×10^8	4.5×10^7	Cloudy underlay

TABLE 17. MICROBIOLOGICAL GROWTH IN JP-4 AND B-H MEDIUM IN CONTACT WITH TOPCOATING MATERIALS; FIFTH EXPOSURE

Test	Coating Exposed	Viable Cell Count/ml in Underlay after Indicated Days				Visual Observation at Fourteen Days
		0	3	7	14	
1	Polyurethane	2.2×10^5	4.9×10^8	5.8×10^7	6.8×10^6	Cloudy, fungal mat; 90% coverage
2	Furan-Coated; Air-Washed	2.2×10^5	3.3×10^8	5.3×10^7	5.8×10^8	Cloudy, fungal mat; 95% coverage
3	Furan-Coated; Water- and Fuel-Washed	2.2×10^5	2.1×10^8	6.6×10^7	2.9×10^8	Cloudy, fungal mat; 95% coverage
4	Glass (Control)[a/]	2.2×10^5	2.6×10^8	7.1×10^7	1.7×10^7	Cloudy, no mat

a. Mixed culture from Ramey AFB diesel water bottom used as inoculum.

3. Biocidal Action of Ethylene Glycol Monomethyl Ether (EGME) on a Fungus

To determine whether the fungus Cladosporium, which grew slowly in the fourth exposure test of the furan-coated tank, was resistant to EGME, the nutrient-containing water bottom from this test was homogenized in a blendor for one minute and EGME added to give 1, 10, and 20 per cent concentrations. As shown in Table 18, this culture was not resistant and EGME was biocidal.

TABLE 18. EXPOSURE OF CLADOSPORIUM TO EGME

	Viable Count per ml after Indicated Days				
EGME, %	0	3	7	14	21
1	2.2×10^7	1.1×10^7	1.7×10^7	2.1×10^7	19.4×10^6
10	2.2×10^7	1.0×10^3	5.4×10^3	277	123
20	2.2×10^7	4.8×10^3	1.1×10^2	0	0

4. Furan Drum Tests

A more practical type of evaluation was performed with a 55-gallon drum coated with furan at the factory. In that test, the following assumptions were made:

1) Initially, a recoated tank would have a clean bottom of non-nutrient water.

2) A biocide in a coating would have some time to equilibrate with the aqueous phase before appreciable quantities of noncarbon nutrients were extracted into the aqueous phase from the fuel.

3) The degree of microbial contamination would be low during the initial phases of nutrient buildup and biocide extraction from the coating.

4) Nutrients extracted from the fuel are not present in the quantity usually used in an ideal laboratory mineral salts medium. In this test, the maximum quantity of mineral salts used was one-fifth normal strength B-H salts.

5) Water would not be completely drained, so that any biocide present would not be exhausted with each draining.

An evaluation incorporating these assumptions and the results are described below.

Two gallons of tap water and 25 gallons Searsport nonadditive JP-4 were added to a furan-coated drum. A 20-gallon uncoated drum was filled with one gallon of tap water and nine gallons of Searsport JP-4 as a control. After one week, plate counts were run on the water in each drum. Dry B-H salts were then added to each drum to give one-tenth the normal B-H strength, which was 2.64 grams to the 55-gallon drum and 1.32 grams to the 20-gallon drum. Five ml and 2.5 ml of a mixed-culture inoculum were then added to the furan-coated and uncoated drums, respectively. Weekly plate counts were run on the underlay from both drums. At the end of the second week at ambient temperature, counts were run on the water, then dry B-H salts were added in the same amounts as above, bringing the B-H strength to one-fifth normal. After three partial drain-and-fill cycles, the furan-coated drum permitted fungal growth at the interface. The results of this treatment are shown in Table 19.

TABLE 19. EFFECT OF FURAN COATING ON MICROBIAL GROWTH IN NUTRIENT BOTTOMS IN A 55-GALLON DRUM

Drum	Bacterial Count/ml of Underlay at Indicated Weeks						
	1[a]	2	3[b]	4[c]	5[c]	6	7[c]
Furan-Coated	2.0×10^3	3.5×10^5	30	5.0×10^3	0	2.5×10^3	0[d]
Uncoated (Control)	5.0×10^2	1.0×10^7	3×10^6	2.0×10^6	4.0×10^6	4.5×10^6	8.0×10^6

a. After the addition of unsterile tap water, the uninoculated drums were allowed to stand for one week, then dry B-H salts were added to give a 1/10 normal B-H medium concentration and the tank bottoms were inoculated with 0.1% by volume of Ramey AFB mixed culture from a diesel storage tank bottom.
b. Dry B-H salts were added to bring the concentration of the underlays to 1/5 normal medium concentration.
c. One-half of the underlays were drained and replaced with 1/5 normal strength B-H medium.
d. Fungal mat developing at interface of fuel and water in furan-coated tank. Isolated clumps at interface of uncoated tank.

IV. ANTI-ICING ADDITIVE

A. ADAPTATION OF MICROORGANISMS TO ETHYLENE GLYCOL MONOMETHYL ETHER

Efforts have been continued to obtain organisms resistant to EGME to anticipate future difficulties that might arise in the use of this material.

1. Isolation of Organisms Capable of Utilizing EGME as a Sole Carbon Source

Suspensions of 11 soil samples were used to inoculate flasks of B-H medium containing 0.25, 1.0, 1.5, and 2.5 per cent EGME. A number of bacteria of the Pseudomonas type and fungi grew in the original flasks containing up to one per cent EGME. At 1.5 per cent and above, only fungi grew. Transfers of these cultures to concentrations of EGME above 2.5 per cent failed to grow.

2. Soil Enrichment Technique

A soil enrichment technique was employed in an effort to isolate or develop strains of microorganisms resistant to EGME. To four small pots of a rich garden soil, EGME was added and mixed at a rate of 2 ml per week for 11 consecutive weeks. Two subsamples of each of the soil samples were kept moist in plastic bags at room temperature and two were held at 35 C. One sample at each temperature also contained a small quantity of a slow-release 20-10-10 fertilizer as an additional mineral supplement. After 11 weeks, a fungus was obtained that was capable of growing in a B-H salts medium containing 5 per cent EGME as a sole carbon source. Subsequent transfers of this culture to higher concentrations of EGME failed to grow. When this culture was inoculated into B-H medium containing 5 per cent EGME and overlaid with JP-4, it also failed to grow. Bacterial utilization of certain glycol ethers, but not EGME, at concentrations of 0.25 per cent have been reported.[3]

In view of these observations and the results previously reported,[1] it appears unlikely that organisms will be found capable of growing in fuel/water bottoms containing quantities of additive now present at Air Force installations.

B. ANTI-ICING ADDITIVE ANALYZED BY FREEZING POINT

Chemical analysis of anti-icing additive in water bottom samples is tedious and time-consuming. Since the additive partitions to the water phase, concentrations of EGME may range as high as 40 per cent. To

determine whether a method based on freezing point could be adopted for additive determinations of the water bottom samples, several AFB water bottom samples that had been chemically analyzed were assayed. The comparison of chemical analysis and determination of concentration by freezing point is shown in Table 20. The concentration of EGME was extrapolated from a graph prepared by using known standards.* The data were checked against laboratory standards and the comparisons are shown in Table 21. The data in Tables 20 and 21 show the freezing point determination of EGME levels in water bottom samples. The freezing point and the chemical method of determination correlated well in fuel/water bottom samples. The data are applicable only to EGME because other ethers of ethylene glycol have different values for concentration at freezing point.

C. EVALUATION OF ETHERS OF ETHYLENE GLYCOL AS BIOCIDES IN AQUEOUS SOLUTIONS

1. Comparison of Methyl and Ethyl Ethers of Ethylene Glycol

The biostatic action of methyl and ethyl ethers of ethylene glycol was tested in four different media using five ml of Searsport nonadditive JP-4 as an overlay on ten ml of aqueous medium. One drop of mixed culture from Ramey AFB diesel bottoms known to contain Pseudomonas, Cladosporium, Candida, and Bacillus species was used as the challenge inoculum. Results, after one month of incubation, are shown in Table 22. Both glycols were equally effective in B-H medium that contained no soluble organic matter, but the ethyl ether was more bacteriostatic than fungistatic. In the presence of large quantities of water-soluble organic materials, the ethyl ether at 6 per cent or more was an effective biostatic agent, but the methyl ether was not. These results explain why the biostatic action of EGME is difficult to detect if the usual agar diffusion assay, which employs a rich organic medium, is used.

2. Comparison of Biocidal Properties of Glycol Ethers as Fuel Additives

The comparative biocidal action of three glycol ethers was first tested in a variety of aqueous media and then in a fuel, nutrient, and water system to simulate conditions in use. As shown in Table 23, in predominantly aqueous systems the quantity of glycol ether required to inhibit microorganisms was dependent upon the nutrient level of the medium. The butyl ether was by far the most effective under these test conditions. However, when these three ethers were tested under simulated in-use conditions in which the biocide was dissolved in the fuel and had to partition to the aqueous phase to be effective, the methyl ether was the most effective (Table 24). The butyl ether, because of its low partition coefficient, was useless as a biocide under these conditions, and the methyl ether was the most effective.

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* Minutes of the Meeting of the Aircraft Fuel Tank Corrosion Group of the Coordinating Research Council (CRC) of the Aviation Fuel, Lubricant, and Equipment Research Committee in January 1963 contained data on the freezing points of aqueous EGME solutions.

TABLE 20. COMPARISON OF CHEMICAL ANALYSIS AND FREEZING POINT TECHNIQUE TO DETERMINE ETHYLENE GLYCOL MONOMETHYL ETHER CONTENT OF WATER SAMPLES

Sample	Per Cent of Additive	
	Chemical Analysis	Freezing Point Technique
TR 70	29.35	28
PR 147	20.66	21.5
153	22.64	21.5
161	15.64	15
173	13.92	15
119	12.22	13
159	25.46	28

TABLE 21. COMPARISON OF FREEZING POINTS BY TWO INDEPENDENT LABORATORIES

Additive Concentration, wt per cent	Freezing Point, C	
	CRC Minutes April 1963	Fort Detrick
10	-2.78	-2.5 to -3.0
20	-7.22	-7.5
30	-13.3	-13.0
40	-22.8	-23.0
50	-37.2	-41.0
60	-60.0	-60.0

TABLE 22. GROWTH OF A MIXED CULTURE IN VARIOUS CONCENTRATIONS OF METHYL AND ETHYL ETHERS OF ETHYLENE GLYCOL IN FOUR MEDIA AFTER ONE MONTH OF INCUBATION AT ROOM TEMPERATURE

Ether in Underlay, %	Medium Used as the Underlay							
	Bushnell-Haas Medium		B-H Medium plus 1% Glucose		B-H Medium plus 1% Peptone		B-H Medium plus 1% Glucose and 1% Peptone	
	ME[a]	ET[b]	ME	ET	ME	ET	ME	ET
1	+[c]	+F[d]	+	+F	+	+	+	+
2	+	+F	+	+F	+	+F	+	+
3	+	+F	+	+F	+	+F	+	+F
4	+	+F	+	+F	+	+F	+	+F
5	+	+F	+	+F	+	+F	+	+F
6	-	-	+	-	+	+F	+	+F
7	-	-	+	-	+	-	+	-
8	-	-	+	-	+	-	+	-
9	-	-	+	-	+	-	+	-
10	-	-	-	-	+	-	+	-
0 Control	+	+	+	+	+	+	+	+

a. Ethylene glycol monomethyl ether.
b. Ethylene glycol monoethyl ether.
c. + indicates positive growth.
d. F indicates a predominance of, or only fungal growth.

TABLE 23. VIABILITY OF A MIXED CULTURE IN NUTRIENT BOTTOMS CONTAINING THREE MONOETHERS OF ETHYLENE GLYCOL OVERLAID WITH JP-4 AFTER FOUR WEEKS' INCUBATION

% Glycol Monoether in Aqueous Phase[a]	Composition of Aqueous Nutrient Layer														
	10% B-H Medium			Normal B-H			B-H + 1% Dextrose			B-H + 1% Peptone			B-H + 1% Peptone + 1% Dextrose		
	Me[b]	Et[c]	Bu[d]	Me	Et	Bu	Me	Et	Bu	Me	Et	Bu	Me	Et	Bu
2	+[e]	+	-	+	+	-	+	+	-	+	+	-	+	+	+
4	+	+	-	+	-	-	+	+	-	+	+	-	+	+	-
6	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
8	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
10	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
12	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-
14	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 (Control)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

a. Water-to-fuel ratio was 2:1.
b. Ethylene glycol monomethyl ether.
c. Ethylene glycol monoethyl ether.
d. Ethylene glycol monobutyl ether.
e. Viability determined by streaking 0.1 ml on agar plates. The 10-ml tubes were inoculated with one drop of a mixed culture of P. aeruginosa, C. resinae, and C. lipolytica containing 2 x 10^6 cells per ml.

TABLE 24. GROWTH OF A MIXED CULTURE IN B-H MEDIUM OVERLAID WITH JP-4 CONTAINING METHYL, ETHYL, OR BUTYL ETHERS OF ETHYLENE GLYCOL

Ethylene Glycol Ether in Fuel Phase,[b] %	Growth[a] after 4 Weeks' Incubation		
	Mono-methyl Ether	Mono-ethyl Ether	Mono-butyl Ether
0.10	-[c]	-	+
0.08	-	-	+
0.06	-	+	+
0.04	+	+	+
0.02	+	+	+
0 (Control)	+	+	+

a. The original inoculum containing P. aeruginosa, C. resinae, and C. lipolytica assayed 21 x 10^6 cells per ml.
b. Fuel-to-water ratio was 1000:1.
c. Growth determined by visual observation.

V. IDENTIFICATION STUDIES

A. PSEUDOMONAS CULTURES AND ISOLATES

As an aid to identification, 16 Pseudomonas cultures of known identity were received from the USDA Regional Laboratory at Peoria, Illinois, for inclusion in the classification scheme. About 80 bacterial cultures, the majority of which are probably Pseudomonas cultures isolated from integral fuel tanks, were also received from Sharpley Laboratories. Of these, only 47 were viable. Several fungal cultures were also received. Identification studies were also started on seven isolates obtained from plates streaked in Bermuda (Project BEARS) by a representative of the U.S. Army Engineering and Development Laboratory.

A study was initiated on the 16 Pseudomonas cultures (USDA) and on a culture obtained from the Stanford Research Institute (SRI-4C). The latter was reported to produce large amounts of a glucose polysaccharide when grown in B-H medium with 3 per cent n-alkanes (C_6-C_{16}) as the source of carbon and energy. The objective was to obtain information on known and previously studied Pseudomonas cultures for comparison with data obtained with isolates from JP-4 tank bottoms. The cultures were tested for their ability to produce mercaptans, oxidize carbohydrates, oxidize gluconate, produce slime after growth in gluconate medium, grow in JP-4 with B-H medium, and grow at 37±1 C and 41±1 C. Mercaptan production was indicated by the production of white vapors when one or two drops of the iodine-azide reagent (3 grams of sodium azide in 100 ml of 0.1 N iodine) were added to 1 ml of a broth culture (1% trypticase, 0.01% cysteine, 0.05% Na_2SO_4) or a loopful of the iodine-azide reagent was introduced above the surface of a slant of trypticase soy broth agar.[4] Determinations were made after 2, 4, 7, and 14 days. Mercaptan production seems to be a property of the majority of the Pseudomonas strains and possibly more characteristic than pigment production. Several of the Pseudomonas strains previously isolated from JP-4 tank bottoms also were found to produce mercaptans when grown in 1 per cent trypticase broth and tested by the iodine-azide reagent. When the isolates were grown in a 1 per cent trypticase broth that contained additional sulfur compounds (0.1 gm per liter cysteine and 0.5 gm per liter sodium sulfate), more than one half of the isolates, including several P. aeruginosa strains, produced mercaptans.

Carbohydrate oxidation was observed by the reactions in Hugh-Leifson medium (0.2% trypticase, 0.5% NaCl, 0.03% K_2HPO_4, 0.3% agar, 0.003% bromthymol blue, 1.0% carbohydrate, pH 7.1) when the following carbohydrates were substituted: glucose, mannitol, xylose, galactose, sucrose, maltose, and lactose.

Gelatin hydrolysis was indicated by covering single streaked plates (nutrient agar plus 0.4% gelatin incubated for 15 days) with a solution of 15 grams of $HgCl_2$ in 100 ml of distilled water and 200 ml of concentrated HCl and noting the presence of a clear zone around the streak. Organisms that do not hydrolyze gelatin have a white opaque precipitate around them.

Gluconate oxidation was determined by inoculating 100 ml of a medium (1.5 grams tryptone, 1 gram yeast extract, 40 grams sodium gluconate, 1000 ml distilled water, pH 7.0) with the culture. After the medium was incubated at room temperature on a shaker for 3, 7, and 14 days, it was tested for the presence of a reducing compound, probably 2-ketogluconate, with a copper sulphate sugar reagent.[5] This same type of medium was used to determine the ability of these organisms to produce slime. On the completion of observations for oxidation of potassium gluconate, the flasks were allowed to stand at room temperature until maximum slime formation occurred. This was usually four days after the flasks were removed from the shaker. A characteristic of the slime is its ability to show the reverse swirl phenomenon. (When a flask containing slime is vigorously swirled and then set on a table, the swirling liquid slows to a stop and then abruptly reverses direction.)

Temperature relationships were determined on agar slants of the following composition: 5 grams tryptone, 1 gram glucose, 5 grams yeast extract, 1 gram K_2HPO_4, 15 grams agar, 1000 ml distilled water, pH 6.8 to 7.0. Slants of each strain were then incubated at each of the following temperatures: 28 C (air temperature), 37±1 C (waterbath), and 41±1 C (waterbath). If growth occurred, three transfers at that temperature were made before transferring to a higher temperature. Of course all the cultures grew at 28 C. The results are presented in Table 25. Those on several of the cultures do not agree with the temperature characteristics as described in Bergey's Manual. For example, P. chloraphis is reported to grow poorly, if at all, at 37 C. However, culture B-2074, described as P. chloraphis, does grow quite readily at 37 C. Culture SRI-4C appears to be P. aeruginosa, since it fulfills the three criteria for this organism as defined by W.C. Haynes:[6] (i) ability to grow at 41±1 C; (ii) ability to oxidize gluconate; and (iii) production of slime in static culture in a medium containing gluconate as the principal carbon source.

At present it seems undesirable to attempt to identify any organism in the Pseudomonas group with the possible exception of P. aeruginosa, P. chloraphis, and a few others that have outstanding characteristics according to Bergey's Manual. All that can be done is to attempt to identify as many of the "significant" biochemical and physiological characteristics as possible in cultures from various collections and attempt to correlate these characteristics with those of unidentified organisms. At present, there is considerable doubt on what the "significant" characteristics are. A composite of the results of these tests is shown in Table 25.

TABLE 25. REACTIONS OF SOME PSEUDOMONAS STRAINS

Culture Number	Culture Identification	Growth		Oxidation of Carbohydrates								Slime Production from Gluconate	Production of Mercaptans	Gelatin Hydrolysis
		37±1 C	41±1 C	Glucose	Mannitol	Xylose	Galactose	Sucrose	Maltose	Lactose	Gluconate			
B- 8	P. ovalis	+	-	+	-	+	+	sl[a]	+	±	-	-	+	-
B- 10	P. fluorescens	+	-	+	+	+	+	+	+	+	+	-	+	-
B- 13	P. putida	+	-	+	-	+	+	+	+	+	+	+	+	-
B- 14	P. testrolens	-	-	+	+	+	+	-	+	+	+	-	+	-
B- 25	P. fragi	-	-	+	-	+	+	+	+	+	+	-	+	+
B- 311	P. riboflavinus	-	-	+	+	+	+	+	+	+	-	-	-	-
B- 769	P. indoloxidans	+	-	-	sl	-	-	+	sl	-	-	-	+	sl
B- 778	P. olevorans	+	+	+	sl	sl	sl	+	-	sl	-	-	+	-
B- 780	P. xanthia	+	-	+	+	+	+	+	+	+	+	-	+	+
B- 927	P. stutzeri	+	-	+	+	+	+	-	+	-	-	±	-	+
B-1028	P. denitrificans	+	-	-	-	+	+	-	sl	sl	-	-	-	-
B-1123	P. perolans	+	+	+	+	+	+	+	+	+	-	-	+	+
B-1885	P. spp.	+	-	+	+	+	+	+	±	+	-	-	+	+
B-2040	P. syncyanea	+	-	+	-	-	+	-	sl	+	+	+	+	-
B-2075	P. chlororaphis	+	-	+	+	+	+	+	sl	+	+	+	+	+
B-2462	P. pavonacea	+	+	-	-	sl	sl	-	sl	sl	-	-	-	-
SRI-4C	P. aeruginosa	+	+	+	+	+	+	±	sl	+	+	+	-	+

a. sl = slight.
± = trace.

All of the USDA Northern Utilization Research and Development Division cultures that were introduced into JP-4 grew poorly or not at all. Culture B-1123 (P. perolans) is the only one that has discolored the fuel (very pale yellow) after two months. A few of the other cultures show light turbidity and sediment, which may be part of the original inoculum. None of the cultures has shown growth at the interface comparable to the initial growth of the isolates that utilize JP-4 from fuel tank bottoms.

B. COMPARISON OF P. AERUGINOSA CULTURES FROM CLINICAL SPECIMENS AND JP-4 TANK BOTTOMS

Eight P. aeruginosa isolates from human clinical infections at Fort Detrick* were introduced into JP-4 and B-H medium in June 1963. After five months, two of the eight had shown slight turbidity and sediment but no discoloration of the fuel. There was no visible evidence of any growth at the interface. Of 30 P. aeruginosa isolates from tank bottoms, all had grown very well with JP-4 and B-H medium and most had discolored the JP-4. In observing the hemolysin reaction of their isolates, MB Division found that almost all of the clinical isolates gave a very strong hemolysin reaction and only one or two isolates of 22 from the JP-4 water bottoms produced a very weak but positive reaction.

* Obtained from R.A. Altenbern, MB Division.

VI. CORROSION STUDIES

With the advent of the wet-wing aircraft system and increased use of turbine fuels, corrosion of integral tanks became a significant problem to both military and commercial operators. The association of water, microbial growth, and corrosion was noted. In previous work, a careful examination of corroded areas revealed in certain cases a communal system involving Pseudomonas, Cladosporium, and Desulfovibrio. Kereluk[7] observed that growth and survival of Pseudomonas and Cladosporium were enhanced by aqueous extracts of Buna N topcoatings. There are numerous references associating Desulfovibrio with the pitting corrosion of iron pipes in oil fields and underground transport lines. The difficulties encountered in isolating and cultivating these organisms, and the disputed mechanism of the corrosion process involving these organisms, stimulated a general study of the sulfate reducers.

A. STUDIES ON DESULFOVIBRIO

At present, no satisfactory medium has been developed to cultivate Desulfovibrio on the surface of petri plates. Techniques for counting these organisms usually employ most-probable number determinations, which involve a fluid medium or colony counts in tubes using 1.5 per cent agar or less as recommended by Postgate.[8] The media generally used consist of (i) a hydrogen donor such as sodium lactate; (ii) inorganic salts, which include sulfa es and an iron salt (usually ferrous ammonium sulfate to act as an indicator for hydrogen sulfide); (iii) one or more organic supplements, usually including yeast extract, which is reported to stimulate growth; and (iv) one or more reducing agents such as ascorbic acid, cysteine, or thioglycollate. Two such media are API (American Petroleum Institute) agar,[9] which is used routinely in the petroleum industry in detecting and counting sulfate reducers, and a medium recently described by Postgate.[8] The compositions of these two media are shown in Table 26.

1. Development of Assay Medium

Attempts to grow pure cultures of D. desulfuricans on the surface of several of these media under hydrogen almost always resulted in growth of only a few large colonies, with blackening below or around the colonies. Transfer from these colonies to other plates yielded about the same results. Incubating plates of TSBA that had been streaked with a heavily contaminated water bottom showed small isolated colonies that were later identified as D. desulfuricans. An API strain of D. desulfuricans was likewise found to grow extremely well on this medium. The colonies grew to about 1 mm in diameter, were quite transparent, and were pale yellow when observed against a dark background (Figure 1). A pure culture of the API strain and the

TABLE 26. TWO TYPICAL MEDIA USED FOR GROWING AND COUNTING D. DESULFURICANS

	Postgate	API
Sodium lactate (70% sol)	3.5 ml	4 ml
K_2HPO_4	-	0.01 gm
$K\ H_2PO_4$	0.5 gm	-
NH_4Cl	1.0 gm	-
Na_2SO_4	1.0 gm	-
$MgSO_4 \cdot 7H_2O$	2.0 gm	0.20 gm
$CaCl_2 \cdot 6\ H_2O$	1.0 gm	-
$FeSO_4 \cdot 7\ H_2O$	0.5 gm	-
$FeSO_4(NH_4)_2SO_4 \cdot 6\ H_2O$	-	0.10 gm
NaCl	-	10.00 gm
Yeast extract	1.0 gm	1.00 gm
Ascorbic acid	0.1 gm	0.10 gm
Thioglycollic acid	0.1 gm	-
Distilled water	1 liter	1 liter
Agar	15.0 gm	15.0 gm
pH	7.6	7.2 to 7.5

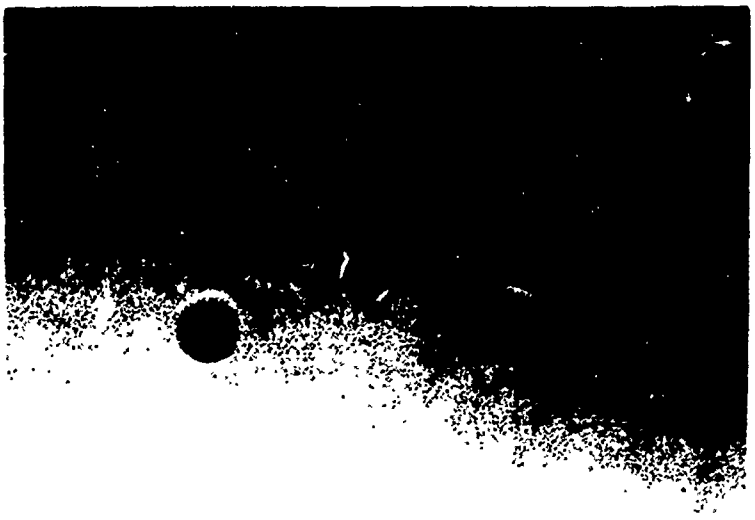

Figure 1. Colonies of Desulfovibrio on Trypticase Soy Broth plus Agar (2%). Colonies were originally about 1 mm in diameter.

strain isolated from fuel water bottom have been transferred for two years at monthly intervals on this medium under hydrogen without any apparent loss of ability to reduce sulfate. A third strain of D. desulfuricans isolated from a corrosion pit grew extremely well on the surface of this medium. Contamination of these cultures can be easily recognized by the appearance of different colony types while under hydrogen and by observation of the plates after they are removed from the hydrogen and allowed to incubate further under aerobic conditions. Efforts to demonstrate sulfate reduction (production of H_2S as indicated by blackening around or under the colonies) by incorporating lactate, magnesium sulfate, and ferrous ammonium sulfate in the TSBA medium were not very successful. Some blackening around the initial inoculum on the plates occurred, but the growth along the streak path was considerably reduced.

In determining which materials in the TSB were essential to growth of the API strain, 2 per cent agar plates of TSB and TSB minus one ingredient were streaked with the API strain and incubated under hydrogen in a desiccator jar (evacuated and refilled with hydrogen 3 times) at room temperature. Growth was determined by visual inspection of colony development along the streak path. The results are shown in Table 27.

TABLE 27. EFFECT OF OMISSION OF INDIVIDUAL COMPONENTS OF TRYPTICASE SOY EROTH AGAR ON THE GROWTH OF D. DESULFURICANS

Medium	Relative Growth[a] 7 Days	21 Days
TSBA (control)	2	4
TSBA minus trypticase	-	-
TSBA minus glucose	4	4
TSBA minus K_2HPO_4	3	4
TSBA minus NaCl	-	4
TSBA minus phytone	1	4

a. No growth along streak.
1 slight growth along streak path.
2 fair growth along streak path.
3 good growth along streak path.
4 excellent growth along streak path.

Initially, growth was favored by the elimination of glucose and hindered by the elimination of sodium chloride.

a. Effect of Nitrogen Source

A number of nitrogen sources were examined individually for their ability to support growth on the surface of 2 per cent agar plates incubated under hydrogen (Table 28).

TABLE 28. GROWTH OF D. DESULFURICANS ON SEVERAL NITROGEN SOURCES

Nitrogen Source, 2%	Growth[a] 7 Days	21 Days
Trypticase (BBL)	2	3
Yeast extract (Difco)	4	4
Lactalbumin hydrolyzate*	-	-
N-Z Case**	-	-
N-Z Amino**	-	-
Casamino Acids. Vitamin free (Difco)	1	1

a. No growth along streak path.
1 several isolated colonies along streak path.
2 fair growth along streak path.
3 good growth along streak path.
4 excellent growth along streak path.

Although the growth on trypticase was slower at first, the amount of growth was eventually about as good as that on the TSB plus 2 per cent agar. Growth on the yeast extract was even better than on the TSB plus 2 per cent agar. Several colonies grew on vitamin-free casamino acids, indicating that one or more amino acids might be sufficient for growth of this strain under hydrogen. Since the absence of glucose tended to favor growth and the absence of sodium chloride tended to retard growth, TSA seemed to be a good medium to use in further studies. Growth on this medium appears to be even slightly better than on TSBA that contains glucose.

b. Selection of Trypticase Soy Agar

When magnesium sulfate (2 gm/liter), ferrous ammonium sulfate (0.5 gm/liter), and sodium lactate (4 ml/liter) were added to TSA and the agar content was increased to 2 per cent (pH 7.2 to 7.4), excellent

* Nutritional Biochemicals Co., Cleveland 28, Ohio.
** Sheffield Chemical Co., Norwich, N.Y.

colonial development and blackening occurred along the entire streak path. It was also noted that comparable growth and blackening could be obtained without adding lactate (hydrogen evidently served as the sole donor). This medium would therefore serve as a detector for a coupled hydrogenase - sulphate reductase system. When these plates are removed from the hydrogen, the black color is replaced by yellow, evidently caused by the oxidation of sulfides to sulfur. This medium seems to hold promise for use in plate counts and shows a good correlation between dilution and number of visible colonies. Figure 2 shows 68 colonies on a streak plate (10^{-6} dilution) of a suspension of a pure culture of the API strain. The duplicate plate had 45 colonies. Plates inoculated with 10^{-7} dilution had 4 and 5 colonies, respectively, and plates inoculated with 10^{-5} dilution had colonies too numerous to count.

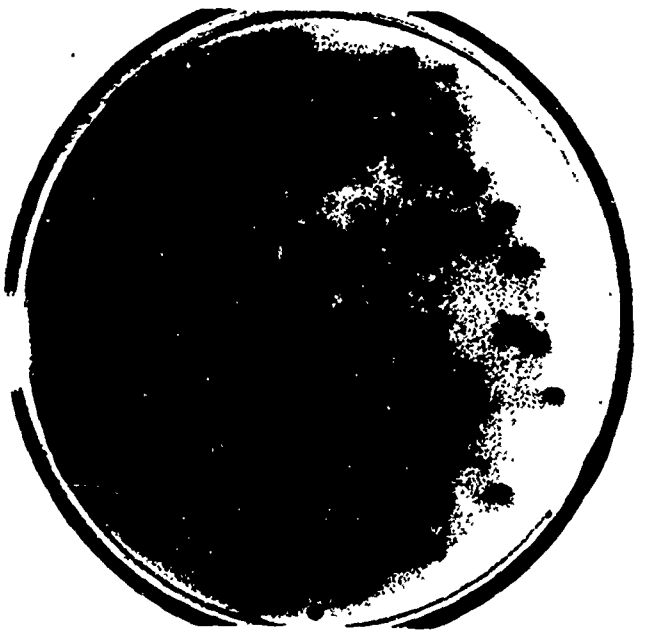

Figure 2. Colonies of Desulfovibrio on Trypticase Soy Agar plus Sodium Lactate (4 ml/liter), Magnesium Sulfate (2 gm/liter), Ferrous Ammonium Sulfate (0.5 gm/liter), Agar (2%).

2. Attempt to Develop a Selective Medium

A study was conducted to make TSBA more selective for Desulfovibrio by use of antibiotics and other inhibitory agents. Sensitivity disks were placed on the surface of TSBA plates streaked with a concentrated suspension of D. desulfuricans (API strain). After incubation under hydrogen for two weeks at room temperature, the zones of inhibition were measured:

Antibiotic	Concentration	Zone of Inhibition, mm
Erythromycin	15 µg	35
Magnamycin	15 µg	11
Bacitracin	20 units	0
Colymycin	10 µg	0
Tetracycline	30 µg	>40
Streptomycin	10 µg	0
Chloromycetin	30 µg	11
Viomycin	100 µg	0
Kanamycin	30 µg	0
Neomycin	30 µg	20
Penicillin	10 units	15
Polymyxin	30 µg	0

A few nonantibiotic compounds were also tried. Rose bengal at a concentration of 50 mg/ml in TSBA did not appear to inhibit the sulfate-reducing strain. When 10 mg of streptomycin per ml was added to the TSBA medium, growth was definitely inhibited. Sodium azide in a concentration of 0.075 mg/ml, potassium tellurite at 0.1 mg/ml, and sodium sulfite in a concentration of 5 per cent added to rose bengal and TSBA were completely inhibitory to the strain. When 5 per cent sodium sulfite was added separately to TSBA, a definite stimulation of growth was noted. Because of the twofold effect of sodium sulfite (its growth enhancement of the sulfate-reducing strain and its inhibitory action on other microorganisms) we decided to use this compound in TSBA for further study and to make this medium more selective by adding an antibiotic that would be active against Pseudomonas and coliform strains associated with sulfate reducers in fuel tank bottoms. Polymyxin and colymycin were selected. TSB medium to which 3 per cent sodium sulfite and 2 per cent agar were added was divided in half in concentrations of 10, 25, 50, and 100 mg/ml; polymyxin was added to the second half at the same concentrations.

The medium was dispensed into plates and allowed to solidify. After a few days for sterility check, the plates were streaked with Desulfovibrio and placed in an atmosphere of hydrogen at room temperature. Observations after two weeks showed that a concentration of 25 mg of polymyxin per liter was inhibitory; 50 mg of colymycin per liter was slightly inhibitory, and 100 mg per liter was completely inhibitory.

On the basis of these results, colymycin at a concentration of 25 mg per liter was tested under realistic conditions against soil and tank bottom suspensions. These tests showed that the selective medium did not produce the desired results. Further experimentation and testing with TSA instead of TSBA should be initiated and a selective medium developed.

Since excellent growth was obtained with 2 per cent yeast extract, we thought that this might be even better as a counting medium. Two per cent agar plates that contained 2 per cent yeast extract, sodium lactate (4 ml/liter), magnesium sulfate (2 gm/liter), and ferrous ammonium sulfate (0.5 gm/liter) were streaked with a pure culture of the API strain. After incubation under hydrogen they showed only slight blackening and fair growth. The salts appeared to have an inhibitory effect on the growth and sulfate reduction by this organism on yeast extract agar. Such inhibitory effects might explain why attempts to grow uniformly discrete colonies of D. desulfuricans on the surface of plates have so far been unsuccessful. In this case, most of the media used had contained yeast extract. It is possible that glucose also might have the same effect, because the attempts mentioned previously to incorporate a source of sulfate and an indicator salt in TSBA have not been satisfactory.

B. CORROSION STUDIES ON ALUMINUM

1. Simulated Tanks

Two simulated fuel tanks, fabricated by The Boeing Company* of 7178 alodine-treated alloy, were topcoated by Products Research Company.** One tank was coated with PR-1560 of the regular composition and one with PR-1560 with a high chromate content. The topcoatings were tested with a mixed culture of Ramey AFB inoculum in a dilute B-H water bottom and a Ramey JP-4 overlay. Growth was evident after the first week and subsequently became quite heavy. After six weeks, the tanks were emptied and reinoculated. The tanks were removed from test after 90 days' total time at 80 to 90 F. The topcoatings were in excellent condition. Chromates had not been extracted by the aqueous phase in contact with the high-chromate coating in quantities required to exert a biostatic effect.

A Boeing simulated fuel tank made of 7178 alodine-treated alloy was coated with Buna N. The tank was inoculated with Ramey water bottom, overlaid with JP-4, and set aside at room temperature to be used to demonstrate our topcoating test procedure. It was examined at infrequent intervals. After 11 months, five black tubercles that covered corrosion pits in the aluminum were found on the bottom of this tank. In contrast

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* The Boeing Company, Wichita, Kansas.
** Products Research Company, Gloucester City, New Jersey.

to the tubercles previously reported, which were light yellow or tan and fluoresced with ultraviolet light, the tubercles above the pits in this tank were black and did not fluoresce. Microscopic examination of the pits revealed yeastlike forms of Cladosporium that are characteristic of this fungus when grown under semi-anaerobic conditions. When material from the pits was streaked aerobically on TSA plates, the predominant organism was P. aeruginosa. When material from the pits was inoculated on TSBA and then in bottles of API medium (anaerobic), hydrogen sulfide production and blackening of the medium were observed after two weeks' incubation. Microscopic examination of the culture revealed vibrio and spiral forms characteristic of Desulfovibrio species. This culture has now been obtained in pure form.

An uncoated Boeing simulated fuel tank fabricated of 7178 alodine-treated alloy was inoculated with pure cultures of P. aeruginosa, Desulfovibrio species, and Cladosporium in JP-4 and B-H medium in December 1961. This tank contained several tubercles similar to those previously obtained with mixed Ramey AFB water bottom cultures. Corrosion pits were found under the tubercles. The predominating aerobic organisms recovered were P. aeruginosa and the Cladosporium species. One or two other contaminants were present in very small numbers. D. desulfuricans was not isolated from the pits, but another nonspore-forming anaerobic rod was isolated.

A small aluminum tank (7178 alloy, alodine-treated), which had been manufactured by Fairchild Stratos Corporation,* was sealed with polysulfide, topcoated with No. 1039 epoxy resin, and inoculated with a Ramey inoculum in November 1961. It was found to have a large blistered area in the bottom. Removal of the blisters disclosed two large corroded areas. Material from those areas was cultured for aerobic and anaerobic bacteria. Fungal filaments were not noted, but microscopic observation indicated that sulfate reducers were present in the corroded areas of this tank.

2. Corrosion Cells

It had been previously reported[1] that sulfate-reducing bacteria had been isolated from a corrosion pit under a tubercle in one tank and highly suspected of being present in corrosion pits under tubercles in a second tank, so it seemed of interest to determine what role they were playing in the corrosion process. One possibility was that the sulfate reducers, along with the other microorganisms in the tubercles, were producing oxygen concentration cells, so that the area directly under the tubercle (free of oxygen) was the anode and the area around the tubercle (slight amount of oxygen) was the cathode.

* Fairchild Stratos Corporation, Hagerstown, Maryland.

Four 8-ounce jars, two of them containing magnetic stirrers, were nearly filled with API medium (minus lactate) for sulfate reducers. The jars were sterilized, agar bridges were inserted between each pair of jars, aluminum alloy (7075-T6 alclad) strips approximately 102 mm by 12 mm were suspended in each jar, and sterile vent tubes with cotton plugs were suspended in one jar of each pair (Figure 3). After one jar was inoculated with a strain of D. desulfuricans obtained from a corrosion pit in an oil storage tank, sterile melted vaspar (equal parts of vaseline and paraffin) was poured over the surface of the liquid in each of the jars and allowed to cool. The aluminum strips were held in place by the vaspar, which also prevented oxygen from entering the liquid in the anaerobic cells. In the pairs, or half cells, that were to be oxygenated, some medium was withdrawn to allow an air space below the vaspar seal. An electrode in the oxygenated half of each cell was connected to an electrode in the oxygen-free half cell (i.e., electrode 3 to electrode 2 and electrode 3i to electrode 2i) and the current was measured with a d.c. vacuum tube voltmeter (Hewlett-Packard Model 412A).* After one week, sodium lactate was added to each of the half cells in each pair and growth of the sulfate reducers was indicated by blackening of the inoculated half cell four days later. Complete blackening of the jar was not noted until about the 21st day. At that time the corrosion current, which had been greater in the control cell, became slightly greater in the inoculated cell and remained so for about 27 days (Table 29). During that period of time, the corrosion current of the inoculated cell (Cell B) was about 1.5 to 5 microamperes greater than that of the control cell (Cell A).

Following this period, the corrosion current became greater in the control cell and remained greater for about 13 more days. Shortly thereafter, the inoculated cell showed a drop in current and a change in polarity. This may have been caused by contamination in the oxygenated half cell, since it was later found that the glass envelopes around the magnetic stirrers in both oxygenated cells were broken. Visual examination of all the electrodes in both oxygenated half cells showed them to be extensively corroded. Electrodes 1 and 1i, which were not part of the electrochemical circuit, showed no noticeable evidence of corrosion. Electrode 2 and 2i showed similar areas of corrosion. These results indicate that the aluminum alloy can corrode when it is part of an oxygen concentration cell and that sulfate reducers seem to accelerate this effect.

* Hewlett-Packard Co., 1501 Page Mill Road, Palo Alto, California.

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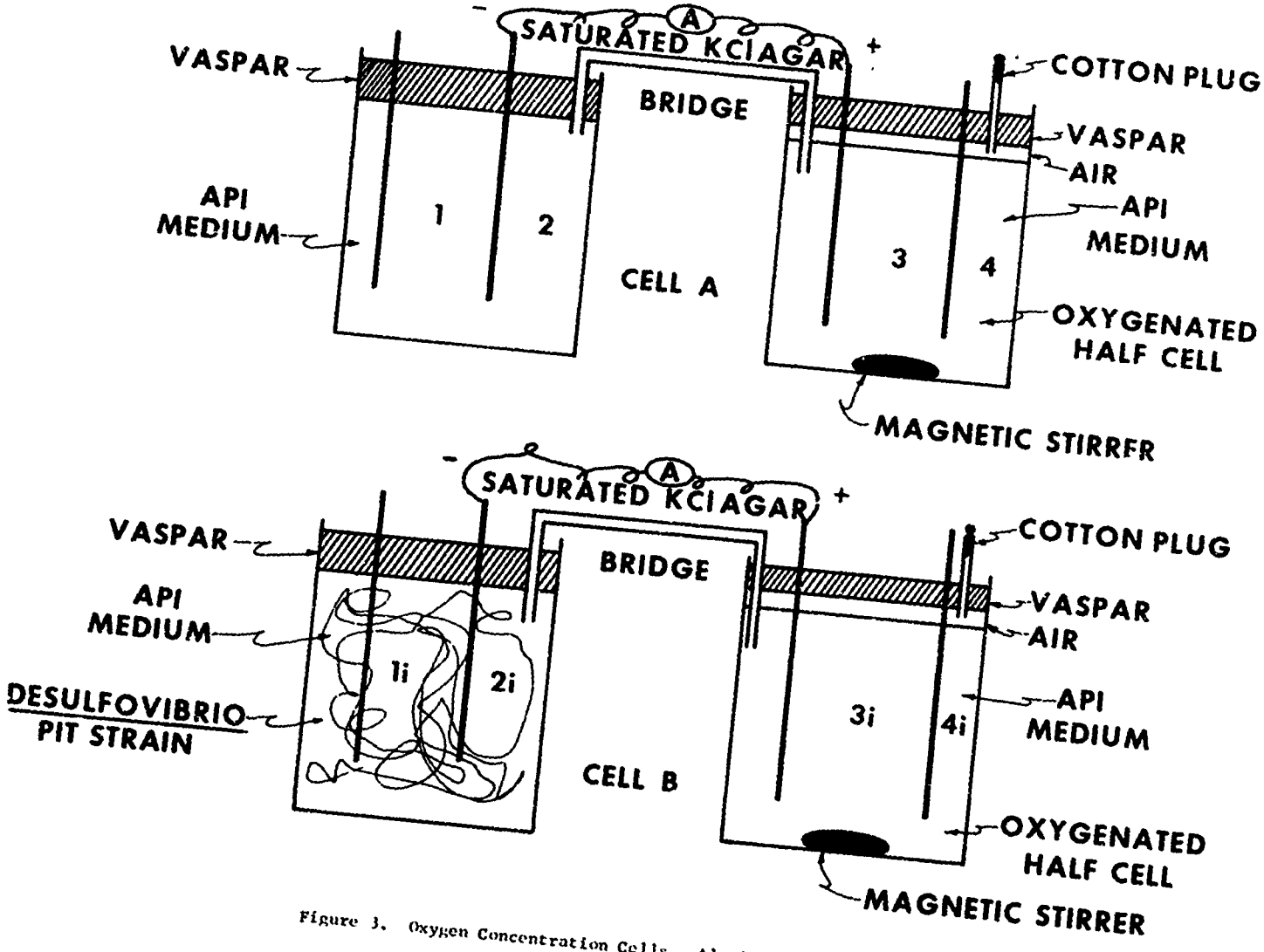

Figure 3. Oxygen Concentration Cells. Aluminum Electrodes.

TABLE 29. CORROSION CURRENT IN OXYGEN CONCENTRATION CELLS

Days	Corrosion Current, microamperes	
	Inoculated (Cell B)	Control (Cell A)
Initial	130.0	120.0
1	11.0	18.0
4	6.0	2.0
7	2.5[a]	4.7[a]
8	11.0	22.0
11	25.0 (lower 1/8 black)	50.0
16	14.0 (low 1/2 black)	21.0
18	12.0 (3/4 black)	12.0
19	12.0	12.0
21	15.0	13.0
22	13.0	11.0
25	15.0	13.0
26	16.0	12.0
28	17.0	12.0
36	17.0	14.5
42	17.5	16.0
48	17.0	18.0
53	20.0	22.0
57	20.0	23.5

a. Sodium lactate added.

VII. MISCELLANEOUS

A. STANDARDIZATION OF METHOD FOR BIOCIDE TESTING

The Committee on Microbiological Deterioration of Fuels of the Society for Industrial Microbiology adopted the following procedure as a tentative method for laboratory screening of fuel-soluble biocides on 23 April 1963.

1) B-H medium at pH 6.8 will be used as the underlay.

2) ASTM grade cetane or another suitable hydrocarbon fuel will be used as the test hydrocarbon.

3) The fuel-to-water ratio will be 100:1.

4) Tests will be conducted in the presence of 1020 mild steel and 2024 aluminum coupons ½ by 6 inches in size. Coupons will be acetone-washed and stored under cetane before use.

5) The inoculum (1%) will consist of a mixture of the following organisms developed in the presence of cetane:

P. aeruginosa - supplied by Fort Detrick

C. resinae - QM 7998, furnished by U.S. Army Natick Laboratories

C. lipolytica - ATCC 8662

6) Four coded biocides will be forwarded to the investigators by the chairman. They will include representatives of a phenolic, a nitrogen-containing, a metal-containing, and a sulfur-containing compound as active groups.

7) The concentration of the biocide to be tested will range from 100 to 1000 ppm in the fuel.

8) Test bottles will be incubated at 25 C and agitated daily.

9) The end point of the test will be the time required to sterilize the underlay. One, 7, and 14 days were suggested as suitable time intervals.

It is of interest that the adopted procedure would not detect the biostatic properties of EGME because a ratio of fuel to water of at least 300:1 is required to demonstrate the biostatic action of EGME in fuel at 1000 ppm.

As a participating member of the Petroleum Committee of the Society for Industrial Microbiology for standardizing a test method for microbial inhibitors in fuels, our laboratory at Fort Detrick tested four compounds on an "as-supplied" basis in a comparative evaluation with other participating laboratories. The tests were performed by the method outlined by the committee. In the analysis of the data, laboratories were coded so they would remain anonymous. The results were reported after 14 days' incubation and show the lowest concentration that caused complete inhibition with a ratio of fuel to B-H medium of 100:1. The compounds were tested in a range of 100 to 1000 ppm in increments of 100. The results are shown in Table 30.

TABLE 30. MINIMUM BIOCIDAL CONCENTRATIONS OF TEST COMPOUNDS IN FUEL USING FUEL-TO-WATER RATIOS OF 100:1

	Concentration, ppm			
Laboratory	Organic Sulfur	Chlorinated Phenol	Organic Boron[a]	Quaternary Ammonium
A	100	100	>1000	<100
B	300	200	700	200
C	200	-	200	<200
D	400	200	400	<100
E	100	200	800	<100
F	200	200	>1000	<100
G	200	600	>1000	<200

a. This was the only compound tested that was not corrosive to iron.

The agreement of results between laboratories was good considering that three of the compounds were not soluble in the fuel at the concentrations tested and the challenge inoculum was not standardized. Since this was the first cooperative test, the committee revised the procedures with the consent of all participants. The standard procedure will be published in the future.

B. PATHOGENICITY OF TEST CULTURES FROM COORDINATING RESEARCH COUNCIL

The CRC-sponsored Aircraft Fuel Tank Corrosion Group that met 10 April 1963 had proposed a preliminary test method to determine microbial penetration of integral fuel tank coatings. In this procedure, the test organisms Hormodendron cladosporioides and a species of P. aeruginosa were the inoculum. Dr. Bejuki recommended to Dr. Kereluk that Biological Laboratories determine the pathogenicity of these cultures. Dr. Kereluk provided slant cultures of each organism. These organisms were grown in the laboratory on Sabouraud's medium and in a system of B-H and JP-4. One-half-ml quantities of undiluted cultures containing more than 10^6 cells of each of the cultures were not lethal when injected intraperitoneally into mice. During the preparation of cultures to be used for mouse challenge, extreme care was taken to avoid the presence of JP-4 in the inoculum. Mice that had been given 0.5 ml of filtered JP-4 intraperitoneally died the same day they were challenged.

C. INSPECTION OF BOMARC MISSILE FUEL TANKS AT LANGLEY AND OTIS AFB

Examination of the dry side of a missile R/J fuel tank revealed condensation products such as gasoline, water, and a gummy substance that evidently leaches from the sealant around the fasteners in the fore portion of the tank. These products were mixed with talc and pieces of sealant. Laboratory examination of this mix did not reveal any biological contamination. Examination of the missile decontamination system revealed large quantities of rust: the iron pipelines showed severe scaling, and a filter showed a heavy deposit of rust on the element. It may be feasible for the Air Force to change from the woven wire cloth filter to a metal-edged filter that would not clog as fast and could be easily cleaned after each decontamination. The samples of the water from the decontamination system had a pH range of 7.47 to 7.86. Biological counts on the water samples before filtration were approximately 1000 cells per ml; after filtration, they were 100 or less cells per ml.

VIII. CONCLUSIONS

Microbiological contamination in JP-4 fuels, bulk storage tank water bottoms, and aircraft sump drainings at six Air Force bases was surveyed 12 months after the incorporation of an anti-icing additive. As in previous surveys, the level of biological contamination was extremely low. Many samples were sterile or approached sterility. Resistant strains capable of growing in water bottoms containing the quantities of additive that partitioned under field conditions did not develop. Samples from other sources, examined after this survey, showed similar results.

Attempts to isolate and adapt microorganisms to grow in a mineral salts medium containing more than 5 per cent ethylene glycol monomethyl ether as the sole carbon source were not successful. At lower concentrations, this compound was readily metabolized.

In predominantly aqueous fuel/water systems, the biocidal properties of ethylene glycol monoethers increased with increasing chain length of the alcohol moiety. However, when tested under more realistic fuel-to-water ratios of 1000:1, the methyl ether was most effective as a biocide. The presence of soluble organic nutrients in a water bottom markedly reduced the biocidal action of the methyl ether of ethylene glycol.

Of several compounds evaluated as potential or standby fuel-soluble biocides, arsenosobenzene was the most promising and was effective under realistic test conditions at a concentration of three ppm in the fuel. In active tanks where the water bottoms are exposed to frequent changes of overlaid fuel, a concentration of one ppm or less would probably be effective.

A total of 54 Pseudomonas cultures, including 16 strains of known identity, 8 from clinical specimens, and 30 from JP-4 water bottoms, were partially characterized. The pathogenic Pseudomonas grew poorly, if at all, on JP-4 as a carbon source and were readily differentiated from those occurring in fuel bottoms by their strong hemolytic reaction on blood. Mercaptan production in a medium containing proteins and cysteine is a predominant characteristic of many pseudomonads.

Three strains of D. desulfuricans were found to grow well and could be enumerated on the surface of modified trypticase soy agar plates incubated in hydrogen. D. desulfuricans was isolated from a corrosion pit in a simulated aluminum wing tank. Cladosporium spp. and P. aeruginosa were isolated from the tubercle that covered the pit. An oxygen concentration cell using aluminum coupons as electrodes and containing a Desulfovibrio culture in the anodic anaerobic half-cell produced a corrosion current slightly higher than that of an unseeded control when biological activity was at its peak.

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