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ANNUAL PROGRESS REPORT

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ACTIVITIES OF HYDROGENASE IN MARINE ENVIRONMENTS AND THE CORROSION OF IRON

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MICROBIAL CORROSION OF IRON

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OBJECTIVES

To study (1) the distribution and activity of hydrogenase and hydrogen uptake by marine microorganisms, and (2) microbial hydrogenase activity and its effect on the depolarization of metallic iron surfaces.

Anaerobic enrichment cultures

Data reported in earlier progress reports have shown that certain marine bacteria, one strain of Desulfovibrio sp. and a facultatively anaerobic pseudomonad, promote the corrosion of iron. These two organisms were used in further studies, the results of which have implicated the enzyme, hydrogenase, as possibly being responsible for the depolarization and resulting corrosion of metallic iron in the anaerobic environment.

In an effort to acquire a broader spectrum of organisms which might show similar corrosive properties, emphasis has been placed on obtaining enrichment cultures from samples taken from various marine environments. Substrates examined to date can be devited into five basic types: (1) water; (2) sediments and muds; (3) sand; (4) metals; (5) wood. General areas sampled include portions of the lower peninsula of Florida and the Bahamas.

Methods

Medium

The medium M10-E, previously described in the 1962 Progress Report, was used not only for isolation but also enumeration of anaerobic bacteria by extinction dilution technique. Exactly 9.3 ml of the freshly prepared medium (hot) were added to half-ounce prescription bottles used in a dilution series, the capsoplaced on loosely, and autoclaved. Immediately following sterilization, the caps were tightened and the medium allowed to cool. Routine loss of 0.3 ml of water during sterilization produced the desired volume of 9.0 ml per bottle. Bottles were conveniently packaged for serial dilutions in the field.

Sampling

Waters to be examined were usually collected with either sterile bottles or plastic bags. Other materials were collected either with a sterile spatula or "sawed-off" glass syringe (for sediments or muds). The sample volume used for enumeration was one ml for both liquids and solids.

Enumeration

The extinction dilution method was used to determine the most probably number of both sulfate and non-sulfate reducing bacteria. Dilutions (1:10) were made to 10^{-8} in the prescription bottles containing M10-E medium. Immediately following the dilution procedures, each dilution bottle was aseptically filled to capacity with reduced medium of the same composition and the cap tightly closed to provide optimum anaerobic conditions. This was facilitated by the use of the device shown in Fig. 2 described in a later section of this report.

Results

Bacterial numbers were recorded after incubation at room temperature for three weeks. All samples showing sulfate reduction (actually, FeS precipitation) were transferred to tubes of M10-E medium and assigned mixed culture numbers. The enrichment cultures obtained to date using the described method are shown in Table I. Included in this table are the collection areas and substrate from which each culture was collected. The 51 mixed cultures listed were obtained from a total of 60 samples. Samples were from the five substrate types listed earlier and had the following distribution: water, 36.7%; sediments or muds, 45.0%; sand, 3.3%; metals, 13.3% and wood, 1.7%.

On primary cultivation, 83% of the samples apparently contained sulfate reducing bacteria. The enumeration procedures showed the average number of sulfate reducers in waters to be the same as for non-sulfate reducing anaerobes (1×10^2 per ml) while values for sulfate and non-sulfate reducers in the remaining substrate types were 1×10^3 and 1×10^4 per ml, respectively.

The mixed cultures obtained are currently being grown in a defined medium in order to confirm the presence of sulfate reducers. The medium is one similar to M10-E but lacks yeast extract and neopeptone. Those mixed cultures corrosive to iron and containing sulfate reducers will be examined in pure culture.

Evaluation of corrosion test methods

A critical examination of our anaerobic corrosion data has shown a rather high incidence of unexplained weight loss of test coupons where such would not be expected. It has been our experience to find not only a greater weight loss in controls than in the test themselves, but also variation in replicate tests. This seems to be true in tests involving both biological as well as chemical agents (see previous reports as well as Table II, for example).

Corrosion test: coupons with and without leads

As an initial approach to the above problem, the importance of the cathodic lead in assaying microbial corrosion was investigated. Microbial growth in the presence of completely submerged iron test coupons normally is present over the entire surface. A thin strip of metal of the same consistence as the test coupon extending out of the medium would therefore produce at the water air interface an area that could act as the cathode in respect to the anodic immersed metal, although microgalvanic cells may be present on the immersed metal surfaces. It was surmised that should the extension or lead be necessary for producing microbial corrosion, the relative contact between the lead and coupon should be of vital importance to insure uniform weight loss of the coupons in replicate tests. In order to determine this effect an experiment was de-

TABLE I. Anaerobic Enrichment Cultures

Culture No.	Collection Area	Substrate
1	Virginia Key (Fla.)	Bay water
2	Pigeon Key (Fla.)	Marine mud
3	Virginia Key	Marine mud
4	Eleuthera (B.W.I.)	Carbonate core I Surface
5	"	" " " 3 in.
6	"	" " " 6 in.
7	"	Surface water core I area
8	"	Carbonate core II Surface
9	"	" " " 3 in.
10	"	Surface water core II area
11	Cochrane Anchorage (B.W.I.)	Surface water
12	Eleuthera	Surface water
13	Hatchet Bay, Eleuthera	Marine mud
14	Six Pence Channel (B.W.I.)	Surface water
15	Gulf Stream	Surface water
16	Biscayne Bay, Miami, Fla.	Trawled mud
17	Bermuda	Submerged steel buoy Scale
18	"	" " " Blister
19	Virginia Key	Sewage sludge
20	Gulf Stream	Surface carbonate core
21	Biscayne Bay	Iron nail (submerged)
22	" "	Siliceous sand
23	" "	Sediments in Thalassia bed
24	Soldier Key (Fla.)	Carbonate sediment
25	Key Biscayne (Fla.)	Shallow marine mud
26	Virginia Key	Iron at mud surface
27	" "	Wood at mud surface
28	" "	Lead at mud surface
29	" "	Mud near lead (above)
30	Banks of W. Andros , B.W.I.	Carbonate core III Surface
31	" " " " "	" " " 3 in.
32	" " " " "	Water over core III area Surface
33	" " " " "	" " " " " Bottom
34	" " " " "	Iron scrap in mud
35	Bimini Bay, B. W. I.	Carbonate core IV Surface
36	" "	" " " 3 in.
37	" "	Water over core IV area Surface
38	" "	" " " " " Bottom
39	Virginia Key	Wet beach sand
40	" "	Dry beach sand
41	" "	Surface water
42	" "	Brackish pond water
43	Estero, Fla.	Brackish pond mud
44	Ft. Myers, Fla.	Marine mud
45	Tampa Bay (Fla.)	Shallow mud
46	" "	Deep mud
47	Palmetto, Fla.	Marine mud
48	Alligator Point (Fla.)	Marine mud
49	" "	Surface water
50	Dunedin, Fla.	Marine mud Surface
51	" "	" " 3 in.

signed whereby a comparison could be made between the amount of corrosion produced when leads were present or absent from test coupons.

Fifty-six iron test coupons were prepared for use (see earlier reports) and divided into four groups of 14 each. Two groups of 14 were fitted with leads, omitting leads on the remaining two groups. All coupons (both with and without leads) sterilized with ethylene oxide, were added to individually cotton-stoppered culture tubes of M10-E medium. One ml of a 96 hour culture of Desulfovibrio sp. was used to inoculate one group of each set with and without leads. The two uninoculated sets served as controls. Test and control tubes for each respective test condition were placed together in a desiccator, evacuated, filled with nitrogen three times, and incubated at room temperature for 15 days under the final atmosphere of nitrogen.

Results of these tests, shown in Table II, present a confusing pattern. Internal variation of coupon weight loss was greater in test and control sets of both conditions being tested than the average for the test or control. Also, the difference between the two control sets was much more than would be anticipated if protective polarization does indeed occur in the absence of a depolarizer such as the appropriate bacteria or oxygen. Most disturbing was the greater overall weight loss in the control set of coupons without leads over that of the inoculated set.

It is assumed that no one factor caused the observed variation in these tests, as several possible interacting effects may be responsible. These could include, particularly in the test using leads, a difference in the oxygen concentration in the desiccators as well as per tube, constitution of the inoculum and variation in the anode/cathode ratio of each test coupon. In tests where no leads were provided, these same conditions could have been active in producing variations but to a more subtle degree.

A variation in oxygen concentration, as each set (leads and no leads) was incubated in a separate chamber, could have affected the corrosion rates by producing micro-oxygen differential effects. This oxygen difference could also have been a localized result of the activity of the microorganisms in the inoculum. However, variations such as those two already mentioned were believed at this point to be secondary as compared with the anode/cathode ratio of each individual test coupon itself. The coupons used were thick and during fabrication stress points due to bending were formed. It is known that such stress points can become anodic.

Test to determine anodic and cathodic areas of coupons

Iron coupons were examined for uniformity of anodic and cathodic sites by watching the reactions of triphenyltetrazolium chloride (TTC) that is reduced at the cathode to a red color, and Turnbull's blue (ferrous ferricyanide) that is produced at the anode where the liberated Fe^{++} reacts with $K_3Fe(CN)_6$, potassium ferricyanide.

Five coupons previously used in corrosion tests were randomly selected and cleaned by the usual methods. The coupons were placed in screw cap tubes and covered with a 0.1% solution of TTC containing 0.1% potassium ferricyanide and 1% agar at 45 degrees. Almost immediately the red color of reduced TTC showed the presence of cathodic areas while at the same time the blue color of ferrous ferricyanide (Turnbull's blue) occurred at the anodic areas. The results indicated a rather high variation in the percentage of anodic and cathodic sites on the individual coupons.

TABLE II. Anaerobic corrosion test using Desulfovibrio sp.. Incubation time was for 15 days at 27°C; coupons used were of the old 40 gauge type.

	Coupons with Leads		Coupons without Leads	
	Coupon Number	Coupon Wt. Loss ($mg/10\ cm^2$)	Coupon Number	Coupon Wt. Loss ($mg/10\ cm^2$)
INOCULATED TESTS	1	11.4	29	6.0
	2	10.8	30	0.6
	3	11.8	31	5.0
	4	10.8	32	0.6
	5	9.8	33	1.9
	6	3.1	34	0.4
	7	12.8	35	0.9
	8	4.3	36	1.7
	9	9.9	37	0.7
	10	7.7	38	0.7
	11	8.3	39	0.6
	12	9.5	40	1.4
	13	15.4	41	2.0
	14	13.1	42	0.8
		Avg. 9.89		Avg. 1.66
STERILE CONTROLS	15	5.7	43	3.7
	16	8.2	44	2.8
	17	5.3	45	2.4
	18	6.3	46	2.2
	19	4.4	47	2.2
	20	5.7	48	1.8
	21	6.0	49	2.3
	22	3.5	50	2.7
	23	7.4	51	2.3
	24	5.7	52	2.6
	25	6.3	53	3.0
	26	3.8	54	2.0
	27	5.3	55	1.5
	28	3.8	56	2.4
		Avg. 5.52		Avg. 2.42

Even though all of the coupons had been cut from the same sheet of cold rolled steel, stresses known to have been produced during fabrication were thought to have caused the anode-cathode differences. To test this the experiment was repeated, this time twisting half of the test coupons to be used in the experiment. The results were as expected in which a larger percentage of each twisted coupon was anodic in contrast to the non-twisted. In light of the above findings, it was assumed that during usual fabrication of coupons (viz., sheared by hand), unequal stresses are imposed which alter the anode/cathode ratio of each coupon. In that the latter was believed to account for significant variation in corrosion test results, new coupons were fabricated. A single sheet of 24 gauge (0.025 in.) cold rolled steel was cut into uniform coupons having an overall surface area of 10 cm^2. All cutting was done with a hydraulic shearing machine with hopes that any stress (believed to be reduced to a minimum) would be uniform. Thin strips were also cut from the same stock to serve as leads to be attached to the coupons.

Tests using the above procedures for location of anodic and cathodic areas on metal surfaces were made again, this time with new 24 gauge coupons which had been unchanged and those which had been unchanged and those which had been twisted. New, twisted coupons showed a greater anodic surface than new, unchanged coupons. The variation in the relative ratio of anodic/cathodic areas was uniformly low (relative to other coupons) for the unchanged coupons, thereby justifying the use of the more sophisticated fabrication procedures as opposed to the old.

Control test: new coupons

In an effort to further test the uniformity of the new coupons, an experiment was performed in which measurement of weight loss from test coupons in sterile medium was made after 15 days.

Forty coupons and leads were selected from those newly prepared from 24 gauge stock. Since it was possible that the cleaning procedures used in the past could produce variation in the data, an improved method was used. Both coupons and leads were first dipped in 50% HCl for seven seconds, rinsed in warm tap water, methanol, and finally in acetone. The coupons and leads were then tumbled separately for 24 hours in a ball mill containing a 50-50 mixture of 250 mesh carborundum-silica sand as the abrasive. Following the abrasive treatment, both coupons and leads were again dipped in 50% HCl for seven seconds and rinsed with the above solvents. The final acetone dip was followed by immediate flaming of the coupon which was then placed in a previously heated and numbered test tube. The coupons were allowed to cool to weighing-room temperature before taring.

Tared coupons were fitted with cleaned leads and added to screw-cap tubes of M10-E medium. These were then autoclaved with the caps slightly loosened and closed tightly immediately after being removed from the autoclave. The forty units thus prepared were maintained at room temperature for 15 days. At the end of the 15 day period, the following characteristics were noted: color of the medium and precipitate, the appearance of a white material on the lead at the gas-liquid interface (listed as "white lead"), and the relative amount of oxidized iron at the gas-liquid interface.

As each tube was opened, a one ml sample of the liquid was placed into fresh M10-E medium for a sterility check. A sample of any "white material" mentioned above was cultured in M10-E as well as examined microscopically. After removing each coupon from its tube, any appearance of a black film on the metal was recorded and the pH of the medium measured. Weight loss of each coupon, cleaned by the following procedure, was determined. The coupon (with lead removed) was dipped in a 1% sodium arsenite solution prepared in 15% HCl for seven seconds, rinsed with warm tap water and then scoured with

cleansing abrasive. The coupon was rinsed with water, dipped in methanol and finally in acetone and flamed dry. Treatment was then identical to the initial procedure previously outlined above.

The results of these tests are given in Table III. Upon first examination of the data it is seen that three of the coupons had exceptionally high weight loss. In that there was no evidence of contamination revealed by the sterility tests, bacteriological activity was ruled out as an explanation for these weight losses. From further examination of the data, several points of interest can be noted. In the cases of high weight loss, five factors are found to be common: (1) a grey medium, (2) a precipitate some shade of green (the darker the green, the higher the weight loss), (3) a lead with white material at the gas-liquid interface (shown to be a sterile, amorphous material), (4) the absence of any oxidized iron and (5) the presence of a black film over the coupon and portions of the lead exposed to the liquid. The latter was shown to be a sulfide by virtue of an odor of H_2S upon acidification. It is particularly striking that tests showing all of the above characteristics accounted for 33% of the total amount of corrosion occurring in the tests. Those showing all but the sulfide production were responsible for 39% of the total.

Of the parameters recorded, only pH did not seem to be a factor common to the high weight loss of iron in these tests.

Also to be noted is the low weight loss from coupons when the following were encountered: a clear medium, cream or tan precipitate (characteristic of fresh, sterile M10-E medium), no white material on the lead, no evidence of oxidized iron and no sulfide film on the coupon or lead. Of the three tests showing this combination of characteristics, deviation from the average weight loss of 1.65 mg/10 cm^2 was only 0.05 mg.

In attempting to find an explanation for the results of this set of tests, the following was postulated. Variation in weight loss from coupon to coupon was a result of the varying oxygen concentration per tube. It is assumed that some variation in oxygen concentration could have occurred at the time of closing the caps on the tubes. However, it is more probable that the variation was produced as a result of the varying degree of leakage around each test tube cap seal. This might effectively vary the amount of oxygen drawn into each respective tube as its vacuum developed during cooling. Should this be the case, then variation in the amount of iron lost per coupon could have been effected in three ways:

(1) Little, initial weight loss as a result of complete anaerobiosis. This could be effected by the complete sealing of a tube to such a degree that no oxygen was available for combination with the polarizing hydrogen or with iron in solution. That any iron loss was detected can be explained on the basis of the natural solution pressure of iron in the medium and a resulting polarization of the coupon by hydrogen. With no oxygen available for depolarization, weight loss was held to a minimum (see tests 7, 29 and 36 of Table III).

(2) Intermediate, variable weight losses resulting from excessive availability of oxygen: In this particular instance, oxygen would be available not only to serve as a depolarizer but also as a reactant in the formation of ferric hydroxide. The extreme variation produced by this circumstance (represented by most of the other tests) may be explained as being a result of varying combinations of conditions mentioned in (1) and (3).

(3) Excessive weight loss resulting from limited access of oxygen: Here would be a case in which the amount of oxygen available, or actually its rate of entry into the

TABLE III. Fifteen day test on sterile controls using new 24 gauge iron coupons in M10-E medium (initial pH = 7.6) at 25°C.

Coupon Number	Wt. Loss ($mg/10cm^2$)	Appearances and Characteristics at end of test					
		Medium Color*	Color of ppt.	White lead	Oxidized Fe at gas-liquid interface	pH	Black film on metal
1	3.9	br.	br.		+ + +	6.9	
2	4.4	br.	br.		+ + + +	7.3	
3	4.7	br.	br.		+ + + +	7.2	
4	2.1	cl.	tn.		+ +	7.6	
5	2.6	br.	br.		+	7.3	
6	3.4	br.	br.		+ +	7.1	
7	1.7	cl.	cr.		0	7.5	
8	2.9	br.	br.		+ +	7.4	
9	4.0	br.	br.		+ + +	7.6	
10	5.1	br.	br.		+ +	6.6	
11	2.5	cl.	br.		+	NR	
12	4.6	gy.	dk. br.		0	NR	
13	4.1	gy.	dk. br.		0	NR	
14	2.7	br.	br.		+	7.6	
15	8.9	gy.	dk. gr.	+	0	7.4	+
16	3.2	br.	br.		+	7.3	
17	3.4	br.	br.		+ +	7.4	
18	15.2	gy.	dk. gr.	+	0	6.7	+
19	3.6	br.	dk. br.		+ + +	7.6	
20	2.6	br.	br.		+ +	7.0	
21	4.4	gy.	lt. gr.		0	7.5	
22	4.5	gy.	dk. gr.	+	0	7.8	
23	2.5	cl.	br.		+	7.4	
24	4.6	gy.	lt. gr.	+	0	7.5	
25	3.6	br.	br.		0	7.6	
26	6.5	gy.	dk. gr.	+	+	7.6	
27	14.7	gy.	dk. gr.	+	+	7.1	+
28	3.5	br.	br.		+ +	7.3	
29	1.7	cl.	cr.		+	7.4	
30	5.1	cl.	br.		+ + + +	7.2	
31	3.7	br.	br.		+	7.4	
32	3.7	br.	br.		+	7.6	
33	13.4	gy.	dk. gr.	+	0	6.7	+
34	2.3	cl.	tn.		+	7.6	
35	3.0	br.	br.		+ +	7.7	
36	1.6	cl.	cr.		0	7.4	
37	2.7	br.	br.		+	7.2	
38	8.4	gy.	gr.	+	0	7.4	+
39	3.2	br.	dk. br.		+ +	7.6	
40	4.5	br.	br.		+ + +	7.3	

* br. = brown
cl. = clear
gy. = grey
gr. = green
dk. = dark
lt. = light
cr. = cream
tn = tan
NR = no reading

tube, would be sufficient only to react with the polarising hydrogen and not with any ferrous ion in solution. As a result of the depolarization of the iron by the oxygen only at a "demand" rate, more iron would go into solution. If the rates of hydrogen production at the cathode, and oxygen entrance, were equal, the weight loss should be at a maximum (see tests 15, 18, 27, 33 and 38 in Table III). In the absence of excess oxygen the dissolved iron would remain in equilibrium as reduced ferrous hydroxide, $Fe(OH)_2$, (accounting for the observed green color of many of the tests). The occurrence of the sterile amorphous material on leads in isolated cases in this set of tests is unexplained at this writing. It has been shown, however, that the material is slowly soluble and produces no CO_2 in 1% HCl.

An explanation for the appearance of sulfide on five of the test coupons is also lacking, although it is probable that the iron surface with hydrogen atoms catalyzed the reduction of sulfate in the medium. That the sulfide was not produced by bacterial reduction of sulfate can be argued in several ways. Sterility tests made on the questionable coupons using reduced M10-E medium showed no evidence of any microbial forms. In addition, if sulfate reducers had been present, H_2S formation would have been homogeneous. This H_2S would then be expected to combine with iron or other metallic ions in the medium as well as on the coupons. Because the latter only was true, the biological production of sulfide is ruled out.

The occurrence of sulfide in sterile controls has also been observed by other investigators. They, too, have been unable to explain this phenomenon. From the results reported here, it is concluded that the "abiological" formation of sulfide is directly related to the magnitude of coupon weight loss or corrosion.

Standardization of corrosion test techniques

Aside from the effect of oxygen concentration on variability of corrosion tests (as pointed out in the above section), it should be mentioned that variation is also possible as a result of coupon handling, cleaning, treatment, etc. In order to reduce all of these effects to a minimum before pursuing corrosion theories further, a standard method for performing corrosion tests was initiated. This method, to be used until shown unsatisfactory, is given below.

STANDARDIZED ANAEROBIC CORROSION TEST METHODS

I. Coupon fabrication: Cut 24 gauge (0.025 in.) cold rolled steel to a uniform size of 1.774 X 0.405 in. (producing a total surface area of 10 cm^2). Using a 1/8 in. die, punch a hole in each coupon 1/8 in. from the end along the central axis (for lead attachment).

II. Leads: These should be cut from the same metal stock as the coupons and measure 0.1 X 3.0 in.

III. Initial cleaning prodedure:

A. Dip coupon and leads in 50% HCl for 7 seconds.

B. Rinse in warm tap water, then methyl alcohol and finally in acetone; flame dry.

C. Tumble both leads and coupons in a ball mill containing a 50-50 mixture of 250 mesh carborundum-silica sand for 24 hours.

D. Rinse with water, then dip in 50% HCl for 7 seconds; rinse with the

above solvents and flame dry while holding coupon with stainless forceps. (Leads can be spread on foil to air dry; store in desiccator over silica gel).

E. Place dry coupons in preheated, numbered test tubes and place in a desiccator over silica gel.

F. Allow coupons to cool to weighing-room temperature.

G. Remove coupons with clean, dry forceps and determine their weight to the nearest 0.1 mg and return them to their tubes.

H. Store all coupons to be used over silica gel (vacuum) for no longer than 24 hours.

IV. Lead attachment:

A. Remove a lead from the desiccator with needle-nose pliers.

B. Using same, make a 90° bend in one end of the lead while holding the other with gloved hand.

C. After removing a tubed coupon, insert the bent end of the lead through the hole and lift the coupon from the tube.

D. Holding the unit vertically by the lead, crimp the bent end of the lead another 90° allowing approximately 1/8 in. of the lead to overlap on itself.

E. Press the lead firmly to make good contact on the coupon, then press the overlapped portion together to lock lead in place.

F. Place the joined unit in its numbered tube.

V. Sterilization of the coupon units:

A. Prepare the following:

1. A 0.1% solution of ascorbic acid in warmed distilled water.

2. Preheated screwcap tubes (20 X 150 mm); numbered as other unit tubes.

B. Fill the heated tubes with the ascorbic acid solution.

C. Using forceps, place respective coupon units in their appropriately numbered tubes of hot ascorbic acid.

1. Do not disturb the "bead" of overflowing liquid.

2. Screw caps into place loosely and autoclave at 15 lbs for 20 minutes.

D. Close tube caps tightly immediately after autoclave is opened.

1. Ascorbic acid solution should be above the top of the lead.

2. Units should be used within 2 hours after sterilization.

VI. Initiation of corrosion tests and controls:

A. Preparation of anaerobic media:

1. Mix together all ingredients of any medium to be used except the reducing agent.

2. Heat to 80° while stirring and add the reducing agent.

3. Adjust pH to value required.

4. Eh should be -100 mV or below.

B. Treatment of tubed medium:

1. While stirring, deliver 20 ml of reduced medium to numbered 20 X 150 mm culture tubes.

2. Cotton-stopper the tubed medium and autoclave at 15 lbs for 20 minutes.

3. As soon as medium is sterilized, remove from the autoclave and:

a. Place tubes in desiccator.

b. Pull slight vacuum, then fill with nitrogen.

c. Repeat "b" above in gradual steps, increasing about 100 mm Hg per flushing; one flush every 10 minutes for 30 minutes.

C. Inoculation of tests:

1. After the medium has been sufficiently saturated with nitrogen remove all tubes from the desiccator.

2. Inoculate all tubes to serve as tests with 1 ml of a 96 hour culture of the organism to be tested.

3. Using sterile forceps, remove the appropriate sterile coupon units from the ascorbic acid solution and add them to the inoculated and uninoculated (control) tubes in numerical order.

4. Place both test and control tubes in a common desiccator and add 50 ml of Fieser's oxygen absorbent solution.*

5. Incubation should be at room temperature under a little less than one. atmosphere of oxygen-free nitrogen (scrubbed through Fieser's solution).

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* 15 g sodium hydrosulfite and 2 g sodium anthroquinone -beta- sulfonate in 100 ml of 20% KOH.

D. Oxygen contamination.

Oxygen contamination plays a very important role in any study of anaerobic corrosion. Small amounts of residual oxygen can act as a depolarizer, thus producing oxygen differential cells resulting in anode-cathode galvanic corrosion. In the outlined procedure residual amounts of oxygen causing oxygen differential cells can be accounted for and corrections made. It is necessary, however, to keep all tests in uniform controlled conditions so as to avoid differences in oxygen contamination.

VII. Termination of test:

A. Sterility check on controls:

1. Remove all tubes from desiccator and transfer a 1 ml sample from each control to a tube containing the same medium.

2. Incubate under nitrogen at room temperature for 10 days.

B. Determine the pH of the medium of both tests and controls.

C. Cleaning procedure:

1. Withdraw the coupon unit from a tube and remove the lead.

2. Using stainless forceps, stir the coupon in a 1% solution of sodium arsenite prepared in 15% HCl for 7 seconds, then rinse in warm, running tap water.

3. With a small amount of scouring powder (e.g. Bon Ami) on the fingers, clean all sides of the coupons with about 10 strokes per side.

4. Rinse well in tap water, then swirl the coupon again for 7 seconds in the HCl-Na arsenite solution; rinse again in tap water.

5. Dip coupon in container of absolute methyl alcohol, then place it in a numbered tube containing the same solvent.

D. Weight loss determination:

1. One at a time, remove each coupon from the tubes of methyl alcohol (using forceps) and dip into acetone.

2. Flame dry and place in preheated, numbered tubes.

3. Place all tubed coupons in a desiccator over silica gel and allow them to cool to weighing-room temperature. Weigh.

Corrosion tests using MC 1

In order to evaluate the newly formulated methods for examining the corrosive nature of anaerobes, an experiment was initiated in which the mixed culture, MC 1, was used as the test organism. This culture, isolated from surface sea water, is known to contain an

anaerobe capable of reducing sulfate. Its corrosive nature, however, has not before been examined.

The method used for the test followed that outlined above. The number of individual test replicates was 20 and was matched by an equal number of controls. The medium used was M10-E and incubation was at room temperature for 10 days under 1 atmosphere of nitrogen.

The results of this test are shown in Table IV. Four of the controls were found to be contaminated and were disregarded. The average weight loss of the remaining 16 uninoculated controls was 1.49 mg/10 cm^2; the average value of the inoculated set was 2.13. This indicates an "average" corrosion rate for MC 1 of 0.64 mg/10 cm^2 per 10 days. This may or may not represent a significant value for a test of this nature. One would be inclined, however, to believe that there is no difference between the tests and controls. This is emphasized when it is noted that a variation of values between inoculated tests is 0.9 and in control is as high as 1.2 mg/10 cm^2.

It is gratifying, however, to find that variation between replicates in this test dropped by approximately 80% from that found previous to the fabrication of new coupons and standardization of the test method.

Contact corrosion: screening for corrosive forms

The reassuring results from the above corrosion test using MC 1 (viz., low replicate variation) prompted an examination of 37 of the 50 other mixed cultures for possible high corrosive abilities.

The new corrosion test method already given was again used, this time limiting the number of controls to three and making an individual test represent a single mixed culture. The contact corrosion tests were incubated at room temperature in M10-E medium under an atmosphere of nitrogen. Both tests and controls were contained in the same desiccator.

Table V gives a summary of the data obtained after 30 days. Mixed culture numbers given in the table correspond to those listed in Table I. In the tests made here, a significant number (32%) of the cultures showed an ability to produce more than 1.0 mg/10 cm^2 weight loss over controls. Controls in this test series showed a weight loss of 1.6 ± 0.1 mg/10 cm^2. One culture, MC 28, seemed to have produced an unusually high weight loss of 17.0 mg/10 cm^2 (over controls). This culture was isolated from a lead (Pb) test panel located at the mud-water interface in Biscayne Bay. Subculture of the original MC 28 in M10-E medium containing lead shot did not noticeably effect the culture in that sulfide was produced as usual. More will be said about the use of this culture in later tests.

Several notes should be made at this point in regard to various data in Table V. In the light of these data, previous data take on more significance. Of special interest is the close agreement between the three controls in this test and those of other tests. If Table III is re-examined, it will be found that weight losses in tests 7, 29 and 36 were basically the same as the three controls reported in Table V. It is significant that all six of these controls showed not only similar weight losses, but also the same characteristics seen in tubes of sterile, anaerobic M10-E medium. It is believed that the cause for this duplication of weight loss, even under different test conditions (particularly incubation period) was as follows.

TABLE IV. Ten-day anaerobic corrosion test using culture MC 1. Temperature of incubation was 27° and the initial pH of the M10-E medium used was 7.6.

Inoculated Tests			Sterile Controls		
Coupon Number	Wt. Loss ($mg/10cm^2$)	Final pH	Coupon Number	Wt. Loss ($mg/10cm^2$)	Final pH
1	2.3	6.8	21	2.0	7.1
2	2.4	6.8	22	3.2 *	7.1
3	2.0	7.1	23	1.7	6.9
4	2.6	6.8	24	1.4	7.1
5	2.3	6.8	25	1.9 *	6.9
6	2.1	7.0	26	1.2	7.1
7	2.4	7.0	27	1.1	7.0
8	2.1	7.0	28	1.2	7.1
9	1.7	7.1	29	2.0	7.1
10	1.7	7.1	30	1.2	7.0
11	2.3	7.0	31	1.4	7.0
12	2.0	7.0	32	1.5	7.1
13	1.8	7.0	33	2.2	7.0
14	2.5	6.7	34	1.5 *	7.8
15	2.1	6.9	35	1.6 *	7.0
16	2.2	6.8	36	1.7	7.2
17	1.9	7.0	37	1.4	7.2
18	2.0	6.9	38	1.0	7.0
19	2.0	7.0	39	1.6	7.0
20	2.2	7.0	40	1.3	7.0

* = contaminated

TABLE V. Thirty-day anaerobic corrosion test using the indicated mixed cultures. Tests were made in M10-E medium (pH 7.6) at 27°.

Coupon Number	Mixed Culture	Wt. Loss ($mg/10cm^2$)	Final pH
1	C *	1.8	7.9
2	C	1.6	8.0
3	C	1.7	7.8
4	51	3.0	7.8
5	50	2.1	7.5
6	49	2.8	7.5
7	48	3.8	7.7
8	47	3.5	7.6
9	46	2.2	8.0
10	45	2.2	7.8
11	44	2.3	7.3
12	43	2.6	7.7
13	42	2.2	7.6
14	41	2.5	7.9
15	40	1.9	7.6
16	39	2.2	7.4
17	38	2.3	7.6
18	37	1.5	8.1
19	36	5.5	7.6
20	35	3.3	7.4
21	34	6.8	7.6
22	33	1.9	7.7
23	32	2.2	7.2
24	31	3.4	8.0
25	30	2.1	8.2
26	29	1.7	7.7
27	28	18.7	8.5
28	27	2.1	7.7
29	26	3.0	8.2
30	25	2.0	7.5
31	24	3.2	7.6
32	23	2.4	7.6
33	22	2.0	7.4
34	21	1.8	7.5
35	20	2.7	8.1
36	19	1.9	7.5
37	18	2.0	7.8
38	17	1.6	7.9
39	16	2.0	7.8
40	15	2.0	8.0

C = uninoculated control

Metal coupons contained in a completely anaerobic and sterile aqueous environment are believed to become polarized. In the absence of a depolarizer, such as oxygen, the weight loss is held to a minimum, dependent upon the characteristics of the metal. Should similar coupons be kept under identical conditions (as controls), weight loss would be expected to be uniform. Only when the hydrogen, polarizing the metal, is removed by the action of a depolarizing agent, does further weight loss occur. Exactly what constitutes a depolarizer under biological anaerobic conditions such as those with which this work is involved has not yet been conclusively determined.

Suspected depolarizers in the systems worked with here are the sulfate reducing bacteria, hydrogen utilizing anaerobes, reducible organic and, in several cases, inorganic compounds. Further work will continue to single out various of these possible depolarizing agents for closer consideration.

One other point which can now be examined is the frequency of high weight loss by any of the new 24 gauge coupons used thus far. In the three tests whose results are seen in Tables III, IV and V, the same coupons were used. Of the 40 coupons tested, only #27 experienced a high weight loss in two different experiments. The remaining four coupons which had shown high weight loss in a single instance failed to do so in any of the other two tests.

Anaerobic corrosion theory

Nature of polarizing hydrogen on metallic surfaces

In our previous work the assumption had been made that the hydrogen, which polarizes the surface of metals in aqueous, anaerobic solutions was essentially a thin film in the molecular form. The origin of this monomolecular layer of hydrogen was believed to originate as a result of the reactions

$$Fe \longrightarrow Fe^{++} + 2e^{-} \quad \text{(anode)} \qquad (1)$$

$$2\ HOH \rightleftharpoons 2\ H^{+} + 2\ OH^{-} \qquad (2)$$

$$2\ H^{+} + 2e^{-} \rightarrow H_2 \quad \text{(cathode)} \qquad (3)$$

in which reactions (1) and (3) occur at the anodic and cathodic areas of the metal, respectively.

As it is necessary for a metal to be depolarized before it can corrode, mechanisms other than, or perhaps common with, bacteria were sought which might fulfill this requisite. One such mechanism investigated in regard to depolarization of metals under anaerobic conditions was that of enzymatic activity.

That the enzyme, hydrogenase, found in certain anaerobic bacteria might effect depolarization of metallic surfaces has been investigated and reported in other progress reports. In the process of developing experiments to test the theory of enzymatic depolarization, the terminal anaerobic hydrogen acceptor chosen was the redox indicator dye, methyl viologen (colorless when oxidized, blue when reduced).

Various hydrogenase preparations were used in corrosion tests (made under nitrogen) in which methyl viologen was used as the hydrogen acceptor. The results of these tests proved to be somewhat doubtful. It was noticed, however, that in many (but not all) cases, the liquid in the tests would appear as various intensities of blue (reduced indicator).

It was not until methyl viologen was used as the hydrogen acceptor in a corrosion test using the coenzyme, DPN (earlier report), that it became obvious that the color intensity of methyl viologen was not indicative of enzymatic depolarization alone. Tests made using clean iron coupons in a sea water solution of 0.002 molar methyl viologen affirmed the belief that the hydrogen acceptor (methyl viologen), independent of the enzyme, could cause weight loss of the iron specimen with a consequent reduction of the dye.

From the above, it was theorized that the mechanism responsible for the weight loss of iron coupons in anaerobic solutions of methyl viologen was essentially depolarization of the metal surface by the indicator dye. If, according to some previously held assumptions, the hydrogen of polarization is in the molecular form, the methyl viologen must react in some way with molecular hydrogen to convert it to the atomic state before it can be taken up and thus effect depolarization.

As a means of checking the above supposition, experiments were designed which attempted reduction of methyl viologen with molecular hydrogen in various ways. Of significance here is the fact that only in the presence of an inorganic catalyst (such as platinum oxide or bacterial hydrogenase could molecular hydrogen reduce this, as well as similar dyes.

Molecular hydrogen is known to be converted to atomic hydrogen in the presence of metallic iron, and current theory holds that hydrogenase is capable of the same. One could, then, from the foregoing facts, conclude that the hydrogen (capable of dye reduction) initially produced on a clean iron surface under anaerobic conditions is not in the molecular, but atomic, form. Under these terms, the reactions given earlier should now show the cathode reaction as

$$2\ H^{+} + 2e^{-} \longrightarrow 2\ H \quad \text{(atomic)}$$

In the absence of an anaerobic hydrogen acceptor, this atomic hydrogen would be expected to proceed to the molecular form by the combination of two hydrogen atoms. This would then result in sufficient accumulation of molecular hydrogen to polarize the cathodic areas and therefore prevent further dissolution of iron (corrosion) from the anodic areas.

The significance of the theory of intermediate, atomic hydrogen formation of iron surfaces should be noted. The discussion has thus far been exclusively in terms of anaerobic conditions. However, it is interesting to note that solutions of redox indicators (methyl viologen, methylene blue, triphenyltetrazolium chloride, as well as others) which show a color change upon reduction in the absence of oxygen, can also be observed to do so under aerobic conditions. This reduction (as indicated by the color change) occurs, however, only in the immediate area of the iron coupon. Since the absence of molecular oxygen is a requisite for the reduced color to remain and be observed, it can be assumed that the oxygen in the vicinity of the coupon has been removed or inactivated.

It is possible that the inactivation of oxygen in the area of the coupon is effected by the dye itself. That is, as the atomic hydrogen is produced it immediately reduces a

layer of the dye near the coupon. This is followed by rapid oxidation of the reduced dye by the dissolved oxygen in the reaction area (coupon surface). After a short time the oxygen in the area could be completely removed, thereby effectively rendering the area around the metal anaerobic. With the latter condition established, the color of any dye further reduced could then be observed.

In the theory above, the metal is assumed to be stripped of the atomic hydrogen produced at its surface as a result of the dye acting as an atomic hydrogen acceptor and a "mediator" between the iron and dissolved oxygen. The basic nature of such a hydrogen mediator would be the relative ease with which it, itself, is reduced and oxidized. That compounds exist in natural environments which are similar in nature to a theoretical hydrogen mediator proposed here is known - - - those most noted being various enzymes and co-enzymes. Should such compounds be available to serve either as hydrogen mediators (as in aerobic situations mentioned above) or simply as hydrogen acceptors (in either aerobic or anaerobic conditions), formation of polarizing hydrogen would be inhibited and corrosion would proceed.

In cases where no such depolarizing agents are available and a metal becomes polarized with molecular hydrogen, those microorganisms capable of utilizing molecular hydrogen may play the key role in initiating, and perhaps continuing, metallic corrosion.

Corrosion cells

It was mentioned earlier in this report that corrosion of iron is believed to be accelerated when the hydrogen of polarization is removed from the metal surface. It was pointed out that origin of this hydrogen was from the combination of electrons (leaving the anodic surfaces of a specimen and migrating to the more cathodic areas) with the hydrogen ions being derived from the dissociation of water.

It has also been pointed out that hydrogen-utilizing microorganisms (notably the sulfate reducers) may be active as biological depolarizers of cathodic hydrogen. That the hydrogen polarizing metallic iron surfaces is essentially at the cathode is shown by the reactions on page 11.

In corrosion tests made thus far, tared iron coupons have been routinely exposed to cultures being examined for their ability to serve as depolarizers. However, when one considers the principle of oxygen differential corrosion and then examines the methods used in our endeavors, a question arises as to the accuracy of our measurements of microbiological corrosion.

If oxygen has access to certain parts of a metal, the polarizing hydrogen will be removed more readily from these areas and the potential will be more negative here than on the other parts. As a result, the latter parts will tend to dissolve because an E.M.F. has been set up. "It seems strange, at first, that the parts of the metal which dissolve are those where the oxygen has not access, whereas the more highly oxygenated areas do not dissolve." (Glasstone, S. 1940. Textbook of Physical Chemistry, 2nd ed. D.Van Nostrand Co., Inc., N. Y. p. 1035.)

If in an anaerobic bacterial corrosion test, the bacteria are to serve as the acceptor of cathodic hydrogen, it becomes important to examine the location of said cultures in relation to the metal specimens to be measured for weight loss.

One method used for examining the corrosion process was as follows. Two 16 cm chambers were fashioned from plexiglas tubing (4 cm diam.) and assembled as shown in Figure 1. Iron coupons were prepared (using the new method), attached to the leads and inserted into the empty plastic chambers. The system was then flushed for five minutes with oxygen-free nitrogen (passed through Fieser's solution). The system was then sterilized for 16 hours by allowing a final flushing of ethylene oxide to remain overnight. Following the gas sterilization, nitrogen (oxygen-free) was passed through the system for eight hours to remove residual fumigant.

Medium M10-E was prepared and added to each cell by using the methods outlined below. Special methods for these procedures were necessary since traces of oxygen in the system could act as a depolarizer. To accomplish the exclusion of all oxygen in the medium itself and to prevent its introduction into the cells while making the medium addition, an apparatus such as that shown in Figure 2 was used.* The medium was added to an Erlenmeyer flask which contained a magnetic stirring bar and had been fitted with a stopper having one liquid and one gas vent (cotton filter type). To the liquid vent was attached the hose which connected the flask to an automatic pipetting syringe.

The gas vent and canula were covered and the entire unit autoclaved with the syringe elevated above the level of the medium. Immediately following removal of the unit from the autoclave, a rubber, serum bottle-stopper was forced into the gas vent opening. A balloon which had been fitted with an 18 gauge hypodermic needle was filled with oxygen-free nitrogen. The needle of this unit was then inserted through the serum stopper. As the flask of medium cooled (aided by magnetic stirring action), any vacuum which would have normally formed was filled with nitrogen.

When the medium had cooled to room temperature, 80 ml were pipetted into each cell through the serum bottle stoppers at the bottom of each cell. By maintaining an equalizing pressure of oxygen-free nitrogen (from the balloon) on the medium dispensing unit, the medium was able to flow freely into the pipette without the formation of a vacuum inside the flask.

Once the chambers were filled with medium at room temperature, wire attachments were made to the coupon lead protruding from each chamber and connected to the Model 80 YSI recording microamp/volt meter (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The cell was then short-circuited and allowed to equilibrate in this sterile condition for six days.

Following the equilibration period the YSI recorder was set to zero and the left chamber inoculated with four ml of a 96 hour culture of MC 27 in M10-E medium. Inoculation resulted in a 0.02 V potential. The particular mixed culture used in this test was the one shown in Table V as having caused the highest weight loss in corrosion tests performed thus far.

The corrosion cell, as designed, failed to maintain the anaerobic conditions imposed.

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* This apparatus was mentioned earlier as being used in anaerobic enrichment techniques. When used in these procedures, the plastic shield shown in Figure 2 is used to prevent airborne contamination of the dilution bottles being filled (see page 2).

Figure 1. Anaerobic corrosion cell using KCl - agar bridge between half-cells.

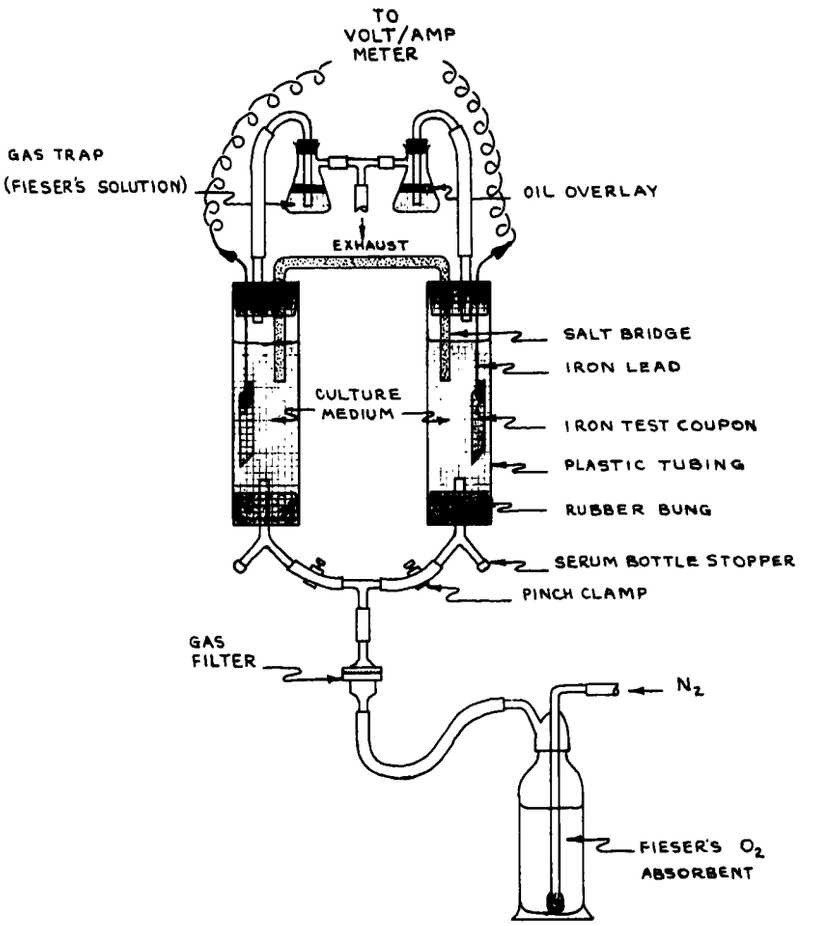

TO
VOLT/AMP
METER
GAS TRAP
(FIESER'S SOLUTION)
OIL OVERLAY
EXHAUST
SALT BRIDGE
IRON LEAD
CULTURE
MEDIUM
IRON TEST COUPON
PLASTIC TUBING
RUBBER BUNG
SERUM BOTTLE STOPPER
PINCH CLAMP
GAS
FILTER
N_2
FIESER'S O_2
ABSORBENT

Figure 2. Anaerobic medium dispenser.

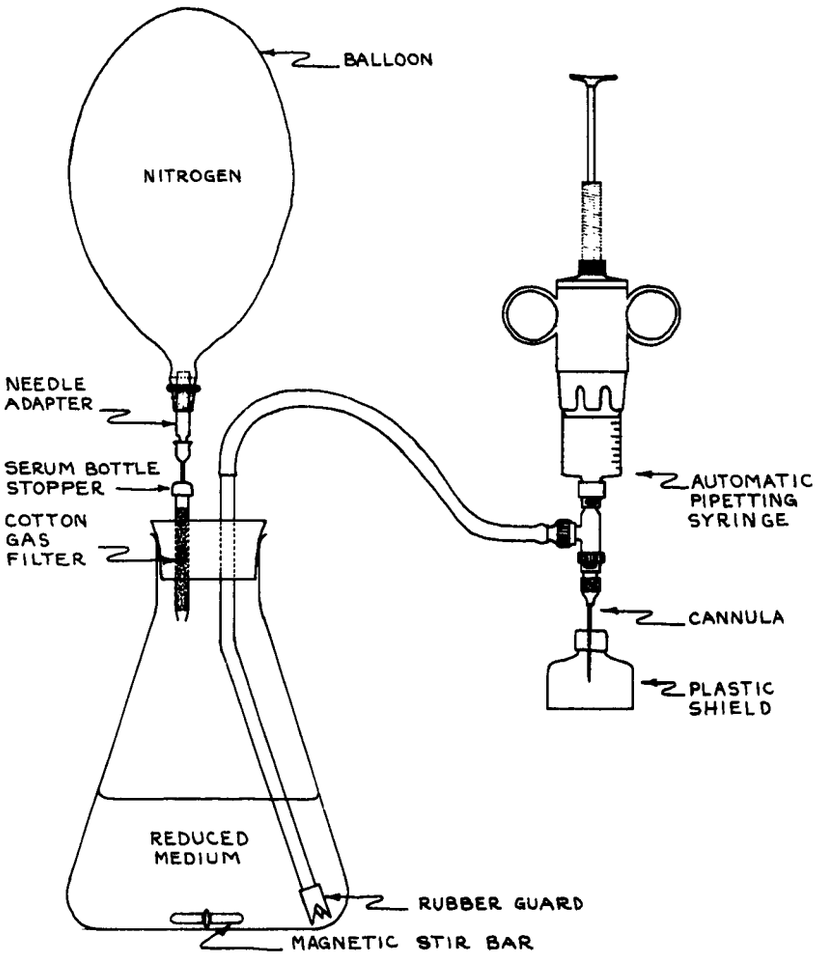
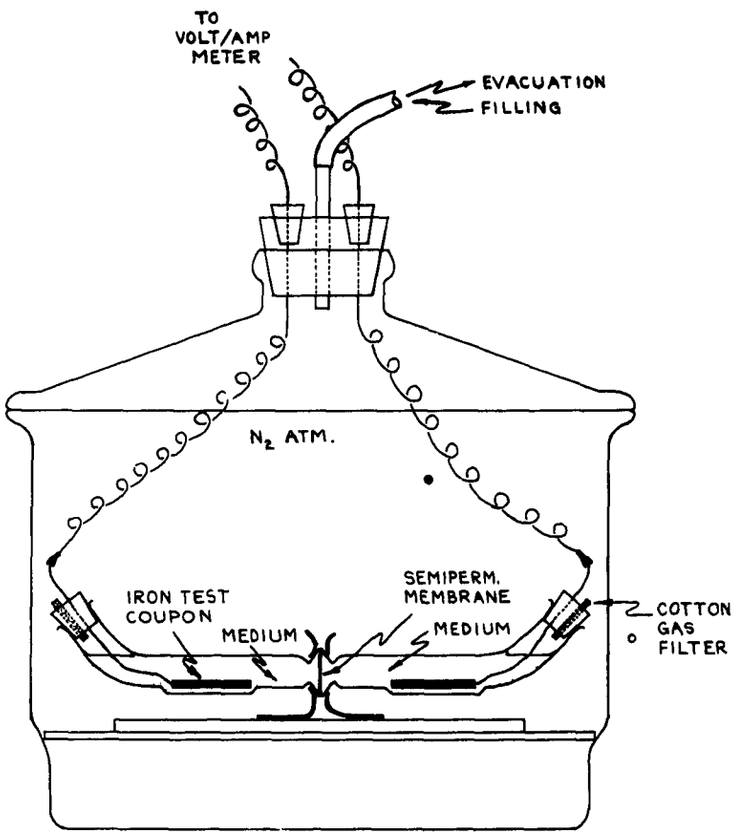

BALLOON
NITROGEN
NEEDLE ADAPTER
SERUM BOTTLE STOPPER
COTTON GAS FILTER
AUTOMATIC PIPETTING SYRINGE
CANNULA
PLASTIC SHIELD
REDUCED MEDIUM
RUBBER GUARD
MAGNETIC STIR BAR

Figure 3. Anaerobic corrosion cell using semi-permeable membrane between half-cells.


TO
VOLT/AMP
METER
EVACUATION
FILLING
N_2 ATM.
IRON TEST
COUPON
MEDIUM
SEMIPERM.
MEMBRANE
MEDIUM
COTTON
GAS
FILTER

In both chambers of the corrosion cell, leakage of oxygen was indicated by the appearance of trivalent iron oxide at the liquid-gas interface and on lead. It is of interest, however, that the chamber which seemed to have been the most oxidized contained the iron coupon which had lost the least weight (5.6 mg/10 cm^2) after 14 days. The least oxidized chamber contained the coupon which lost 11.7 mg/10 cm^2 during the same period, indicating that an oxygen differential corrosion cell had been established.

In order to overcome the possibility of development of an oxygen differential corrosion cell when an anaerobic cell is desired, a modification of the cell design has been made, although not as yet tested. The new corrosion cell, shown in Figure 3, will be contained in a desiccator filled with an atmosphere of oxygen-free nitrogen. Essentially, the cell will be made of a parabiotic chamber with a 2.2 μ Millipore filter located between each half of the chamber units. This semi-permeable membrane will replace the salt bridge in the cell schematic shown in Figure 1. Both halves will contain a tared iron coupon and be filled with medium. One side will be inoculated with a known corrosive organism and the weight loss of each coupon (and perhaps the potential developed) will be measured. It is hoped that duplication of weight loss produced by using a system thus outlined may serve as a means for accurately screening a variety of microorganisms for the ability to promote the corrosion of iron.