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Methane in Lake Erie: Analysis of Mechanisms of Production and of Amounts Produced

Ohio State Univ, Columbus Water Resources Center

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and
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Department of Microbiology
The Ohio State University

1977

United States Department
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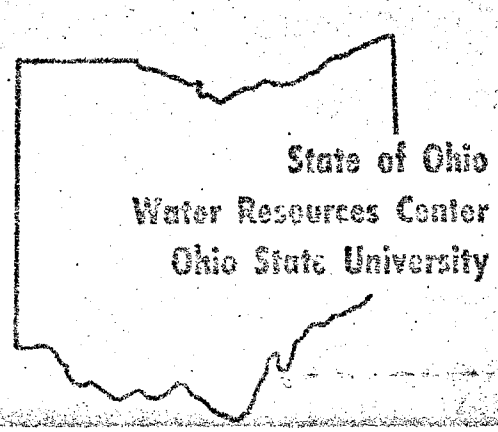

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16. Abstract

Microbial methanogenesis in the sediment and water column of the Western and Central Basins of Lake Erie was studied. Year to year methane production varies, but relative methanogenesis by geographical location is constant with near shore and harbors having highest production. The Western Basin generally supports more methane production than the Central Basin.

Four species of methanogenic bacteria, Methanobacterium ruminantium, Methanobacterium strain M.o.H., Methanospirillum hungatii, and Methanosarcina barkerii, were shown by the direct fluorescent antibody technique to be distributed in specific locations in the Lake and its harbors.

Laboratory study of these same methanogenic species showed that they produce ~~methane at the rate of~~ an average of 1.2 umoles of methane per hour per 10^8 cells. Methane accumulation does not inhibit methane production; hydrogen depletion terminates production.

17a. Descriptors *Methane, *Methane bacteria, Gases, Carbon cycle, Cycling nutrients, Anaerobic digestion, Biodegradation, Aquatic bacteria, Sediments, Anaerobic bacteria, Gas chromatography, *Lake Erie, Microbial degradation, Water pollution sources, *Path of pollutants, Distribution, Lake sediments,

~~17b. Identifiers~~ *Methane production rates, *Methanobacterium, *Methanospirillum, *Methanosarcina, Fluorescent antibody, ~~*Distribution of methane bacteria~~

17c. COWRR Field & Group ~~H-H~~ 05B, 02H, 05C

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METHANE IN LAKE ERIE: ANALYSIS OF MECHANISMS OF PRODUCTION AND OF AMOUNTS PRODUCED

by
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and Gail E. Mallard
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March 1977

WATER RESOURCES CENTER
Engineering Experiment Station
THE OHIO STATE UNIVERSITY

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Office of Water Research and Technology,
U.S. Department of the Interior under
Project A-040-OHIO

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PROJECT OBJECTIVES

Objectives set for the project were:

a. Analysis of the temporal and geographic production and distribution of methane in water and sediments.

b. Evaluation and development of techniques for the isolation and maintenance of the various types of bacteria involved (including the analysis and modification of present procedures and the development of new ones).

c. Characterization of the methanogenic isolates.

d. Analysis of the temporal and geographic distribution of methanogenic isolates.

These objectives encompass a large long term study. Within the two year plan for the study reported herein, only partial attainment of the stated objectives was anticipated, and the anticipated level of objective attainment was accomplished.

This report will be presented in three sections. Parts of this report were not sponsored by OWRT but are included to give subject continuity to the report.

SECTION I

INTRODUCTION

The role methane plays in the carbon cycle of an aquatic system is being investigated. The significance of methane oxidation and the factors controlling the rate of oxidation in the fresh water system have been discussed and, similarly, the effects of temperature on methanogenesis in Lake Mendota sediment samples and the effects of temperature and substrate on methane production in Lake Erie sediment samples have been reported.

The distribution of dissolved methane in the aquatic system has received less attention. Since methane has assumed an increasingly significant role in the carbon budget of the aquatic system, we felt that it was necessary to monitor the amount of dissolved methane that was available to the system.

Two aspects were of special interest:

1. The concentration of dissolved methane throughout the water column.
2. The potential for methane production in sediments.

The aquatic system studied was the Central and Western Basins of Lake Erie. The concentrations of methane in the water column and the potential for sediment methane production were periodically monitored from early spring until mid winter during 1974 and 1975.

MATERIALS AND METHODS

Location of Sampling Sites

Fifty-one sampling stations were located throughout both the Western and Central Basins, with thirty stations being located in the Central Basin proper, six stations in the Central-Western Basin junction and fifteen stations in the Western Basin proper (Figs. 1 and 2).

The sampling depths at each station were one meter below the water surface, mid depth and one meter above the sediments. If a thermocline was present, samples were collected at one meter above and below the stratification. At some shallower regions of the Western Basin only surface and bottom samples were required.

Sample Collection and Preparation

Gas tight water samples were collected from a one inch tygon tubing which was supplied with a continuous water flow from a submersible pump. A 50-cc disposable plastic syringe adapted with a three-way disposable stopcock and 1.5 inch 18-G needle was used to collect air tight 25 ml water samples. The samples were transferred to previously evacuated 60-cc serum bottles capped with standard serum bottle stoppers. The samples were equilibrated with ultra high purity helium and an additional 25-cc of helium was introduced. The samples were vigorously shaken to purge the dissolved methane from the water. After equilibration, the serum bottles were inverted and returned to atmospheric pressure by venting the bottles with an 18-G needle. The resulting gas samples were preserved with 0 2 ml of 50% trichloroacetic acid and were stored at ambient temperature until analysis.

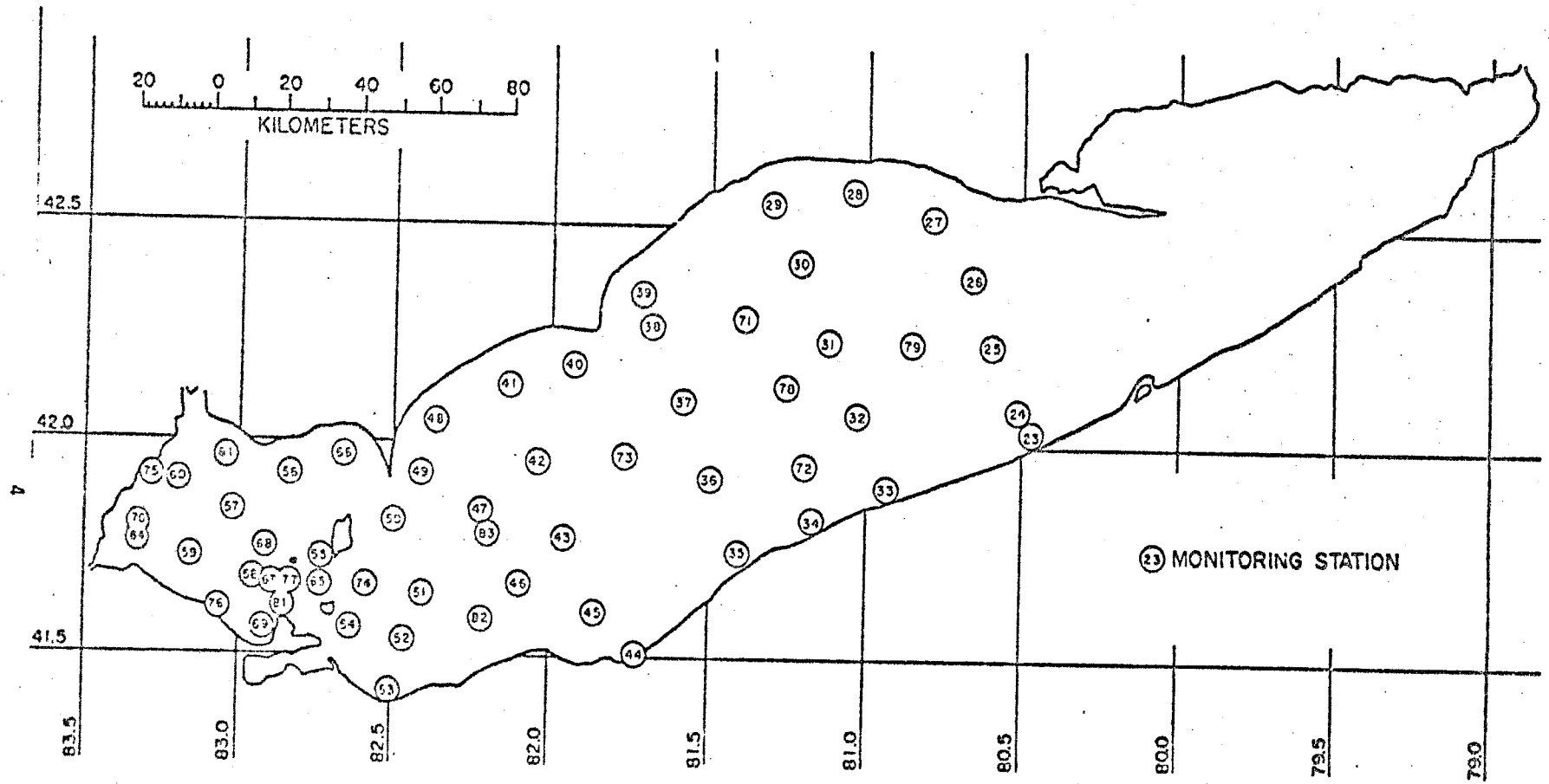


FIGURE I-1. Monitoring Stations Location Map for the Central and Western Basins of Lake Erie

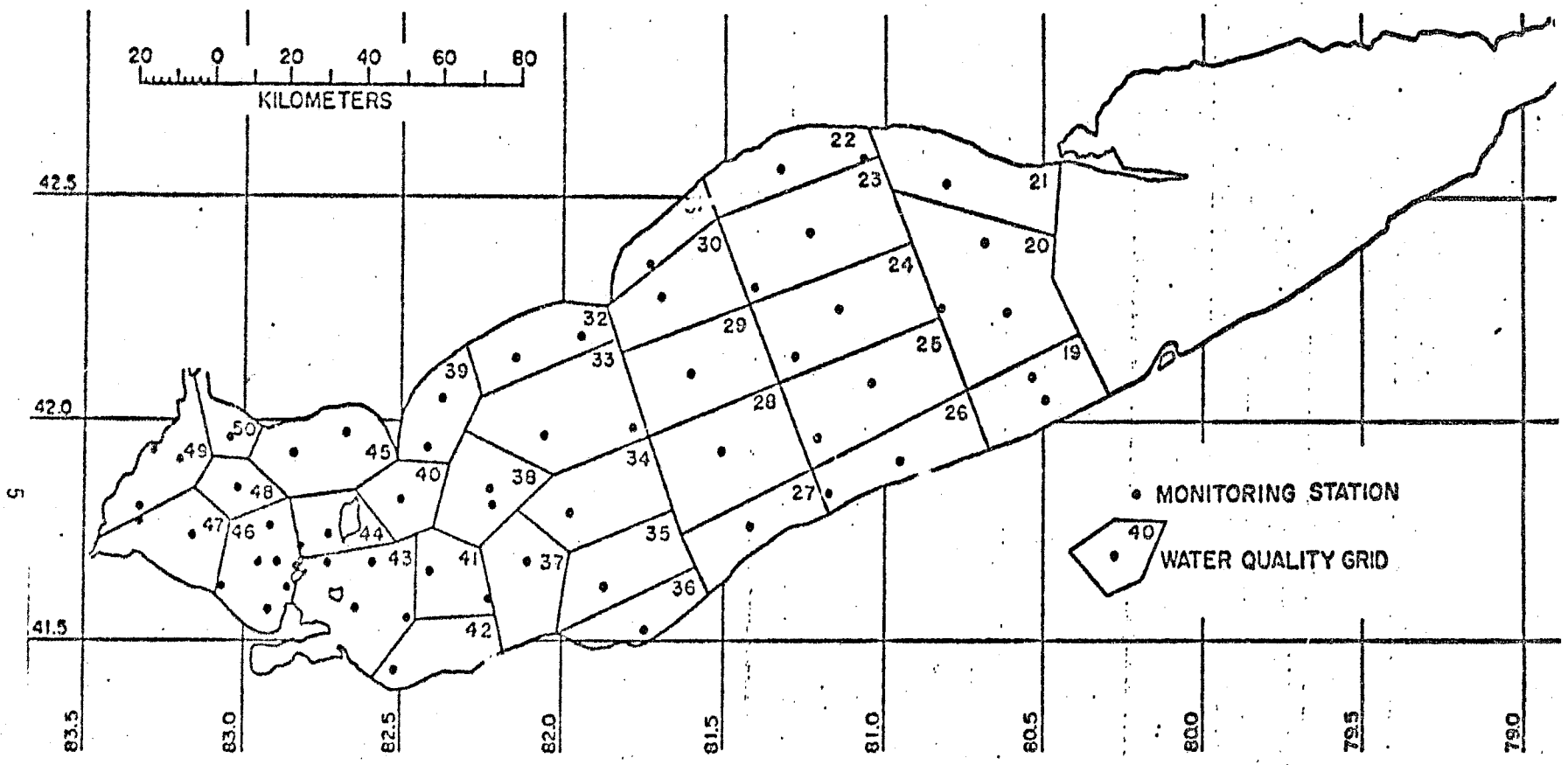


FIGURE I-2. Water Quality Grids Used for Area- and Volume-Weighted Calculations in the Central and Western Basins of Lake Erie

Sample Collection and Preparation for Methane Analysis

Gas tight water samples were collected from a one inch tygon tubing which was supplied with a continuous water flow from a submersible pump. A 50-cc disposable plastic syringe adapted with a three-way disposable stopcock and 1.5 inch 18-G needle was used to collect air tight 25 ml water samples. The samples were transferred to previously evacuated 60-cc serum bottles capped with standard serum bottle stoppers. The samples were equilibrated with ultra high purity helium and an additional 25-cc of helium was introduced. The samples were vigorously shaken to purge the dissolved methane from the water. After equilibration, the serum bottles were inverted and returned to atmospheric pressure by venting the bottles with an 18-G needle. The resulting gas samples were preserved with 0.2 ml of 50 percent trichloroacetic acid and were stored at ambient temperature until analysis.

Sediment samples were obtained with a Ponar sediment sampler and were handled as carefully as possible so that the sediment sample was not disturbed any more than required.

Subsamples were collected from the Ponar benthos sample by means of a modified disposable 10-cc syringe which permitted a cylindrical sample approximately 1.5 cm x 5 cm (approximately 8.8 - 10.0-cc sample) to be obtained. This subsample was transferred to a 30-cc serum bottle and was carefully overlayed with a small volume (2-3 ml) of water collected with the benthos sample and then was stoppered with a serum bottle stopper. One sample served as a control and another was monitored for both methane production after incubation.

It must be noted that even though stringent procedures were followed, the results of this sediment analysis must be interpreted very carefully due to the sampling conditions. Methanogenesis has been shown to be a strictly anaerobic process and this sampling technique may have severely altered the anaerobic

nature of the sediments. Therefore, the results of this portion of the survey cannot be accepted as equivalent to that which occurs in situ. This series of experiments demonstrates only whether or not an active methanogenic population is present.

Sample Analysis

Methane quantitation was performed with a Varian 2740 series gas chromatograph which was equipped with dual hydrogen flame ionization detectors. The chromatographic columns were 6' x 1/8" glass columns packed with 100-120 mesh Porapak N and were operated at 105°C. Prepurified N_2 was used as a carrier gas. The methane standard employed was a 315 V/V methane in nitrogen. A permanent copy of the methane peaks and the corresponding integration units were recorded with a Linear Instrument integrating recorder model 8384.

Results and Discussion

The data for the distribution of dissolved methane and the potential for methanogenesis from the sediments may be variously analyzed. First, the values obtained for the dissolved methane (mg/m^3 of water which is equivalent to parts per billion, ppb) may be contoured. The contours for the various strata indicate mass water movements, currents and particularly organic loading from point sources or intra-basin water exchanges. Unlike most other anaerobically generated nutrients, methane is relatively stable or inert to further biological utilization. Only a few species of bacteria are able to metabolize methane and these species are usually found concentrated at the sediment water interface or at the lower knee of the thermocline under stratified conditions. These localized regions provide a microaerophilic environment ($\leq$ part per million, ppm, O_2) which is optimal for microbial methane oxidation. Thus, once the methane escapes these regions, the dissolved methane is relatively

inert biologically and can be utilized as a stable parameter to monitor eutrophic conditions.

Secondly, the methane data may be shown as volume weight per specified grid region. This method of analysis permits the total estimated quantity of dissolved methane (reported as metric tons) to be shown. This total permits one to evaluate the methanogenic activity of a specified region of the lake. A similar grid analysis allows one to compare potential production rates of the sediments. (The potential production rates observed for the sediments are reported as weight of methane produced per gram dry sediment per day.)

This survey concerned the Western and Central Basins of Lake Erie. A total of fifty-one sampling stations were used of which thirty stations were located in the Central Basin proper, six stations were in the Central-Western Basin junction and fifteen stations were in the Western Basin proper. These fifty-one stations were further associated with thirty-two water quality grids (see Figs. 1 and).

For further convenience in interpreting the data, specific water quality grids may be grouped according to similar biological and physical characteristics as well as geographical location. The Central and Western Basins are divided into additional sub-basins according to these water quality grids:

Sub-Basin	Water Quality Grids
Eastern Central Basin	19-31
Western Central Basin	32-39
North Shore Central Basin	21, 22, 31, 32, 39
South Shore Central Basin	19, 26, 27, 36
"B" (Between) Basin, Sandusky	40-43
Western Basin	44-50

Water Column Methane 1974 (Contours)

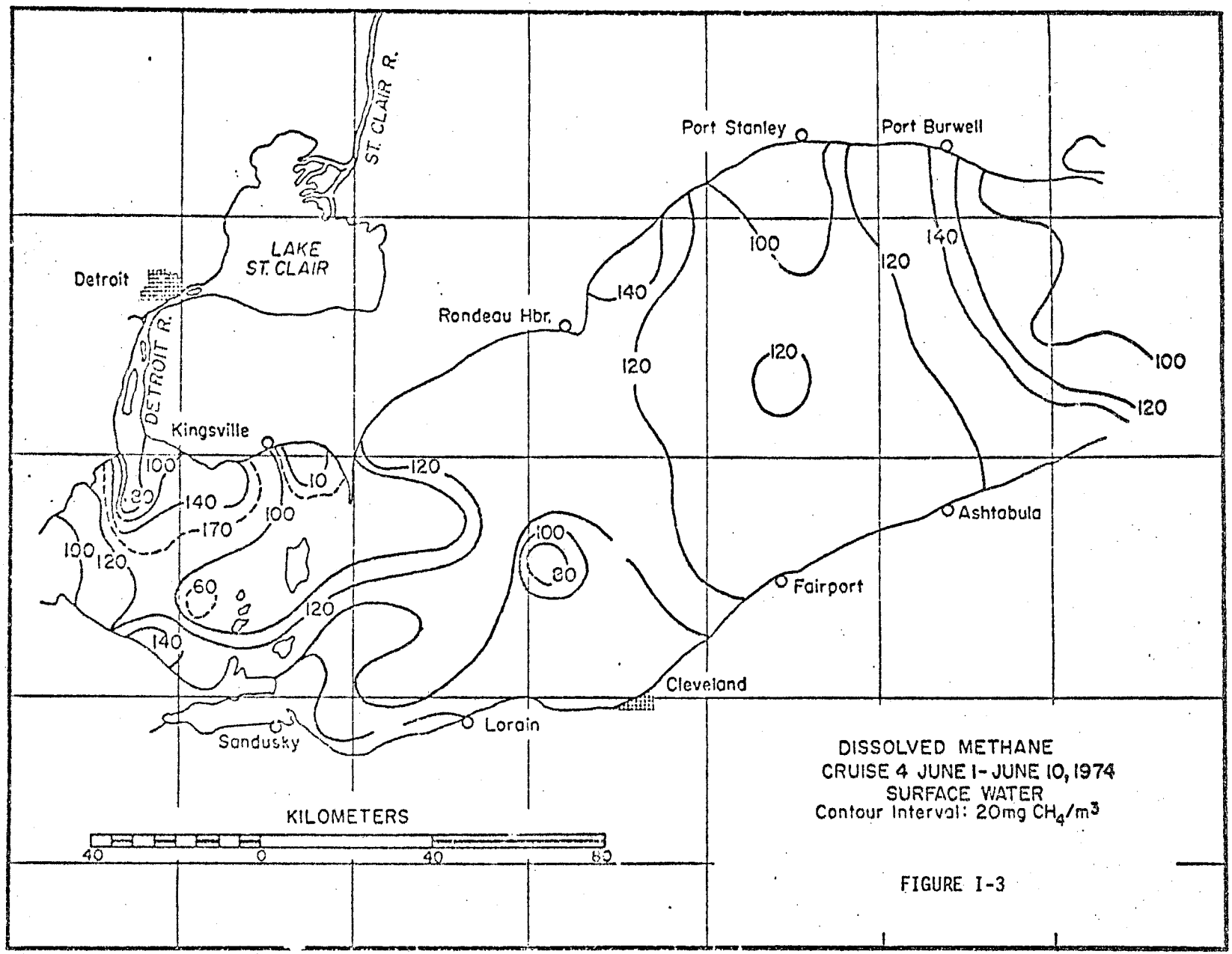
The nine cruises surveyed during 1974 can be conveniently divided into six biologically active periods:

a)	Cruise 1	4/07 - 4/17/74
b)	Cruise 2	4/25 - 5/04/74
	Cruise 4	6/01 - 6/10/74
c)	Cruise 5	6/28 - 7/07/74
	Cruise 6	7/26 - 8/04-74
d)	Cruise 7	8/12 - 8/19/74
	Cruise 8	8/26 - 9/07/74
e)	Cruise 10	10/21 - 11/01/74
f)	Cruise 11	12/11 - 12/14/74

The data from these six activity periods will be contoured and discussed. The data obtained during 1975 will only be briefly mentioned following discussion of the 1974 data.

The early spring period (a) demonstrated a minimal quantity of methane throughout the entire water column. The concentration reported, less than 10 mg/m^3, is equivalent to less than 10 parts per billion (ppb) which was the lower detection limit of the chromatographic system utilized. The low concentration was probably due primarily to the low temperature and oxygenated conditions in the entire lake.

A rapid and drastic increase of dissolved methane occurred during the late spring - early summer period (b). This period demonstrated consistent increases in methane concentrations and culminated in the yearly maximum during cruise 4. This yearly maximum can be attributed to the warming of the water column, to the residual organic build up during the winter and to the increase in available organics caused by the spring diatom bloom. A graphical analysis of this early summer data, cruise 4, is presented in Figures 3, 4, 5, and 6.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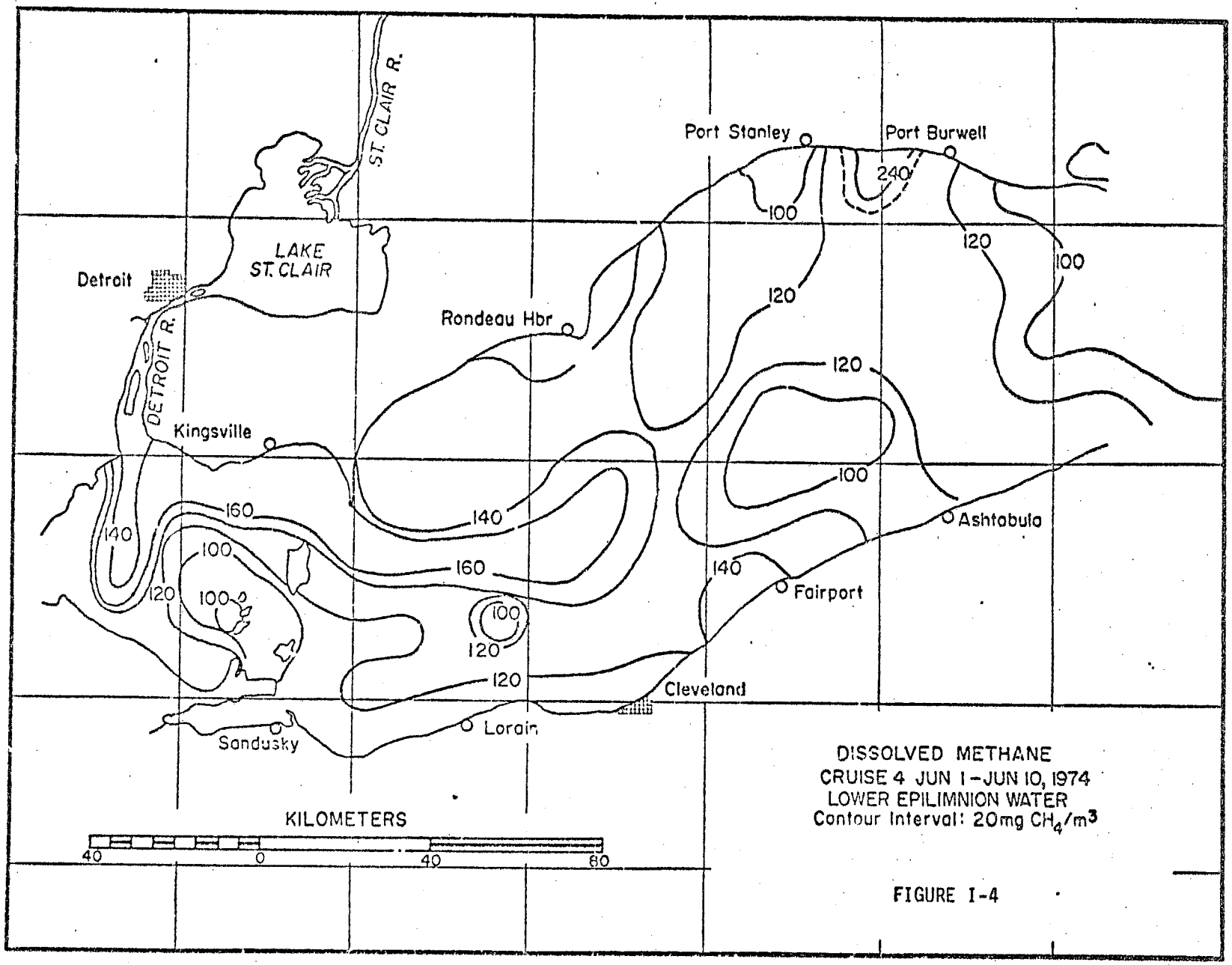
DISSOLVED METHANE
CRUISE 4 JUNE 1 - JUNE 10, 1974
SURFACE WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-3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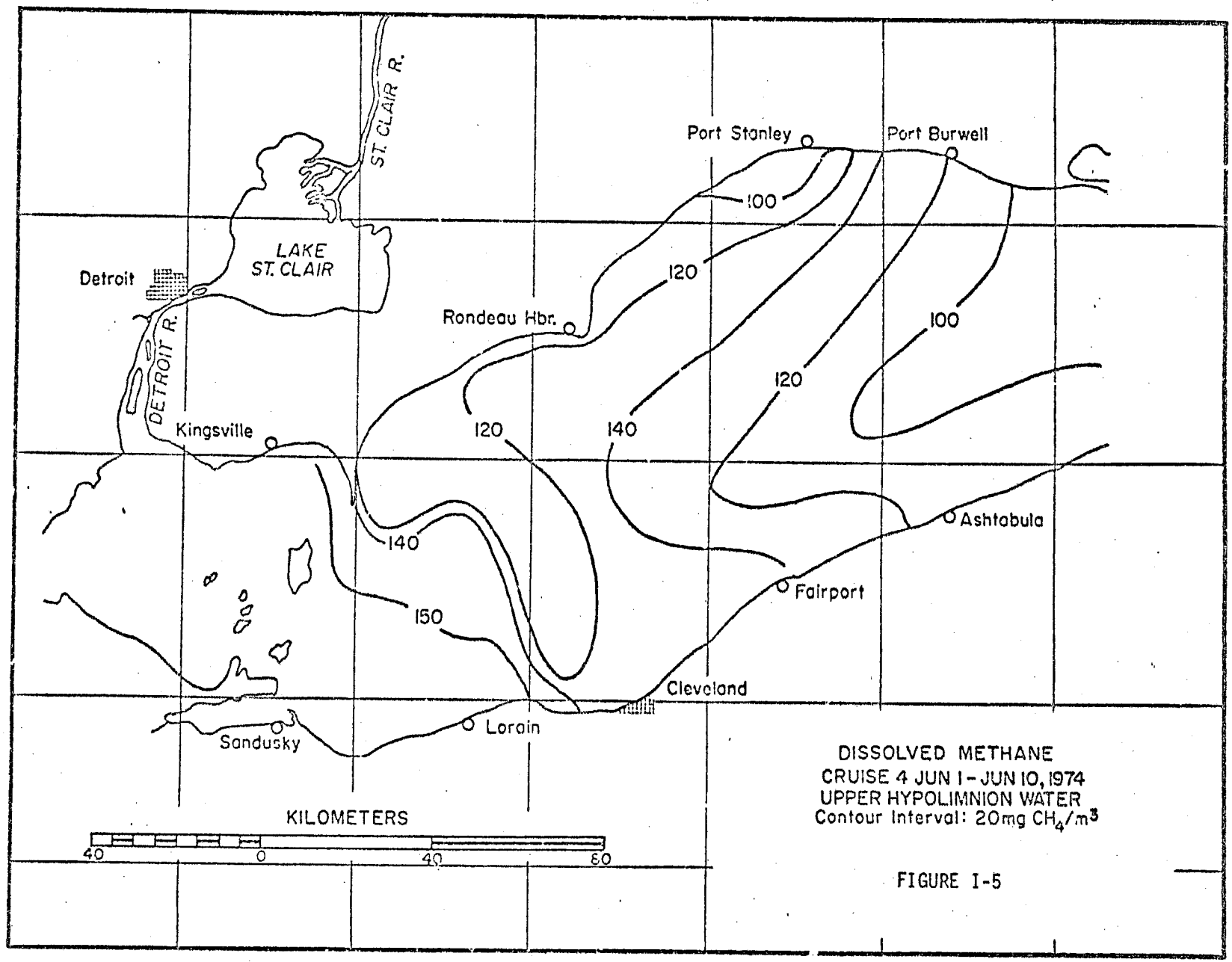
DISSOLVED METHANE
CRUISE 4 JUN 1 – JUN 10, 1974
LOWER EPILIMNION WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-4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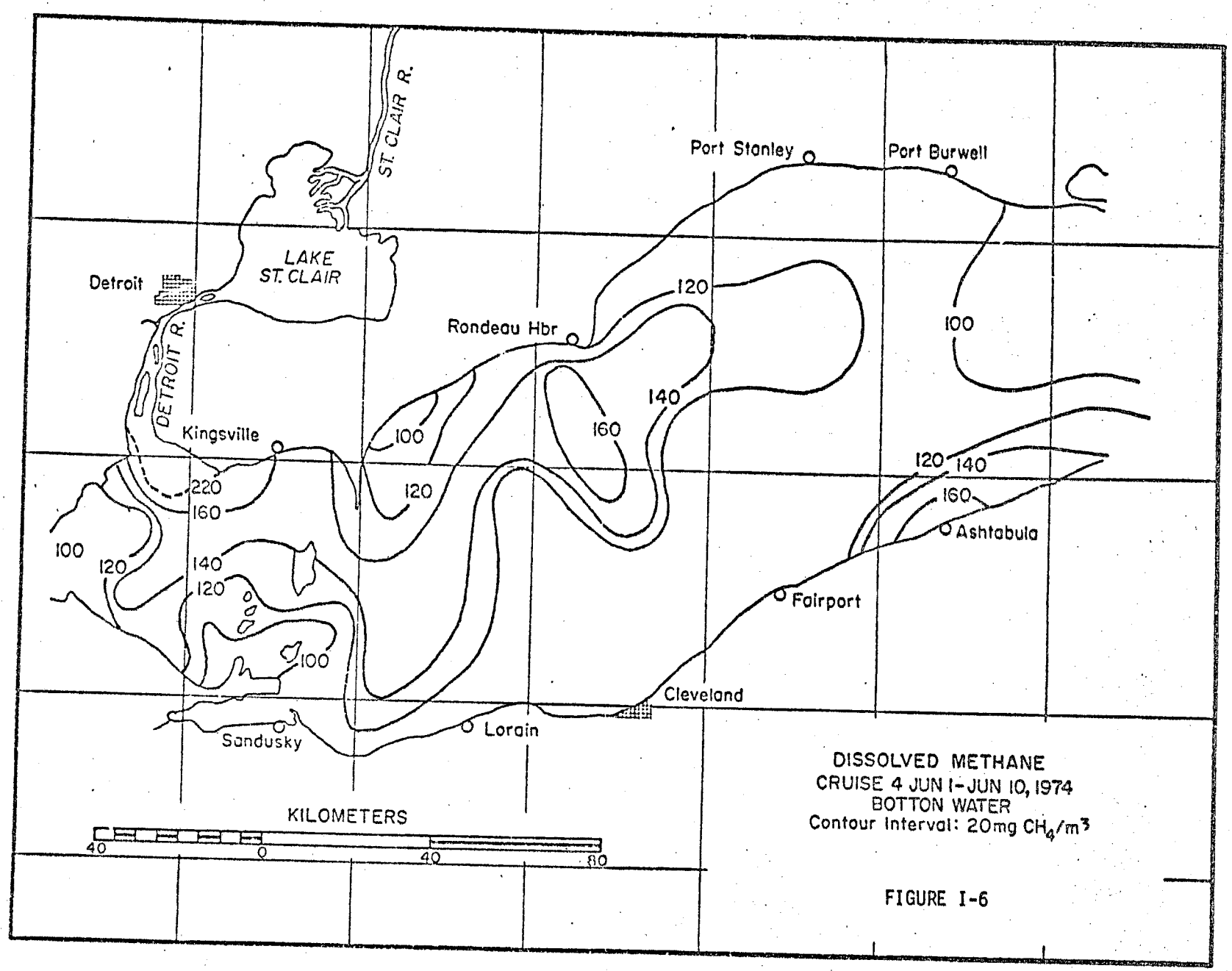
DISSOLVED METHANE
CRUISE 4 JUN 1 - JUN 10, 1974
UPPER HYPOLIMNION WATER
Contour Interval: 20mg CH_4/m^3

FIGURE 1-5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DISSOLVED METHANE
CRUISE 4 JUN 1-JUN 10, 1974
BOTTON WATER
Contour Interval: 20mg CH_4/m^3

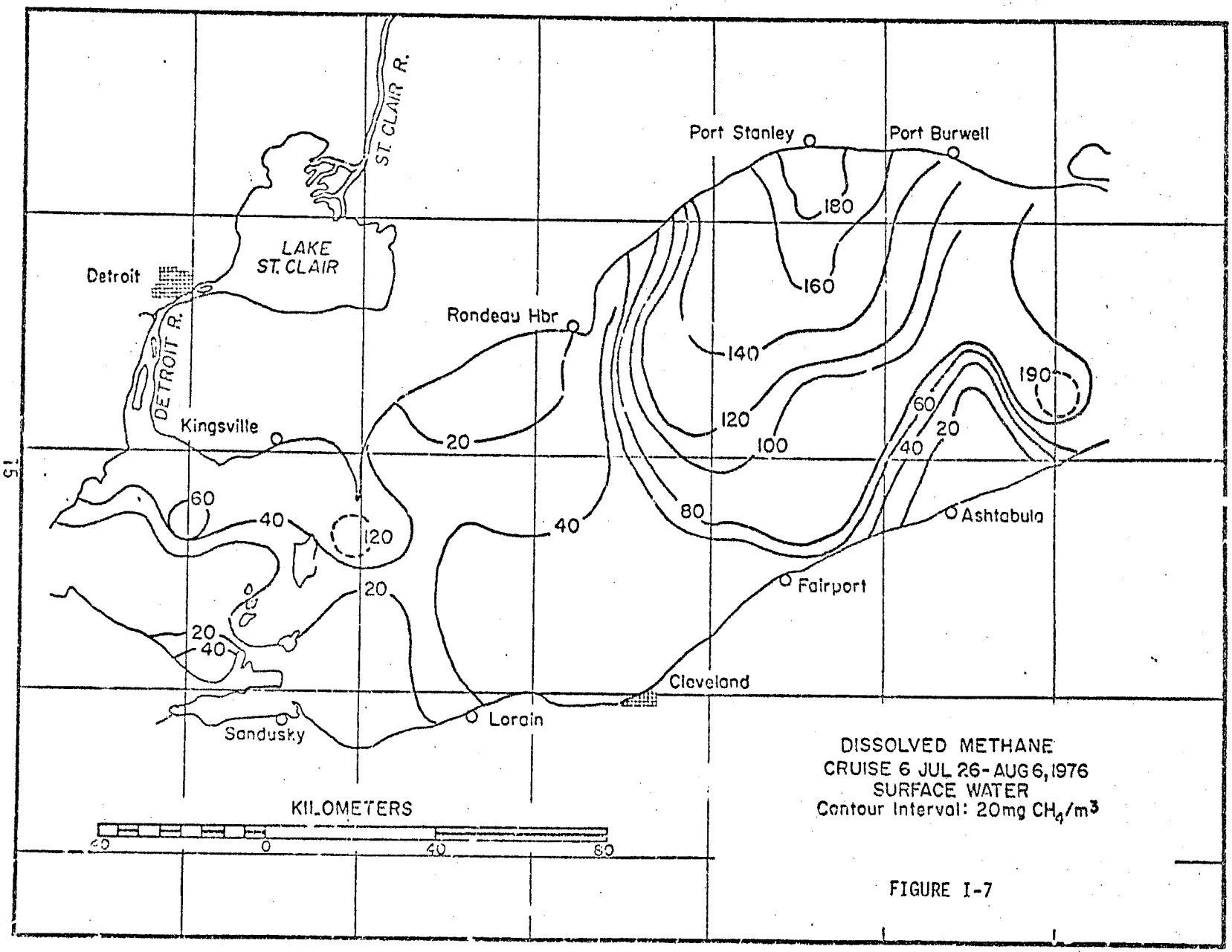
FIGURE I-6

A number of geographic or basin effects are evident from these contours. The entrainment of colder Eastern Basin water into the Central Basin is demonstrated by the low but noticeable impulse of methane in the bottom and particularly in the upper hypolimnion waters. The warmer surface and lower epilimnion waters are unaffected by the colder entrained water.

The surface water of the Central Basin proper possesses a uniform horizontal distribution of methane but there were observed two apparent Western Basin effects. One is the low flux of methane observed entering through Pelee Passage, the second is the significantly higher impulse recorded in the Sandusky region. These effects are amplified in the lower epilimnion water with the exception that a drastically higher impulse is noticed at Pelee Passage. The colder hypolimnion water is not affected by the Western Basin water, but is significantly influenced by the previously mentioned Eastern Basin water.

The Western Basin has two major contributors--the Maumee River and more importantly the Detroit River. The influence of the Detroit is found well out into the lake proper and can often be noticed along the south shore line. Extensive survey by others demonstrated that the Detoit influence was observed as far south as the Ohio shore and often joined in the flow of the Maumee easterly along the south shore, northward just west of the island region and then southeasterly into the Central Basin via Pelee Passage.

The mid-summer cruises (c) represent a significant decrease in the concentration of dissolved methane. Cruise 6 data is displayed in Figures 7, 8, 9, and 10. The surface water had a negligible input from the Eastern Basin but a peculiarly high impulse of methane was recorded at the Port Stanley region. Western Basin effects were again observed at Pelee Passage and to a lesser extent at the Sandusky region. The contributions through Pelee Passage were greatest in the surface water. The unique activity recorded during this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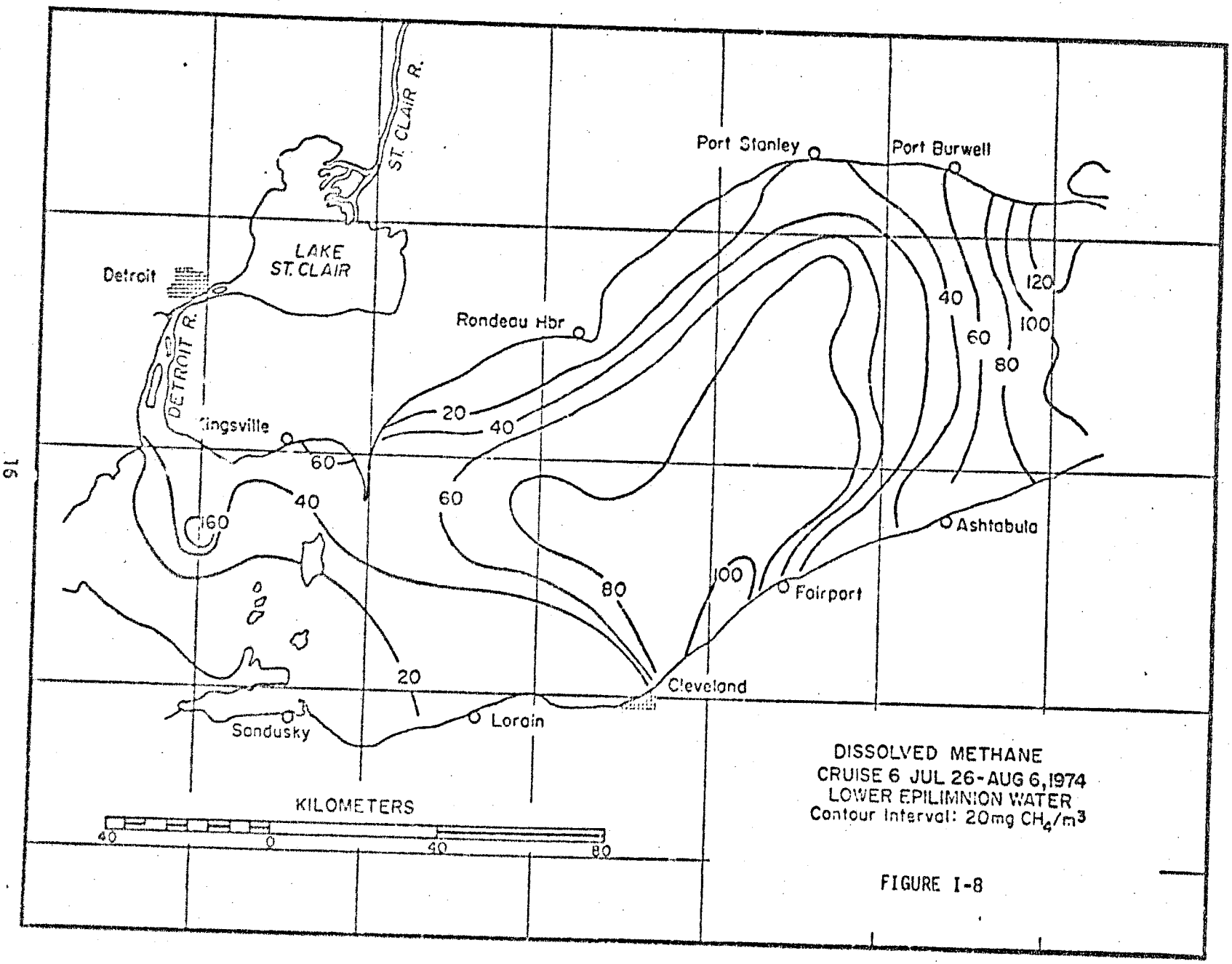
DISSOLVED METHANE
CRUISE 6 JUL 26 - AUG 6, 1976
SURFACE WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-7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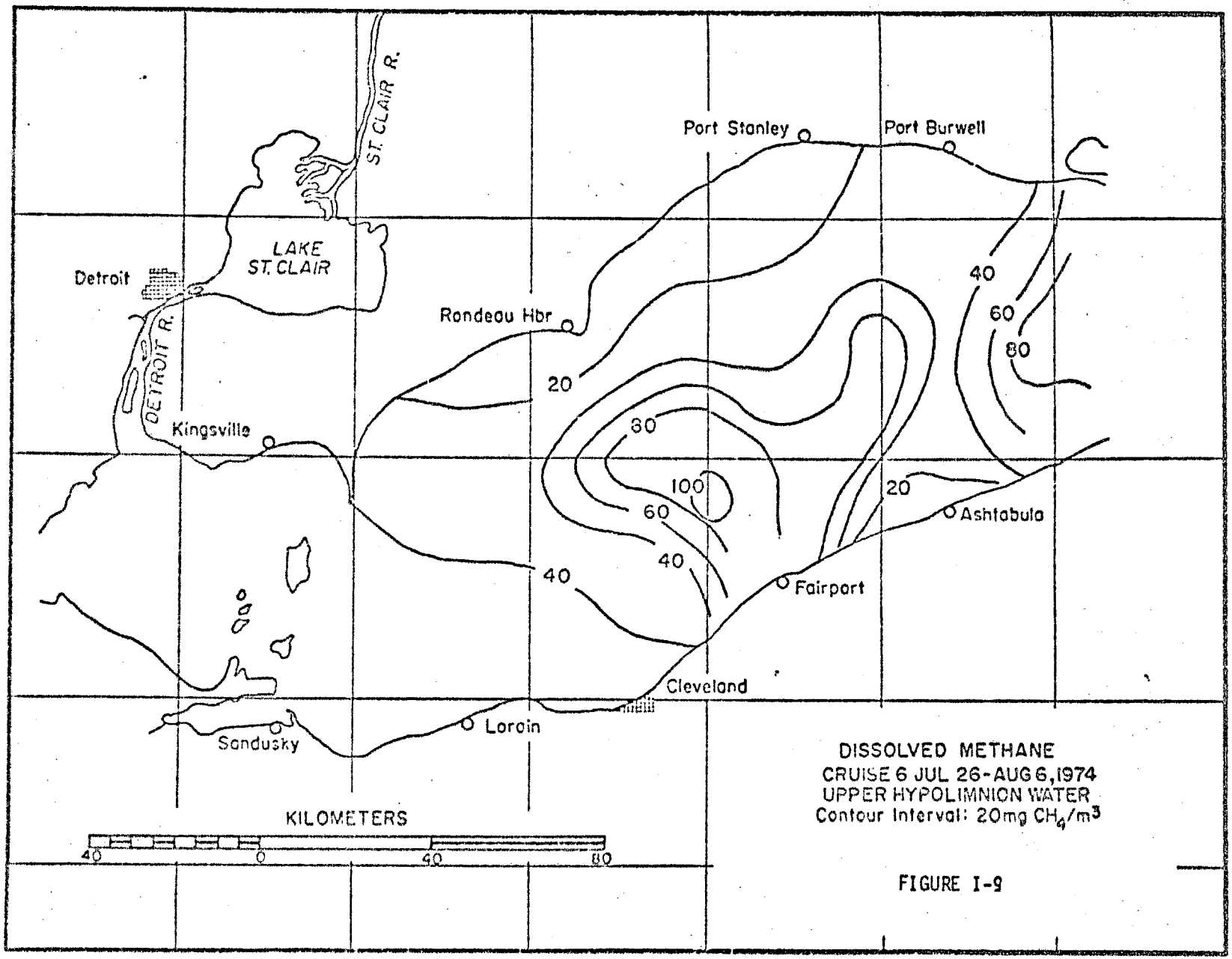
DISSOLVED METHANE
CRUISE 6 JUL 26-AUG 6,1974
LOWER EPILIMNION WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-8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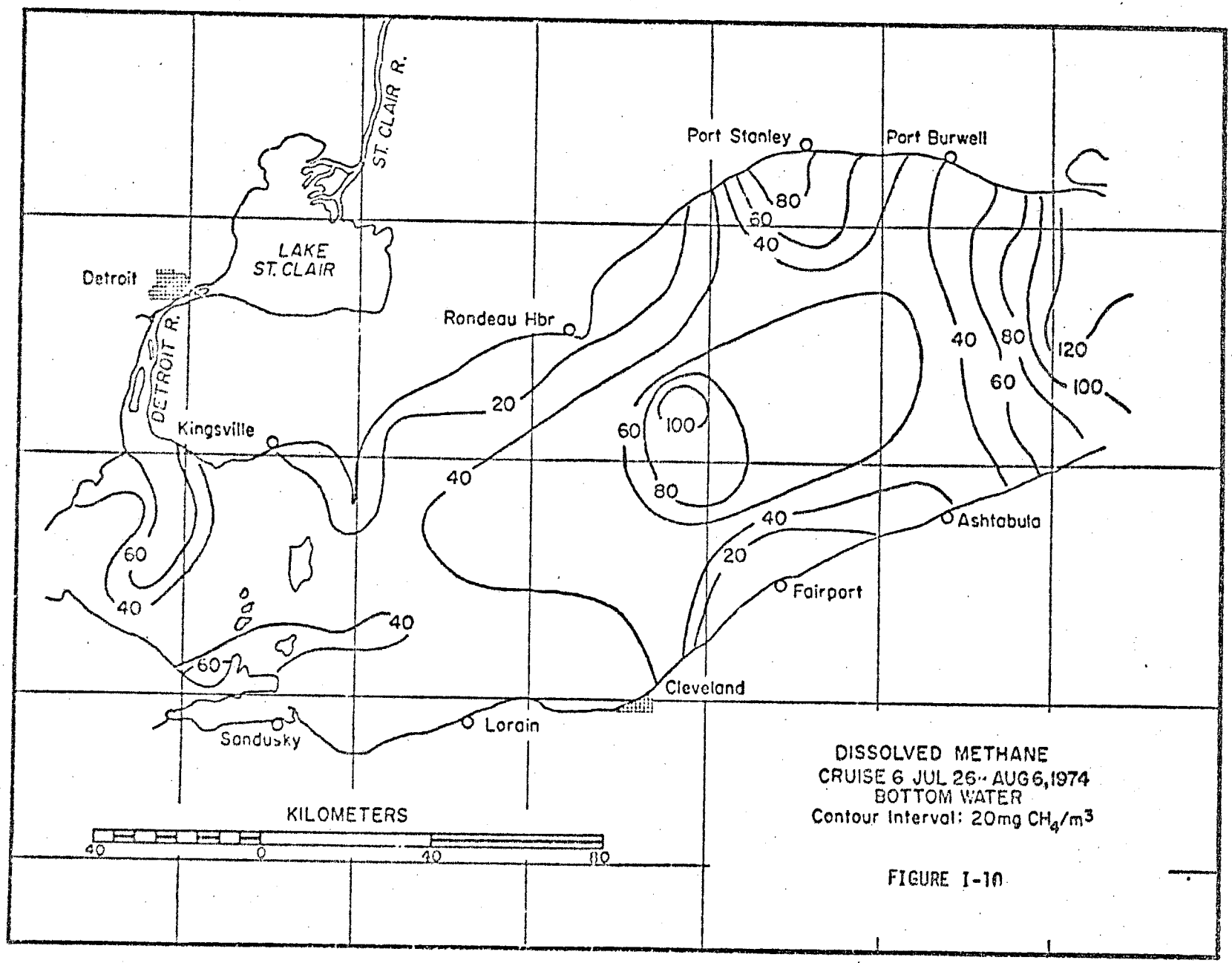
DISSOLVED METHANE
CRUISE 6 JUL 26-AUG 6, 1974
UPPER HYPOLIMNION WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-9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DISSOLVED METHANE
CRUISE 6 JUL 26 - AUG 6, 1974
BOTTOM WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-10

cruise occurred at the Eastern-Central Basin junction where the lower epilimnion and hypolimnion waters indicated a large pulse of methane possible originating from this region.

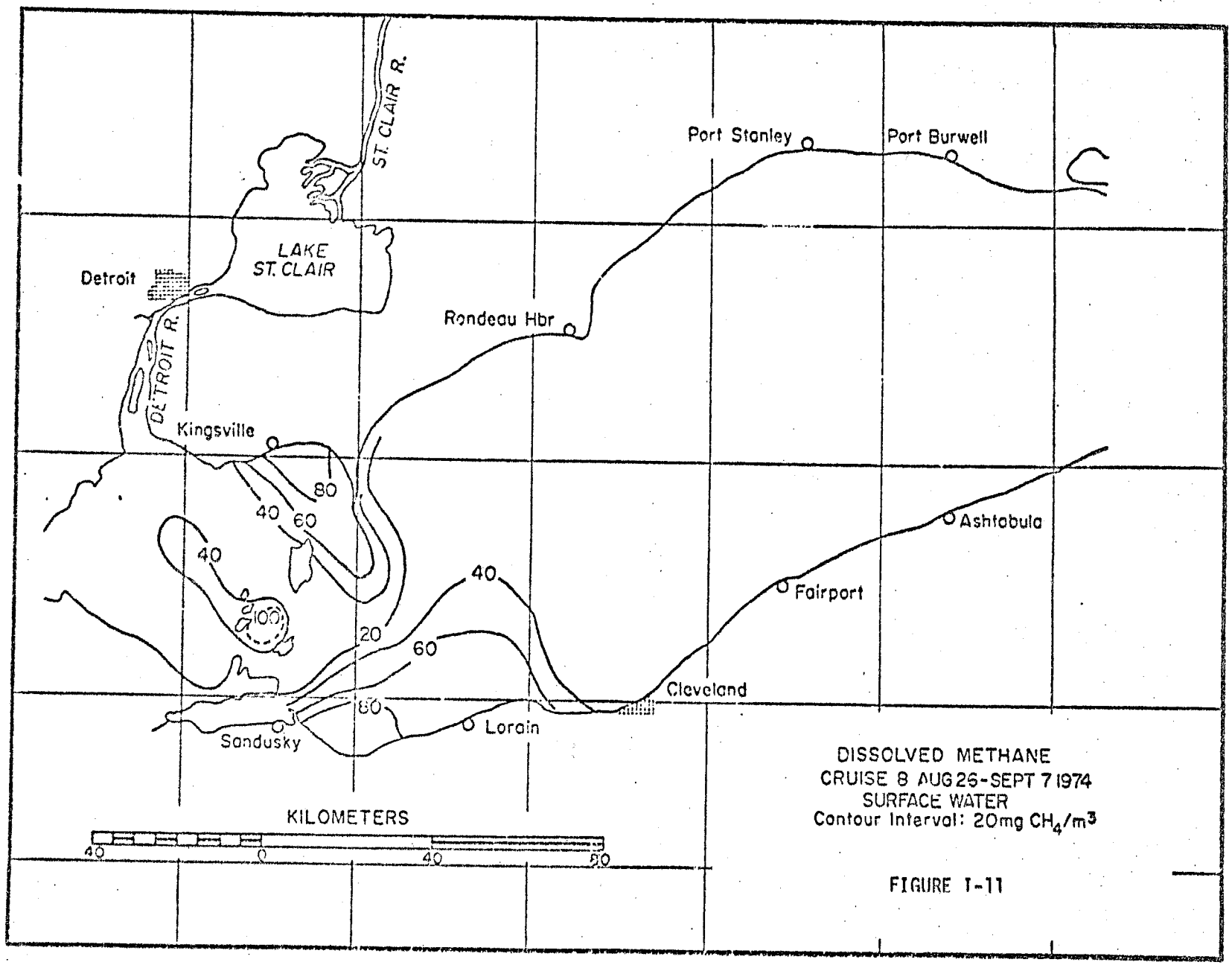
During the development of anoxia (d) and at the time of maximum anoxia, cruise 8, the methane concentrations were surprisingly low. The data of cruise 8 is graphically presented in Figures 11, 12, 14, and 14.

The surface water showed a uniform horizontal distribution with a relatively large methane impulse occurring along the Sandusky shore with a smaller Western Basin influence originating in the Kingsville region and extending southeasterly into the Central Basin.

The gradients observed in the lower epilimnion and hypolimnion waters of the Eastern-Central Basin junction were those expected. There was a low methane impulse entering from the Eastern Basin with a corresponding increase in dissolved methane as one progressed westward. The localized high concentration at Station 54 (mid depth and bottom water) can be attributed to the development of anoxia and localized eutrophic conditions.

Just prior to completion of the cruise of the Central Basin the thermocline broke up and the series of 40 stations were only monitored at three depths. The bottom water at the Western-Central Basin junction again demonstrated Western Basin loading of the Central Basin.

The annual fall turnover and post-turnover periods (e) resulted in a two fold increase in the methane concentrations of the water column as compared to the late summer cruise (Figs. 15, 16, 17; Table 4). As during the mid-summer cruise, an impulse of methane in the surface water was recorded at the eastern end of the Central Basin. Tnis impulse is amplified in the mid-depth water. A distinct zone of high methane concentrations was monitored along the north shore and extended westward toward Pelee. The largest concentrations were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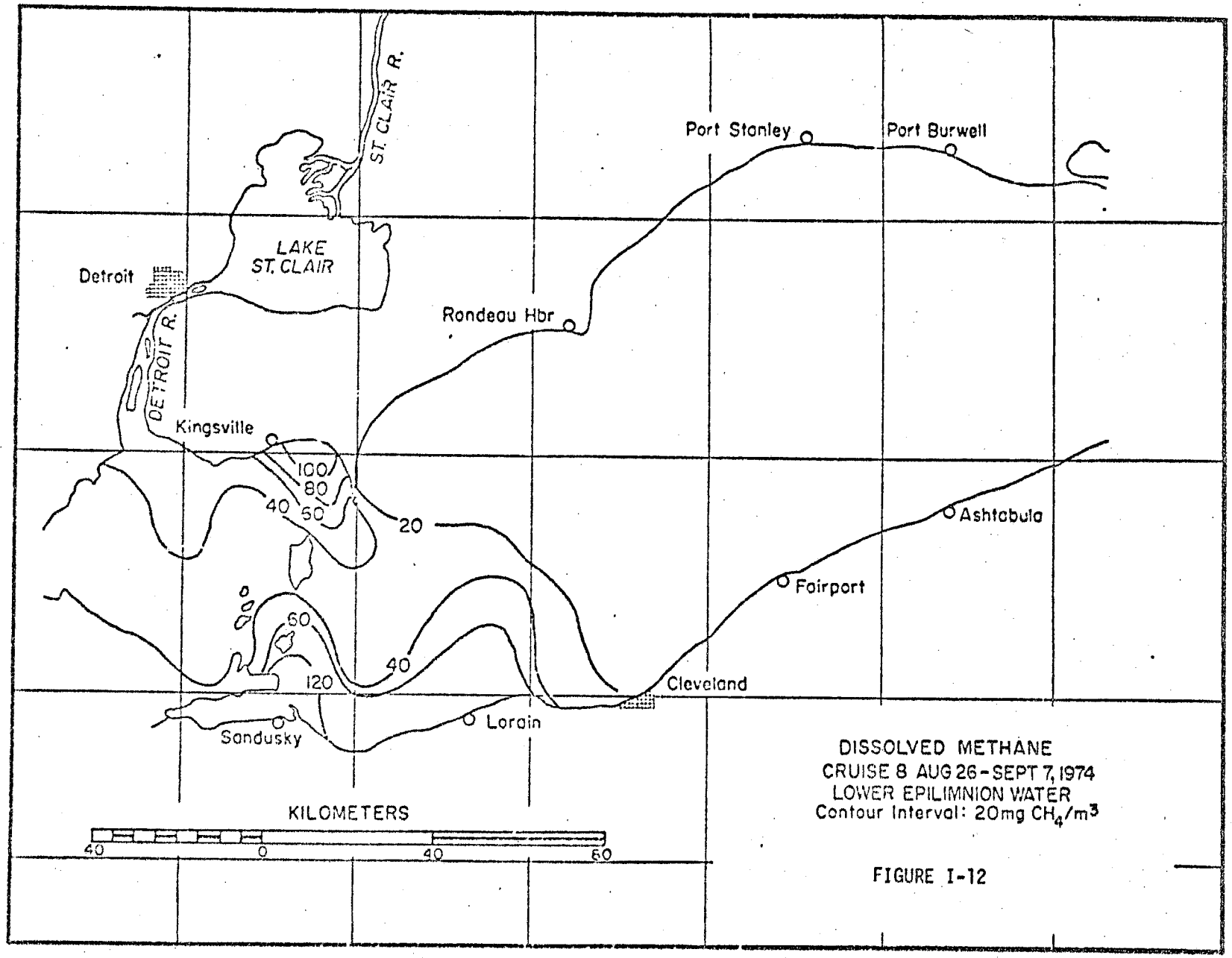
DISSOLVED METHANE
CRUISE 8 AUG 26-SEPT 7 1974
SURFACE WATER
Contour Interval: 20mg CH_4/m^3

FIGURE 1-11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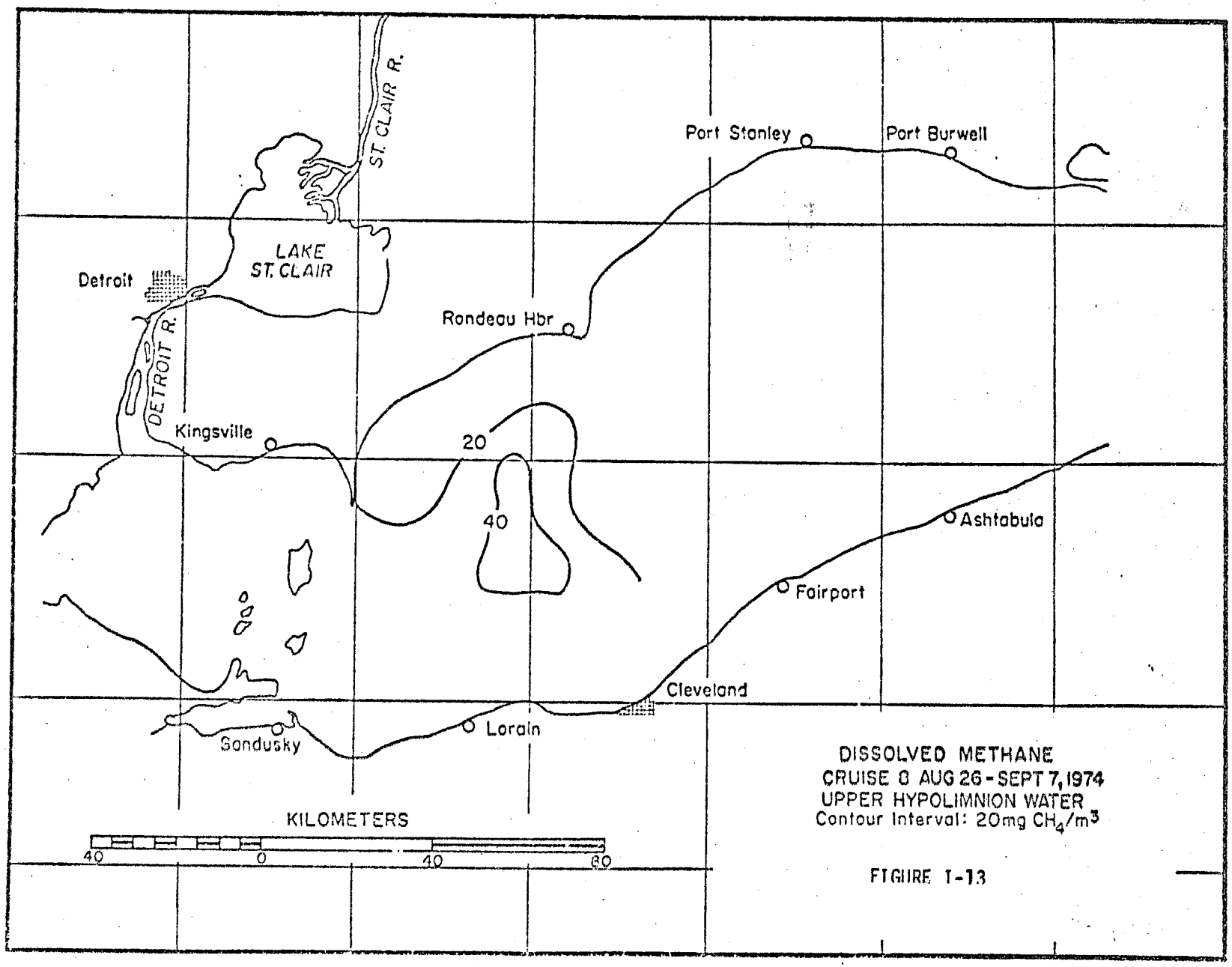
CRUISE 8 AUG 26 - SEPT 7, 1974
LOWER EPILIMNION WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-12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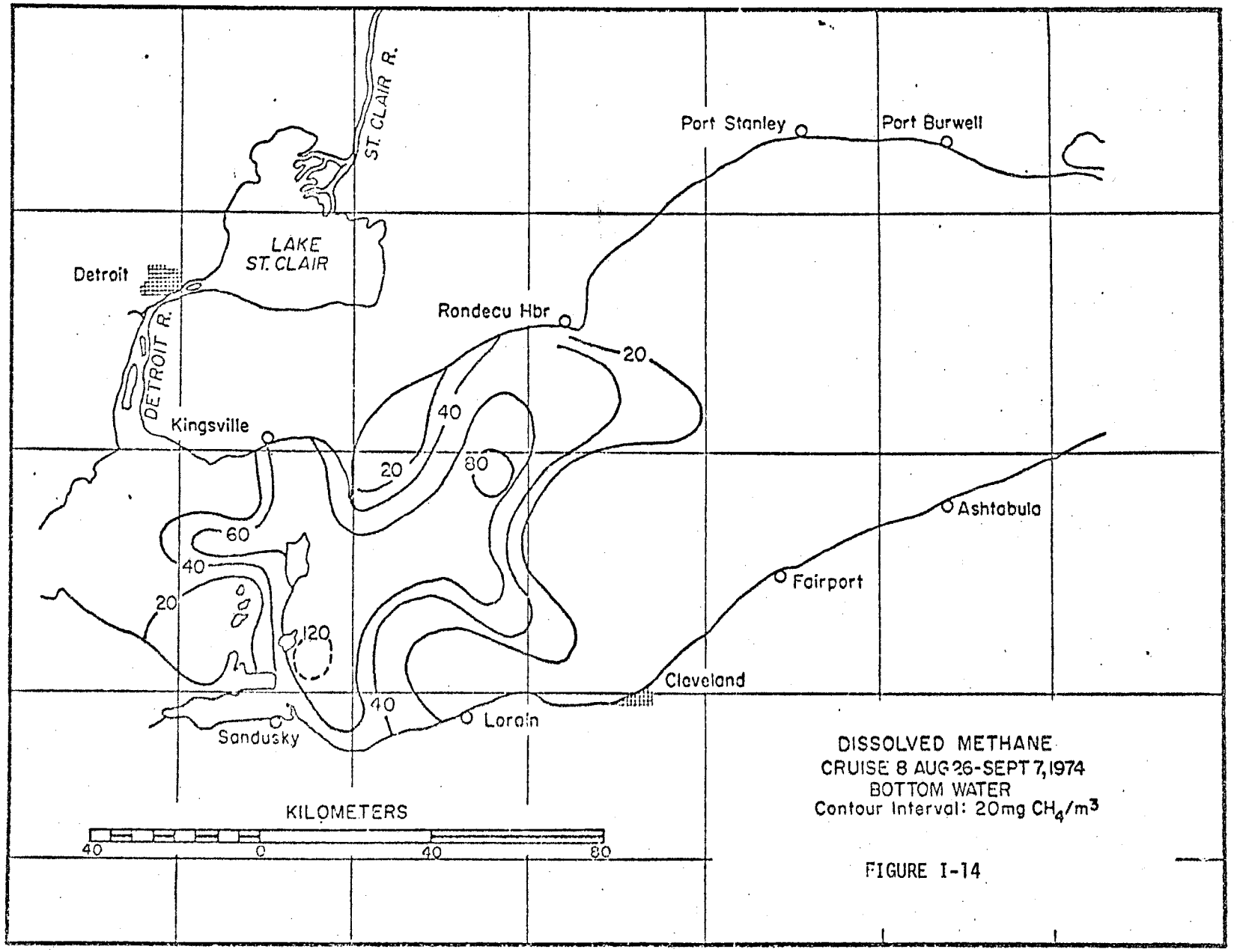
CRUISE 8 AUG 26 - SEPT 7, 1974
UPPER HYPOLIMNION WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-13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DISSOLVED METHANE
CRUISE 8 AUG 26-SEPT 7, 1974
BOTTOM WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-14

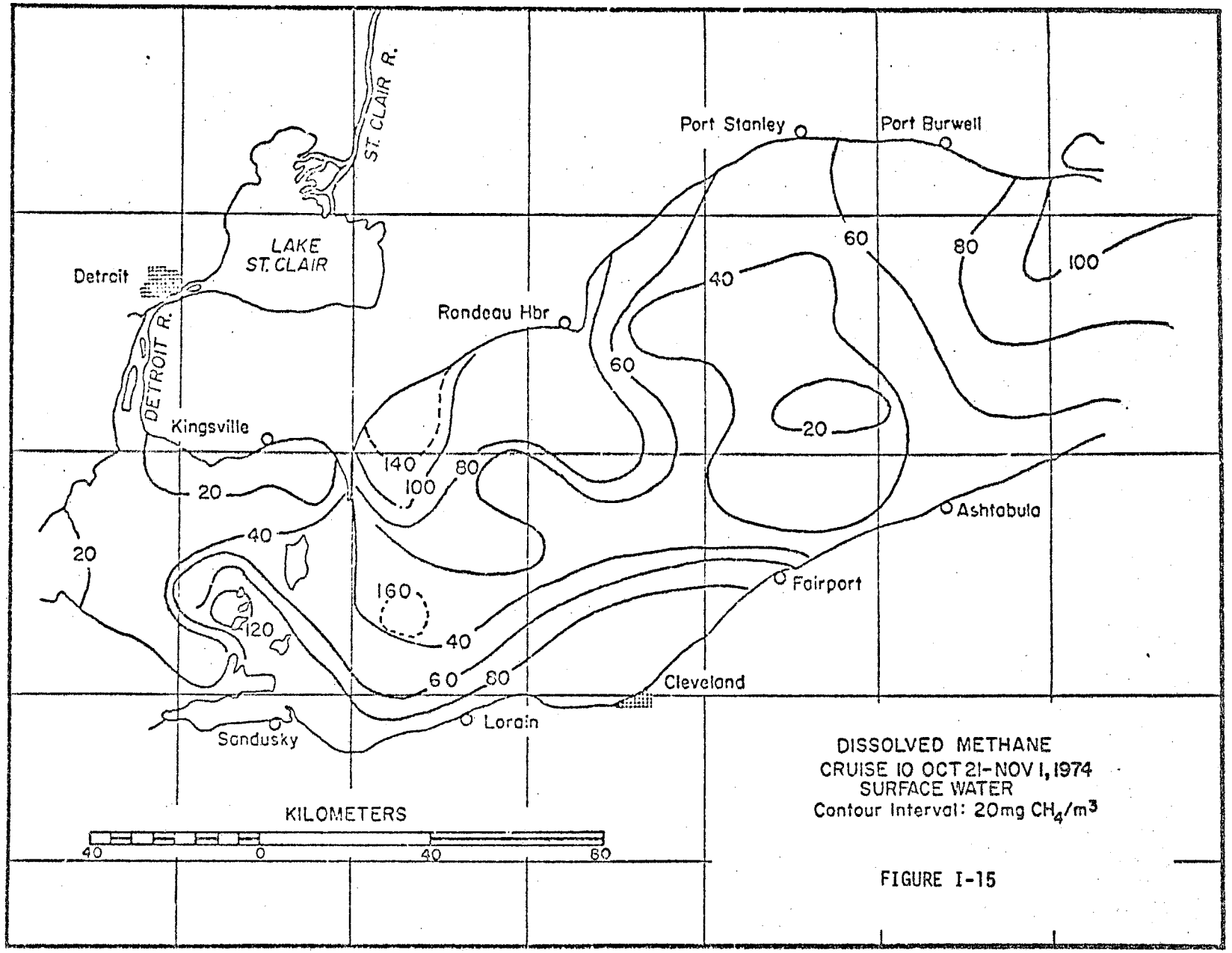
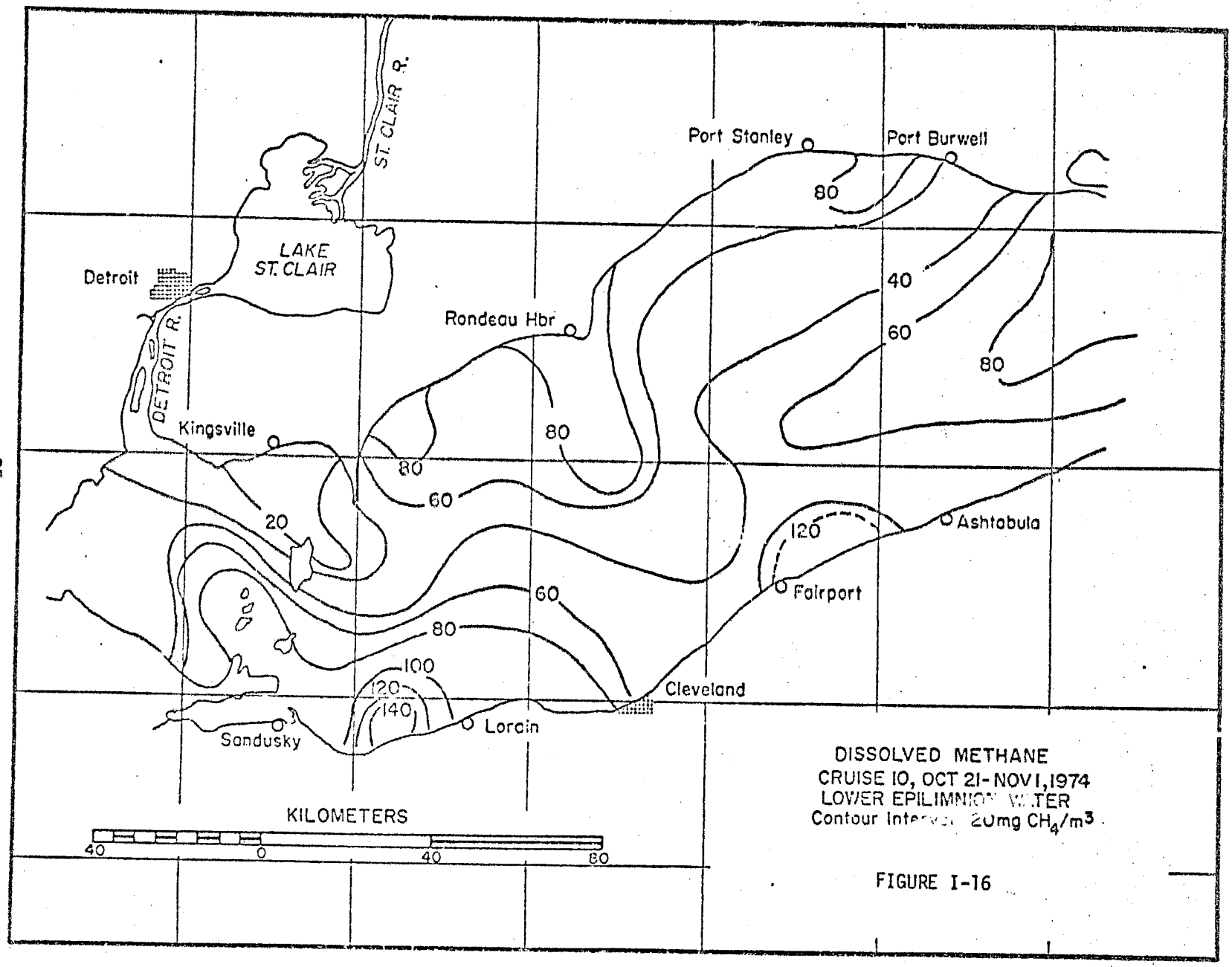

ST. CLAIR R.
Port Stanley
Port Burwell
LAKE ST. CLAIR
Detroit
DETROIT R.
Rondeau Hbr
Kingsville
Ashtabula
Fairport
Cleveland
Lorain
Sandusky
20
40
60
80
100
120
140
160
KILOMETERS
40
0
40
80
DISSOLVED METHANE
CRUISE 10 OCT 21-NOV 1, 1974
SURFACE WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-15


ST. CLAIR R.
Port Stanley
Port Burwell
80
LAKE
ST. CLAIR
Detroit
DETROIT R.
Rondeau Hbr
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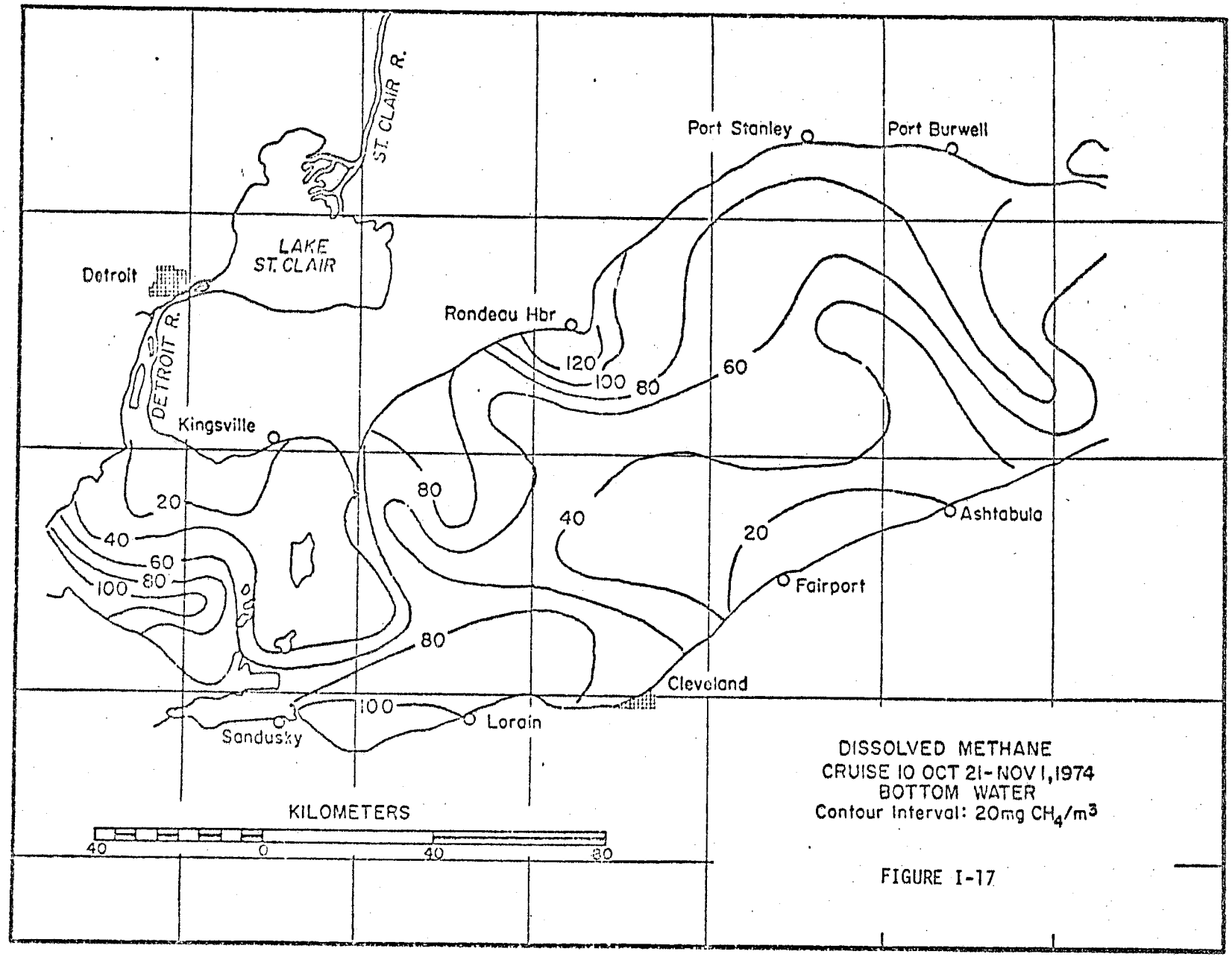
Kingsville
80
80
60
20
120
Ashtabula
Fairport
60
80
100
120
140
Cleveland
Sandusky
Lorain
KILOMETERS
40
0
40
80
DISSOLVED METHANE
CRUISE 10, OCT 21- NOV 1, 1974
LOWER EPILIMNION WATER
Contour Interval 20mg CH_4/m^3

FIGURE I-16


ST. CLAIR R.
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Rondeau Hbr
Port Stanley
Port Burwell
Ashtabula
Fairport
Cleveland
Lorain
Sandusky
120
100
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40
20
KILOMETERS
40
0
40
80
DISSOLVED METHANE
CRUISE 10 OCT 21-NOV 1, 1974
BOTTOM WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-17

observed in the surface waters of Stations 48 and 49 and were due probably to mixing of the high organic sediments in this region or to upwelling of bottom waters. The highest reported methane values occurred near Sandusky at Stations 51, 52, 53, and 54. No significant variations occurred in the water column. Again, the high values were indicative of localized eutrophic conditions.

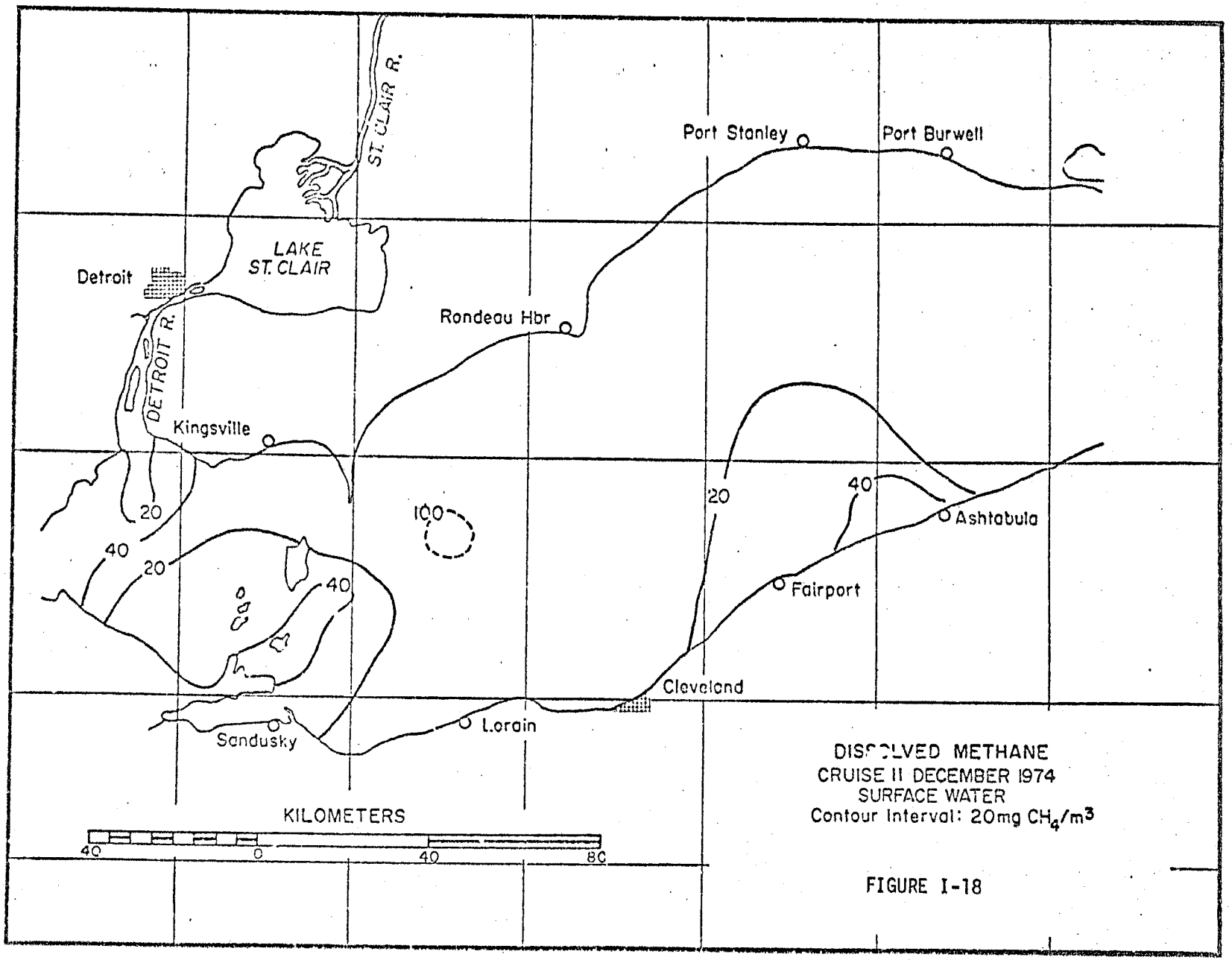
One isolated station located near Rattlesnake Island (Station 67) demonstrated an unusually large methane concentration in both the surface and mid-depth water. Apparently this activity was due to the sediments of the region since neighboring stations had relatively lower values.

Of the two rivers, only the bottom water of the Maumee made a significant contribution to methane input to the Western Basin. The radical change in the methane concentration in the surface and mid-depth water to that in the bottom indicates that the warmer Western Basin water is back flushing down the Maumee forcing the colder river water underneath. The contours 15, 16, and 17 demonstrate these observations. The Western Basin possesses a smooth linear gradient between the Detroit and the Maumee Rivers. The data indicate an organic loading from the Detroit which is being degraded and results in methane production along the south shore.

The mid-winter cruise (f) and Figures 18, 19, and 20 represent a stabilization of the methanogenesis of the Central Basin. Two isolated stations, 47 surface and 78 mid-depth, possess unusually high values both of which would tend to indicate washed in organics or dissolved methane. Sandusky Bay, Fairport and Ashtabula harbors represent locales of obvious organic loading to the Lake's surface water.

Water Column Methane 1975

The dissolved methane values were unusual in that they rarely exceeded 50 ppb (50 mg/m^3). The majority of the concentrations during stratification fell


ST. CLAIR R.
Port Stanley
Port Burwell
LAKE ST. CLAIR
Detroit
DETROIT R.
Rondeau Hbr
Kingsville
20
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20
100
20
40
40
Ashtabula
Fairport
Cleveland
Lorain
Sandusky
KILOMETERS
40
0
40
80
DISSOLVED METHANE
CRUISE II DECEMBER 1974
SURFACE WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-18

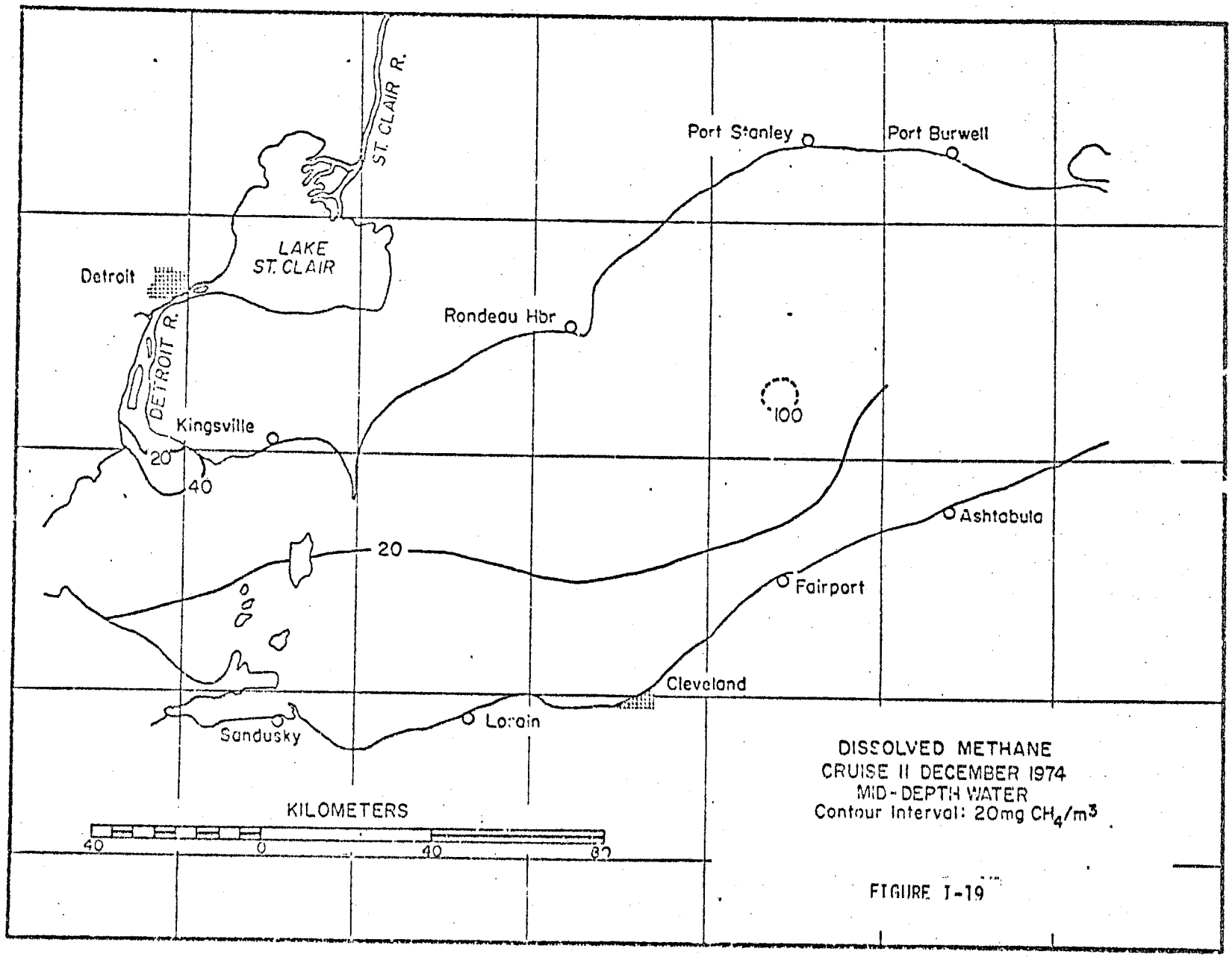
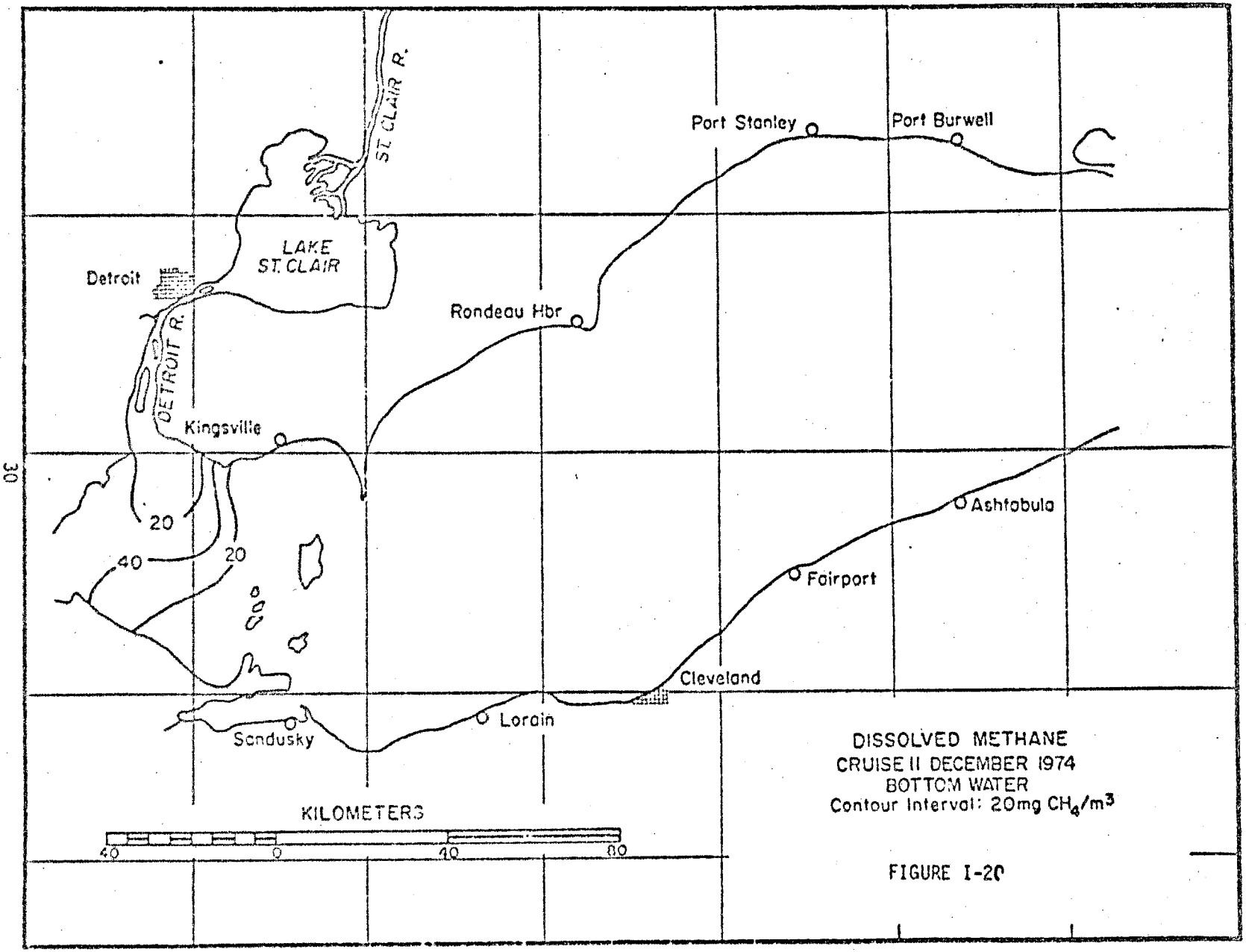

ST. CLAIR R.
Port Stanley
Port Burwell
LAKE ST. CLAIR
Detroit
DETROIT R.
Rondeau Hbr
100
Kingsville
20
40
20
Ashtabula
Fairport
Cleveland
Lorain
Sandusky
KILOMETERS
40
0
40
80
DISSOLVED METHANE
CRUISE II DECEMBER 1974
MID-DEPTH WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-19


ST. CLAIR R.
Port Stanley
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Rondeau Hbr
DETROIT R.
Kingsville
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Ashtabula
Fairport
Cleveland
Lorain
Sandusky
KILOMETERS
40
0
40
80
DISSOLVED METHANE
CRUISE II DECEMBER 1974
BOTTOM WATER
Contour Interval: 20mg CH_4/m^3

FIGURE I-20

between the 10-30 ppb range. No definite gradients were observed between strata as was often the case during the 1974 season.

The only major correlation between the 1974 and 1975 data is the documentation of obvious organic loading at most of the major ports along the lake's shore line. Along the south shore lire continual loading is observed at Sandusky, Cleveland and Fairport-Ashtabula regions. On the Canadian shore line Port Stanley-Port Burwell, Rondeau Harbor, and Point Pelee influences are shown.

The major difference in the methane values in the open lake for 1975 vs. 1974 is the lack of massive transport of methane between basins. The data indicate that methanogenesis was extremely limited in both the Central and Western Basins and that massive methane wash into the Central Basin from the Western did not occur.

Volume Weight of Methane

Yearly summary sheets for the methane volume weights for the sub-basins are presented in Tables 1 and 2. As discussed previously, the total methane weight observed during 1975 was drastically lower than the 1974 values. To clarify the data, a percent comparison of the sub-basin volume weights was made to demonstrate general trends concerning methane distribution in the lake. The following data will present a summary of the percentage distribution observed per sub-basin during both 1974 and 1975 seasons. The percentage values listed represent the fraction of the total lake methane observed in the specified sub-basin.

TABLE I-1

Summary of Sub-Basin Volume Weights 1974

Sub-Basin	1	2	4	5
Eastern Central	t=2014.3	16,073.1	23,391.5	21,261.1
	E=2014.3	16,073.1	14,969.1	16,642.9
	M= 0	0	1,842.4	879.1
	H= 0	0	6,580.0	3,739.1
Western Central	881.1	10,428.8	10,417.8	7,130.4
	881.1	10,428.8	6,104.2	5,609.6
	0	0	534.4	282.2
	0	0	3,779.2	1,238.6
North Shore CB	396.0	3,377.9	4,664.5	3,917.5
	396.0	3,377.9	2,740.6	2,819.0
	0	0	392.1	195.7
	0	0	1,531.8	902.8
South Shore CB	301.5	2,673.1	3,727.5	2,685.3
	301.5	2,673.1	2,561.1	2,251.1
	0	0	216.3	60.6
	0	0	950.1	373.6
"B" Basin CB	225.5	2,007.0	2,694.3	2,387.2
	225.5	2,007.0	1,726.9	2,387.2
	0	0	319.0	0
	0	0	648.4	0
Western Basin	238.5	1,528.3	3,180.0	2,621.4

TABLE I-1. (Cont'd)

Summary of Sub-Basin Volume Weights 1974

Sub-Basin	6	7	8	9
Eastern Central	t=15,536.2	10,036.4	2,806.6	10,394.8
	E=11,800.3	7,480.3	1,979.5	10,394.8
	M= 932.2	833.8	205.3	0
	H= 2,812.7	1,722.3	621.8	0
Western Central	(2,708.5)[a]	5,453.4	1,883.0	5,586.0
	(2,111.5)	3,742.9	1,616.1	5,586.0
	(139.6)	402.9	66.5	0
	(457.4)	1,307.6	200.4	0
North Shore CB	1,884.8	2,741.2	493.9	2,875.5
	1,295.6	2,308.2	349.9	2,875.5
	90.5	129.6	23.4	0
	498.7	303.4	120.6	0
South Shore CB	(1,746.7)	1,289.1	405.0	1,656.7
	(1,482.1)	1,173.0	327.1	1,656.7
	(64.4)	58.2	17.0	0
	(200.2)	57.9	60.9	0
"B" Basin CB	637.0	(871.3)	1,057.1	1,348.6
	612.9	(536.0)	1,057.1	1,348.6
	8.3	(56.7)	0	
	15.8	(278.6)	0	
Western Basin	899.8	566.4	793.2	831.6

a = parenthesis indicate insufficient data points.

TABLE I-2

Summary of Sub-Basin Volume Weights 1975

Sub-Basin	Cruise 1	2	3	4	6
Eastern-Central Basin	t=3005.8	(665.7)	3946.5	(2016.5)	2896.1
	E= 0	(387.5)	2125.3	(1572.2)	0
	M= 0	(48.5)	505.7	(98.5)	0
	H= 0	(229.7)	1315.5	(345.8)	0
Western-Central Basin	891.4	1472.2	1884.6	(1168.2)	1249.2
	891.4	791.9	1038.7	(602.3)	1249.2
	0	151.2	222.4	(99.3)	0
	0	529.1	623.5	(466.6)	0
North Shore CB	430.4	(431.8)	701.1	(60.4)	550.4
	430.4	(219.2)	412.7	-	550.4
	0	(54.5)	77.5	-	0
	0	(158.1)	210.9	-	0
South Shore CB	563.1	(247.4)	561.5	378.3	391.6
	563.1	(247.4)	420.9	358.4	391.6
	0	0	123.6	6.8	0
	0	0	17.0	13.1	0
"B" Basin CB	315.5	300.8	275.2	319.8	268.5
	315.5	217.1	210.4	319.8	268.5
	0	27.1	19.3	0	0
	0	56.6	45.5	0	0
Western Basin	323.3	427.0	454.0	350.9	239.4

Sub-Basin	Year	Percentage Range	Average %
East Central	1974	37.7 - 66.4	49.3
West Central	1974	11.6 - 28.9	22.2
North Shore Central	1974	6.6 - 13.1	9.9
South Shore Central	1974	5.4 - 7.8	7.0
"B" Basin	1974	2.7 - 14.2	6.2
Western Basin	1974	2.7 - 10.7	5.5
East Central	1975	47.0 - 54.4	50.9
West Central	1975	16.1 - 27.2	22.4
North Shore Central	1975	7.8 - 9.8	8.9
South Shore Central	1975	7.0 - 10.2	8.0
"B" Basin	1975	3.5 - 5.7	4.7
Western Basin	1975	4.3 - 5.8	5.3

As the data indicate the ranges are relatively consistent, but more significantly the yearly averages per sub-basin are identical. This reinforces the idea that the sub-basin lake methanogenesis was equally affected during the 1975 season. With the present data and with the lack of any previous methane data for the lake it is impossible to explain the significant inhibition of methanogenesis during the 1975 season. One can only conclude that the same general trends occurred but only to a more limited extent.

Methane Production Potentials

The sediment methane production potentials for 1975 showed a similar decrease in methanogenesis over that observed during the 1974 season. Table 3 presents the potential production rates recorded during 1974 and 1975 by sub-basin regions. The more critical data on Table 3 is the 1974/1975 ratios. For the Central Basin regions a consistent result is observed. The shore stations appeared much less active during the 1975 season. For example in the Eastern Central Basin grids 19, 20 and 30 possessed a high 74/75 ratio indicating a more active methanogenic rate during 1974. Only the mid-lake grid 28 showed a high 74/75 ratio indicating again a very active sediment during 1974. The Western Central Basin demonstrated similar shore effects. Grids 32 and particularly grid 36 indicated drastic differences in production rates during 1974

TABLE I-3

Methane Production Potentials

Values Are $x10^{-4}$mg CH_4/gm Sediment/Day

Basin	Grid	Station(s)	1974 Range	1975 Range	74/75 Ratio
Eastern Central Basin	19	23,24	0-211.0	0-0.4	527.500
	20	25,26,79	0-641.0	0-0.6	1068.300
	21	27	0.3-0.5	0.2-30.0	0.017
	22	28,29	0-1.4	0.4-220.0	0.006
	23	30,78	2.6-118.7	0.5-113.4	1.047
	24	31	0.5-390.0	0.9-2.7	1.444
	25	32	2.1-34.2	0-10.0	3.420
	26	33,34	0-410.0	0-190.0	2.158
	27	35	0-300.0	0-66.9	4.484
	28	36	5.2-1424.0	0-26.0	54.769
	29	37	22.8-182.7	1.1-610.0	0.300
	30	38	0.2-159.3	0.4-12.9	12.349
	31	39	0-158.0	0-24.0	6.583
Western Central Basin	32	40,41	0-240.0	0-24.0	10.000
	33	42,73	0.6-585.0	0-130.0	4.500
	34	43	6.1-204.0	0-440.0	0.464
	35	45	0-110.0	4.6-114.3	0.962
	36	44	0-348.0	0-2.7	128.889
	37	46	0-72.5	0-12.5	5.800
	38	47	0-72.8	0-77.0	0.945
	39	48,49	0.3-132.4	0.7-136.7	0.969
North Shore Central Basin	21	27	0.3-0.5	0.2-30.0	0.017
	22	28,29	0-1.4	0.4-220.0	0.006
	31	39	0-158.0	0-24.0	6.583
	32	40,41	0-240.0	0-24.0	10.000
	39	48,49	0.3-132.4	0.7-136.7	0.969
South Shore Central Basin	19	23,24	0-211.0	0-0.4	527.500
	26	33,34	0-410.0	0-190.0	2.158
	27	35	0-300.0	0-66.9	4.484
	36	44	0-348.0	0-2.7	128.889
"B" Basin	40	50	0-12.1	0-1.0	12.100
	41	51	0.4-146.6	0-52.9	2.771
	42	53	0-2250.0	0-120.0	18.750
	43	52,54,65,74	0-583.1	0-40.0	14.578
Western Basin	44	55	0-140.0	0-0.3	466.667
	45	56,66	0-164.5	4.5-97.8	1.682
	46	58,67,68, 69,76	0-1110.0	0-78.0	14.231
	47	59	0-460.0	0-7.1	64.789
	48	57	0-203.2	0-0.2	1016.000
	49	60,70,75	0-566.9	0-628.0	0.903
	50	61	0-269.7	0-16.0	16.856

and 1975.

When considering the north and south shore grids more accurate analysis of the shore line is possible. Of the north shore grids only grids 31 and 32 demonstrated high 74/75 ratios. Thus the most microbially active region of methanogenesis appears to be Rondeau Harbor. On the south shore two isolated regions of high activity are grids 19 (Erie, Pa.) and 36 (Cleveland, Ohio).

The "B" Basin, in general, shows a more active production potential throughout the 1974 season.

The Western Basin possesses high 74/75 ratios at grids 44, 47, and 48. These grids are located centrally in the Western Basin and document very active methanogenesis in the Island region of the basin. The shore line grids (45, 49 and 50) show a decreased 74/75 ratio indicating similar methanogenesis during 1974 and 1975.

Relationship Between Water Column Methane and Sediment Production Potentials 1974-1975

When a comparison is made between dissolved methane values and production potentials there is a trend observed. During 1974 massive wash in of methane was observed at Cleveland, Fairport-Ashtabula, Erie, Port Stanley-Port Burwell and Rondeau Harbor regions. These correspond to regions of active sediment methanogenesis during 1974 season. The 74/75 sediment ratios demonstrate that similar methanogenesis occurred in mid lake regions during the two seasons.

These shore grids consistently demonstrated lower production rates during 1975 which was drastically reflected in lower water column methane. Thus the most significant methanogenesis appears to be occurring at near shore stations were the resulting methane is subsequently washed to mid lake regions. This speculation is consistently born out in the 1974 water column contours.

Similarly, since the Western Basin grids demonstrated more active methanogenesis during 1974 at the Island region and since the current patterns are such that the water in the Island region is often washed via Pelee Passage into the Western Central Basin this may explain the massive wash in methane recorded in the Central Basin during 1974. The lack of significant methanogenesis at these same regions during 1975 results in lack of intra basin methane exchanges.

SECTION II

INTRODUCTION

Methane is produced biologically by a group of strictly anaerobic bacteria which derive energy principally from the oxidation of hydrogen coupled to the reduction of carbon dioxide. There are three main environments in which these organisms seem to survive: the mud at the bottom of ponds and lakes, the digestive tract of ruminants and other animals, and sewage treatment systems. Features these places have in common are anaerobic conditions, a relatively high level of organic matter, and a large population of microorganisms. The factors affecting methanogenesis in these environments are, however, poorly understood.

Although hydrogen has been established as the main energy source for methanogenic organisms, several questions remain about the interaction of methanogenic organisms and their substrate. How much of the hydrogen produced in an anaerobic environment is available to these organisms? Are methane producers depressed in their methane generation and growth by low levels of hydrogen?

Beyond doubt the number of methane producing organisms in any environment will play an important role in defining methane generation. There are three different methods currently available to estimate the number of methanogenic bacteria in a sample of mud, sewage, or rumen contents. These numbers mean little, however, if there is no way to relate them to the potential for methane production. The rate at which methane is generated is an important parameter which should be investigated before the whole process of methanogenesis in natural systems can be well understood.

A final issue which needs to be resolved is whether the type of methanogenic bacteria present in any environment is important. Based on general cell

morphology and ultrastructure there are four types of methane producing organisms. Are hydrogen utilization and rate of methane production different for these different cell types?

It was the purpose of this study to answer the questions raised here about hydrogen utilization and rate of methane generation. The work was carried out with four species of methane producing bacteria in pure culture. This was done to allow for comparisons to be made between the various cell types.

MATERIALS AND METHODS

Culture Techniques--General

The general cultural techniques reported here were developed for these studies. The final goal was to grow organisms which are extremely sensitive to the slightest trace of oxygen. This is a problem which has faced many investigators who have responded with many different solutions. Some information about specific techniques or approaches was borrowed from these investigators. Some techniques had to be developed independently. In any case it is very doubtful that the exact system reported here is in use in any other laboratory.

Even the simplest method, manipulation, or technique had to be perfected by a series of trials and errors. The process of learning anaerobic methods was complex and the false trails were numerous. An overview of the development of these techniques is presented in the Results section. The techniques described in this section are those which ultimately proved to be successful.

All reagents and apparatus used in this study are listed in the Appendix according to the company from which they were obtained.

Composition of Media

Rumen fluid medium. Stocks of all cultures were maintained on agar slants of the rumen fluid medium of Bryant et al. (6) which contained the following ingredients in distilled water at the indicated final percentage compositions (wt/vol):

Trypticase	0.2
Na_2CO_3	0.2
KH_2PO_4	0.0225
K_2HPO_4	0.0225
$(NH_4)_2SO_4$	0.0450
NaCl	0.0450

$MgSO_4 \cdot 7\ H_2O$	0.0045
$CaCl_2 \cdot 2\ H_2O$	0.0045
Resazurin	0.0001

In addition to the above reagents, the medium contained 30% (v/v) clarified rumen fluid and 1% (v/v) cysteine sulfide reducing agent (7). The final pH of this medium after autoclaving and with an atmosphere of H_2:CO_2 (80:20) was 6.9-7.0.

Preparation of rumen fluid. Rumen contents were obtained from a fistulated sheep. The contents were placed in an Erlenmeyer flask which was filled nearly to the top, stoppered loosely, and brought immediately to the laboratory. Rumen contents are semi-solid. After processing the yield of the clear fluid which is the medium ingredient was approximately 50%.

The fresh rumen contents were centrifuged at 25,000 X G for 20 minutes in a Sorvall RC2-B refrigerated centrifuge. The supernatant was drawn off and centrifuged again at 25,000 X G for 20 minutes. At this point the fluid had no pieces of solid matter floating in it but was still very turbid. In order to clarify it, the fluid was then passed through a Millipore filter (pore size 0.45 μm) fitted in a Gelman pressure filter. The filter was attached to a tank of 100% CO_2. Ordinarily the clarified fluid was used immediately for medium preparation. It could, however, remain in the refrigerator overnight if stoppered and provided with an atmosphere of oxygen-free carbon dioxide.

Growth medium. The preparation of rumen fluid was a very time consuming process and once prepared the ingredients contributed by the rumen fluid were completely unknown. Thus it was desirable to have a simpler and more defined medium for most of the experiments. Work by Bryant et al. (8) on the nutrient requirements of methanogenic bacteria indicated that the growth of these organisms is stimulated by addition of acetate, certain vitamins, and ammonium

ion. The original medium had ammonium ion at the optimal level and it was modified by adding the other two ingredients at optimal levels. The rumen fluid was omitted. The medium contained the following ingredients in distilled water at the indicated final percentage compositions (wt/vol):

Trypticase	0.4
Na_2CO_3	0.2
Sodium acetate	0.2
Yeast extract	0.2
KH_2PO_4	0.0225
K_2HPO_4	0.0225
$(NH_4)_2SO_4$	0.0450
NaCl	0.0450
$MgSO_4 \cdot 7\ H_2O$	0.0045
$CaCl_2 \cdot 2\ H_2O$	0.0045
Resazurin	0.0001

Cysteine-sulfide reducing agent (7) was added at a level of 1% (v/v). The final pH of this medium after autoclaving and with an atmosphere of H_2:CO_2 (80:20) was 6.9-7.0.

Methanospirillum medium. Methanospirillum hungatii grew poorly in both rumen fluid medium and growth medium. Therefore stocks of this organism were maintained on agar slants of the medium described by Ferry, Smith, and Wolfe (14). This medium contained the following ingredients in distilled water at the indicated final percentage compositions (wt/vol):

Trypticase	0.2
Na_2CO_3	0.4
Sodium formate	0.2
Yeast extract	0.2

KH_2PO_4	0.023
K_2HPO_4	0.023
$(NH_4)_2SO_4$	0.023
NaCl	0.046
$MgSO_4 \cdot 7\ H_2O$	0.009
$CaCl_2 \cdot 2\ H_2O$	0.006
Resazurin	0.0001

Cysteine-sulfide reducing agent (7) was added at a level of 1% (v/v). The final pH of this medium after autoclaving and with an atmosphere of H_2:CO_2 (80:20) was 7.

Nutrient broth medium. Most of the isolation procedures were carried out on a nutrient broth medium which contained the following ingredients added to distilled water at the indicated final percentage compositions (wt/vol):

Nutrient broth	0.4
$NaHCO_3$	0.5
Resazurin	0.0001

In addition, cysteine-sulfide reducing agent (7) was added at a level of 1% (v/v). The final pH after autoclaving and with an atmosphere of H_2:CO_2 (80:20) was 7.1.

Culture Vessels

Four different types of culture vessels were used in the various experiments reported here. Many experiments were done with tube cultures in either roll tubes with rubber stoppers or Hungate-style anaerobic screw cap tubes. In general these tubes contained 9 ml medium. These tube culture methods are very widely used in cultivating anaerobes (4, 17, 22, 25).

Prescription bottles were sometimes used with a layer of agar across the flat side of the bottle on which it was possible to streak for colony

isolation. This method of cultivation had been previously suggested by Bryant (4) and Skinner (41). In some cases the bottles were closed with black rubber stoppers. Sometimes it was desirable to be able to sample inside the bottle for methane production. For these occasions a different seal was designed. A slotted butyl rubber stopper was inserted in the neck of the bottle. This was held in place by the bottle cap. The liner of the cap had to be removed in order for it to fit over the stopper. A hole was drilled in the center of the cap to allow access to the inside of the bottle via a syringe.

An attempt was made to design an anaerobic culture dish of plexiglass which would allow the growth of colonies on a flat agar surface and give a better view of the colonies than was possible with the bottle cultures. This chamber is illustrated in Figure 1.

For the studies on rates of methanogenesis and hydrogen depletion it was necessary to grow the organisms as batch cultures in flasks. In these cultures it was necessary to sample repeatedly for both gas and liquid without oxidizing the media or allowing gas exchange with the atmosphere. The previously published systems for batch culturing of anaerobes (6, 24, 29) and for gas-tight cultures (31) involve either specially constructed glassware or have a somewhat complex design. Thus it was necessary to design a new system which proved to be both effective and simple to construct.

The important component of this system was a black rubber stopper fitted with a Hungate-style screw cap tube which was cut off below the stopper (Figure 2). The construction of the stopper required only a cork borer and a file for scoring the tube and took about 5-10 minutes. Once constructed the septum of the tube could be replaced and the unit used repeatedly. Since the glass of the tube was in contact with a relatively large area of the stopper, leakage of gases was minimal. This stopper was fitted to a 250 ml Erlenmeyer

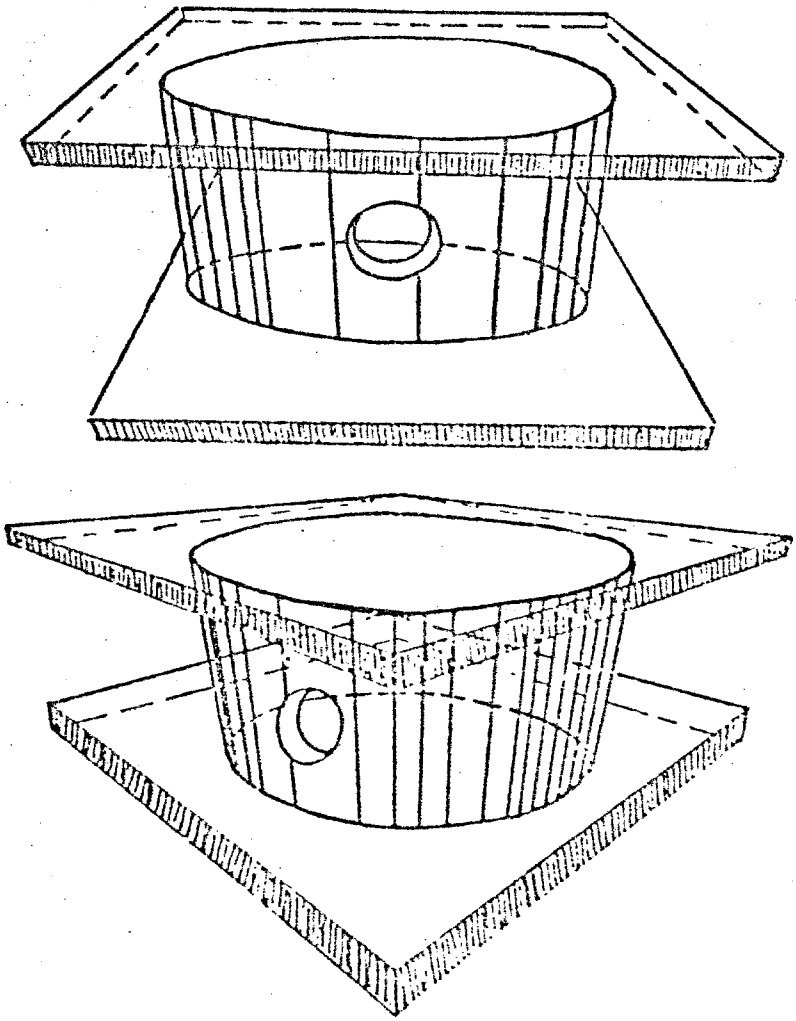

FIGURE II-1. Anaerobic Culture Dish

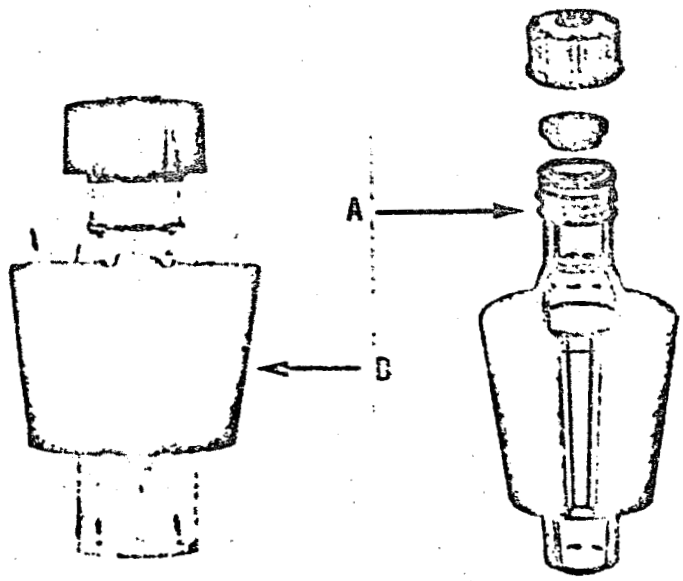

FIGURE II-2. Gas-tight Stopper.

(A) Hungate-style anaerobic tube cut in half.
(B) Black rubber stopper with a hole bored through it.

flask for all the batch culture experiments.

Media Preparation

Medium was prepared by mixing all ingredients except the reducing agent. At this point no anaerobic techniques had been followed and the medium was oxidized. It was then dispensed into tubes or bottles with the apparatus illustrated in Figure 3.

Using this apparatus the medium was in contact with oxygen-free gas before, during, and after dispensing. The culture vessel was placed under a stream of oxygen-free gas for several seconds which swept the oxygen from it. The medium was then pumped into the vessel using a foot pedal controlled pump. The oxygen-free gas then passed over the medium for a few seconds longer before the stopper was seated into the culture vessel without access of air from the atmosphere. The reducing agent was added at this point and the medium was autoclaved. When removed from the autoclave the medium was reduced.

All media were pumped into the tubes or bottles as a liquid. When a solid medium was needed, the agar was added to the culture vessel before the medium. This was accomplished by using a spoon made from a piece of bent glass tubing. This spoon held approximately 0.15 grams of agar which was sufficient to solidify 10 ml of medium. The agar melted in the autoclave and was evenly distributed in the medium by inverting the culture vessel several times.

The procedure followed for dispensing media in shake flasks was somewhat different. The liquid medium was poured into the flasks and a stream of H_2:CO_2 (80:20) from a 13 gauge needle passed over the medium for 2 minutes. The septum and the screw cap of the tube were put into place and the flask set aside for 10-15 minutes. Once again H_2:CO_2 (80:20) was passed into the

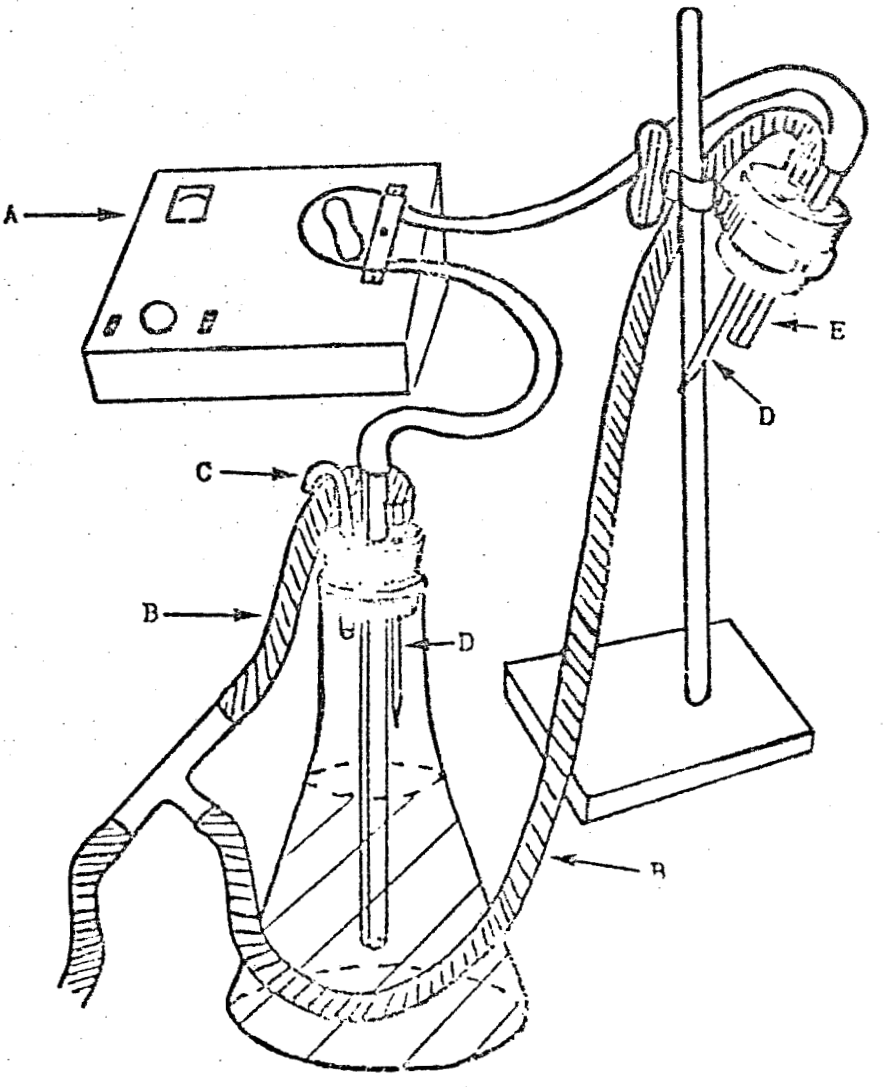

FIGURE II-3. Apparatus for Dispensing Media:

(A) Peristaltic Pump; (B) Rubber Tubing Carrying oxygen-free CO_2 or H_2:CO_2; (C) Vent;
(D) 13 GA Hypodermic Needle Delivering Oxygen Free Gas;
(E) Cut-off Pasteur Pipette Delivering Media.

flask by a 21 gauge needle inserted through the septum. Another 21 gauge needle served as the exit port. After 2 minutes of purging the exit needle was withdrawn followed by the gassing needle. This caused a slight positive pressure to remain in the flask. At this point the reducing agent was added and the flask was ready to be autoclaved.

All media were autoclaved for 20 minutes at 121 C. Roll tubes and some bottle cultures were autoclaved in a press to keep the stoppers from coming out Screw cap tubes and bottles needed no restraint other than the caps. The stoppers of shake flasks were tied on with string. The anaerobic culture dish was sterilized empty and pre-reduced and sterile medium was added anaerobically and aseptically.

Transfer of Cultures

All liquid cultures were transferred with a tuberculin syringe without opening the culture. This method presented the least danger of oxidation and/or contamination of the medium. Occasionally agar slants in tubes or agar layers in bottles were inoculated by injecting a few drops of inoculum into the surface of the agar. The inoculum was spread over the surface of the agar by gently tipping the culture vessel. Transfers from one slant to another and streaking for isolation were done using the methods developed by Hungate (19). The gas used during transfer of cultures was H_2:CO_2 (80:20) which was passed through a sterile Swinney filter device fitted with a 0.45 μm pore size Millipore filter disc. In general all cultures were purged with sterile H_2:CO_2 for 2 minutes and the culture vessels were left with a slight positive pressure after transfer and before incubation.

Cultures

Four pure cultures of methanogenic bacteria were provided by M.P. Bryant,

University of Illinois, Urbana, Illinois. These were the following:

Methanobacterium ruminantium strain PS

Methanobacterium M.O.H.

Methanosarcina barkeri strain MS

Methanospirillum hungatii strain JF

A methanogenic organism was also isolated from Lake Erie sediment. The sediment came from a point 33 miles east of Cleveland, 2 miles offshore where the water is 18 feet deep. The isolation was carried out over a period of 9-10 months. Anaerobic techniques were being developed at the same time as the isolation procedure was being attempted which was a contributing factor to the long period of time necessary.

The isolation was attempted by carrying out a series of dilutions in liquid media on the assumption that a tube with a high concentration of methane would have a high concentration of methane producing organisms. This proved not to be the case and resulted in many blind paths being followed. A total of 12 dilution series were made in the path leading to the isolate. The last two or these were made in a mineral salts medium similar to that of Zeikus and Wolfe (52) except that 100 mg/liter of yeast extract was substituted for the vitamin solution. This removed many of the contaminants which were unable to survive on this minimal medium. Finally the highest dilution in this synthetic broth which showed methane production was centrifuged to concentrate the cells. These cells were then used to inoculate agar bottles. This resulted in isolated colonies from which the isolate was obtained.

All cultures were maintained on agar slants of either rumen fluid medium, growth medium, or *Methanospirillum* medium. Stocks were routinely transferred at approximately four week intervals.

Characterization of Isolate

There are only a limited number of methanogenic species. All of them apparently share the same energy yielding process. The most important feature by which they are distinguished from each other is morphology. Within any morphological group the characteristics used to differentiate strains are requirements for growth factors present in rumen fluid, ability to use different substrates for methane generation, and temperature optimum. With these considerations in mind a partial characterization of the Lake Erie isolate was carried out.

According to morphology this organism is definitely of the bacillus type. Within this group the only substrates used are hydrogen gas and formate. The organism was tested for the ability to produce methane from these two substances. The organism was inoculated into three tubes each of nutrient broth medium with 1% formate added and an atmosphere of 100% CO_2, nutrient broth medium with an atmosphere of H_2:CO_2 (80:20), and nutrient broth medium with an atmosphere of 100% CO_2. All tubes were incubated at 37 C and monitored for methane by gas chromatography.

The optimum temperature for growth and methane generation was determined by inoculating tubes of growth medium with the isolate. The tubes were purged and incubated with an atmosphere of H_2:CO_2 (80:20) at the following temperatures: 4, 15, 23, 30, 37, and 45 (all temperatures in degree C). There were five tubes at each temperature. These were monitored routinely for methane production.

Gas Analysis

Gas analysis was done with gas chromatography. Three recorders were used in conjunction with the chromatographs: a Westronics LD/11A strip chart

recorder, a Linear Instruments recorder Model 252, and a Linear Instruments recorder Model 252A. The two recorders from Linear Instruments were equipped with electronic integrators.

In most cases gases were injected into the chromatograph with a microliter gas syringe. In some cases where it was necessary to prevent any possible danger of contamination of the cultures, gases were sampled and injected into the chromatograph with sterile 1cc tuberculin syringes.

Methane. Methane production was monitored with a Varian Aerograph 2740 gas chromatograph equipped with hydrogen flame ionization detectors. The carrier gas was nitrogen. Since the experiments reported here extended over a rather long period of time, several different columns and packing materials were used. For some of the early isolation work silica gel was used. It was packed into a 1/8 inch by 8 foot stainless steel column. The later studies were done with Porapak N (100/120 mesh) and Porapak Q (100/120 mesh) which were packed into 1/8 inch by 6 foot glass columns.

Carrier gas flow rate and injector, detector, and column temperatures were always optimized for the detection of methane. In all cases the retention time of methane was determined by comparison to a standard. The methane peaks were quantitated by comparison with standards containing a known amount of methane. Standards were run at the same time and at the same attenuations as the unknowns.

Hydrogen. Hydrogen was detected with a Carle 8004 gas chromatograph. It was equipped with thermistor detectors and a 1/8 inch by 8 foot stainless steel column packed with 30/60 mesh silica gel. The column temperature was 120 C. Nitrogen carrier gas was used at a flow rate of approximately 14 ml/min. Using these conditions the response of the chromatograph was linear over a wide range of hydrogen concentrations.

Calibration of chromatograph for hydrogen detection. It had been reported by several authors that hydrogen could be detected using a thermal conductivity gas chromatograph if nitrogen were the carrier gas (21, 23, 50). None of these authors, however, mentioned the sensitivity of this method or whether the response of the chromatograph was linear over a wide range of hydrogen concentrations.

Czerkawski and Clapperton (11) reported that hydrogen could be detected accurately at a range of concentrations of 0.1-70.0% by volume. They used a thermal conductivity detector, argon carrier gas, and a series of three columns to obtain the gas separation necessary for their work. Since the system used in these studies differed in several respects from that used by Czerkawski and Clapperton, it was necessary to determine its reliability and sensitivity. Three experiments were performed in this calibration process.

In the first experiment different amounts of H_2:CO_2 (80:20) were injected into the chromatograph at two machine attenuations. The results of this experiment are presented graphically in Figure 4.

In the second experiment various concentrations of hydrogen were set up in serum bottles by the following procedure. The total volume of the serum bottles was determined by filling them with water. The volume was slightly different for each bottle and this information was noted. The bottles were filled with 10 ml water, stoppered, and the atmosphere purged with nitrogen for approximately 3 minutes. This was done to eliminate oxygen and carbon dioxide which would give extra peaks during chromatography. After purging there was a slight positive pressure remaining in the bottle. The contents of the bottle were then equilibrated to atmospheric pressure by allowing water to drain out through a needle. A known volume of hydrogen was then injected into the bottle with a gas syringe. Once again the bottle was equilibrated to atmospheric

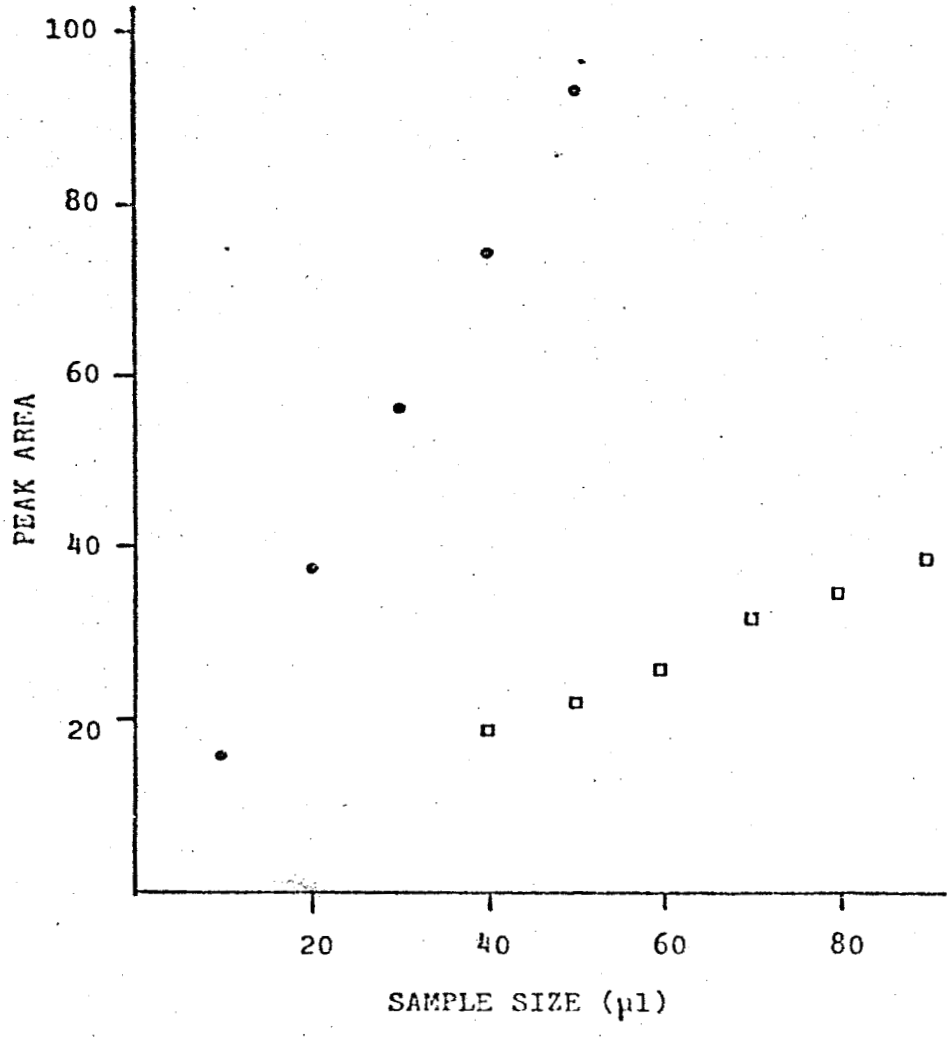

FIGURE II-4. Relationship Between Sample Size and Peak Area.
80% hydrogen. Attenuation 25 = • .
Attenuation 100 = □

pressure by allowing water to drain out. The water removed in this manner was measured. The total gas volume and the amount of hydrogen were then known and the percent hydrogen in the atmosphere could easily be calculated. Various sample sizes of these concentrations were then injected into the chromatograph. These results are presented in Figure 5.

The third experiment was set up in the same way as the second except that higher concentrations of hydrogen were used. In this experiment chromatographic analysis was done with 20 μl samples. The results of this experiment are presented in Figure 6.

The sensitivity of the system was determined by making various concentrations of hydrogen in nitrogen and testing 20 μl samples with the chromatograph. The lowest concentration which gave a definite and reproducible peak was 0.11% hydrogen in nitrogen.

The conclusions which can be reached from these experiments are that use of a chromatograph with thermal conductivity detectors with nitrogen carrier gas is an effective way to determine the concentration of hydrogen in a gas sample. The response of the chromatograph is linear over a wide range of sample concentrations extending down to a concentration of 0.11%. It was also concluded that the minimum sample size which gives reproducible results and a linear response is 20 μl.

Microscopy

Cultures were routinely examined with a Nikon phase microscope with a 100X phase contrast objective. The photomicrographs were taken with the same microscope fitted with a Nikon prism reflex attachment.

Preparation of Culture Tubes with a Known Amount of Hydrogen

Several experiments were performed in which an important variable was the

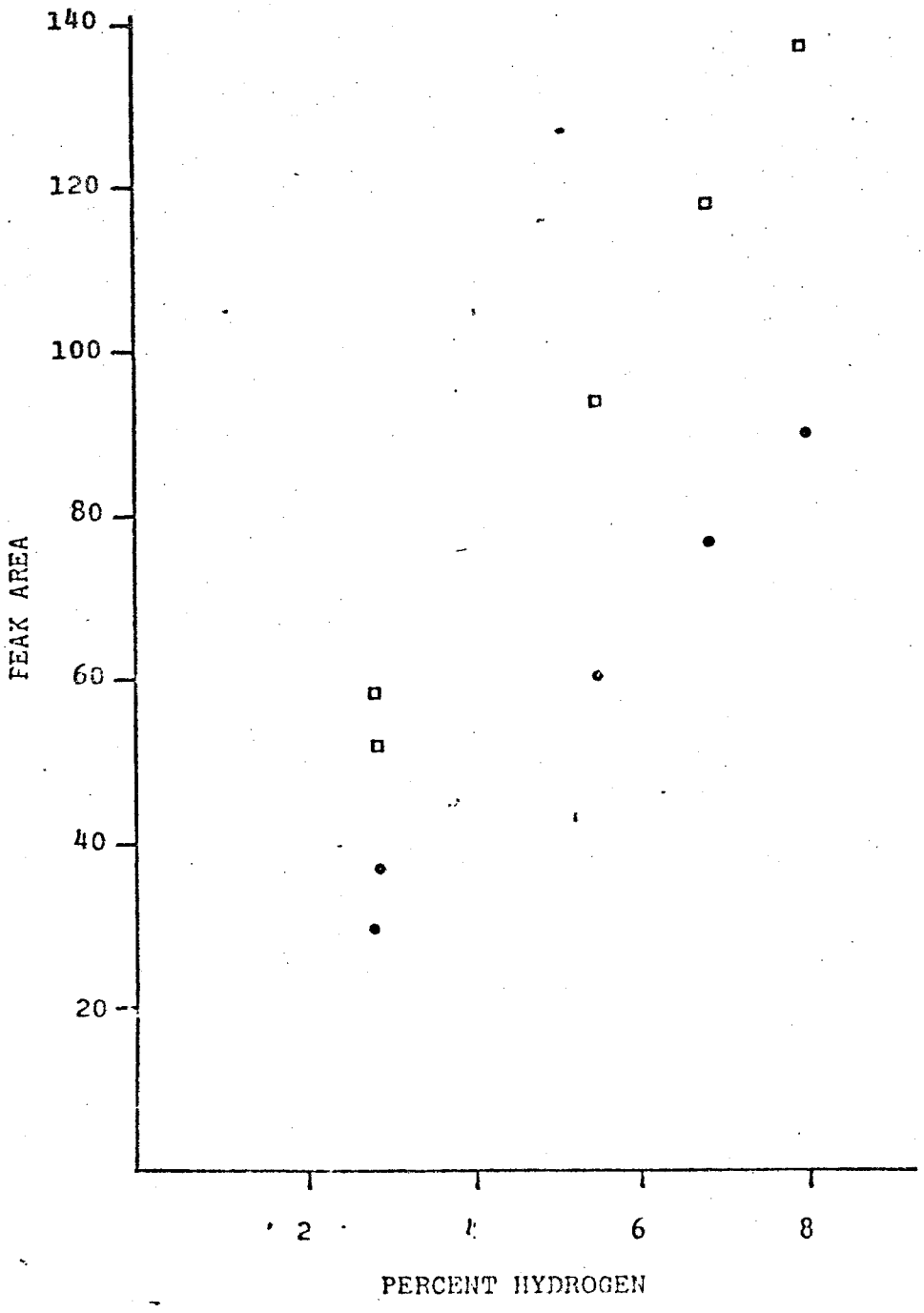

FIGURE II-5. Relationship Between Peak Area and Percent Hydrogen (by volume) in Nitrogen. 20 μl samples = ●. 30 μl samples = □.

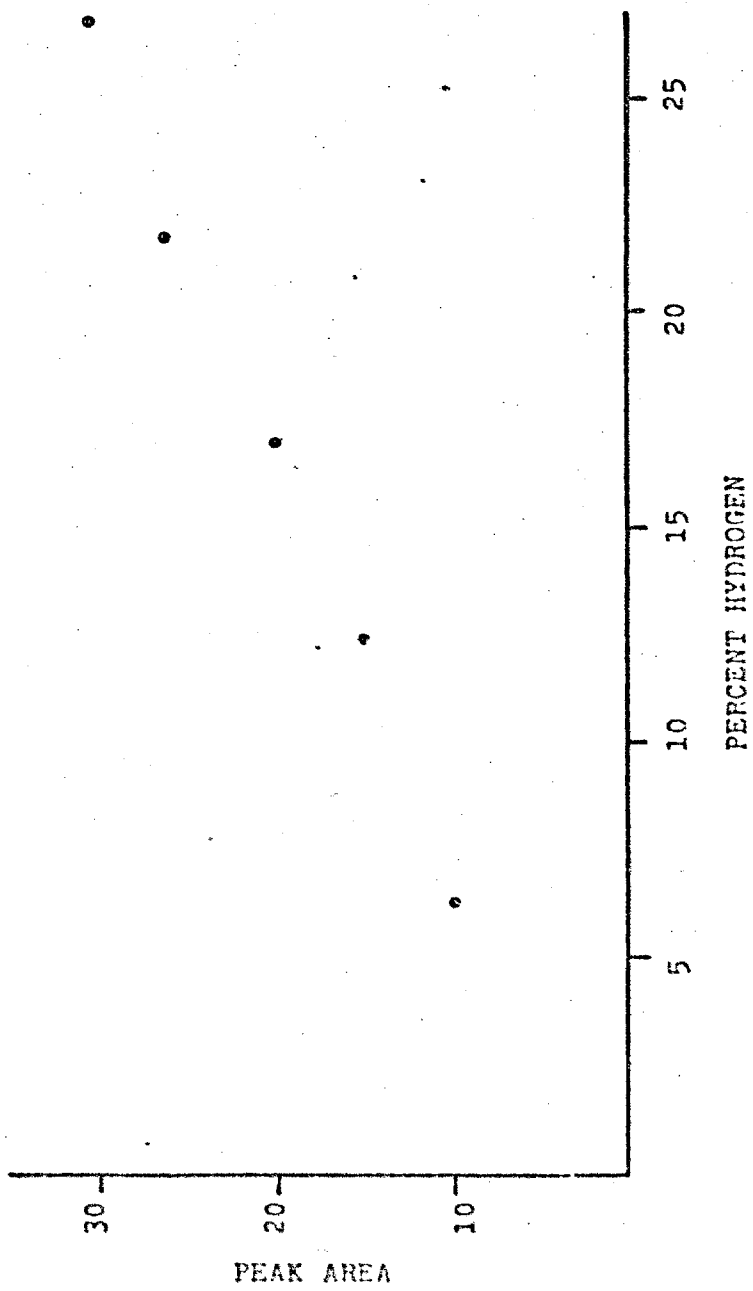

FIGURE II-6. Relationship Between Hydrogen Concentration and Peak Area. 20 μl samples.

amount of hydrogen in the atmosphere. In order to determine the percentage of hydrogen in the atmosphere, the following procedure was used. The total volume of the culture tube was determined by filling with water. Medium was added to empty tubes with a pipette, the caps were set in place, and the tubes were purged with 100% CO_2 for 1-2 minutes. Carbon dioxide dissolves slowly into the medium which results in decreased pressure inside the tube, so after about an hour CO_2 was again added. At this point a known amount of 100% hydrogen was added to each tube with a syringe. The tube was immediately equilibrated to atmospheric pressure by allowing some medium to drain out of the tube via a needle. Since some of the medium was to be expelled it was necessary to add more than was actually desired and to vary this so that all tubes would have approximately the same volume of medium and gas phase when they were inoculated. The volume of hydrogen added and the amount of medium expelled were noted so that the actual percent hydrogen in the atmosphere could be calculated.

For example, the total volume of the tubes used was 17 ml. If the desired final volume of medium was 7 ml and the hydrogen concentration desired was 5%, the tube would be set up as follows. With a final gas volume of 10 ml of which 5% is hydrogen it is necessary to add 0.5 ml hydrogen to the tube. When this is added, 0.5 ml of medium will drain out during the equilibration process. So the tube was originally set up with 7.5 ml medium, 0.5 ml hydrogen added, and 0.5 ml medium allowed to drain out for equilibration to atmospheric pressure.

After addition of hydrogen and equilibration, the reducing agent was added to the tubes and they were autoclaved. After cooling the tubes were ready to be inoculated with no further disturbance to the gas phase.

Effect of Hydrogen Concentration on Methane Production

A series of experiments was carried out in an attempt to discover the effect of initial hydrogen concentration on methane production. In all of these experiments the hydrogen concentrations were set up in the culture vessels according to the procedure previously described. The incubation temperature for all experiments was 37 C.

The first experiment was carried out in serum bottles with red rubber serum stoppers. The medium was water from Lake Erie buffered with sodium bicarbonate. No indicator or reducing agent was added. The inoculum was a mixture of water and sediment taken from Pond Lick Lake, a small lake in southeastern Ohio. The hydrogen concentrations ranged from 11% to 80% (expressed as percentage of total gas volume). In addition, two controls were set up. A sterile control had 80% hydrogen in the atmosphere and no inoculum. Another control allowed for measurement of endogenous methane production. It had cells but no added hydrogen. There were three replicates of the controls and of each hydrogen concentration in the experimental bottles. The bottles were monitored for methane production over a two week period.

The remaining three experiments in this series were carried out in Hungate-style tubes with nutrient broth medium. A pure culture of Methanobacterium ruminantium was used as the inoculum. Experiment 1 consisted of 114 tubes with hydrogen concentrations of 0% to 100% (percentage of total gas volume) with three tubes at each concentration. Methane production was monitored over a period of 12 days. Experiment 2 was essentially a repetition of Experiment 1. Experiment 3 consisted of 30 tubes with hydrogen concentrations of 27.5% to 80% (percentage of total gas volume) with three tubes at each concentration. Methane production was monitored over a period of 90 hours.

Hydrogen Depletion Experiments

An attempt was made to determine how much of the hydrogen available to batch cultures could be used by the cells. In flask cultures which had been monitored for methane production over a period of time it was possible to tell when methane production had leveled off. At this point the total amount of methane in the flask was determined by gas chromatography. Roberton and Wolfe (37) reported that 3.7 moles of hydrogen are needed for each mole of methane generated. They believed that the theoretical value of 4 was not reached because a small amount of reducing power was obtained from the medium. Using the value 3.7 an estimate of the amount of hydrogen consumed could be calculated. This calculated value was verified by measuring hydrogen depletion in a totally different way. A pressure gauge was introduced into the flask and the pressure measured. This figure could also be used as a basis for the calculation of hydrogen depletion. Unfortunately when the pressure gauge was introduced into the flask, the air inside the gauge entered the flask and caused the medium to be oxidized. Thus no further experiments could be done with that particular culture.

Using these calculations there was an indication that methane production had ceased because all hydrogen in the flask had been consumed. In an attempt to verify this, two cultures of Methanobacterium ruminantium were chosen in which methane production had reached its maximal level. At this point H_2:CC_2 (80:20) was added to each flask and methane production was monitored over a period of time with the gas chromatograph.

In an attempt to achieve better analysis, a series of experiments was performed in which both methane production and hydrogen depletion were monitored by gas chromatography. These experiments were done in 250 ml Erlenmeyer flasks capped with modified rubber stoppers to allow for gas sampling. The

flasks contained 100 ml growth medium and were inoculated with pure cultures of methanogenic organisms. The atmosphere in the flasks at the beginning of the experiment was H_2:CO_2 (80:20). When the hydrogen had decreased to a level below the sensitivity of the chromatograph, H_2:CO_2 (80:20) was added to atmospheric pressure as measured by the pressure gauge. The flask was then retested with the chromatograph to determine the exact percentage of hydrogen in the atmosphere. Methane was also quantitated by chromatography. The culture was then reincubated and the whole process repeated when the hydrogen was again depleted. This experiment was done with two flasks each of M.O.H., Methanobacterium ruminantium, Methanospirillum hungatii, and Methanosarcina barkeri.

Cell Number vs Methane Production

An experiment was performed with Methanobacterium ruminantium to determine if there was a direct relationship between the number of methanogenic cells and the amount of methane produced in a given period of time. Hungate tubes containing 9 ml of nutrient broth medium were the culture vessels used in this experiment. The first tube was inoculated with 0.1cc of a growing culture of M. ruminantium. This tube was well mixed, 1cc was withdrawn, and added to the second tube. The procedure was repeated with 8 tubes to give a dilution series of 10^{-2} and 10^{-9} of the original culture. All dilutions were done anaerobically with sterile 1 ml tuberculin syringes. The tubes were then purged for 30 seconds with H_2:CO_2 (80:20) and incubated at 37 C. The H_2:CO_2 was delivered through a sterile Swinney filter apparatus fitted with a 0.45 μm Millipore filter. The tubes were sampled for methane at 48 and 72 hours of incubation.

Rate of Methane Production

Pure cultures of methanogenic bacteria were grown in 250 ml Erlenmeyer flasks with rubber stoppers modified to allow access to the contents. The flasks contained 100 ml of growth medium with an atmosphere of H_2:CO_2 (80:20). The flasks were placed on a rotary shaker at 37 C. The flasks were sampled routinely for methane and for cell number.

Three of the species of methanogenic bacteria studied produce clumps when grown in this manner. Methanospirillum hungatii cells do not clump together but grow as very long chains of cells. It is necessary to disrupt these cell aggregates in order to obtain accurate cell counts. It was therefore necessary to find a method of disrupting the cell clumps without breaking the cells. Sonication was tried but this resulted in a decrease in cell counts by about half. A method which proved to be successful was disruption of the clumps with a tissue homogenizer. Counts made before and after this treatment were essentially the same. These before and after counts were done with young cultures in which the clumping affect was not yet noticeable.

The procedure for cell counting then was as follows. One ml of the culture was withdrawn from the flask with a sterile 1cc syringe fitted with a sterile 21 G, 1½ inch needle. The needle was large enough to allow all clumps to enter and the sample size of 1 ml was sufficient to get a representative sample of the culture. This sample was then stirred with the tissue homogenizer for approximately 30 seconds. At this point the clumps were no longer visible. A sample of this suspension was then counted with a Petroff-Hauser cell counter. In no case were fewer than 100 squares counted and most of the time all 400 squares on the grid were counted. An average of the number of cells per square was used to calculate the number of cells per ml in the culture.

Methane was analyzed by gas chromatography. At each sampling a standard of 315 ppm methane in nitrogen was run and peak areas of the culture were compared to those of the standard.

In the beginning of the growth curve, the amount of methane in the culture flask could be directly compared to the standard by analyzing both at the same attenuation settings on the chromatograph. Methane in the flask soon became so concentrated that it could no longer be directly compared to the standard. To solve this problem, a sample of the gas in the culture was diluted in a known gas volume and this diluted sample was injected into the chromatograph and compared to the standard.

In actual practice all samples injected into the chromatograph were 20 μl. When dilution was necessary, either 20, 40, or 70 μl were withdrawn from the culture and placed in a 7 ml vial containing H_2:CO_2 (80:20). The vial was fitted with a butyl rubber serum stopper and a cap with a hole drilled in the center. This vial was able to hold a pressure of 5 pounds as measured with a pressure gauge without leaking. Originally two independent dilutions were made at each reading. However, both gave essentially the same results and since the second sampling resulted in extra stress on the culture, this practice was discontinued.

The experiment and analysis was performed for 3 flasks each of M. ruminantium, M. barkeri, and M.O.H. This was done twice using a different inoculum for the second set of three flasks. The experiment and analysis was performed twice for M. hungatii. The first flask had Methanospirillum medium and the second had growth medium. This organism can use the formate in Methanospirillum medium as a substrate for methane production. Since the rates for the other organisms were obtained with hydrogen as the energy source, it was desirable to obtain a rate for M. hungatii under similar conditions. There was also an

attempt to obtain this information for the organisms isolated from Lake Erie. The data was collected using the same methods as for all other organisms. Unfortunately this organism grows rather poorly in liquid media and so the analysis of the data had to be done in a slightly different fashion.

RESULTS OF EXPERIMENTATION

Development of Anaerobic Techniques

The development of techniques for the growth of strict anaerobes is a long and frustrating process. Yet if one wishes to work with methanogenic organisms in pure culture, it is absolutely necessary first to develop good anaerobic methods. It is instructive to briefly review here this developmental process. In retrospect it is easy to explain the failures at certain points and to see the mistakes which were being made. Yet the knowledge necessary for this understanding was gained only through this process of trial and error.

Mixed cultures producing methane had been cultivated in this laboratory for quite some time in serum bottles with mud and water from Lake Erie (27). These serum bottles were capped with red rubber stoppers and had an atmosphere of 95% N_2:5% CO_2. These were mixed cultures containing the organisms normally present in the natural aquatic environment. The step from a mixed culture to a pure culture is very critical with respect to anaerobic methods. As long as a few facultative heterotrophic organisms are present in the culture, they will consume the oxygen and reduce the redox potential to a point where the methanogenic organisms will be able to survive. Once these contaminating organisms are removed, the anaerobic methods employed must be much better.

The first attempts at isolation involved streaking enrichment cultures from lake sediment containing bottles onto agar plates and incubating them in an anaerobic environment. It was recognized early that GasPak generators would not be good enough to allow growth of methane producers. An anaerobic incubator in which the atmosphere was evacuated with a vacuum pump and filled with N_2:CO_2 (95:5) three times was tried as an anaerobic container. After incubation, many colonies appeared; however, it was difficult to determine if

any of these were producing methane since it was impossible to analyze the atmosphere in the incubator for methane.

This problem was solved by modifying an anaerobic jar. A plexiglass lid was cut for the jar and fitted with a rubber gasket so that it would be air-tight. This lid had two holes drilled in it into which red rubber serum stoppers were fitted. The jar could then be filled with H_2:CO_2 (80:20) via a needle and purged to remove oxygen. Agar plates streaked with methanogenic cultures were placed in this jar. Samples of the atmosphere in the jar were withdrawn from time to time and tested for methane. Methane was present but it was unknown which colony on which plate was producing this methane. The only way to determine this was to systematically pick every isolated colony and inoculate it in a bottle where the methane generated by those organisms could be trapped for analysis. This proved to be unsuccessful.

It was believed that this failure was due to the organisms in these isolated colonies being exposed to lethal amounts of oxygen during the process of picking colonies off of the plate since the anaerobic jar had to be opened to do this. At this point a glove bag was obtained with the intention of picking colonies in it. At first this glove bag was filled with N_2:CO_2 (95:5) but when a methylene blue indicator turned blue, it was concluded that there was too much oxygen in the gas tank. To eliminate this a trap of pyrogallol and NaOH was constructed and the gas was bubbled through it. Considerable oxygen could get through this trap and it proved unworkable in several ways. The whole idea of using the glove bag was abandoned.

At this point the development of rigorous anaerobic techniques was undertaken. Some guidance was available in the literature but there is a vast difference between reading about something and actually doing it. Thus what would seem to be the simplest and most straight-forward technique and/or

manipulation often required hours, days, or weeks for mastery. Eventually this process led to the methods described in the Materials and Methods section.

The growth of anaerobes involves two main concerns. The first is to obtain a suitably reduced medium and an oxygen-free environment. The second is to maintain these conditions during the growth of the organisms.

The first method tried to obtain reduced conditions was boiling the medium. Holdeman and Moore (16) indicate that boiling alone will reduce media (as judged by resazurin indicator). Bryant (4) and Hungate (22) boil media to drive off oxygen before dispensing in tubes. In actual practice after approximately 30 minutes of boiling with a chimney under a stream of oxygen-free carbon dioxide as suggested by Moore (30), the resazurin in the nutrient broth medium indicated a reduced condition. Unfortunately it was not possible to maintain this condition during dispensing of the medium.

It is also possible to obtain reduced conditions by biological means. Smith (42) suggested that growth of Escherichia coli would lower the redox potential of a medium enough to allow methane producers to grow. E. coli cultures did reduce the medium as judged by the redox indicator. The problem then became to kill the E. coli without oxidizing the medium. There were some problems with this but eventually it was possible to autoclave cultures without overly oxidizing them. However, a problem remained in that the dead cells were still in the medium. Thus when a culture was examined microscopically it was difficult to know whether it had become contaminated or whether the cells seen were just dead E. coli.

The method which was finally settled upon was reducing the medium chemically. The cysteine-sulfide reducing agent described by Bryant and Robinson (7) has proved very reliable. Not only is the problem of the dead cells gone but the reducing agent has some capacity to remove traces of oxygen introduced

into the tube during transfer or sampling. This latter characteristic is a distinct advantage over reduction by biological means.

The second major concern in the cultivation of anaerobes is maintaining reduced and oxygen-free conditions. In large part this is a matter of care in transfer and sampling but it is also necessary to have an appropriate culture vessel. In relation to this it should be pointed out that red rubber stoppers are permeable to oxygen and cause reoxidation of the medium. This fact was pointed out by Hungate (20) but is rarely mentioned in the anaerobic literature. Most articles on methods or techniques do, however, call for the use of butyl rubber stoppers. These stoppers are usually black or gray which indicates that although it is not mentioned, most people working with strict anaerobes discriminate against red rubber stoppers.

Attempts at Differentiation of Methanogenic Colonies

Methanogenic bacteria are generally found in habitats where there is a great variety of different organisms. This is a contributing factor to the difficulty of the isolation process. One is frequently faced with the situation of a bottle or tube which has methane in the atmosphere and a variety of organisms growing on the agar surface. Obviously the methane was produced by organisms growing in that culture. But which colony or which part of the zone of confluent growth contains the methanogenic organisms? One is faced with the prospect of picking each colony and placing it in either broth or agar and waiting to see if methane is generated. This is a very time consuming and difficult process. If there were some way to differentiate these organisms from contaminants on the agar surface, the task would be easier. Two approaches were tried at solving this problem. Unfortunately neither proved to be successful.

Methanogenic organisms produce a product, methane, which is a gas. If this gas could be trapped in the form of a gas bubble, it would be a convenient way to pinpoint the colony of interest. At the time that this was under consideration, no methane producing cultures were available. As a test Enterobacter aerogenes was cultivated in a fermentation medium containing glucose so that considerable amounts of carbon dioxide would be generated.

It was hoped that the carbon dioxide produced by each colony would be caught and trapped in the agar. To accomplish this a dilution series was made in agar tubes which were then rolled in an ice bath so that the agar solidified as a thin layer along the sides of the tube. Some bubbles were observed after incubation but all colonies were not associated with gas bubbles. Also the gas bubbles moved around in the tube so that a bubble might be over one colony at one time and over another colony half an hour later. It was concluded that this would not be a good way to differentiate methanogenic organisms from other types of bacteria.

Cheeseman et al. (9) reported that methanogenic bacteria contain a compound which fluoresces when excited by ultraviolet light. It was thought that perhaps it might be possible to detect methanogenic colonies by taking advantage of this property. In fact Edwards and McBride (13) have recently reported that it is possible to identify methanogenic colonies in this way. In order to do this they used an anaerobic chamber within a glove box.

Since this requires specialized and expensive equipment an attempt was made to design a system which would eliminate these problems. Ultraviolet light can pass through plexiglass so a culture chamber of this material was constructed. This is illustrated in Figure 1. After some initial problems getting an airtight seal on the chamber, methanogenic cultures were grown in this chamber. Unfortunately it was not possible to see fluorescence of the

colonies in these chambers.

A hand-held UV light did not provide enough illumination to really see any fluorescence from the colonies on the agar surface. A more powerful UV lamp gave so much illumination that any fluorescence from the colonies was washed out. If an intermediate between these two could be found, this might yet prove to be a means of differentiating methanogenic colonies.

Characterization of Isolate

A methane producing organism was isolated from sediment obtained from Lake Erie. Since this was accomplished by carrying out a series of dilutions, it is probable that this organism was the most numerous methanogenic organism in the sediment at that time. It was a 10^{-5} dilution of sediment from which this organism was ultimately obtained. It is unlikely that a methanogenic organism present in numbers lower than this isolate would have been recovered by the isolation process. Once the appearance of this organism was known, it could be recognized in virtually all tubes in the direct line to this isolate. Also at the end of the process many tubes which had large amounts of methane were examined microscopically even if they were not in a dilution series which led to this isolate. In all cases this organism was present.

There are only a limited number of species of methanogenic organisms which are divided into four main groups on the basis of morphology. Phase contrast microscopy of this organism showed that it is definitely of the bacillus type. It is a long, thin, frequently curved or twisted rod which often occurs in long chains. Morphologically it is similar to Methanobacterium M.O.H. It is probable that these organisms are the same species. Bryant (5) considers M.O.H. to be a variant of M. formicicum which is unable to use formate as a substrate for growth and methane production.

The results of the substrate utilization studies are presented in Table 1. Although there is some variation among the three replicates it can be seen that the best substrate for methane generation is hydrogen. Methane production on nutrient broth with 100% CO_2 is very low and methanogenesis with formate present is only slightly better. There was some concern that the pH of the medium might be lowered with formate present and with an atmosphere of 100% CO_2. At the end of the incubation the pH was checked. These results are also presented in Table 1. The pH was approximately the same for all tubes.

In addition to substrates utilized for methane generation, another feature used to differentiate species is temperature optimum. The results of the temperature studies are presented in Table 2. It is quite clear that the optimum temperature for methane generation by the isolate is in the range 30-37 C.

Effect of Hydrogen Concentration on Methane Production

The first experiment was conducted in an attempt to determine whether the addition of hydroger would be a stimulus to methane production by a natural population. A mixture of water and sediment taken from a small lake was used as the inoculum. This mixture would have all organisms normally present in both the lake water and the sediment. The medium used was water taken from Lake Erie which had been buffered with sodium bicarbonate and autoclaved. The culture vessels used in this experiment were serum bottles capped with red rubber stoppers. Concentrations of hydrogen were added at levels of 11-80% (v/v). A sterile control had 80% hydrogen but no inoculum. Endogenous levels of methane generation were obtained from bottles with inoculum but no added hydrogen.

TABLE II-1. Substrate Utilization by Lake Erie Isolate

Medium	Methane (Relative Units) Day 2	Day 24	Final pH
NB, No Additions	7	29	7.1
100% CO_2	18	24	7.0
	27	34	6.9
NB + Formate	13	34	6.8
100% CO_2	44	72	6.85
	55	88	6.8
NB, No Additions	24	164	6.95
H_2:CO_2 (80:20)	49	414	7.0
	12	115	6.9

Methane produced after 2 and 24 days of incubation at 37 C. The medium is nutrient broth medium (NB) with and without added formate. The atmosphere above the culture was either 100% carbon dioxide or hydrogen: carbon dioxide (80:20). There were three replicates for each set of conditions. The relative units for methane are related to peak height and are the same for all measurements.

TABLE II-2. Effect of Incubation Temperature on Methane Production

Temperature C	Day 2	Day 15
4	22.6	22.6
15	38.4	25.2
23	28.0	28.6
30	49.6	405.9
37	54.3	304.2
45	50.7	32.8

Lake Erie isolate after 2 and 15 days of incubation. Average methane generation expressed in mmoles.

The bottles were checked for methane after 24 hours of incubation (Figure 9) and at the end of two weeks (Figure 10). At the end of the experiment the pH was checked in all bottles. The range was from 6.75 to 7.6.

At the 24 hour point there does not seem to have been any stimulation of methane production with increasing hydrogen concentration. The amount of methane detected in the sterile control is really very little and might be due either to methane in the atmosphere contaminating the syringe or more probably to release of some methane from the lake water. Even the lowest amount of hydrogen used stimulated methane production over the endogenous levels. In this short period of time it would seem that any amount of hydrogen is to some extent stimulatory.

The situation at two weeks is somewhat confusing. All hydrogen bottles had much more methane than the endogenous control and the sterile control. Both controls remained at essentially the 24 hour level of methane. The units for methane in Figure 10 are 1000 times greater than those in Figure 9 so it can easily be seen that during this two week period there were very great increases in the amount of methane in nearly all bottles. There are, however, large discrepancies in the amount of methane produced in bottles with the same amount of hydrogen. This may be due to the fact that the inoculum for these bottles was a slurry of sediment and water. It would be easy to get an uneven distribution of organisms in such a situation. The amount of methane produced in the bottles may be a function of the number of methanogenic organisms present rather than the amount of hydrogen present. The number of facultative organisms present may also be a determining factor. Since no reducing agent was used in these bottles, it was the facultative organisms which lowered the redox potential to a point where the methanogenic organisms could survive.

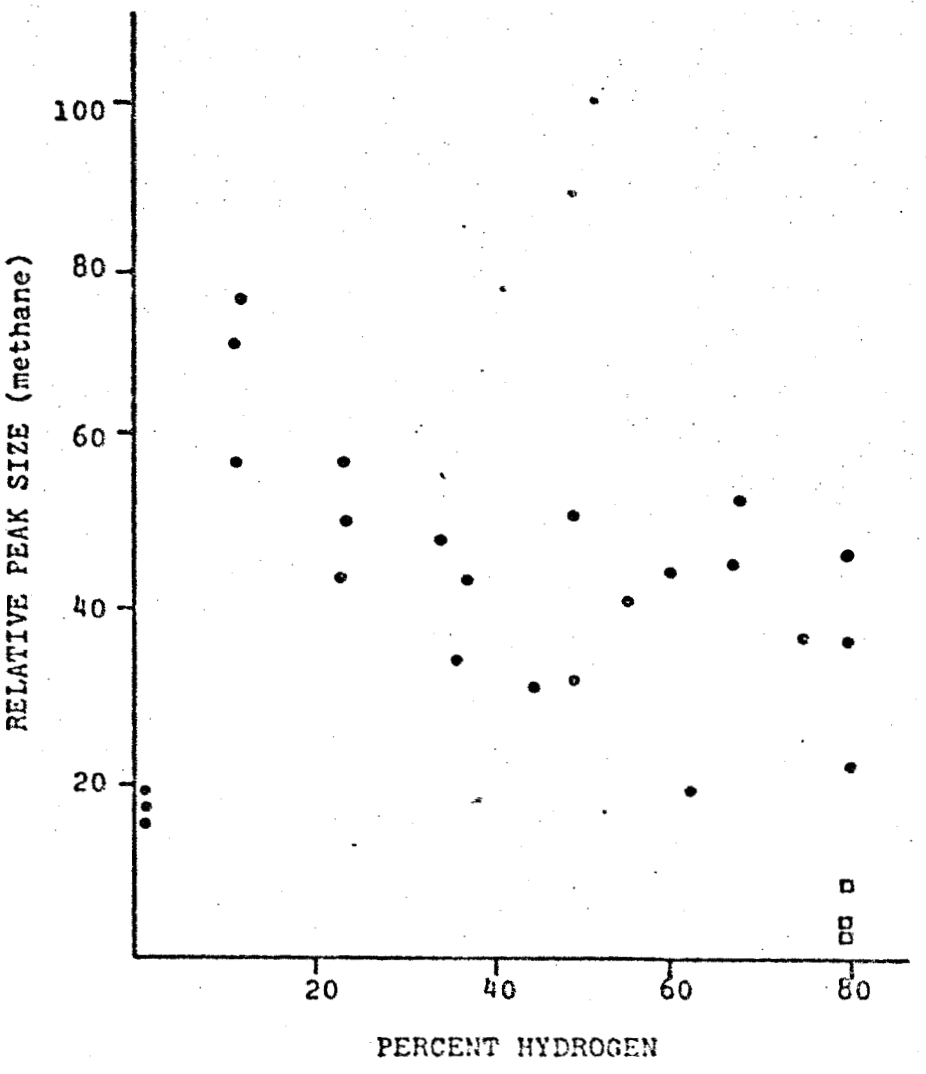

FIGURE II-9. Hydrogen Concentration vs Methane Produced
Mixed culture from sediment.
24 hours of incubation.
□ = sterile control.

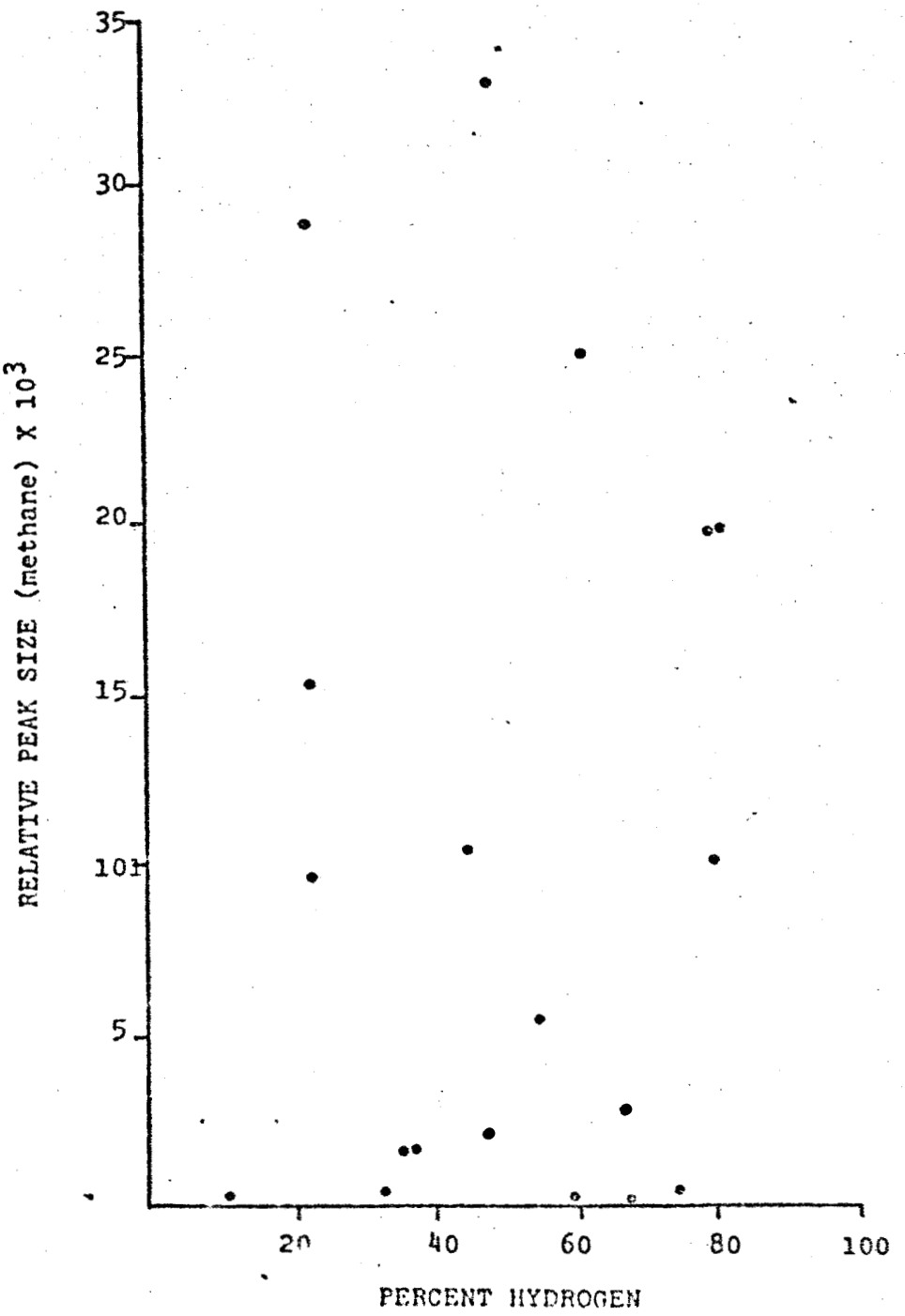

FIGURE II-10. Hydrogen Concentration vs Methane Produced.
Mixed culture from sediment.
2 weeks of incubation.

A rough estimate of the amount of methane produced (in μmoles) for each bottle indicates that in no case was the potential for methane production realized. One bottle which had approximately 23% hydrogen produced a very large amount of methane. But this was still only half of the methane which would have been possible if all available hydrogen had been converted to methane. Two factors, however, prevent one from forming any firm conclusions in this regard. First there is a possibility that some of the methane produced was being oxidized. These were mixed cultures and methane-oxidizing bacteria were certainly present in the system. Also it was discovered after the conclusion of the experiment that the red rubber stoppers used to close the bottles were not an effective barrier to hydrogen. Thus there is a good possibility that some of the nydrogen added to the bottles was unavailable to the methane producers due to leakage.

To better control the variables, a second experiment was set up using a pure culture of Methanobacterium ruminantium. The hydrogen concentrations used varied from 0-100% with 0.1% increments in the range 0-1%; 1% increments in the range 1-10%; and approximately 5% increments in the range 10-100%.

The lowest amount of hydrogen added to any tube was 10 μl. Using Henry's law to calculate the solubility of a gas in a liquid, this amounts to a hydrogen concentration of 0.786×10^{-6} molar. This is close to the Km value of 1×10^{-6} molar calculated by Hungate et al. (23). At this concentration less than half of the rate limiting enzyme for the conversion of hydrogen to methane should be saturated. If this Km value is correct, the methanogenic organisms should be able to convert even this low level of a ded hydrogen to methane. Increasing amounts of hydrogen above this level should cause an increase in the rate of methane generation.

If the organisms convert all 10 ml of the hydrogen to methane, the yield of methane would be approximately 2.70 µl. This figure is based on a value of 3.7 moles hydrogen needed for each mole methane generated (37). This amount of methane should be reliably detected with the gas chromatographic methods used.

Thus this experiment could theoretically give information about the effect of increasing hydrogen concentration on the rate of methane production. On the basis of the information available methane production should increase with increasing hydrogen concentrations up to a point at which the hydrogen would be saturating and some other variable, probably numbers of methane producers, would limit the reaction.

After one week of incubation the amount of methane in all tubes was small and essentially the same. The results of the samples taken on day 12 are presented in Figure 11. Many of the points in the 0-10% range are not plotted since there were many tubes and all were essentially the same. There seems to be an increase in methane in the range 40-60% hydrogen. There was, however, some doubt about the validity of these results due to the lag period which was much longer than normal and may indicate that there was a problem either with the cells in the inoculum or with the medium. In any case the experiment was repeated.

The set up of the second experiment was essentially the same as the first. The results of the sampling done at 48 hours of incubation are presented in Figure 12. Many points in the 0-10% range are not plotted since there were many tubes and all were essentially the same. There is an increase in methane produced at about the 30% level of hydrogen. This trend was also seen at the 24 hour and 72 hour sampling. Contrary to the original expectation, all the tubes with less than 20% hydrogen had about the same amount of methane at 24 hours and this situation did not change very much upon 72 hours of incubation.

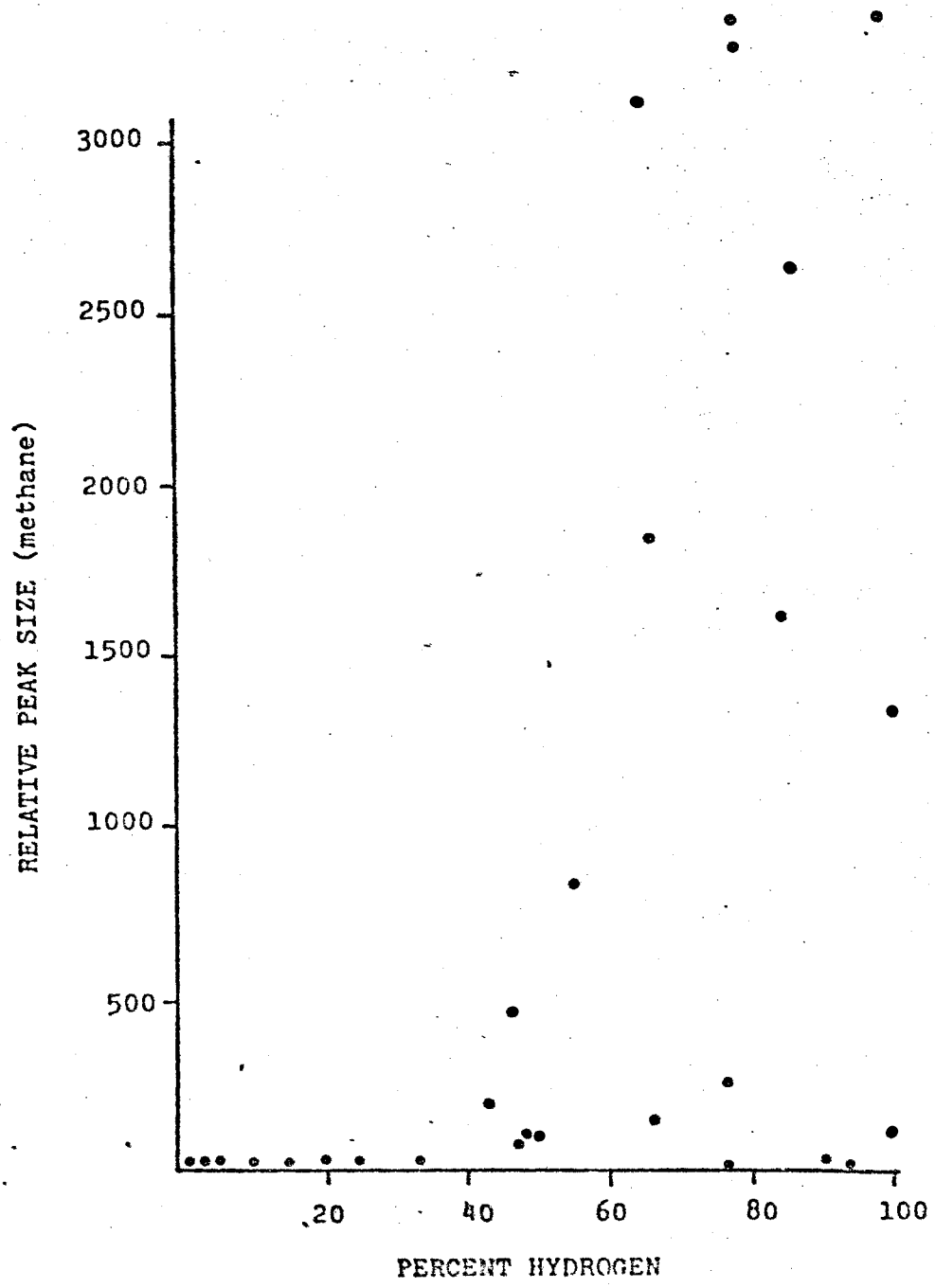

FIGURE II-11. Hydrogen Concentration vs Methane Produced by M. ruminantium After 12 Days of Incubation

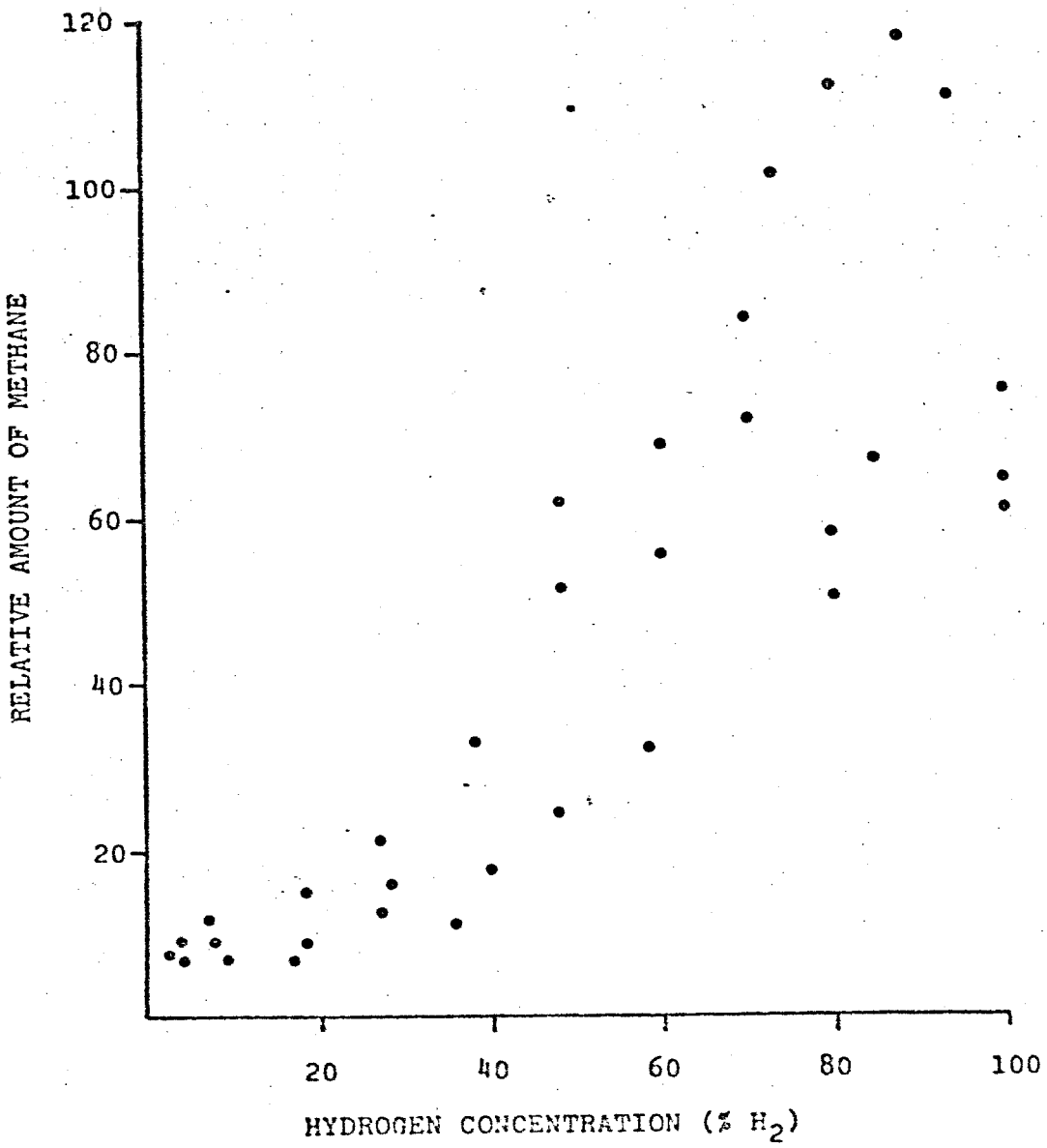

FIGURE II-12. Hydrogen Concentration vs Methane Produced by M. ruminantium After 48 Hours of Incubation
Relative amount of methane units are related to peak size.

The methane produced at these low levels of hydrogen was not very different from controls with no added hydrogen. Also the amount of methane was much smaller than would be expected if all the hydrogen added had been utilized for methane production.

On the basis of these results a third experiment was set up in which hydrogen concentration varied over a narrower range of 27.5-80%. The results obtained after 90 hours of incubation are presented in Figure 13. There is no increase in amount of methane generated with increasing concentrations of hydrogen. These cultures were also checked at 20 hours and 36 hours with the same results. The amount of methane in each culture increased with time but there was no apparent relationship between methane production and hydrogen concentration. It should also be pointed out that in no case did the amount of methane produced approach that theoretically possible with the amount of hydrogen provided. The results of this experiment then seem to contradict those obtained in the previous experiment. On the basis of that experiment one would expect a rise in methane production somewhere in the range of hydrogen concentrations provided.

With regard to the original question of response of the cultures to low amounts of hydrogen, at least some tentative conclusions can be made. First, the results of these experiments disagree with those of Czerkawski et al. (12). Hydrogen does not appear to have been converted to methane as fast as it could diffuse into the media. If this were the case, there should have been some difference in the amount of methane produced at different hydrogen concentrations. Also, if Hungate et al. (23) were right in their calculation of the Km for the conversion of hydrogen to methane, there should have been an increase in the rate of methanogenesis (and therefore the amount of methane generated in a given time) in the range of hydrogen concentrations 0.1-10%. On the

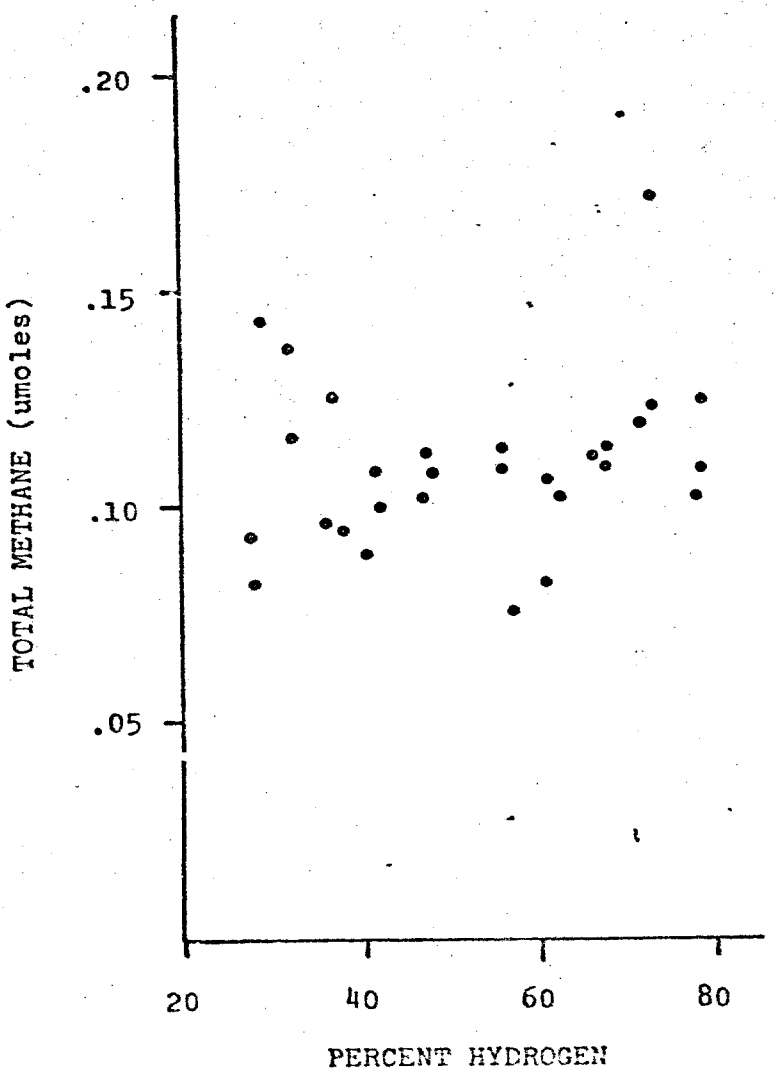

FIGURE II-13. Hydrogen Concentration vs Methane Produced by *M. ruminantium*--90 Hours of Incubation

basis of dissolved hydrogen this range extends from a value close to the Km to 100 times the Km.

There is one major difference between the experiments reported here and those done previously which may bear on the differing results obtained. The work done by Czerkawski et al. (12) and Hungate and coworkers (21, 23) was with rumen contents which have a very large population of methanogenic bacteria. Smith and Hungate (44) have reported counts as high as 2×10^8 methanogenic bacteria per ml of rumen contents. The results obtained here must be interpreted in light of the fact that the numbers in these cultures were much lower. Because of these uncertainties the question of hydrogen utilization by methanogenic bacteria was approached in a totally different way.

Hydrogen Depletion Experiments

In flask cultures which were monitored for methane over a period of time it was possible to tell when methane production had leveled off. The amount of methane present at this time was determined by gas chromatography. Using this value one can obtain an estimate of the amount of hydrogen consumed. It was also possible to measure hydrogen depletion by pressure changes inside the flask. Since 3.7 moles of hydrogen are consumed for every mole of methane generated (37), the pressure inside the flask drops during the growth of a culture. The results of these measurements and calculations are presented in Table 3.

At the time of inoculation each flask had a gas volume of 160 ml. This was filled with 80% hydrogen which would give a value of 5.12 mmoles hydrogen or 128 ml hydrogen (at 1 atm). In actual practice when the flasks were purged with hydrogen: carbon dioxide, the exit needle was withdrawn first. This caused the flasks to have a slight positive pressure. So in actual fact the

TABLE II-3. Methane Production and Hydrogen Consumption and Resulting Pressure Change

	mmoles CH_4	mmoles H_2 Used	ml CH_4	ml H_2	Press Calc	Press Real
MS1	1.26	4.66	31.5	116.5		
MS2	1.51	5.59	37.7	139.7		
MR1	1.08	4.00	27.0	100.0		
MR2	1.05	3.88	26.3	97.0	.56	.33
MR3	1.15	4.25	28.7	106.4	.52	.30
MR4	1.05	3.88	26.3	97.0	.56	.33
MR5	1.46	5.40	36.5	135.0	.38	.30
MOH	1.31	4.85	32.7	121.2	.45	.40

Shake flask cultures with 100 ml medium and 160 ml gas.
MS = M. barkeri
MR = M. ruminantium
MOH= M.O.H.

flasks probably contained somewhat more hydrogen than the above values indicate. It can be seen from Table 3 that in most cases a very large percent (if not all) of the hydrogen available was converted to methane. The values in the table for volume methane produced and hydrogen consumed are calculated on the basis of 1 atm pressure. In reality the gases expand to fill all 160 ml available in the flask causing the pressure to drop. There is a reasonable agreement between the pressure actually measured and that calculated.

When stationary phase is reached, the amount of methane present in the flasks is approximately the same for the three species of methane producers. Furthermore, this amount of methane accounts for essentially all of the hydrogen initially present in the flask. This suggests that the cells stop producing methane because of hydrogen depletion.

To test this hypothesis, flasks of M. ruminantium were inoculated and placed on a shaker. The methane was monitored over time with a gas chromatograph (Figure 14). It can be seen that at about 140 hours the methane curve for flask C begins to level off and that approximately the same amount of methane is present in flask A. The amount of methane present at 165 hours represents the consumption of essentially all the hydrogen in the flasks. At 166 hours the hydrogen in the flasks was replenished.

After the addition of hydrogen the amount of methane in the flask was again monitored over time. Unfortunately during the addition of hydrogen, some gas escaped from flask A resulting in a drop in total methane at hour 169.

As can be seen in Figure 14 there is a rapid increase in the amount of methane in both flasks. This is to be expected considering the fact that a large population of methanogenic orgarisms is present in both flasks. It can also be seen that methane is being produced logarithmically once again.

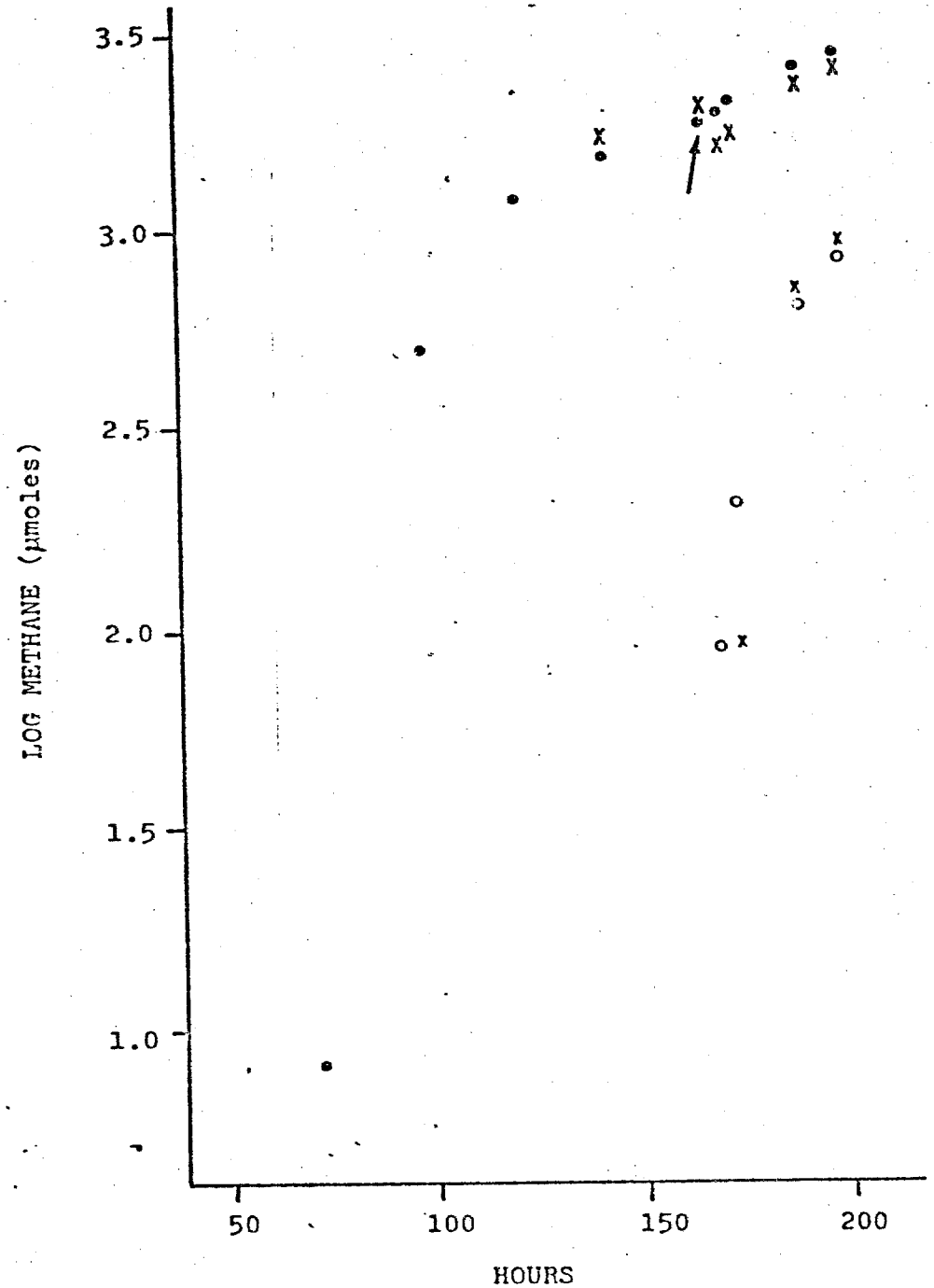

FIGURE II-14. Methane Production by M. ruminantium. Flask A = X. Flask C = •. Hydrogen added at arrow. "New" methane produced after hydrogen added indicated by x = Flask A ; o = Flask C.

Since there are so many cells present in the flask at this time, the hydrogen is consumed very rapidly. As can be seen from Figure 14, after 120 hours of growth the organisms in flask C had produced a total of 1.17 mmoles of methane. Thirty hours after the addition of hydrogen culture C had produced 0.90 mmoles of methane. It would thus appear that cultures which have stopped producing methane have done so because of hydrogen depletion and that if hydrogen is replenished, these cells are capable of rapidly converting it into methane.

There still remained, however, several questions about hydrogen utilization. How many times would the cells consume the hydrogen given to them? Could the cells really use all the available hydrogen as data from Table 3 would suggest? Is there some level at which methane would become inhibitory?

In an attempt to answer these questions, the experiment with the flasks of M. ruminantium was continued. The flasks were incubated until the hydrogen added had been depleted. At this point the amount of methane in the flask was determined by chromatography and hydrogen: carbon dioxide was added to a level of 1 atmosphere (as judged by the pressure gauge).

This process was repeated several times and the results are presented in Tables 4 and 5. The mmoles H_2 and mmoles CH_4 present in the flask were calculated on the basis of the volume occupied by these gases. At one atmosphere of pressure a mmole of gas occupies 25.45 ml at 37 C and 24.46 ml at 25 C. The flasks were incubated at 37 C and measured at room temperature so an intermediate value of 25 ml/mmole was chosen for the calculations presented here. In fact the difference between these values is slight. In calculating the mmoles available in a flask containing 80% hydrogen, the value is 5.03 if 25.45 ml/mmole is used and 5.23 if 24.46 ml/mmole is used. The value obtained using 25 ml/mmole is 5.12 moles. The methane theoretically generated from

TABLE II-4. Hydrogen Depletion by *M. ruminantium* Grown in Shake Flask Culture--Flask A

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
7	--	--	--	25.3	1.98 (1.62)	1.98
7	59.7	3.82	1.03			
9	--	--	--	35.8	2.60	.98
9	51.4	3.72	1.00			
10	--	--	--	48.3	3.09	.49
10	34.8	2.23	.60			
12	--	--	--	57.6	3.68	.59
12	28.4	1.82	.49			
15	--	--	--	63.2	4.04	.36
15	22.6	1.45	.39			
17	--	--	--	68.4	4.38	.34
17	19.5	1.25	.34			
19	--	--	--	83.8	5.36	.98
19	13	.83	.22			
21	--	--	--	85.7	5.48	.12
21	10	.64	.17			
23	--	--	--	88.1	5.64	.16

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

TABLE II-5. Hydrogen Depletion by M. ruminantium Grown in Shake Flask Culture - Flask C

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
7	--	--	--	28.9	1.85	1.85
7	56.9	4.36	1.18			
9	--	--	--	46.1	2.95	1.10
9	44.3	2.83	.77			
10	--	--	--	53.4	3.42	.47
10	30.4	1.95	.53			
12	--	--	--	No Accurate Data		
12	Hydrogen Added					
15	--	--	--	65.2	4.17	.75
15	24.16	1.55	.42			
17	--	--	--	72.6	4.65	.48
17	16.2	1.04	.28			
19	--	--	--	83.8	5.36	.71
19	15.6	.99	.27			
21	--	--	--	86.6	5.54	.18
21	10	.64	.17			
23	--	--	--	88.1	5.64	.10

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

any given amount of hydrogen was calculated on the basis of 3.8 mmoles hydrogen needed for each mmole methane. To illustrate the procedure and the results consider flask A of M. ruminantium (Table 4). The amount of hydrogen added on day 7 (166 hours) was calculated on the basis of available volume not filled with methane or carbon dioxide. By day 9 the hydrogen in this flask had dropped below a level detectable by the chromatograph (0.11%). The methane generated in the interval from day 7 to day 9 was 0.98 mmoles. This agrees very well with the theoretical value of 1.03 mmoles calculated from the amount of hydrogen (3.82 mmoles) added on day 7.

On day 9 hydrogen was once again added to the flask to a level of zero on the pressure gauge (1 atm). By day 10 the hydrogen was reduced below a detectable level and was replenished. This was repeated on day 12, 15, 17, 19, and 21. The values given for hydrogen added on day 10, 12, 15, and 17 are calculated from available space not occupied by methane and carbon dioxide. Each time the hydrogen was depleted, the methane was measured to determine how much had been generated. In general this amount of methane produced agreed fairly well with that predicted if all added hydrogen were metabolized to methane.

Hydrogen was also added repeatedly to M. ruminantium flask C (Table 5). As with flask A, some additions of hydrogen were made without quantitating by chromatography the amount of hydrogen added. The values given in Table 5 for these cases were determined by calculations of available space.

The same experiment was done for two flasks each of the other three species of methanogenic bacteria. These results are presented in Tables 6-11. After the hydrogen in the flask had dropped to a level at or below the limit of sensitivity of the chromatograph, hydrogen was added to a pressure of zero on the pressure gauge (1 atm). Hydrogen was then rechecked with the chromatograph and peak sizes compared with a standard to determine exactly how much

TABLE II-6. Hydrogen Depletion by M.O.H. Grown in Shake Flask Culture

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
13	--	--	--	28.6	1.83	1.83
13	56.9	3.64	.98			
15	--	--	--	45.35	2.90	1.07
15	44.23	2.83	.76			
16	--	--	--	61.5	3.94	1.04

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

TABLE II-7. Hydrogen Depletion by M.O.H. Grown in Shake Flask Culture

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
13	--	--	--	26.9	1.72	1.72
13	55.5	3.55	.96			
15	--	--	--	43.8	2.80	1.08
15	45.7	2.92	.79			
16	--	--	--	62.8	4.02	1.21

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

TABLE II-8. Hydrogen Depletion by *M. barkeri* Grown in Shake Flask Culture

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
5	--	--	--	25	1.60	1.60
5	55.5	3.55	.96			
8	--	--	--	47.3	3.03	1.43
8	45.5	2.91	.78			
11	--	--	--	51.8	3.32	.29
11	39.2	2.51	.68			
16	--	--	--	64.3	4.12	.80
16	29.8	1.90	.51			
20	--	--	--	71.8	4.60	.48
20	24.0	1.54	.42			
22	--	--	--	80.1	5.12	.53

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

TABLE II-9. Hydrogen Depletion by M. barkeri Grown in Shake Flask Culture

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
5	--	--	--	25.5	1.63	1.63
5	55.5	3.55	.96			
8	--	--	--	45.45	2.91	1.28
8	51.7	3.31	.89			
11	--	--	--	47.5	3.04	.13
11	42.2	2.70	.67			
16	--	--	--	47.9	3.06	.02

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

TABLE II-10. Hydrogen Depletion by M. hungatii Grown in Shake Flask Culture

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
7	--	--	--	28.9	1.85	1.85
7	62.3	3.99	1.08			
10	--	--	--	46.5	2.98	1.13
10	47.1	3.01	.81			
13	--	--	--	53.8	3.44	.46
13	38	2.43	.66			
15	--	--	--	65.1	4.16	.72
15	30.6	1.96	.53			
16	--	--	--	74.5	4.77	.61
16	24.5	1.57	.42			
18	--	--	--	78.0	4.99	.22

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

TARIF II-11. Hydrogen Depletion by M. hungatii Grown in Shake Flask Culture

Day	$\%H_2$	mmoles H_2	CH_4 mmoles theory	$\%CH_4$	mmoles CH_4	Change in CH_4
0	80	5.12	1.38			
7	--	--	--	30.4	1.94	1.94
7	63.4	4.06	1.10			
10	--	--	--	43.3	2.77	.83
10	47.5	3.04	.82			
13	--	--	--	57.3	3.67	.90
13	37.5	2.40	.65			
15	--	--	--	68.3	4.37	.70
15	29.7	1.90	.51			
16	--	--	--	74.9	4.79	.42
16	23.8	1.52	.41			
18	--	--	--	80.0	5.12	.33

Symbol (--) indicates a hydrogen level below the limit of the chromatograph. Values for percent hydrogen and mmoles hydrogen represent what was added to the flask on that day.

hydrogen had been added. As described previously methane was also monitored and the amount produced in the intervals between hydrogen additions was calculated.

It can be seen that in all cases the amount of methane produced agrees rather well with the amount calculated from the known amount of hydrogen added. Also this data clearly show that the methanogenic organisms can repeatedly utilize any hydrogen which is made available to them. Hydrogen was added to cultures as much as nine times and each time it was removed and reduced to a concentration either at or below the limit of detectability with the gas chromatograph. This indicates that the methanogenic organisms have a tremendous affinity for their substrate, hydrogen. This is especially impressive when one considers that the pressure in the flasks was in most cases considerably less than 1 atm. At the time of the first hydrogen depletion the pressure change is the greatest because there was not very much methane in the flask. Assuming a pressure drop to 0.3 atm and a hydrogen depletion to the level of detectability of 0.11% hydrogen (v/v) in the flask, a calculation of the solubility of hydrogen gives a dissolved hydrogen concentration of 2.36 nmoles per 100 ml. It should be remembered that the Km value calculated by Hungate and coworkers (23) was 100 nmoles per 100 ml.

A final conclusion which can be drawn from these experiments is that there does not appear to be any inhibition as a result of the accumulation of methane up to the level of 88% methane in the atmosphere which was the highest level reached in these studies. With regard to this it should be pointed out that methane is not a very soluble gas in aqueous systems. With a concentration of 80% methane in the gas phase and a total pressure of 1 atm, the amount of methane dissolved in 100 ml would be 0.0915 mmoles or 2.29 ml. The one ill effect of methane accumulation is that as the percentage of methane increases,

the amount of volume occupied by hydrogen must decrease at any given pressure.

Cell Number vs Methane Production

From the studies on the relationship between initial hydrogen concentration and methane production there appeared to be a relationship between numbers of methanogenic bacteria and the amount of methane produced. In an attempt to determine just how close this relationship was, an experiment was performed in which ten-fold dilutions were made of a culture of M. ruminantium.

The results of this experiment are presented in Table 12. In most cases there is a substantial increase in the amount of methane in the interval 48 to 72 hours which indicates that during this period the cells remained viable and were respiring. There also seems to be a direct relationship between the number of cells initially present and the amount of methane generated in a given period of time. This is illustrated in Figure 15 in which the $\log_{10}$ of the amount of methane is plotted against the negative $\log_{10}$ of the dilution of the original culture. The data for this graph were taken from the 48 hour sampling.

The fact that there was a direct relationship between cell number and quantity of methane is not too surprising since methane is the end product of the respiration of these cells. The question of how much methane could be produced in a given period of time by these cells remained to be investigated.

Rate of Methane Production

The purpose of this set of experiments was to determine the rate of methane production for pure cultures of methanogenic organisms. A review of the literature shows that no investigator has attempted to calculate a rate of methane production for a given number of cells. As discussed previously, the only available data suggest a rate of 6×10^{-9} μmoles methane per hour per cell for M.O.H.

TABLE II-12. Relationship Between Number of Methanogenic Cells and Amount of Methane Produced

Negative Log Dilution	Relative Amount of Methane 48 hr	72 hr
2	68,000	250,000
3	2,200	4,200
4	140	260
5	63	300
6	14	28
7	14	34
8	9	22
9	12	38

M. ruminantium test tube cultures incubated at 37 C. Units for relative amount of methane represent peak height and are the same units in all cases.

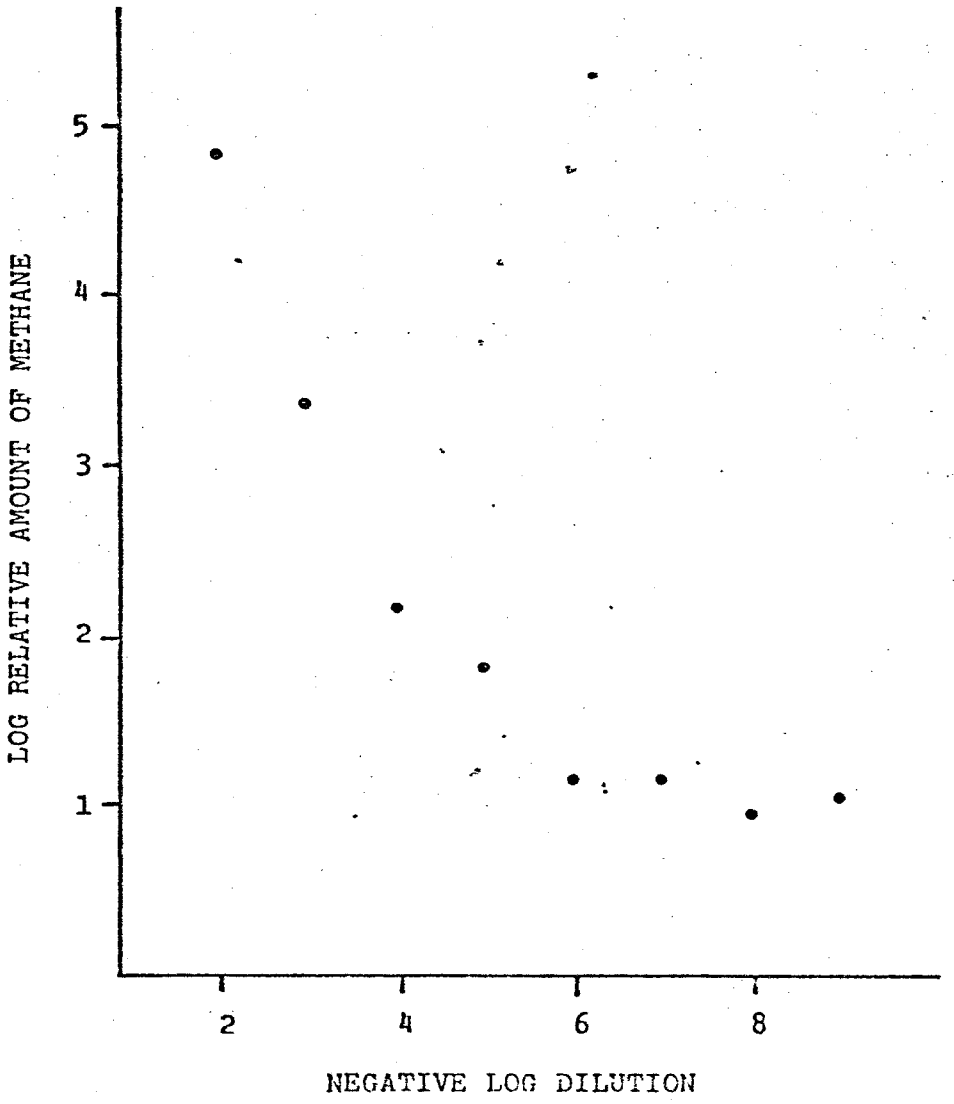

FIGURE II-15. Methane Production vs Dilution of M. ruminantium--48 hours Units for relative amount of methane represent peak height.

The method used here to determine rate of methanogenesis per cell per unit time utilizes a direct count to determine cell numbers and chromatographic analysis to determine the amount of methane. The quantity of methane in a culture at any given time depends on two variables: time and cell number. The number of cells is increasing over time and it is assumed all of these cells are continuously producing methane.

In the growth of a culture of methanogenic organisms there is a period of time during which both the cell number and the amount of methane in the culture are increasing exponentially. This means that not only are cell number and methane increasing over time but they are increasing at a constantly increasing rate. When the logarithms of the measurements of methane and cells are plotted against time, a straight line results. From this straight-line portion of the graph one can estimate the amount of methane generated per unit time and also the number of cells present at the beginning and end of the time period.

The fact that the relationship of log cells and log methane to time is linear made it possible to calculate the amount of methane and the number of cells from the equation of the line. This eliminates the necessity to take readings directly from the graph which might introduce error.

From these values of methane generated per unit time and numbers of cells in that unit of time, one can construct a graph with these two parameters as the axes. The slope of this graph will be methane per time per cell.

The experiment and the analysis of data were done for a total of 20 flask culture of the four methanogenic organisms. The data and their analyses will be presented for one flask culture of each organism. The data presented for M. hungatii are those obtained with hydrogen as sole energy source. The actual experimental measurements for these flasks are presented in Tables 13-16. The

TABLE II-13. Actual Measurements of Cells and Methane--
M. ruminantium

Time hrs.	Total CH_4 µmoles	Log CH_4	Total Cells	Log Total Cells
4	1.4	.146	9.5×10^7	7.978
14	14	1.146	2.1×10^8	8.322
18	20	1.301		
20.5			1.2×10^9	9.079
22.5	82	1.914		
24			1.4×10^9	9.130
27	231	2.364		
28.5			5.9×10^9	9.770
39.5	642	2.808		
43			1.96×10^{10}	10.293
48			1.73×10^{10}	10.238
49	628	2.798		
69.5	1309	3.117		
71.5			2.64×10^{10}	10.422

TABLE II-14. Actual Measurements of Cells and Methane--
M. barkeri

Time hrs.	Total CH_4 μmoles	Log CH_4	Total Cells	Log Total Cells
9.5	3.4	.526		
10			3.00×10^7	7.477
22.5	10.4	1.021		
23			8.00×10^7	7.903
27.5	14.6	1.164		
28			1.40×10^8	8.146
33.5	22.3	1.350		
34.5			5.60×10^8	8.748
47	83	1.920		
47.5			7.60×10^8	8.881
53	140	2.146		
54			1.94×10^9	9.288
59	258	2.412		
59.5			1.52×10^9	9.182
72.5	486	2.687		
75			4.96×10^9	9.696

TABLE II-15. Actual Measurements of Cells and Methane--M.O.H.

Time hrs.	Total CH_4 μmoles	Log CH_4	Total Cells	Log Total Cells
48	2.6	.408		
48.5			5.50×10^7	7.740
72	14	1.146		
72.5			1.60×10^8	8.204
80	21	1.320		
80.5			3.25×10^8	8.512
94.5	83.5	1.922		
95.5			3.35×10^8	8.525
104.5	164	2.215		
105			7.00×10^8	8.845
119.5	445	2.648		
120.5			1.52×10^9	9.182

TABLE II-16. Actual Measurements of Cells and Methane--
M. hungatii

Time hrs.	Total CH_4 µmoles	Log CH_4	Total Cells	Log Total Cells
39	1.8	.258		
50			1.45×10^8	8.161
62.5	8.7	.937		
63			3.00×10^8	8.477
73.5	42.5	1.196		
74			2.95×10^8	8.470
85.5	40	1.602		
86			7.50×10^8	8.875
96.5	110	2.041		
97			1.35×10^9	9.130
109.5	356	2.551		
110.5			3.52×10^9	9.546

graphs of time vs total cell number and total methane generated are presented in Figures 16-23.

It can be seen from these graphs that both methane generation and cell number go through a period of exponential increase. A linear regression analysis was done for all graphs to determine the equation of the straight line portion of each of the curves. The form of this equation is $y = mx + b$. A summary of the values for slope and intercept for all four flasks is given in Table 17.

Using this equation, the amount of methane and the number of cells present at any given time can be calculated (Tables 18-21). For this analysis an interval in the middle of the straight-line portion of the curve for cells and methane was chosen. It is important to use the same interval for both graphs since these values are going to be combined and must be directly comparable. It should be noted that the equation of the line gives the log methane and log cells. The antilogs or actual concentration of methane and cells are used for the remaining calculations. By subtracting each value for methane from that following it, it is possible to calculate the hourly increase in methane. The cell count at the beginning of each hour is obtained directly from the graph or the equation of the line on the graph.

All that remains is to plot the rate of methane generation versus the cell count. This has been done twice for each culture as shown in Figures 24-27. In the first case the number of cells present at the beginning of the hour is considered to have produced the methane. In the second case it is the cells present at the end of the hour which are considered to have produced the methane. This gives two slopes representing two different rates of methane produced per cell. The actual value will lie somewhere between the two. In practice it is not really necessary to plot the data since a linear regression

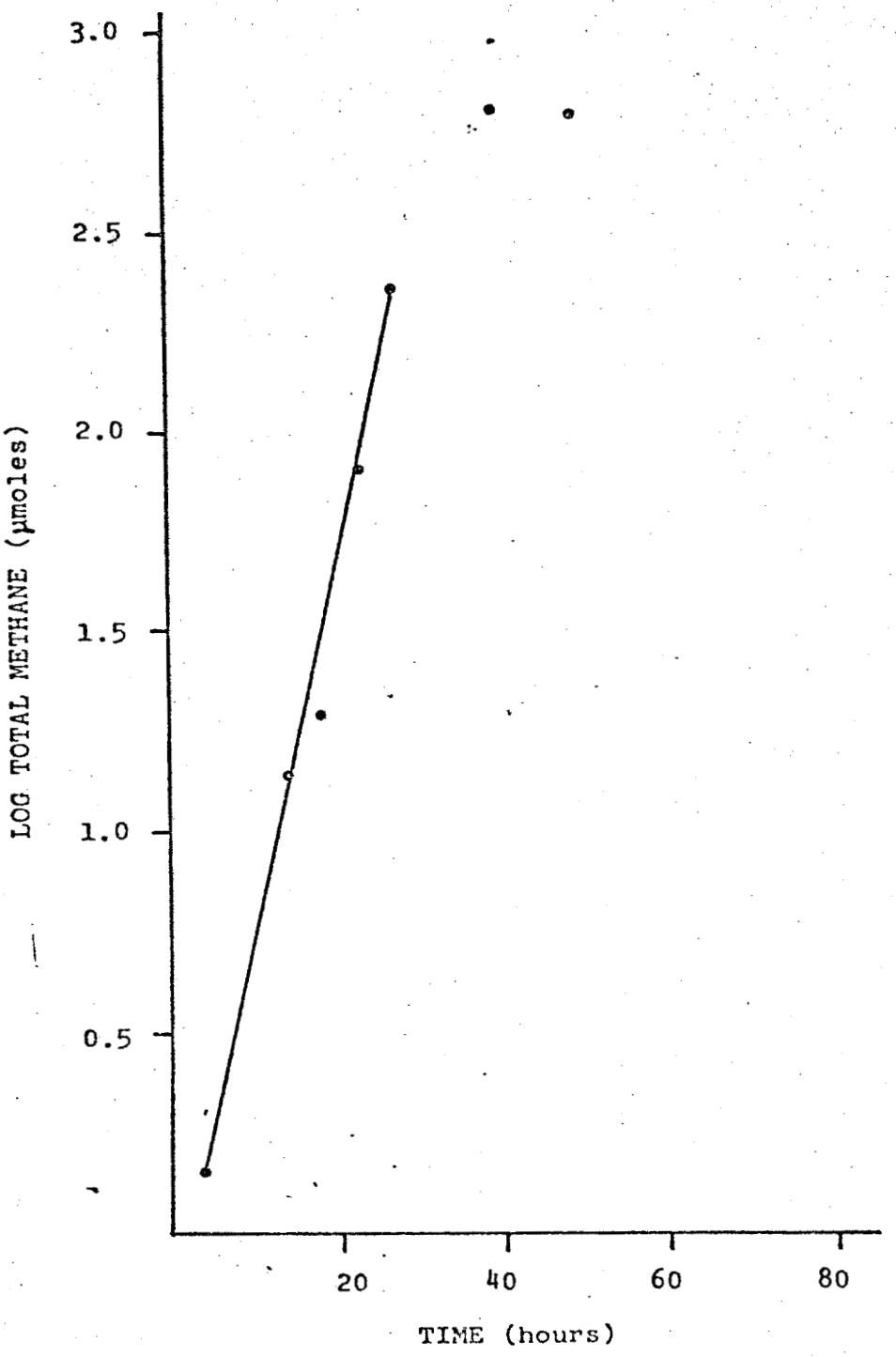

FIGURE II-16. Total Methane vs Time--M. ruminantium

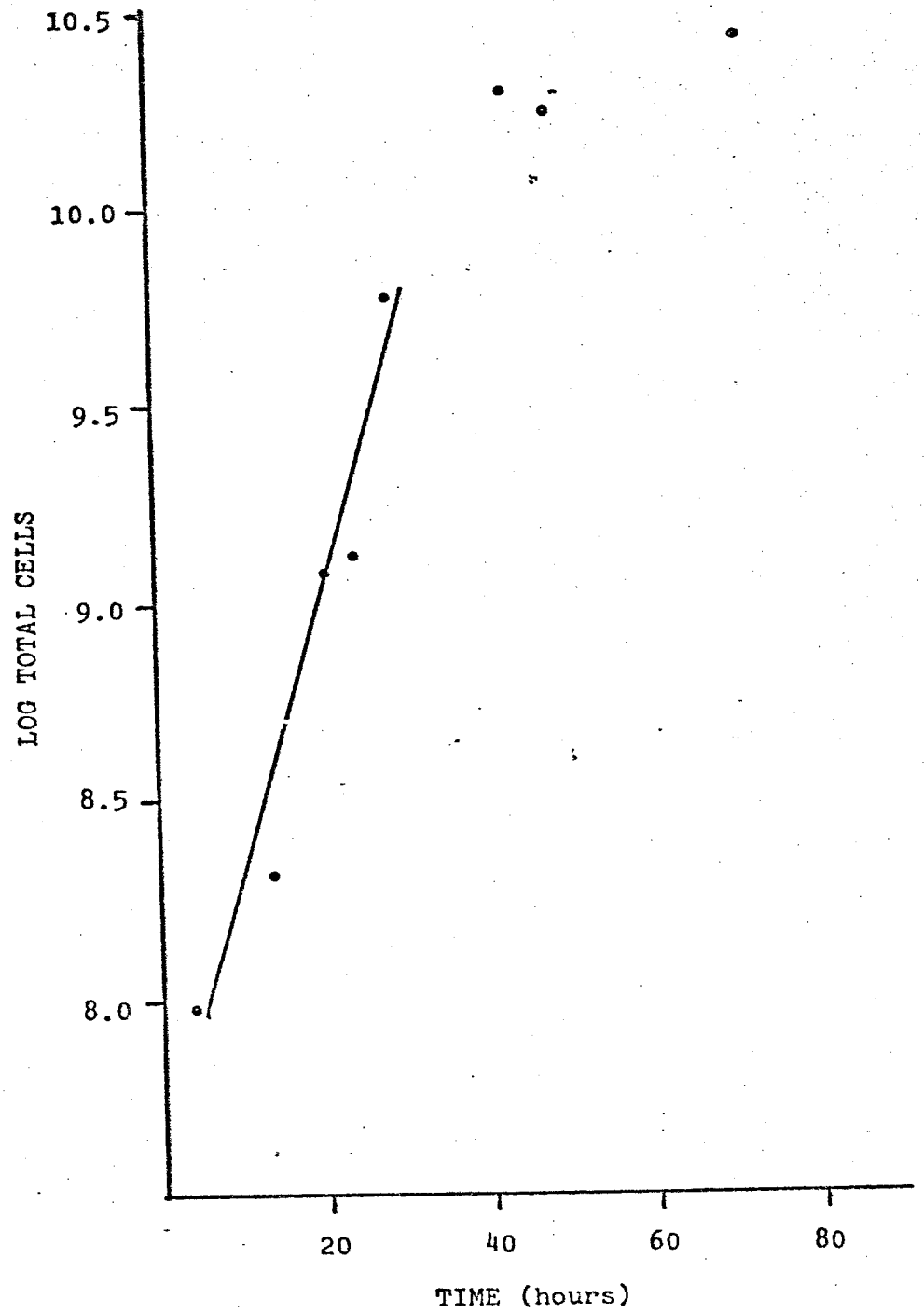

FIGURE II-17. Total Cells (100 ml) vs Time--M. ruminantium

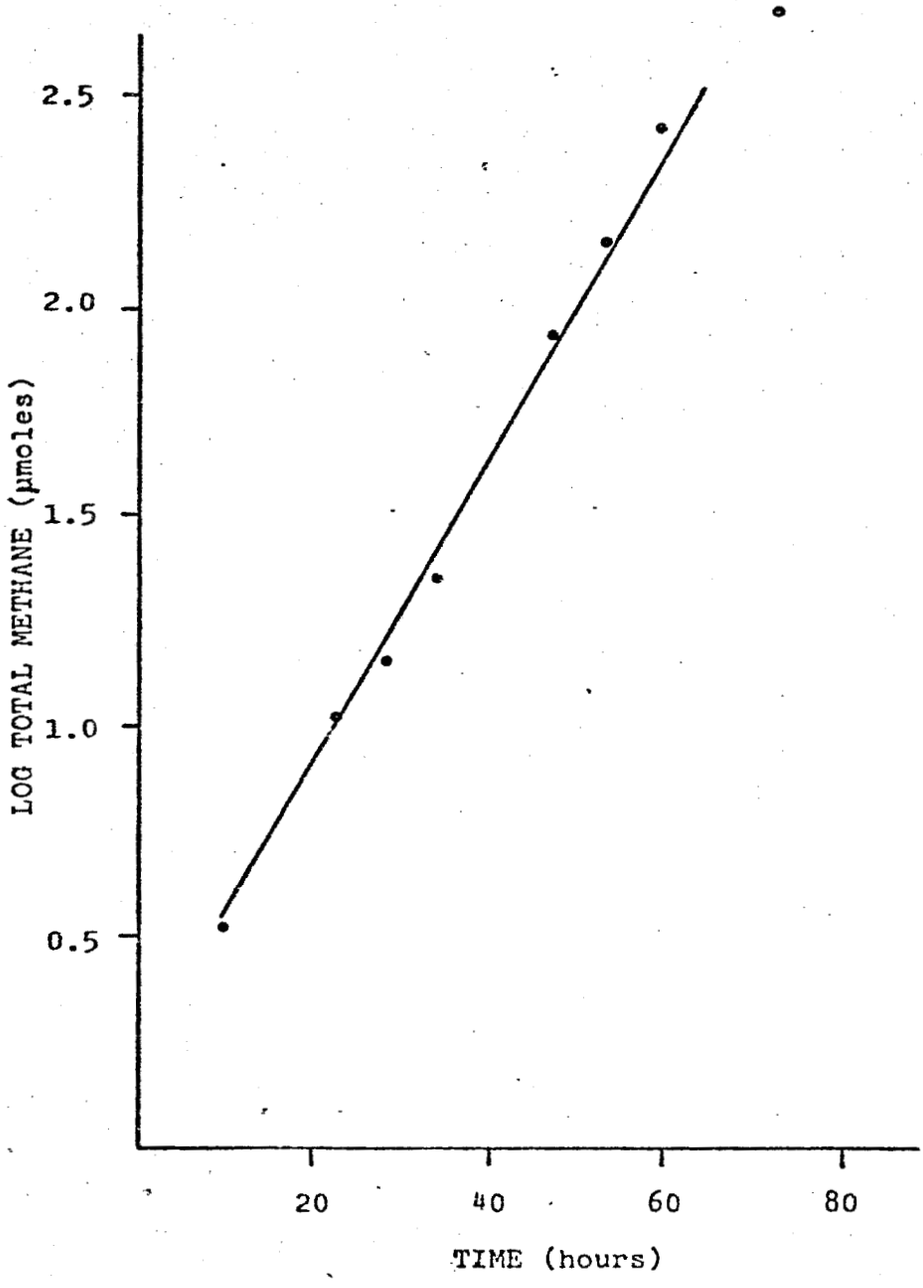

FIGURE II-18. Log Total Methane vs Time--M. barkeri

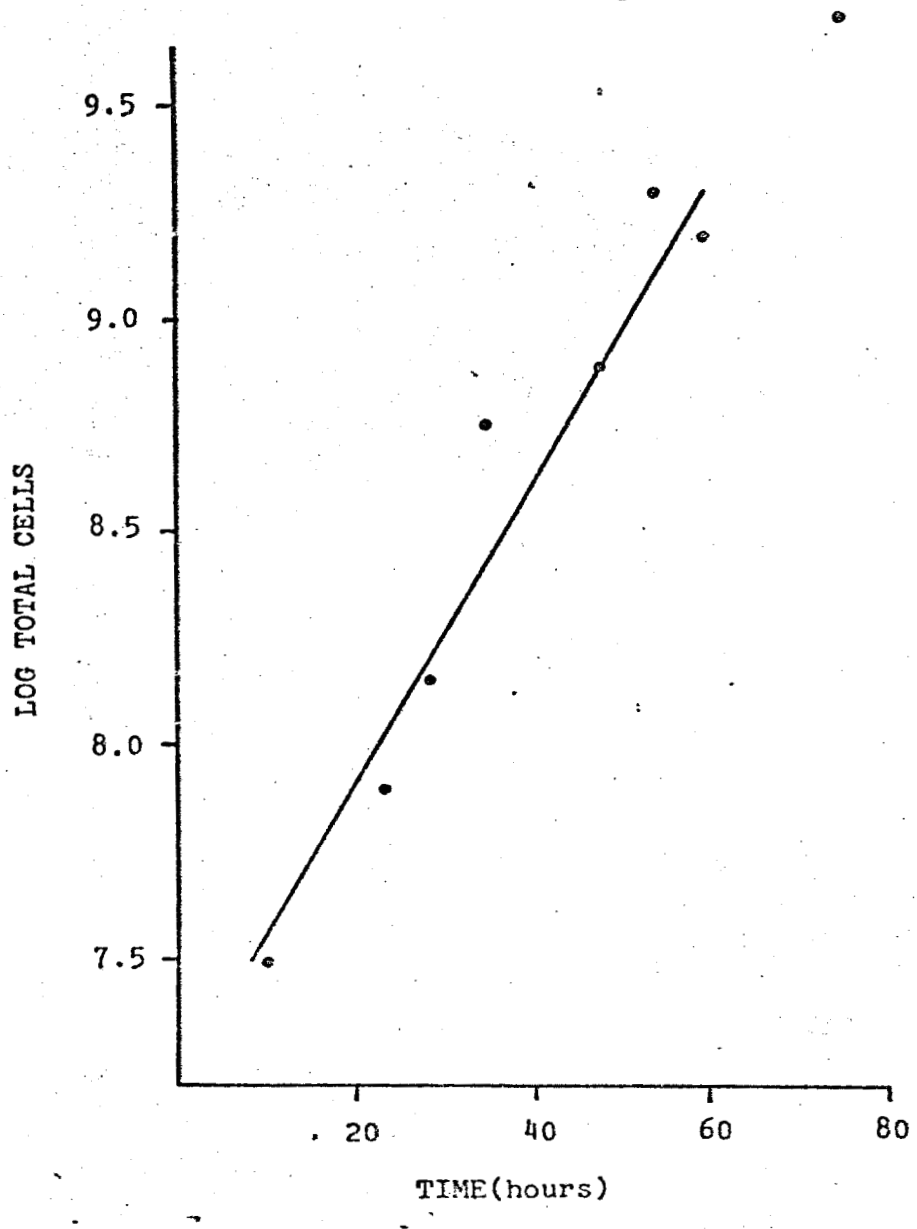

FIGURE II-19. Total Cells (100 ml) vs Time--M. barkeri

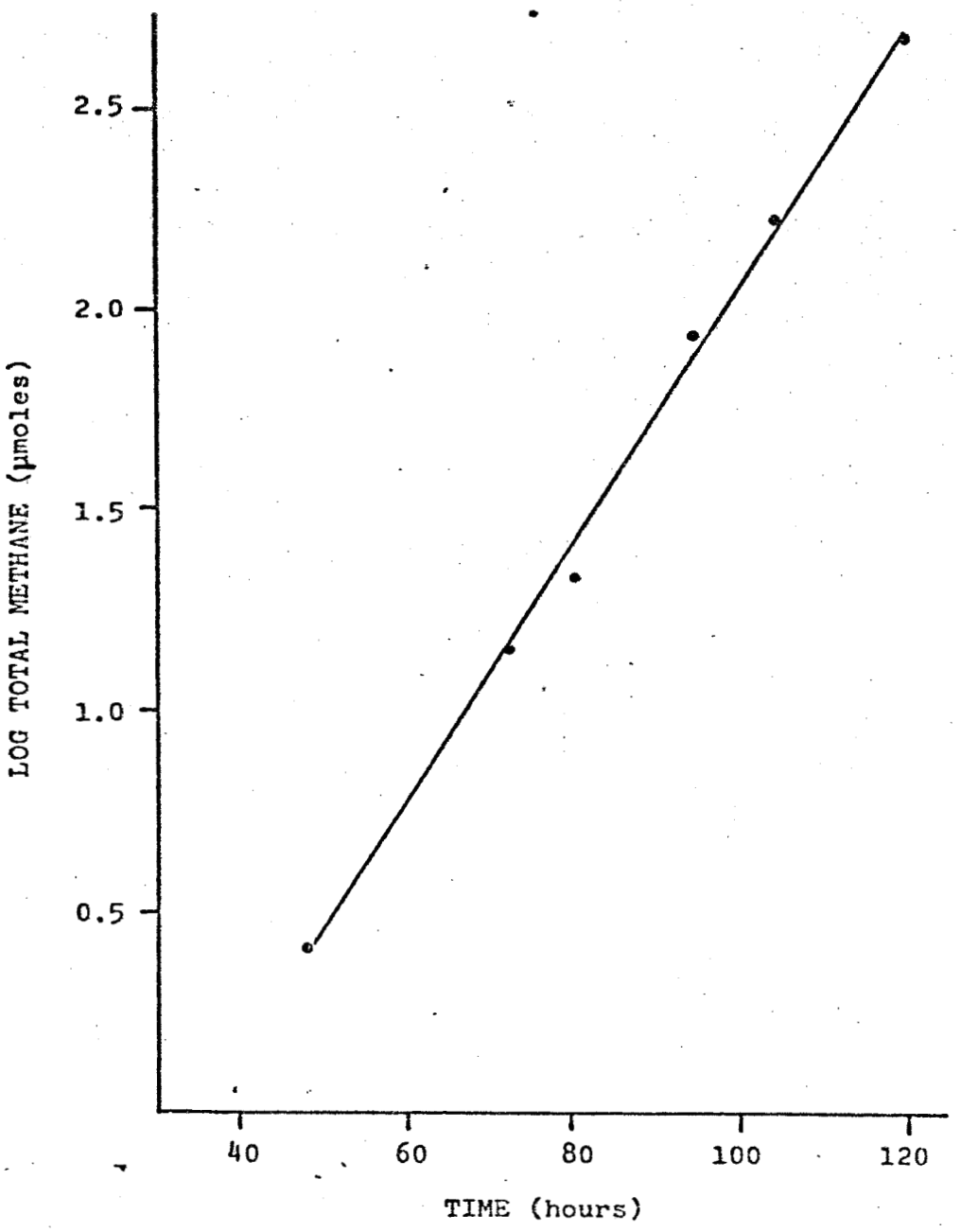

FIGURE II-20. Log Total Methane vs Time--M.O.H.

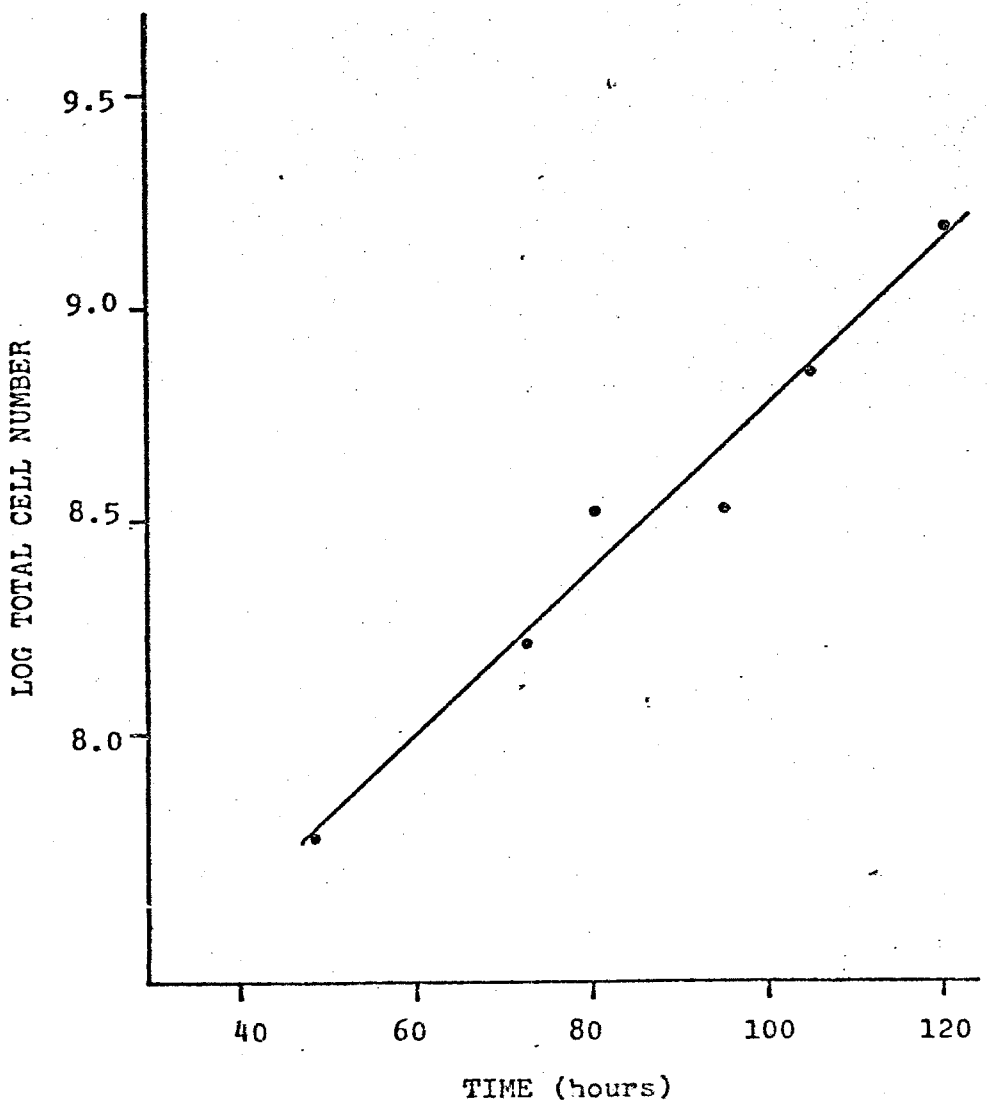

FIGURE II-21. Log Total Cell Number (100 ml) vs Time--M.O.H.

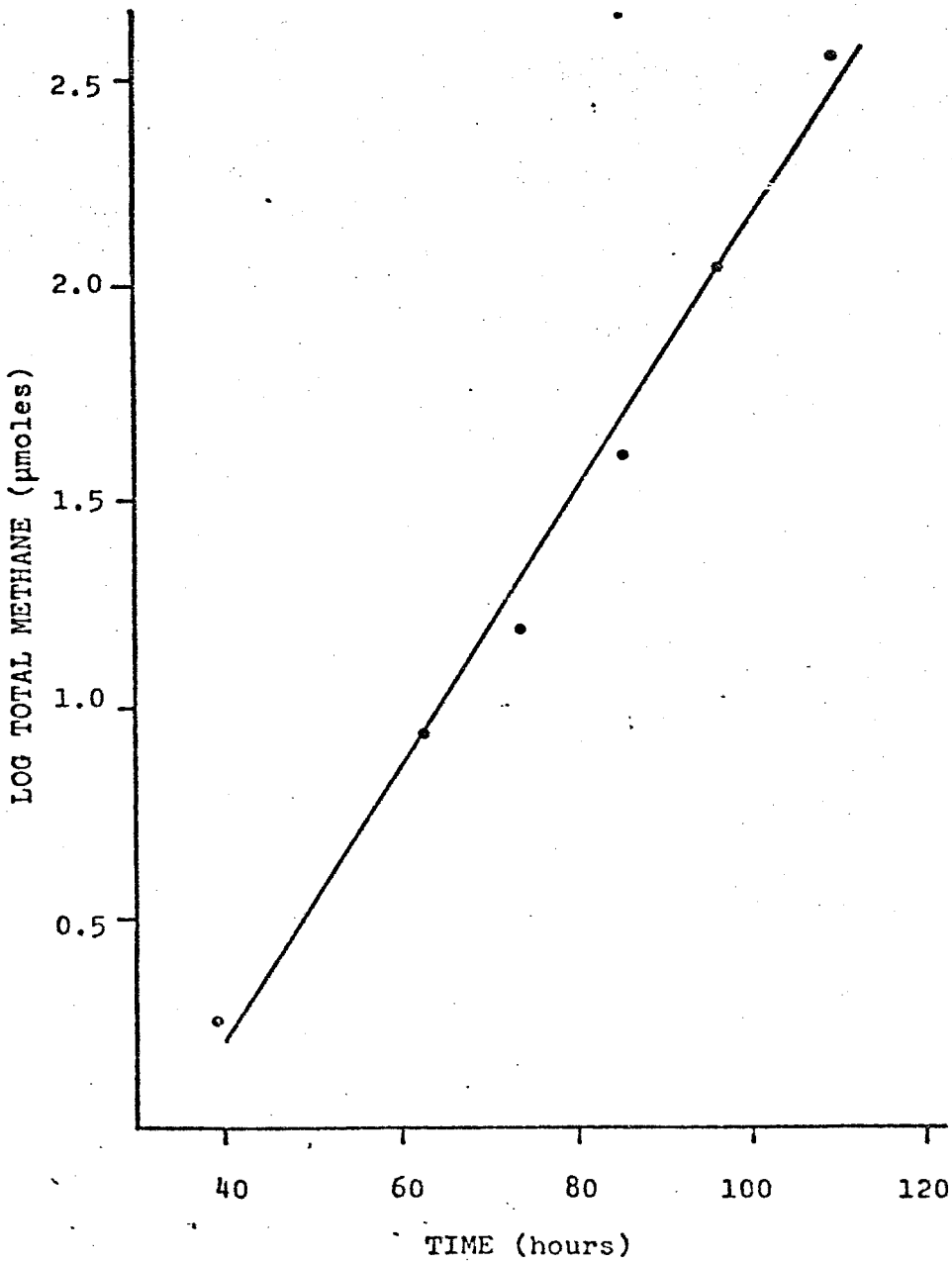

FIGURE II-22. Log Total Methane vs Time--M. hungatii

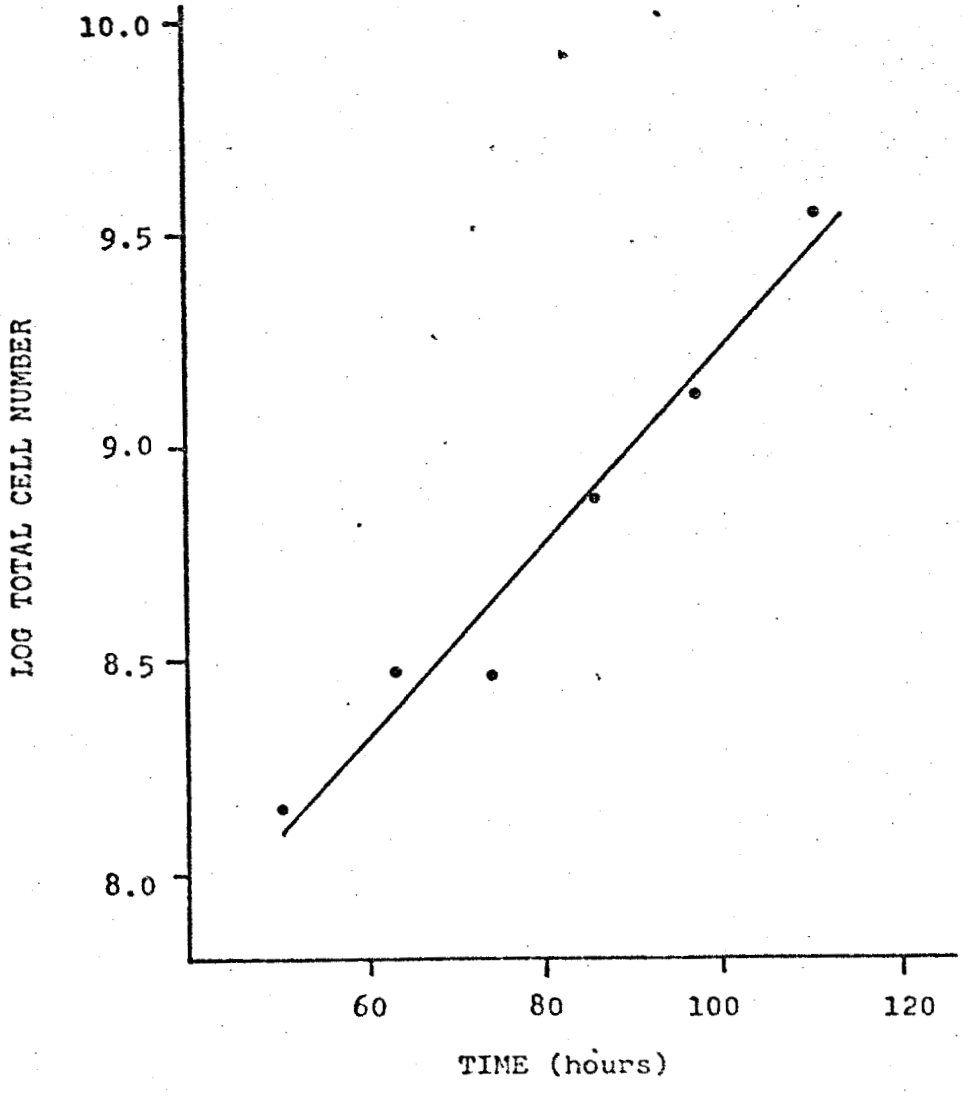

FIGURE II-23. Log Total Cell Number (100 ml)--M. hungatii

TABLE II-17. Summary of Slopes and Intercepts for Methane and Cell Curves-All Cultures

Culture	Rate of Increase		y Intercept	
	Log Cells/hr	Log CH_4/hr	Cells	CH_4
M rum	.0719	.0952	7.5472	-.2537
M bark	.03459	.03578	7.2316	.2018
M.O.H.	.01928	.0319	6.8225	-1.1488
M hung	.02240	.03226	6.9849	-1.0775

M rum = M. ruminantium
M bark = M. barkeri
M.O.H. = M.O.H.
M hung = M. hungatii

TABLE II-18. Amount of Methane and Cell Number Used to Calculate Rate
M. ruminantium

Time (hr)	Log CH_4 Total μmoles	Total CH_4 μmoles	ΔCH_4 μmoles/hr	Log Cells	Cells X 10^8
15	1.1743	14.94	-	8.6257	4.22
16	1.2695	18.60	3.66	8.6976	4.98
17	1.3647	23.16	4.56	8.7695	5.88
18	1.4599	28.83	5.67	8.8414	6.94
19	1.5551	35.90	7.07	8.9133	8.13
20	1.6503	44.70	8.80	8.9852	9.67
21	1.7455	55.65	10.95	9.0571	11.40
22	1.8407	69.30	13.65	9.1290	13.46

TABLE II-19. Amount of Methane and Cell Number Used to Calculate Rate
M. barkeri

Time (hr)	Log CH_4 Total μmoles	Total CH_4 μmoles	ΔCH_4 μmoles/hr	Log Cells	Cells X 10^8
30	1.2753	18.85	-	8.2694	1.86
31	1.3111	20.47	1.62	8.3040	2.01
32	1.3469	22.23	1.76	8.3386	2.18
33	1.3827	24.14	1.91	8.3732	2.36
34	1.4185	26.21	2.07	8.4078	2.56
35	1.4543	28.46	2.25	8.4424	2.77
36	1.4900	30.91	2.45	8.4770	3.00
37	1.5258	33.56	2.65	8.5115	3.25
38	1.5616	36.44	2.88	8.5461	3.52
39	1.5974	39.57	3.13	8.5807	3.81
40	1.6332	42.97	3.40	8.6153	4.12

TABLE II-2n. Amount of Methane and Cell Number Used to Calculate Rate M.O.H.

Time (hr)	Log CH_4 Total µmoles	Total CH_4 µmoles	ΔCH_4 µmoles/hr	Log Cells	Cells X 10^8
80	1.4050	25.41	-	8.3648	2.32
81	1.4369	27.35	1.94	8.3841	2.42
82	1.4689	29.44	2.09	8.4034	2.53
83	1.5008	31.68	2.24	8.4227	2.65
84	1.5327	34.10	2.42	8.4419	2.77
85	1.5647	36.70	2.60	8.4612	2.89
86	1.5966	39.50	2.80	8.4805	3.02
87	1.6285	42.51	3.01	8.4998	3.16
88	1.6604	45.76	3.25	8.5191	3.30

TABLE II-21. Amount of Methane and Cell Number Used to Calculate Rate
M. hungatii

Time (hr)	Log CH_4 Total μmoles	Total CH_4 μmoles	ΔCH_4 μmoles/hr	Log Cells	Cells X 10^8
75	1.3423	21.99	-	8.6647	4.62
76	1.3745	23.69	1.70	8.6871	4.86
77	1.4068	25.52	1.83	8.7095	5.12
78	1.4391	27.48	1.96	8.7319	5.39
79	1.4713	29.60	2.12	8.7543	5.68
80	1.5036	31.88	2.28	8.7767	5.98
81	1.5359	34.34	2.46	8.7991	6.30
82	1.5681	36.99	2.65	8.8215	6.63
83	1.6004	39.85	2.86	8.8439	6.98

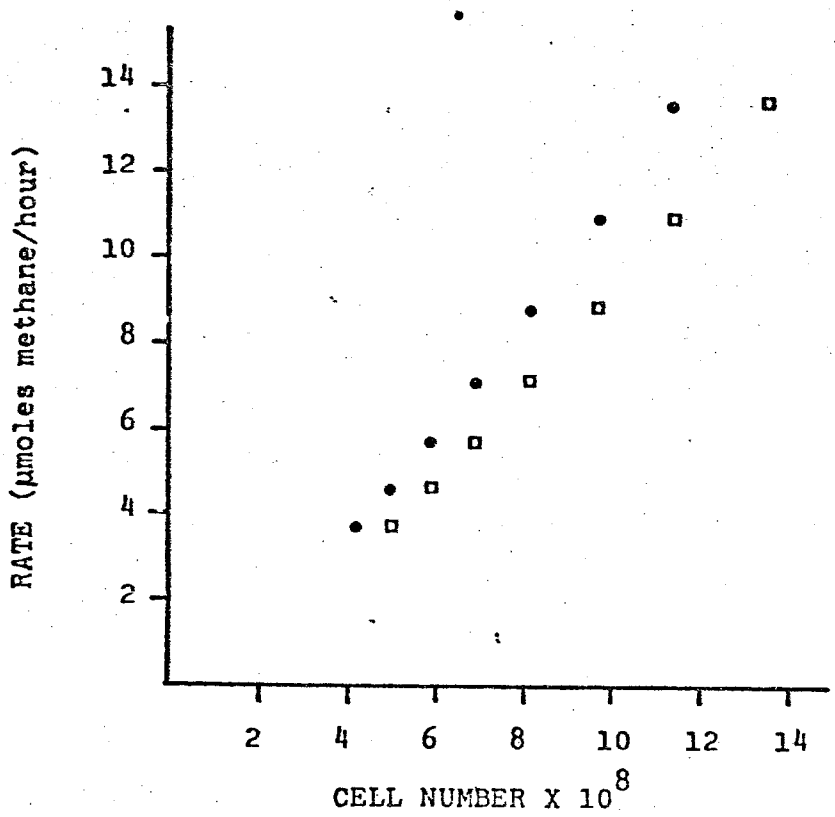

FIGURE II-24. Rate of Methane Production vs Cell Number--M. ruminantium
● = cells present at beginning of hour interval considered to have produced the methane.
□ = cells present at end of hour interval considered to have produced the methane.

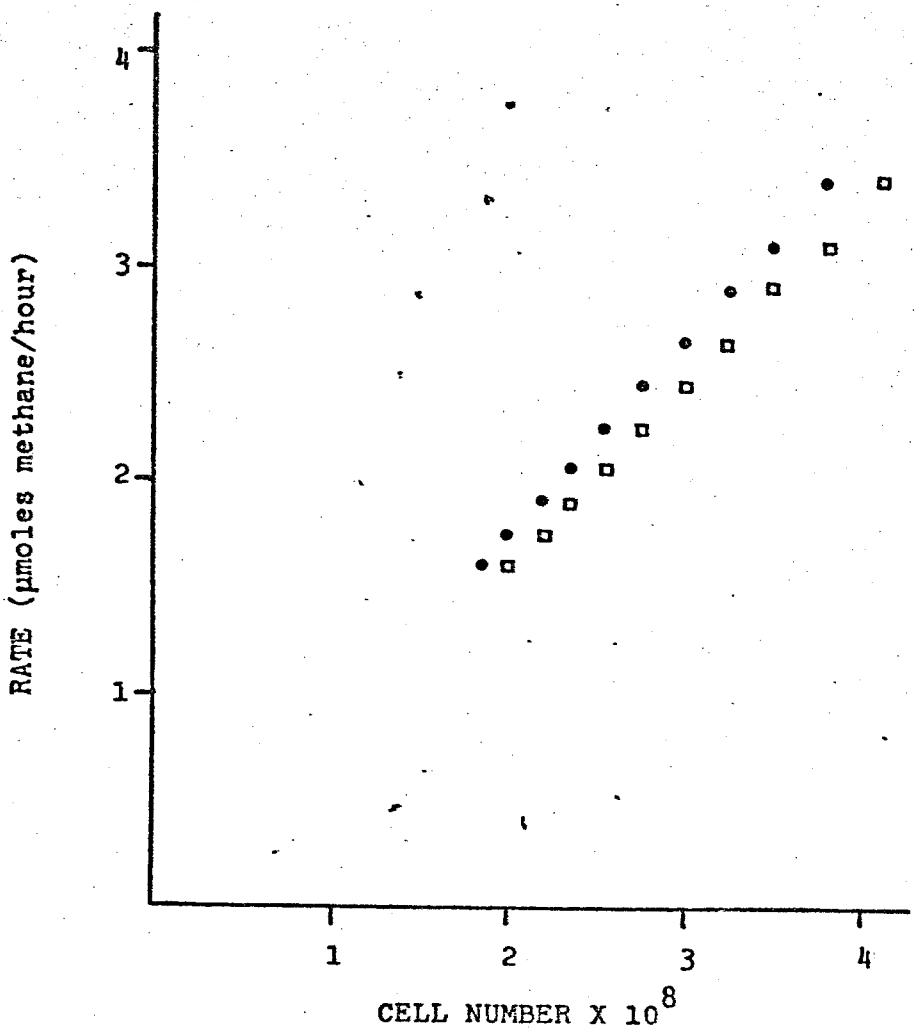

FIGURE II-25. Rate of Methane Production vs Cell Number--M. barkeri
● = cells present at beginning of hour interval considered to have produced the methane.
□ = cells present at end of hour interval considered to have produced the methane.

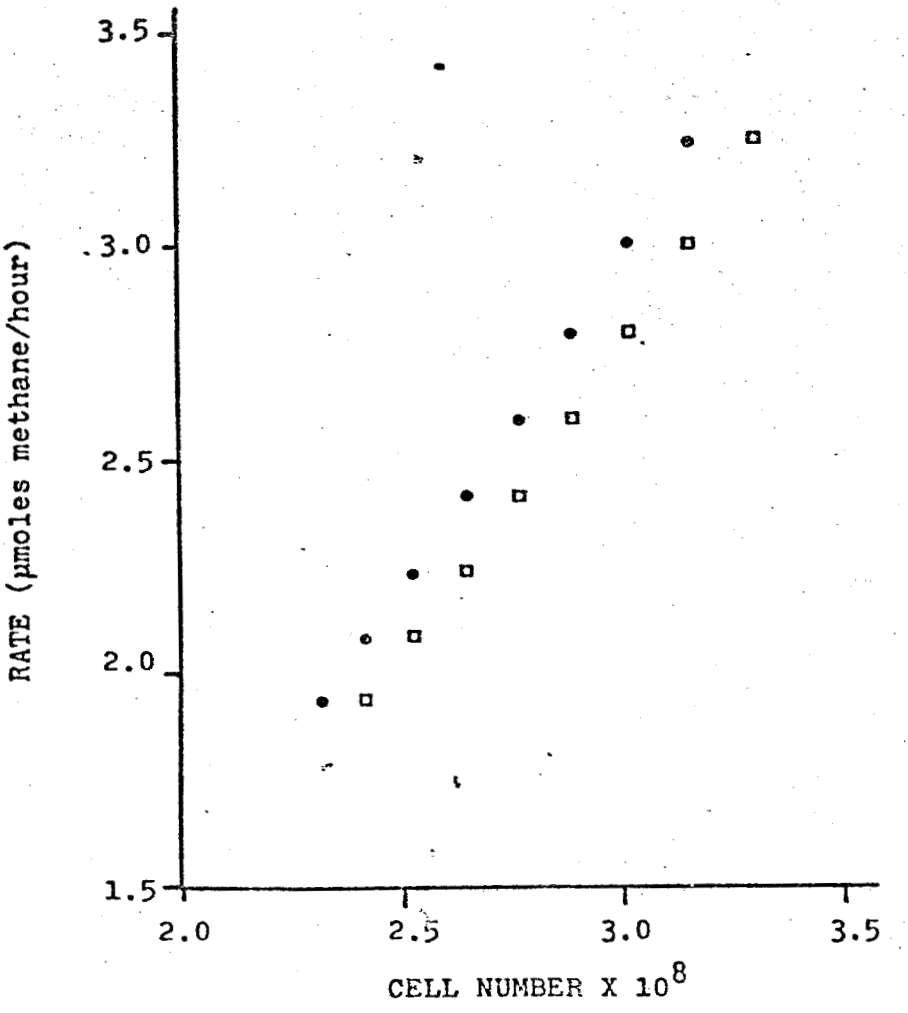

FIGURE II-26. Rate of Methane Production vs Cell Number--M.O.H.
● = cells present at beginning of hour interval considered to have produced the methane.
□ = cells present at end of hour interval considered to have produced the methane.

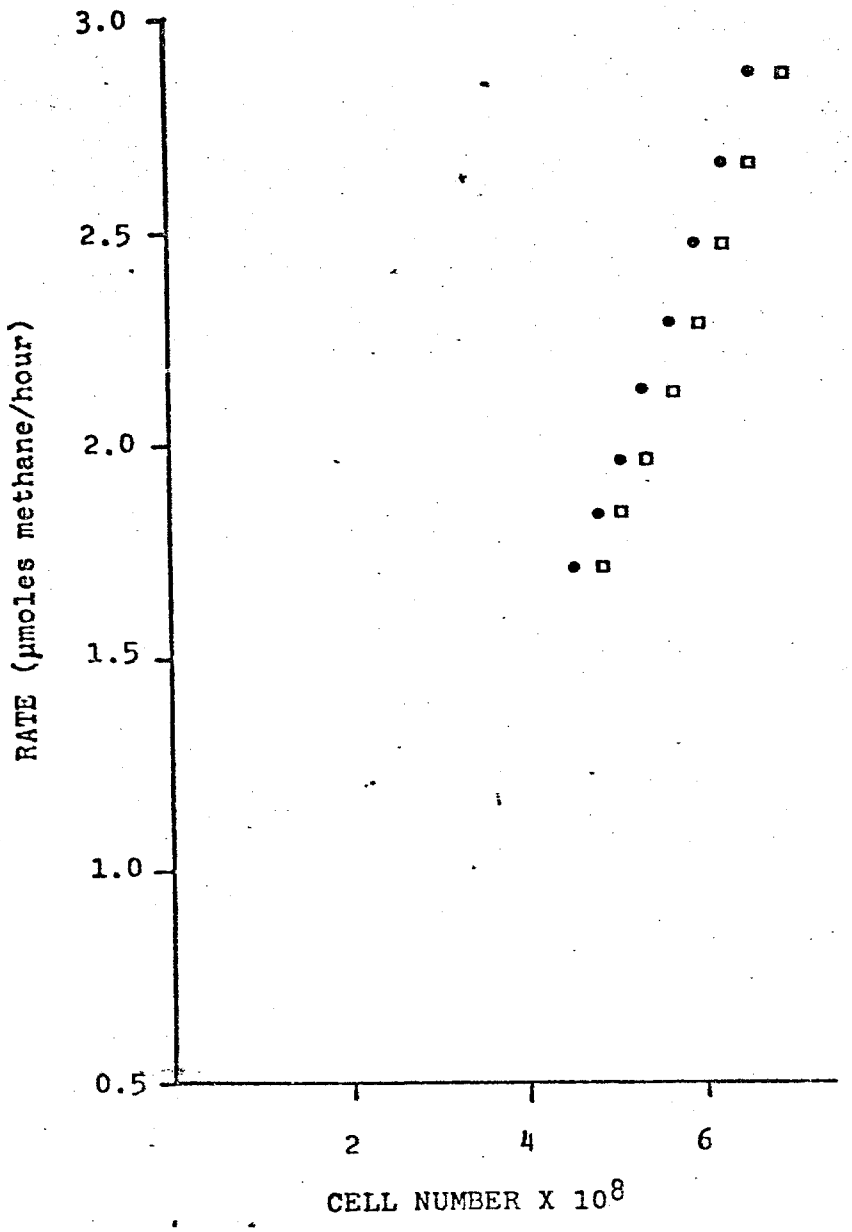

FIGURE II-27. Rate of Methane Production vs Cell Number--M. hungatii
● = cells present at beginning of hour interval considered to have produced the methane.
□ = cells present at end of hour interval considered to have produced the methane.

analysis will give the "best-fit" equation of the line and therefore the best slope. A summary of the rates of methane production for all flasks is presented in Table 22.

Analysis of Sampling Errors

One of the advantages of the method used is that it tends to reduce the effect of any sampling error. However, it is important to determine the effect of errors in the original measurements of methane and cells.

In an attempt to do this an analysis of the flask of M. ruminantium described previously was undertaken. A 95% confidence belt was generated around the least squares line (Figures 28 and 29). If the mean of the population of y values lies on the least squares line, then when the population is sampled 95% of the samples will lie within this confidence belt.

Rate of Methane Generation for Lake Erie Isolate

Although the organism isolated from Lake Erie resembled M.O.H. in every other respect, it grew very slowly, especially in liquid medium. The experiment on rate determination was attempted on many occasions with this organism. But on each occasion, even with a very large inoculum, it took these cells days to reach the same level of methane generation and cell number reached by other species in a matter of hours. Because of these considerations a somewhat different method of analysis was used to arrive at the rate of methane generation for these cells.

A flask culture of this organism which had been growing for approximately one month had produced considerable methane and reached sufficient mass that the cell clumps could be seen. This flask was purged with H_2:CO_2 (80:20) for 5 minutes and placed on a rotary shaker at 37 C for 30 minutes. It was then purged for another 5 minutes with H_2:CO_2 (80:20). This was done to remove

TABLE II-22. Rates of Methane Production for All Flasks

Culture	Inc. Cells log/hr	Inc. CH_4 log/hr	µmoles/hr/10^8 Range	Cells Avg
M bark				
1.1	.0240	.0238	1.2537-1.3258	1.2897
1.2	.0219	.0265	.6847- .7204	.7025
1.3	.0286	.0269	.9419-1.0058	.9738
2.1	.0346	.0358	.8429- .9129	.8779
2.2	.0259	.0375	.9540-1.0129	.9834
2.3	.0399	.0382	.7760- .8508	.8134
avg	.0291	.0314		.9401
M rum				
1.1	.0216	.0778	2.3831-2.5064	2.4447
1.2	.0431	.0562	.7769- .8589	.8179
1.3	.0458	.0957	1.0845-1.2054	1.1449
2.1	.0930	.0963	1.1402-1.4132	1.2767
2.2	.0725	.0974	.9536-1.1271	1.0403
2.3	.0719	.0952	1.1771-1.3915	1.2843
avg	.0580	.0864		1.3348
M.O.H.				
1.1	.0086	.0300	2.8713-2.9288	2.9000
1.2	.0164	.0253	2.3027-2.3918	2.3472
1.3	.0148	.0262	2.1907-2.2667	2.2287
2.1	.0193	.0319	1.4791-1.5461	1.5126
2.2	.0269	.0313	1.2301-1.3090	1.2695
2.3	.0285	.0350	1.5537-1.6581	1.6054
avg	.0190	.0299		1.9772
M hung				
1	.0224	.0323	.5485- .5775	.5630
2	.0294	.0329	.5100- .5572	.5336
avg	.0259	.0326		.5483

M bark = M. barkeri, M rum = M. ruminantium, M.O.H. = M.O.H., M hung = M. hungatii.
Culture series 1 for each species inoculated with same inoculum.
Culture series 2 with a different inoculum.

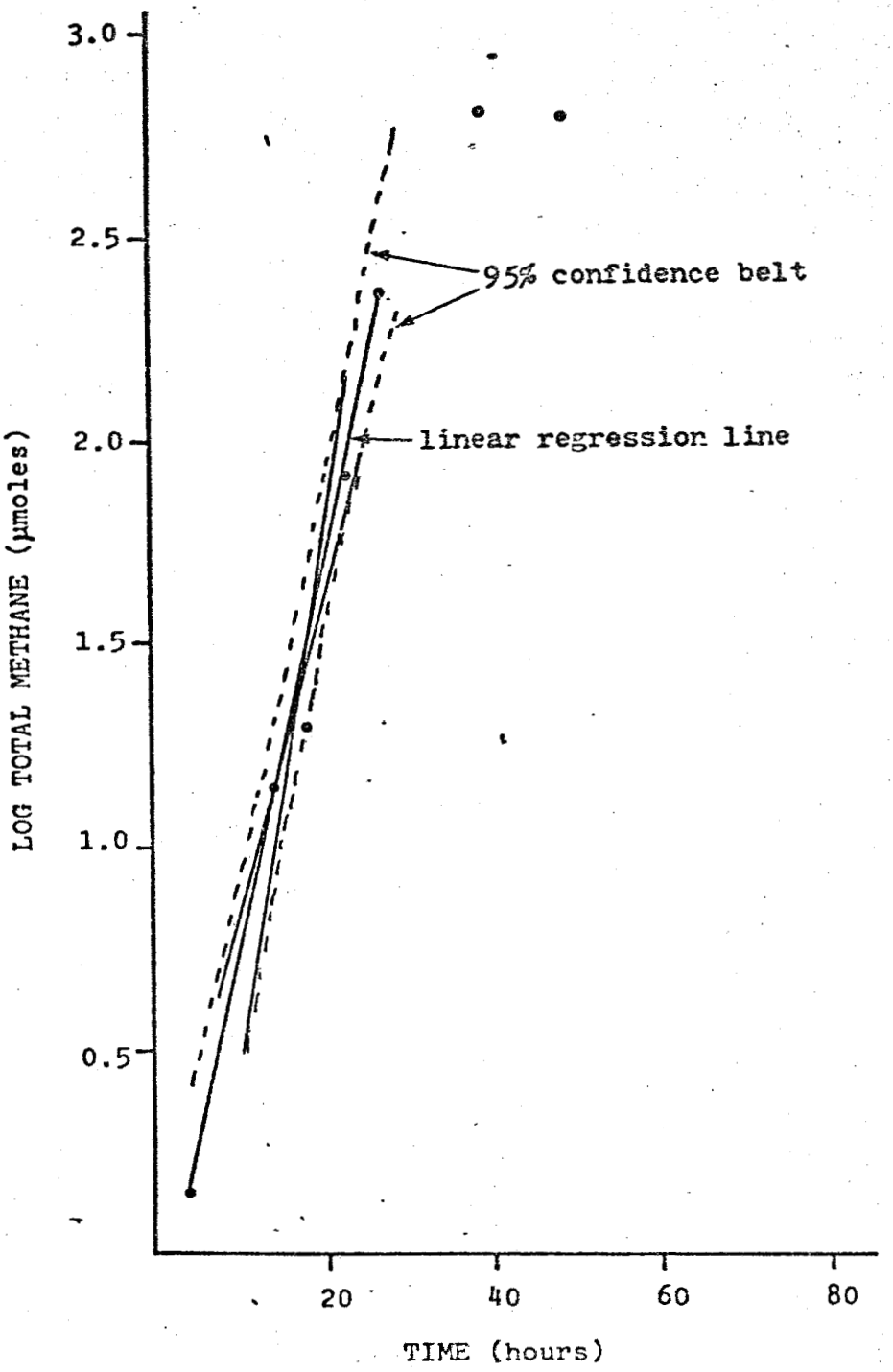

FIGURE II-28. Log Total Methane vs Time for M. ruminantium with 95% Confidence Belt and Worst Sampling Errors Indicated

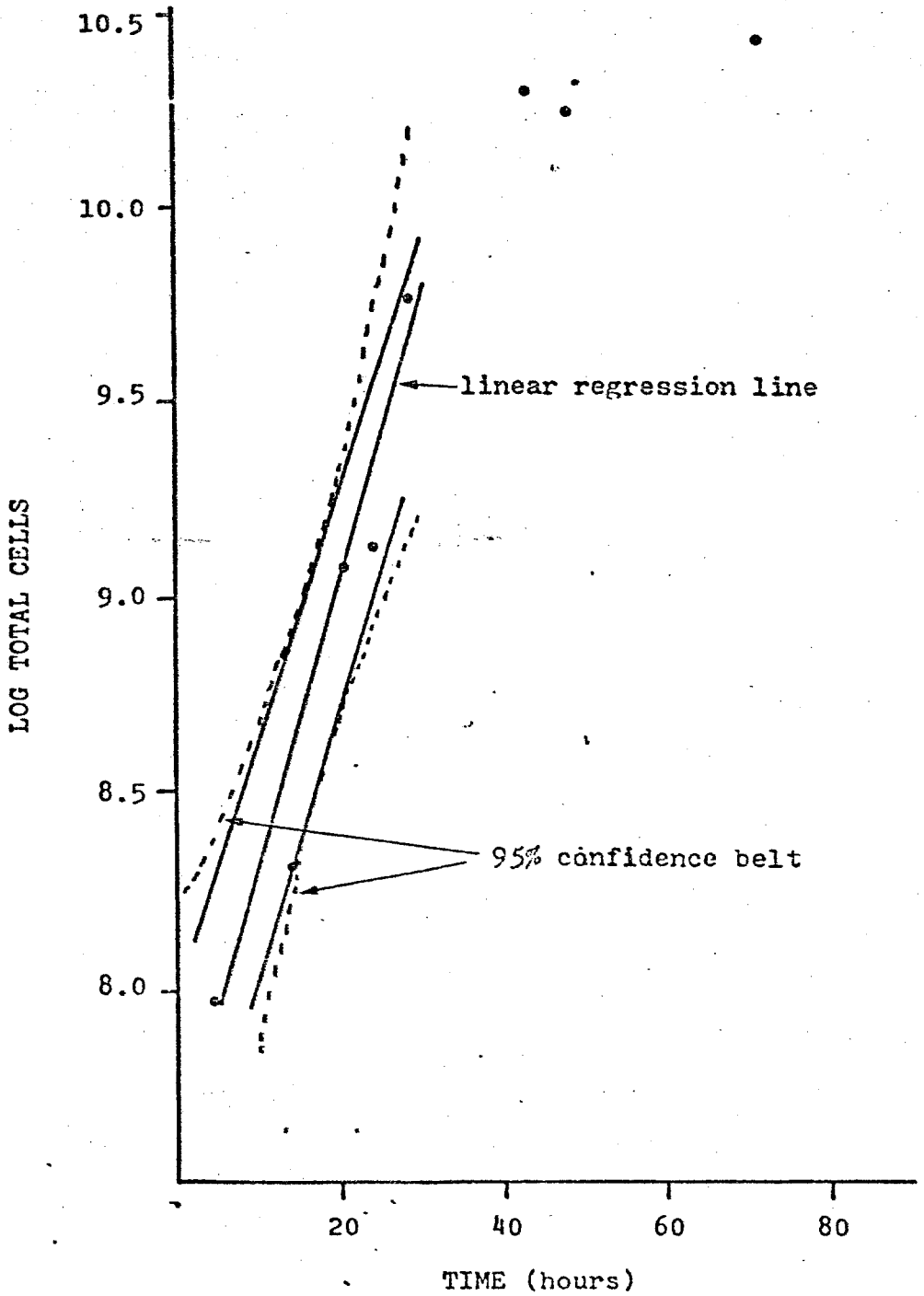

FIGURE II-29. Log Cell Number vs Time for M. ruminantium with 95% Confidence Belt and Worst Sampling Errors Indicated

methane in the gas as well as the liquid phase of the culture. It was then replaced in the incubator and monitored for methane and cell number over time. Time zero was taken as the end of the second purging.

The measurements of cells and methane are presented in Table 23. It can be seen that there is a slow but steady increase in both methane and cell number. Rate of methane generation vs cell number is plotted in Figure 30. It can be seen that as the cell number increases, the rate of methane production increases. A linear regression analysis was done for these points. The "best-fit" line has a slope of 1.0686 μmoles methane per hour per 10^8 cells. This rate is somewhat lower than that of the cultures of M.O.H. It is still, however, well within the range of rates of methanogenesis calculated for all cells (Table 22).

TABLE II-23. Actual Measurements for Time, Cell Number, and Amount of Methane for a Flask Culture of Lake Erie Isolate

Time hr	CH_4 μmoles	Change Time	Change CH_4	Rate CH_4/hr	Cell Number X 10^8
4	4.01	-	-	-	-
18	18.02	14	14.01	1.00	2.5
24	28.65	6	10.63	1.77	-
43	82.19	19	53.54	2.82	5.25
68	188.62	25	106.43	4.26	4.30
90.5	282	22.5	93.38	4.15	6.35
115	430	24.5	148	6.04	6.75
166	1003	51	573	11.23	11.85

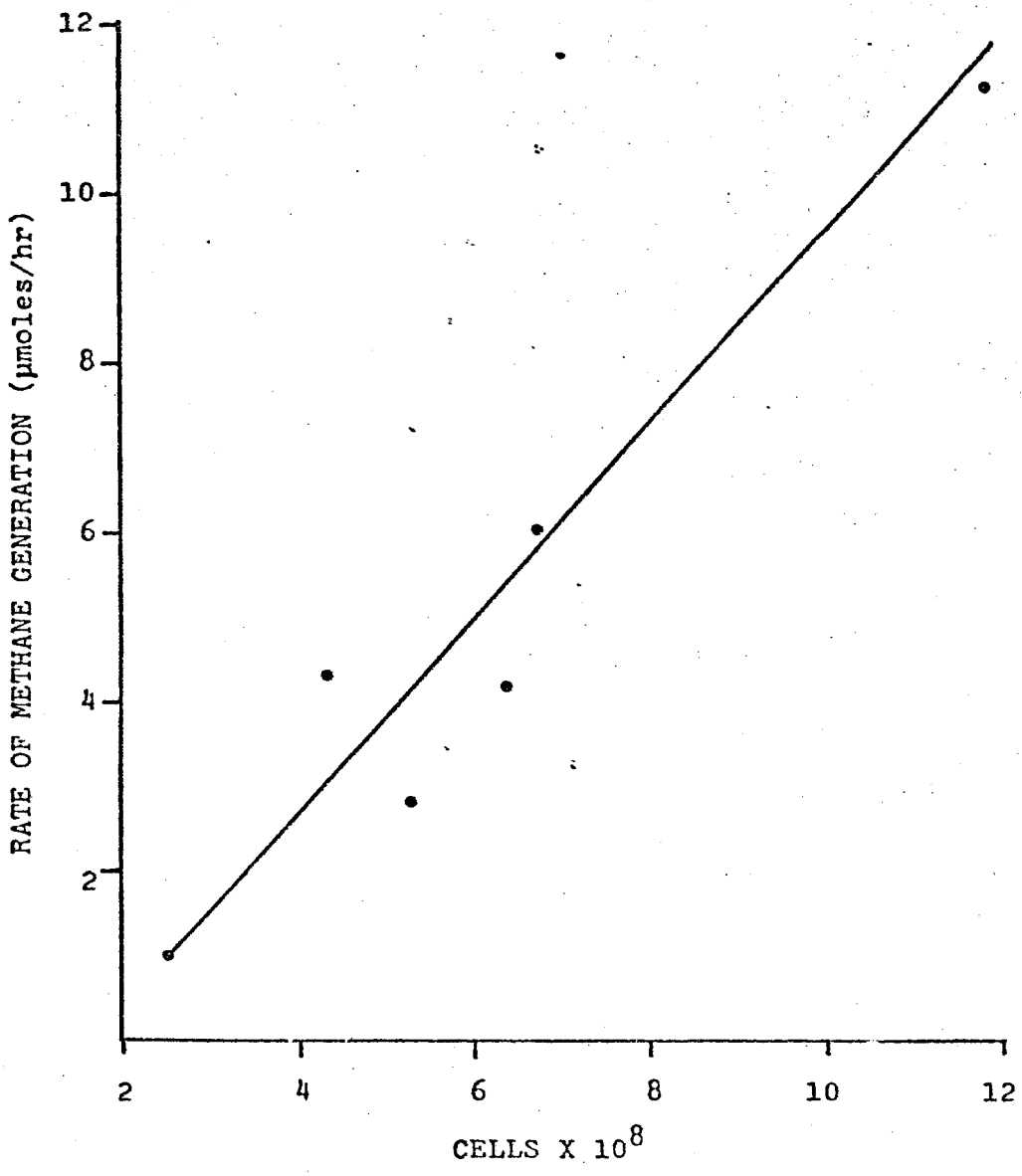

FIGURE II-30. Rate of Methane Generation vs Cell Number--Lake Erie Isolate

CONCLUSIONS

Perhaps before beginning a discussion of this work it would be well to summarize the importance of methane production. The generation of methane is the final step in the anaerobic degradation of organic matter. Among the products of heterotrophic metabolism of organics are acetate, carbon dioxide, and hydrogen. These are turned into methane and cells by the methanogenic species. Since methane gas is poorly soluble, its formation has the effect of removing carbon from the system. Methane generation also "pulls" the chain of reactions by removing hydrogen and thus displacing the equilibrium in favor of degradation of the organic matter.

There are three main environments in which this process takes place. A truly impressive amount of methane is generated in the rumen of a large cow which may produce 200 liters of methane per day (45). Man has taken advantage of the anaerobic digestion process in sewage treatment plants which contain huge fermentation vats. These produce large amounts of methane which are often used to provide heat and/or power for the plants. The sediment at the bottom of ponds and lakes is the last environment in which methane generation is important. Foree and McCarty (15) working with laboratory cultures reported that methane fermentation was responsible for the stabilization of algal organics. Methane generation is therefore vital in this ecosystem for the cycling of carbon.

One accomplishment of this study was the isolation of a methanogenic organism from Lake Erie. This organism is morphologically very similar to Methanobacterium M.O.H. which is in turn probably a variant of Methanobacterium formicicum (5).

Both the existence of methane in aquatic systems and the potential for methane generation are well documented (2, 3, 18, 25, 27, 36, 46). In spite

of this, most pure cultures have been isolated from either rumen contents or sewage systems (14, 23, 32, 42, 43, 44). One exception to this is a report by Prins et al. (34) of the isolation of an organism they identified as Methanobacterium M.O.H. from a mud sample taken from a small sweet water pond. It is to be expected that the same organisms which generate methane in the rumen and in sewage systems are present in aquatic environments. However, more organisms need to be identified in lake systems in order to strengthen this hypothesis.

As discussed previously, work with methanogenic organisms has been retarded by the extreme difficulty encountered with pure culture work. At present there are eight well characterized species of methane producing organisms (5). The nutritional requirements of two of these organisms have been recently reviewed by Bryant et al. (8) and are rather well understood. Investigators at the University of Illinois have undertaken an intensive study of the biochemistry of the terminal steps in the methanogenic process. This work has been most recently summarized by Wolfe (49). These physiological studies are, of course, valuable but some of the more elementary questions about rate of generation of methane and hydrogen uptake also needed to be answered.

Perhaps the most unexpected result of this study was the great similarity in the metabolism of the species studied. These represent all four morphological types of methane producing organisms. All are quite different which has suggested to Zeikus and Bowen (51) that the ability to produce methane is the end point of great evolutionary divergence.

There are, however, reports in the literature which suggest that methane producers although morphologically dissimilar have a very similar metabolism. Prins et al. (34) have demonstrated that chlorinated methane analogs inhibited methane production in both M. ruminantium and M.O.H. There is presently some doubt as to the exact mechanism of inhibition by the chloromethanes but it

seems to competitively inhibit a methyl transfer reaction (49). At any rate it is instructive to note that two very different species are inhibited by the same compounds.

Another piece of evidence for the similarity of the physiology of methane producers is in a recent article (48) demonstrating that both M. ruminantium and M.O.H. contain F_{420}, an important cofactor in the hydrogenase system of these organisms.

Results of this study strengthen the concept of metabolic similarity. All species tested had a tremendous affinity for hydrogen, the main energy source for methanogenesis. Also when hydrogen was present in excess, all of these bacteria produced methane at approximately the same rate. These two facts allow one to speculate about the factors influencing methane production in any system even if the dominant type of organism is unknown.

The studies of hydrogen metabolism demonstrate the affinity of these cells for their substrate. The cells were able to repeatedly remove essentially all of the hydrogen from a flask, causing the pressure to drop to 0.5 atmospheres or lower in the process. It is unlikely that any methanogenic organism in nature would ever be faced with the levels of hydrogen presented in these depletion experiments. Hydrogen arises in nature as the result of anaerobic decomposition of organic matter. As such it is being continually generated by the heterotrophic organisms present in the same environments as the methanogenic bacteria. However, the ability of the methane producers to utilize all available hydrogen in the shake flasks indicates that they can quite probably do the same thing in a natural environment.

One reason why hydrogen does not normally accumulate in anaerobic environments such as sewage plants, the rumen, and the mud at the bottom of lakes is that methane producers use it as fast as it is produced. This has been

demonstrated by methane inhibition experiments in the rumen (1) and in sewage systems (47). Another reason why hydrogen does not accumulate is that those organisms which produce hydrogen may produce it in much lower levels if it is not being immediately consumed for methanogenesis. This has been demonstrated by Reddy et al. (35) and more recently by Scheifinger et al. (40).

These considerations suggest that monitoring levels of methane production in an aquatic ecosystem might be a way to gauge the water quality. When organics are added to a water column the process of decomposition begins. One of the products of this is hydrogen gas. Any hydrogen produced in this fashion would be rapidly turned into methane. Methane is a poorly soluble gas which is only attacked biologically in the presence of oxygen. It is therefore likely that methane will remain in the water above the sediment or bubble up from the sediment to be detected. The idea is that increased levels of organic carbon would be transformed into methane which would serve as the indicator. Preliminary unpublished data from Lake Erie reinforce this concept. There seems to be higher levels of dissolved methane and higher production rates in areas of the lake near heavily populated areas where higher levels of organics would be found.

The assumption behind this is that the methanogenic bacteria are always present in sufficient numbers to assimilate all hydrogen and that hydrogen is rarely present in excess. Work with both the rumen (1) and sewage systems (47) demonstrates that hydrogen does not accumulate unless methanogenesis is inhibited indicating that the methane bacteria use hydrogen as fast as it is produced. It is probable that this is also the case in aquatic systems. In this study when hydrogen was added to lake sediments, there was a vast increase in methane production over endogenous levels.

Rates of methane production have been obtained for several cell types. These range from 0.53 to 2.9 μmoles per hour per 10^8 cells. The high and low values were obtained with a flask of M.O.H. and M. hungatii respectively. The overall average for all four species is 1.2 μmoles per hour per 10^8 cells. This should be considered the maximum rate of methanogenesis since it was obtained from log phase cells in the presence of excess hydrogen.

If methane is generated at a rate of 1.2 μmoles per hour per 10^8 cells, then the rate of hydrogen uptake should be approximately 4.4 μmoles per hour per 10^8 cells. It is interesting to compare this rate with rates published for other cells using hydrogen as an energy source. Uptake values for Hydrogenomonas facilis are 17.8, 28, and 28.8 μmoles per hour per 10^9 cells (28, 38, 39). A value for Hydrogenomonas ruhlandii is 5.6 μmoles H_2 per hour per 10^9 cells (33). These values were obtained in manometric studies with resting cells with oxygen in the atmosphere. A sulfate reducing species was reported to consume hydrogen at the rate of 4 μmoles per hour per 10^9 cells using sulfate as the terminal electron acceptor (10). This value was also obtained with manometric techniques. All of these values were reported on the basis of mg dry weight of cells and were converted to cell number using 10^9 cells per mg.

The value obtained for the methanogenic species is somewhat higher than the values for the other cells using hydrogen. It must be kept in mind that the rates for the other cells types were obtained from resting cell techniques while that for the methanogenic species was derived from growing cells. Nevertheless the values for rate of utilization of hydrogen for all three cells are reasonably close together.

This is especially interesting when one considers that these two species are very different from each other and are using three different compounds as electron acceptors. An interesting topic for further research would be an

investigation of the rate limiting step in methane generation. These data lead to speculation that it might be associated with the hydrogen utilization side of the picture.

Using the average rate of 1.2 μmoles CH_4 per hour per 10^8 cells one can calculate the potential for methane production if the number of methanogenic bacteria is known. For example Belyaev (2) has reported finding 2.5×10^3 to 2.5×10^5 methane producing cells per ml in the mud of several Russian lakes. The maximal rate of methane production should then be 0.03-3.0 nmoles per hour per ml sediment.

The converse of this line of reasoning also allows for some speculation. If hydrogen is present in excess, then the rate of methane generation should be determined by cell numbers. If one measured the rate of methane production over time it would be possible to make an estimate of the number of methane producers present. For example Macgregor and Keeney (25) obtained 400 nmoles per ml sediment as the maximum rate of methane generation in Lake Mendota sediment. They incubated the sediment for 48 hours under at atmosphere of helium at 10 C. This is a rate of 8.3 nmoles per ml sediment per hour. The minimum number of methanogenic organisms in that mud sample should be 7×10^5 bacteria per ml sediment. The numbers may in fact be higher than this since the temperature was rather low and the atmosphere was helium rather than hydrogen. Both of these facts would tend to depress the rate of methane generation. To accurately judge numbers from rates one would have to add excess hydrogen to the system. Due to the great affinity of methane producers for hydrogen, it is likely that they could effectively compete for the hydrogen even if many other cells of different types were present which would be the case in a sediment sample. It is also encouraging to note that in this study the rate obtained for the Lake Erie isolate was very close to that obtained for the

known pure cultures even though its growth rate was much slower. So it would not even be necessary for the cells in a sediment sample to be growing rapidly for a reasonably accurate rate to be obtained.

In conclusion there are several implications of this study which need to be reviewed and discussed. First of all the studies on hydrogen utilization show quite clearly that methane producing organisms are able to utilize essentially all hydrogen available to them. These studies also demonstrated that the accumulation of methane does not appear to be inhibitory. These two facts, in addition to the rate statements, might be important considerations in the design of anaerobic digestors for the purpose of the generation of methane for commercial purposes.

The possibility of using methane generation as an indicator of water quality has already been discussed as has the use of rates of production to determine numbers and numbers to estimate potential rate.

Methane generation is of major importance in anaerobic sewage treatment and is also important in stabilization of organics and carbon cycling in the aquatic environment. These results could therefore be useful to people in both these fields with respect to the design of models.

Finally it must be emphasized that since the rate of production of methane and the ability to utilize hydrogen are very similar for all species studied, the considerations mentioned above should hold for all systems regardless of which organism may be dominant.

Some areas for further work are the isolation of more methanogenic species from aquatic environments and investigation into the rate limiting step in methane generation. Also, reliable estimates of numbers of methanogenic organisms in an environment need to be correlated with rate of methane generation in order to establish the ability to estimate one from the other in mixed culture situations.

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SECTION III

INTRODUCTION

The biochemistry of methane production and bacterial methanogenesis has been the focus of several studies while other studies have examined bacterial methanogenesis in natural environments. The distribution and population of methanogenic species in aquatic sediments have not been investigated.

Various methods for the enumeration of methane bacteria in the natural environment have been reported but these methods have been dependent on Most Probable Number (MPN) techniques or on enrichment procedures. Even though these procedures provide valuable data concerning the total approximate number of methanogens, these methods cannot differentiate the genera of methane bacteria present. Also considering the fastidious nature of these strict anaerobes, difficulties may be incurred during sampling and/or subsequent handling during inoculation into the appropriate media. Thus an accurate method is needed which can determine the actual number and type of methanogens present in an untreated sample. The method of choice is the fluorescent antibody (FA) technique.

The purpose of this survey was to determine the rate of methane evolution from the sediments of selected sampling sites in Cleveland Harbor and to determine the distribution of several known genera of methanogenic bacteria in the harbor sediments. To supplement this data, methane production potentials were estimated from a series of in vitro experiments, and the sensitivity of the FA technique was increased by organic and inorganic enrichment of the sediment samples.

MATERIALS AND METHODS

Methane evolution rates were monitored by a specially constructed inverted sheet metal (18 ga.) cone giving a surface area of 0.5m^2 and associated gas trap. The gas trap consisted of an appropriately modified 100 ml plastic graduate cylinder stoppered with #6 neoprene stopper into which the top half of a Hungate anaerobic test tube was inserted. At a limited number of stations in Cleveland Harbor the cone was lowered on a steel cable to approximately 0.5 m above the harbor sediments. Evolved gases collected for an appropriate period of time after which the cone was retrieved and the volume of total gases evolved was measured. The gaseous samples were transferred to Hungate anaerobic test tubes which had been previously helium purged and evacuated. The samples were analyzed on location with a Carle model 8000 Basic Gas Chromatograph (silica gel column 60/80 mesh, 105°C, helium carrier gas) or were transported to the laboratory and were analyzed with a Varian 2740 flame ionization gas chromatograph (Porapak Q 100/120 mesh, 105°C, nitrogen carrier gas). The methane peaks were quantitated by a Linear Instruments integrating recorder model 252.

Methane production potentials were evaluated by obtaining a sediment sample (Ponar sediment sampler), a small quantity (5-10 cc) of which was placed into a 30 cc stoppered serum bottle. The subsamples were incubated at ambient temperatures for 5 days and then refrigerated until analyzed for methane as previously described.

The fluorescent antibody method used was similar to that of Schmidt et al. (2). Pure cultures of Methanobacterium ruminantium, Methanobacterium strain M.o.H., Methanosarcina barkeri, and Methanospirillum hungatii were grown in organic medium. Cells were harvested, washed in saline and resuspended to 10^9 cells/ml in sterile saline.

Antibodies were produced in ~3 kg white New Zealand rabbits by intravenous injection according to schedule reported by Schmidt et al. (2). The final tube agglutination of all species was in excess of 1:1000.

Blood samples were obtained by cardiac puncture and were centrifuged to harvest the serum. The serum was fractionated by ammonium sulfate precipitation similar to the methods of Schmidt et al. (2).

The protein was determined by the biunet method and the final solution was adjusted to 1% protein with phosphate buffer. The resulting protein solution was conjugated to fluorescein isothiocyanate (FITC). Conjugation was performed at 4°C overnight. Unreacted FITC was removed by exhaustive dialysis in phosphate buffer at 4°C. The final FITC conjugate was filtered sterilized and 2 ml aliquots were stored at -20°C.

Specificity of the conjugate was evaluated by staining a variety of bacterial species (Table 1).

Slide Preparations

The sediment samples to be analyzed were prepared as a slurry. A 0.05 ml suspension of this sample was heat fixed to a glass microscope slide and a sufficient volume of 1:1.5 rhodamine conjugated bovine serum albumin (Rh-BSA) (Microbiological Associates) was applied as a counter stain. The Rh-BSA was permitted to dry completely at 37°C, after which free Rh-BSA was removed by washing in 0.01 M phosphate buffer (pH 7.2). The specific FA stain was applied (0.05 ml volume) and the slide was incubated in a moist chamber for 1 hour at 37°C. After completing the incubation, the unreactive FA was removed by washing as before in phosphate buffer. The resulting slide was air dried (37°C), covered with coverslip and immersion oil and viewed with a Zeiss Universal microscope adapted with an ultraviolet (UV) epiilluminator.

TABLE III-1. Evaluation of Specificity of FITC Conjugates FA Staining Reaction*

Bacterial Species*	M.o.H.	M.R.	M.S.	J.F.
1) Azotobacter vinelandii (62)	-	-	-	-
2) Bacillus megaterium (21)	-	-	-	-
3) Bacillus megaterium (125) ATCC 19213	-	-	-	-
4) Bacillus subtilis (27)	-	-	-	-
5) Clostridium pasteurianum (441) ATCC 6013	-	-	-	-
6) Escherichia coli (395)	-	-	-	-
7) Methylococcus capsulatus (452) ATCC 19069	-	-	-	-
8) Pseudomonas denitrificans (564)	-	-	-	-
9) Rhodospirillum rubrum (828)	-	-	-	-
10) Thiobacillus novellus (407)	-	-	-	-
11) Methanobacterium strain M.o.H.	+4	-	-	-
12) Methanobacterium ruminantium	-	+4	-	-
13) Methanosarcina barkeri	-	-	+4	-
14) Methanospirillum hungatii	-	-	-	+4

* Staining was evaluated as +4 for strong reaction observed when homologous antigen and antibody were used. (-) indicated no apparent cross reaction when compared to homologous antigen-antibody reaction.

To increase the sensitivity of this FA technique both organic and inorganic media were used for enrichment procedures. Sterile enrichment media was inoculated with the appropriate sediment, purged with H_2:CO_2 (80:20) and incubated with shaking at 37°C for 3 weeks. The resulting, turbid broth cultures were checked for methane production, of which, significant quantities were observed in all enrichment cultures, organic and inorganic. These broth cultures were used directly for preparing heat-fixed smears and were processed by methods cited previously.

RESULTS

The methane production potentials (Table 2) indicate that very active methanogenesis is occurring in the entire Cleveland Harbor region. The most active sediments were those collected at station 1 which is in the Cuyahoga River proper. Stations 3 and 4 which are located at the junction of the Cuyahoga and the harbor and at a short distance east of the river input also demonstrate an extremely active methanogenic population. The slightly lower production potential observed in the west break wall region (station 2) may reflect the reduced effects of the Cuyahoga input due to the predominantly east ward flow of the river water. This difference is less apparent when one compares the in situ evolution rates (Table 2) monitored at stations 2 and 4. These evolution rates demonstrate the methanogenesis in situ are essentially identical at the two stations. Significantly lower production potentials and evolution rates are observed at stations 5 through 8. The sediments at these stations are similar in that they are predominantly fine silt with the exception of station 8 which is fine sand.

Even though there was a measurable gradient of methnogenesis occurring in the harbor region, the data indicated that an active methanogenic population was present.

Of the known methanogens for which specific FA had been prepared, only M. ruminantium and Methanobacterium strain M.o.H. were observed in untreated sediment samples (Table 3). M. ruminantium was demonstrated to be present only in the Cuyahoga River at station 1. The resident population was determined as $9.8 \cdot 10^8$ cells per gram of dry sediment. Methanobacterium strain M.o.H. was documented at two locales, stations 6 and 8, at population densities of 5.1 and $1.1 \cdot 10^6$ cells per gram of dry sediment respectively. The lack of this organism at the intermediate station 7 was surprising.

TABLE III-2. Methane Evolution and Production Potentials

Station Number	Evolution[a]	Production Potentials[b]
1		16.8
2	27.0	3.0
3		7.0
4	24.0	10.4
5		1.6
6	5.5	3.0
7		1.4
8	0.23	ND[c]

[a] Evolution rates wer mg methane per m^2 per hour

[b] Production potentials are recorded as 10^{-2} mg per gram of dry sediment per day

[c] No data

TABLE III-3. Methanogenic Population in Cleveland Harbor Sediments

Station	Natural Population[a]	Bryant's Organic	Smith's Inorganic
1	$9.8 \cdot 10^{8}$[b]	+[d]	0
2	0	+[d]	0
3	0	+[d]	0
4	0	+[d]	0
5	0	0	0
6	$5.1 \cdot 10^{6}$[c]	0	+[e]
7	0	0	+[e]
8	$1.1 \cdot 10^{6}$[c]	0	+[e]

[a] Natural population reported as number of cells per gram of dry sediment

[b] Cell number designates M. ruminantium

[c] Cell number designates Methanobacterium strain M.o.H.

[d] Indicates presence of Methanospirillum hungatii strain JF

[e] Indicates presence of Methanobacterium strain M.o.H.

Since there were 5 stations for which no methanogenic populations were observed and since there was measurable methanogenesis occurring at these stations, it was apparent that our FA system was not sensitive enough or else a natural resident methanogenic population, undetectable by our specific FA, was responsible for this methane generation.

It was impossible to evaluate the presence of a natural resident population with our specific FA, but it was possible to increase the sensitivity of our FA methods by resorting to various enrichment procedures (Table 3). The results indicate that our original FA techniques did lack sufficient sensitivity to detect some of the methanogens present. The organic enrichment procedures demonstrated that M. hungatii strain JF was present in the original sediment but was in such low numbers that a concentration or enrichment procedure was needed to evaluate its presence. Surprisingly M. ruminantium and Methanobacterium strain M.o.H. were not observed in the organic media. Inorganic enrichment resulted in a completely different population. The M. hungatii documented in the organic enrichment media at stations 1-4 did not appear in the inorganic salt media, whereas Methanobacterium strain M.o.H. did prevail at stations 6-8.

The major point of interest in that station 7 which demonstrated lack of a population of Methanobacterium strain M.o.H. in the untreated samples yielded an active population in the inorganic salt media. This fact could be explained by a low population in the sediment or faulty sampling during collection of the original sediment sample.

DISCUSSION

The data indicate tremendous methanogenesis is occurring in Cleveland Harbor and the Cuyahoga River sediments. Maximum evolution rates of 27.0 mg $CH_4/m^2/hr$ and production potentials of $16.8 \cdot 10^{-2}$ mg/g dry weight of sediment/day were recorded. The maximum production potential recorded is very similar to the maximum value of methanogenesis occurring in Lake Mendota sediment samples reported by Zeikus and Wenfry (3).

The gradient of methanogenesis occurring in the harbor sediments indicates an apparent environmental or nutritional diversity within a relatively defined locale. This unknown factor may be related to the contribution of the Cuyahoga River to the lake and harbor regions.

This diversity is also evident in the distribution observed for the methanogens. Only M. ruminantium and M. hungatii were observed naturally or were enriched in stations 1-4. M. hungatii could be enriched only in the organic medium and was restricted to stations 1-4. This may indicate that an organic factor may limit the growth or distribution of this methanogen. The sediments at stations 1-4 were fine silt and may have contained significant organic matter.

The absence of M. ruminantium in both enrichment media requires further investigation. Since, M. ruminantium was restricted to one specific locale, there is a possibility that a particular growth factor is required which is not found elsewhere in the sediments or enrichment cultures.

The occurrence of Methanobacterium strain M.o.H. at stations 6 and 8 in natural samples and at stations 6-8 in basal salt media strongly suggests that this species is competitive in a low organic environment. Earlier work had demonstrated that M. ruminantium requires acetate for growth and as a carbon source, and that Methanobacterium strain M.o.H. does not require but is

stimulated by acetate. This acetate requirement for M. ruminantium may be one of the factors effecting its distribution in the lake sediments. Similarly, formate can serve as substrate for M. hungatii. The lack of Methanobacterium strain M.o.H. in the organic media and at stations 1-5 demonstrates that other heterotrophs can easily dominate in organic environments. Zeikus and Winfrey (3) demonstrated the predominance of a Methanobacterium species in organically rich sediments of Lake Mendota. From the data presented here it would seem that the Mendota species is similar physiologically to M. ruminantium. However, our data is being interpreted without knowledge of the organic content, microbial metabolites or toxic chemicals which may be influencing this harbor population.

Unlike the sediments of Lake Mendota, the Methanosarcina species, M. barkeri, was not observed naturally nor could it be enriched in our sediment samples.

Station 5 was the only one which supported methanogenesis but which demonstrated absence of the specific methanogens surveyed. From this, one can speculate that an active, indigenous methanogen(s) is present. This hypothesis is further supported by the rapid methane evolution rate which indicates the presence of a methanogenic population. Similarly, an evolution rate as low as 0.23 mg $CH_4/m^2/hr$ corresponded to at least a methanogenic population of $1.1 \cdot 10^6$ cells/g dry weight; thus the maximum rate observed, 27.0 $mg/m^2/hr$, indicates a 100 fold increase, but at the maximum evolution station, only M. hungatii was observed after organic enrichment, further suggesting the presence of an unknown indigenous methanogen(s).

Even though the known methanogens are capable of growth on basal salts and $H_2:CO_2$ (McBride and Wolfe, 1971) this trait is not always expressed in enrichment cultures from the natural environment. The ability of some of

these methanogens to be stimulated by various small chain organic acids appears to be a selective factor in determining distributions in the natural system. Similarly, Methanobacterium strain M.o.H. seems to be more competitive in lower organic environments.

The lack of the known methanogens at some of the active stations monitored indicates the presence of an indigenous methanogen(s) which may represent a significant if not major member of the total methanogenic population.

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