PREDOMINANT AEROBIC BACTERIA OF FISH AND SHELLFISH

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Abstract

Determinative outlines, methods and materials are presented for classifying the majority of the aerobic, heterotrophic bacteria associated with fish and shellfish. Methods for nucleic acid base ratio analysis and a computor program for numerical taxonomy are included.

Preface

Microbial activities play major roles in limiting the value of fishery products by influencing their flavor and keeping quality and in limiting their availability by causing diseases in the aquatic animals. If the limiting factors are to be minimized, the responsible bacteria will need to be recognized and understood. However, much of the information on the indigenous bacteria of fish and shellfish is presented in terms too general to be of practical value to the diagnostician. The present work is an effort to provide means for rapid, tentative identification of many bacteria associated with fish and shellfish. More details on characteristics of the organisms may be obtained from appropriate references listed in the bibliography section.

Since opinions differ on the classification of aquatic bacteria, the determinative schemes of the present work are somewhat arbitrary and are intended to be neither authoritative nor final. The schemes were developed from data on type strains studied in the Department of Veterinary Microbiology at Texas A&M University and from characteristics listed in original and updated literature descriptions. Many of the bacteria classified by the schemes presented are found in brackish water environments and some of the media require saline additives for growth and proper identification of the bacteria.

Nucleic acid base analysis and numerical methods are being

used more frequently in microbial taxonomy. While those techniques are not currently practical for primary identification, the accuracy and reliability of primary identification schemes could be increased if the schemes were based upon information provided by those techniques. Consequently, methods for base analysis and numerical taxonomy are included in the present work to encourage the use of those techniques so that perhaps the bacteria of aquatic animals may be better understood.

Comments, favorable or unfavorable or suggestions for improvement of the document will be received by the author with utmost gratitude.

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Determinative Outlines

List of Abbreviations Used in Outlines

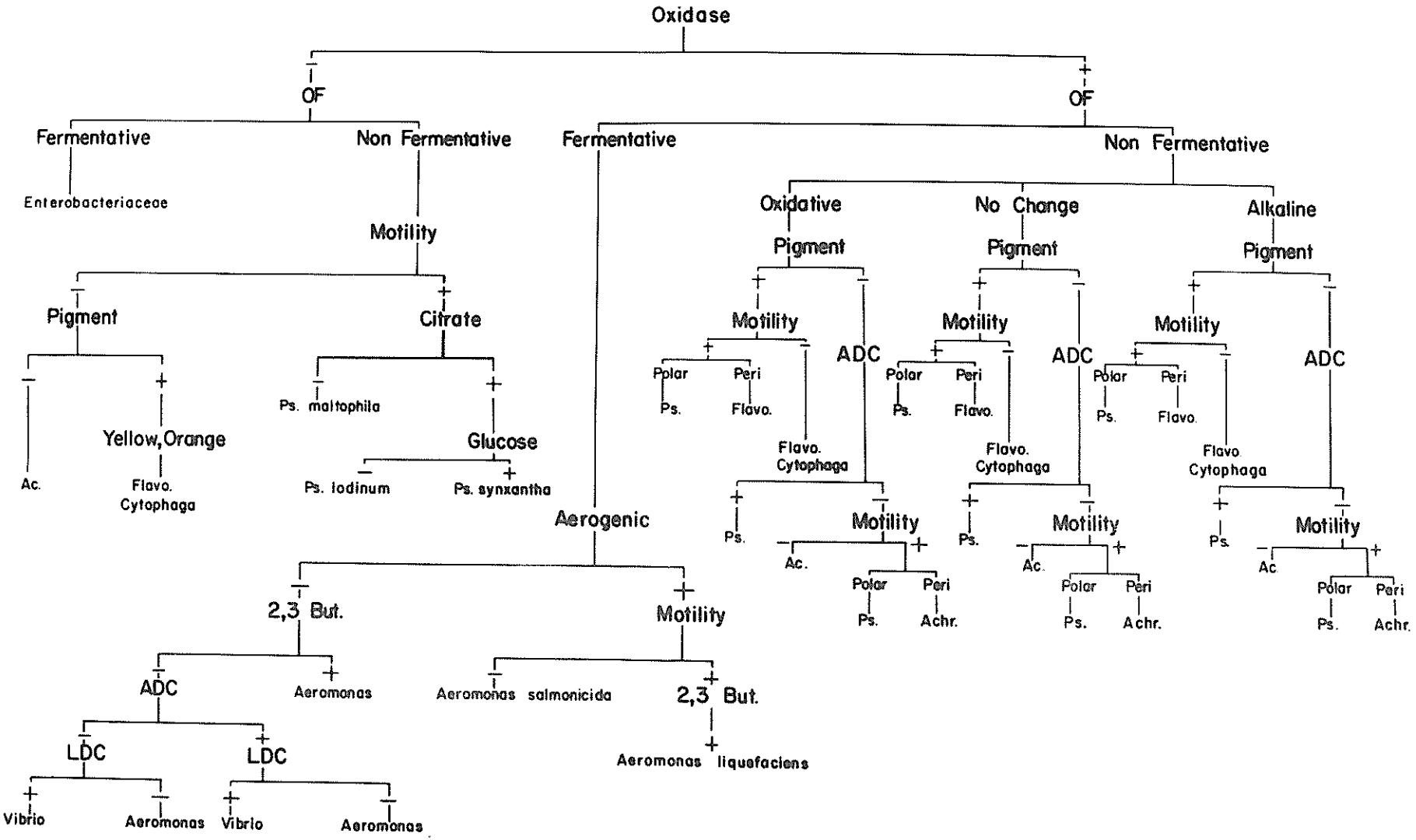
Details of the appropriate media, reagents and tests are listed in the paragraphs indicated in the Media, Reagents and Tests portion of the document.

The abbreviations listed below are used in the determinative outlines:

A = acid
Ac. = Acinetobacter
Achr. = Achromobacter
ADC = arginine decarboxylase
AK = acid becoming alkaline
Ben. = Beneckea
Brev. = Brevibacterium
2,3 But. = 2,3 butanediol
Fl. = fluorescein
Flavo. = Flavobacterium
K = alkaline
LDC = lysine decarboxylase
Lit. Milk = litmus milk
Micro. = Micrococcus
M. = Mycobacterium
NC = no change
NO_3 = Nitrate reduction
OF = oxidative or fermentative activity
Peri. = peritrichous flagella
Ph. = Photobacterium
Polar = polar flagella
Ps. = Pseudomonas
RC = reduced coagulated
RP = reduced peptonized
VP = voges proskauer
V. = Vibrio

Gram Negative Rods

DETERMINATIVE SCHEME FOR GRAM NEGATIVE RODS



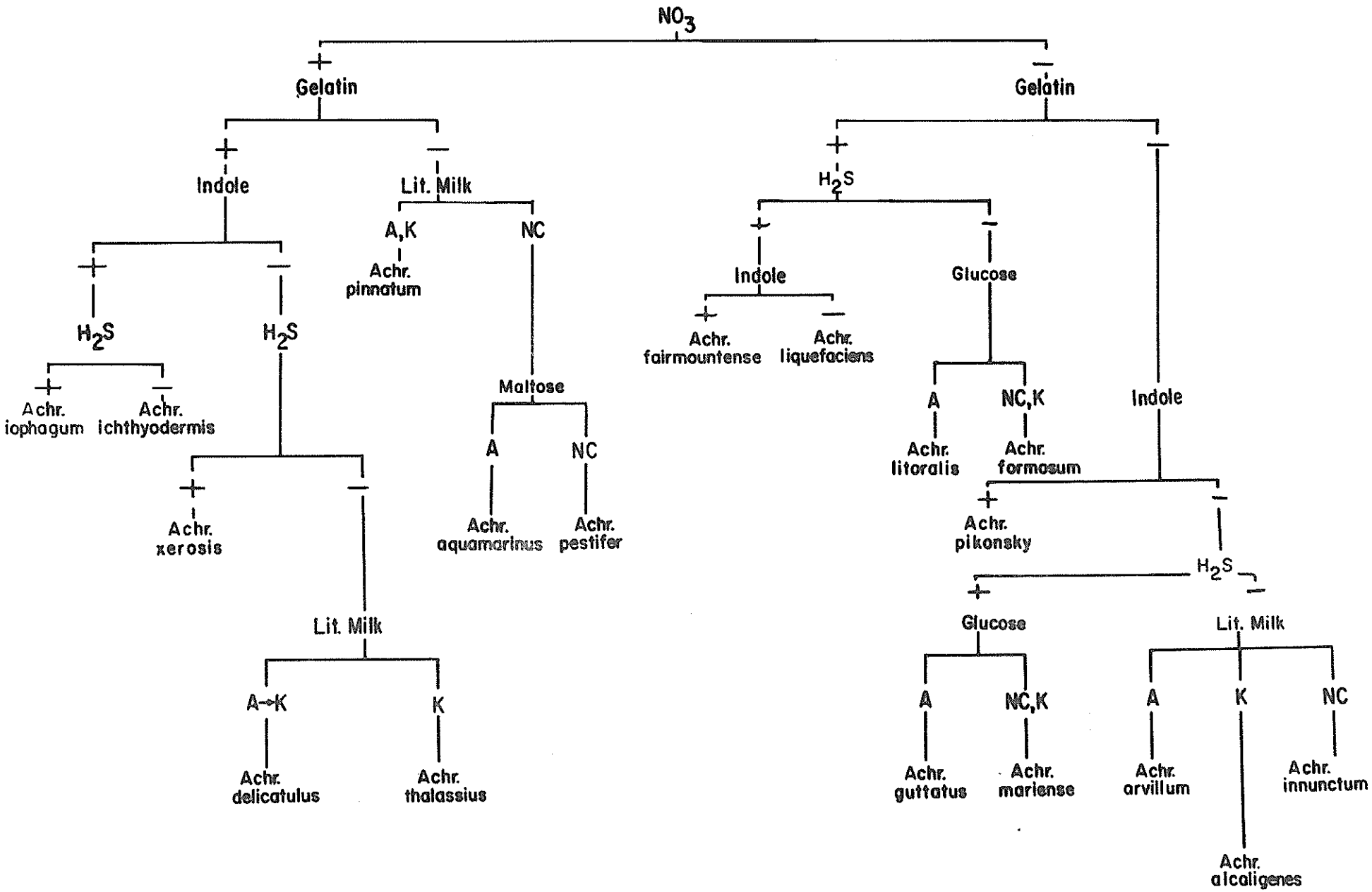
The classification of gram-negative rods to the genus level according to the determinative scheme presented, requires the inoculation of the following media:

1. Agar slant for determination of oxidase activity (paragraph 57), darkfield motility, flagella staining (paragraph 33), pigment production and for subsequent transfers.
2. Two tubes of oxidation-fermentation medium containing 1% glucose to determine mode of action on glucose.
3. Glucose fermentation medium (paragraph 32) with Durham tubes.
4. Arginine decarboxylase (paragraph 6).
5. Lysine decarboxylase (paragraph 6).
6. Glucose nutrient broth for determination of 2,3 butanediol production (paragraph 10).
7. Citrate medium (commercially available).

Nonmotile flavobacteria and cytophaga are not clearly delineated in the literature. Cytophaga sp. often possess a slime layer (paragraph 65), are capable of lysing bacterial cells (paragraph 7) and generally do not tolerate higher temperatures as well as do flavobacters.

Certain groups of gram-negative bacteria described in the scheme of the next page e.g. Beneckea sp., Chromobacterium sp., luminous bacteria and Pasteurella sp. possess unique features which are usually sufficient for preliminary categorization of those organisms. Hemophilus sp. are too fastidious for recovery on media not containing appropriate supplements (paragraph 38).

Achromobacter (Achr.)

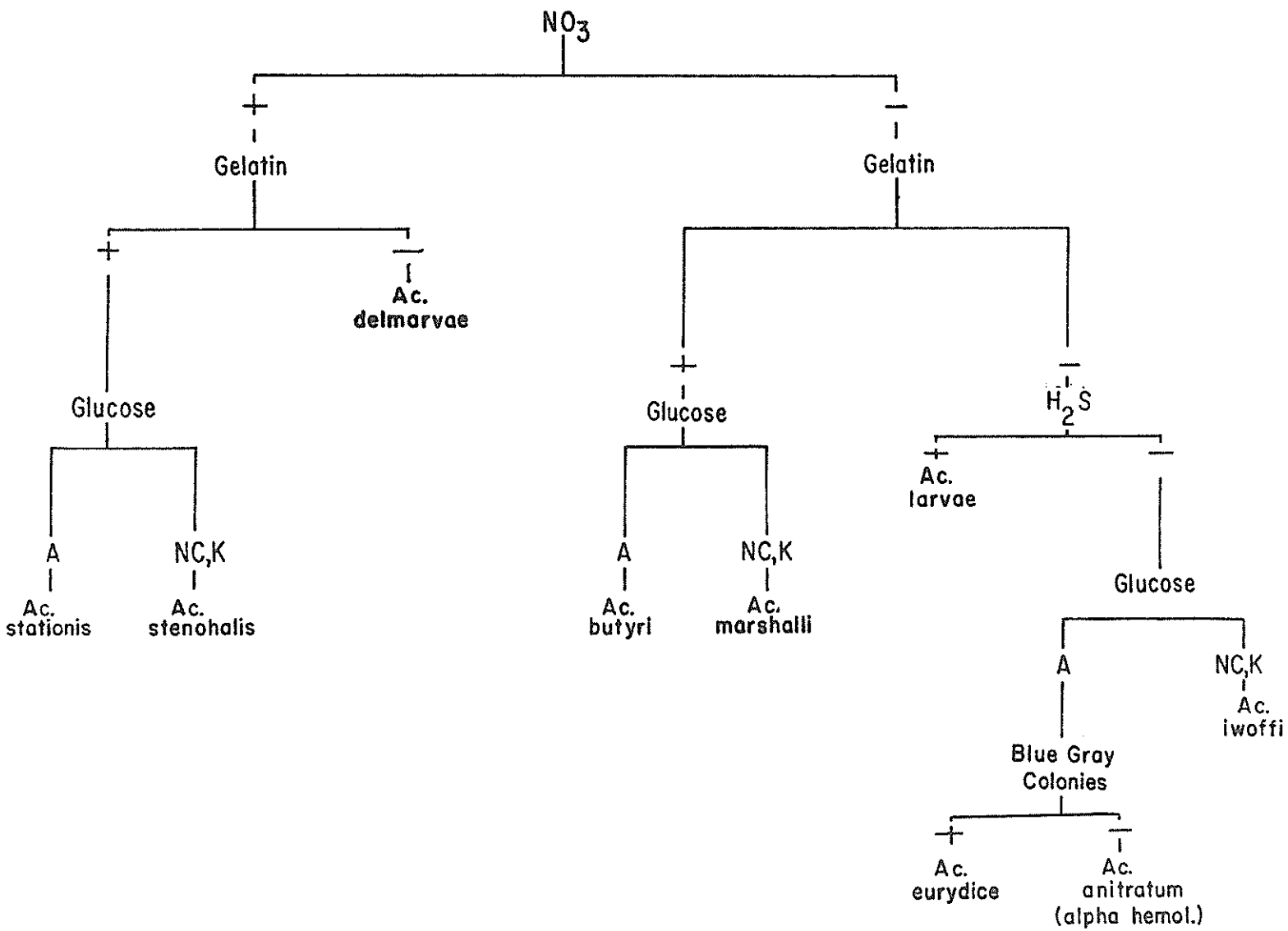


Achromobacter

Classification of Achromobacterium sp. into suggested species centers require inoculation of the following media:

1. Indole - Nitrate medium (commercially available) for performing indole test and nitrate reduction test (paragraph 54).
2. Gelatin agar plate for performing gelatin hydrolysis test (paragraph 34).
3. Triple sugar iron agar (commercially available) for determining H_2S production.
4. Litmus milk (paragraph 44)
5. Glucose fermentation broth
6. Maltose fermentation broth

Acinetobacter (Ac.)



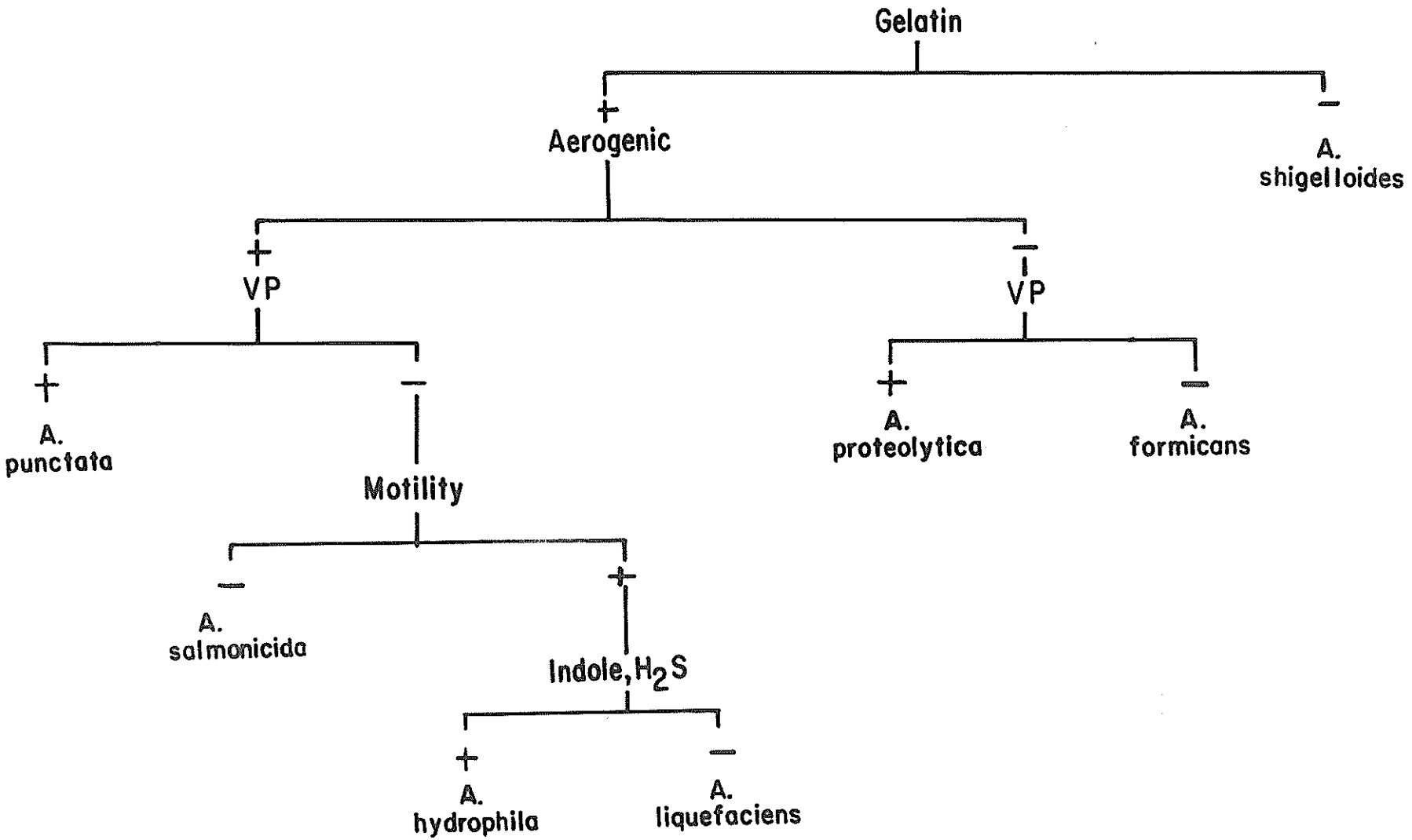
Acinetobacter

Classification of Acinetobacters into proposed species centers requires that the following media be inoculated:

1. Nitrate medium for performing nitrate test (paragraph 54).
2. Gelatin agar plate for performing gelatin hydrolysis test (paragraph 34).
3. Triple sugar iron agar for detecting H_2S production.
4. Glucose fermentation broth.
5. Trypticase soy agar for detecting blue gray colonies.

Members of the Acinetobacter sp. often occur as extremely short, thick rods closely resembling diplococci.

Aeromonas (A.)

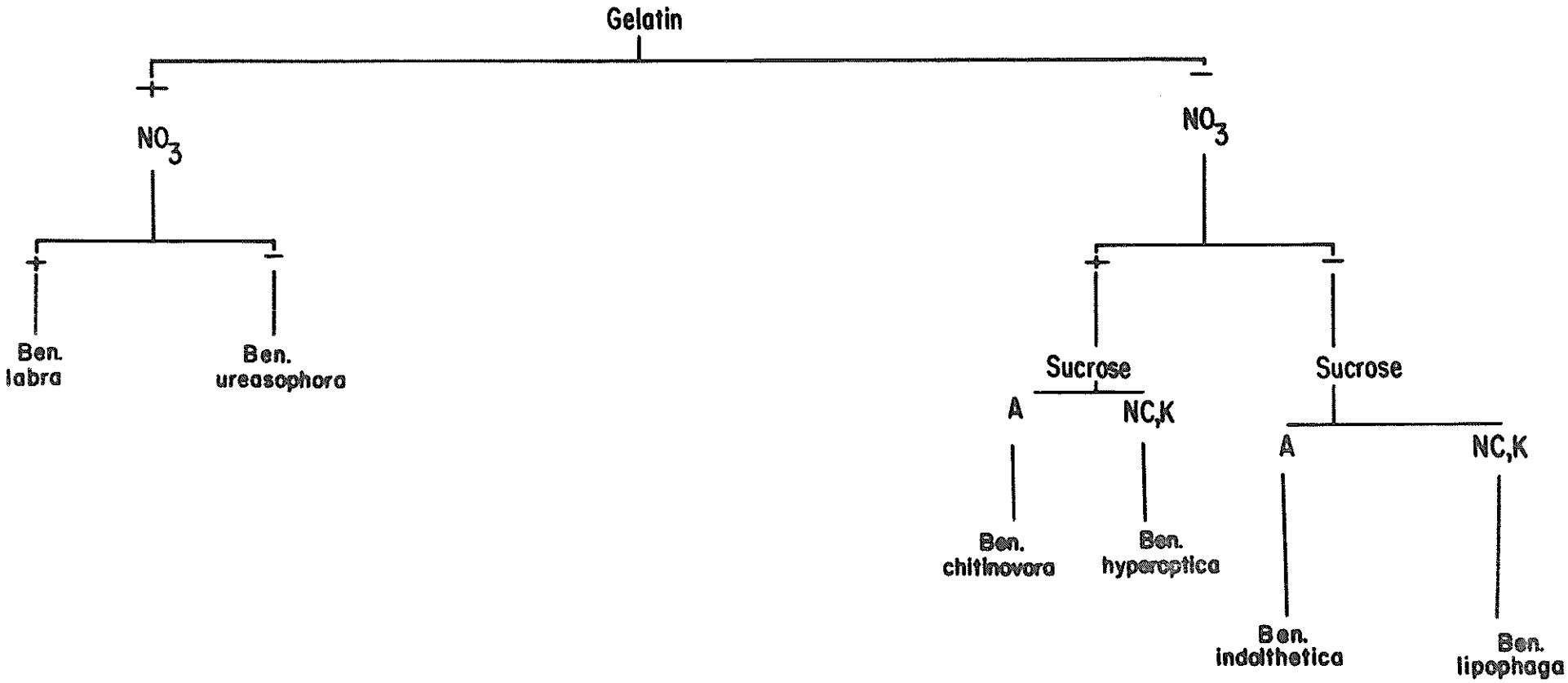

Gelatin
+
Aerogenic
−
A. shigelloides
+
VP
−
VP
+
A. punctata
−
Motility
+
A. proteolytica
−
A. formicans
−
A. salmonicida
+
Indole, H_2S
+
A. hydrophila
−
A. liquefaciens

Aeromonas

Classification of bacteria tentatively identified as Aeromonas sp. requires that the following media be inoculated:

1. Gelatin agar plate for gelatin hydrolysis test (paragraph 34).
2. Glucose fermentation broth containing Durham tubes for detecting aerogenesis.
3. MR-VP broth (commercially available) for Voges-Proskauer test.
4. Indole broth.
5. Triple sugar iron agar for detecting H_2S production.

Beneckea (Ben.)



Bacteria belonging to the genus Beneckea are characterized by their ability to degrade chitin (paragraph 17). Further identification requires inoculation of the following media:

1. Gelatin agar plate for gelatin hydrolysis test (paragraph 34).
2. Nitrate medium for performing nitrate test (paragraph 54).
3. Sucrose fermentation broth.

Chromobacterium

The chromobacteria are characterized by the production of an ethanol soluble, water and chloroform insoluble violet pigment. Sneath (119, 120) suggests that the test for violacein (paragraph 74) is specific for the genus Chromobacterium.

Characteristics of the genus Chromobacterium include:

Motile by both polar and lateral flagella
Utilize citrate but not malonate
Acid, but no gas from glucose
Nitrates reduced to nitrogen gas
Indole, VP, Urease negative
Ammonia produced from peptones

	Growth at 4 C	Growth at 37 C	Lecinthinase	Trehalose	Aesculin	
Chromobacterium violaceum	-	+	+	A	-	
Chromobacterium lividum	+	-	-	-	-	

Cytophaga

	Gel	NO_3	Catalase	Glucose	Starch	Lactose	Sucrose	H_2S	Indole	Pigment	
Cytophaga sp. 1	+	+	+	A	+	A	–	+	–	yellow	
Cytophaga marinoflava	+	+	+	A	–	A	A	–	–		
Cytophaga sp. 2	+	+	+	–	–			–	–		
Cytophaga krzemienskae	+	+		A	+	A	–	–	–	pink	
Cytophaga latercula	+	+	–		–				+	red	
Cytophaga diffluens	+	+	–	A	–	–		–	–	orange	
C. diffluens var aprica	+	–	–	A	+			+			
Cytophaga salmonicolor	+		+	A	+	A	A			red	
Cytophaga fermentans	+	+	+	A	+	A	A			grey	
Cytophaga lytica	+	+	+		+			–		yellow	
Cytophaga succinicans	+	–	+	A	+	A	A			orange	
Cytophaga sp. 3	+	–	+	A	+	–	–	+	–		
Cytophaga sp. 4	+	–	+	–	+	–	–	–	–		
Chondrococcus columnaris	+	–	+	–	–	–	–	+	–	yellow	
Cytophaga johnsonii	+	–	–	A	+	A	A		–	yellow	Decomposes chitin
Cytophaga sp. 5	+	–	–	–	+	–	–	–	–		
Cytophaga psychrophila	+	–	–	–	–	–	–	–	–		
Cytophaga sp. SKG	–	+	+	A	+				–		Brownish Diff. Pig.
Cytophaga sp. 6	–	–	+	–	–				–		Brownish Diff. Pig.
Cytophaga sp. 7	–	–	+	–	+				–		Brownish Diff. Pig.
Cytophaga sp. 8	–	–	+	–	–				–		Non pigmented
Cytophaga sp. 9	–	–	+	–	+				–		Non pigmented
Cytophaga sp. 10	–	–								yellow	

Enterobacteriaceae

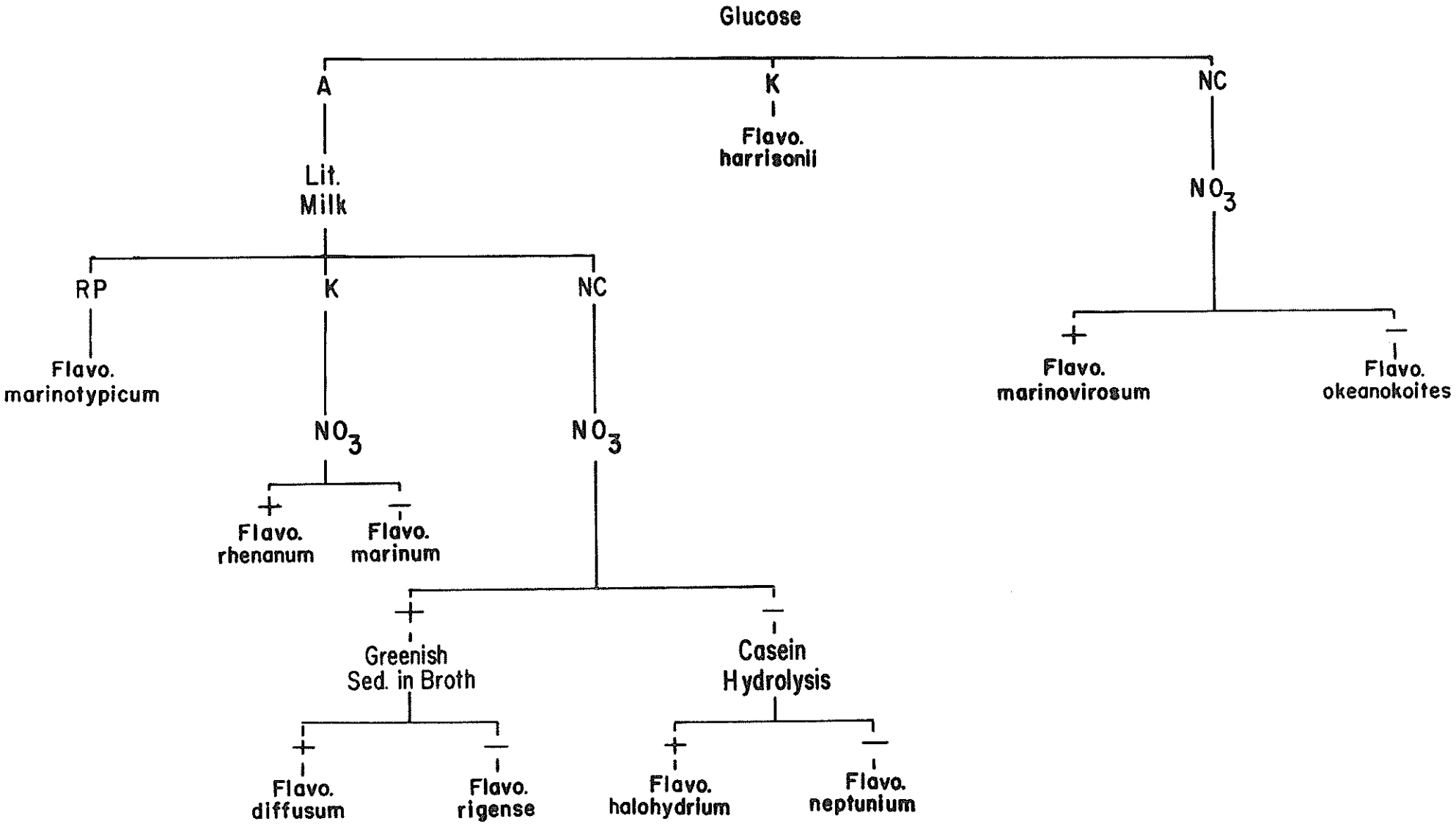
	H_2S	Indole	MR	VP	Citrate	Urease	Lactose	Mannitol	Dulcitol	LDC	ADH	ODC	
Escherichia	-	+	+	-	-	-	+	+	v	v	v	v	
Klebsiella	-	-	-	+	+	+	+	+	v	+	-	-	Malonate +
Citrobacter	+	-	+	-	+	-	v	+	v	-	v	v	
Arizona	+	-	+	-	+	-	v	+	-	+	+	+	Gelatin +
Salmonella	+	-	+	-	+	-	-	+	+	+	+	+	
Proteus	+	v	+	-	v	+	-	-	-	-	-	v	Gelatin +
Edwardsiella	+	+	+	-	-	-	-	-	-	+	-	+	
RM bacterium	-	-	+	-	+	-	-	+	-	+	-	+	Gelatin +

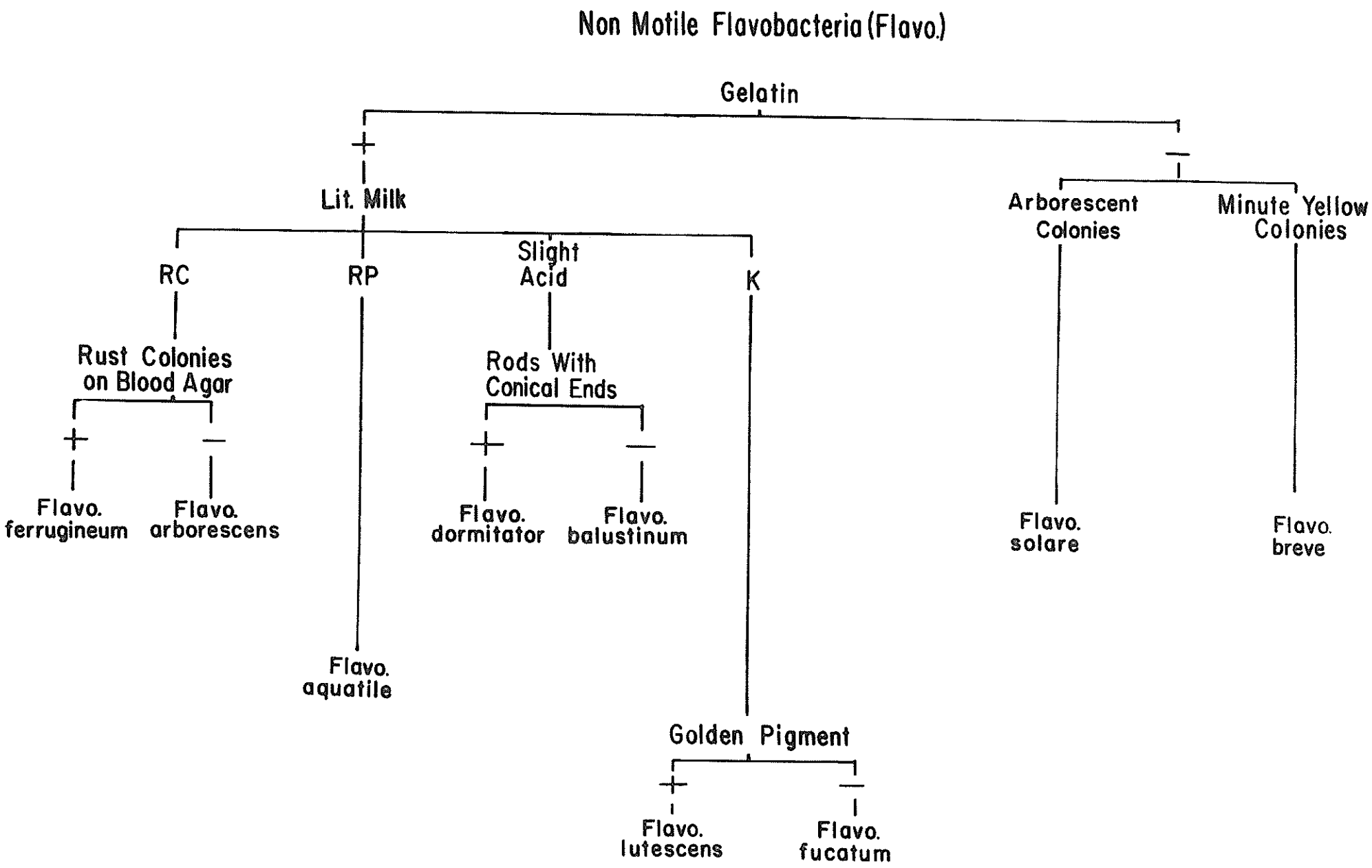
Flavobacteria

Classification of bacteria tentatively identified as Flavobacterium sp. is performed by inoculating the following:

1. Glucose fermentation broth
2. Litmus milk (paragraph 44)
3. Nitrate medium for performing nitrate test (paragraph 54).
4. Broth
5. Casein agar for detecting casein hydrolysis (paragraph 14).

Motile Flavobacteria (Flavo.)




Non Motile Flavobacteria (Flavo.)
Gelatin
+
−
Lit. Milk
Arborescent Colonies
Minute Yellow Colonies
RC
RP
Slight Acid
K
Rust Colonies on Blood Agar
Rods With Conical Ends
+
−
+
−
Flavo. ferrugineum
Flavo. arborescens
Flavo. dormitator
Flavo. balustinum
Flavo. solare
Flavo. breve
Flavo. aquatile
Golden Pigment
+
−
Flavo. lutescens
Flavo. fucatum

Hemophilus piscium (121)
(Ulcer Disease Organism)

Hemophilus piscium will not grow on routine culture medium, but can be cultivated on Hemophilus agar (paragraph 38). Characterization tests are performed in a basal medium consisting of the following components in 0.5% amounts in distilled water: tryptic digest of casein, yeast extract, proteose peptone no. 3 (Difco) and sodium chloride. The basal medium is enriched with potato extract as used in Hemophilus agar.

Characteristics of the organism include:

Optimum growth temperature = 20-25 C
Colonies small, 2 - 3 mm after one week
Facultative anaerobe
Glucose, fructose, sucrose, acid, no gas
Weak acid from Mannose, Galactose, Cellobiose, Dextrin
No change in Arabinose, Xylose, Rhamnose, Lactose, Melibiose, Raffinose, Melizitose, Insulin, Glycerol, Adonitol, Mannitol, Dulcitol, Esculin, Inositol
Nitrates not reduced
Indole negative
H_2S negative
Methyl Red positive
Acetyl Methyl Carbinol produced
Starch hydrolyzed
Gelatinase not present
Beta hemolysis (rabbit erythrocytes)
No change in Litmus milk

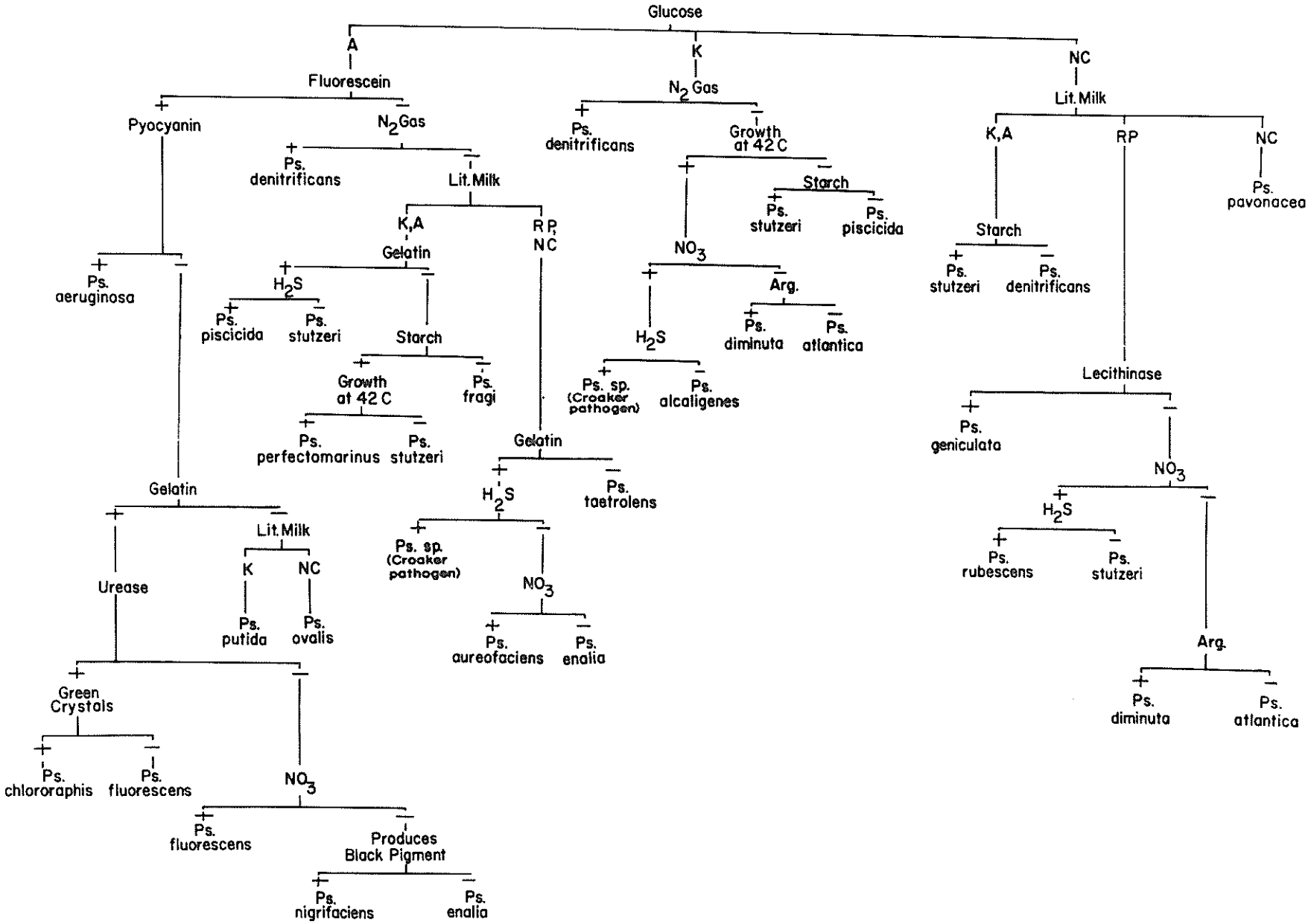
Luminous Bacteria

	Gel	Indole	Suc	Lac	VP	2-3 But	
Lucibacterium harveyi	+	+	A	A	-	-	
V. albensis	+	+	A	-	+	+	
Ph. splendidum	+	+	-		-		
Ph. mandapamensis	+	-	-	-	+	-	
V. fischeri	+	-	-	-	-	-	
Ph. pierantonii	-	-	A	A	-	-	
Ph. phosphoreum	-	-	-	-	+	+	oxidase negative

Pasteurella

	Motility	Hemolysis	Indole	Litmus Milk	NO_3	VP	MR	H_2S	Glucose	Sucrose	Lactose	
Yers. philomiranga	-	-	-	R	-			-	A	A	-	
Past. novicida	-	-	-	NC	-	-	-	+	A	A	-	
Past. piscicida	-	-	-		-	+	-	-	A	A	-	Requires at least 0.5% NaCl and no growth at 37C.
Past. sp. (Galveston)	+		+	NC	+	+	+	-	A	-	-	
Past. tularensis	-		-	NG				+	A	-	-	No growth in nutrient broth or agar without special enrichment.
Past. multocida	-	-	+	NC	+	-	-	-	A	A	-	

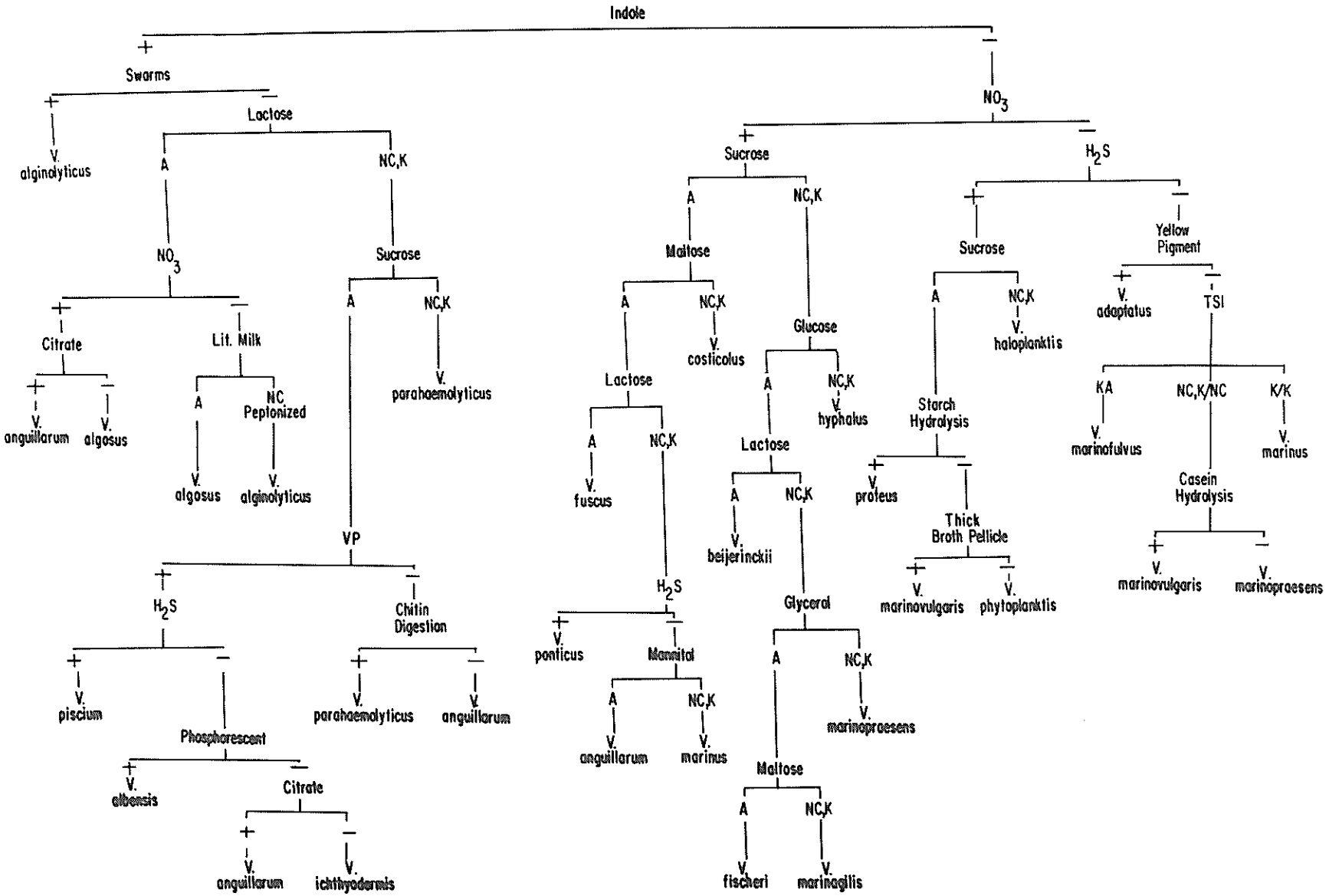
Pseudomonas



Classification of Pseudomonas into proposed species centers requires that the following media be inoculated:

1. Glucose fermentation medium
2. Pseudomonas P medium (commercially available)
3. Pseudomonas F medium (commercially available)
4. Litmus milk (paragraph 44)
5. Triple sugar iron agar for detecting H_2S production
6. Broth to be incubated at 42C
7. Egg yolk medium for determining lecithinase activity (paragraph 43)
8. Arginine decarboxylase medium (paragraph 6)
9. Nitrate medium (paragraph 54)
10. Gelatin agar plate for performing gelatin hydrolysis test (paragraph 34)
11. Urea agar (commercially available) for detecting urease activity

Vibrio (V.)


Indole
+
Swarms
+
V. alginolyticus
−
Lactose
A
NO3
+
Citrate
+
V. anguillarum
−
V. algosus
−
Lit. Milk
A
V. algosus
NC Peptonized
V. alginolyticus
NC,K
Sucrose
A
VP
+
H2S
+
V. piscium
−
Phosphorescent
+
V. albensis
−
Citrate
+
V. anguillarum
−
V. ichthyodermis
−
Chitin Digestion
+
V. parahaemolyticus
−
V. anguillarum
NC,K
V. parahaemolyticus
−
NO3
+
Sucrose
A
Maltose
A
Lactose
A
V. fuscus
NC,K
H2S
+
V. ponticus
−
Mannitol
A
V. anguillarum
NC,K
V. marinus
NC,K
V. costicolus
Glucose
A
Lactose
A
V. beijerinckii
NC,K
Glycerol
A
Maltose
A
V. fischeri
NC,K
V. marinagilis
NC,K
V. hyphalus
NC,K
V. marinopraesens
−
H2S
+
Sucrose
A
Starch Hydrolysis
+
V. proteus
−
Thick Broth Pellicle
+
V. marinovulgaris
−
V. phytoplanktis
NC,K
V. haloplanktis
−
Yellow Pigment
+
V. adaptatus
−
TSI
KA
V. marinofulvus
NC,K/NC
Casein Hydrolysis
+
V. marinovulgaris
−
V. marinopraesens
K/K
V. marinus

The classification of Vibrios into proposed species centers requires inoculation of the following media:

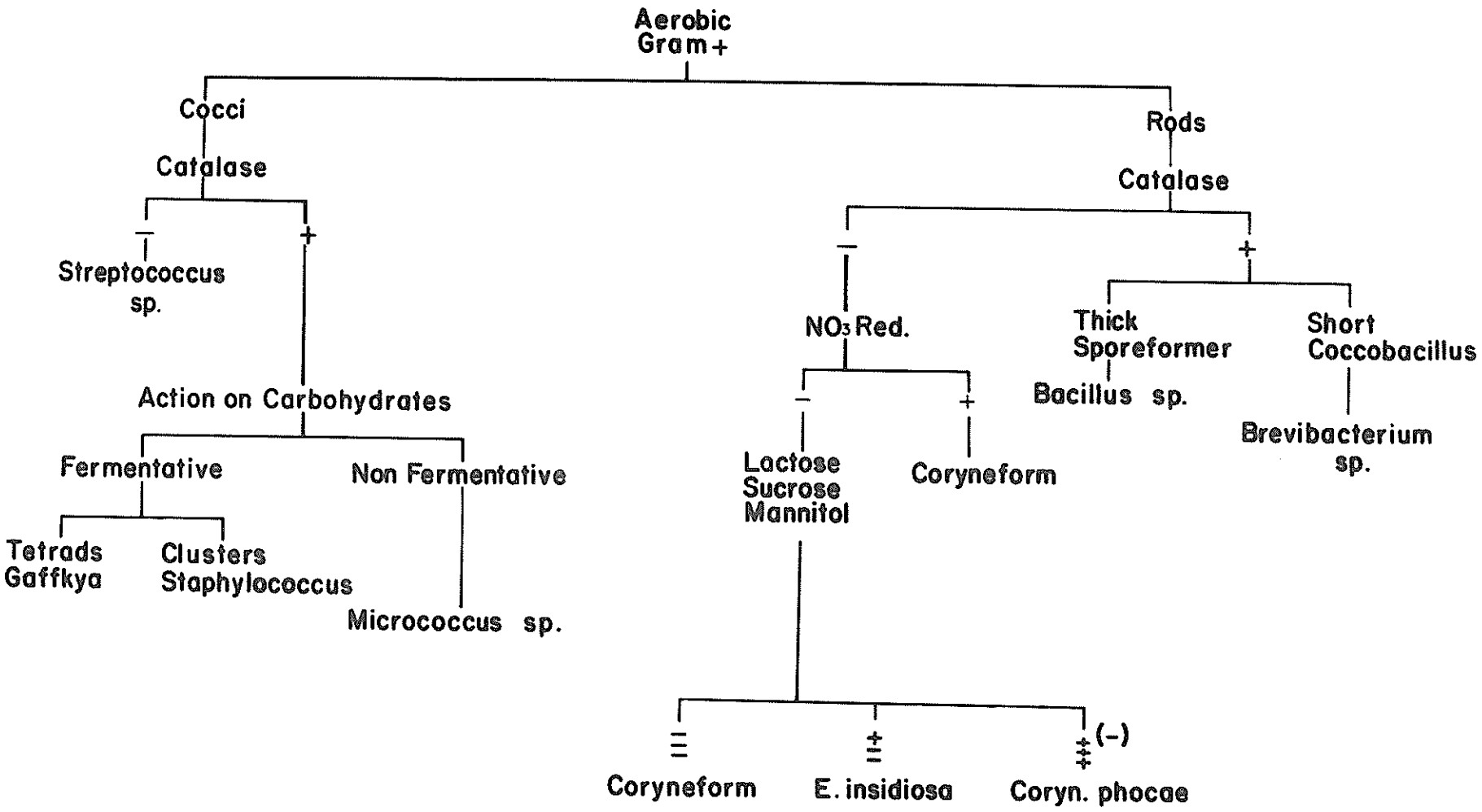
1. Indole-Nitrate medium for detecting indole production and ability of the organisms to reduce nitrate (paragraph 54)
2. Glucose fermentation broth
3. Maltose fermentation broth
4. Lactose fermentation broth
5. Sucrose fermentation broth
6. Mannitol fermentation broth
7. Glycerol fermentation broth
8. Salicin fermentation broth
9. Triple sugar iron agar for detecting H_2S production
10. Voges-Proskauer broth
11. Casein agar for detecting casein hydrolysis (paragraph 14)
12. Chitin agar for detecting chitinoclastic activity (paragraph 17)
13. Broth
14. Litmus milk (paragraph 44)

Gram Positive and Acid Fast Bacteria

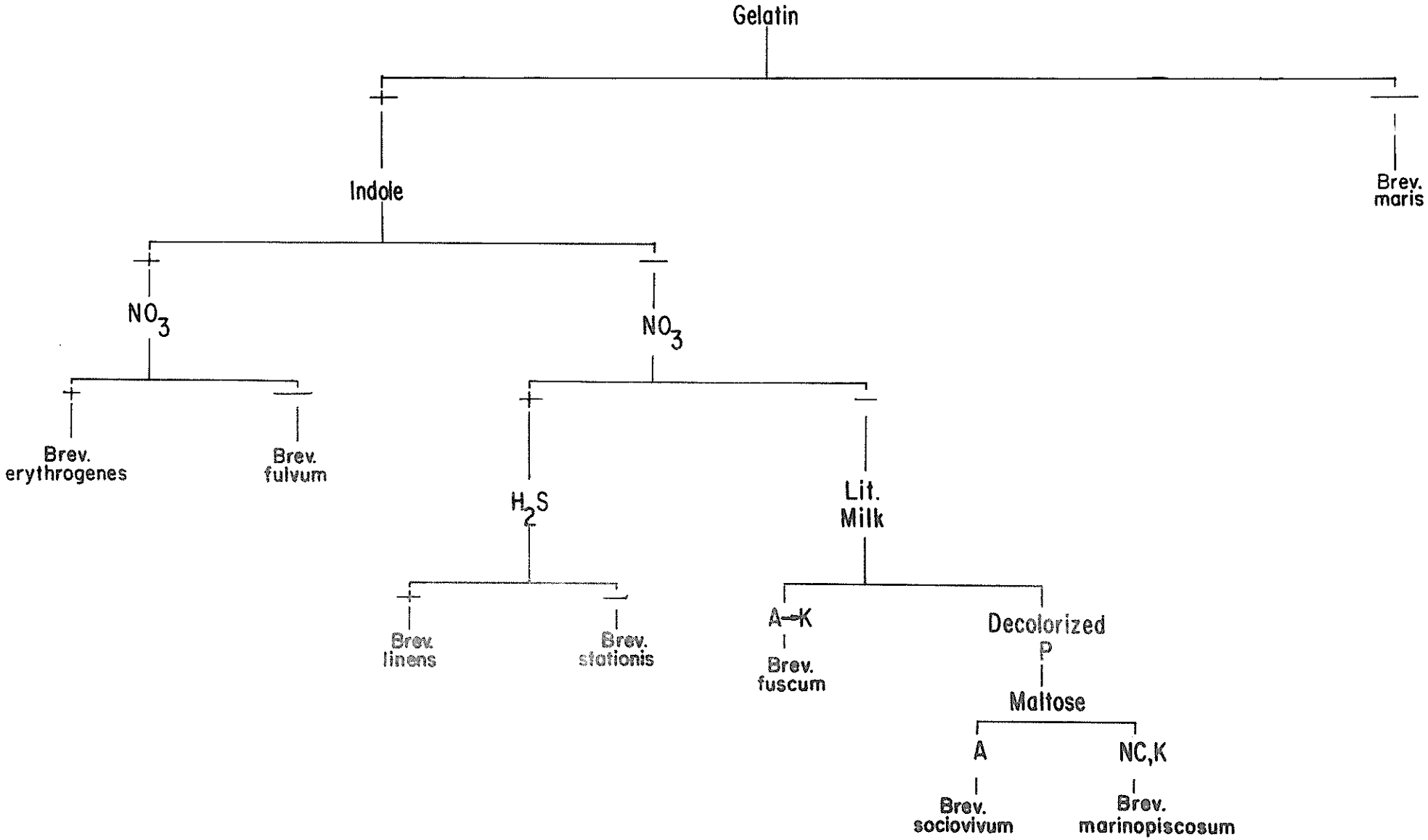
Gram Positive and Acid Fast Bacteria

As with many of the gram-negative organisms, information on the distribution and taxonomy of the gram-positive organisms in aquatic environments is fragmentary at present. The gram-positive organisms included in the following charts therefore are not intended to constitute a complete listing. The charts reflect organisms most likely to be encountered in fish and shellfish while some genera of bacteria, eg. Bacillus, have been deferred for later consideration.

Determinative Outline For Gram Positive Bacteria



Brevibacterium (Brev.)



Coryneform Bacteria

	Gel	Indole	H_2S	Glucose	Glycerol	Arabinose	Sucrose	Mannitol	Salicin	
Corynebacterium phocae	-	-	-	A	A	-	A	A	A	Doesn't Grow on potassium tellurit
Erysipelothrix insidiosa	-	-	+	A	-	A	-	-	-	Grows on potassium tellurite

Small, Gram positive diplobacilli found in kidneys of certain diseased fish require special medium (paragraph 42) for cultivation. This group of bacteria has not been adequately studied, but is believed to be a member of Corynebacterium sp. (92).

Micrococcus

	Gel	Glucose	Salicin	Motility	Pigment	
Micro. conglomeratus	+	A		-	Yellow	
Micro. varians	-	A	-	+	Yellow	
Micro. agilis	-	A	-	+	Rose	
Gaffkya homari	-	A	+	-	White	Fermentative, B. hemolytic
Micro. halodurans	-	A		-	White	Fermentative, non hemolytic
Micro. colpogenes	-	-	-	-	Yellow	Attacks chitin

Mycobacteria
(Rapid Growers)

	Growth rate											
	45°C	52°C	Dextrose	Trehalose	Starch	Nit. red.	Xylose	Dulcitol	Raffinose	Inositol	Sucrose	
M. fortuitum	-	-	A	A	+	+	-	-	-	-	-	Off white to yellow pigment
M. salmoniphilum	-	-	A	A	-	-	-	-	-	-	-	Light purple, amber diffusible pigment
M. smegmatis	+	-	A	A	+	+	A	A	-	A	-	Yellow to orange
M. ranae			A	A			A	-	-	A	-	
M. phlei	+	+	A	A	+	+	A	-	A	-	-	Yellow to orange
M. marinum	-	-	A	-	-	-	-	-	-	-	-	Yellow, thin beaded or barred rods
M. platypoecilus	-	-	A	-			-	-	-	-	-	Orange
M. thamnopheus	-	-	A	A	+		-	-	-	-	A	Pink

Streptococcal Fish Pathogen (103)

The streptococcal fish pathogen described by Robinson and Meyer (103) was grouped serologically as Lancefield Group B and was negative for the following tests:

H_2S, NH_3, Gelatin, Urea, Amylase, Methylene Blue Milk reduction, Nitrate to Nitrite reduction, 10%, 40% bile tolerance, 4% and 6.5% NaCl tolerance.

In 14 carbohydrates tested, acid was produced in only maltose and sucrose.

Nucleic Acid Base Analysis

Future descriptions of bacteria will most likely include quantitative expressions of the deoxyribonucleic acid (DNA) base composition of each new isolate. It is desirable therefore, that methods for estimating this quantity be available to investigators likely to be encountering undescribed bacteria. Currently, the molar percent guanine plus cytosine (%GC) is used for assessing the relatedness of various genera of bacteria and values for the various genera are listed in several references (13,26,27,28,31, 59,75,76,77). Current methods used in estimating %GC are based upon absorbancy measurements (32,44,138), measurements of thermal denaturation (34), and buoyant density (63,84,107) of DNA solutions.

Extracting Bacterial DNA

Methods for extracting bacterial DNA are based upon Marmur's procedure (78) and require approximately 2 - 3 g (wet weight) of bacterial cells. Generally, 1 - 2 mg DNA may be derived from 1 g cells.

Reagents

Lysozyme (Armour crystalline) is used to lyse cells resistant to detergent action. The lysate is then subjected to sodium lauryl sulfate treatment.

Sodium lauryl sulfate, 25%, an anionic detergent is used to lyse cells, inhibit enzyme action and denature some proteins.

Saline-EDTA, 0.15M NaCl (8.76g/L) and 0.1M ethylene diamine tetra acetate (41.62g/L) at pH 8. The EDTA and/or high pH inhibits DNAase activity.

Saline-citrate, 0.15M NaCl and 0.015M trisodium citrate (4.41g/L) pH 7.0 $\pm$ 0.2 maintains the ionic strength of dissolved DNA and chelates divalent ions.

Dilute Saline-citrate is prepared by diluting saline citrate 10X.

Water saturated with phenol is used to extract nucleic acids.

Chloroform-isoamyl alcohol, 24:1 is used for deproteinization. The chloroform causes surface denaturation of the proteins. Isoamyl alcohol reduces foaming, aids separation and maintains the stability of the layers of the deproteinized solution.

Ethanol, 95% is used to precipitate nucleic acids following deproteinization.

Ribonuclease, 0.2% crystalline in 0.15M NaCl, pH 5.0

digests the RNA and facilitates its separation from DNA.

Acetate-EDTA, 3.0M sodium acetate (408g/L) and 0.001M EDTA (0.416g/L), pH 7.0 provides the proper ionic environment in the isopropanol step for the separation of DNA from RNA.

Isopropanol, 100% is used to precipitate DNA.

Procedure

1. Cells grown in liquid medium are harvested in late log phase and washed once in 50 ml saline-EDTA. Afterwards, the cells are suspended in 25 ml saline-EDTA for lysing.

2. Lysing the cells:

a. Gram positive cells: Add 400 ug to 1 mg lysozyme/ml cell suspension in 25 ml saline-EDTA. Incubate at 37 C until lysis is complete (20 - 30 minutes). Add small amount of saline-EDTA to reduce viscosity then proceed as for Gram negative organisms.

b. Gram negative cells: Add 2 ml sodium lauryl sulfate to 25 ml cell suspension and incubate 15 minutes at 60 C.

3. Dissociating DNA from nucleoprotein:

a. Cool the lysate to 4-6 C in an ice bath and add an equal volume of cold water saturated with phenol.

b. Centrifuge 15 - 20 minutes in a refrigerated centrifuge approximately 5000 to 10000X G and remove the upper layer containing DNA.

c. Transfer the interface to a separate tube and add 5 ml cold saline-EDTA solution. Add an equal volume of cold water saturated with phenol, shake to obtain a homogenous emulsion then centrifuge as before. Remove the upper DNA containing layer and combine with the first extract.

d. Add an equal volume of chloroform-isoamyl alcohol, shake 3 minutes, then centrifuge as before for 15 minutes and remove the upper layer containing DNA.

4. Precipitating the DNA:

a. Layer 2 volumes of 95% ethanol on top of the DNA solution.

b. Mix the two layers by stirring with a glass rod inserted just below the interface. The nucleic acids spool on the rod as fibers and are easily removed.

c. Redissolve the fibers in 5 - 10 ml dilute saline-citrate, add ribonuclease to a final concentration of 50 mg/ml and incubate the mixture for 30 minutes at 37 C.

d. Deproteinize with chloroform-isoamyl alcohol as in "3d" above until an interface is barely visible.

e. Remove the upper DNA-containing layer and add 1 ml acetate-EDTA for each 9 ml of DNA solution.

f. Add 0.54 volumes 100% isopropanol one drop at a time while stirring with a glass rod. Wash DNA with fresh isopropanol.

g. The DNA may be stored in saline-citrate with a drop of chloroform added as a preservative, or may be kept in alcohol.

Estimating Percent Guanine-Cytosine (%GC) by Absorbancy Ratios (31,32)

The technique of estimating the percentage of guanine and cytosine to total bases (%GC) by the absorbancy ratios $E_{260/280}$ is not recommended for precision %GC determinations. The technique is useful however, for categorizing an undescribed bacterium or for a quick approximation of the thermal denaturation point (T_m) or buoyant density (α) of an unknown DNA solution (30,31).

Procedure

The purified DNA is dissolved in 3 ml 0.1N acetic acid to a concentration that yields an optical density of 0.5 - 1.0 (25 - 50 µg DNA/ml) at 260 mµ. The values should be corrected for blank optical density at both 260 mµ and 280 mµ. Between 38% and 72% GC, the moles %GC is approximated by the relationship:

$$\%GC = 168.6 - 87.4\ (E_{260}/E_{280})$$

Values for the relationship %GC and E_{260}/E_{280} are presented graphically in figure 1.

The relationship between T_m and the absorbancy ratio is as follows:

$$T_m = 138.4 - 35.8\ (E_{260}/E_{280})$$

The relationship between α and the absorbancy ratio is as follows:

$$\alpha = 1.825 - 0.0857\ (E_{260}/E_{280})$$

These relationships are graphically presented in figures 2 and 3.

Figure 1

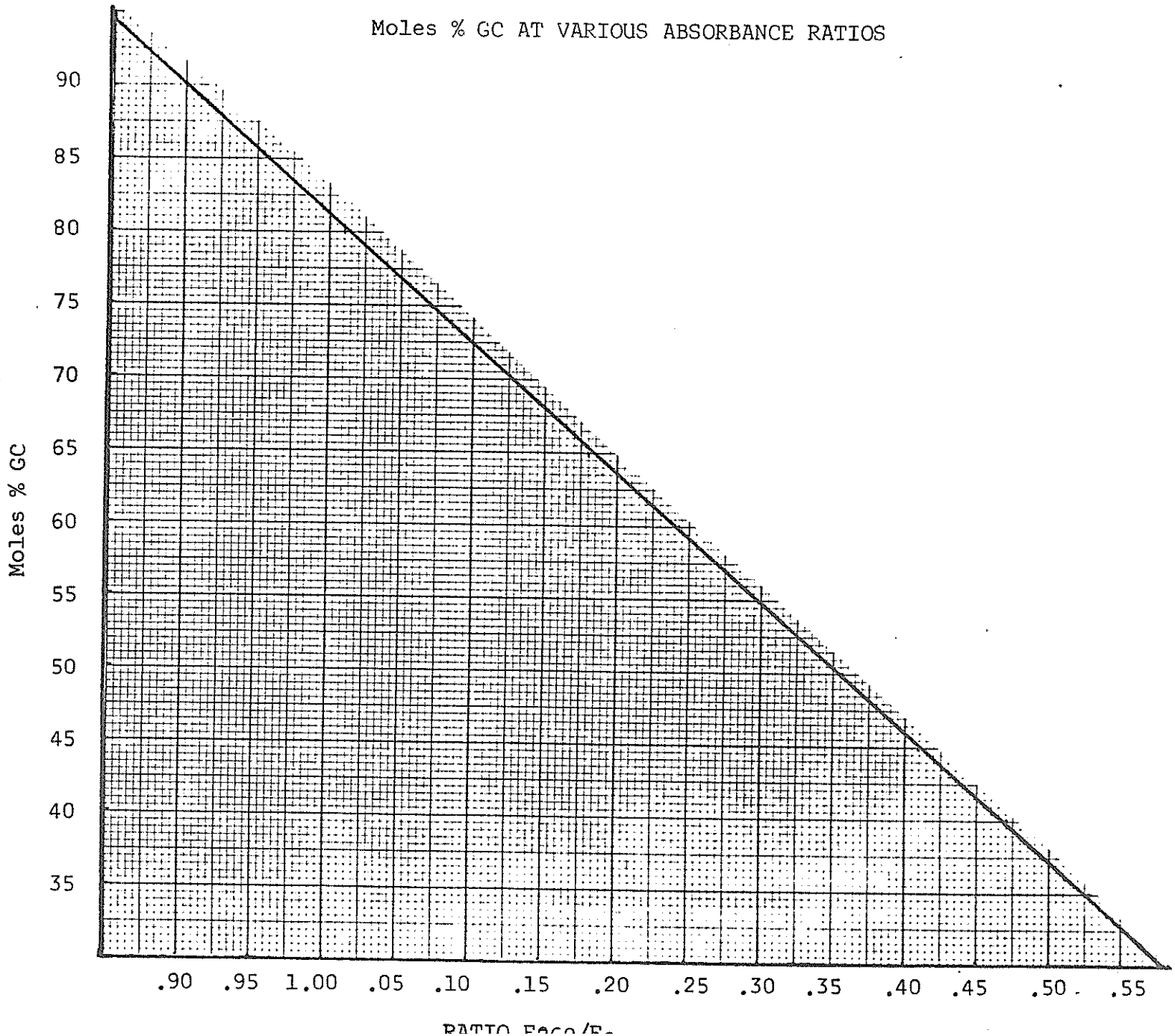

Moles % GC AT VARIOUS ABSORBANCE RATIOS
Moles % GC
90
85
80
75
70
65
60
55
50
45
40
35
.90
.95
1.00
.05
.10
.15
.20
.25
.30
.35
.40
.45
.50
.55

Figure 2

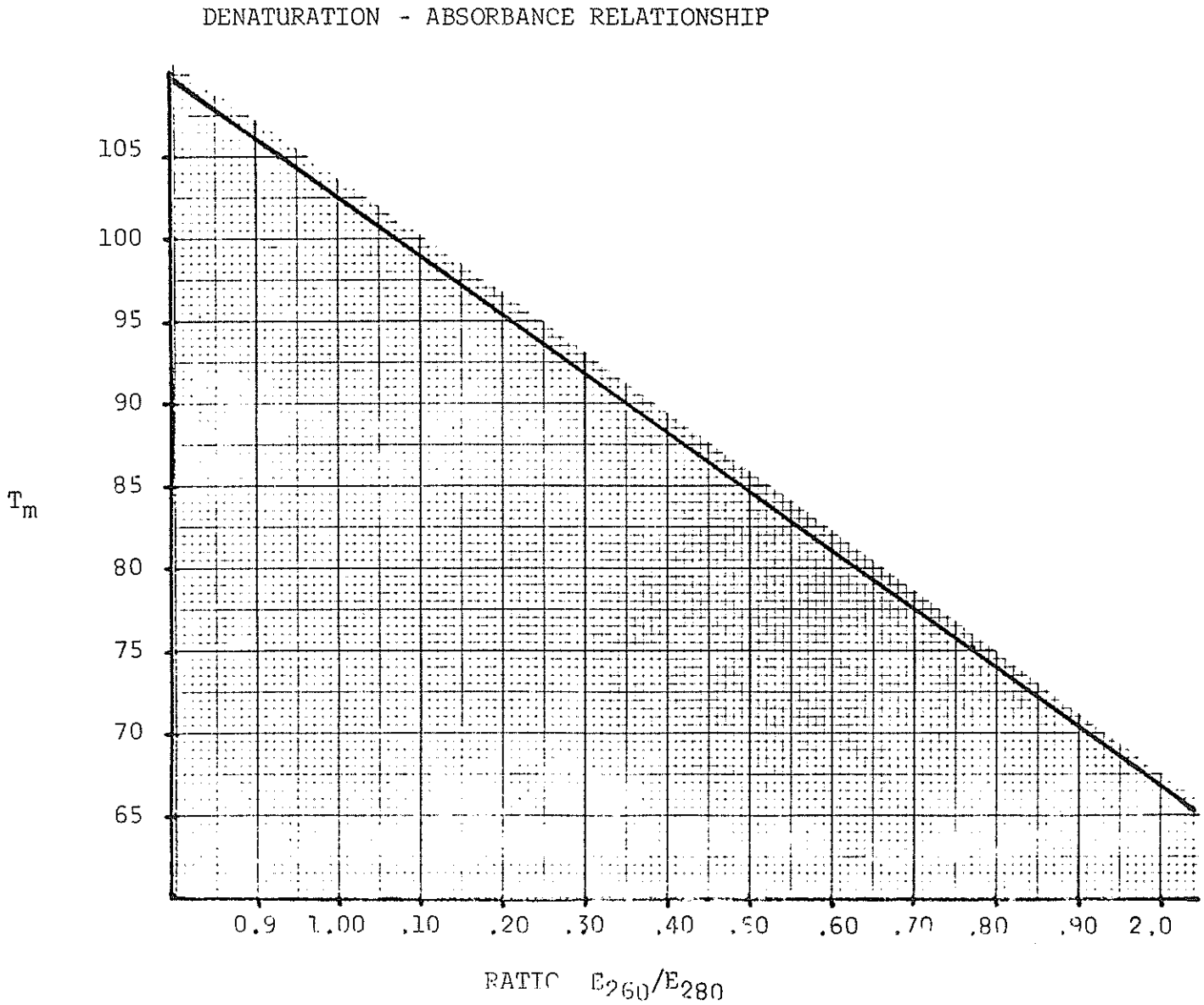
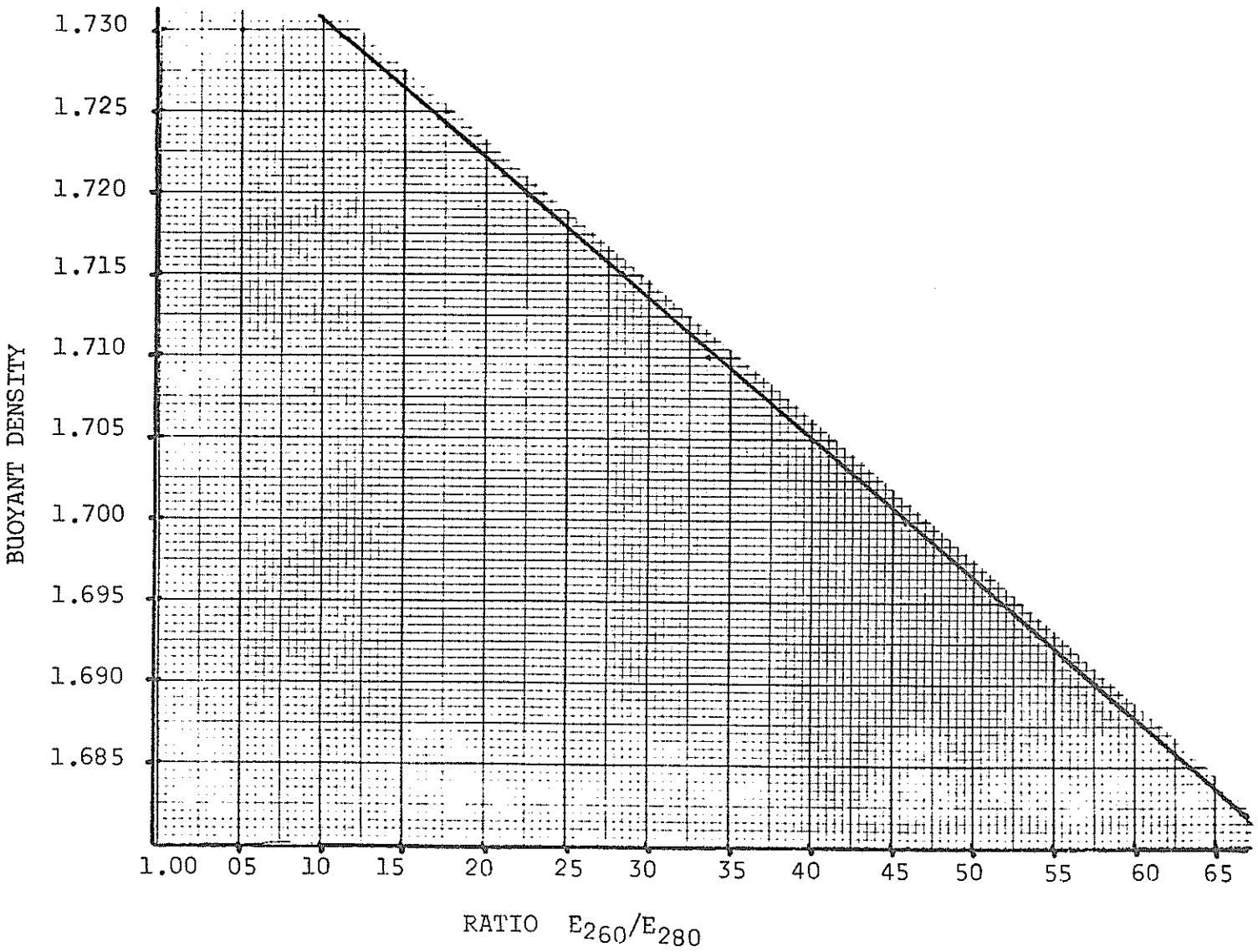

DENATURATION - ABSORBANCE RELATIONSHIP
T_m
105
100
95
90
85
80
75
70
65
0.9
1.00
.10
.20
.30
.40
.50
.60
.70
.80
.90
2.0
RATIO E_{260}/E_{280}

Figure 3

BUOYANT DENSITY - ABSORBANCY RELATIONSHIP



Estimating %GC by Bromination (138)

The bromination technique for estimating DNA base composition is based upon the quantitative reaction between N-bromoacetamide and all bases except adenine (136). These reactions lead to changes in absorbancy and the changes provide a means for determining the DNA base composition.

Reagents

Sulfuric Acid, 1 N H_2SO_4 diluted 1:36

N-bromoacetamide (NBA), 0.006 M solution. This solution is unstable to heat and light and should be kept in a flask wrapped in aluminum foil in a refrigerator.

Procedure

1. Dissolve precipitated DNA in 1N H_2SO_4 by stirring overnight, remove any remaining insoluble material and dilute DNA solution with H_2SO_4 to give maximum absorbancy readings of 0.4 - 0.8 at 270 mu.

2. Measure the absorbancy of the test solutions and blank solutions of 1N H_2SO_4 at 270 and 360 mu prior to bromination. Subtract the 360 mu reading from the 270 mu value (ODB reading).

3. Add 0.10 ml and 0.075 ml NBA to test and blank solutions respectively.

4. Cover the cuvettes and allow them to stand 120 minutes.

5. Measure the absorbancy of the test solutions at 270 mu and 360 mu. The difference of the two values gives the optical density reading after bromination (ODA reading).

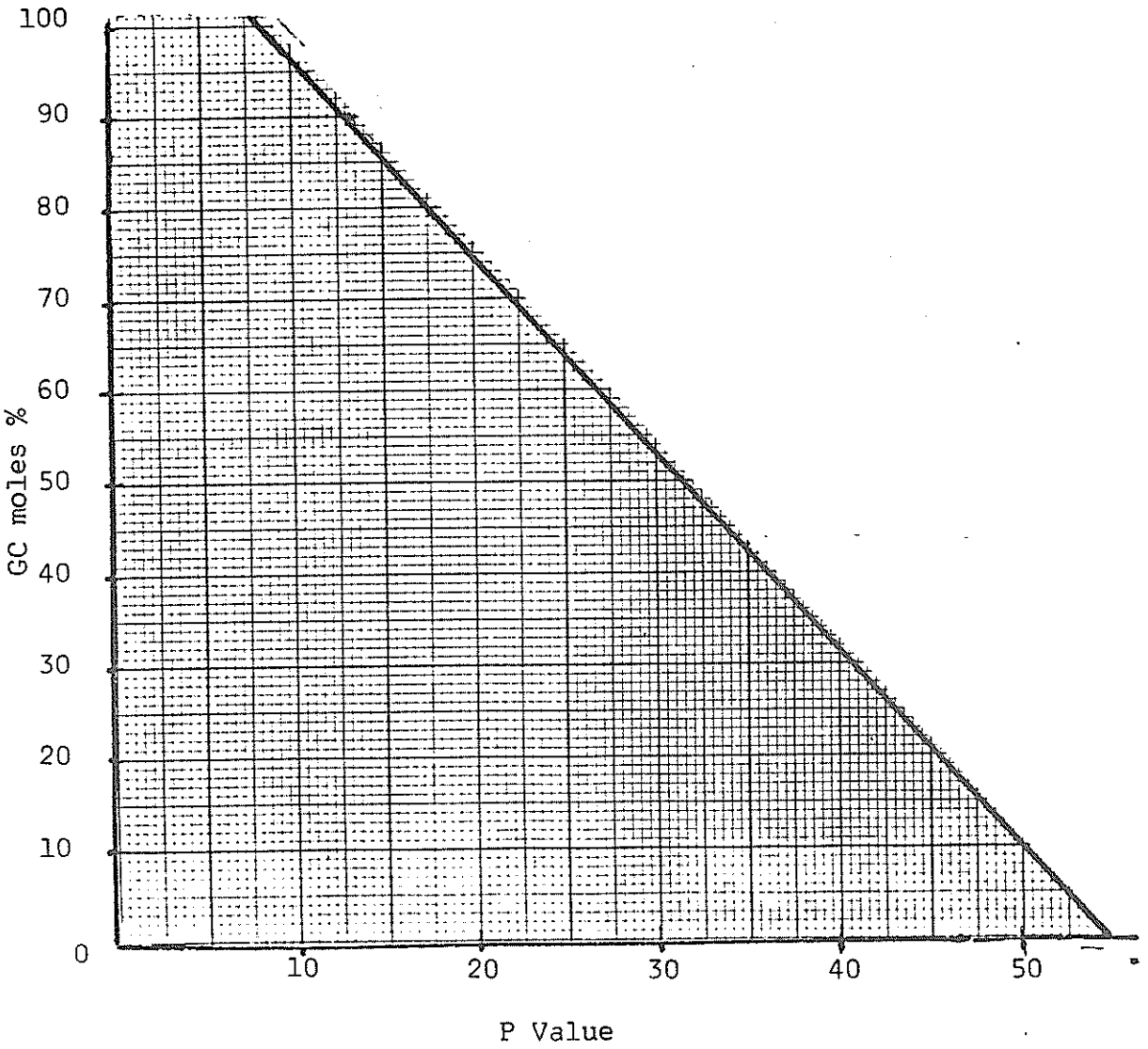
6. The percent absorbancy remaining (P) is given by:

$$P = \frac{ODA}{ODB} \times 100$$

7. The standard P values related to %GC are depicted in figure 4.

Figure 4

GC moles% AT VARIOUS P VALUES



Estimating %GC by Thermal Denaturation of DNA (78)

When a solution of DNA is heated, the double stranded helix is denatured to single stranded DNA. The optical density of the DNA solution increases as a result of denaturation and the temperature at which optical density is increased (hyperchromicity) is related to the base composition by the formula:

$$\%GC - 2.44\ T_m - 169.25$$

These relationships (78) are depicted graphically in figure 5.

Reagents

Saline-citrate, 0.15M NaCl - 0.015M sodium citrate (as described for extracting DNA) adjusted to pH 7.0.

Procedure

1. Saline-citrate and saline-citrate containing 15 - 50 ug DNA are placed in stoppered quartz cuvettes and the cuvettes placed in a thermostatically controlled cell holder.

2. Measure the optical density, then raise the temperature of the cells to approximately 5 C below the estimated onset of hyperchromicity.

3. After equilibrium, raise the temperature in 1 C increments, allowing about 10 minutes equilibration at each temperature. A sharp increase in absorbance occurs in the range during which DNA denatures. When no further increase occurs on raising the temperature, the denaturation can be considered complete.

4. The optical density at each temperature, is divided by the value at 25 C and the ratio (relative absorbance) plotted

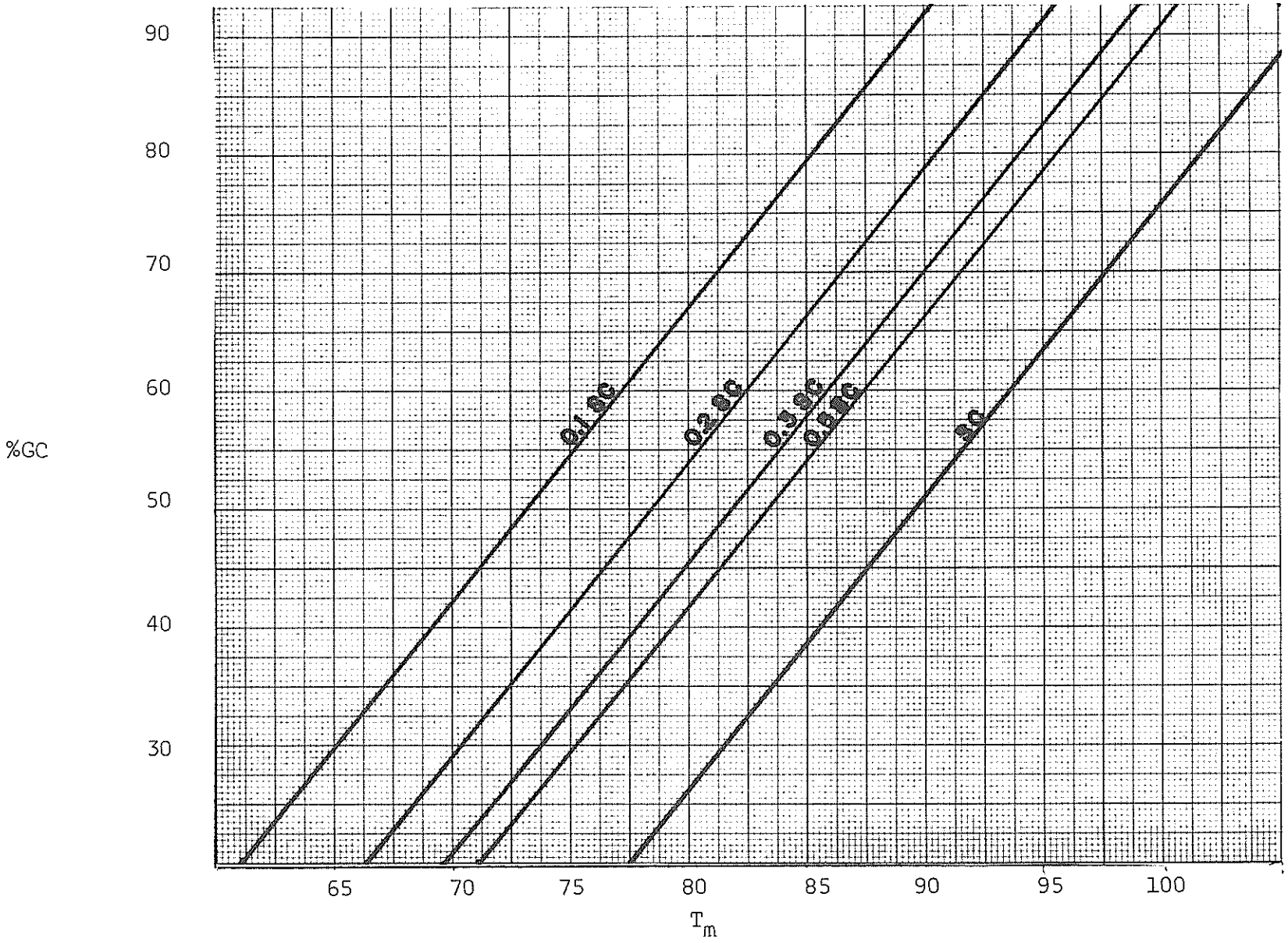
to the temperature of the solution. The temperature corresponding to half the increase in relative absorbance is designated T_m (melting temperature).

The thermal transition of DNA bears a linear relationship to the ionic strength of the solution. Dilute solvents yield a lower T_m for DNA than do concentrated solvents, thus the experimental T_m would then be extrapolated to standard conditions.

The three variables (T_m, %GC and ionic strength of solvent expressed as molarity) are depicted in figure 5.

Figure 5

Moles % GC - Denaturation Temperature (T_m)



Estimating %GC by Buoyant Density in Cesium Chloride (63,84,107)

Increased GC content in solutions of DNA is reflected by increased buoyant density (ρ) of those solutions and the equation:

$$\%GC = 1020.6\ (\rho - 1.6606)$$

relates the %GC to buoyant density in g/cm^3 when DNA solutions are centrifuged in cesium chloride (108). This relationship may be depicted graphically as in figure 6.

Procedure

Concentrated cesium chloride (CsCl) is prepared by dissolving optical grade CsCl, 130 g in 70 ml 0.02M Tris buffer pH 8.5. The solution is held at room temperature overnight and then filtered through a glass filter to remove particles. The density of the CsCl solution is 1.886 g/ml. In order to estimate %GC by buoyant density in CsCl, the DNA solution is adjusted to approximately 1.7 g/ml with CsCl (0.84 ml concentrated CsCl stock and 0.23 ml solution containing 5 - 10 ug unknown DNA and 5 - 10 mu solution containing 5 - 10 ug unknown DNA and 5 - 10 mu reference DNA). The solution may be overlayered with mineral oil to completely fill the centrifuge tube. The sample is then centrifuged 48 hrs. at 33,400 rpm; 24 hrs, at 44,700 or 18 hr. at 56,100 rpm in a Beckman L2 refrigerated ultracentrifuge using the SW-65 rotor.

After centrifugation, the contents of the tubes are analyzed spectrophotometrically. A close estimate of the distribution of the DNA can be derived by measuring the optical density of aliquots obtained by piercing the bottom of the tubes. The density of the aliquots can be estimated by measuring the refractive index of the various aliquots and relating the measurements to the graph presented in figure 7.

By using appropriate reference DNA preparations, the density of the unknown DNA may be estimated by extrapolation.

Figure 6

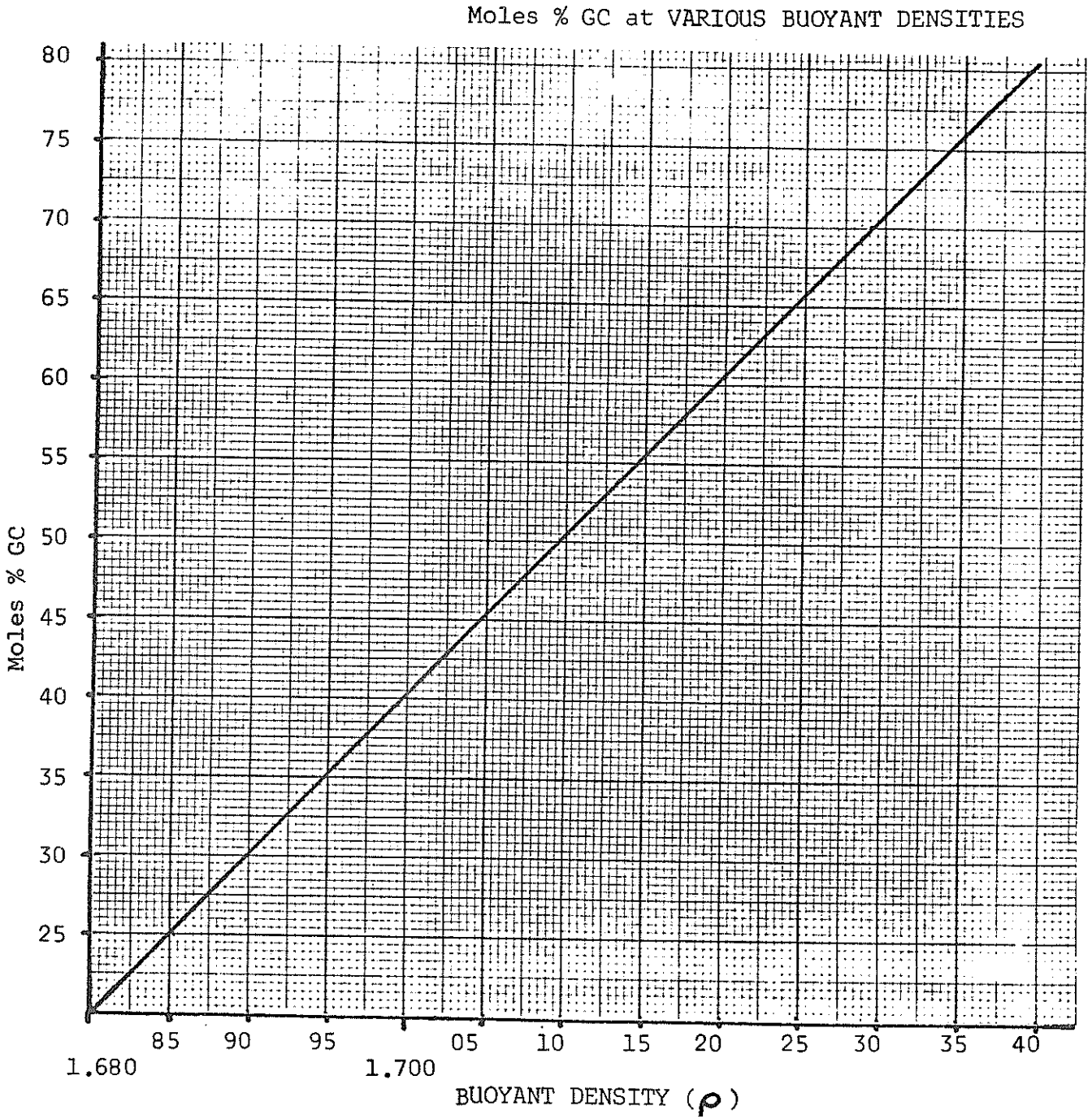
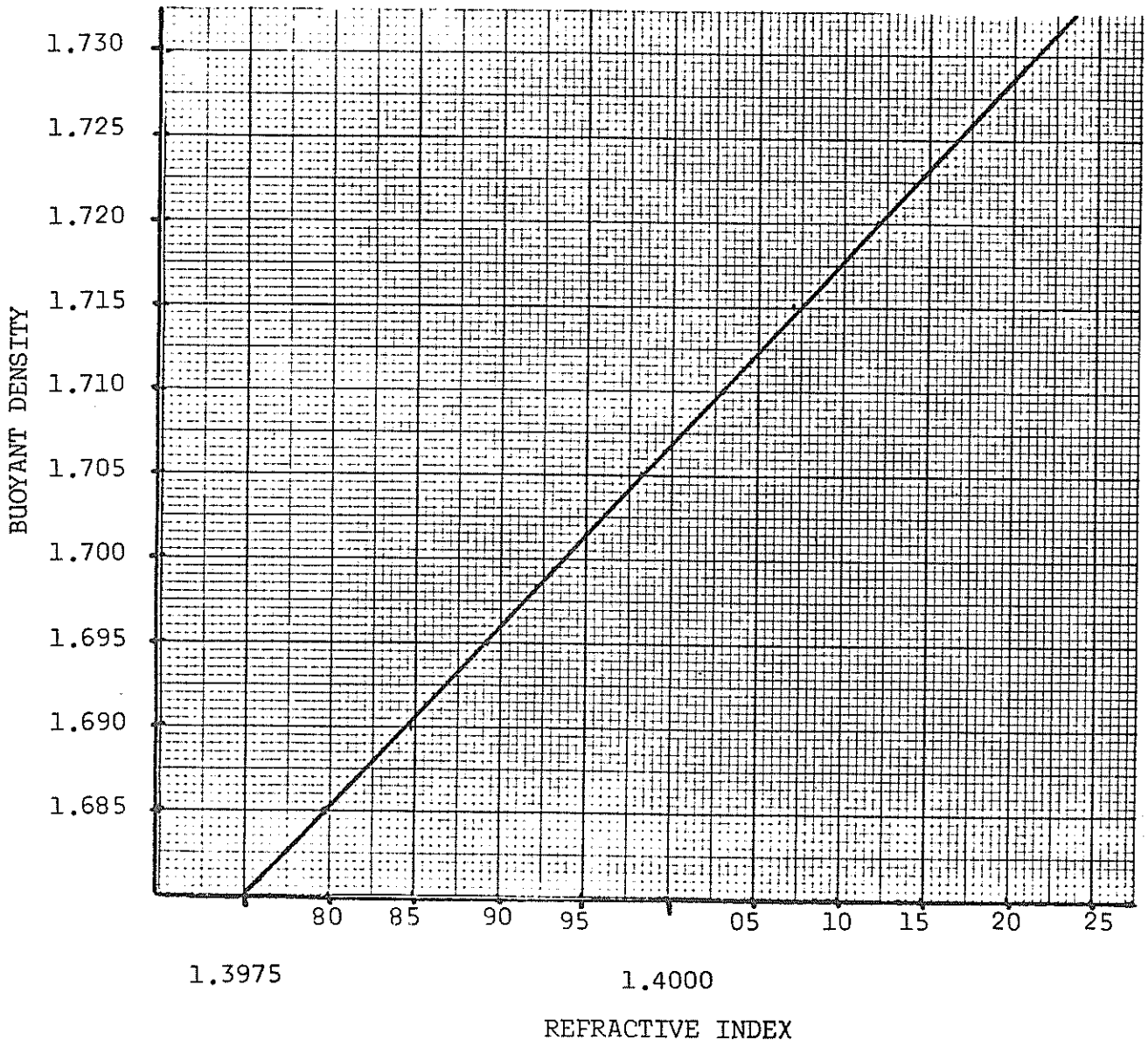

Moles % GC at VARIOUS BUOYANT DENSITIES
Moles % GC
80
75
70
65
60
55
50
45
40
35
30
25
85
90
95
05
10
15
20
25
30
35
40
1.680
1.700
BUOYANT DENSITY (ρ)

Figure 7

BUOYANT DENSITY REFRACTIVE INDEX RELATIONSHIP



Numerical Taxonomy

Numerical taxonomy is an attempt to assess the overall similarity of one isolate, strain or operational taxonomic unit to other entities of a group being studied (23,25,26,123). Each individual is defined by a series of morphologic, serologic, physiologic, cultural or biochemical features. For the purpose of numerical classification, all features possess equal weight. Those individuals with the highest degree of similarity are arranged into taxonomic groups known as phenons. The phenons correspond to genera, species, etc., of conventional nomenclature.

Numerical taxonomy is particularly appropriate for the classification of bacteria from fish and shellfish for a variety of reasons. Isolates from these sources are poorly described in the literature, thus identification of field isolates is almost impossible. Numerical taxonomic methods require no pre-arranged classification and because all features possess equal weight, individual variation does not greatly influence the classification of a particular strain. Furthermore, the numerical method which utilizes a multitude of features, can be used to produce determinative keys based upon a few most probable discriminate features.

Computer Program for Assessing the Relatedness of Various Isolates

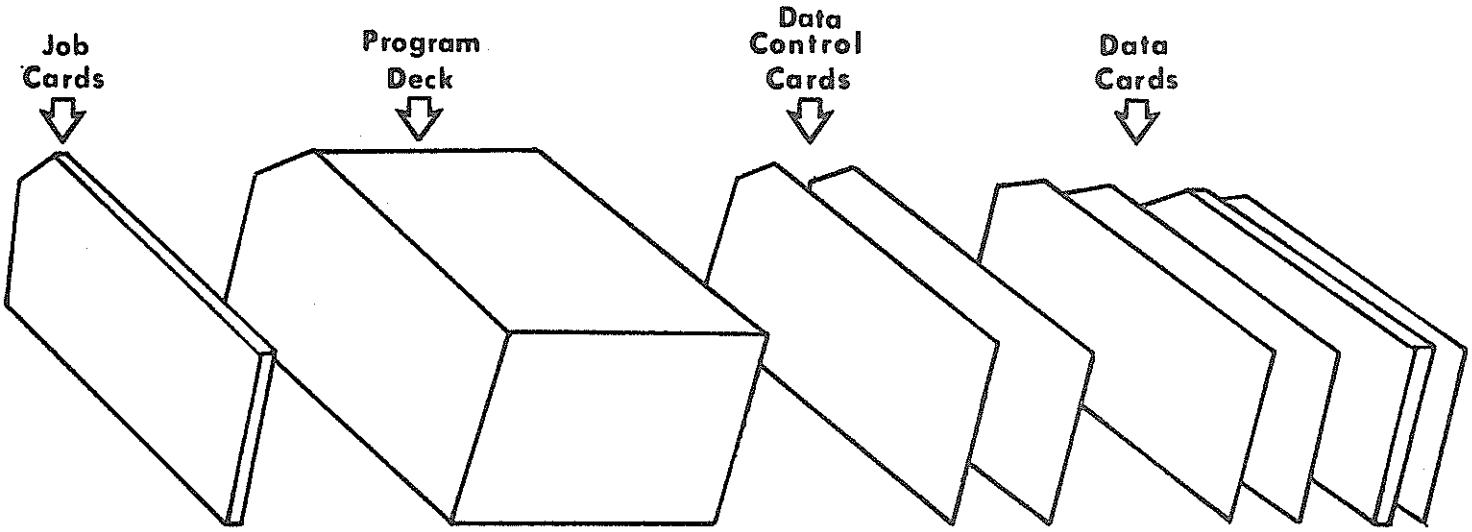
The computer program presented, written in Fortran IV, assesses the relatedness of a group of bacteria by comparing all recorded features of each isolate to the features of all other isolates of the study. Two types of features are tabulated: 1) those features whose state can be specified by presence or absence, positive or negative and 2) noncomparable features, i.e. those features whose status is unknown in the isolate. When known, the features may be recorded as "+" or "-" or may be recorded with some numerical code eg "1 = +"; "2 = -", etc. Noncomparable characters are either left blank or recorded as numerical "0".

The relatedness is calculated by deriving the "S" value (123) of two isolates being compared:

$$S_{1,2} = \frac{\text{Number of Matches}}{\text{Number of Comparisons - Noncomparable Characteristics}}$$

A. Recording Data. Forms should be prepared which may be used in the laboratory and from which data may be punched directly. Data on each isolate is recorded on two 80 column lines. Descriptive data is recorded on the first line (representing the first IBM punch card) and biochemical test results are recorded in appropriate columns on the second line (representing the second IBM punch card). The key punch operator should note that the first card contains alpha numeric characters and begins in column 7. On the second card, column "5" is left blank and if additional continuation cards are not needed, a numeric character is punched in column "6" of that card. Columns "73 - 78" are used for identifying the isolate and columns "79 - 80" are used for card sequencing.

B. Input. The cards are loaded as follows:



1. Job Cards - The arrangement of the job cards vary at different institutions. When used at the Data Processing Center at Texas A&M University, the program may be loaded as a large WATFIV JOB.

2. The Program Deck - The program as illustrated below is capable of processing up to 60 isolates, up to 400 features on each isolate utilizing 120K - 240K storage. The operational parameters can be altered very easily, the only limitation being the storage capacity of the computer.

```
//$WATFIVX JOB (146J4,4-L  ,001,001,  ),
      IMPLICIT INTEGER (A-Z)
      REAL FLOAT
      REAL*8 NAME(60), S(60,60), TITLE(9), TYPEX, TYPE
      INTEGER*2 TEST(60,400), SEQ(1720,2), SEQ2(60)
      EQUIVALENCE (TEST(1,1), SEQ(1,1), SEQ2(1))
      DATA TYPEX /'NAME'/
      INTEGER*2 BLANK, ZERO
      DATA BLANK, ZERO /' ','0'/
C
C
    1 READ(5,501,END=999) NSTRNS, NTESTS, TITLE
  501 FORMAT (2I3,9A8)
      IF (NSTRNS .LT. 2 .OR. NSTRNS .GT. 60) GO TO 1000
      IF (NTESTS .LT. 1 .OR. NTESTS .GT. 400) GO TO 1000
      DO 10 I = 1,NSTRNS
      READ(5,502,END=900) TYPE, NAME(I)
  502 FORMAT (A6,A8)
      IF (TYPE .NE. TYPEX) GO TO 1001
   10 READ(5,503,END=900) (TEST(I,J), J = 1,NTESTS)
  503 FORMAT (72A1)
C
C        DATA IS ALL READ. PRINT OUT DATA FOR VISUAL INSPECTION.
C
      DO 20 I = 1,NSTRNS
      WRITE(6,600) TITLE
  600 FORMAT (1H1,30X,9A8)
      WRITE(6,601) NAME(I)
  601 FORMAT ('-INPUT DATA LISTING FOR STRAIN ',A8/)
      NN = (NTESTS - 1)/25 + 1
      DO 20 J = 1,NN
      STOP = J*25
      START = STOP - 24
      IF (STOP .GT. NTESTS) STOP = NTESTS
   20 WRITE(6,602) START, STOP, (TEST(I,K), K = START,STOP)
  602 FORMAT (1X,'TESTS ',I3,' TO ',I3,5X,25A2)
C
C        NOW COMPUTE TABLE OF 'S' VALUES.
C
      NN = NSTRNS - 1
      DO 100 I = 1,NN
      II = I + 1
      DO 100 J = II,NSTRNS
      INVLID = 0
      MATCH = 0
      MISS = 0
      DO 101 K = 1,NTESTS
      IF (TEST(I,K) .EQ. BLANK .OR. TEST(J,K) .EQ. BLANK .OR.
     1    TEST(I,K) .EQ. ZERO .OR. TEST(J,K) .EQ. ZERO) GO TO 110
      IF (TEST(I,K) .EQ. TEST(J,K)) GO TO 120
      MISS = MISS + 1
      GO TO 101
  110 INVLID = INVLID + 1
      GO TO 101
  120 MATCH = MATCH + 1
  101 CONTINUE
      NGOOD = NTESTS - INVLID
      S(I,J) = 0.
      IF (NGOOD .NE. 0) S(I,J) = FLOAT (MATCH) / NGOOD
  100 CONTINUE
C
```

```
      C        NOW PRINT AND PUNCH THE TABLE OF 'S' VALUES.
      C
   51       ICNT = 51
   52       DO 200 I = 1,NN
   53       S(I,I) = -1.
   54       II = I + 1
   55       DO 200 J = II,NSTRNS
   56       S(J,I) = S(I,J)
   57       IF (ICNT .LE. 50) GO TO 201
   58       WRITE(6,600) TITLE
   59       WRITE(6,603)
   60   603 FORMAT ('-TABLE OF ''S'' VALUES (UNSORTED)'/)
   61       ICNT = 1
   62   201 WRITE(6,604) NAME(I), NAME(J), S(I,J)
   63   604 FORMAT (1X,2A12,3X,F20.6)
   64   200 CONTINUE
   65       S(NSTRNS,NSTRNS) = -1.
      C
      C        NOW SORT 'S' VALUES, AND PRINT THE SORTED LIST.
      C
   66       NCNT = 1
   67       DO 300 I = 1,NN
   68       II = I + 1
   69       DO 300 J = II,NSTRNS
   70       SEQ(NCNT,1) = I
   71       SEQ(NCNT,2) = J
   72   300 NCNT = NCNT + 1
      C
   73       NCNT = NCNT - 1
   74       NN = NCNT - 1
   75       LAST = NN
   76       DO 301 I = 1,NN
   77       STOP = LAST
   78       LAST = 1
   79       DO 302 J = 1,STOP
   80       I1 = SEQ(J,1)
   81       I2 = SEQ(J,2)
   82       J1 = SEQ(J+1,1)
   83       J2 = SEQ(J+1,2)
   84       IF (S(I1,I2) .GE. S(J1,J2)) GO TO 302
   85       SEQ(J,1) = J1
   86       SEQ(J,2) = J2
   87       SEQ(J+1,1) = I1
   88       SEQ(J+1,2) = I2
   89       LAST = J
   90   302 CONTINUE
   91       IF (LAST .EQ. 1) GO TO 400
   92   301 CONTINUE
      C
      C        THE KEYS FOR THE 'S' VALUES ARE NOW SORTED.
      C
   93   400 ICNT = 51
   94       DO 401 I = 1,NCNT
   95       I1 = SEQ(I,1)
   96       I2 = SEQ(I,2)
   97       IF (ICNT .LE. 50) GO TO 402
   98       ICNT = 1
   99       WRITE(6,600) TITLE
  100       WRITE(6,605)
  101   605 FORMAT ('-TABLE OF ''S'' VALUES (SORTED)'/)
  102   402 WRITE(6,604) NAME(I1), NAME(I2), S(I1,I2)
```

```
103     401 ICNT = ICNT + 1
      C
      C         DO NON-CROSSING DENDROGRAM.
      C
104         NN = NSTRNS - 1
105         DO 810 I = 1,NSTRNS
106         ICNT = 51
107         DO 811 J = 1,NSTRNS
108     811 SEQ2(J) = J
109         LAST = NN
110         DO 812 I1 = 1,NN
111         STOP = LAST
112         LAST = 1
113         DO 813 J = 1,STOP
114         J1 = SEQ2(J)
115         J2 = SEQ2(J+1)
116         IF (S(I,J1) .GE. S(I,J2)) GO TO 813
117         SEQ2(J) = J2
118         SEQ2(J+1) = J1
119         LAST = J
120     813 CONTINUE
121         IF (LAST .EQ. 1) GO TO 820
122     812 CONTINUE
123     820 DO 821 J = 1,NN
124         IF (ICNT .LE. 50) GO TO 822
125         WRITE(6,600) TITLE
126         WRITE(6,606) NAME(I)
127     606 FORMAT ('-NON-CROSSING DENDROGRAM FOR ',A8/)
128         ICNT = 1
129     822 J1 = SEQ2(J)
130         WRITE(6,607) NAME(J1), S(I,J1)
131     607 FORMAT (1X,A8,3X,F20.6)
132     821 ICNT = ICNT + 1
133     810 CONTINUE
      C
      C         NOW, START NEXT SET OF DATA, IF PRESENT.
      C
134         GO TO 1
      C
      C
      C
      C         ERROR ROUTINES.
      C
135    1000 WRITE(6,6000) NSTRNS, NTESTS, TITLE
136    6000 FORMAT ('1HEADER CARD ERROR'/1X,2I4,1X,9A8)
137         STOP
138     900 WRITE(6,6001)
139    6001 FORMAT ('1END OF DATA REACHED WHEN NOT EXPECTED.')
140         STOP
141    1001 WRITE(6,6002) TYPE, NAME(I)
142    6002 FORMAT ('1EXPECTED ''NAME'' CARD. FOUND ',A6,A8)
      C
143     999 WRITE(6,6003)
144    6003 FORMAT (1H1)
145         STOP
146         END

      //$DATA
```

3. Data Control Card - Data control card precedes recorded data and in certain operations is preceded by an operational control card eg //$Data, $Data etc. The number of isolates being examined is recorded in columns 1-3 and the number of features in columns 4-6 of the Data Control Card.

4. Data Cards - The data deck consists of at least two cards for each isolate being studied. On the first card, descriptive information pertaining to the isolate is recorded in columns 7-72. On the second card data on the isolate is recorded in columns 1-4 and 7-72. If more than 70 features are to be recorded, the same format will be utilized in succeeding cards. In the last card of the series a numeric character eg "3" is recorded in column 6 . On all cards columns 73-78 contain identifying numbers and columns 79-80 are used for card sequencing.

C. Output. The program generates three types of output:

1. Listing of characteristics on each isolate for inspection:

TAXONOMY OF AEROMONAS -LIKE ORGANISMS

INPUT DATA LISTING FOR STRAIN MS 374

TESTS 1 TO 25 + + - + + + - + + + + + - - - + + + - + - + - - +
TESTS 26 TO 50 + + - - + - - 0 + + 0 0 0 0 0 0 - + - 0 0 0 0 0 0
TESTS 51 TO 71 0 0 0 0 + + + 0 - 0 0 0 + + + + 0 0 + 0 -

2. Tables of "S" values. The sorted table is arranged from highest to lowest level of similarity.

TAXONOMY OF AEROMONAS -LIKE ORGANISMS

TABLE OF 'S' VALUES (SORTED)

MS 360	CARD MS	0.847826
MS 374	CARD MS	0.782609
MS 360	MS 375	0.727273
MS 375	CARD MS	0.720930
MS 374	MS 375	0.707317
MS 374	MS 360	0.666667

3. Tables for construction of dendrogram.

TAXONOMY OF AEROMONAS -LIKE ORGANISMS

NON-CROSSING DENDROGRAM FOR MS 374

CARD MS	0.782609
MS 375	0.707317
MS 360	0.666667

Media, Reagents and Tests

Media, Reagents and Tests

The following descriptions are arranged alphabetically in enumerated paragraphs according to their intended use for studying aquatic bacteria. Routine microbiologic procedures may be found in standard textbooks.

Bacteria derived from salt water environments should be tested in media supplemented with stock salt solution (paragraph 67). In some instances a precipitate forms in the medium after adding stock salt solution to it; but in most cases, precipitate formation does not interfere with growth and testing of the bacterium. Sodium chloride (1-3%) often fulfills the ionic requirements of marine bacteria, thus may be substituted for stock salts as a supplement to media designed for non-halophilic bacteria.

In certain instances product names are mentioned. This does not necessarily imply endorsement of those products.

1

Aesculin Hydrolysis (118)

Chromobacterium, Xanthomonas and Pseudomonas are often positive for this feature.

Medium: Bacto-peptone.....1%
Aesculin.....0.1%
Ferric citrate.....0.05%
Agar.....1.5% (may be omitted)

Test: Inoculate tubes or plates. Incubate 14 days for tubes, 4 days for plates.

Results: Blackening and loss of fluorescence indicates positive result.

2

Acid Fast Stain (53)

Carbol fuchsin
Basic Fuchsin (90% dye).....0.3 g
Ethanol (95%).....10 ml
Phenol, 5% aqueous.....100 ml

Dissolve dye in the alcohol and add the phenol solution
Alkaline Methylene Blue
Dissolve 0.3 g methylene blue (90% dye) in 30 ml ethanol. Add to 100 ml 0.01% KOH.

1. Heat fix smears; flood with carbol fuchsin and heat gently to steaming for 3 minutes.

2. Decolorize with acid alcohol
HCl.....2 ml
Ethanol.....98 ml

The smear is decolorized until the thinner parts are decolorized and the heavier portions remain pink.

3. Counterstain with methylene blue 1 minute.

4. Wash in water, blot and dry.

Result: Acid-fast bacteria are stained red and tissue cells and other bacteria are blue.

3

Agar Decomposition (50,124)

Agar derived by hot water extraction of marine Rhodophyta consists of agarose and agaropectin. Agarose contains repeating units of 1:3 B-D-galactopyranose and 1:4 linked 3,6 anhydro- -L galactopyranose. Agaropectin, a relatively minor constituent is composed of a complex containing sulfuric and uronic acids. Several genera are capable of attacking agar.

Medium: Bacto-peptone.....0.5%
K_2HPO_4.....0.2%
$CaCO_3$.....2.0%
Agar.....1.0%

Dissolve in aged seawater, artificial seawater, or tapwater with 3% NaCl.

Test: Inoculate freshly poured plates and incubate 5-14 days. Flood with KI - I solution.

Results: Cleared yellowish halos contrasted with reddish-violet unattacked agar indicates positive result.

4

Alginase Activity (71)

Medium: 3% potassium alginate (Grade 1 KR, Kelco, San Diego, Calif.).

Test: Alginase activity is indicated by liquefaction after 7 days.

5

Ammonia Production From Peptones (53)

The deamination of peptones to ammonia is a feature common to but not limited to Pseudomonas sp.

Test: Nutrient or peptone broth is inoculated and incubated 5 days. A small strip of filter paper is wetted with Nessler's reagent and placed in the upper part of a culture tube. The tube is warmed to 50-60°C in a water bath.

Result: Filter paper turns brown or black if ammonia is present.

6

Arginine and Lysine Decarboxylase

Decarboxylase reactions are useful in distinguishing a variety of aquatic bacteria.

Medium: Add 1% L-amino acid to commercially available (2% DL-amino acid) basal decarboxylase medium. The reaction of the medium should be pH 6.0-6.5 after sterilization. The basal medium less amino acid is used as a control.

Test: Inoculate control (basal) medium and test medium and aseptically overlay the broths with sterile mineral oil. Aeration may sometimes be prevented by tightening screw caps soon after heating, in which case proper reactions can be obtained without adding mineral oil.

Result: Control and negative reactions are acid. Positive results are indicated by alkaline reactions. If ammonium is detected in the arginine test and the organism does not produce urease the reaction is considered due to an arginine dihydrolase.

7

Bacteriolytic Activity

Specific feature of Myxobacteria
Procedure A (57)

Medium: Agar.....1.5% in water. Use washed Klebsiella cells from 1 nutrient agar slant/2 plates. Spread cells evenly over surface of plate. Dry surface at 45°C.

Test: Spot or streak test culture and examine for 14 days for lytic zones.

Procedure B (80)
Washed E. coli cells (24 hr. BHI).....0.1%
$MgSO_4 \cdot 7H_2O$.....0.05%
NaCl.....0.6%

Autocalve. Add 0.5 ml to sterile 1.5% agar plates. The plates should be inverted over lids and placed in incubator 45-60 min. prior to adding cells.

Spot test organisms and observe for lysis.

8

Benedict's Solution (86)

Used for gluconate test

Sodium citrate.....17.3 g
Na_2CO_3.....10.0
$CuSO_4.5H_2O$..... 1.7 g
Distilled Water qs to 100 ml

Dissolve citrate and carbonate in 60 ml water. Dissolve copper sulphate in 20 ml of water and add with constant stirring to first solution. Adjust to volume with water. Crystallization occurs at low temperatures.

9

BTB-Teepol Agar (106)

For selective isolation of Vibrio parahemolyticus

Yeast Extract.....0.3%
Peptone.....1.0%
Sodium Chloride.....4.0%
Sucrose.....2.0%
Bromthymol Blue.....0.004%
Thymol Blue.....0.004%
Teepol.....0.2%
Agar.....1.5%

Adjust pH to 9.0 and autoclave.

10

2-3 Butanediol Production (18)

Specific feature of Aeromonas sp.

Medium: Nutrient broth containing 0.5% glucose; dispense in 9 ml amounts.

Test: Incubate inoculated broth 48 hr or longer, then pipette

1 ml 2.3% periodic acid (kept in brown bottle). Shake tubes and allow them to stand at room temperature 30 minutes. Add 1.5 ml piperazine solution (prepared by dissolving 25 g piperazine hexahydrate in 99 ml distilled water). Add 1.3 ml 87% formic acid and 0.5 ml 4% sodium nitroprusside solution. Prepare piperazine solution fresh each day.

Result: Positive result is indicated by development of blue color within 2 minutes.

11

Calcium Carbonate Medium (114)

Utilized to differentiate Pseudomonas sp. from acetic acid bacteria. Pseudomonas sp. oxidize calcium lactate to calcium carbonate, whereas Acetomonas sp. do not.

Yeast extract.....1.0%
Calcium lactate.....1.0%
Agar.....1.5%

Test and Result: Inoculate plate and observe for precipitation of crystals of $CaCO_3$ under growth.

12

Carbohydrate Basal Medium for Rapidly Growing Mycobacteria (48,104)

$(NH_4)_2HPO_4$.....0.1%
KCl.....0.02%
$MgSO_4$.....0.02%
Agar.....1.5%

Adjust to pH 7.0 and add 1.5% (v/v) of a 0.04% brom cresol purple. Dispense in 5 ml amounts and sterilize. Add 0.5 ml of filter sterilized 1.0% carbohydrate per tube.

13

Carrageenan Psychrophilic Medium (95)

Used for recovery of psychrophilic organisms.

Eugon broth.....3.0%
Yeast extract.....0.5%
Agar.....0.2%
Carrageenan.....1.5%

(Marine Colloids Inc., Springfield, N. J.)

Mix and boil for proper distribution, carrageenan will not dissolve completely until after autoclaving and its solidifying temperature is 30 C. The medium is not as rigid as agar, thus it must be incubated upright.

14

Casein Hydrolysis (53,117)

Medium: Nutrient agar containing 10% skim milk

Test: Inoculate plate and incubate

Results: Clear zone suggests casein hydrolysis (may be confirmed by adding $HgCl_2$ test reagent)

15

Cellulose Activity (71)
(Carboxymethyl Cellulose Digestion)

Medium: 3% carboxymethyl cellulose (high viscosity) in Tryptose broth.

Test: Inoculate tubes and incubate 1 week. Cellulase activity is indicated by liquefaction.

16

Cell Wall Stain (36)

1. Heat-fix smear
2. Add 3 drops 0.34% cetyl pyridinium chloride
3. Add 1 drop saturated aqueous Congo red
4. Wash
5. Counterstain for a few seconds with methylene blue
6. Wash and examine

Cell wall stains red.

17

Chitinase Activity (124)

Preparing colloidal chitin:
1. Prepare 3% chitin solution in 50% H_2SO_4
2. Filter through glass wool to remove undissolved particles.

3. Add acid mixture to distilled water (carefully) to effect a 1:5 acid water solution. Allow sedimentation to proceed overnight.
4. Discard supernatant and resuspend sediment in approximately 100 ml of water per gm original chitin.
5. Centrifuge and wash twice.
6. Resuspend in 5 ml water/gm original chitin, incorporate in basal medium at final concentration of 30%. Dispense in 5 ml amounts and autoclave.
7. Heat in boiling water to melt agar and pour over basal medium.

Test: Inoculate and incubate under optimum conditions

Result: Chitinoclastic activity is indicated by clear zones around areas of growth.

18

Columnaris Medium (42)

Used for the isolation of Chondrococcus columnaris

Tryptone	0.05%
Yeast Extract	0.05%
Beef Extract	0.02%
Sodium Acetate	0.02%
Agar	0.9%

Adjust to pH 7.2 - 7.4, autoclave. Add neomycin and polymyxin B to a final concentration of 5 ug/ml.

19

Corynebacterium Medium

See Kidney Disease Medium (paragraph 42)

20

Cytophaga Basal Hydrolysis Medium (92)

Basal medium used to test C. psychrophila's ability to hydrolyse starch, gelatin, casein, tributyrin, tyrosine, xanthine and albumin.

Tryptone	0.1%
Yeast Extract	0.05%
Beef Extract	0.05%
Metabolite	0.5%
Agar	1.1%

21

Cytophaga Basal Nutrient Medium (92)

Basal medium used for determining the nutritional requirements of C. psychrophila.

$(NH_4)_2HPO_4$	0.1%
KCl	0.02%
$MgSO_4.7H_2O$	0.02%
Hydrolyzed Casein (vitamin free)	0.2%
Metabolite	0.1%

22

Cytophaga Elective Media (134)

Described for isolation of C. fermentans var. agarovarans and C. salmonicolor var agarovarans.

Sodium Chloride	3.0%
KH_2PO_4	1.0%
$MgCl_2.6H_2O$	0.05%
NH_4Cl	0.1%
$CaCl_2$	0.004%
$NaHCO_2$	0.5%
$Na_2S.9H_2O$	0.01%
0.004M Ferric Citrate	0.5%
Yeast Extract	0.03%
Agar	0.5%

Adjust to pH 7.0.

23

Cytophaga Fermentation Broth (2)

Originally used for determining carbohydrate fermentation by C. succinicans.

Peptone	0.2%
Yeast Extract	0.1%
Beef Extract	0.1%
K_2HPO_4	0.05%

Adjust to pH 7.1. Sterilize by autoclaving then add filter sterilized $NaHCO_3$(0.025%) and 0.4% carbohydrate.

24

Cytophaga Medium (2,93)

Described for isolation and routine plating of C. succinicans.

Tryptone	0.05%
Yeast Extract	0.05%
Beef Extract	0.02%
Sodium Acetate	0.02%
Agar	0.9%

Adjust to pH 7.2 - 7.4.

Stock cultures are incubated for about one week at 18 C, then stored at 4 C.

25

Cytophaga Mineral Base (92)

Basal medium used to test C. psychrophila

$MgSO_4.7H_2O$	0.02%
$NH_4H_2PO_4$	0.1%
$K_2H_2PO_4$	0.1%
NaCl	0.2%
Salt of organic acid	0.2%
Bromthymol blue	0.001%
Agar	1.5%

26

Cytophaga Selective Medium (21,80)

Add the following to Cytophaga mineral base:

Penicillin	15 units/ml
Chloramphenicol	5 ug/ml
Sodium taurocholate	5 mg/ml
Sodium cholate	5 mg/ml

Cytophaga rubra and C. tenuissma are sensitive to the bile salts.

27

Dihydroxyacetone from Glycerol (114)

Used in differentiating Pseudomonas sp. from acetic acid bacteria. Acetomonas sp. produce and accumulate dihydroxyacetone from glycerol.

Yeast Extract.....1.0%
Glycerol.....2.0%
Agar.....1.5%

Test: Inoculate slants or plates and incubate for optimum growth. Place in cold and flood with Fehling solution.

Result: A positive result is indicated by the production of yellow to red CuO in 10 minutes.

28

Ethanol Agar (114)

Acetomonas sp. produce acid from ethanol.

Yeast Extract.....1.0%
Ethanol.....2.0%
$CaCO_3$.....2.0%
Agar.....1.5%

Inoculate medium and observe for clear zones around growth.

29

Fatty Acid Ester Hydrolysis (115)

Peptone.....1.0%
NaCl.....0.5%
$CaCl_2$.....0.01%
Tween.....1.0%
Agar.....1.5%

Tweens are manufactured by Atlas Chemical Industries, Inc.

Tween 20 = lauric acid ester
Tween 40 = palmitic acid ester
Tween 60 = stearic acid ester
Tween 80 = oleic acid ester

Inoculate and observe for opaque zones around growth.

30

Fat Hydrolysis (53)

Warm fresh unsalted butter in a 50 C waterbath. Separate butterfat from curd and dispense into screw-capped tubes. Sterilize.

Medium: Nutrient agar and 5% butterfat emulsion

Test: Inoculate, incubate under optimum conditions. Flood growth with saturated copper sulfate and react 15 min. Pour off reagent and wash to remove excess copper sulfate.

Result: Bluish-green zone appears around growth due to formation of insoluble copper salts of free fatty acids.

31

Fat Hydrolysis (97) (Margarine)

Preparing margarine:

1. Mix 100 ml molten margarine and 100 ml 0.1% Victoria blue*.
2. Add few ml saturated sodium carbonate solution. Keep mixture hot by standing flask in boiling water and shake well to mix.
3. Transfer to separatory funnel and allow the fat to separate (fat will be red); discard aqueous blue portion.
4. Wash with hot distilled water and discard washings.
5. Distribute in screw-capped tubes and sterilize by autoclaving.

Preparing basal medium:

Heat nutrient agar or other appropriate media to liquefy. Cool to 45 C. Add stained fat to a concentration of 5% and emulsify by shaking one minute.

*Victoria blue has been demonstrated to be toxic for certain bacteria thus other dyes have been used. Those dyes and their reactions are listed below:

	Neutral Red	Methylene Blue	Malachite Green
Fats:	Orange-yellow	Red	Olive Green
Fatty Acids:	Red	Blue	Blue-Green

Inoculate and incubate under optimum conditions. Deep blue zones surrounding areas of growth indicate lipolytic activity.

32

Fermentation Broth (Basal)

Used in detecting carbohydrate fermentation by those organisms which produce alkaline by-products from ordinary fermentation medium, eg. Pseudomonas sp.

Yeast Extract.....0.1%
Peptone.....0.1%
NaCl.....0.5%
Phenol Red.....0.4 ml of a 0.5% solution/100 ml

Adjust to pH 7.2 prior to autoclaving. After autoclaving, add sterile carbohydrate solution to 1.0% final concentration.

33

Flagella Stain (102)

Prepare the following solutions:

1. 12% tannic acid in 10% Kal $(SO_4)_2 12\ H_2O$
2. 2% $FeNH_4(SO_4)_2 \cdot 12H_2O$
3. 2% $AgNO_3$ (prepare fresh as needed)
4. Concentrated NH_4OH

Ammoniacal silver (make fresh as needed) - add enough "4" to "3" to precipitate; then continue to add 4 until precipitate just dissolves. Add "3" dropwise until mixture is faintly cloudy.

Mordant - mix equal portions of "1" and "2", let stand 10 minutes to 24 hr. before use.

Procedure:

1. Suspend 1 loopful of organisms from a fresh slant in 1 ml of distilled water.
2. Incubate 1 hr. at 37 C.
3. With a Pasteur pipette, gently withdraw a small amount fluid from the top of the suspension and apply to top of a slide tilted 45° or greater.
4. Allow liquid to run to the edge of the slide and air-dry the preparation (precleaned slides are necessary).
5. Apply mordant for 10 minutes; rinse gently but thoroughly.
6. Add ammoniacal silver 10 minutes and rinse.
7. Air-dry.

34

Gelatin Hydrolysis (53)

Medium: Nutrient agar + 0.4% gelatin, pH 7.2.

Test: Inoculate plate and incubate under optimum conditions. Flood plate with $HgCl_2$ test reagent (paragraph 46).

Result: Clear zone around area of growth indicates gelatin hydrolysis.

35

Gluconate Oxidation (86)

Serves to distinguish certain Pseudomonas sp. (positive result) from Aeromonas, Vibrio, and Enterobacteriaceae (negative result).

Medium:
KH_2PO_4.....0.2%
$MgSO_4.7H_2O$.....0.01%
NaCl.....0.5%
$(NH_4)_2SO_4$.....0.05%

Adjust to pH 6.5. Add 1% potassium gluconate solution. Dispense 1 ml into test tubes and autoclave.

Test: Grow test organism on slant, transfer heavy inoculum into 1 ml of medium and incubate at 37C 6 hr. Add 3 ml of 1% aqueous ammonium molybdate and 0.2 ml glacial acetic acid and heat mixture in boiling water 5 minutes. Cool quickly under running tapwater.

Result: Positive result is indicated by a deep blue color.

Alternative procedure:
K_2HPO_4.....0.04 (pH 6.5)
KNO_3.....0.2%
Potassium gluconate.....2.0%

Store over chloroform at 4^oC; tube in 1 ml amounts for use. Steam tubes 10 minutes and cool.

Test: Inoculate and after incubation add 5 drops of double strength Benedicts solution. Steam tubes 10 minutes.

Result: Reddish-brown precipitate of reduced copper indicates positive result.

36

Glucose-Salt-Teepol-Broth (106)

Selective enrichment medium for V. parahemolyticus.

Beef extract.....0.3%
Peptone.....1.0%
Sodium chloride.....3.0%
Glucose.....0.5%
Methyl violet.....0.0002%
Teepol.....0.4$

Adjust to pH 9.4 and autoclave.

37

HCN (Cyanide) Production (119)

Medium: Blood Agar

Indicator paper-saturated solution of benzidine acetate is prepared in boiling water. Cool and filter. To the filtrate add 0.1 volume of 3% cupric acetate solution. Dip tip of filter paper in the mixture.

Test: Inoculate blood agar plate solid and incubate 2 days. Place indicator paper inside, but not touching medium and replace lid.

Results: Indicator paper turns blue in presence of HCN. Cl, Br, HCl, give false positive test. SO_2 and H_2SO_4 inhibit reaction. Paper turns brown in 10 minutes due to ammonia from cultures.

Alternative Procedure:

Inoculate slant in large bottle and incubate 2 days. Flush with air by bubbling through 1 ml 10% NaOH mixed with 0.1 ml 1% $FeSO_4.7H_2O$. After 10 minutes, heat to boiling and add 1 drop 1% $FeCl_3$ and cautiously acidify with 5M HCl. Prussian blue reaction represents positive results.

38

Hemophilus Agar (121)

For isolation of Hemophilus piscium.

Potato extract (Cocarboxylase). Mix 2 parts distilled water with 1 part potato and homogenize in blender. Extract overnight in refrigerator. Withdraw and clarify extract. Sterilize by

filtration.

Mueller Hinton medium.....3.8%
Yeast Extract.....0.5%
Maltose.....0.5%

Heat to dissolve, autoclave. Cool to 50C. Add potato extract to a final concentration of 5%.

39

Hippurate Cleavage (53)

Medium:

Sodium Hippurate.....0.3%
Phenol Red (0.2%).....0.5%
Agar.....1.5%

Place in tubes and slant

Test: Lightly inoculate slant and examine daily.

Result: Hydrolysis is indicated by growth and pink color.

40

Iodoacetate Test (74)

Iodoacetate prevents the fermentative dissimilation of glucose and is useful for distinguishing microorganisms capable of dissimilating carbohydrates oxidatively (eg. Pseudomonas sp.) from those that dissimilate carbohydrates fermentatively.

Medium:
Bacto-tryptone.....0.1%
Yeast extract.....0.1%
NaCl.....0.5%
K_2HPO_4.....0.03%
Glucose.....1.0%
Aqueous Brom
Thymol Blue (1.5%).....0.2%

Adjust to pH 7.2. Sterilize, and add sterile 0.001M monoiodoacetate.

Test: Inoculate iodoacetate carbohydrate broth and control (broth without iodoacetate).

Results: Acid reaction in iodoacetate carbohydrate broth represents oxidative dissimilation; acid in control only represents negative reaction.

41

Ion Requirements (24)

Used to test for Na+, Ca++, K+, Mg++ requirements of marine organisms.

Medium:

Basal Medium:

Casamino acids.....0.1%
Yeast extract.....0.05%

Salt solutions (incorporate one of the following):

$CaCl_2$.....0.1M
$MgSO_4$.....0.028M
KCl.....0.01M For Na+ requirement

NaCl.....0.4M
MgCl.....0.028M For Ca++ requirement
MCl.....0.01M

NaCl.....0.4M
$CaCl_2$.....0.01M
$MgSO_4$.....0.028M For K+ requirement

NaCl.....0.4M
$CaCl_2$.....0.01M For Mg++ requirement
KCl.....0.01M

NaCl.....0.4M
$CaCl_2$.....0.01M For mixed salt control
$MgSO_4$.....0.01M
KCl.....0.01M

Omit salt solution for other control

Ion agar.....1.5%

Adjust to pH 7.4 with Tris Buffer.

42

Kidney Disease Medium (91)

Tryptose.....1%
Beef Extract.....0.3%
Sodium Chloride.....0.5%
Yeast Extract.....0.05%
L-Cysteine HCl.....0.1%
Blood.....20%

43

Lecithinase Activity (12)
"Egg Yolk" Reaction

Useful in distinguishing Pseudomonas sp. (Ps. fluorescens +; Ps. aeruginosa -).

Medium: Scrub shell of fresh hen's egg then soak in 70% ethanol 1 hr. Separate egg yolk, place in flask and add 4 parts distilled water. Mix and heat in waterbath at 45 C for 2 hr. Deposit particulate matter by centrifugation or by overnight incubation at 4-6 C. Decant supernatant. Add 1 ml per 10 ml basal medium.

Basal Medium

Yeastrel	0.3%
Peptone	0.5%
NaCl	1.0%
Agar	1.5%

Sterilize. Add yolk preparation. Turbidity in medium is related to salt concentration.

Test: Inoculate medium and observe for opacity and/or precipitate around growth.

44

Litmus Milk Reactions (53)

Medium: Skim milk with sufficient litmus to give pale mauve color. Sterilize by steaming 30 minutes. Commercial medium is available.

Test: Inoculate as for broth, incubate and examine daily.

Result: Reactions and combinations of reactions involves lactose, casein and other milk constituents:

Acid production (A): Litmus turns pink
Acid clot (AC): Milk clots with production of sufficient acid.
Reduction (R): Reduction of litmus and loss of color; may precede or follow other changes.
Alkaline clot (KC): Coagulation of milk, litmus remains blue.
Peptonization (P): Hydrolysis of casein causes clearing and loss of opacity in milk medium (as opposed to coagulation).

45

McCurdy's Dilution Medium (80)

For isolating Myxobacteria

$MgSO_4.7H_2O$.....0.5%
K_2HPO_4.....0.025%
Soluble Starch.....0.5%

46

Mercuric Chloride Test Reagent (53)

$HgCl_2$.....15 g
Conc. HCl.....20 ml
Dist. H_2O.....100 ml

Precipitates proteins, thus is used to recognize protein hydrolysis (e.g. gelatin).

47

Minimal Medium C. Source Pseudomonas (111)

$(NH_4)_2SO_4$.....0.5%
K_2HPO_4.....0.5%
Carbohydrate to be tested.....1.0%

48

Minimal Medium Organic Acid Pseudomonas (111)

K_2HPO_4.....0.5%
$(NH_4)_2SO_4$.....0.5%
$MgSO_4$.....0.05%
Organic Acid (Salt).....0.3%
0.002% Phenol Red.....0.6%

Salts of organic acids often used:

Formate	Succinate
Oxalate	Malonate
Acetate	Malate
Benzoate	DL Tartrate
Lactate	Citrate

Test: Inoculate with washed cells and incubate at least 7 days before reading.

Result: Alkaline reaction and growth greater than control indicates positive result.

49

Motility - Denitrification (98,99)

For identification of certain Pseudomonads and other denitrifing bacteria.

Tryptose.....1%
Infusion Agar.....0.8%
KNO_3.....0.1%

Stab inoculate and observe for gas production.

50

Mucoid Colony: Levan Production (45)

Certain Pseudomonas sp. may be distinguished from others on basis of this feature. Ps. aureofaciens, Ps. chlororaphis and certain strains of Ps. fluorescens give positive results; whereas Ps. putida, Ps. aeruginosa, and certain strains of Ps. fluorescens give negative results.

Medium: Plates are streaked with suspect organism and incubated for optimum growth.

Result: Extremely mucoid colonies are indicative of a positive test.

51

Myxobacteria Agar (21)

Used for isolation and enumeration of Myxobacteria from aquatic habitats.

Tryptose.....0.005%
Yeast Extract.....0.005%
Sodium Acetate.....0.002%
Beef Extract.....0.002%
Peptonized Milk.....0.05%
Agar.....1.5%

Adjust pH to 6.8, autoclave, then add Neomycin 5 ug/ml and cycloheximide 10 ug/ml.

52

Nalidixic Acid Cetrimide Agar (49)

For selective isolation of Pseudomonas aeruginosa.

Peptone	2%
Dipotassium Phosphate	0.03%
Magnesium sulfate (anhydrous)	0.03%
Cetrimide	0.02%
Nalidixic Acid	1.5%
Agar	1.5%

53

Nessler's Reagent (30)

Used for Ammonia Detection.

Dissolve 8g KI and 11.5g HgI_2 in 20 ml water and adjust to 50 ml. Add 50 ml 6N NaOH. Mix and allow to stand 24 hr.

Water must be NH_4 free. Allow reagent to settle before use and protect from light.

54

Nitrate Reduction (30,53)

Medium: A medium containing 0.2% KNO_3 is utilized, which is most suited to growth of the organism.

Test: Four drops of sulphanilic acid reagent (0.8% sulfanilic acid in 0.2N acetic acid) followed by 2-4 drops of alpha naphthylamine acetate reagent (0.5% alpha naphthylamine acetate in 0.2N acetic acid) are added to culture.

Result: Development of pink color is a positive result indicating presence of nitrates. Nitrates may have been reduced beyond nitrites, thus negative test must be confirmed by addition of zinc dust to reduce nitrates, if present. Development of pink color after addition of zinc dust is indicative of a negative result.

55

Ordals Medium (90)

For isolation of Chondrococcus columnaris.

Tryptone.....0.05%
Yeast extract.....0.05%
Beef extract.....0.02%
Sodium acetate.....0.02%
Agar.....0.9%

Adjust pH 7.2 - 7.3

56

Oxalate Decomposition (10)

Medium: Potassium oxalate hydrate.....0.1%
$(NH_4)_2SO_4$.....0.05%
K_2HPO_4.....0.05%
$MgSO_4.7H_2O$.....0.01%
$FeSO_4H_2O$.....0.01%
$CaSO_42H_2O$.....0.001%
Yeast extract.....0.1%
Agar.....1.5%

Add 2 ml sterile 0.1M $CaCl_2$/100 ml medium.

Test: Inoculate and examine for cleared areas around colonies or growth.

57

Oxidase Test (30,53)

Differentiates Enterobacteriaceae from many aquatic bacteria.

Kovacs Oxidase

Prepare 1% solution of tetramethyl paraphenylene diamine HCl in dark bottle. Store in refrigerator. Deteriorates after 2 weeks. Place piece of Whatman No. 1 filter paper in petri plate and drop 2-3 drops of reagent in center of paper. Transfer portion of growth to impregnated paper and smear in a line 3-5 cm long. A purple color in 5-10 seconds indicates a positive test.

Pectinase Activity (95)

Useful in Pseudomonas classification.

Medium: Two solutions sterilize separately.

Calcium Agar Base:

Peptone.....0.5%
Meat extract.....0.5%
Calcium lactate.....0.5%
Agar.....1.5%

Pectin Solution:

(Sodium polypectate "Exchange" Sunkist growers, Oakland, Calif.)

1. Prepare plate of pectin by mixing 2 gm pectin with absolute alcohol.
2. Make up to 100 ml with distilled water and add 0.1 gm disodium salt of EDTA.

Pour pectin solution as a thin layer over plate of Ca++ agar (1 cm). Gel sets within 12-24 hr. at 27°C.

Test: Inoculate plate and observe for decomposition ("pitting") in medium.

59

Pectinolytic Medium (65)

Pectin (BDH apple pectin, demethylated at pH 8.6).....5%
K_2HPO_4.....0.5%
$MgSO_4 \cdot 7H_2O$.....0.01%
NaCl.....0.02%
$CaCl_2 2H_2O$.....0.02%
$FeCl_3 6H_2O$.....0.001%
Yeast Extract.....0.1%
Agar.....2.0%

After inoculation, incubate, add 1% aqueous cetavlon (cetyl trimethyl ammonium bromide).

Cetavlon precipitates polysaccharide---hydrolysis is indicated by clear zones.

60

Phosphatase (5)

Phenolphthalein diphosphate 0.01% incorporate in any basal medium suitable for growth of test organism.

Test: Incubate 3-5 days and expose colonies to ammonia vapors.

Result: Pink colonies or colonies surrounded by pink halos indicate positive result.

61

Assay for Proteolytic Marine Bacteria (116)

Two layers of the medium are poured separately.

Bottom Layer

Nonfat dry milk	4%
Agar	3%

Autoclave separately, then aseptically mix in equal portions.

Top Layer

May be seawater nutrient agar or marine agar poured over solidified basal layer.

Although casein is not a common marine protein, the majority of the aquatic bacteria tested gave similar results with either casein or fish juice agar.

62

Assay for Proteolytic Organisms (79)

Std. Methods Agar (BBL)	2.35%
Pancreatic Digest Casein	0.5%
Yeast Extract	2.5%
Glucose	1.0%
Agar	1.5%
Sodium Caseinate	1.0%
Hydrated Trisodium Citrate	0.441%
$CaCl_2.6H_2O$	0.438%

63

Salt-Colistin Broth (107)

Selective enrichment medium for V. parahemolyticus.

Yeast extract.....0.3%
Peptone.....1.0%
Sodium Chloride.....2.0%
Colistin methanesulfonate.....500 u/ml

The medium should be adjusted to pH 7.4; do not heat after adding colistin.

64

Silica Gel Medium (46)

Used in studying nitrifying bacteria.

Two solutions to make gels

1) 20% aqueous o-phosphoric acid (certified A.C.S. 85%, Fisher).

2) 10 g powedered silica gel (grade 923, 100 to 200 mesh, Well Scientific, Inc., Balt. Med.) or Silica acid (Reagent grade, J. T. Baker, Chem. Co., Philipsburg, N. J.) are brought into solution in 100 ml 7% KOH by heating Dispense "2" into flasks, 20 ml amounts and autoclave.

Mix equal amounts of sterile double strength growth medium and rapidly add an appropriate amount (approx. 4 ml) sterile phosphoric acid, rotate to mix and immediate pour into sterile petri dishes. Gelation begins about 10 minutes after pH is adjusted to 7.0 with acids. Gels become firm in minutes and undergo syneresis. The water of syneresis can be aseptically decanted or evaporated in an incubator.

65

Slime Layer (69)

1. Mix a loop of cultures to be examined on coverslip.
2. Dry in air and place in Chabaud's solution for 10 minutes.
   Alcohol (80%).....60 ml
   Formalin.....5 ml
   Glacial Acetic Acid.....2 ml
   Phenol Crystals.....15 gm

3. Rinse and place in 5% tannic acid 30 minutes.
4. Wash thoroughly and stain in crystal violet for several hr.
5. Mount in stain.

66

Starch Hydrolysis (53)

Medium: Nutrient Agar + 1% soluble starch.

Test: Inoculate, incubate under optimum conditions 2-14 days. Flood plate with Grams iodine solution.

Result: Hydrolyzed starvh appears as a clear zone and is the result of B-amylase activity. Reddish brown zones indicate partial hydrolysis and is the result of α-amylase activity.

67

Stock Salt Solution (10X)

May be used in most conventional growth and biochemical media for growth and testing of marine bacteria.

NaCl	23.4%
KCl	1.5%
$MgSO_4.7H_2O$	1.2%
$CaCl_2.2H_2O$	0.2%

Autoclave. Add salt to a final concentration of 10%.

68

Swarming Medium (54)

For distinguishing Cytophaga sp. from similar microorganisms.

Yeast extract	0.1%
Ion Agar No. 2 (Oxoid)	0.8%

Surface moisture dried before incubation.

Result: Typical spreading growth occurs in the form of a thin film extending rapidly over the surface from site of inoculation.

69

Marine Swarming Medium (54)

Medium:
Beef extract.....0.1%
Agar.....2.0%
Peptone.....0.05%

Swarming is dependent upon peptone concentration. 8%, 4%, 1%, 0.25%, 0.01% are also used.

Dissolve in 3 parts seawater + 1 part distilled water and sterilize. Dry medium 30 minutes at 55 C and store overnight at 20 C before inoculation.

70

Trimethylamine Oxide (TMO) Reduction (20)

Utilized to study organisms associated with fish spoilage, particularly Achromobacter sp., Achr. delicatulum, Achr. amylovorum and Achr. lipidus reduced TMO. Achro. aquamarinus was a non reducing species.

Medium:
Peptone "C".....0.5%
NaCl.....0.5%
$MgSO_4.7H_2O$.....0.1%
KH_2PO_4.....0.1%
Glucose.....0.25%
*Trimethylamine Oxide.....0.1%

Test: Incubate cultures 8 days in above medium. Impregnate absorbant cotton with bromthymol blue which has been adjusted to pH 4 with H_2SO_4. Add 1.5 ml formalin to 5 ml culture and allow tube to stand 3 minutes. Add 3 ml saturated solution of potassium carbonate and quickly close tube with stopper fitted with glass tube slightly constricted and containing small piece of dye saturated cotton; incubate at 45 C for 30 minutes.

Results: If trimethylamine is present, it condenses in cotton causing indicator to turn blue due to alkalinity.

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* 0.5% choline, acetyl choline and betaine has been substituted for TMO.

71

Twedt's Vibrio parahaemolyticus Medium (133)

For selective isolation of V. parahaemolyticus.

Peptone.....2.0%
Yeast Extract.....0.2%
Corn Starch.....0.5%
NaCl.....3.0%
Agar.....1.5%

Adjust pH to 8.0, autoclave, cool and add penicillin 5 units/ml. Vibrio parahaemolyticus colonies are distinguished by their ability to hydrolyze starch.

72

Tween 80 Hydrolysis (22)
Fatty Acid Ester Hydrolysis

Vibrio sp., Aeromonas sp. and some Pseudomonas sp. hydrolyse Tween 80.

Medium: Bacto Agar.....1.5%
Bacto Trypticase Soy Broth.....2.0%
$CaCl_2$ (Sat. Sol'n).....1%
Tween.....1%

Sterilize, adjust to pH 7.0.

Test: Inoculate and allow organisms to grow 48 hr. Remove colonies with water if necessary to observe opacity.

73

Inhibition by Vibriostat (110)

Serves to distinguish Vibrio sp. from similar organisms. 2, 4-Diamino-6, 7-disopropyl pteridine phosphate.....5% in chloroform (available from Gallard-Schlesinger Chemical Mfg. Corp., 584 Mineola Ave., Carle Place, L. I., N. Y. 11514).

Dup blank antibiotic sensitivity discs in solution and allow the preparation to dry.

Test: Apply as usual for antibiotic sensitivity testing.

Result: Most pathogenic virbios are inhibited by this compound.

74

Violacein Test (119,120)

Characteristic for members of genus Chromobacterium.

Suspend a loopful of organisms in ethanol and another in chloroform contained in wells of white tile. Transfer a loopful of the ethanol suspension to another well containing 25% H_2SO_4, which in presence of violacein turns green. Add 10% sodium hydroxide to remainder of ethanol suspension, which in presence of violacein turns green, then reddish.

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