PRODUCTION, LOCATION, AND BINDING OF VIOLACEIN IN *JANTHINOBACTERIUM*

THESIS

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By

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Violacein is a purple pigment typically produced by species of *Chromobacterium* and *Janthinobacterium*. A soil isolate, identified as *Janthinobacterium*, was studied. Maximal pigmentation occurred at 25°C under aerobic conditions in the Keeble and Cross medium. Intracellular pigment was shown to be located in the cell membrane. Comparision of pigment production and growth curves indicated that violacein is synthesized in the cell and released into the environment possibly as a result of cell lysis. Extracellular pigment is water soluble, makes up 60% of the total pigment and shows a blue shift when compared to solvent extracted pigment. Results from purification indicated that the pigment is non-covalently bound to a small protein and aggregated into a larger molecule.

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CHAPTER I

INTRODUCTION

The genus *Chromobacterium* consists of Gram-negative, non-spore forming bacilli with round ends that are motile with polar or lateral flagella. No definite capsule is formed and colonies are non-gelatinous when growing in ordinary medium. They are facultatively anaerobic with an optimal growth temperature of 30-35°C and are commonly found in soil and water of tropical countries. They usually degrade carbohydrates fermentatively and produce HCN, resulting in the production of an ammonium cyanide odor, and a characteristic violet pigment known as violacein. *Chromobacterium violaceum* has been reported to cause infection of mammals including man (31, 48, 64).

Janthinobacterium (formerly *Chromobacterium lividum*) consists of non-spore forming, Gram-negative rods with round ends, and no definite capsule. They are motile by means of both a single polar flagellum and usually one to four subpolar lateral flagella. Colonies on solid media are low, convex, round and violet pigmented. The growth is often gelatinous or rubbery and a tough pellicle appears around the surface of broth cultures. The optimal growth temperature is

25°C. *Janthinobacteria* are strict aerobes, degrade carbohydrates oxidatively and usually produce ammonia and do not produce an ammonium cyanide odor. *Janthinobacterium* strains are commonly found in soil and water of temperate regions and are occasionally involved in food spoilage (11, 34, 65).

In the 8th edition of Bergeys' *Manual of Determinative Bacteriology* (63), organisms which produce typical violacein were included only in the genus *Chromobacterium* in the "Genera of uncertain affiliation" in part 8, the Gram-negative facultatively anaerobic rods. In this edition, there were two distinct groups of *Chromobacterium*: *Chromobacterium violaceum*, consisted of mesophilic facultative anaerobes, and *Chromobacterium lividum*, referred as a psychrophilic strict aerobe based on the work of Sneath (61).

In the new 1st edition of Bergeys' *Manual of Systematic Bacteriology* (65), *Chromobacterium lividum* was elevated to the genus level as *Janthinobacterium* and is included in section 4: Gram-negative aerobic rods and cocci. *Chromobacterium* is classified in section 5: Facultatively anaerobic Gram-negative rods. Further justification for a separate genus is based on the work of De Ley (14) who found that *Janthinobacterium* and *Chromobacterium* differed not only

phenotypically but genotypically by DNA/RNA hybridization and RNA similarity maps.

The main characteristics differentiating *Chromobacterium* and *Janthinobacterium* are shown below in Table 1. Another group of purple pigment producing Gram-negative rods has been reported by Gauthier (26).

TABLE 1.

Characteristics differentiating the genus *Chromobacterium* from the genus *Janthinobacterium*[a]

Characteristics	*Chromobacterium* (%) strain giving positive reaction	*Janthinobacterium* (%) strain giving positive reaction
Glucose is catabolized by: Fermentation	D[b] (80)	-
Oxidation	D (20)	+ (95)
Turbid zone on egg yolk agar (lecithinase)	+	-
Acid from:		
Trehalose	+	-
L-Arabinose	-	+ (95)
Xylose	-	+ (95)
Casein hydrolysis	+	- or weak
Esculin hydrolysis	-	+ (95)

[a]According to the first edition of Bergey's Manual of Systematic Bacteriology (64, 65).

[b]Delayed

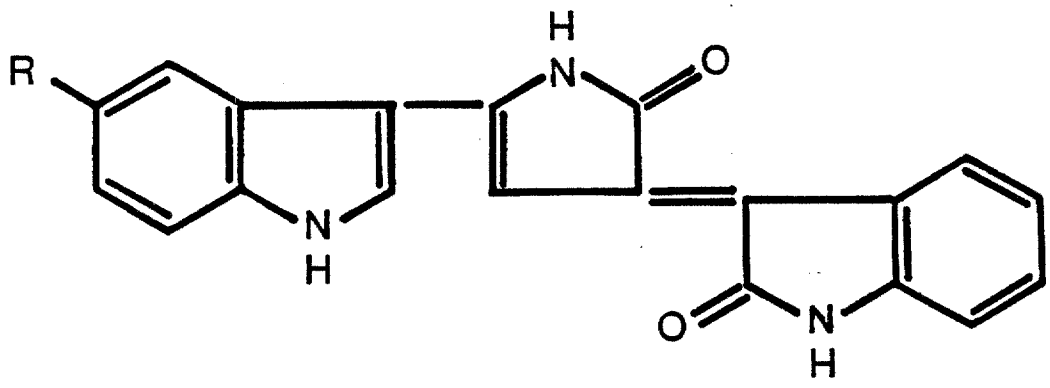
They are of marine origin and their relation to *Chromobacterium* and *Janthinobacterium* is still uncertain. They require a sea water base for growth and have been proposed to be included in a new genus *Alteromonas* with the new species named *leteoviolaceus* (26). All strains were reported by McCarthy et al. (43) to produce violacein.

Although all strains from the genera mentioned appear to produce the same purple pigment, poorly pigmented and non-pigmented variants occur in nature and in laboratory stocks (8, 59). *Chromobacterium* and *Janthinobacterium* are relatively undemanding in their nutritional requirements and grow on ordinary media, but they do not grow well or produce their characteristic pigment so readily in the presence of large numbers of other bacteria (47). The ability to produce pigment depends on many cultural factors such as: medium composition, temperature, degree of aeration and pH (30, 33). Each of these factors greatly affects the synthesis of violacein. Usually, these organisms are best grown on surface-spread plates because oxygen is required for the production of violacein, the pigment by which they are most readily recognized. Pigmentation of non-pigmented variants is often regained by sub-culturing on starch, dextrin, glycogen or potato. It is important to note that Darrasse et

al. (13) reported the stimulation of pigmentation by low concentrations of neomycin and penicillin.

According to Ballantine et al. (1, 2) the chemical structure of violacein is: 5-3-(5-hydroxindoly)3-3-(2-oxindolylidene)-2-oxopyrroline as shown in Figure 1. It has a molecular weight of 343.34 and is insoluble in water, chloroform, benzene and toluene. It is soluble in ethanol, acetone, methanol, glycerol (61) and dimethyl sulfoxide (33). When treated with HCl or 10% H_2SO_4 it becomes blue-green or green. The color is stable and returns to violet on neutralization. With NaOH it becomes green then turns reddish brown and decomposes. The color is not restored by neutralization. The pigment is stable at 20°C or below, but is decolorized at 80°C or exposure to strong light.

The spectrum of violacein has been reported since the end of 19th century. There is a maximal absorption peak at about 570 nm and a minimal peak at 440 nm in ethanol (28). In acetone, the peaks are at 570 nm and 445 nm. The specific extinction coefficient has been determined to be about 44 at 665 nm by DeMoss and Evan (15) and 65.4 at 579 nm by Sneath (61). When a 10% (v/v) solution of H_2SO_4 is added to an ethanolic solution of violacein, the pigment gives a green color with an absorption maximum at 700 nm. Using alumina chromatography for the purification of violacein from



R:OH = VIOLACEIN

5-3(5-hydroxindoly) 3-3(2-oxindolylidene)

- 2-oxopyrroline

$C_{20}H_{13}O_3N_3$

M.wt. : 343.33

R:H = DEOXYVIOLACEIN

Figure 1. Structure of violacein and deoxyviolacein.

Chromobacterium, Sneath (62) observed an unidentified reddish-purple fraction which ran faster than the main purple-blue band.

The biosythetic pathway of violacein formation is still unknown. Mitoma et al. (45) suggested 5-hydroxytryptophan could be an intermediate between tryptophan and violacein. Studies of the carbon requirements for pigment synthesis show that L-tryptophan is the sole source of carbon for violacein formation (15, 16). However, Inniss and Mayfield (30) found that pyruvate and alanine stimulated pigmentation in a psychrotrophic strain of *Chromobacterium lividum* which had an optimal growth temperature range of 15–25°C. Sebek and Jager (55) proposed a divergent pathway for violacein systhesis i.e., L-tryptophan → indole → violacein and suggested that deoxyviolacein was formed in a later stage. In studies of the effect of cultural conditions on violacein formation, Kimmel and Maier (33) showed that the synthesis of violacein in both genera required L-tryptophan and was also enhanced by casein hydrolysate. It was also observed that both genera required oxygen for pigmentation (8, 33). Letendre et al. (36) studied the enzyme responsible for tryptophan hydroxylation that presumably provides the precursor for the violacein.

This purple pigment has been described since the end of 19th century. Many characteristics related to its physical and chemical properties were reported in subsequent studies. Isolation and determination of the molecular weight of violacein was studied by Reilly and Pyne (50). Attempts to further purify the pigment were reported by Tobie (70). Ehrismann and Noethling (22) tried to determine the molar extinction coefficient. A better purification method was developed by Strong (68). The attempts at determining the structure of violacein were not successful until the degradation and synthetic studies of Ballantine et al. (1, 2). He reported the structure of violacein and another minor component which is blue in color identified as deoxyviolacein. DeMoss and Evan (15) reported that these two pigments could be separated by paper chromatography or on alumina columns. The violacein/deoxyviolacein ratio was reported to be 15.9:5.9.

The function of violacein in the cell is unknown. Friedheim (25) noticed a stimulation of respiratory activity on addition of violacein to non-pigmented cells. DeMoss (18) suggested a function in protecting against photodynamic killing because this pigment is non-diffusible and has a high extinction coefficient at 580 nm. DeMoss (18) also suggested that the pigment could be just an accidental end product of

tryptophan degradation, keeping the intracellular level of tryptophan at a relatively low, non-toxic concentration, since the normal pathway of tryptophan catabolism; conversion to kynurenine and anthranilic acid, does not occur to an appreciable extent in this organism (45).

Antibiotic properties of violacein have been reported by Lichstein and Van De Sand (37, 38). They observed the marked inhibitory effect of purified violacein on the growth of Gram-positive but not Gram-negative bacteria and fungi. Violacein is toxic to soil protozoa (56). Groscop and Brent (29) reported that violacein is toxic to all tested strains of free-living amoebae.

Recent studies from Duran et al. (20, 21) showed that the photodynamic effect of this pigment at wavelengths > 450 nm is similar to that of crystal violet which is used as a photochemotherapeutic agent. Also the photolysis products are active against Gram-positive and Gram-negative bacteria *in vitro*.

Most studies that relate to the pigment have dealt with its purification, structure, possible biosynthetic pathway association with tryptophan metabolism, and possible functions. Little work has been done on the location and the association of this pigment with other components in the cell, although, it has been observed that with a soil isolate

this pigment appears in both the cell and medium when grown in a liquid medium (24). In studies on the purification of an extracellular metallo-proteinase from this organism, Etherington et al. (24) noticed that the ammonium sulfate precipitate of crude enzymes was always intensely pigmented. The water insoluble pigment became soluble in liquid medium indicating that it was bound to some water soluble compound.

The purpose of this study, using a soil isolate identified as a species of *Janthinobacterium,* was to: [1] Find the optimal conditions for both growth and pigmentation; [2] Determine the relationship between growth and pigment production and the distribution of both cellular and extracellular pigment; and [3] Locate the cellular positon of this pigment. The investigation of the binding and location of violacein should help to improve our understanding of the interaction between the pigment and other cellular constituents. Also, the metabolism of violacein which is closely linked to tryptophan metabolism, and the function of this pigment in the cell can be better understood.

CHAPTER II

MATERIALS AND METHODS

The Organism

The strain used for this study was an isolate from a local soil. It was isolated by using the selective medium of Ryall and Moss (51) which consists of beef extract, 0.1%; yeast extract, 0.1%; Bacto-casitone, 0.2%; glucose, 1%; cycloheximide, 50 μg/ml; mycostatin, 50 μg/ml; agar, 1.8%. Plates sprinkled with soil or spread with samples of a serial dilution of soil were incubated at room temperature in the dark. A purple pigmented colony was isolated and identified using the tests developed by Sneath (62) which are widely accepted and included in the first edition of Bergey's Manual of Systemic Bacteriology (64).

Cultures were maintained on a similar improved agar medium based on Bennett's agar developed by Keeble and Cross (30) and were subcultured every two weeks by selecting dark purple colonies. Cultures also were maintained in 0.1% peptone water at 4°C or at -20°C in nutrient broth containing 50% glycerol for long term preservation.

Culture Conditions

Cultures were grown in a broth medium containing beef extract, 0.1%; glucose, 0.5%; yeast extract,0.1%; Bacto-casitone, 0.2%; and sodium thioglycollate, 0.05%. The final pH was 7.0. Flasks containing no more than one fourth their volume of medium were incubated at 25°C on a reciprocal shaker.(5 cm stroke, 100 stroke min^{-1}) (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey). Purity of cultures was checked microscopically and by streaking on plates of the same medium as described above without sodium thioglycollate and with 1.8% agar. A 1% inoculum of a culture adjusted to an absorbance of 0.3 at 450 nm on a Spectronic 20 (Bausch & Lomb, Inc., Rochester New York) was used for test flasks. Growth was monitored by measuring absorbance at 450 nm. Viable cell counts were performed using the standard spread plate technique. Cell dry weight was determined on cells washed two times in distilled water and dried at 70°C to constant weight in a vacuum oven.

Extraction of Pigment

Cultures were centrifuged at 5900 × g for 10 minutes in a Sorvall RC 2-B superspeed centrifuge (Ivan Sorvall, Inc., Newtown, Connecticut). Both the pellet and supernatant fluid were frozen and thawed before extraction. Thawed cell pellets

were repeatedly extracted by vortexing with 96% ethyl alcohol until colorless. The extracellular supernatant fluid was treated with acetone, then partitioned with ethyl ether until colorless. The pigmented ethyl ether extract was evaporated to dryness and redissolved in 96% ethyl alcohol. Absorbance of extracellular and cellular pigment extracts were measured at 576 nm in a Perkin-Elmer Lambda 3A spectrophotometer. (Perkin- Elmer Co. Norwalk, Connecticut). Sneath (60) determined the specific extinction coefficient of violacein to be 65.4 in 96% ethyl alcohol. Using this figure the concentration of pigment was determined as follows:

$$\text{Pigment Concentration (mg/mL)} = (OD_{579}) / 65.4$$

Characterization of Pigment

The absorption spectrum of crude pigment from the medium supernatant fluid and solvent extracted pigment from the cell pellet and supernatant fluid were examined from 300–750 nm in the Lambda 3A spectrophotometer. Also, the color change reaction and absorption spectrum of the pigment in 96% ethyl alcohol upon adding 10% (v/v) sulfuric acid and adding 10% (w/v) sodium hydroxide were examined. The solubility of the free pigment in water, chloroform, ethyl ether and ethyl alcohol was also determined.

Preparation of Mureinoplasts

Mureinoplasts is the term used to describe a Gram-negative bacterium, which has the outer membrane removed and is surrounded only by the peptidoglycan layer and the cytoplasmic membrane (19). A procedure used for producing such forms of cells was described by Costerton et al. and DeVoe et al. (10, 19). Pigmented cell cultures (30 hours old) were harvested by centrifugation at 5090 × g for 10 minutes. The pigmented cell pellets were resuspended in 100 mL of 0.5M NaCl and washed three times in this solution. Washed cells were collected by centrifugation at 7710 × g for 10 minutes and resupended in 50 mL of 0.5M sucrose. The sucrose suspension was incubated at 30°C for at least one hour on a rotary shaker. Cells were harvested by centrifugation at 7710 × g for 10 minutes and washed immediately with same volume of 0.5 M sucrose. During this procedure, each pellet and supernatant was examined for the presence of pigment. Light and electron microscopy were used to monitor the formation of mureinoplasts.

Preparation of Protoplasts

The principles for preparing protoplasts were described by DeVoe et al. (19). Mureinoplasts were suspended by vortexing in protoplast buffer containing lysozyme, 100 µg;

NaCl, 0.4 M; KCl, 0.01 M; $MgSO_4$, 0.03 M and Tris-HCl buffer, 1 mM, (pH 7.5), and incubated at 25°C for at least 5 hours on a shaker. The formation of protoplasts was monitored by phase contrast microscopy. The efficiency of formation was greater than 70%. The cell suspension was centrifuged at 7710 × g for 10 minutes and diluted in distilled water of 1/10 volume of the orginal culture. The diluted suspension was sonicated for six 10 seconds period at 25 watts with a sonifier cell disruptor Model W1850 (Heat System-Ultrasonic Inc., Plainview L.I., New York).

Unruptured cells and protoplasts were separated by centrifugation at 3020 × g for 20 minutes. The supernatant fluid was then centrifuged at 25,300 × g for 35 minutes to sediment the cell membrane. Each fraction, including supernatant and pellet were examined for the presence of the pigment. Phase contrast and electron microscopy were used to monitor the procedure.

Electron Microscopy

Ten mL samples of whole cell, mureinoplast or protoplast suspensions were collected. Each sample was resuspended and washed twice with 5 mL of 0.1 M cacodylate washing buffer (pH 7.2) and then incubated overnight at 4°C in 2 mL of 4% glutaraldehyde to fix the specimens. Fixed cell pellets were

enrobed in 3% agar and post-fixed in 1% OSO_4 for one hour. After fixation, samples were dehydrated with acetone and embedded in epon. Thin sections were cut with a Sorvall MT 6000 ultramicrotome and stained with uranyl acetate and lead citrate. Stained sections were examined with a JEOL 100CX electron microscope.

Extraction of the Extracellular Pigment Complex

Extracellular supernatant fluid obtained by centrifugation of the cell culture at 7710 × g for 10 minutes was evaporated under reduced pressure to near dryness. One percent aqueous Triton X-100 was used to solubilize the supernatant concentrate and insoluble material separated by centrifugation at 7710 × g for 10 minutes. The Triton X-100 extract was dialyzed in 8,000-10,000 MW cut-off dialysis tubing against water overnight with frequent changes of dialysis water. The dialyzed sample was assayed for protein and pigment and analyzed by polyacrylamide gel electrophoresis.

Purification of the Extracellular Pigment Complex

The crude pigmented complex was precipitated from the culture supernatant fluid by the addition of saturated $(NH_4)_2SO_4$ solution (pH 7) to 65% saturation. The pigmented complex was collected by centrifugation at 5900 × g for 10 minutes and dialyzed against water. Dialyzed pigmented samples were chromatographed on Sephadex G-200 in 0.5 M phosphate buffer (pH 7) in a Pharmacia C 16/40 permeation chromatography column (Pharmacia Fine Chemicals, Piscataway, New Jersey). The method of Lowry et al. (37) was used to determine protein concentration with bovine serum albumin as the standard. All samples were stored at -25°C prior to analysis.

Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the crude culture supernatant fluid, the ammonium sulfate precipitate and the gel exclusion column fraction were carried out on 7.5% separating gels with 3% stacking gels. The SDS-disc discontinuous system described by Maizel (40) was used. The stacking (spacer) gel buffer, resolving gel buffer and electrode buffer were made according to the procedures described by Maizel (40). After

electrophoresis, a solution of methanol/acetic acid/water (30:10:60 by vol.) was used for fixing and destaining the gels. Protein bands were stainned with 0.1% of coomassie brilliant blue R in the fixing solution. Sample buffer (8 mL) was composed of distilled water, 4 mL; 0.5M Tris-HCL buffer (pH 6.8), 1 mL; glycerol, 0.8 mL; 10% SDS, 1.6 mL; 2-mercaptoethanol, 0.4 mL; 0.05% (w/v) bromophenol blue, 0.2 mL. The second polyacrylamide gel electrophoresis system used was the non-denaturing discontinuous system described by Chrambach et al.(6). Samples were resolved on 5% separating (resolving) gels with 3% stacking gels. Tubes of 12 cm in length with an inside diameter of 5 mm were used. Sample containing approximately 25 µg protein were loaded on the tube gel and run at a constant power of 3 mA per tube. Pigment bands were located in unstained gels. Protein bands were identified by staining with coomassie brilliant blue R. Electrophoresis was performed on a Bio-Rad Model 150A Gel Electrophoresis Cell equipped with a Model 500 power supply (Bio-Rad Laboratories, Richmond, California).

Extraction of the Pigment Complex from the Non-Denaturing Gel

The purple pigmented bands at the tops of non-denaturing gels were cut off, crushed and homogenized in 0.5 M phosphate

buffer (pH 7). The gel particles were sedimented by centrifugation at 7710 × g for 30 minutes. This procedure was repeated three times to extract the pigmented complex from the gel until the gel became colorless. The purple complex was analyzed for protein by the Lowry assay and the protein compositon was read by SDS-PAGE.

CHAPTER III

RESULTS

Isolation and Identification of *Janthinobacterium*

Fourteen soil samples were collected from different locations. Both sprinkle and spread plate techniques were used. A pale purple pigmented colony was isolated and purified from a spread plate culture incubated at room temperature. The differential tests suggested by Sneath (62) and listed in Bergeys' *Manual of Systematic Bacteriology* (64) to differentiate *Chromobacterium* and *Janthinobacterium* were used. The results from these tests for *Chromobacterium* and *Janthinobacterium* are shown in Table 2. The oxidation/fermentation (glucose) test was delayed and acid from sugar was obscure. Also, the growth at 37°C and 4°C is not a dependable test. Egg yolk agar, casein hydrolysis esculin hydrolysis and HCN production are consistent with the characteristics of *Janthinobacterium*. Since this organism is strict aerobe, it should be classified as a species of *Janthinobacterium* rather than *Chromobacterium*.

TABLE 2.

Result of differentiation tests used to classify soil isolates

TESTS	Soil isolate	*Janthino-bactuim*[a]	*Chromo-bacterium*[a]
Anaerobic growth			
in GAS-PAK jar	-	-	+
Growth at 37°C	-	-	+
Growth at 4°C	-	+	-
O - F test[b] (glucose)			
fermentation	-	-	D[c] (80)
oxidation	± (D)	+ (95)	D (20)
Turbid zone on egg yolk agar	weak	-	+
Acid from:			
trehalose	weak	-	+
L-arabinose	+	+ (95)	-
xylose	-	+ (95)	-
Casein hydrolysis	+	- or +	+
Esculin hydrolysis	+	+ (95)	-
HCN production	-	-	+

[a]Results are those given in the first edition of Bergey's Manual of Systematic Bacteriology (64, 65).

[b]Oxidative or fermentative attack on glucose.

[c]Delayed.

Numbers in parentheses indicate the % of strains giving a positive reaction.

Optimal Medium for Growth and Pigmentation of *Janthinobacterium*

The media tested for optimal growth and pigmentation included different strengths of tryptic soy broth (TSB), brain heart infusion broth (BHIB), nutrient broth (NB), the modified Keeble and Cross medium (30) and the Ryall and Moss medium (51). Broth cultures were checked visually for growth and pigmentation as a function of time. The results after 48 and 72 hours incubation are shown in Table 3, and indicate the best medium for *Janthinobacterium* is that of Keeble & Cross (30). Adding 0.4-0.5% starch or dextrin instead of glucose and adding 5-25 $\mu g\ mL^{-1}$ neomycin did not enhance pigmentation of *Janthinobacterium* when compared to the control medium.

Growth Curve

The growth curve of *Janthinobacterium* in the modified broth medium of Keeble & Cross (30) was determined by plotting absorbance at 450 nm (1 cm light path) versus time as shown in Figure 2. Four hundred and fifty nm was used since this is the minimal absorbance of the pigment in the medium. The growth was also measured by plotting the viable cell count versus time as shown in Figure 2. The results

indicated that growth reached a maximum in 20 hours and shows little or no loss in viability for 60 hours.

TABLE 3.

Effect of medium composition on growth and pigmentation of *Janthinobacterium*.

Tested medium		48 Hours Pigmentation	72 Hours OD_{450}[a] (growth)	72 Hours Pigmentation
TSB[b]	F.S[c]	±[d]	2.6	±
	H.S.	±	0.5	±
	Q.S.	±	0.4	±
NB[b]	F.S	-	1.0	-
	H.S.	±	0.3	±
	Q.S.	±	0.4	±
BHIB[b]	F.S.	-	0.2	-
	H.S.	±	1.0	±
	Q.S.	±	0.7	±
Ryall & Moss broth		+	0.7	+
Keeble & Cross broth		+	2.4	+

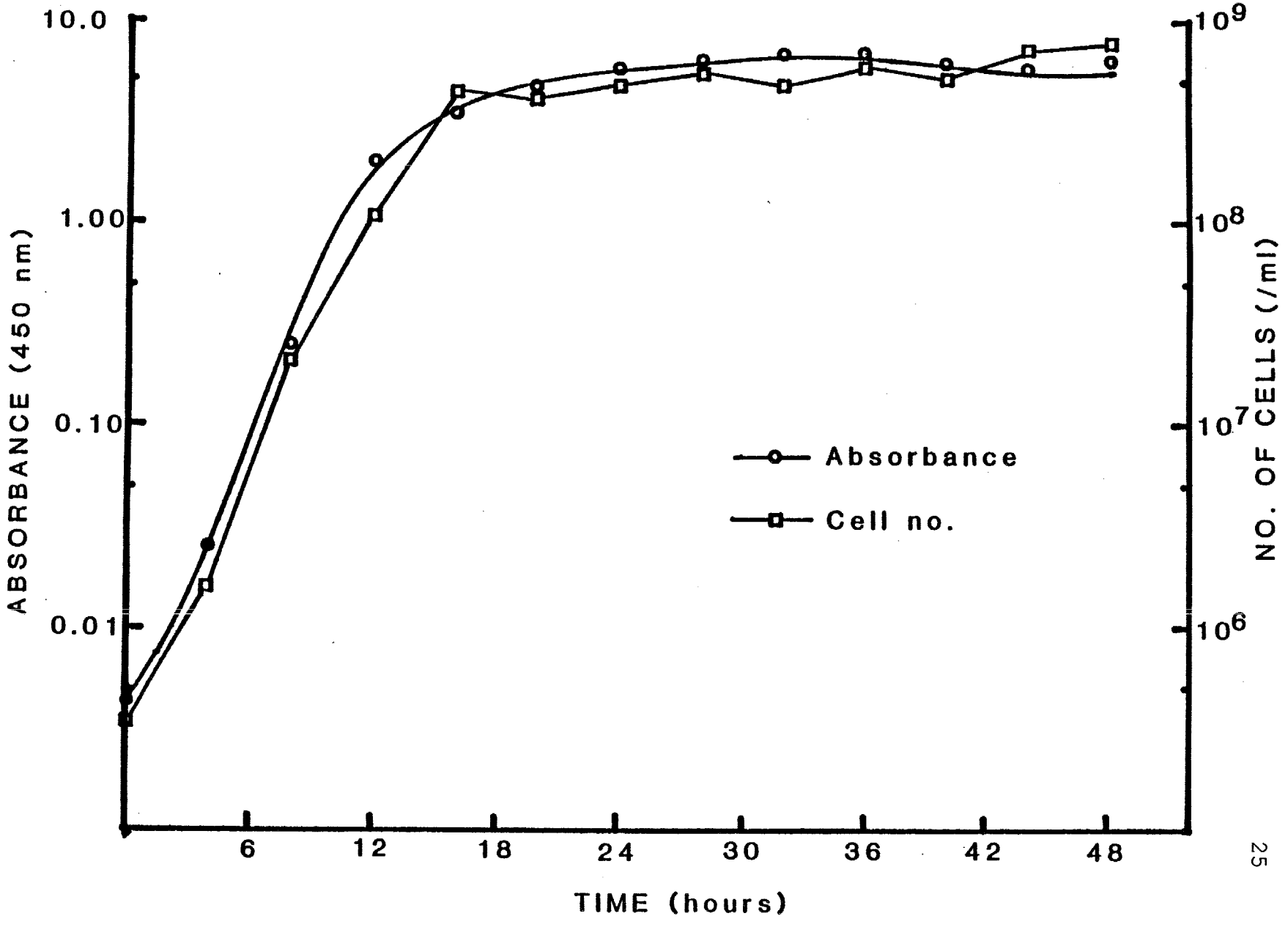
[a]absorbance at 450 nm

[b]TSB, Tryptic Soy Broth; NB, Nutrient Broth; BHIB, Brain Heart Infusion Broth.

[c]F.S., Full Strength; H.S., 50% Strength; Q.S., Quarter Strength.

[d]+, good; ±, weak; -, no.

Figure 2. Growth curve of *Janthinobacterium* as measured by absorbance at 450 nm and viable count in the Keeble & Cross medium (30). Cultures were inoculated with a 1% inoculum adjusted to an absorbance of 0.3 an incubated on a reciprocal shaker at 25°C.


10.0
1.00
0.10
0.01
ABSORBANCE (450 nm)
10^9
10^8
10^7
10^6
NO. OF CELLS (/ml)
Absorbance
Cell no.
6
12
18
24
30
36
42
48
TIME (hours)

Cell Dry Weight

In order to establish the relationship between pigment production and cell mass, a plot of cell growth as measured by absorbance at 450 nm versus cell dry weight was made. The plot in Figure 3 shows a linear relationship between absorbance and cell dry weight.

Production of Extracellular and Intracellular Pigment

Violacein was found to be non-diffusible in cells grown on agar media, but both extracellular and intracellular pigment were observed in liquid medium. Both extracellular and intracellular pigments were extracted and the concentrations determined. The production curve of both as a function of time is shown in Figure 4. The curve shows that measurable cellular pigment production began in the late logarithmic phase (12 hours) and increased rapidly for about 60 hours after which it leveled off. Measurable extracellular pigment was produced later than cellular pigment and increased rapidly during the first 72 hours of growth and then leveled off. More than 60% of the total pigment was found to be in the extracellular liquid medium and remained stable even after cell growth began declining.

Figure 3. The relationship between the cell dry weight and growth of *Janthinobacterium* measured by absorbance at 450 nm. Cultures were inoculated with a 1% inoculum adjusted to an absorbance of 0.3 and incubated on a reciprocal shaker at 25°C.

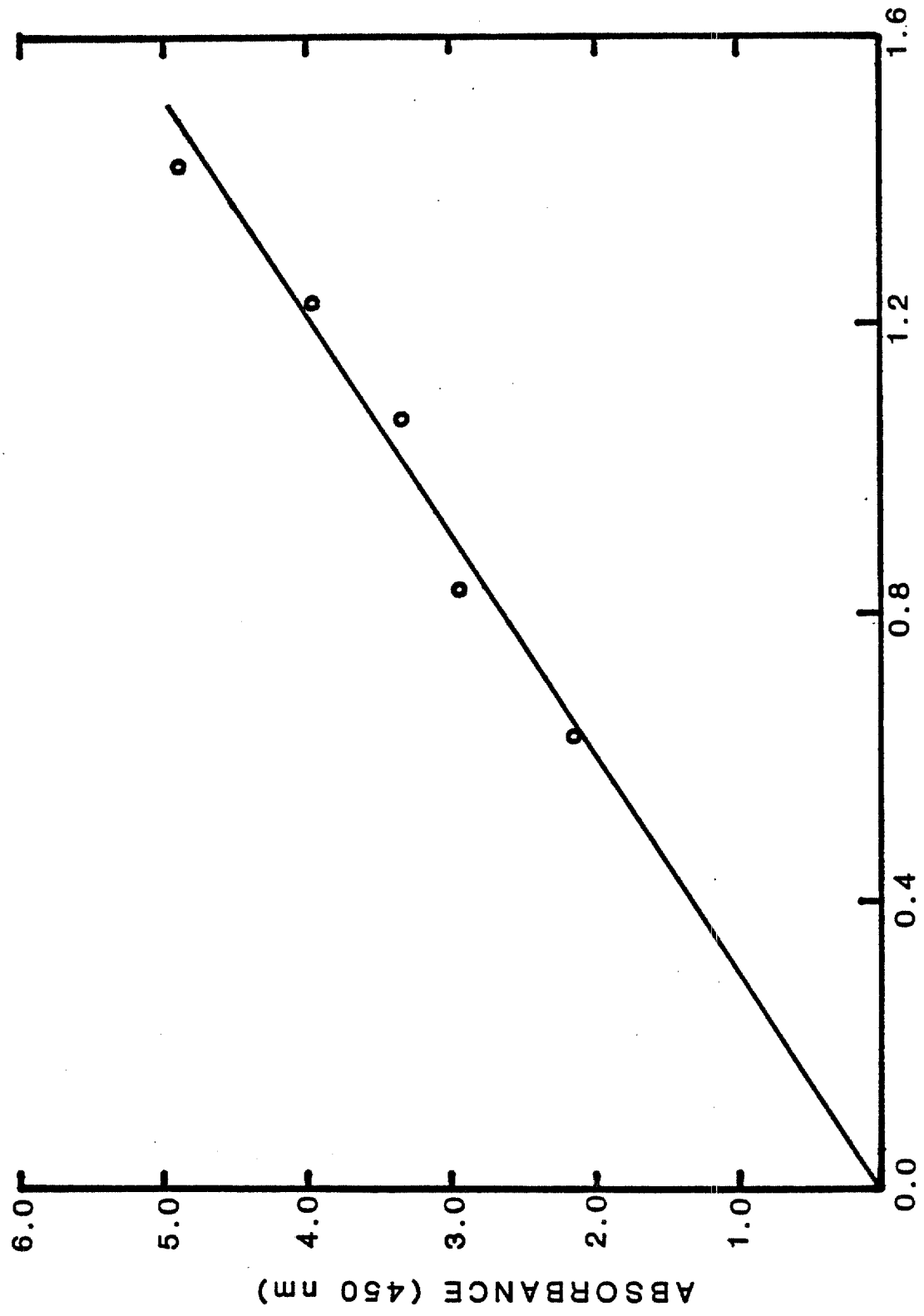
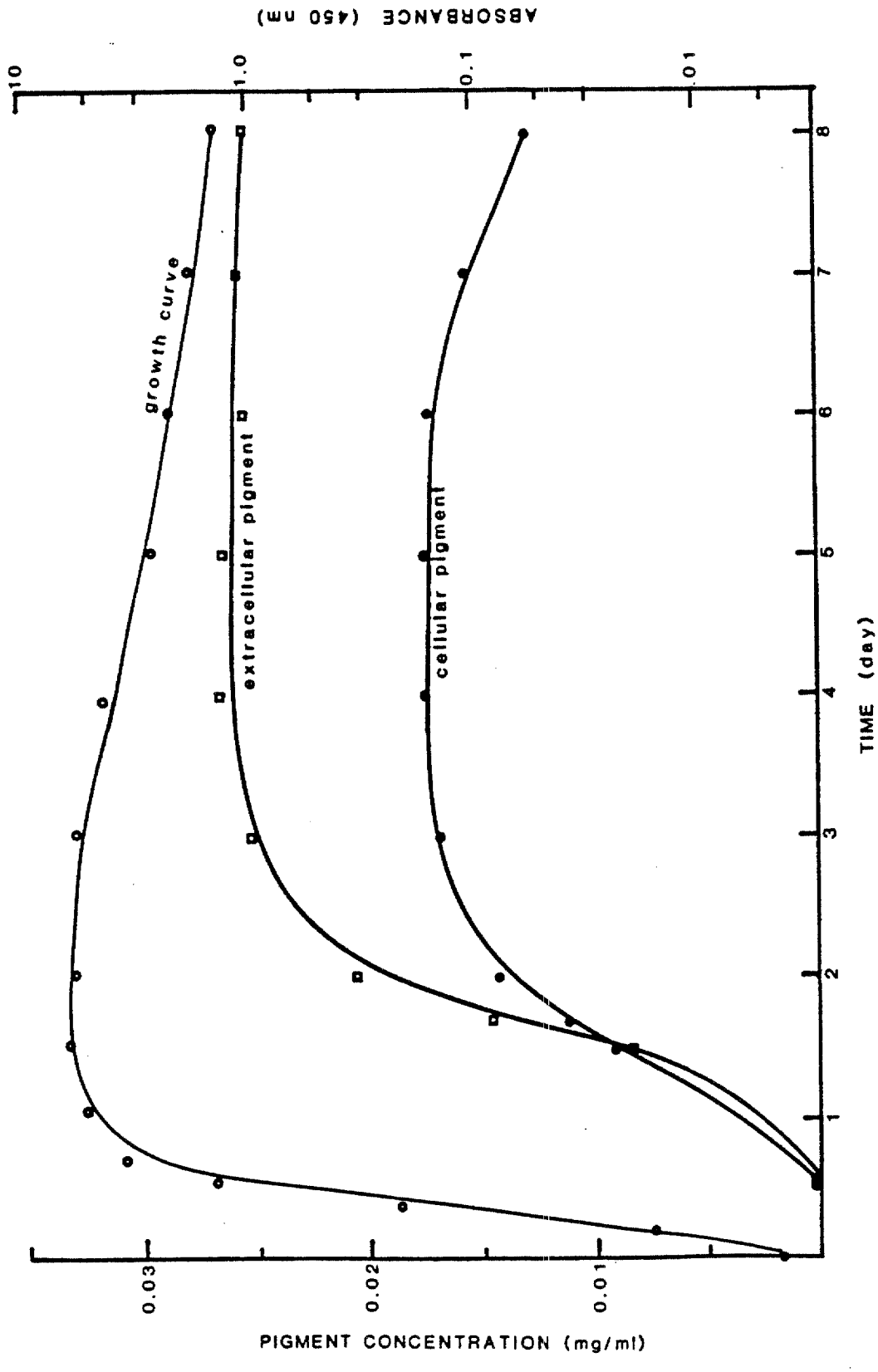

ABSORBANCE (450 nm)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.4
0.8
1.2
1.6
CELL DRY WEIGHT (mg/ml)

Figure 4. Production curve of extracellular and intracellular pigment by *Janthinobacterium*. Cultures were inoculated with a 1% inoculum adjusted to an absorbance of 0.3 and inoculated on a reciprocal shaker at 25°C. Concentration of pigment was determined by using the specific extinction coefficient (61). Extraction and calculation of the pigment were as described in Materials and Methods. Growth was measured by absorbance at 450 nm.


ABSORBANCE (450 nm)
10
1.0
0.1
0.01
growth curve
extracellular pigment
cellular pigment
TIME (day)
1
2
3
4
5
6
7
8
0.03
0.02
0.01
PIGMENT CONCENTRATION (mg/ml)

Extracellular pigment production was also measured by absorbance at 550 nm which is the absorbance maximum of the pigmented supernatant fluid. The production curve was consistent with that obtained by measuring extracted pigment (Figure 4).

Characteristics of the Pigment

Both extracellular and cellular pigment extracted with 96% ethyl alcohol gave the same visible light absorption spectrum, (Figure 5), and the spectrum is identical to that of violacein reported by Sneath (61). This pigment is non-diffusible in cells grown on agar plates. The crude pigment extracted with ethyl alcohol was found to be insoluble in water and chloroform. On addition of 10% (v/v) aqueous sulfuric acid, the purple ethanolic solution became green and gave a spectral shift. If 10% (w/v) sodium hydroxide was added, it became green at first, but quickly turned reddish brown. All these observed reactions are consistent with those reported by Sneath (62) for violacein. Soluble, unextracted pigment in the supernatant fluid of broth cultures (bound pigment, Figure 5) showed a blue shift in the main peak when compared to the extracted pigment.

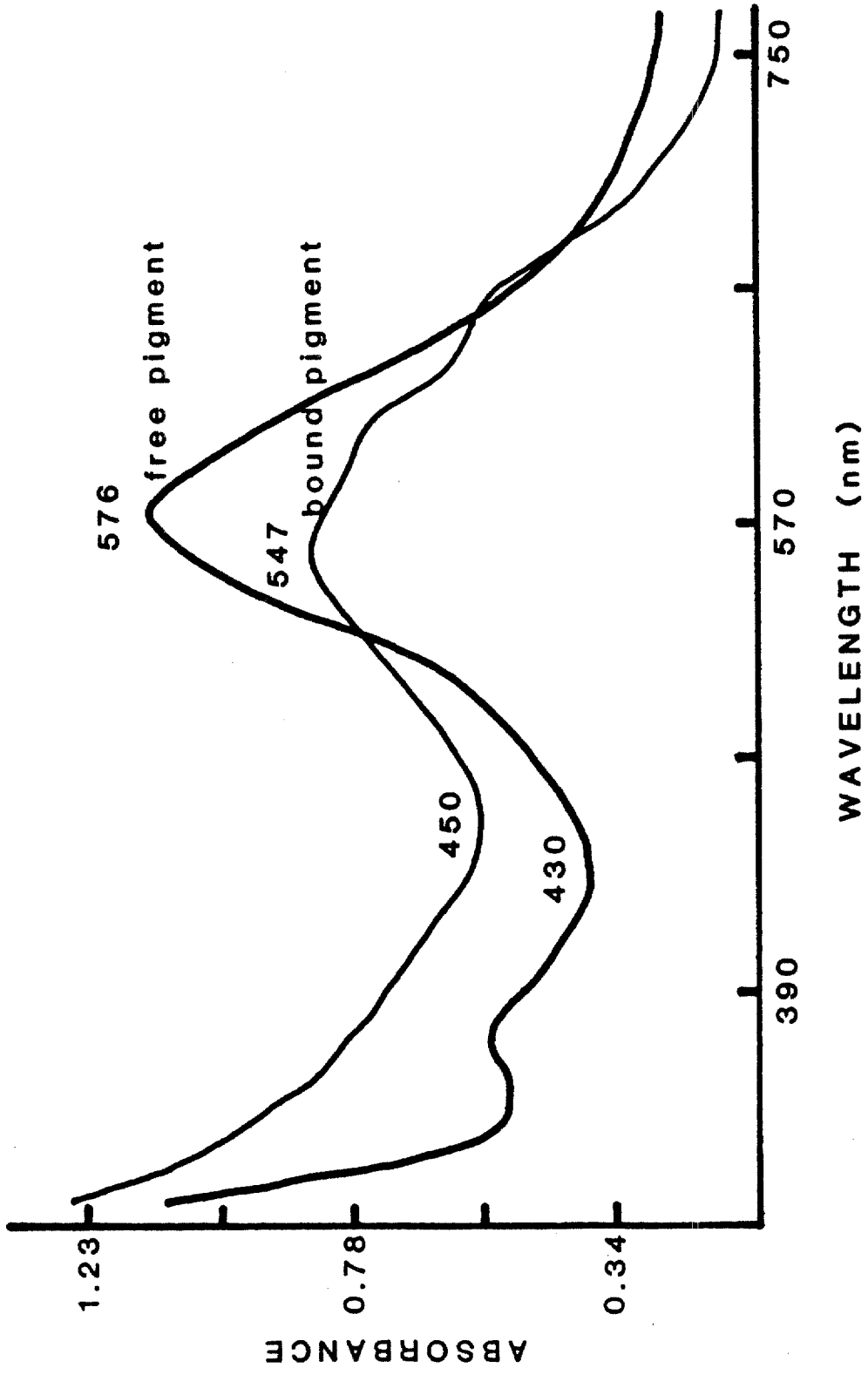


Figure 5. Visible absorption spectra of free and bound pigment of *Janthinobacterium*.

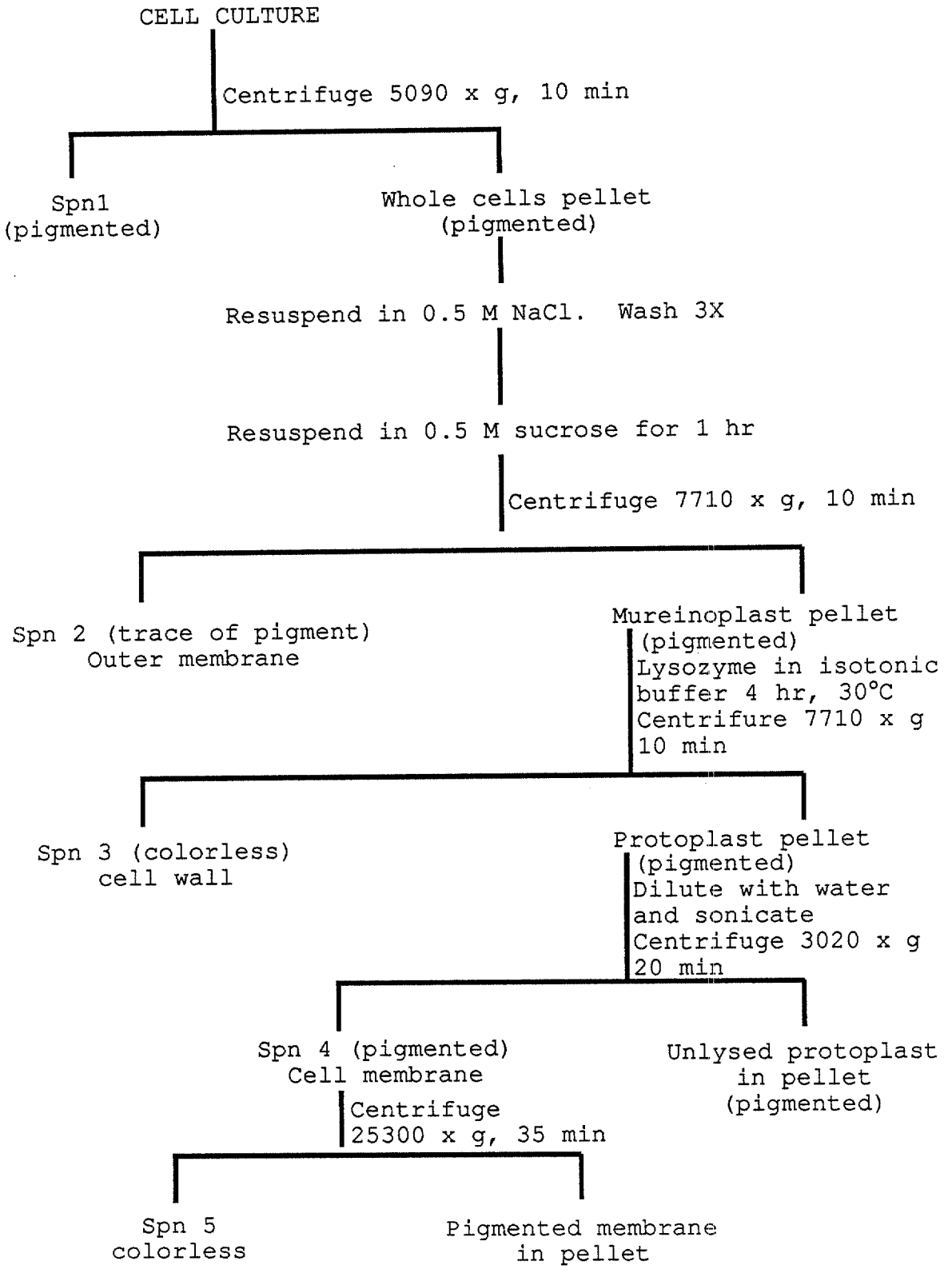
Location of Cellular Pigment

There are several possible locations in the cell where the pigment might be found, i.e., the outer membrane, cell wall, cytoplasmic membrane or the cytoplasm. Figure 6 shows the location of cellular pigment which was determined by the preparation of protoplasts and cell membranes.

Most of the intracellular pigment was found to be in the final cytoplasmic membrane preparation even though traces of pigment appeared in other fractions. In the case of agar cultures, it is associated only with the cell.

Since young cultures of 24 hours did not produce enough pigment, the analysis was performed on 30 hours cultures. The cell pellet from broth culture obtained by centrifugation always gave a very tough mass which was difficult to disperse. This resuspension problem could be reponsible for the low (70%) protoplast yield. Use of α-amylase in 0.02 M phosphate buffer (pH 6.9), 6.7 mM NaCl did not seem to reduce the extracellular polysaccharides or increase the protoplast yield.

Figure 6. Flow Chart for the preparation of mureinoplasts, protoplasts and cell membranes. Thirty hour cultures were used. Cultures, conditions and procedures for preparing cell fractions were as described in Materials and Methods Spn, Supernatant.


CELL CULTURE
Centrifuge 5090 x g, 10 min
Spn1
(pigmented)
Whole cells pellet
(pigmented)
Resuspend in 0.5 M NaCl. Wash 3X
Resuspend in 0.5 M sucrose for 1 hr
Centrifuge 7710 x g, 10 min
Spn 2 (trace of pigment)
Outer membrane
Mureinoplast pellet
(pigmented)
Lysozyme in isotonic
buffer 4 hr, 30°C
Centrifure 7710 x g
10 min
Spn 3 (colorless)
cell wall
Protoplast pellet
(pigmented)
Dilute with water
and sonicate
Centrifuge 3020 x g
20 min
Spn 4 (pigmented)
Cell membrane
Unlysed protoplast
in pellet
(pigmented)
Centrifuge
25300 x g, 35 min
Spn 5
colorless
Pigmented membrane
in pellet

Electron Microscopy

The formation of mureinoplasts and protoplasts were monitored by phase contrast and electron microscopy. The protoplast yield reached 70% or more as estimated by phase contrast microscopy. Electron micrographs of whole cells, menreinoplasts and protoplasts are shown in Figure 7, 8, 9. Figure 7 shows that the whole cells before treatment have an intact double layered envelope and a typical rod-shaped morphology. Mureinoplasts, shown in Figure 8, to have lost the outer membrane layer. Protoplasts, shown in Figure 9, have lost the outer envelope and cell wall. The shape of the cell has also changed because of the loss of the rigid peptidoglycan layer.

Nature of the Extracellular Pigment Complex

More than 60% of the pigment was found in the extracellular liquid medium even though this pigment is insoluble in water, and is non-diffusible on agar media. In addition, the absorption spectrum of this water soluble form of pigment showed a blue shift (Figure 5) compared to the free pigment. This suggests the pigment is bound to some water soluble compound to form a pigment complex existing in the liquid medium.

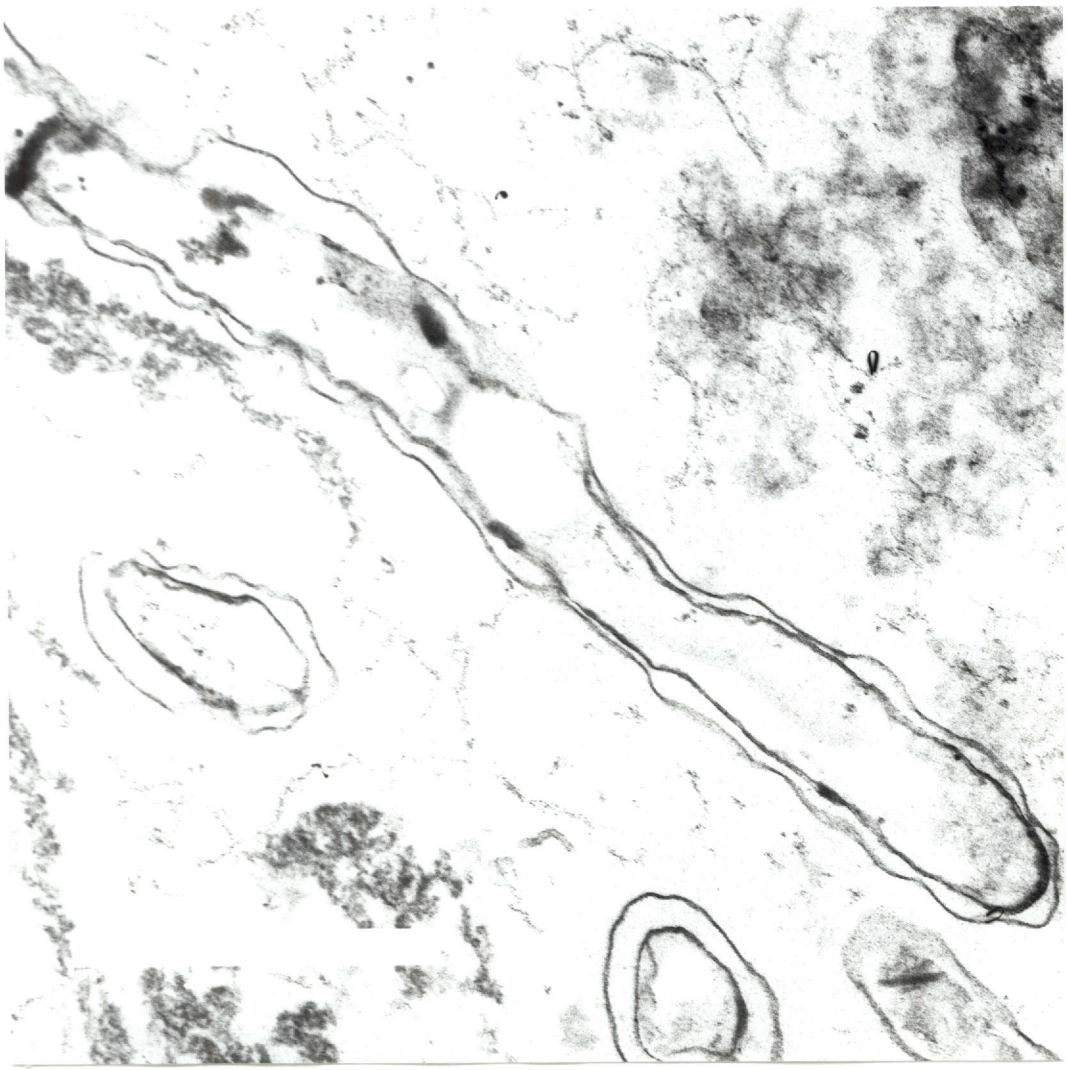


Figure 7. Electron micrograph of a thin section of whole cells of *Janthinobacterium*. Bar = 1.4 µm.

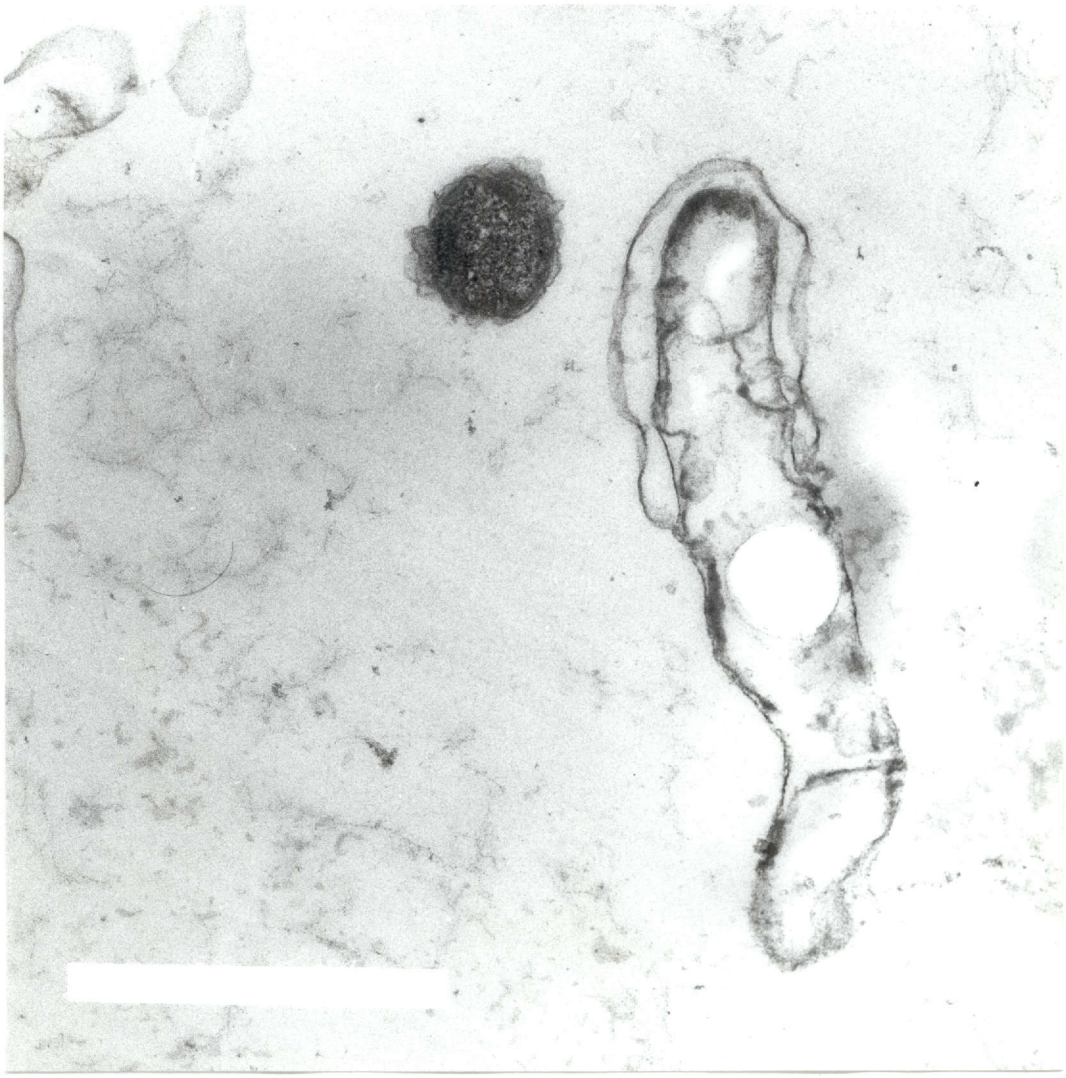


Figure 8. Electron micrograph of a thin section of mureinoplasts of *Janthinobacterium*. Bar = 1.4 µm.

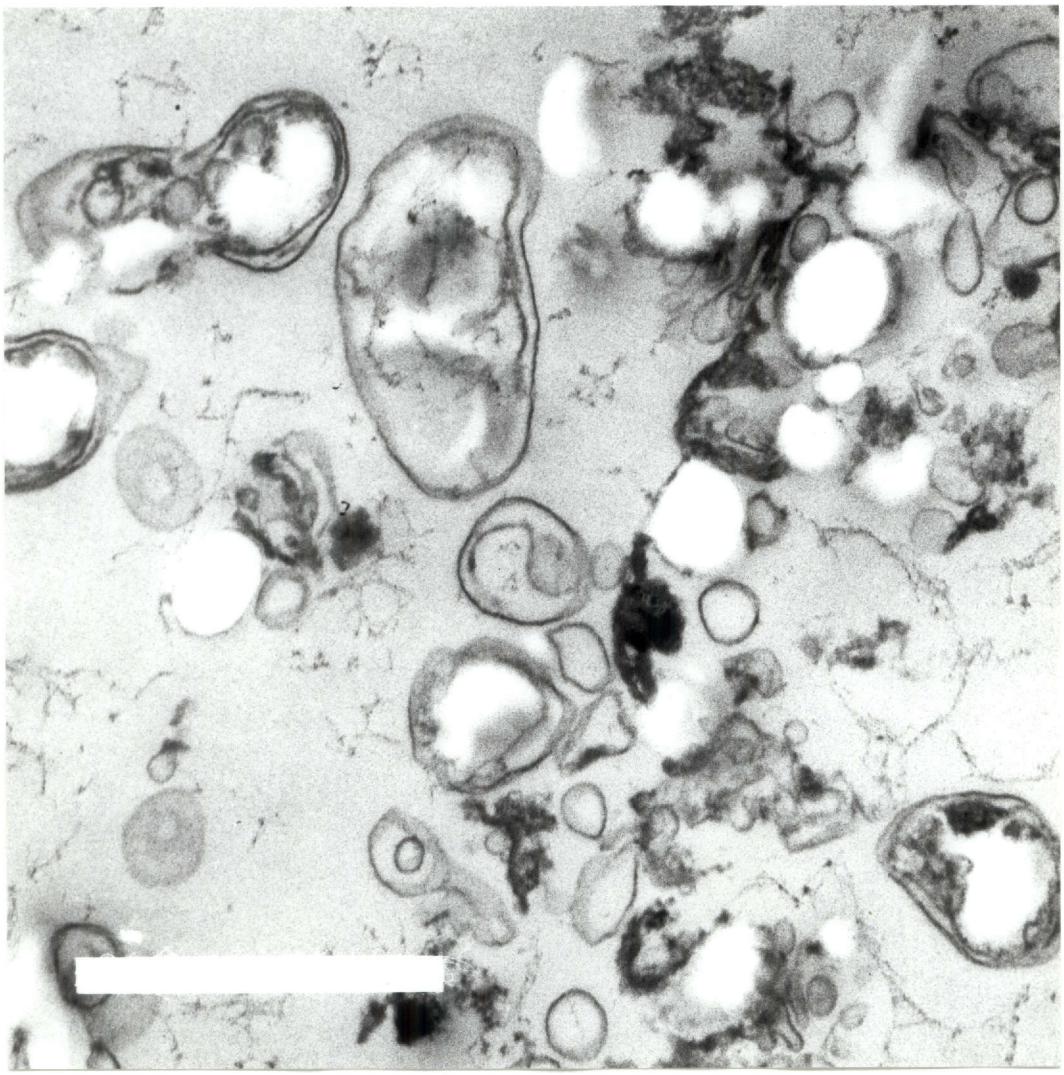


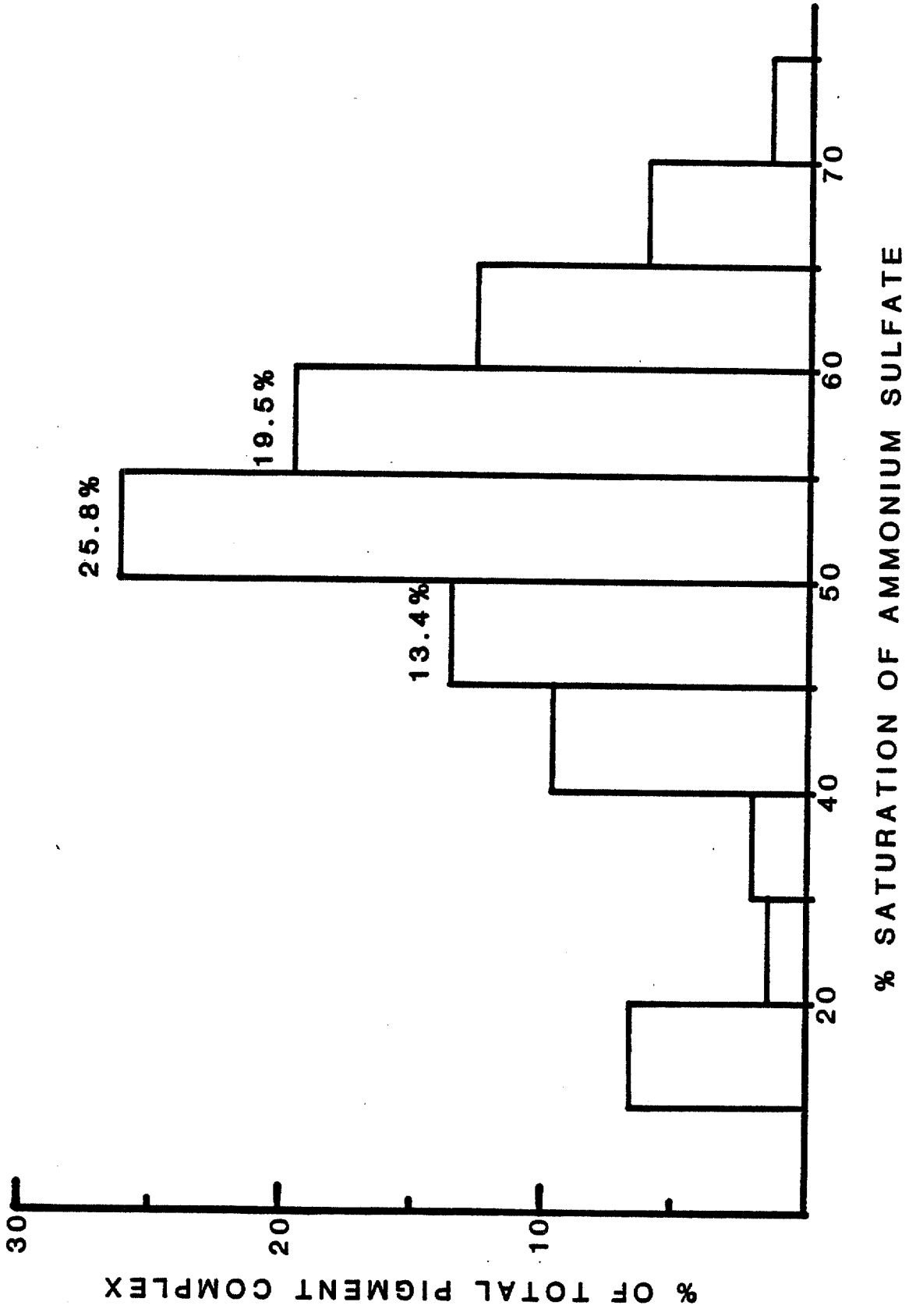
Figure 9. Electron micrograph of a thin section of protoplasts of *Janthinobacterium*. Bar = 1.4 μm.

Ammonium Sulfate Precipitation of the Extracellular Pigment Complex

Supernatant fluids from 72–96 hours cultures were always used for ammonium sulfate precipitation. The optimum saturation of ammonium sulfate was determined by increasing its concentration and measuring the amount of pigment complex precipitated spetrophotometrically at 550 nm. The results of the percentage of total pigment complex precipitated are shown in Figure 10. The addition of ammonium sulfate to a final saturation of 70% precipitates most of the pigment complex and leaves the supernatant fluid almost colorless.

The pigment complex, collected by centrifugation, was easily redissolved in 0.5 M phosphate buffer (pH 7.0) or 1% Triton X-100. This resolubilized pigment complex was found to stay inside dialysis tubing with a 8,000–10,000 molecular weight cutoff. Also, the resolubilized pigment complex clogged the pores of a 0.22 μ membrane filter and was sedimented by ultracentrifugation for one hour at 160,000 × g. This crude pigment-complex assayed positively for protein.

Figure 10. Ammonium sulfate precipitation ranges of the extracellular pigment complex of *Janthinobacterium*. A solution of 100% saturated ammonium sulfate (pH = 7.0) was used to vary the ammonium sulfate concentration.


25.8%
19.5%
13.4%
30
20
10
% OF TOTAL PIGMENT COMPLEX
20
40
50
60
70
% SATURATION OF AMMONIUM SULFATE

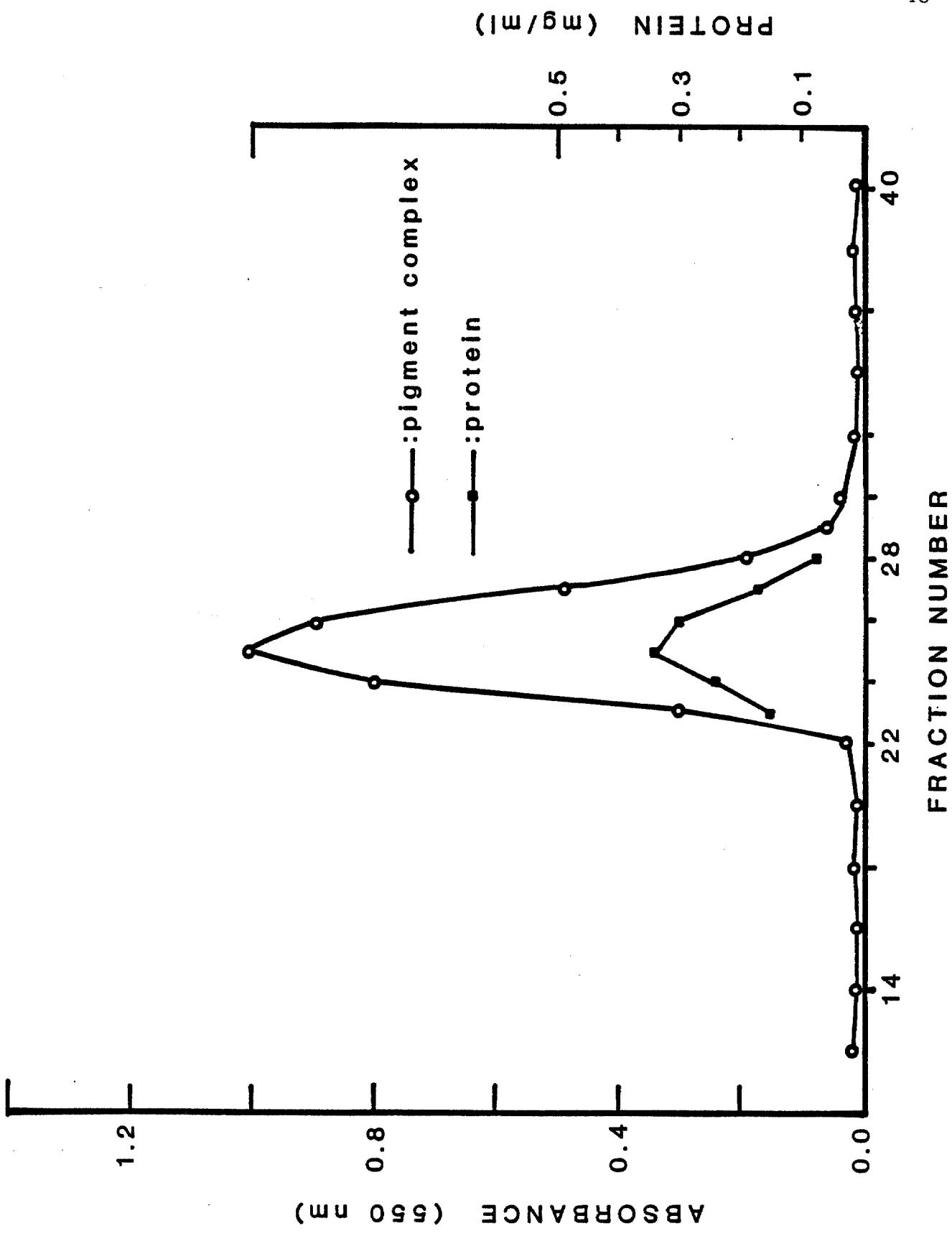
Gel Permeation Chromatography

The gel elution profile of the dialyzed ammonium sulfate precipitate in 0.5M phosphate buffer when passed through a Sephadex G-200 column is shown in Figure 11. The bed packing of gel was checked with 0.2% blue-dextran 2000 (Pharmacia Fine Chemical Inc., Piscathway, New Jersey). This pigment complex fraction was excluded and came off in the void volume. Protein co-eluted with the pigment (Figure 11).

Separation of the Pigment-Protein Complex by Polyacrylamide Gel Electrophoresis

In addition to ammonium sulfate precipitation and gel chromatography, attempts were made to purify the pigment-protein complex in the extracellular supernatant by gel electrophoresis. With SDS-PAGE (7.5% acrylamide) of the crude extracellular pigment, one purple pigment band, which ran ahead of the tracking dye, was observed before staining. When the gel was stained with coomassie blue, several protein bands were observed above the tracking dye and none of them were in the position occupied by pigment. Two particularly

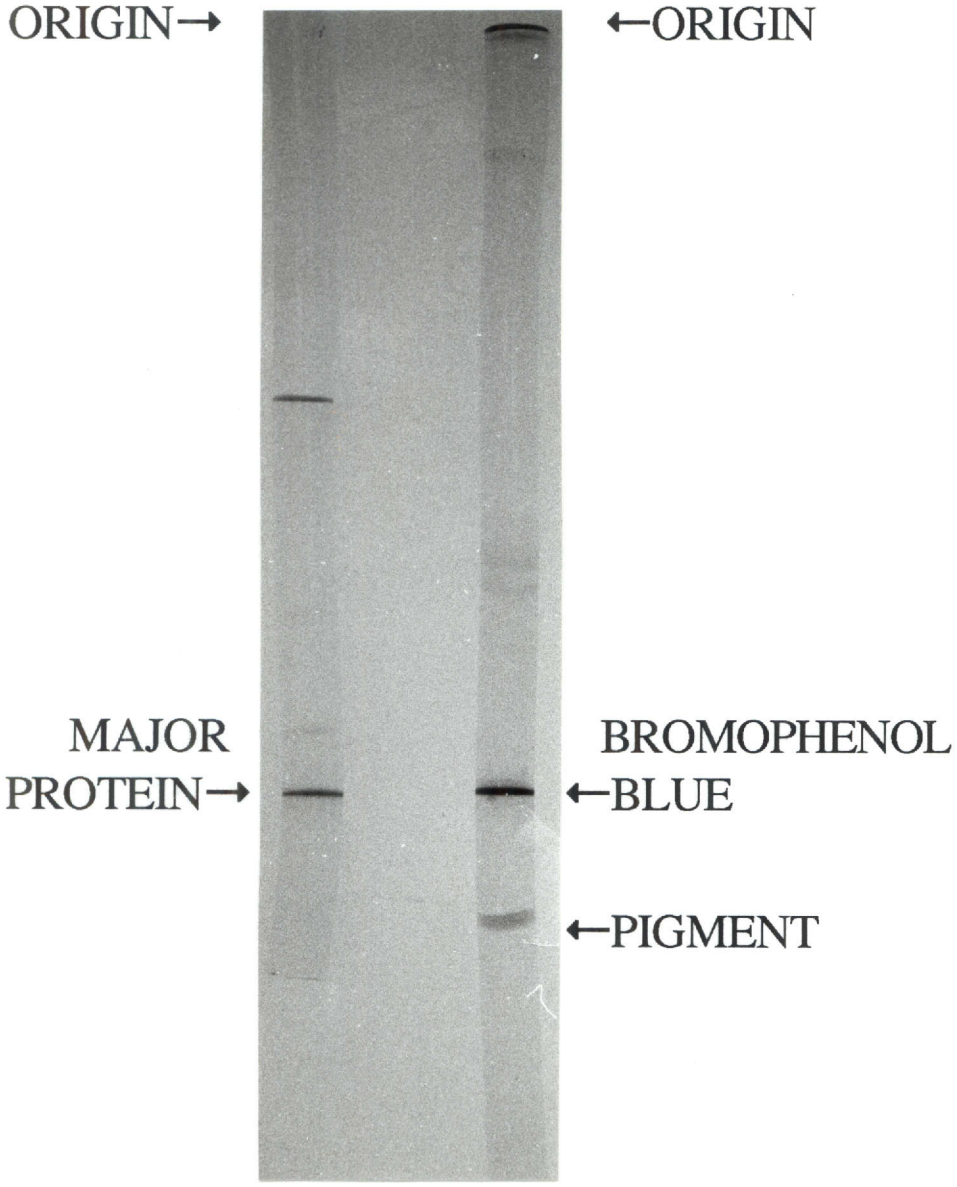
Figure 11. Elution profile for the extracellular pigment complex of *Janthinobacterium* on Sephadex G-200. Sample volume, 2 mL; Eluant, 0.5 M phosphate buffer (pH = 7.0); Column, Pharmacia K16/40; bed height, 36.5 cm; flow rate, 3.6 mL/hr. Pigment was measured by absorbance protein was assayed by the method of Lowry (37).


ABSORBANCE (550 nm)
0.0
0.4
0.8
1.2
PROTEIN (mg/ml)
0.1
0.3
0.5
:pigment complex
:protein
14
22
28
40
FRACTION NUMBER

dense bands were observed as shown in Figure 12 (left lane) with the fastest moving protein being the most dense. With a Triton-PAGE (7.5% acrylamide) system, the intact pigment complex could not be resolved. Distorted protein bands and two separate pigment bands were observed; one was red and the other blue, and both ran ahead of the tracking dye. With a non-denaturing PAGE (5% acrylamide) system, most of pigment complex was found to clog the top of the stacking gel. No pigment band was found on the gel before staining but two protein bands were observed after staining.

Since these protein bands were non-pigmented, the non-denaturing gel electrophoresis could serve as an electrophoric separation step. The pigmented top of non-denaturing gel was cut off, the pigment-protein complex extracted and re-electrophoresed on a SDS gel (7.5% acrylamide) as shown in Figure 12 (right lane). The fastest moving protein band was found to be predominant and the other major protein band was diminished.

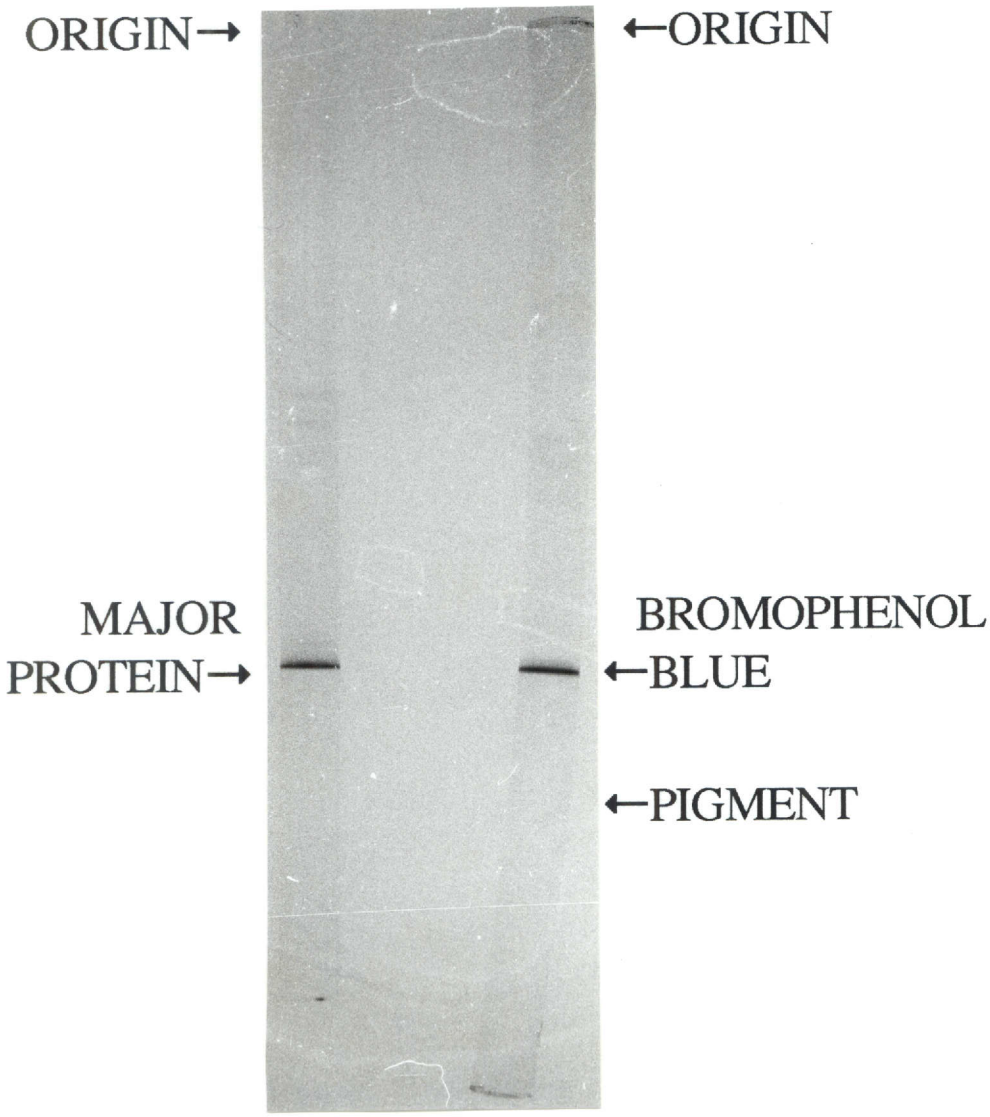
Figure 12. Electrophoretic analysis of the extracellular pigment complex of *Janthinobacterium*. Left lane; SDS-PAGE of the crude pigment complex from the concentrated extracellular supernatant fluid. Right lane; SDS-PAGE of the pigment complex extracted from Non-Denaturing gels.


ORIGIN→
←ORIGIN
MAJOR
PROTEIN→
BROMOPHENOL
←BLUE
←PIGMENT

SDS PAGE of the Ammonium Sulfate Precipitated Complex and the Pigmented Fraction from Gel Permeation Chromatography

The dialyzed ammonium sulfate precipitate was electrophoresed on SDS gel (7.5% acrylamide) as shown in Figure 13. The only major protein band found corresponded to the fastest moving one found in the SDS gel of the crude extracellular supernatant fluid (Figure 12). SDS-PAGE of the pigmented fraction excluded by Sephadex G-200 yielded a major protein band in the same position was found as shown in Figure 13.

Figure 13. Electrophoretic analysis of partially purified extracellular pigment complex from *Janthinobacterium*. Left lane; SDS-PAGE of the pigment complex precipitated by ammonium sulfate. Right lane; SDS-PAGE of the pigment complex from a Sephadex G-200 column.


ORIGIN→
←ORIGIN
MAJOR
PROTEIN→
BROMOPHENOL
←BLUE
←PIGMENT

CHAPTER IV

DISCUSSION

The classification of violacein producing bacteria has been uncertain because they possess many similar characteristics to the *Pseudomonadaceae*, the *Enterobacteriaceae* and even the *Vibrionaceae* (43). *Chromobacterium lividum* was first proposed by Sneath (61) and Eltinge (23) to be a separate group of psychrophilic non-fermentative species distinct from the mesophilic, fermentative species *Chromobacterium violaceum*. Later, based on the study of De Ley et al. (14), it was proposed that *Chromobacterium lividum* be classified as a new genus, *Janthinobacterium*.

The production of violacein has become less important as a distinguishing characteristic since the recognition of some marine violacein producing isolates. These could not be satisfactorily included in the genus *Chromobacterium*, and have been referred to as *Alternomonas* (26). Furthermore, it is possible to isolate non-pigmented strains of *Chromobacterium violaceum* (57) and *Chromobacterium lividum* (24).

The observations reported here have shown that the soil isolate is an obligate aerobe, forms a rubbery colony and gives a positive reaction for esculin hydrolysis and a negative reaction for HCN production. All these observation favor the classification of this isolate as *Janthinobacterium*. Although, casein hydrolysis is a property usually associated with mesophilic fermentative species and was used to differentiate *Chromobacterium* from *Janthinobacterium*, strong proteolytic strains of *Janthinobacterium* have been described by Etherington et al. (24). Growth at different temperatures and acid from carbohydrates were found to be variable. Moss and Ryall (47) found that non-acid producing strains are more frequently isolated from soil, and are less reactive in tests for acid from sugars.

The problem of variation in pigmentation was also observed in our strain. Methods to stimulate pigmentation, such as addition of neomycin and starch or dextrin were described by Sneath (61). However, addition of these compounds did not stimulate pigmentation in our strain. Good pigmentation was obtained with our strain if it is grown on the medium of Keeble and Cross (32) at 25°C and with adequate aeration. In addition to the medium and culture conditions, it is important to use a fresh culture on agar medium for inoculum and dark pigmented colonies for subculturing. Also,

it has been observed that liquid cultures, refrigerated or frozen do not give stable pigmentation on subculturing. The best way to maintain the pigmentation ability of this organism is to subculture frequently on an agar medium.

Stationary cultures of this organism always produced a tough colony or pellicle no matter if it was grown on an agar plate or in broth. Samples of these are difficult to obtain without removing the entire colony or pellicle with a needle or loop. Well dispersed cell suspensions incubated on a shaker gave a tough cell pellet after centrifugation which was difficult to resuspend even with vortexing. This organism has been reported by Corpe (9) and Martin & Richards (41) to produce a considerable amount of extracellular polysaccharide which is highly resistant to degradation and persists after the bacteria producing it have died. It has been suggested that it may be important in the formation of soil aggregates. The formation of a tough pellet upon centrifugation which could not be completely dispersed caused difficulties in preparing mureinoplasts and protoplasts. This was overcome in part by using low speed centrifugation and washing the cells only a few times. Also, too vigorous vortexing was avoided because it caused early cells lysis and gave rise to a very viscous solution.

From the comparison of the pigment production and growth curves, it was also observed that extracellular pigment

production was delayed and the level became constant after 72 hours, while intracellular pigment increased in the first 60 hours of growth and then decreased. The delayed production of extracellular pigment and the decline of intracellular pigment indicated that violacein is synthesized in the cell and released into the extracellular environment possibly as a result of cell lysis.

The formation of protoplasts reached, 70%, was not high. There could be several direct or indirect reasons for the low yield of protoplasts. The age of the culture, cultural medium, concentration of lysozyme (49) the production of extracellular polysaccharides, and the method used for the test may account for the low ratio of formation.

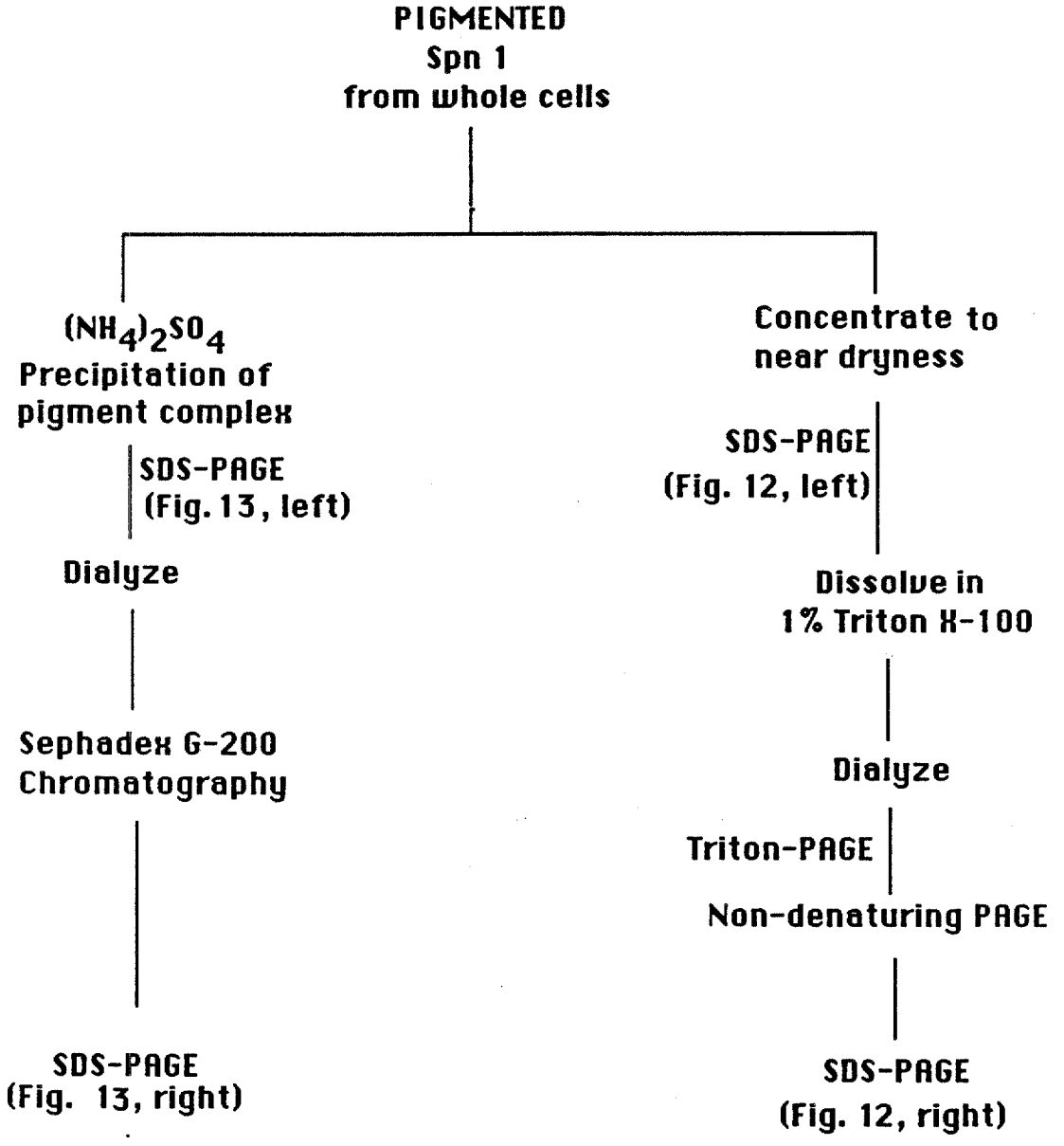
Most of pigment produced in broth cultures was extracellular (60%) and was soluble in the extracellular liquid medium. The fact that the free pigment is not water soluble suggested that the pigment was bound to a polar molecule, possibly a protein, which made the pigment become soluble in the aqueous solution. The protein molecule could be a protein orginally derived from cell membrane. Membrane proteins usually have low polarities or have non-polar regions which can bind non-polar molecule (5, 69). In this study the results with SDS-PAGE gels showed the extracellular pigment to be non-covalently bound to a small protein which aggregated into a large complex. Evidence for aggregation

included the clogging of non-denaturing polyacrylamide gels and membrane filter, sedimentation by ultracentrifugation at 160,000 × g and its exclusion by Sephadex G-200. The high degree of aggregation could be due to hydrophobic interaction of the non-polar pigment bound to the protein molecule. It was also observed that a heavily pigmented culture would become pale and when that happened most of pigmented complex accumulated at the bottom of the flask after a period of time without shaking.

Limited solubility and aggregation of this pigment complex could be the reasons for the leveling-off of the extracellular pigment production curve, i.e., the extracellular fluid was saturated with the complex. Separation steps used for purifing the pigment complex from the extracellular supernatant fluid are briefly summarized in Figure 14. SDS-PAGE gels of different fractions were shown in Figure 12 and Figure 13. This complex could be solubilized by 1% Triton X-100 which; in many cases, has been used to solubilize hydrophobic membrane proteins without denaturing the native form. The milder Triton X-100 PAGE system was first used to try to separate the intact pigment complex. However, pigment dissociated from the protein and ran as two separate bands ahead of the tracking dye. This suggested that the binding between the pigment and the protein is weak and non-covalent. A similar observation of

two pigment bands on alumina chromatography was described by Sneath (61). The pigment complex also could not be resolved with a non-denaturing gel and was found to clog the top of the gel. Thus, the Triton X-100 PAGE system and the non-denaturing PAGE system were not satisfactory for separating the intact pigment complex. SDS-PAGE (Figure 12, left lane) of the crude pigment complex from concentrated extracellular supernatant fluid suggests that several proteins could be associated with the pigment but the intact pigment complex was not resolved on SDS-PAGE. It also suggests dissociation of the pigment from the protein and the binding between them is non-covalent, and either one of these two major proteins could bound to the pigment. SDS-PAGE (Figure 12, right lane) of the extracted pigment complex from the pigmented top of non-denaturing gel showed a similar result to that obtained from the crude pigment complex except the fast moving protein became predominant. Ammonium sulfate precipitation and gel permeation chromatography were used to separate this complex without denaturation. SDS-PAGE of these fractions always showed only one major protein band and the free pigment migrating ahead of the tracking dye. The results indicate the pigment is bound to the fast migrating protein of the SDS gel.

Figure 14. Scheme for the separation of the extracellular pigment complex from *Janthinobacterium*. Results of SDS-PAGE analysis are shown in Figures 12 and 13.


PIGMENTED
Spn 1
from whole cells
$(NH_4)_2SO_4$
Precipitation of
pigment complex
SDS-PAGE
(Fig. 13, left)
Dialyze
Sephadex G-200
Chromatography
SDS-PAGE
(Fig. 13, right)
Concentrate to
near dryness
SDS-PAGE
(Fig. 12, left)
Dissolve in
1% Triton X-100
Dialyze
Triton-PAGE
Non-denaturing PAGE
SDS-PAGE
(Fig. 12, right)

In summary, the purple pigment (violacein) of *Janthinobacterium* is synthesized in the cell membrane after growth reaches a maximum and is released into liquid medium, probably as a result of cell lysis. The pigment is non-covalently bound to a small protein molecule which tends to aggregate into a large complex. Antimicrobial and phototherapeutic properties of the pigment were recently studied by Duran and Faljoni-Alario (20, 21). Similar conclusions were reached by DeMoss (18). The observations and studies reported could help in understanding the physiological role of this pigment in the cell and the environment.

BIBLIOGRAPHY

1. **Ballantine, J.A., R.J.S. Beer, D.J. Crutchley, G.M. Dodd,** and **D.R. Palmer.** 1959. The synthesis of violacein and related compounds. Proc. Chem. Soc. 232-237.

2. **Ballantine, J.A., C.B. Barrett, R.J.S. Beer, S. Eardly, A. Roberson, B Shaw,** and **T.H. Simpson.** 1958. The chemistry of bacteria. Part VII. The structure of violacein. J. Chem. Soc. 755-760.

3. **Bettelheim,, K.A., J.F. Gordon,** and **J. Taylor.** 1968. The detection of a strain of *Chromobacterium lividum* in the tissues of certain leaf nodulated plants by the immunofluorescence technique. J. Gen. Microbiol. **54**:177-184.

4. **Capaldi, R.A.** 1977. Membrane proteins and their interactions with lipids. In: Membrane Proteins. Marcel Dekker, Inc. **1**:1-120.

5. **Capaldi, R.A.,** and **G. Vanderkooi.** 1972. The low polarity of many membrane proteins. Proc. Nat. Acad.. Sci USA **69**:930-932.

6. **Chrambach, A., T.M. Jovin, P.J. Svendsen,** and **D. Rodberd.** 1976. Analytic and prepartive polyacrylamide gel electrophoresis. An objectively defined fractionation route, apparatus and procedures. In: Method of Protein Separation. M Catsimpoolas(ed.), Plenum Publishing Corp., NY. **2**:27-144.

7. **Corpe, W.A.** 1951. A study of the wide spread distribution of *Chromobacterium* species in soil by a simple technique. J. Bacteriol. **62**:515-517.

8. **Corpe, W.A.** 1953. Variation in pigmentation and morphorogy of colonies of gelatinous strains of *Chromobacterium* species from soil. J. Bacteriol. **66**:470-477.

9. **Corpe, W.A.** 1960. The extracellular polysaccharides of gelatinous strains of *Chromobacterium violaceum*. Can. J. Microbiol. **6**:153-163.

10. **Costerton, J.,W. Forsberg, T.I. Matula, F.L.A. Buckmire,** and **R.A. MacLeod.** 1967. Nutrition andmetabolism of marine bacteria XVI. Formation of protoplasts, spheroplasts and related forms from a Gram-negative marine bacterium. J. Bacteriol. **94**:1764-1777.

11. **Cox, N.A.** 1975. Isolation and identification of a genus, *Chromobacterium*, not previously found on processed poultry. Appl. Microbiol. **29**:864-867.

12. **Dainty, R.H., D.J. Etherington, B.G. Shaw,** and **J. Baylow.** 1978. Studies on the production of extracellular proteinases by a non-pigmented strain of *Chromobacterium lividum* isolated from Abattoir Effluent. J. Appl. Bacteriol. **45**:111-124.

13. **Darrasse, H., R. Mazaud, P. Giudicelli** and **R. Camain.** 1955. Un cas humain mortel d'infection a *Chromobacterium violaceum*. Bull. Soc. Pat. EXOT.,**48**:704-713.

14. **De Ley, J., P. Segers,** and **M. Gills.** 1978. Intra-and intergeneric similarities of *Chromobacterium* and *Janthinobacterium* ribosomal ribonucleic acid cistrons. J. Syst. Bacteriol. **28**:154-168.

15. **DeMoss, R.D.,** and **N.R. Evan.** 1959. Physiological aspects of violacein biosynthesis in nonproliferating cells. J. Bacteriol. **78**:583-566.

16. **DeMoss, R.D.,** and **N.R. Evan.** 1960. Incorporation of C^{14}-labeled substrates into violacein. J. Bacteriol. **79**:729-733.

17. **DeMoss, R.D.,** and **M.E. Happel.** 1959. Nutritional requirements of *Chromobacterium violaceum*. J. Bacteriol. **77**:137-144.

18. **DeMoss, R.D.** 1967. Violacein. In: Antibiotics. D. Gottlieb, and PD Shaw (ed.). Springer-Verlag, NY. **2**:77-81.

19. **DeVoe, I.W., J. Thompson, J.W. Costerton,** and **A.M. Robert**. 1970. Stability and comparative transport capacity of cells, mureinoplasts, and true protoplasts of a Gram-negative bacterium. J. Bacteriol. **101**:1014-1026.

20. **Duran, N.,** and **A. Faljoni-Alario**. 1980. Bacterial chemistry. I. Studies of potential phototherapeutic substance from *Chromobacterium violaceum*. An. Acad. Bras. Cienc. **52**:297-302.

21. **Duran, N.,** and **A. Faljoni-Alario**. 1980. Bacterial chemistry. II. Antimicrobial photoproduct from pigment of *Chromobacterium violaceum*. An. Acad. Bras. Cienc. **55**:231-234. 1983.

22. **Ehrismann, O.,** and **W. Noethling**. 1936. Die Absorptionsspektren des Pyocyanins, Prodigiosins und Violaceins. Biochem. Z. **284**:376-382.

23. **Eltinge, E.T.** 1957. Status of the Genus *Chromobacterium*. Internat Bull Bact.Nomen. Taxon. **7**:37-44.

24. **Etherington, D.J., P.B. Newman, R.H. Dainty,** and **S.M. Partridge**. 1976. Purification and properties of the extracellular metallo-proteinases of *Chromobacterium lividum* (NCIB 10926). Biochim. Biophys. Acta. **445**:739-752.

25. **Friedheim, E. A-H.** 1932. La fonction respiratoire du pigment *Bacillus violaceus*. C. R. Soc. Biol. Paris **110**:353-356.

26. **Gauthier, M.J.** 1976. Morphological, physiological and biochemical characteristics of some violet-pigmented bacteria isolated from sea water. Can. J. Microbiol. **22**:138-149.

27. **Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg,** and **G.B. Phillips**. 1981. Manual of Methods for General Bacteriology. ASM Washington DC.

28. **Gilman, J.P.** 1953. Studies on certain species of bacteria assigned to the genus *Chromobacterium*. J. Bacteriol. **65**:48-52.

29. **Groscop, J.A.**, and **M.M. Brent**. 1964. The effects of selected strains of pigmented microoganisms on small free-living amoebae. Can. J. Microbiol. **10**:579-584.

30. **Inniss, W.E.**, and **C.I. Mayfield**. 1979. Effect of temperature on violacein production in a psycrotrophic *Chromobacterum* from lake Ontrio sediment. Microb. Ecol. **5**:51-56.

31. **Johnson, W.M., A.F. DiSalvo**, and **R.R. Steuer**. 1971. Fatal *Chromobacterium violaceum* septicemia. Am. J. Clin. Pathol. **56**:400-406.

32. **Keeble, J.R.**, and **T. Cross**. 1977. A improved medium for the enumeration of *Chromobacterium* in soil and water. J. Appl. Bacteriol.**43**:325-327.

33. **Kimmel, K.E.**, and **S. Maier**. 1969. Effect of culture condition on the synthesis of violacein in mesophilic and psychrophilic strains of *Chromobacterium*. Can. Microbiol. **15**:111-116.

34. **Koburger, J.A.**, and **S.O. May**. 1982. Isolation of *Chromobacterium* spp. from foods, soil and water. Appl. and Environ. Microbiol. **44**:1463-1465.

35. **Leifson, E.** 1956. Morphological and physiological characteristics of the genus *Chromobacterium*. J. Bacteriol. **71**:393-400.

36. **Letendre, C.H., G. Dickens**, and **G. Guroff**. 1974. The tryptophan hydroxylase of *Chromobacterium violaceum*. J. Biol. Chem. **249**:7186-7191.

37. **Lichstein, H.C.**, and **V.F. Van De Sand**. 1945. Violacein, an antibiotic pigment produced by *Chromobacterium violaceum*. J. Infect. Disease **76**:47-51.

38. **Lichstein, H.C.**, and **V.F. Van De Sand**. 1946. The antibiotic activity of violacein, prodigiosin, and phthiocol. J. Bacteriol. **52**:145-146.

39. **Lowry, O.H., J.N. Roseberough, L.A. Farr**, and **R.J. Randall**. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.

40. **Martin, H.H.** 1963. Bacterial protoplast-a review. J. Theoret. Biol. **5**:1-34.

41. **Martin, J.P.**, and **S.J. Richards**. 1963. Decomposition and binding action of polysaccharides from *Chromobacterium violaceum* in soil. J. Bacteriol. **85**:1288-1294.

42. **Maizel, J.V.** 1971. Gel electrophoresis of proteins. In: Methods Virol. **5**:179-245.

43. **McCarthy, S.A., T. Sakata, D. Kakimoto**, and **R.M. Johnson**. 1985. Studies on marine purple pigment. I. Production and isolation of purple pigment by *Alteromonas luteoviolacea*. Nippon Suisan Gakkaishi **51**:479-484.

44. **Michaels, R.**, and **W.A. Corpe**. 1965. Cyanide formation by *Chromobacterium violaceum*. J. Bacteriol. **89**:106-112.

45. **Mitoma, C., H. Weissbach**, and **S. Udenfriend**. 1956. 5-hydroxytryptophan formation and tryptophan metabolism in *Chromobacterium violaceum*. Arch. Biochem. Biophys. **63**:122-130.

46. **Moss , M.O., C. Ryall**, and **N.A. Logan**. 1978. The classification and characterization of *Chromobacterium* from a low land river. J. Gen. Microbiol. **105**:11-21.

47. **Moss, M.O.**, and **C. Ryall**. 1981. The genus *Chromobacterium*, In: MP Starr, H Stolp, HG Truper, A Balows, and HG Schlegel (ed.), The prokaroytes. Springer-Verlag, Berlin. 1355-1364.

48. **Ognibene, A.J.**, and **E. Thomas**. 1970. Fatal infection due to *Chromobaterium violaceum* in Vietnam. Am. J. Clin. Pathol. **54**:607-610.

49. **Osborn, M.J.**, and **R. Munson**. 1974. Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria. Methods Enzymol. **31**:642-653.

50. **Reilly, J.**, and **G. Pyne**. 1927. On the pigment produced by *Chromobacterium violaceum*. Biochem. J. **21**: 1059-1064.

51. **Rice, J.W.** 1938. A distribution study of *Chromobacterium lividum*. J. Bacteriol. **36**:667-668.

52 **Ryall, C.**, and **M.O. Moss**. 1975. Selective media for the enumeration of *Chromobacterium spp* in soil and water. J. Bacteriol. **38**:53-59.

53. **Salton, M.R.** 1976. Methods of isolation and characterization of bacteria membrane. In: Methods in Membrane Biology. ED Korn (ed.), Plenum Press, NY. London. Volume 6, Chapter 2.

54. **Scopes, R.K.** 1982. Protein Purification: Principles and Practice. Spring-Verlag, NY.

55. **Sebek, O.K.**, and **H.K. Jager**. 1962. Divergent pathways of indole metabolism in *Chromobacterium violaceum*. Nature **196**:793-795.

56. **Singh, B.N.** 1942. Toxic effects of certain bacterial metabolic products on soil protozoa. Nature **149**:168.

57. **Sivendra, R., H.S. Lo**, and **K.T. Lim**. 1975. Identification of *Chromobacterium violaceum* pigmented and non-pigmented strains. J. Gen. Microbiol. **90**:21-31.

58. **Sivendra, R.**, and **S.H. Tan**. 1988. Pathogenicity of non-pigmented cultures of *Chromobacterium violaceum*. J. Clin. Microbiol. **5**:514-516.

59. **Sneath, P.H.A.** 1956. Cultural and biochemical characteristics of genus *Chromobacterium*. J. Gen. Microbiol. **15**:70-98.

60. **Sneath, P.H.A.**, J.P.F. Whelan, R.B. Singh, and D. Edwards. 1953. Fatal infection by *Chromobacterium violaceum*. Lancet **2**:276-277.

61. **Sneath, P.H.A.** 1960. A study of the bacterial genus *Chromobacterium*. Iowa State J. Sci. **34**:243-500.

62. **Sneath, P.H.A.** 1966. Identification methods applied to *Chromobacterium*. In: Identification methods for microbiologist, Gibbs BB, Skinner FA (eds.), New York, London: Academic Press. 15-20.

63. **Sneath, P.H.A.** 1974. *Chromobacterium* Bergonzini 1881, In: Berger's Manual of Determinative Bacteriology. Buchanan RE, Gibbons NE (eds.): The Williams & Wilkins Co., Baltimore, MD. **8**:354-357.

64. **Sneath, P.H.A.** 1984. Genus *Chromobacterium* Bergonzini 1881, 153[AL]. In: Bergeys' Manual of Systematic Bacteriology. N.R. Krieg and J.G. Holt (ed.), The Williams & Wilkins Co., Baltimore, MD. **1**:580-582.

65. **Sneath, P.H.A.** 1984. Genus *Janthinobacterium* Deley, Segers and Gillis 1978, 164[AL]. In: Bergeys' Manual of Systematic Bacteriology, NR Krieg and JG Holt (ed.), The Williams & Wilkins Co., Baltimore, MD. **1**:376-377.

66. **Stahly, G.L., C.L. Sesler,** and **W.R Brode.** 1941. A method for measuring bacterial pigments by the use of the spectrophotometer and photoelectric colorimeter. J. Bacteriol. **43**:149-154.

67. **Sykes, J.** 1971. Centrifugal techniques for the isolation and characterization of subcellular components from bacteria, In: JR Norrisand, DW Ribbons (ed.), Methods in Microbiology. Academic Press, Inc., New York. **5B**:55-207.

68. **Strong, F.M.** 1944. Isolation of violacein. Sci. **100**:287.

69. **Tanford, C.** 1980. The Hydrophobic Effect: Formation of micelles and biology membrane. John Wiley & Sons, Inc.

70. **Tobie, W.C.** 1934. The pigment of *Bacillus violaceus* I. The production, extraction, and purification of violacein. J. Bacteriol. **29**:223.

71. **Wegman, J.,** and **I.P. Crawford.** 1968. Tryptophan synthetic pathway and its regulation in *Chromobacterium violaceum*. J. Bacteriol. **95**:2325-2335.

72. **Whiteside, T.L.,** and **W.A. Crope.** 1968. Effect of enzymes on the composition and structure of *Chromobacterium violaceum* cell envelopes. J. Bacteriol. **97**:1449-1459.

73. **Whiteside, T.L.,** and **W.A. Corpe.** 1969. Extracellular enzymes produced by a *Pseudomonas spp* and their effect on cell envelopes of *Chromobacterium violaceum*. Can. J. Microbiol. **15**:81-92.