



<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study,  
without prior permission or charge

This work cannot be reproduced or quoted extensively from without first  
obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any  
format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author,  
title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>  
[research-enlighten@glasgow.ac.uk](mailto:research-enlighten@glasgow.ac.uk)

A. C. Wardlaw.

MICROBIOLOGY LIBRARY

ProQuest Number: 10662740

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10662740

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code  
Microform Edition © ProQuest LLC.

ProQuest LLC.  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106 – 1346

To Alison

PYOCYANINE FORMATION

BY

PSEUDOMONAS AERUGINOSA

ALAN D. MCGILLIVRAY

Presented for the Degree of Doctor of Philosophy  
in the Faculty of Science, University of Glasgow

Department of Microbiology

October, 1972

ACKNOWLEDGEMENTS

I would like to thank Professor A.C. Wardlaw for his constant advice, helpful suggestions and encouragement throughout the supervision of this research. The guidance of the late Professor I.W. Lominski, who suggested the project, was greatly appreciated.

Also I wish to thank Dr. J.P. Arbuthnott for his critical appraisal of the work and Dr. C.A. Fewson of the Biochemistry Department, for his consultation and interest in the project. Thanks are due to Dr. W.H. Holms of the Biochemistry Department, for the use of laboratory facilities.

I would like to express my appreciation to Dr. J.H. Freer for the electron micrographs; to Mrs. A. Strachan and Miss J. Hooper for secretarial assistance; and to Mr. I. McKie for photographic services.

## OBJECT OF THE RESEARCH

Although the production of a diffusible blue-green pigment, pyocyanine by Pseudomonas aeruginosa is one of the common place observations of bacteriology, many aspects of the phenomenon remain to be explained.

Burton, Eagles and Campbell (1948) and Ingledew and Campbell (1969a) showed, with the ATCC 9027 strain, that phosphate deficiency in the culture medium is one of the factors which induce pyocyanine synthesis.

The objects of this investigation were :

- (a) To confirm the earlier work on the role of phosphate deficiency and to explore the effect of altering other constituents of the medium. This was done with NCTC 6750, the type strain of P. aeruginosa.
- (b) To attempt to explain how the phosphate level of the medium regulates pyocyanine production.
- (c) To investigate the possible role of pyocyanine in the metabolism of the parent organism.

To provide a chemical basis for the investigation, pyocyanine and some related phenazines were synthesized and considerable effort taken to characterise their absorption spectra in the ultra-violet, visible and infra-red regions.

CONTENTS

	<u>Page</u>
<u>ACKNOWLEDGMENTS</u>	i
<u>OBJECT OF THE RESEARCH</u>	ii
<u>LIST OF TABLES</u>	xi
<u>LIST OF FIGURES</u>	xiii
<u>LIST OF PLATES</u>	xvi
<u>LIST OF ABBREVIATIONS</u>	xviii

INTRODUCTION

SECTION I :	<u>PYOCYANINE</u>	1
A.	Original Observation of Pyocyanine	1
B.	Properties, Synthesis and Structure	2
C.	Isolation and Determination	4
D.	Biosynthesis of Pyocyanine	5
E.	Oxidation Reduction Potentials of Pyocyanine	10
F.	Biological Activity of Pyocyanine	12
G.	Other Bacterial Phenazines	20



	<u>Page</u>
3. Measurement of Bacterial Growth	43
a. Opacity of Cell Suspension	43
b. Viable Cell Count	44
c. Bacterial Dry Mass	44
SECTION II : <u>BIOCHEMISTRY</u>	44
A. Preparation and Assay of Pyocyanine	44
1. Synthesis of Pyocyanine and Phenazines according to Surrey (1955)	44
2. Identification	48
a. Thin layer Chromatography	48
b. Ultra-violet and Visible Spectrophotometry	50
c. Infra-red Spectrophotometry	50
3. Assay procedure	50
a. Extraction and Gravimetric Estimation	50
b. Spectrophotometric Estimation according to Kurachi (1958b)	51
B. Cells and Cell Envelopes	51
1. Biochemical Measurements on Bacterial Cells	51
a. Protein	51
b. Lipopolysaccharide	51
c. Total Organic Matter	52
d. Lipid	52
e. Total Phosphorus	53
f. Deoxyribonucleic and Ribonucleic acid	53
g. Adenosine triphosphate	54

	<u>Page</u>
2. Preparation of Cell Free Extracts	55
a. Enzymatic Lysis	55
b. Mechanical Lysis	56
3. Electron Microscopy	57
a. Thin Sectioning	57
b. Negative Staining	57
4. Polyacrylamide Disc Gel Electrophoresis	58
C. Biochemical Activities of Pyocyanine	59
1. Oxygraph Measurement of Respiration	59
a. The Biological Oxygen Monitor	59
b. Endogenous Respiration	60
c. Succino-oxidase activity	61
2. Succinate Dehydrogenase Assay	61

## RESULTS

SECTION I	: <u>SYNTHESIS AND IDENTIFICATION OF PYOCYANINE</u>	63
	<u>AND OTHER PHENAZINES</u>	
A.	Yields and melting points of Pyocyanine, 1-Phenazinol and 1-Methoxyphenazine	63
B.	Chromatography of synthesized compounds	64

	<u>Page</u>
C. Ultra-violet and visible spectro- photometric characterisation of synthesized compounds	66
D. Infra-red spectra of synthesized compounds	74
SECTION II : <u>THE EFFECT OF CULTURAL CONDITIONS UPON</u> <u>PYOCYANINE PRODUCTION</u>	82
A. Pyocyanine production by <u>P. aeruginosa</u> using solid and liquid media	82
B. Pyocyanine production and determination in Resuspension Medium	83
1. The Resuspension Medium	83
2. Choice of method for measuring Pyocyanine in culture supernatants	85
3. Consistency of observation of Pyocyanine in Standard Resuspension Medium Supernatant	87
4. Ultra-violet and visible absorption spectra of Standard Supernatant	87
5. Spectrophotometric detection of Pyocyanine added to Standard Supernatant	90
6. Chromatography of culture supernatant preparations	95
7. Spectrophotometric detection of l-Phenazinol added to Standard Supernatant	97

C.	Effect of changing composition of Resuspension Medium	99
1.	Effect of inoculum size on growth and Pyocyanine production	99
2.	Essential and non-essential components of Resuspension Medium	102
3.	Pyocyanine production and growth in Resuspension Medium with replaced components	105
4.	Spectrophotometric analysis of the supernatants from Resuspension Medium with replaced components	108
5.	Growth in Resuspension Medium under atmospheres of hydrogen and nitrogen	111
D.	Pyocyanine production in Minimal Resuspension Medium	114
SECTION III :	<u>BIOCHEMICAL CHARACTERISATION OF P. AERUGINOSA</u>	118
	<u>CELLS</u>	
A.	Kinetics of growth and Pyocyanine production	118
B.	Changes in the chemical composition of cells and supernatants	121
	a) Nucleic acid	122
	b) Protein	124
	c) Lipid and Phospholipid	126
	d) Lipopolysaccharide	129
	e) Adenosine triphosphate pool	129

	<u>Page</u>
C. Electrophoretograms of cell envelope proteins	132
D. Electron Micrographs of <u>P. aeruginosa</u> cells	135
SECTION IV : <u>INHIBITION OF PYOCYANINE PRODUCTION</u>	151
A. Effect of phosphate addition	151
B. Effect of chloramphenicol addition	154
C. Effect of adding synthesized Pyocyanine	157
SECTION V : <u>EFFECT OF PYOCYANINE ON P. AERUGINOSA</u>	159
A. Endogenous respiratory activity of whole cells	159
B. Cell envelope bound succino-oxidase	165
C. Cell Envelope bound succinate dehydrogenase	168
<u>DISCUSSION</u>	171
A. Determination of Pyocyanine	171
B. Cultural conditions for Pyocyanine production	173
C. Characterisation of <u>P. aeruginosa</u> during Pyocyanine production	177
D. Effect of phosphate deprivation	181
E. Control mechanisms in Pyocyanine production	183
F. Function of Pyocyanine in <u>P. aeruginosa</u>	188
G. Further perspectives	192

PageREFERENCES

194

SUMMARY

212

APPENDICES

Appendix 1	-	Preparation of media	1A
Appendix 2	-	Preparation of buffers	3A
Appendix 3	-	SDS Disc-gel Electrophoresis	5A
Appendix 4	-	Preparation of reagents	8A

<u>LIST OF TABLES</u>		<u>Page</u>
I	Literature reported biosynthesis studies on Pyocyanine	6
II	Literature reported biological activity of Pyocyanine	14
III	Phenazine derivative produced by <u>Pseudomonas aeruginosa</u>	22
IV	Media for Pyocyanine production (after MacDonald, 1966)	35
V	Yields and properties of synthesized Pyocyanine and other Phenazines	65
VI	Comparison of experimental and reported wavelengths of maximum absorption ( $\lambda_{max}$ nm) and molar extinction coefficients ( $\epsilon$ l. mole <sup>-1</sup> cm <sup>-1</sup> ) for	
	(a) Pyocyanine	69
	(b) l-Phenazinol and l-Methoxyphenazine	70
VII	Characteristic infra-red absorption frequencies of	
	(a) Pyocyanine	80
	(b) l-Phenazinol and l-Methoxyphenazine (from Corbett, 1964)	81
VIII	Range of Pyocyanine levels estimated in "Standard Supernatant" by $E_{690}$	88
IX	Absorption $\lambda_{max}$ (nm) and extinction coefficients for Standard Supernatant and synthesized Pyocyanine	91
X	Comparison of extinction ratios for absorptions of Pyocyanine and of Standard Supernatant supplemented with known amounts of synthesized Pyocyanine.	94

	<u>Page</u>
XI	Composition of Resuspension Medium 103
XII	Effect of changes in the components of Resuspension Medium on Pyocyanine production and growth 104
XIII	Effect of replacing essential components of Resuspension Medium on Pyocyanine production and growth 107
XIV	Absorbance values for visible and ultra-violet spectra of supernatants from (a) Resuspension Media with replaced nitrogen and sulphur sources 109 (b) Resuspension Media with replaced carbon sources 110
XV	Effect of anaerobic incubation on Pyocyanine production and growth 113
XVI	Effect of varying inoculum size in Minimal Resuspension Medium on Pyocyanine production and growth 116
XVII	Absorbance values for visible and ultra-violet spectra of supernatants from Minimal Resuspension Media with varying inoculum sizes 117
XVIII	Summary of Electron Micrograph observations on <u>P. aeruginosa</u> cells from Resuspension Medium 136
XIX	Summary of observations on <u>P. aeruginosa</u> cells grown on solid media 160
XX	Effect of Pyocyanine addition on cell envelope bound succino-oxidase 166

LIST OF FIGURES

	<u>Page</u>
1. Biosynthetic pathway leading to aromatic amino acids (from Ingledew and Campbell, 1969b)	9
2. Oxidation-reduction potentials for pyocyanine at various pH values (from Friedheim and Michaelis, 1931)	11
3. Oxidation-reduction potentials in respiratory chain carriers (from Hawker and Linton, 1971)	13
4. Reaction scheme leading to Pyocyanine	47
5. Purification scheme for Pyocyanine	49
6. Ultra-violet and visible spectra of	
(a) Pyocyanine in 50 mM Tris, pH 8.5	71
(b) Pyocyanine in 0.1M HCl	71
(c) 1-Phenazinol in 0.1M NaOH/50% MeOH	72
(d) 1-Phenazinol in 0.1M HCl/50% MeOH	72
(e) 1-Phenazinol in 50 mM Tris, pH 8.5	73
(f) 1-Methoxyphenazine in 50 mM Tris, pH 8.5	73
7. Infra-red absorption spectrum of	
(a) Pyocyanine in KBr, in CCl <sub>4</sub> and in CHCl <sub>3</sub>	75
(b) 1-Phenazinol in KBr and in C <sub>2</sub> Cl <sub>4</sub>	77
(c) 1-Methoxyphenazine in KBr and in C <sub>2</sub> Cl <sub>4</sub>	79
8. Flow diagram of a typical series of manipulations leading to variation in Pyocyanine production	84

	<u>Page</u>
9. Flow diagram for the preparation of the inoculum for Resuspension Medium	86
10. Ultra-violet and visible spectra of	
(a) Pyocyanine in 50 mM Tris, pH 8.5	89
(b) Standard Supernatant	89
11. Ultra-violet and visible spectra of supernatants with added Pyocyanine	92
12. Thin layer chromatograms of supernatant preparations and known Phenazines	96
13. Ultra-violet and visible spectra of supernatant with added 1-Phenazinol	98
14. Effect of inoculum size on growth and Pyocyanine production in Resuspension Medium	101
15. Ultra-violet and visible spectra of Resuspension Media supernatants with replaced carbon sources	112
16. Ultra-violet and visible spectra of supernatants from Minimal Media with different inoculum sizes	115
17. Kinetics of growth and Pyocyanine production	120
18. Nucleic acid content of cells and supernatant	123
19. Protein content of cells and supernatant	125
20. Lipid content of cells	127

	<u>Page</u>
21. Lipopolysaccharide content of cells	128
22. Adenosine triphosphate pool in cells	131
23. Electrophoretograms of cell envelope proteins in 11.7% acrylamide gels	133
24. Electrophoretograms of cell envelope proteins in 7.0% acrylamide gels	134
25. Cell suspension opacity and Pyocyanine production in the presence of added phosphate	152
26. Minimum inhibitory concentration of chloramphenicol for growth in phosphate-supplemented Resuspension Medium	155
27. Cell suspension opacity and Pyocyanine production in the presence of added chloramphenicol	156
28. Cell suspension opacity and Pyocyanine production in the presence of added Pyocyanine	158
29. Oxygraph trace of 3h cell respiratory rate with added Pyocyanine	162
30. Effect of added Pyocyanine on the rate of endogenous respiration of cells from Resuspension Medium	164
31. Effect of Pyocyanine addition on cell envelope bound succino-oxidase	167
32. Effect of Pyocyanine addition on cell envelope bound succinate dehydrogenase	170

LIST OF PLATES

	<u>Page</u>
1     Effect of inoculum size on the colour of <u>P. aeruginosa</u> cultures in Resuspension Medium.	100
2     Electron micrographs of thin sectioned whole cells of <u>P. aeruginosa</u> fixed with glutaraldehyde in Y-T growth medium before washing as outlined in Inoculum Suspension preparation X 34,500.	138
3     Electron micrographs of thin sectioned whole cells of <u>P. aeruginosa</u> fixed with glutaraldehyde in Y-T growth medium before washing as outlined in Inoculum Suspension preparation X 72,600.	139
4     Electron micrograph of thin sectioned whole cells of <u>P. aeruginosa</u> fixed with glutaraldehyde in Resuspension Medium immediately after inoculation (0h) x 34,500.	140
5     Electron micrograph of thin sectioned whole cells of <u>P. aeruginosa</u> fixed with glutaraldehyde in Resuspension Medium immediately after inoculation (0h)x72,600.	141
6     Electron micrograph of thin sectioned whole cells of <u>P. aeruginosa</u> fixed with glutaraldehyde in Resuspension Medium immediately after inoculation (0h)x117,300.	142

- 7 Electron micrograph of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium at the end of the lag interval (3h) with glutaraldehyde x 34,500. 143
- 8 Electron micrograph of thin sectioned whole cells of P. aeruginosa fixed in the Resuspension Medium at the end of the lag interval (3h) with glutaraldehyde x 42,300. 144
- 9 Electron micrograph of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium at the end of the lag interval (3h) with glutaraldehyde x 117,300. 145
- 10 Electron micrograph of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium at the end of the lag interval (3h) with glutaraldehyde x 117,300. 146
- 11 Electron micrograph of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium after twenty hours (20h) with glutaraldehyde x 34,500. 147
- 12 Electron micrograph of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium after twenty hours (20h) with glutaraldehyde x 72,600. 148
- 13 Electron micrograph of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium after twenty hours (20h) with glutaraldehyde x 117,300. 149
- 14 Electron micrograph of negatively stained (3h) envelopes from P. aeruginosa after extensive washing in 50 mM Tris. x 72,600. 150

LIST OF ABBREVIATIONS

ATCC	-	American Type Culture Collection
ATP	-	adenosine triphosphate
C	-	centigrade
cm	-	centimeter
cyt	-	cytochrome
DCIP	-	2,6-dichlorophenol-indophenol
DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease
E	-	extinction
EDTA	-	ethylene diamine tetra-acetate, disodium salt
FAD	-	flavine adenine dinucleotide
g	-	gram or gravitational acceleration
h	-	hour
IR	-	infra-red
LPS	-	lipopolysaccharide
M	-	molar
m amp	-	milliamp
Me	-	methyl
mg	-	milligram
MIC	-	minimum inhibitory concentration
min	-	minute
ml	-	millilitre
m M	-	millimolar
$\mu$	-	micro
NAD	-	nicotinamide adenine dinucleotide
NCTC	-	National Collection of Type Cultures
nm	-	nanometer

O.D.	--	optical density (opacity)
PL	--	phospholipid
PMS	--	phenazine methosulphate
psi	--	pounds per square inch
RNA	--	ribonucleic acid
rpm	--	revolutions per minute
SDH	--	succinate dehydrogenase
SDS	--	sodium dodecylsulphate
Tris	--	tris (hydroxymethyl) aminomethane
UV	--	ultra-violet
v/v	--	volume for volume
w/v	--	weight for volume

INTRODUCTION

## SECTION I : PYOCYANINE

### A. Original Observation of Pyocyanine

The observation of blue green pus in infected wounds was made some years before Pseudomonas aeruginosa itself had been characterised. Pyocyanine was first isolated from Pseudomonas cultures by Fordos in 1860. He achieved this by adding ammonia to cultures of 'Bacillus pyocyaneus' and then extracting the blue pigment with chloroform. He demonstrated the alkaline nature of aqueous pyocyanine solution after evaporating the chloroform and harvesting the long blue needle shaped crystals.

Gessard (1882 and 1890) improved the extraction procedure by shaking the chloroform solution with aqueous acid. The red aqueous layer, after restoring the blue pigment with alkali, re-extracted with chloroform to yield the purified blue pigment. Gessard (1890) showed that two substances were responsible for pigmentation of the culture supernatant, namely

(a) Pyocyanine :- a bluish green, non-fluorescent compound formed in peptone medium. It was water-soluble and, from alkaline solution, could be extracted into chloroform;

(b) Fluorescin\* :- a greenish yellow fluorescent compound formed only in the presence of phosphate. It could not be extracted into chloroform.

Gessard noted that some of the cultures were achromogenic.

Jordan (1899) studied seven strains of P. aeruginosa and found that one produced only pyocyanine, five produced both pyocyanine and fluorescin and the remaining one gave only fluorescin. He reported that fluorescin production required phosphate and sulphate while pyocyanine required neither.

\* This is distinct from Fluorescein, which is the oxidised form of Fluorescin.

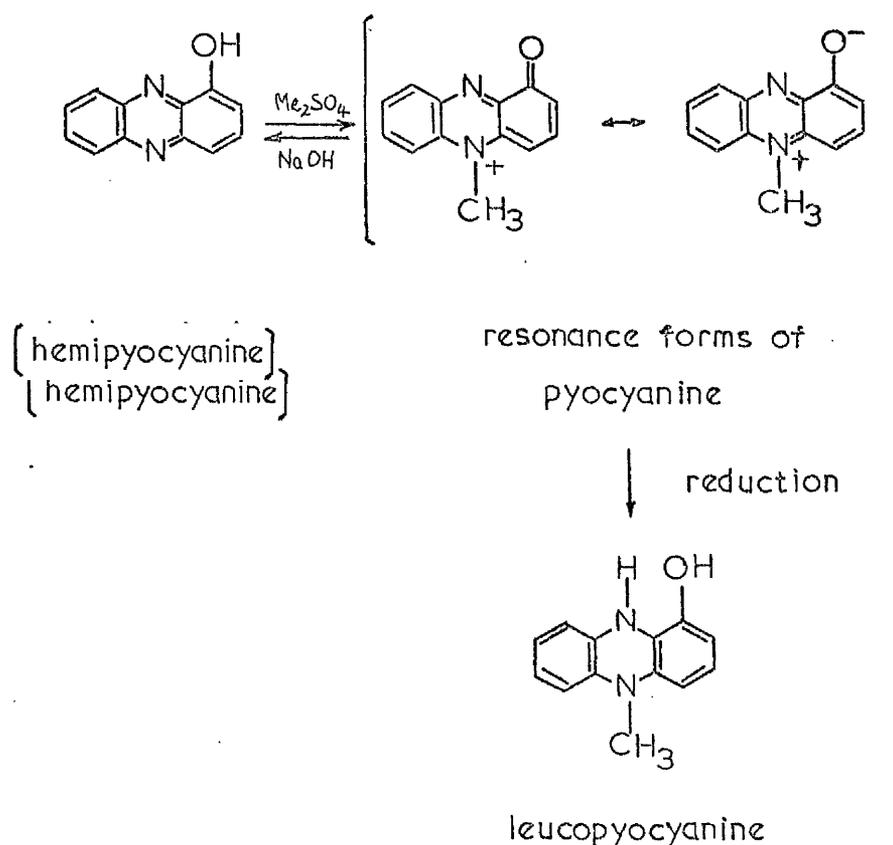
Using synthetic media he noted in old cultures the formation of dark degradation products and yellowish brown pigment whose origin he ascribed to the oxidation of fluorescein.

#### B. Properties, Synthesis and Structure

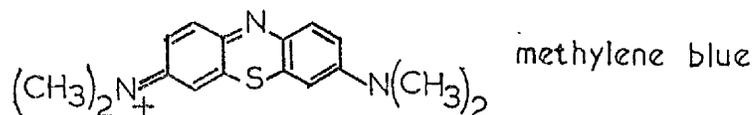
Wrede and Strack (1924) showed that pyocyanine, allowed to stand in dilute alkali exposed to air, changed colour from blue to red. The product of this change was not chloroform-soluble. Addition of acetic acid to this liquor followed by concentration gave yellow needle-shaped crystals as precipitate. Addition of mineral acid caused the colour to revert to red. This yellow compound was identified as 'hemipyocyanine' (Wrede and Strack, 1928) and was also obtained by heating dry pyocyanine. The empirical formula was given as  $C_{12}H_8ON_2$ . 'Hemipyocyanine' and 'leucopyocyanine' were described as the oxidation products of pyocyanine in alkali exposed to air. In an effort to determine the structure of the pigment, Ledderhose (1880) had synthesised the first derivative, the picrate, and determined the empirical formula  $C_{14}H_{14}ON_2$ . Little further investigation took place till McCombie and Scarborough in 1923 synthesised a series of different salts and concluded that the formula for pyocyanine was  $C_{28}H_{28}O_3N_4$ . Wrede and Strack (1929a) revised their proposal and invoked the existence of a dimeric structure having the formula  $C_{26}H_{20}N_2O_4$ . Earlier work (1924) had shown that glucose or dithionite could be used to reduce the pigment to a colourless 'Leucopyocyanine' which was reoxidised readily by the atmosphere. Their suggestion of a dimeric form stemmed from their evidence that reduction with hydrogen over platinum, followed by benzoylation, gave a compound  $C_{13}H_{11}ON_2$  ( $C_7H_5O$ ). Insight into the structure of the aromatic nucleus of pyocyanine was derived from the

isolation of the tricyclic nitrogenous base, phenazine, following distillation of 'hemipyocyanine' with zinc dust (Wrede and Strack, 1925). Later Wrede and Strack (1929b) achieved the first chemical synthesis of pyocyanine and showed that 'hemipyocyanine' was in fact, 1-phenazinol, the 1-hydroxy derivative of phenazine. An identical synthetic route was described in greater detail by Surrey (1955) and, as will be described in Materials and Methods (see page 47 ), was used in this investigation.

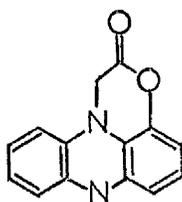
Although the molecular weight estimations of McCombie and Scarborough (1923) implied that pyocyanine existed as a dimer, Michaelis (1931) pointed out that this was unnecessary and outlined the presently accepted formula according to the scheme of reactions as follows :



The degradation of pyocyanine to 1-phenazinol has a parallel in the oxidative demethylation of methylene blue in alkali (Swan and Felton, 1957):



The relative position of the methyl and hydroxyl group on the pyocyanine molecule was established by Hilleman (1938), employing the oxalyl chloride treatment of 1-phenazinol to give a cyclic addition product thus confirming the structure.



Resonance hybrid structures have been proposed for pyocyanine from dipole moment measurements (Jensen and Holten, 1949). This dipolar bond character may have been the cause of erroneous estimation of empirical formulae quoted earlier.

### C. Isolation and Determination

Spectrophotometry of pyocyanine in solution is the basis of reported methods of detection, although solvent and calibration material varies with different workers. Early literature on spectrophotometry of pyocyanine included the work of Babes (1890) who noted two spectroscopic absorptions at 350 and 660 nm. Cluzet (1921) reported a UV maximum at 377 nm and Ehrisman and Noethling (1936) found  $\lambda_{\max}$  values in alkaline solution at 326 and 660 nm. These values represent incomplete studies and more recent reports dating from the work of Kurachi (1958b) are compared in Table VIa (see page 69) with those  $\lambda_{\max}$  values found in this investigation.

Pyocyanine can be detected by its broad absorption in the 700 nm visible region but there is variation in their exact  $\lambda_{\max}$  position : 690 nm (Kurachi, 1958b) and 740 nm (Corbett, 1964). The two methods normally employed by workers in the field are as follows :

- a) (MacDonald, 1966 and Ingledew and Campbell, 1969a) Exhaustive extraction with chloroform outlined in Figure 5 (page 49 ; based on the Wrede and Strack method, 1924), followed by spectrophotometric standardisation ( $E_{690}$ ) against pyocyanine perchlorate ( $\epsilon = 3400$ )
- b) (Kurachi, 1958b) Spectrophotometry ( $E_{690}$ ) of a bacteria free aqueous alkaline solution followed by calibration with freshly prepared pyocyanine ( $\epsilon = 4100$ ).

Both methods of assay avoid the possible interference, suggested by Kurachi (1959c), which may arise from the oxidative demethylation of pyocyanine to give l-phenazinol at low redox potentials. The extraction procedure in (a) removes l-phenazinol through its insolubility at acid pH and in (b), Kurachi demonstrated that  $E_{690}$  is not influenced by contamination with l-phenazinol.

#### D. Biosynthesis of Pyocyanine

Studies on the biosynthesis of pyocyanine have been hampered by the fact that the labelled substrate is catabolised before its incorporation into pyocyanine. The work outlined in Table I is based on the study of the percentage of  $^{14}\text{C}$  incorporated into pyocyanine relative to the quantities found in cellular or respiratory components.

The problems of precursor metabolism were circumvented by the work

TABLE I

## Literature reported Biosynthesis Studies on Pyocyanine

Author	Main Conclusion
Blackwood and Neish (1957)	$^{14}\text{C}$ -glycerol most effect substrate giving labelled pyocyanine. Other substrates were glucose, fructose, alanine, leucine, pyruvate and acetate.
Frank and De Moss (1959)	$^{14}\text{C}$ -alanine grown cells gave labelled pyocyanine. After transfer of these cells to "cold" alanine, unlabelled pyocyanine was produced indicating direct use of amino acids. Mixed $^{14}\text{C}$ -alanine plus pyruvate or glycerol changed the percent incorporation as did variation of phosphate concentration and growth. 1-phenazinol- $^{14}\text{C}$ was not a pyocyanine precursor.
Kurachi (1959d)	Some strains increased pyocyanine production when anthranilic acid was added. In cultures where growth was inhibited by acetanilide, supernatant accumulation of anthranilic acid occurred plus 'labile' compound (not characterised). 'Labile' compound stimulated pyocyanine production by other strains. Suggested as Ch orismic acid derivative.

---

Author	Main Conclusion
Millican (1962)	$^{14}\text{C}$ -shikimic acid substrate gave high activity pyocyanine and extensive "scrambling".
MacDonald (1963)	Substrates providing more pyocyanine carbon than cellular carbon were fructose, ribose, $\alpha$ -keto-gluconate, glucose, glycerate, N-acetylglucosamine, shikimic and quinic acids. Glycerol was shown not to provide pyocyanine building blocks directly. Most pyocyanine carbon was derived from quinic acid although it was also best substrate for growth. Amino acids, malonate and acetate were not directly condensed to give pyocyanine.

---

of Ingledew and Campbell (1969b) using a 'Q negative' strain (one which could not metabolise quinate or shikimate). Competition experiments using labelled 2-ketogluconate and shikimate or quinate revealed that 98% and 44% of pyocyanine carbon came from the two aromatic acids respectively. The assignment of precursor status to either shikimate or quinate was prevented by the detection of labelled shikimate in cultures supplied with quinate- $^{14}\text{C}$ . The latter was shown to be in equilibrium with dihydro shikimate and dihydro quinate, any of which could act as building blocks for pyocyanine.

Assuming a methionine donation of the N-methyl group (Kurachi, 1959a and Shiek and MacDonald, 1964), Ingledew and Campbell (1969b) proposed the biosynthesis of pyocyanine to occur via the condensation of two nitrogenous derivatives of shikimate. Ingledew suggested the participation of a precursor from the branch point between dihydroquinate and chorismate outlined in Figure 1 .

Other workers have proposed the condensation of hydro aromatic compounds anthranilate (Carter and Richards, 1961) and of kynurenine sulphate (Hollstein, Burton and White, 1966) from the degradation of tryptophan shown below :

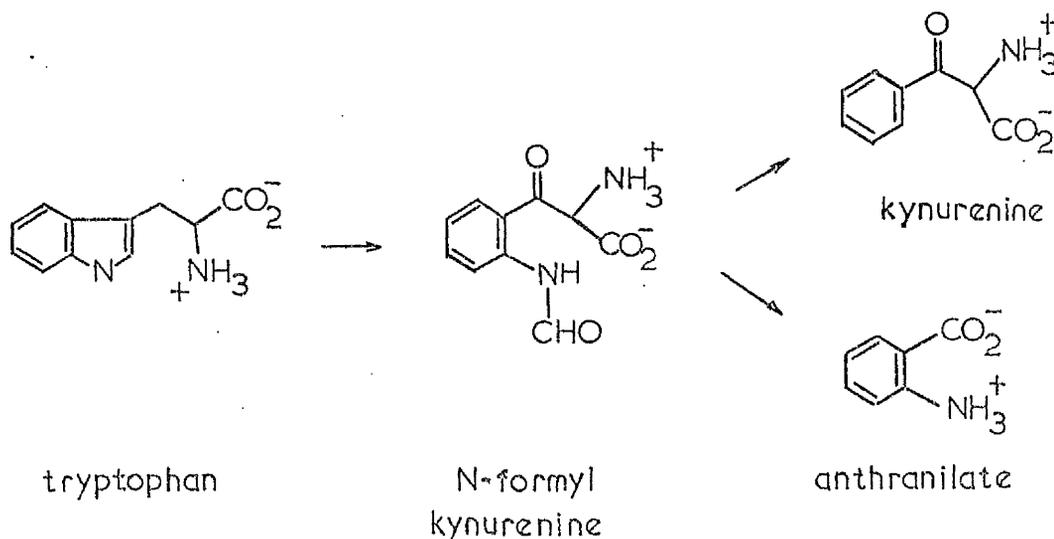
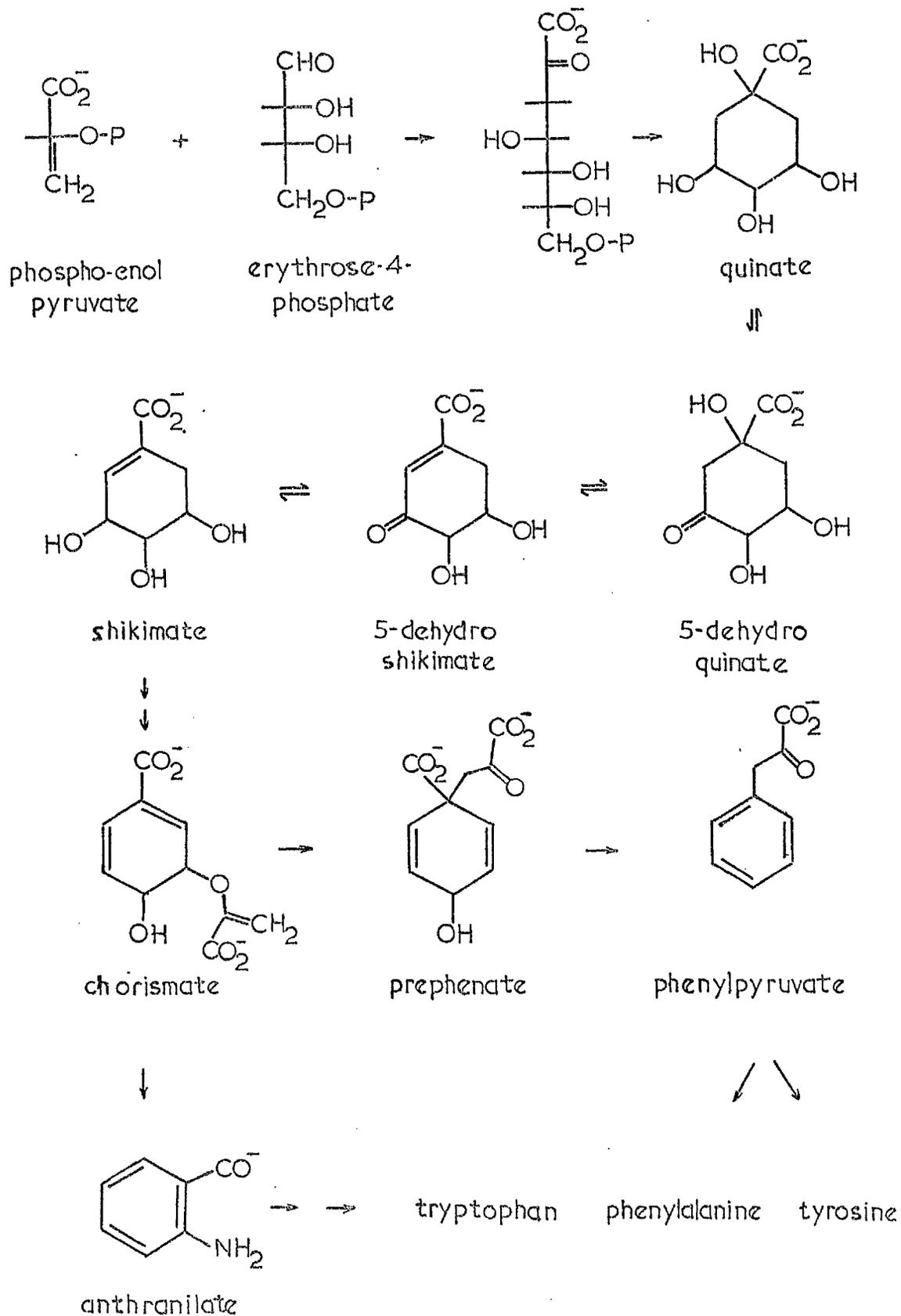


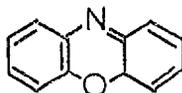
Figure 1

## Biosynthetic pathway leading to Aromatic Amino Acids

(from Ingledew and Campbell, 1969b)



Such schemes are analagous to the biosynthesis of the actinomycin chromophore (Katz and Weissbach, 1962) :



which has been shown to result from the condensation of two molecules of 4-methyl-3-hydroxy anthranilate.

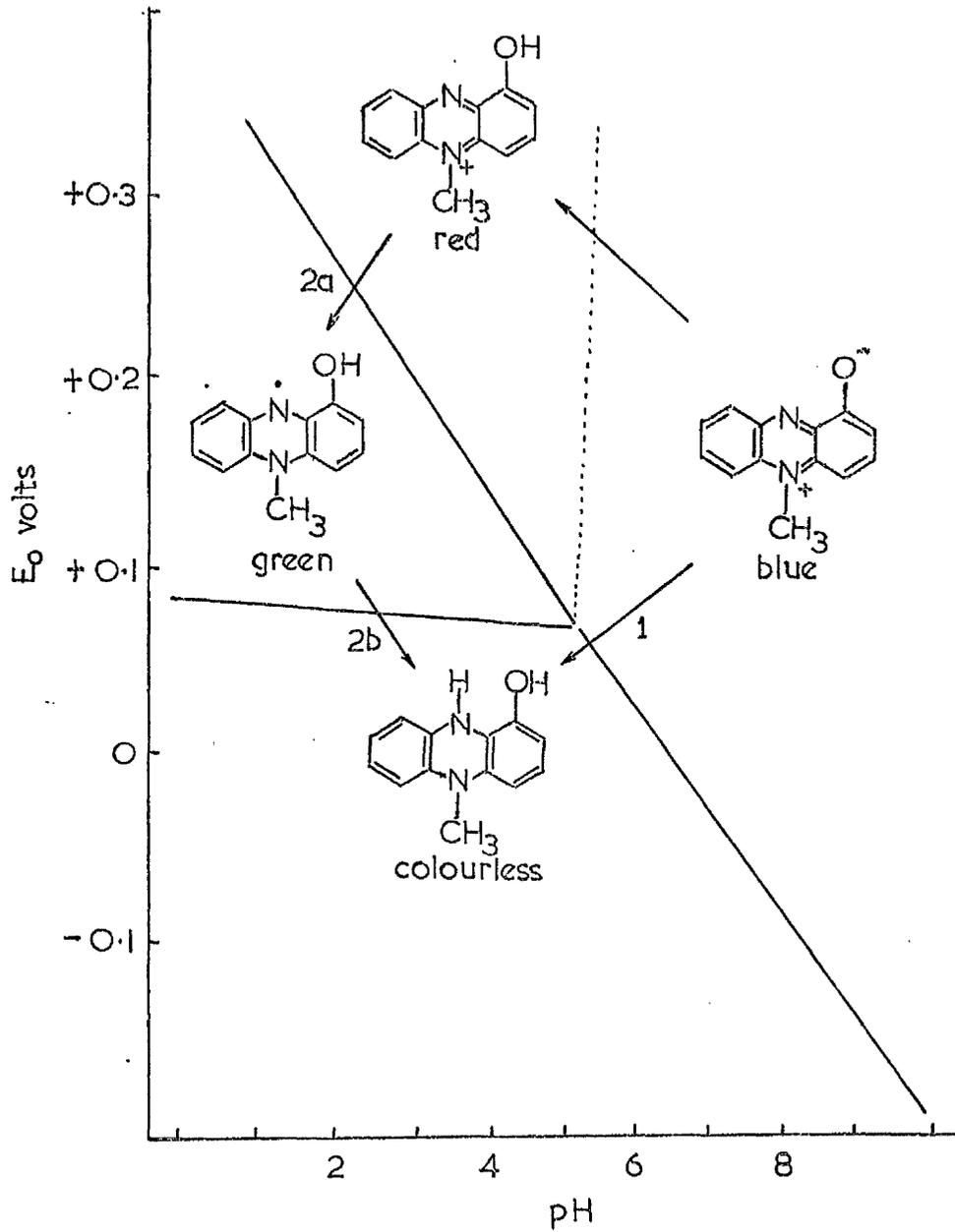
#### E. Oxidation Reduction Potentials of Pyocyanine

The structure of pyocyanine is such that it is autoxidisable i.e. capable of undergoing reversible oxidation and reduction, the structures involved having  $E_0$  values and colour changes recorded in Figure 2 .

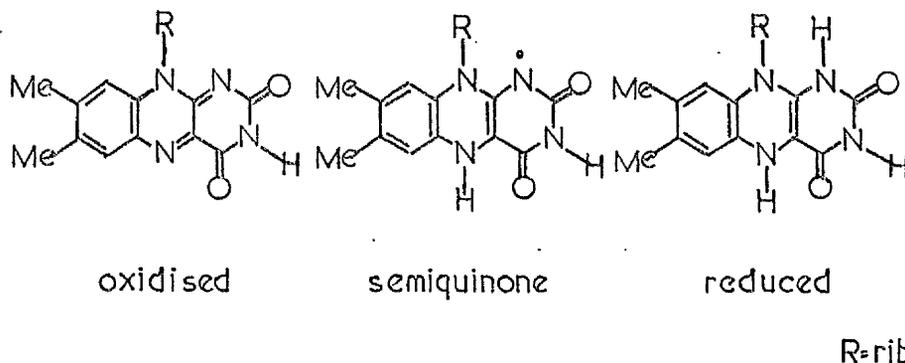
According to Friedheim and Michaelis (1931), the redox reactions of pyocyanine fall into two categories. At pH above 6 it behaves as a normal quinoid type dye, two electrons being added in the reduction process (route 1) and the blue oxidised form changing to colourless reduced pyocyanine. At pH below 6 the oxidised form exists as the red protonated cation. Reduction is achieved by the one electron steps (2a) and (2b) through the green semiquinone form to the colourless reduced compound. Michaelis (1932) suggested that pyocyanine could function in a manner similar to the N-9-isoalloxazine ring of riboflavine, the prosthetic group in flavoprotein, reacting via a one-electron intermediate product (semiquinone).

( $E_0'$  - Standard Reduction Potential for autoxidisable couple when the molar concentration of oxidised and reduced species at pH 7 are the same. According to thermodynamics the electron flow is from the negative (reducing) to the positive couple until equilibrium is attained).

Oxidation-Reduction potentials for Pyocyanine at various  
pH values (from Freidheim and Michaelis, 1931)



The structural similarity is illustrated below :



This aspect of pyocyanine activity has not yet been demonstrated in biological systems although Zuagg (1964) detected pyocyanine semiquinone at low pH.

The prediction of biological activity of a redox couple from its  $E_0'$  values is complicated by binding and permeability effects in an *in vivo* situation (Hewitt, 1950). In further consideration of the redox activity of pyocyanine, it is useful to compare its  $E_0' = -0.034$  volts with those of reactions occurring in the mitochondrial system (Figure 3). The potential in volts (on ordinate) shows the correlation of electron transfer to oxygen in the respiratory chain. It should be noted that the flavo-protein ( $E_0' = -0.30v$ ) is lower than free riboflavine ( $E_0' = -0.20v$ ) and that pyocyanine ( $E_0' = -0.03v$ ) is in a similar position to the fumarate-succinate couple. Zuagg proposed that chemical association of pyocyanine with the enzyme-substrate complex or with the lipid phase of the reaction could lead to stabilisation of the free radical intermediate.

#### F. Biological Activity of Pyocyanine

Pyocyanine prepared by synthesis has been shown to affect a variety of biological systems. Table II contains a summary of the observations

Oxidation-reduction potentials in respiratory chain carriers

(from Hawker and Linton, 1971)

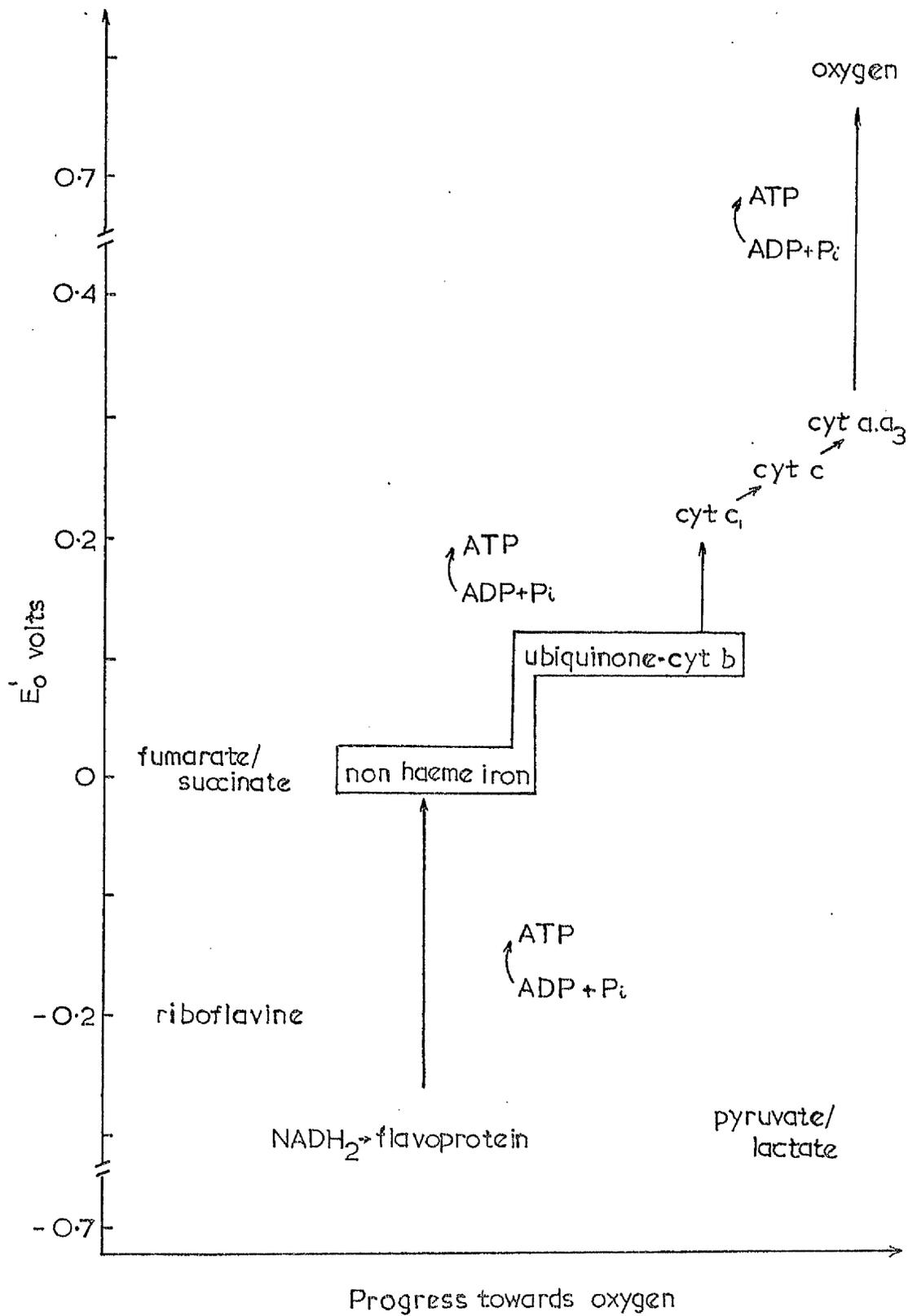


TABLE II

Literature reported biological activity of Pyocyanine

Author	Date	System being investigated	Effect of Pyocyanine
Ehrismann	1934	Variety of Bacterial Cells	Respiratory stimulation which was not counteracted by cyanide or fluoride. Catalysis of respiration in acetone dried cells.
Friedheim	1934	Tumour Cells	Stimulated oxygen uptake of cells suspended in glucose. Effect not seen in glucose-bicarbonate medium.
De Meio, Kissin and Barron	1934	Tumour Cells	Restored respiration of cyanide and fluoride inhibited systems.
Runnstrom and Michaelis	1935	Haemolysed Blood Preparation	Hexose phosphate synthesis from added glucose and NAD or glucose and pyocyanine.
Dickens	1936	Tumour and Normal Cells	Stimulated oxygen consumption and depressed aerobic glycolysis.
Weil-Malherbe	1937	Brain Tissue Cells	Stimulated $\alpha$ -keto glutarate utilisation.
Young	1937	Cerebral Cortex	Glycolysis and respiration on glucose accelerated, followed by irreversible inhibition.

Author	Date	System being investigated	Effect of Pyocyanine
Dickens and McIlwain	1938	Tumour Cells	Respiratory rate of hexose monophosphate system stimulated while aerobic glycolysis decreased. Anaerobic glycolysis inhibited. Pyocyanine was a less efficient electron carrier than FAD. Lactate and malate dehydrogenase activity stimulated. Evidence suggested structural association between enzyme substrate complex and pyocyanine.
Lennerstrand	1938	Phosphate buffered solution containing glucose/apoenzyme/ NAD and acetaldehyde giving phosphoglyceric acid.	Pyocyanine competed with acetaldehyde and more phosphate became organically bound.
Fazekas, Colyer, Nesin and Himivich	1939	Whole animal (cat)	Cerebral oxygen consumption increased and central nervous system disturbed.
Soresina	1939	Chick Embryo	Rate of growth stimulated.
Kharchenco, Ryabushko and Petrukhova	1947	Whole animal (rabbit)	Increased respiratory rate and depressed CNS activity.
McIlwain	1950	Tissue Cells	Pyocyanine accumulation within the cell.

Author	Date	System being investigated	Effect of Pyocyanine
Case and McIlwain	1951	Mitochondria from brain and liver	Stimulated respiration and lowered the yield of phosphorylated products of metabolism.
Judah and Williams- Ashman	1951		
Owen Jr., Karlson and Zeller	1951	Mycobacterial L-amino acid oxidase	Inhibited oxygen consumption.
Campbell, MacQuillan, Eagles and Smith	1957	Glucose catabolism of <u>P. fluorescens</u> and <u>Pr. vulgaris</u>	Substrate oxidation was stopped at 2-keto gluconate stage. Lactate and malate oxidation was inhibited at pyruvate and oxalacetate respectively. Succinate and fumarate oxidation was stopped at oxalacetate.
Hill and Walker	1959	Chloroplasts	Increased phosphorylation suggested as arising through participation of free radical form of pyocyanine.

Author	Date	System being investigated	Effect of Pyocyanine
Marcus and Feeley	1962	L-amino acid oxidase from snake venom	Inhibition of respiration explained as reduction of FAD-enzyme complex to less active form. Activity was substrate-dependent, actively metabolised substrates being most inhibited. Inhibited substrates were less affected as their concentration decreased. Findings were interpreted as pyocyanine involvement, not at substrate-enzyme site but at flavoprotein-oxygen contact. Semiquinone of flavoprotein at high concentration was most inhibited through conversion by pyocyanine to an inactive reduced form.
Landau, Hastings and Zotter	1963	Rat liver slices	<sup>14</sup> C-glycerol utilisation stimulated to increased CO <sub>2</sub> production and incorporation of <sup>14</sup> C into glycogen and fatty acid.
Harman and MacBrim	1963	SDH from brain tissue and rat liver	Inhibition of SDH activity showing pyocyanine concentration dependency which was taken as evidence for dual site of action (a) enzyme flavoprotein and (b) cytochrome chain.

made on whole animals, isolated organs, dispersed mammalian and bacterial cell suspensions and isolated enzyme systems from mammalian sources. It can be seen that in most systems, pyocyanine stimulated oxygen consumption and/or substrate utilisation.

Various attempts have been made to explain the physiological significance of pyocyanine to its parent organism. From the date of identification of its redox activity, pyocyanine was given the role of accessory respiratory catalyst (Stheeman, 1927). Friedheim (1931) found that the oxygen consumption rate of P. aeruginosa cells grown in non-pigmented culture was stimulated when pyocyanine was added. He attributed the effect to increased utilisation of substrates provided by cell lysis. Lichstein and Saule (1944) showed that azide inhibited respiration of P. aeruginosa was counterbalanced by pyocyanine.

In 1960 Kurachi, interested in the extracellular production of a proposed respiratory catalyst, investigated the problem using a variety of P. aeruginosa cell preparations. Freshly harvested glucose-grown cells were washed and stored (16 h). Their endogenous respiratory activity was unaffected by addition of pyocyanine. However cyanide-inhibited endogenous metabolism was stimulated after addition of pyocyanine. Pyocyanine addition did not affect the rate of metabolism of the cell suspension when exogenous substrate was provided. Kurachi considered it unlikely that pyocyanine played any active part in P. aeruginosa respiration unless the cytochrome chain was inhibited (e.g. by cyanide).

Kurachi showed a more striking effect with the products of cell autolysis in non-pigmented 4 day cultures of P. aeruginosa. On addition of glucose, lactate or succinate the autolysate failed to consume oxygen,

but adding pyocyanine resulted in measurable respiratory rate.

A similar stimulation was reported with acetone-dried preparations from glucose and glycerol-grown cells. With glucose-grown, acetone-dried cells, respiration with gluconate substrate and pyocyanine was twice that with glucose under similar conditions. The lack of respiratory activity in the acetone-dried cells was thought to be due to the removal of  $\text{NADP}^+$  and FAD during sample preparation. As pyocyanine could substitute for these coenzymes, a "bridging" function independent of flavoprotein was inferred. Kurachi concluded that *P. aeruginosa* cells could acquire energy anaerobically through the use of pyocyanine as an electron coupler in glucose oxidation and in fumarate reduction.

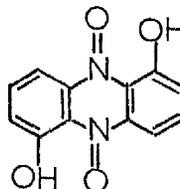
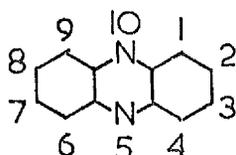
Kurachi also demonstrated an aspect of pyocyanine action other than that of hydrogen acceptor. Although the oxidation of lactate by acetone-dried cell preparations could be stimulated by pyocyanine, and by methylene blue, the lower activity of the latter was not reflected in comparing dye colour reduction rates. This was regarded as evidence of an alternative mode of respiratory catalysis by pyocyanine.

In further examination of pyocyanine activity using cell free lysate preparations, Kurachi found that gluconate and glucose both reduced pyocyanine under anaerobic conditions and that this reaction could be coupled to the reduction of fumarate to succinate. Reduction was not observed under aerobic conditions. This reaction was also demonstrated with glucose-6-phosphate and the malate/lactate couple. This was cited as evidence of a function for pyocyanine a) under anaerobic conditions where the reduction in cytochrome system was inoperative, or b) where phosphate-deprivation led to a shortage of FAD or NAD.

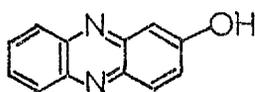
### G. Other Bacterial Phenazines

Pyocyanine is one of many naturally-occurring phenazine derivatives. Those which have been reported in cultures of strains of P. aeruginosa are presented in Table III. Although they are suspected of having a common biosynthetic origin (Chang and Blackwood, 1968) interconversion is not thought probable. The breakdown of pyocyanine in ageing alkaline cultures has already been discussed.

The types of phenazine derivatives produced by the family Pseudomonadaceae include the general class of 5, 10-dioxides the parent structure of which, 1,6-phenazinediol - 5,10-dioxide (Iodinin) has the numbering system shown below, (Clemo and McIlwain, 1938) :-



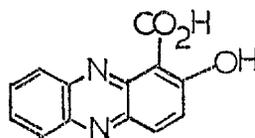
Hydroxy phenazine derivatives have been isolated from P. aureofaciens (Levitch and Rietz, 1966)



2-hydroxyphenazine

2-hydroxyphenazine

and



2-hydroxyphenazine-1-carboxylic acid

Phenazine derivatives are also produced by other micro-organisms. For example Streptomyces griseoluteus produces Griseolutein A and B (Nakamura, Maeda and Umezawa, 1964)



Griseolutein A

(1-methoxy-4-hydroxyacetoxy-  
methyl-9-carboxy phenazine)

and Griseolutein B (1-methoxy-4,6,7,12-tetrahydro-6-hydroxy-6-hydroxymethyl-2H-oxazino 5,4,3-d,e phenazine-11-carboxylic acid).

The most recent novel phenazine obtained by Hanson (1968) from a Sorangium strain is 1-hydroxy-6-methoxyphenazine 5,10-dioxide (Myxin).

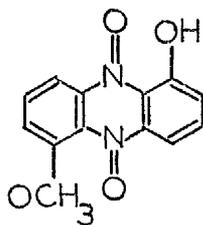
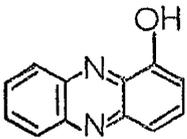
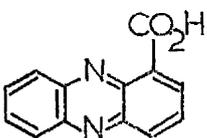
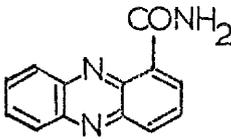
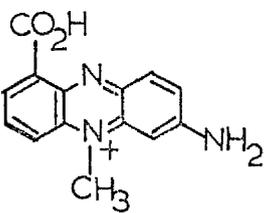
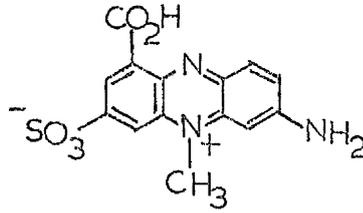


Table III

Phenazine derivatives produced by Pseudomonas aeruginosa

Structure	Colour	Systematic (common) name	Reference
	Yellow	1-hydroxyphenazine (1-phenazinol)	Swan and Felton (1957) Gerber (1967)
	Yellow	phenazine-1-carboxylic acid (tubermycin B)	Toohey, Nelson and Krothov (1965) Gerber (1969)
	Yellow	phenazine-1-carboxamide (oxychlororaphine)	Takeda and Nakashini (1959)
	Red	5-methyl-7-amino phenazine-1-carboxylic acid (aeruginosin A)	Holliman (1957.)
	Red	5-methyl-7-amino-1-carboxy phenazine-3-sulphonic acid (aeruginosin B)	Herbert and Holliman (1969)

SECTION II : PSEUDOMONAS AERUGINOSA

A. General Characteristics of the Organism

The genus Pseudomonas derives its name from the Greek meaning 'false monad'. The same Greek root has been used for the family Pseudomonadaceae and the order Pseudomonadales to which the organism belongs. The characteristics and nomenclature of the type species were described by Migula (1900): Pseudomonas aeruginosa (Schroeter). He also used the name Pseudomonas pyocyanea (1895). Early identification was attributed to Schroeter (1872) who designated the species Bacterium aeruginosum. The name 'aeruginosum' arose from the Latin meaning 'copper rust' because of the organism's green pigment. Before Migula's classification the organism was isolated in pure culture by Gessard in 1882. He used the name Bacillus pyocyaneus or commonly 'blue pus' organism due to its not infrequent occurrence in wounds.

The detailed analysis of nutritional spectra of P. aeruginosa was made by Stanier, Palleroni and Doudoroff (1966) and represents the accepted taxonomic guide. The organism is Gram-negative and monotrichous, the polar location of the flagellum being included as a criterion for identification. Agar colonies are large and spreading with a translucent irregular edge and greyish dark centre. The culture normally imparts a green or brown pigment and fluorescence to the medium and a characteristic odour of 2-amino-acetophenone is detected.

Many strains on nutrient agar undergo spontaneous lysis giving irridescent erosions on the surface growth. The mechanism of these so-called 'autoplaque' formations (Berk, 1963) is unknown and participation

of bacteriocins, phage or autolytic enzymes are not considered responsible (Berk, 1966). Growth in broth is turbid and a thick pellicle is formed. Pigment descriptions range from yellow green to blue with fluorescence, the colour changing to brown with age.

The characteristic pattern of biochemical activities (Cowan and Steel, 1965) is as follows:

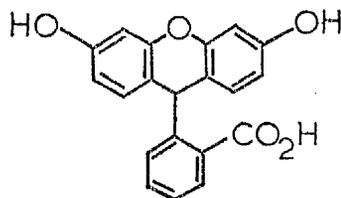
catalase positive; oxidase positive;  
 oxidative metabolism in Hugh and Liefson test (1953) positive;  
 common sugars not fermented (Wilson and Miles, 1964);  
 glucose oxidised to gluconate and 2-ketogluconate (Haynes, 1951);  
 nitrates reduced to nitrite, ammonia or free nitrogen  
 resulting in a facultatively anaerobic metabolism  
 (Robinson, 1932);  
 gelatine liquefied, milk peptonised but indole not formed.

To distinguish P. aeruginosa from other fluorescent aerobic pseudomonads the ability to grow at 42°C and at 4°C are used as differential characters.

The organism lacks a complete Embden-Meyerhoff system (Stern, Wang and Gilmour, 1960) and glucose metabolism proceeds via the Entner-Doudoroff pathway (1952). When P. aeruginosa is grown on tricarboxylic acid intermediates it has a low level of the Entner-Doudoroff enzymes gluconate-6-phosphate dehydrogenase and KDGP aldolase and of the oxidative part of the Pentose Phosphate Pathway. (Hamilton and Dawes, 1960; Von Tigerstrom and Campbell, 1966; Ng and Dawes, 1967; Lessie and Weidhardt, 1967).

Investigations into the extracellular products found in cultures

of P. aeruginosa include the observation of slime formation (Brown and Richards, 1964). This was a substrate-dependent property and has been implicated in the mechanism of the organism's pathogenicity by Liu and Mercer (1963). The diffusible metabolic products regarded as characteristic of P. aeruginosa in specific media, are pyocyanine and fluorescein. King, Ward and Raney (1954) devised synthetic media, Medium A and B, to promote separately the formation of these two pigments. The major difference in these media is the phosphate concentration, a higher than optimal level for pyocyanine resulting in improved yield of fluorescein which was not produced in the absence of phosphate. The conditions for fluorescein production have been investigated by King, Campbell and Eagles (1948) but the literature contains no investigation of its metabolic significance. Fluorescein has been characterised spectrophotometrically by its intense UV absorbance over the range 350 to 200 nm (Zanker and Peter, 1958) and has the structure shown below:



The significance of pyocyanine has been discussed in the previous section. For pyocyanine detection Brown and Foster (1970) have proposed the use of defatted milk medium to illustrate the pigment against a white background. The use of chromogenic ability as a criterion of identification under normally pigment-producing conditions of growth must be viewed in the light of MacDonald's (1966) demonstration that subculturing can lead to loss of pigment production.

## B. Pathogenicity and Resistance

Ringen and Drake (1952) investigated the occurrence of P. aeruginosa and reported its presence in various environments including sewage and the intestinal tract. It is carried by 12% of healthy individuals (Shooter, Walker, Williams, Horgan, Parker, Asheshav and Bullimore, 1966) and is regarded as being an opportunistic pathogen, causing septicaemia, bacterial endocarditis and infections in burn wounds and in the respiratory tract (Curtin, Petersdorf and Bennet, 1961). The use of broad spectrum antibiotics may facilitate its growth in patients (Koch, 1956).

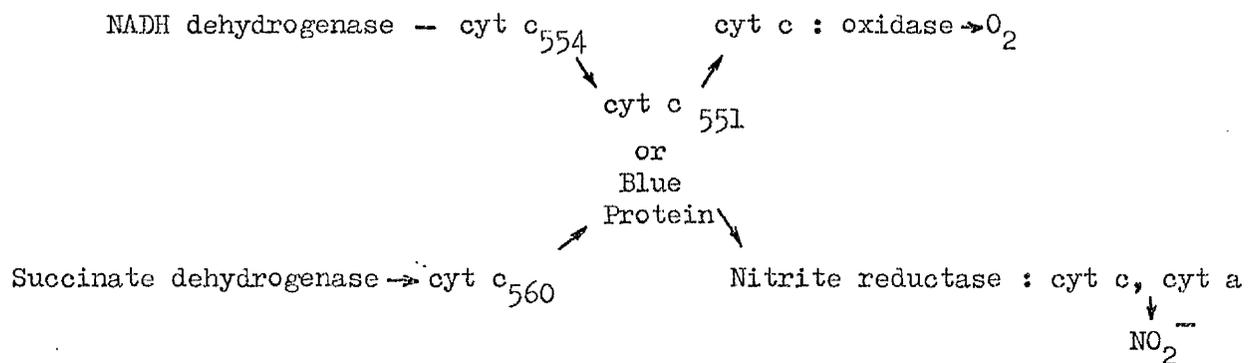
Investigations into the pathogenicity of P. aeruginosa date back to 1899 when Emmerich and Low demonstrated the proteolytic ability of culture filtrates of the organism. The presence of toxic extracellular enzymes contributes significantly to pathogenic activity (Fisher and Allen, 1958). Cell products which may be important are haemolysin, lecithinase and other protease and these have been linked with the destruction of leucocytes (Liu, 1966). Johnson, Morris and Berk, (1967) showed that elastase production was involved in tissue necrosis. The breakdown product of pyocyanine, 1-phenazinol, has been implicated in the inhibition of mitochondrial respiration (Armstrong and Stewart-Full, 1971).

Problems in the treatment of infections caused by P. aeruginosa arise from its drug insensitivity and its ability to utilise a wide range of carbon compounds as nutrients. It is resistant to sulphonamides and penicillin, and at present the effective antibiotics are Polymyxin, Colistin and Gentamicin (Garrod and O'Grady, 1968). There is no satisfactory explanation for the general resistance although an ability to inactivate

some antibiotics has been demonstrated (Doi, Ogura, Tanaka and Umezawa, 1968).

### C. Respiratory System

The respiratory chain in P. aeruginosa according to Yamanaka and Okunuki (1964) is outlined below :



This scheme does not consider the part played by the ubiquinone and naphthoquinone components shown to be present in P. aeruginosa by Bishop, Pandya and King (1962). The ubiquinone was present at higher concentration and the levels of both were unaltered by growth of the organism under anaerobic conditions with nitrate as the terminal acceptor. The presence of ubiquinone and naphthoquinone has been demonstrated in E. coli (Kashket and Brodie, 1963) and they are thought to act between the dehydrogenase and the cytochrome system. The oxidation-reduction activity of the cytochromes in P. aeruginosa has been shown to act along with the enzyme cytochrome c:O<sub>2</sub> oxidoreductase (Azoulay and Couchoud-Beaumont, 1965). In nitrite reductase, cytochromes a and c have been found (Horio and Kamen, 1961). Another component of the respiratory chain is the copper-containing blue protein, with one atom of copper per protein of molecular weight 17,000. It has

maximum absorption at 630 nm in the oxidised form (Sutherland and Wilkinson, 1962; Ambler, 1963).

The dehydrogenases which have been shown to be associated with fragments of cell envelope are aldehyde dehydrogenase (Heydeman and Azoulay, 1963) and alanine and succinate dehydrogenases (Campbell, Hogg and Strasdine, 1962; Norton, Bulmer and Sokatch, 1963). As with the mitochondrial system, flavine nucleotides as prosthetic groups have been inferred from the suitability of artificial electron acceptors (Kornberg and Phizackerley, 1961).

In general the respiratory chain enzymic activity is bound to envelope particles and the following cytochrome-linked systems have been found :

succinate oxidase, Bently and Schlechter (1960)  
glucose oxidase, Campbell et al., (1962b)  
D-alanine oxidase, Norton et al., (1963).

The cytochrome content of the cells has been shown to vary with the cultural conditions and with the phase of growth of the culture, increases being detected after the maximal growth period (Gibson, 1961). Depletion of oxygen in the medium can lead to utilisation of available nitrate via the nitrite reductase system (De Ley and Kersters, 1964).

The literature contains no report of intracellular pyocyanine acting in the respiratory chain described above.

#### D. Antibiotic Products

The antibiotic activity of pyocyanine is one of the oldest

examples of a microbial product which is antagonistic to other bacteria (Emmerich and Low, 1899). These authors made ten-fold concentrated culture supernatants which were lytic for Vibrio cholera and Bacillus anthracis. This type of activity was later attributed to the 'enzyme pyocyanase'. Klimoff (1901) refuted its enzymatic nature because it was heat stable and the preparation was shown to be heterogeneous.

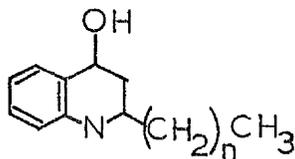
Schoental (1941) was the first to characterise several antibiotic factors in P. aeruginosa cultures based on the comparison with synthesized compounds. Pyocyanine, l-phenazinol and a colourless oily fraction given the name 'pyocyanic acid' were described. These were considered to be components of 'pyocyanase'.

To date, little more is known about the mechanism of P. aeruginosa antibiotic activity. Literature reports are confined to observations of bacteriostatic or bactericidal action and of respiratory inhibition. The various investigators include Schoental (1941) and Waksman and Woodruff (1942), and their results are characterised by variation in the reported inhibitory concentrations. Waksman and Woodruff also demonstrated that pyocyanine activity varied according to the method of growing the test organism.

The bacteriostatic activity against S. aureus is thought to involve binding of pyocyanine to thiol structures such as cysteine - an association which has been demonstrated. However, addition of the thiol did not reverse the effect in bacteria treated with pyocyanine (Cavallito, 1946). Schoental (1941) considered that l-phenazinol obtained from 10 day old cultures of P. aeruginosa was a strong 'bactericidal agent'. By visual comparison of the growth of treated organisms E. coli and Strep. faecalis

were among the few resistant species. Stokes, Peck and Woodward Jr., (1942) examined the bacteriostatic activity of 1-phenazinol in the growth medium. In evaluating these results it must be kept in mind that uniform inocula were not used. S. aureus, Strep. lactis and E. coli were susceptible although the concentration of 1-phenazinol required was twice that of pyocyanine causing a similar effect.

Other antibiotic products of P. aeruginosa were classified under the general term 'pyo' compounds (Hays, Wells, Katzman, Cain, Jacobs, Thayer, Doisy, Gaby, Roberts, Muir, Carrol, Jones and Wade, 1945) having the basic structure shown below:



n = 2,3,4...

These compounds were active against both Gram-positive and Gram-negative organisms.

### SECTION III : INFLUENCE OF CULTURAL CONDITIONS ON PYOCYANINE PRODUCTION

#### A. Components of the Medium

Various synthetic and semi-synthetic media have been used in the study of pyocyanine production by P. aeruginosa. Using his own isolates, Gessard in 1890 studied minor biochemical differences, such as the rate of gelatin liquefaction, to distinguish his strains and noted their pyocyanine production in various liquid media. He found that beef bouillon and egg albumin encouraged both fluorescein and pyocyanine production and that optimum pyocyanine production was achieved using peptone solution supplemented with glycerol. The yields of pigment in agar were inferior. The hydrolysis of albumin increased its utility as a growth and pigment supporting substrate. Gessard (1890) was the first to show that loss of pigment production by a strain might occur spontaneously.

Jordan (1899), in his experiments to find the optimum conditions for pyocyanine production, used seven strains of P. aeruginosa isolated by other workers who had not explored their pigment producing character. Solutions containing asparagine or ammonium salts of lactate, citrate or acetate were successful media for pigment production. Only two pyocyanine producers gave pigment on succinate. Tartarate, oxalate and formate allowed the growth of all strains but did not give pyocyanine. Nutrient gelatin and beef broth were superior media in this respect and inorganic growth factors such as  $\text{PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{Mg}^{2+}$  had no stimulatory or inhibitory effects on pyocyanine synthesis. One of Jordan's strains was described as having spontaneously lost its chromogenic capacity and a second was made to do so by serial transfer in the pigment-producing medium. The pyocyanine system of the latter culture could not be regenerated. This

brought Jordan to the conclusion that

"the pyocyanogenic property was sometimes spontaneously lost".

An interesting aspect of the work was the demonstration that not all cells of the one strain were equally chromogenic; plating out allowed the selection of subcultures of non-pigmented cells which were identical in all other respects. The problem was considered to have arisen through the use of artificial media resulting in chromogenic loss and

"more degraded scions of a vigorous parent stock".

While investigating the minimum inorganic requirements for growth of P. aeruginosa, Robinson in 1932 found that  $\text{PO}_4^{3-}$  and  $\text{Mg}^{2+}$  were necessary, while  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$  were not. The best synthetic medium for pigment production contained  $\text{MgSO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{NH}_4\text{Cl}$  with citrate and acetate as carbon sources. No pyocyanine was produced under anaerobic conditions even when  $\text{NO}_3^{2-}$  was added to permit growth. Robinson concluded that pigment production required the same factors as were needed for growth and that bacto-peptone liquid medium gave the best yields. As will be appreciated later, it is interesting to note that phosphate was kept at a low level to augment pyocyanine formation. Robinson stated the need, in future work, for consideration of the concentrations of components and of the level of dissolved oxygen in the medium.

Seleen and Stark (1943) in their investigation into the nutritive requirements of P. aeruginosa isolated 37 pyocyanine-producing cultures from selective media containing NaCl or KCN. They found glycerol peptone agar the optimal medium for pyocyanine production. The effect of temperature on pyocyanine production paralleled its effects on growth with

optimum at 37°C. Lack of homogeneity among strains of the species is exemplified in Seleen's work where 37 out of 199 strains of fluorescent P. aeruginosa produced pyocyanine. This type of observation coupled with the spontaneous loss of pigment producing ability, probably represents the reasons for the lack of information on the detailed requirements for pyocyanine production up till 1947.

Burton, Eagles and Campbell in 1947 investigated the suitability of various amino acids as sources of carbon and nitrogen. The strain of P. aeruginosa used, ATCC 9027, provided consistent results and it has been the strain most frequently used for the study of pyocyanine production by Burton and his co-workers. Leucine and alanine were found to be suitable sources of nitrogen, and in combination with glycerol and the salt solution specified in Table IV, the medium gave optimal pyocyanine yield in stationary liquid culture. Later Burton, Campbell and Eagles (1948) analysed the inorganic requirements for pyocyanine production. The best results were obtained with particular concentrations of  $MgSO_4$ ,  $K_2HPO_4$  and  $FeSO_4$ . The phosphate concentration was optimal at 0.04 g percent, lower levels giving no growth or pyocyanine, while higher levels gave reduced pyocyanine yields.  $Mg^{2+}$  added as the chloride and  $SO_4^{2-}$  as sodium sulphate, illustrated the simultaneous stimulation of growth and pyocyanine by magnesium and the selective promotion of pyocyanine by sulphate.

Burton's medium has been used to the present day for biosynthesis studies on pyocyanine production. Minor variations have been suggested and these are outlined in Table IV. Hellinger (1951) suggested adding  $CaCO_3$  to overcome the inhibitory effects of lowered pH.

The absolute amounts of pyocyanine produced in the cultures were

measured by MacDonald (1966) under the conditions specified by the original authors quoted in Table IV . Pyocyanine was determined by chloroform extraction and spectrophotometric ( $E_{690}$ ) measurement according to Kurachi (1958b). The quantities for ATCC vary from 100 to 300  $\mu\text{g}/\text{ml}$  depending upon the conditions of inoculation and incubation employed.

It was on the basis of Burton's work that King, Ward and Raney (1954) formulated the diagnostic Medium A for the identification of 107 strains of P. aeruginosa whose chromogenic property was difficult to demonstrate. The medium was made up of  $\text{K}_2\text{SO}_4$ ,  $\text{MgCl}_2$ , glycerol and bacto peptone was used because of its low phosphate content.

The manipulation of the chemical components of the medium is only one aspect of the problem. The physical conditions of the culture have been assumed varying degrees of importance depending on the strain and the substrate. Grossowicz, Hayat and Halpern (1957) stated that aeration of liquid culture failed to stimulate pyocyanine production but that optimum production depended upon the volume of medium and the vessel. Kurachi (1958a) compared static and aerated liquid cultures and found the latter gave 30% more pyocyanine. However the divergence of viable cell numbers and pH change caused by the aeration, could have played a part in pigment synthesis. Kurachi found a temperature optimum of  $37^\circ\text{C}$  and noted a maximum in the graph of pyocyanine produced versus substrate concentration.

MacDonald (1966) emphasized the difficulties in generalising about the effect of cultural conditions. However he concluded that greater consistency of pyocyanine yield was obtained in shaking cultures. The level of phosphate was critical in shaking cultures, 0.01 g percent giving maximum pyocyanine while 0.005 and 0.04 g percent gave half the maximum.



A technical problem in this work was illustrated by the use of two strains subcultured from the original ATCC 9027 strain of Burton et al., (1948). From freeze-dried stock, using subcultures of fewer than three transfers on Difco Maltose Broth, MacDonald obtained the parent 9027 strain. Strain PRIF20 came originally from the parent, but had been subcultured extensively. Under identical conditions of inoculation and growth these two strains exhibited marked differences in their ability to produce pigment. MacDonald concluded that consistently high pigment production was not possible unless the complicating effects of the past history of the organism were carefully controlled.

The work just described laid the foundation for the most recent cultural procedure, which was developed by Ingledew and Campbell (1969a) and which has been used extensively in this investigation. The essential feature of the procedure is that two media are used. The first is a medium which supports heavy growth, but in which the cells do not produce pyocyanine. Stationary phase cultures of this medium are used as the inoculum for the second medium, "Resuspension Medium" in which production of pyocyanine occurs. The Resuspension Medium is nutritionally complete except for the total absence of phosphate (see page 103). The carbon source, 2-ketogluconate, was the same in the growth medium and during incubation of the cells in Resuspension Medium. Low levels of phosphate were detected in the supernatant after pyocyanine synthesis had started and were ascribed to the breakdown of ribosomes in the organism. Some growth, as measured by O.D. increase, accompanied pyocyanine production in the Resuspension Medium. Ingledew proposed that phosphate depletion acted as a "trigger" for initiation and maintenance of pyocyanine synthesis.

## B. Condition of Inoculum

So far, the metabolic state of P. aeruginosa cells at the onset of pigment synthesis has not been established. From Table IV it can be seen that the time for pigment production to reach a maximum varies considerably. There is disagreement on whether the cells of the organism are dividing during the phase of pyocyanine production.

Grossowicz et al., (1957) considered their system to consist of non-proliferating cells. The inoculum was taken from a 24 h nutrient agar plate culture and transferred, after washing, to a liquid medium containing glutamate as carbon and nitrogen source,  $MgSO_4$ , and phosphate buffer at pH 7.4. Maximum pyocyanine yield was obtained using an inoculum of 2.6 mg dry weight of cells per ml. Lower levels of inoculation reduced the pyocyanine production but doubling the inoculum gave no more, or less, pyocyanine.

Frank and de Moss (1959) attempted to reproduce the results of Grossowicz using strain ATCC 9027. The same procedure for preliminary growth of the cells, followed by washing and transfer to fresh medium was adopted. The optimum inoculum was recorded as  $1 \times 10^9$  viable cells/ml and pyocyanine production was shown to be inhibited by phosphate levels higher than  $6 \times 10^{-4}$  M. Frank and de Moss recorded a five-fold increase in the viable cell count and a similar increase in bacterial protein during pigment production.

Halpern, Tench and Grossowicz (1962) published a re-affirmation of the non-proliferating nature of their pyocyanine producing cell suspension and no further work has been reported to clarify the problem.

In conclusion, pyocyanine production is strain dependent and can take place in either simple or complex media. As shown by MacDonald (1966), subculturing can lead to changes in the cultural requirements for pyocyanine production or to apparent loss of the chromogenic ability. The level of phosphate is one of the important variables.

It is noteworthy that most of the recent work has been done with strain ATCC 9027 and comes from the laboratory of J.J.R. Campbell who was an early collaborator of Burton.

### C. Inhibition of Pyocyanine Production

Fujita (1957) was the first to investigate the effect of chloramphenicol on P. aeruginosa which had been subcultured in the presence of the drug to induce resistance, while maintaining the pyocyanine production. The MIC for non-resistant strains of the organism was 60 µg/ml. The concentration of chloramphenicol required to prevent pyocyanine production by the resistant strain was 1,000 µg/ml. Aureomycin, terramycin and streptomycin did not inhibit pyocyanine production.

In the resting cell system of Grossowicz et al., (1959), cyanide and azide ( $10^{-3}$  M) reduced the amount of pyocyanine produced by 60%. Iodoacetate had a similar effect but azide at  $10^{-2}$  M had no effect on pyocyanine production. Using cell inoculum levels below those which gave optimal pyocyanine production, growth was observed and was found to be inhibited along with pyocyanine production on treatment with penicillin, aureomycin and chloramphenicol. Grossowicz concluded that the selective inhibition of pigment synthesis required a respiratory poison. The observed azide insensitivity was not rationalised. Frank and de Moss (1959)

confirmed the inhibitory activity of chloramphenicol on pigment production. Although they had previously recorded cell growth, a link between pyocyanine production and protein synthesis was invoked.

Using structural analogues of metabolites Kurachi (1959d), whose experimental system required anthranilate as substrate, found that acetanilide and aniline blocked pyocyanine synthesis.

An indication of inter-strain variation in the effect of antibiotics was obtained by Schneierson, Amsterdam and Perlman (1960). Standard inocula of young cells of four pathogenic, pigmented strains whose chloramphenicol MIC was 100  $\mu\text{g}/\text{ml}$ , were all found to yield less than 50% of the normal level of pyocyanine in the presence of 20  $\mu\text{g}/\text{ml}$  of the drug. Erythromycin (50  $\mu\text{g}/\text{ml}$ ) and tetracycline (5  $\mu\text{g}/\text{ml}$ ) were effective in the inhibition of pigment production with four and two strains respectively. Growth was reported as being unaltered, although visual comparisons only were made. The above observations could be due to bacteriostatic activity of chloramphenicol, selection of particular mutants, or the direct action on the pyocyanine synthetic system.

Espinosa, Hidalgo and Portoles (1972) tried to solve the problem by comparing the response of normal and antibiotic-resistant strains to 80% of the MIC. Both exhibited the similar inhibition of phenazine synthesis by chloramphenicol and oxytetracyclin, even although no inhibition of growth occurred. The estimates of pyocyanine production were based on dry weight estimations on the supernatant liquor and assumed other extracellular products to be constant. No quantitative data on viability were included.

The activity of the respiratory inhibitors  $F^-$ ,  $CN^-$ ,  $N_3^-$  and iodoacetate remains unexplained. The inhibitory activity of chloramphenicol implicates protein synthesis during pyocyanine production although the bacteriostatic effect has not been properly explained.

MATERIALS AND METHODS

## SECTION I : BACTERIOLOGY

### A. Culture of the Organism

#### 1. Strain of Pseudomonas aeruginosa

The National Collection of Type Cultures (NCTC) 6750 P. aeruginosa, type species of the genus Pseudomonas was used throughout. For maintainance of stock cultures, ampoules of cells which had been freeze dried in Mist Desiccans (3 parts serum plus 1 part 30% glucose nutrient broth) were opened and plated on to Trypticase Soy Agar (TSA; BBL, Bioquest; Appendix 1 ). Single colonies from the pigmented growth were transferred to nutrient agar slopes which were incubated for 18 h at 37° and stored at 4°C. Each slope culture was opened only 4 times and was discarded after 4 weeks.

#### 2. Media

##### a. Growth Medium of von Tigerstrom and Razzell (1968)

Details of the Yeast-Tryptone growth medium are given in Appendix 1 . The medium used here differed in certain respects from that of the above authors :-

- (i) Only disodium succinate was used as carbon source, although von Tigerstrom and Razzell described a variety of strains as having different optimal carbon sources. The cell yields obtained compared well with the 1 gm dry weight reported.
- (ii) The ferrous sulphate solution was sterilised by filtration through a 0.22u 'Millipore' membrane. von Tigerstrom and

Razzell added this component before the medium was sterilised by autoclaving at 15 lb/in<sup>2</sup>.

- (iii) The addition of ethanol after 10 h incubation was usually omitted. The few occasions on which ethanol was added gave higher cell yields but not sufficient to justify a 10 h period in the laboratory.

Hereinafter this medium is referred to as Y-T medium.

b. Resuspension Medium (after Ingledew and Campbell, 1969a)

Resuspension Medium was designed to stimulate pyocyanine production by P. aeruginosa. The following salts (g/100 ml) were dissolved in 50 ml Tris buffer (Appendix 2 ) at pH 7.4 :-

0.02 MgCl<sub>2</sub>.6H<sub>2</sub>O  
 0.047 KCl  
 0.05 NH<sub>4</sub>Cl  
 0.10 Na<sub>2</sub>SO<sub>4</sub>  
 1.0 Disodium Succinate

This medium differed from that of Ingledew and Campbell in that sodium 2-ketogluconate was replaced by an equivalent weight in disodium succinate. (Distilled water was used in all cases). The medium was dispensed in 90 ml quantities in 250 ml Erlenmeyer flasks, with indentations to assist aeration, and was sterilised by autoclaving at 15 lb/in<sup>2</sup>. The cultures were incubated at 37°C on an IH-400 Gallenkamp Orbital Incubator at 150 r.p.m. To minimise contamination all flasks were filled with glass distilled water, autoclaved and rinsed before use.

c. Inoculum for Resuspension Medium

The inoculum for the resuspension medium was obtained by harvesting the stationary phase cells from Y-T growth medium. This had an optical density of 3.8 - 4.2 at 490 nm when measured on an SP600 spectrophotometer (Pye Unicam, Cambridge) in cells with 10 mm light path. Where the O.D.<sub>490</sub> exceeded 0.4 the cell suspension was diluted with 0.85% saline to give a reading in the range 0.1 - 0.4. Distilled water was used as the blank.

The stationary phase cells were harvested and washed by centrifugation (12,000 x g at 4°C for 20 min) in sterile saline. They were resuspended in sterile Tris at 10 times original concentration and 2.5 ml of this suspension was transferred to the Resuspension Medium before it was made up to the final 100 ml volume.

The Inoculum Suspension (IS) had been less than 2 h removed from the growth medium and was retained, after washing at 4°C, for no more than 30 min before inoculation into Resuspension Medium. Incubation was under the conditions described for the growth culture.

3. Measurement of Bacterial Growth

a. Opacity of Cell Suspension

O.D. measurements were made at 490 nm using suitable dilutions of the cell suspension. The latter was prepared from 1 - 5 ml aliquots of culture and removing the cells by centrifugation (12,000 x g at 4°C for 20 min). The pellet was evenly resuspended to its original culture volume and, if necessary, diluted with saline.

b. Viabile Cell Count

The number of viable cells present during the incubation in Resuspension Medium was determined by the surface plating method. Ten-fold doubling dilutions (1 ml in 9 ml sterile saline) were made down to  $10^{-10}$  concentration using fresh pipettes for each dilution. Using a calibrated Pasteur pipette, 0.02 ml of each dilution was spotted onto the surface of a TSA plate. Individual plates contained 4 spots and two plates were used for each dilution. After 24 h incubation, the total number of colonies on each pair of plates showing separate colonies was recorded. Dilutions giving a colony count in the region of 100 per plate were considered best for estimating the concentration of viable bacteria in the original sample.

c. Bacterial Dry Mass

The dry weight of sedimentable bacterial matter was estimated at intervals throughout the incubation period in Resuspension Medium. Triplicate 10 ml volumes of evenly dispersed culture were centrifuged and washed as described above. The washed cell pellets were spun down in tared centrifuged tubes and all supernatant liquid carefully drained off. The cell weight was determined by drying for 48 h to constant weight in a vacuum desiccator over phosphorus pentoxide.

SECTION II : BIOCHEMISTRY

A. Preparation and Assay of Pyocyanine

1. Synthesis of Pyocyanine and Phenazines according to Surrey (1955)

The series of reactions involved is given in Figure 4 . The

starting material (pyrogallol monomethyl ether) was first prepared by oxidation of 2-hydroxy-3-methoxybenzaldehyde shown in reaction 1. Using  $\text{PbO}_2$  to generate the oxidised species (reaction 2) the presence of o-phenylenediamine leads to a condensation reaction yielding 1-methoxyphenazine. The latter is demethylated with HBr to yield 1-phenazinol. Alkylation of the latter with methyl sulphate (reaction 4) gives pyocyanine. The details of individual steps are as follows:

a. Synthesis of Pyrogallol monomethyl ether.

The apparatus consisted of a three necked 'Quickfit' flask (2 l volume) fitted with a nitrogen gas inlet system, a thermometer and a reflux condenser. 60.8 g of 2-hydroxy-3-methoxybenzaldehyde was added, in finely ground aliquots, to 200 ml of 2N NaOH. Stirring was continued throughout and the temperature was kept at  $37^\circ\text{C}$ . Through the thermometer opening 280 ml of 6% aqueous  $\text{H}_2\text{O}_2$  solution was added stepwise, to keep the temperature around  $45 - 50^\circ\text{C}$ . After the reaction mixture had cooled, it was saturated with NaCl and then extracted (4 x 100 ml) with ether. The combined ether extracts were dried over anhydrous sodium sulphate and the ether was removed by vacuum distillation. The product was purified by distillation under reduced pressure and was collected as a yellowish oil at  $135^\circ\text{C}$  and 22 mm pressure.

b. 1-Methoxyphenazine

200 g of lead dioxide ( $\text{PbO}_2$ ) were added to a solution of 10 g of pyrogallol monomethyl ether in 200 ml of dry benzene in a 2 l Erlenmeyer flask. After shaking for 15 min, the resulting reddish brown solid was removed by filtration on a Buchner funnel.

To the filtrate was added, with stirring, 6 g of o-phenylene-diamine in a mixture of 80 ml of glacial acetic acid and 200 ml of benzene. This solution was left for 90 min before being washed as follows :-

3 times with H<sub>2</sub>O (100 ml)

2 times with 5% NaOH (100 ml)

once with H<sub>2</sub>O (100 ml)

The benzene solution was shaken with 5 g of Norit and 50 g of anhydrous Na<sub>2</sub>CO<sub>3</sub> and then filtered on a Buchner funnel. The yellow benzene solution was stirred with 50 g of activated alumina and filtered. The alumina cake was washed with benzene to remove the residual product.

After removal of the benzene by vacuum distillation the light yellow solid was recrystallised from ethanol and water. The air-dried crystals had a melting point of 167°C.

c. 1-Phenazinol

A solution of 4.2 g of 1-methoxyphenazine in 125 ml of 55% hydrobromic acid was placed in a 250 ml round bottomed flask fitted with a reflux condenser. The flask was heated on an oil bath for 3 h at 110°C. The cooled reaction mixture, diluted with 125 ml of distilled water, was neutralised with approximately 100 ml of 10N NaOH and exhaustively extracted using two portions (25 ml) of 2N NaOH. The extract was made acid, using acetic acid, and the product was extracted into 50 ml of ether. Anhydrous sodium sulphate was used to dry the ether extract and the solvent was removed by vacuum distillation. The solid residue was recrystallised from ethanol and water to yield orange-yellow crystals with a melting point of 150°C.



#### d. Pyocyanine

A solution of 2.0 g of 1-phenazinol in 13.4 g of freshly distilled methyl sulphate was placed in a 250 ml round-bottomed flask fitted with an air condenser and an anhydrous calcium chloride drying tube. The reaction mixture was heated for 10 min at 100°C and, after cooling, 75 ml of ether was added. The solid product was filtered and washed with 100 ml of ether. The dry methosulphate was dissolved in 30 ml of water and made alkaline by the addition of 2 - 3 ml of 10% NaOH. The blue aqueous solution of pyocyanine was purified by the extraction procedure given in Figure 5 .

The combined chloroform solutions were dried over anhydrous sodium sulphate and decanted. The chloroform was removed by air evaporation and the solid pyocyanine recrystallised from hot water (60°C). The product, when dried over calcium chloride in a desiccator, melted at 133°C. To avoid photocatalysed decomposition the desiccator was kept in the dark.

## 2. Identification

### a. Thin Layer Chromatography

Thin layer chromatography (TLC) plates were soaked in Decon 90 washed in distilled water and oven dried before being coated with 0.25 mm thickness of Silica Gel G (Merck). The plates were activated by heating in a dry oven at 110°C for 30 min before use.

The samples examined by TLC were (1) standard solutions of synthesized compounds, (2) freeze-dried bacteria-free culture supernatant and (3) chloroform extracts of culture supernatant. Optimum resolution was achieved using chloroform:methanol (9:1) as the developing solvent.

Figure 5

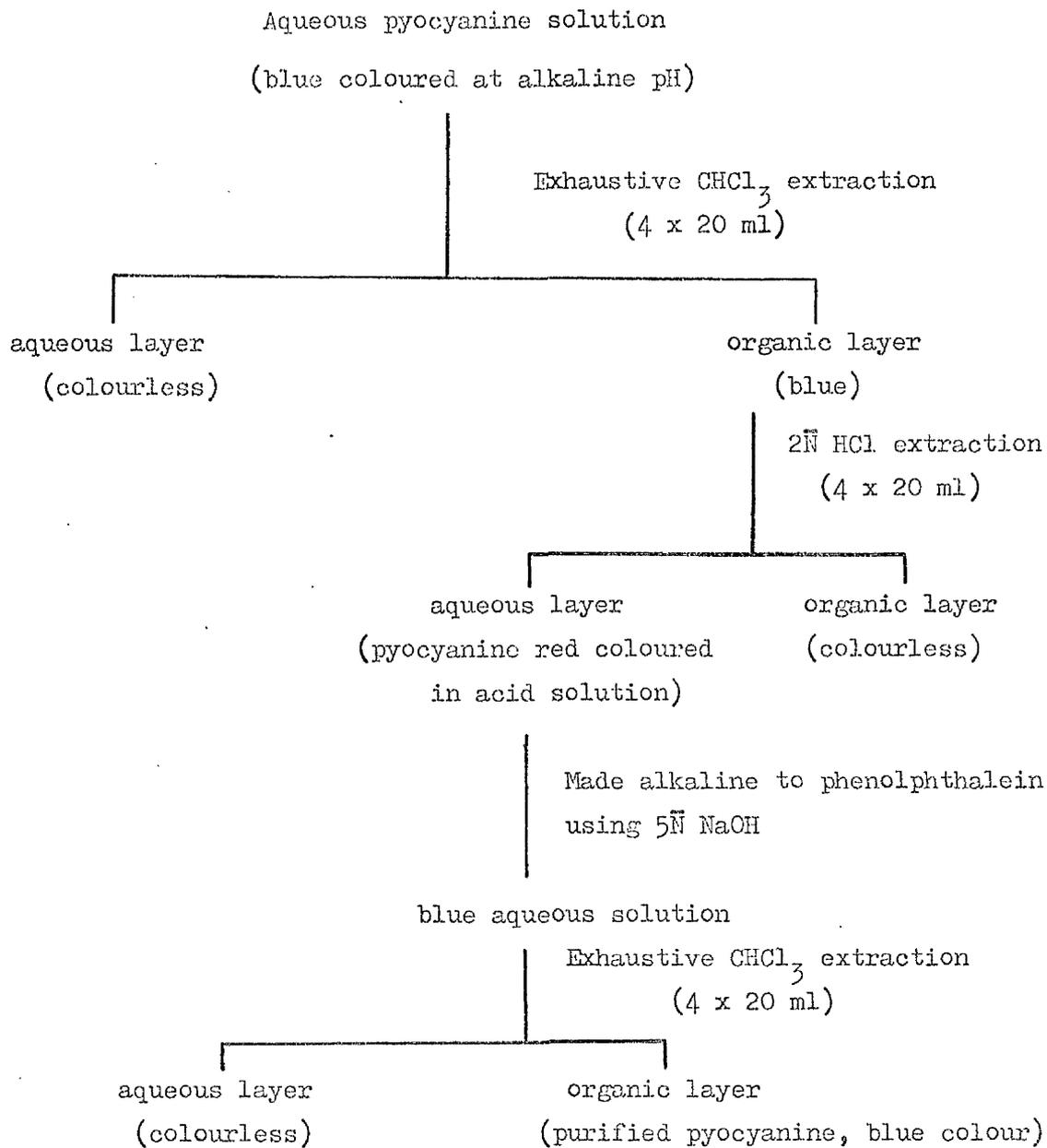
Purification of Pyocyanine

Plate development in the chromatography tank (Shandon, London) was considered complete when the solvent front had travelled 14 cm. The dried plates were observed for visible spots and also under UV light before spraying with 50% sulphuric acid. The plates were dried at 100°C for 1 min and re-examined.

b. Ultra-violet and Visible Spectrophotometry

The UV and Visible Spectra were recorded using an SP 800A spectrophotometer (Pye Unicam, Cambridge, England). Matched quartz cells of pathlength 10 and 2 mm were employed. The slit width was 0.06 mm and all spectra were recorded using solutions free from noticeable turbidity. Except where otherwise stated, the blank cuvette contained distilled water. Before use, the base line of the recorder was set using distilled water in the sample position.

c. Infra-red Absorption Spectra

IR spectra were recorded on a Perkin-Elmer Model 257 spectrophotometer over the range 4000 to 625  $\text{cm}^{-1}$ . Samples were prepared either as KCl discs (1 mg/3 mg) or as solutions in carbon tetrachloride and tetrachlorethylene. The pathlength was 0.1 mm.

3. Assay Procedures

a. Extraction and Gravimetric Estimation

Pyocyanine concentration in supernatants was estimated gravimetrically after exhaustive chloroform extraction and purification procedure outlined in Figure 5. Triplicate samples were taken and the mean dry weight of the residual blue crystals was recorded.

b. Spectrophotometric Estimation according to Kurachi (1958b)

The intensity of the blue colour as measured by  $E_{690}$  has been shown to be proportional to pyocyanine concentration. A standard solution of pyocyanine in Tris allowed calibration of the 690 nm absorptions. The validity of this method is discussed later (page 83 ).

B. CELLS AND CELL ENVELOPES

1. Biochemical Measurements on Bacterial Cells

a. Protein

The modification by Lowry, Rosebrough, Farr and Randall (1951) of the Folin reaction was used to estimate bacterial protein (Appendix 4 ). Crystalline bovine serum albumin was used as standard and the values of  $E_{750}$  were recorded after correction for the reagent blank containing no protein.

To estimate protein in the bacterial cells, the cell pellet was suspended in 0.1M NaOH containing 2%  $\text{Na}_2\text{CO}_3$  and solubilised by sonic vibration at 0°C. This was followed by heating the suspension in a boiling water bath for 10 min to give a clear solution.

b. Lipopolysaccharide

LPS was extracted by the phenol-water procedure of Westphal , Luderitz and Bister (1952) and purified by ultracentrifugation at 100,000 x g. The packed cell yield from 2 l of culture in Resuspension Medium (approx. 1 g dry weight) was suspended at a concentration of 0.01 g/ml in water. An equal volume of 90% (w/v) phenol was added.

The mixture was stirred at 65--68°C for 30 min, chilled to 2°C and centrifuged (10,000 x g for 20 min at 4°C). The opalescent supernatant was decanted and the residue was extracted once more by addition of an equal volume of water. The combined, opalescent aqueous supernatants were dialysed against running water for 4 days and finally against distilled water for 24 h. The extract was concentrated by pervaporation against polyethyleneglycol to 20 ml. The suspension was subjected to high speed centrifugation (100,000 x g for 6 h at 4°C). The opalescent pellet of LPS was taken up in distilled water to give a final 5 ml volume. An aliquot was removed for estimation of nucleic acid contamination at  $E_{260}$ . The suspension was made up to 50 ml with 0.1 M NaCl and recentrifuged to give a final LPS suspension with essentially no absorbance at 260 nm, indicating the absence of any contaminating nucleic acid. An aliquot of the suspension was removed for estimation of total organic matter by Johnson's method (below).

c. Total Organic Matter

The concentration of LPS was measured by the dichromate oxidation method of Johnson (1949; Appendix 4 ) using glucose as standard. Aqueous dilutions of the LPS suspension in 0.8 ml volumes were added with shaking to 1.0 ml Johnson reagent. The reaction mixture was heated for 10 min and cooled prior to the addition of 10 ml of water. Solid  $\text{Na}_2\text{SO}_3$  (10 mg) was added to the blank previously treated in the same way and the  $E_{440}$  of the sample was measured against the reduced blank.

d. Lipid

Extractable lipid was estimated according to Folch, Lees and Stanley

(1957). The cell yield from 500 ml of Resuspension Medium was shaken with 50 ml of chloroform-methanol (2:1) containing 2 ml of 0.05% aqueous calcium chloride. When the emulsion had separated the aqueous phase was removed and 4.8 ml of  $\text{CHCl}_3$ -Methanol-0.05%  $\text{CaCl}_2$  (3:48:47) was added and shaken. The upper layer was carefully removed and the interface washed with 2 volumes of 0.5 ml of the above solution. The isolated organic layer was evaporated to dryness under a stream of nitrogen.

e. Total Phosphorus

Phosphorus was determined by the Allen method (1940; Appendix 4 ). Samples of lipid extract were boiled with  $\text{H}_2\text{O}_2$  (30%) for 20 min to oxidise the organic matter. Periodic addition of  $\text{H}_2\text{O}_2$  was necessary to prevent drying out of the sample. To the contents of the cooled tubes 2.5 ml Amidol reagent plus 1.5 ml ammonium molybdate solution were added. The standards consisted of oven dried  $\text{K}_2\text{HPO}_4$  dissolved in distilled water and stored over chloroform at 4°C. Standards and reagent blanks were run in parallel with samples and  $E_{640}$  readings made at exactly 15 min after the addition of the reagents.

f. Deoxyribonucleic and Ribonucleic Acid

The Munro and Fleck procedure (1966) based on the Schneider (1957) technique of pentose analysis was used to determine nucleic acid. DNA was estimated by the colour reaction described by Burton (1956) involving diphenylamine reagent (Appendix 4 ) for the deoxy sugar. RNA detection depends upon the conversion of purine-bound ribose to furfural which is sensitive to the orcinol reagent (Appendix 4 : Schneider, 1957).

The preliminary treatment of the freshly harvested Resuspension

Medium cells was the same for both procedures. The cell yields from triplicate 5 ml volumes were made 0.25 M with respect of  $\text{HClO}_4$  and held at  $2^\circ\text{C}$  for 30 min. They were then centrifuged ( $10,000 \times g$  for 20 min at  $4^\circ\text{C}$ ) and the supernatant, containing free extracellular nucleotides, was discarded. The cellular deposits were resuspended in 4 ml of 0.5 M  $\text{HClO}_4$  and heated at  $75^\circ\text{C}$  for 25 min to solubilize the nucleic acids. Supernatants from this treatment provided the samples for assay.

DNA estimation required 1 ml of supernatant to which 2 ml diphenylamine reagent was added. Blanks containing  $\text{HClO}_4$  without sample and standard solutions of DNA were treated in parallel. After incubation for 20 h at  $30^\circ\text{C}$ , the O.D. values at 600 nm were recorded.

For the RNA estimation, 3 ml volumes of supernatant were mixed with 0.3 ml orcinol reagent and 3 ml of acidic  $\text{FeCl}_3$ . The samples were heated for 45 min in a boiling water bath, cooled and  $E_{660}$  measured. A standard solution of RNA was used for calibration.

g. Adenosine triphosphate

The ATP was assayed by the Luciferase method of Cole, Wimpenny and Hughes (1967) based on that of McElroy (1963) and modified by Holms, Hamilton and Robertson (1972). ATP (di-sodium salt; Sigma grade), glycylglycine and Firefly Lantern Extract (FLE-250) were obtained from Sigma, London.

The FLE was reconstituted in arsenate magnesium buffer by adding 25 ml of cold distilled water to a vial of lyophilised extract. The contents were constantly but gently mixed for 24 h at  $4^\circ\text{C}$ . Aliquots were stored at  $-10^\circ\text{C}$  until required.

ATP was extracted from the Resuspension Medium culture by transfer of 4 ml into 1 ml 30% perchloric acid at 0°C. After 10 min, 3 ml of 1 M KOH at 0°C was added, and the precipitate of potassium perchlorate was allowed to sediment for 10 min before centrifugation at 12,000 x g and 4°C in glass centrifuge tubes. The cell samples were extracted from duplicate culture supernatants. Extracts of ATP were frozen immediately in a mixture of solid CO<sub>2</sub> and ethanol and were stored at -10°C with internal standards treated in an identical manner.

The analyses for ATP were made on volumes of extract between 10 and 100 µl and on standard ATP solutions, some of which were freshly prepared, others which had been extracted at the time of sampling. All solutions were added at 0°C to 1 ml of distilled water at 0°C and 2 ml of buffer (6 mM MgCl<sub>2</sub> and 75 mM glycylglycine) at pH 7.4 and 37°C. The temperature of the resulting mixture was 24°C. Then 20 µl of FLE was added and the stopwatch immediately started as the components were mixed over parafilm (Gallenkamp, London). The cuvette was placed in front of a photomultiplier tube and, precisely 15 sec after FLE addition, the number of light emissions were counted on a scaler over a period of 10 sec. The count corrected for background was proportional to ATP concentration over the range 1 - 50 p moles. The photomultiplier tube was housed in a light tight box so that the cuvette was placed in its precise position without introducing stray light. The quartz (Spectrosil, Ross Scientific Co. Ltd., London) cuvettes were stored in the dark and the entire apparatus was earthed to avoid static electrical interference. (The assay was performed at 24°C).

## 2. Preparation of Cell-free Extracts

### a. Enzymatic Lysis

The lysosyme treatment was based on the method of Repaske (1958)

who found that EDTA and Tris buffer enhanced the digestion of Gram negative cells by lysozyme. In a typical preparation, 10 g of frozen cells were suspended in 300 ml of 50 mM Tris buffer (pH 7.4) and 48 mg of lysozyme was added in 32 ml of EDTA (4 mg/ml). At the same time 10 mg of DNAase in 2 ml of water was added. The mixture was stirred for 30 min at room temperature during which time it went viscous and then mobile. The mixture was centrifuged at 40,000 x g for 20 min at 4°C to yield a pellet containing cell envelopes, protoplasts and unlysed cells. This was resuspended in 200 ml of Tris and recentrifuged at 40,000 x g. This washing procedure was repeated. The final centrifugation consisted of 15 min at 4,000 x g to remove residual intact cells. The preparations were examined by phase-contrast microscopy and recentrifuged where necessary. The cell envelopes were washed by centrifugation three times in Tris buffer and resuspended at a concentration of 10-20 mg/ml.

b. Mechanical Lysis

Cell free extracts of P. aeruginosa were also obtained using the X-press (Biotec, London) pressure cell. Cells were suspended at 100 mg/ml in Tris and 0.1 ml DNAase (1 mg/ml) was added. 5 ml aliquots of the heavy suspension were subjected to freezing in the chamber of the pressure cell which had been precooled to -15°C in solid carbon dioxide-ethanol mixture. A pressure of 14,000 lbs/inch<sup>2</sup> was applied to force the frozen paste through the cell orifice. The unit was placed back in the cooling mixture before each of the three passages applied to the 5 ml aliquots. The residual whole cells were separated and the cell envelopes washed and resuspended as described under the enzymic lysis.

### 3. Electron Microscopy

#### a. Thin Sectioning

Bacterial samples were taken after different periods of incubation in Resuspension Medium and treated with glutaraldehyde to 5%. The cells were then sedimented by centrifugation (3,000 x g for 30 min at 4°C) and resuspended in fresh fixative in Veronal - acetate buffer (Kellenberger, Ryter and Sechaud, 1958; Appendix 2 ), at pH 6.1 for 2 h. After thorough washing in veronal-acetate buffer, cells were again fixed in veronal-acetate buffered 1% osmium tetroxide for 3 h at room temperature. The twice-fixed and washed pellets were stained with 0.5% magnesium uranyl acetate for 1.5 h at room temperature, washed once in veronal-acetate buffer and embedded in 1.5% Bacto agar. Agar blocks (0.5 mm<sup>3</sup>) were next dehydrated in an ethyl alcohol series (25%, 50%, 75%, 95%, 100% v/v ethanol), infiltrated and embedded with Epon (Luft, 1961) as described for Araldite (Glauert and Glauert, 1958). Sections (silver interference colour) were cut with a diamond knife on an LKB Ultratome III Model 8802A, collected on distilled water and heat stretched (Roberts, 1970) with a Polaron Equipment Ltd. heat pen. After collection on formvar-covered, carbon-coated grids, sections were stained with lead citrate according to Venable and Coggeshall (1965) before examination.

#### b. Negative Staining

Washed cell envelope preparations were examined by the negative staining method of Brenner and Horne (1959) using ammonium molybdate. Samples were diluted with 2% (w/v) ammonium molybdate pH 5.2 to yield a slightly turbid suspension. With a capillary pipette the suspension was

applied to grids covered with carbon-coated formvar and the excess removed using a filter paper point. The grids dried within a few seconds and were examined immediately after preparation on a Philips EM 300 Electron Microscope at operating voltage of 60 kV.

#### 4. Polyacrylamide Disc Gel Electrophoresis

The procedure for electrophoresis in the presence of 1% sodium dodecyl sulphate (SDS) was a modification of the method of Davis (1964). Gels contained 11.7% (w/v) and 7.0% (w/v) acrylamide (B.D.H., Poole) and 0.37% (w/v) NN'-methylenebisacrylamide (bis; B.D.H., Poole). Stacking gels were mechanically strengthened by a modification of the method of De Vito and Santome (1965) omitting EDTA and containing 4% acrylamide and 1% Bis. The gels and buffer system (Appendix 3 ) were made 1% with respect to SDS. The gel tubes 7 x 0.5 cm were run in Tris glycine buffer containing 4 drops of 1% Bromophenol blue as track dye. Electrophoresis was performed at 1 mA per tube until the track dye added to the cathode compartment had migrated to within 1 cm of the bottom of the gel, at which time the current was boosted to 5 mA per tube.

The cell envelope fractions described above were diluted 1:10 and solubilised by sonication at 0°C for 15 min. The samples were made 1% with respect to SDS and, prior to application of 0.1 ml, 3 drops of glycerol were added to prevent mixing. A maximum of 200 µg of protein was applied and in some cases β-mercaptoethanol 1% was added to the sample, as described by Grula and Savoy (1971) to improve the resolution of the bands.

After electrophoresis, gels were fixed and stained overnight in a solution containing 1% (w/v) amido black, 10% (v/v) acetic acid and 50% (v/v) methanol. Gels, prior to electrophoretic destaining, were rehydrated in 7%

(v/v) acetic acid. Rf values were determined by comparison with bromophenol blue. The molecular weights of resolved components were standardised according to the method of Shapiro et al., (1967) using the following marker proteins electrophoresed under identical conditions :-

bovine serum albumin	(Sigma Chemical Company, St. Louis, Missouri, U.S.A.),
molecular weight	= 67,000;
ovalbumin	(Koch-Light, Colnbrook, Bucks.),
molecular weight	= 45,000;
yeast hexokinase	(Sigma Chemical Company, St. Louis, Missouri, U.S.A.),
molecular weight	= 45,000;
bovine pancreatic chymotrypsinogen	(Miles Seravac, Maidenhead, Berks.),
molecular weight	= 25,000;
sperm whale myoglobin	(Koch-Light, Colnbrook, Bucks.),
molecular weight	= 17,800;
and horse heart cytochrome c	(Koch-Light, Colnbrook, Bucks.),
molecular weight	= 12,400.

### C. BIOCHEMICAL ACTIVITIES OF PYOCYANINE

#### 1. Oxygraph measurement of Respiration

##### a. The Biological Oxygen Monitor

The change in concentration of dissolved oxygen in aqueous solution was measured using a polarograph electrode. The diffusion of gas across an

oxygen permeable membrane was recorded, under constant voltage, as a conductivity change in the sensor system. The sensor consisted of a platinum anode and a silver cathode immersed in potassium chloride electrolyte enclosed within the membrane. The sensor was exposed to test solutions in an airtight cuvette surrounded by a thermostatted jacket and provided with a magnetic stirrer. The Biological Oxygen Monitor (Yellow Springs Instrument Co. Inc.) was employed according to the method of Billiar, Knappenberger and Little (1970).

The buffer in which the electrode was immersed and in which enzyme reactions were studied was 50 mM Tris which was established as having a capacity for dissolved oxygen, at the ambient temperature of 37°C, identical to that of Ringer's solution. The latter when fully saturated at 37°C contains 5 µl of dissolved oxygen (Eastabrook, 1967).

As 5 ml volumes were routinely used in the Oxygraph a reduction by half in the dissolved oxygen content of the solution over a period of 1 h represents a rate of consumption of

$$5 \times 5 \times \frac{50}{100} = 12.5 \text{ } \mu\text{l/h.}$$

b. Endogenous Respiration

The rate of oxygen consumption without added substrate was determined as follows. The cells from 1 ml of culture in Resuspension Medium were sedimented (12,000 x g for 20 min at 4°C) and washed 3 times by centrifugation as above, in 10 ml of sterile saline. The packed cells were stored in 50 mM Tris buffer at 4°C for 12 h at 10 times the original concentration, in order to stimulate endogenous respiration (Campbell, Gronlund and Duncan, 1962). Volumes of this stored suspension (0.1 - 0.3 ml)

were transferred to 37°C prior to making up to 5 ml with Tris, equilibrated against air at 37°C, and were placed in the Oxygraph sample chamber. The trace of the decreasing dissolved oxygen content of the solution was recorded over a 20 min period. Where the effect of adding pyocyanine was monitored, the system was first allowed to establish a linear rate of oxygen consumption (10 min) before the addition of pyocyanine (0.1 - 0.3 ml volumes) with a microsyringe. This addition was done without exposing the system to the atmosphere which would interfere with the trace of oxygen consumption. The respiration in the presence of pyocyanine was measured over the linear part of the trace. The Oxygraph gave an almost immediate response to pyocyanine and was linear down to 40% dissolved oxygen.

c. Succino-oxidase Activity

The cell envelope preparation (1 ml volumes) was found to have a negligible rate of respiration when no substrate was provided. The addition of 0.2 M di-sodium succinate solution (0.1 to 0.5 ml) enabled the succino-oxidase enzyme system, demonstrated to be envelope-bound by Campbell et al., (1962b) to change the dissolved oxygen of the suspending buffer. The effect of adding aliquots of pyocyanine to the reaction after stabilisation was tested as described under endogenous respiration. As the Oxygraph was being used to monitor relative rates of oxygen consumption of cell-associated enzymes, the sensor was calibrated with 5 ml volumes of air equilibrated 50 mM Tris at 37°C.

2. Succinate Dehydrogenase Assay

The cell envelope preparations of P. aeruginosa were examined for dehydrogenase activity towards succinate, malate and lactate. Only

succinate dehydrogenase was described by Campbell et al., (1962b) and, in agreement, the other substrates tested here gave no reaction.

The assay procedure was based on the spectrophotometric method of Ellis (1959). The phenazine methosulphate (PMS) served as intermediate electron acceptor at the site of FAD mediated succinate dehydrogenase. As the terminal oxidase was inhibited by added cyanide, the final electron acceptor 2,6-dichlorophenol-indo-phenol (DCIP) is reduced. The change from blue to yellow was monitored as a decrease in the absorbance at 600 nm and was recorded in a Pye Unicam SP 800A spectrophotometer fitted with a 30°C controlled cuvette holder and a slave recorder.

The test cuvette (10 mm pathlength) contained the following reagents :-

0.06 ml of 2.5 mM - DCIP

0.30 ml of 10 mM - KCN

0.15 ml of PMS (3 mg/ml freshly prepared)

0.60 ml of 20 mM di-sodium succinate

(or equivalent mM of alternative)

and 0.1 - 0.2 ml of the envelope suspension.

Tris buffer was added to give a final 3.0 ml volume. All reagents except PMS were present in the blank cuvette so that only the reduction of DCIP via PMS was being measured. The envelope suspension was brought rapidly from 0°C to assay temperature and the reaction was initiated with substrate. Possible effects of pyocyanine were tested by adding it to both reaction and blank cuvettes.

The reaction velocity was measured by  $\Delta E_{600}/\text{min}$  over a linear part which occupied the initial 50% of the reaction. To minimise photo reduction effects the cuvettes were covered with aluminium foil until initiation.

## RESULTS

SECTION I : SYNTHESIS AND IDENTIFICATION OF  
PYOCYANINE AND OTHER PHENAZINES

A.      Yields and Melting Points of Pyocyanine,  
          1-Phenazinol and 1-Methoxyphenazine

Despite the fact that pyocyanine is a relatively simple organic compound it is not commercially available. A probably reason for this is its instability, leading to its partial conversion to 1-phenazinol. The water of crystallisation in pyocyanine promotes this degradation which is not entirely prevented by storage in vacuo over phosphorus pentoxide.

Early in this investigation it seemed desirable to prepare synthetic pyocyanine to act as a reference material. A survey of the literature indicated that the four-stage synthesis starting from 2-hydroxy-3-methoxybenzaldehyde as described by Surrey (1955) was the most suitable. This route had the advantage of providing 1-phenazinol which was also of interest in this investigation. The alternative source of pyocyanine was the degradative route of MacIlwain (1937). His starting material was phenazine methosulphate, the photo-catalysed oxidation of which yields pyocyanine only.

Three separate batches of pyocyanine were prepared by the method of Surrey, except that the preliminary step of oxidation of 2-hydroxy-3-methoxybenzaldehyde to pryogallol 1-monomethyl ether was not monitored for yield. Constancy of boiling point during distillation under reduced pressure (136-138°C/22 mm) was used as the criterion of purity as reported by Surrey. The three steps leading to phenazine products are summarised in the following sequence of reactions :

- 1) Condensation of pyrogallol 1-monomethyl ether with o-phenylene diamine to yield 1-methoxyphenazine;
- 2) Demethylation of 1-methoxyphenazine with hydrobromic acid to give 1-phenazinol;
- 3) Methylation of 1-phenazinol with methyl sulphate to yield pyocyanine.

A slight modification was introduced into the scheme in that 1-methoxyphenazine was recrystallised from ethanol and not pyridine as described by Surrey.

Table V shows that the yields of intermediates varied at different reaction steps and that the principal losses occurred in the preparation of 1-methoxyphenazine. These latter losses occurred mainly during the isolation of the product from the reaction mixture. The yields obtained were similar to the published values. Table V also shows the melting points of the compounds (after three recrystallisations) and a comparison with the literature values.

#### B. Chromatography of synthesized compounds

The synthesized compounds were examined for purity by thin layer chromatography on Silica Gel G using chloroform-methanol (9:1) as solvent. The compounds were resolved as homogeneous spots and the results will be described in a later section (page 95). The  $R_f$  values of pyocyanine, 1-phenazinol and 1-methoxyphenazine were respectively 0.38, 0.64 and 0.58 and in a mixture of the three compounds, the separated components were identified by their colour as described in Table V.

Table V

Yields and properties of synthesized Pyocyanine and other Phenazines

	Pyocyanine	1-Phenazinol	1-Methoxyphenazine
Appearance	Dark blue needles	Orange solid	Yellow needles
Melting Point (°C)	125-126 (133) <sup>a</sup> (133) <sup>b</sup> (124-128) <sup>c</sup>	152-153 (154) <sup>b</sup>	169-170 (168) <sup>b</sup>
Yield <sup>‡</sup>	51 (58) <sup>a</sup>	75 (70) <sup>a</sup>	25 (33) <sup>a</sup>

‡ Yield (mole %) from immediate precursor

The values in brackets are those reported by

(a) McIlwain (1937)

(b) Surrey (1955)

(c) Corbett (1964) using the route described by Surrey (1955)

C. Ultra-violet and visible spectrophotometric  
characterisation of synthesized compounds

Having obtained pure preparations of pyocyanine, 1-phenazinol and 1-methoxyphenazine all three compounds were examined spectrophotometrically.

First it should be stated that the absorbance characteristics of pyocyanine and its phenazine precursors have been investigated in a variety of solvent systems by numerous workers. Table VIa and VIb present the reported wavelengths of maximal absorbance ( $\lambda_{\max}$ , nm) and their associated molar extinction coefficients ( $\epsilon$ ,  $\text{l.mole}^{-1} \text{ cm}^{-1}$ ) expressed as  $\log \epsilon$  of pyocyanine obtained either by extraction of *P. aeruginosa* cultures or by synthesis.

The solvent chosen in this investigation was 50 mM Tris (pH 8.5) although 0.1M HCl and 0.1M NaOH were also used. Emphasis was given to characterising the compounds in Tris because this buffer was used in the culture media for growth and pyocyanine production by *P. aeruginosa*. The absorption spectra from 200 to 700 nm in Tris, HCl and NaOH are presented in the series of figures 6a to 6f. The blank in each instance was a cuvette containing solvent.

Figure 6a shows the ultra-violet and visible spectrum of pyocyanine (0.38 mM). Note that the  $\lambda_{\max}$  values in the ultra-violet are at 238 nm, spectroscopically designated the  $\beta$  band, and at 312 nm and 375 nm, termed p band absorptions. The band designations refer to the type of electron transition that is responsible for the absorption. The associated molar extinction coefficients are 17,800, 25,200 and 5,900 respectively.

The visible spectrum, arising through an  $n-\pi^*$  type transition is

characterised by a broad absorbance with  $\lambda_{\max}$  at 690 nm,  $\epsilon = 3,300$ . For comparison the spectrum of 0.45 mM pyocyanine in acid solution (0.1M HCl) is shown in Figure 6b . The  $\beta$  band at 240 nm,  $\epsilon = 12,600$  is not changed in position although its intensity is reduced. However it tends to be masked by one of the p band peaks  $\lambda_{\max}$  280 nm,  $\epsilon = 42,000$ , which has shifted to shorter wavelength and is increased in intensity. The other p band peak,  $\lambda_{\max}$  387 nm,  $\epsilon = 17,800$  shows less shift but its extinction is almost three times that in alkali. The visible absorbance undergoes a red shift to  $\lambda_{\max}$  520 nm and is reduced in intensity  $\epsilon = 2,200$ .

The spectra under acid and alkaline conditions show close correspondence with those reported by other workers (Table VIa ). The use of Tris does not lead to differences in the  $\lambda_{\max}$  or  $\epsilon$  values from those reported under other alkaline conditions. The range of molar extinction coefficients for the  $n-\pi^*$  band is 3,100 to 4,000. This variation may be due to differences in purity of the natural and synthetic products.

Turning now to l-phenazinol, Figure 6e records the ultra-violet and visible  $\lambda_{\max}$  values of 0.21 mM l-phenazinol in 50 mM Tris. The  $\beta$  band with  $\lambda_{\max}$  260 nm,  $\epsilon = 33,000$  is the largest absorbance. The p band absorbance occurs as a complex series of peaks, the main  $\lambda_{\max}$  being at 370 nm,  $\epsilon = 5,950$ . This peak has shoulders at 350 nm and 340 nm. The pale yellow colour of l-phenazinol in Tris corresponds to a visible  $\lambda_{\max}$  at 420 nm,  $\epsilon = 2,200$ .

The spectrum of 0.22 mM l-phenazinol in 0.1M NaOH containing 50% methanol is that of the anionic form of the molecule (Figure 6c ). The  $\beta$  band occurs with  $\lambda_{\max}$  at 295 nm,  $\epsilon = 41,000$  which is a shift to longer wavelength of 35 nm from its position in Tris. However the p band peaks are found in the same position as those in Tris with  $\lambda_{\max}$  at 364 nm and

375 nm. Their intensity,  $\epsilon = 3,040$  and  $\epsilon = 3,160$  respectively is a reduction to almost 50% of their former level. The red, anionic form of 1-phenazinol has a broad visible  $n-\pi^*$  band with  $\lambda_{\max}$  at 520 nm,  $\epsilon = 2,300$ . Although the colour changes from yellow to red with increasing pH, the strength of the absorption is not affected.

In 0.1M HCl/50% methanol, 0.1 mM 1-phenazinol has absorption peaks in the ultra-violet region which correspond closely to those observed in Tris. The major peak has  $\lambda_{\max}$  at 268 nm,  $\epsilon = 40,000$ . The complex p band absorption peaks occur as one major  $\lambda_{\max}$  at 385 nm,  $\epsilon = 16,000$  with a shoulder at 375 nm,  $\epsilon = 11,000$ . These peaks are more intense than the p band peaks in other solvents. The 1-phenazinol is ionised in strong acid solution to form a cation and a similar shift of visible absorption, as observed in strong alkali towards the red, is found. The  $n-\pi^*$  broad absorption has a  $\lambda_{\max}$  at 505 nm,  $\epsilon = 1,800$  (cf  $\lambda_{\max}$  420 nm in Tris).

When the absorption spectra of 1-phenazinol in strong acid and in strong alkali are compared with those reported in the literature, close agreement is found. The spectrum in Tris pH 8.5 is significantly different from that in the above solvents. The  $\lambda_{\max}$  values and molar extinction coefficients are very similar to those reported by Corbett where methanol was used as solvent (see Table VIb ). The characteristic peaks observed in both methanol and Tris are :

$\beta$  band,  $\lambda_{\max}$  260 nm

p band,  $\lambda_{\max}$  370 nm and

$n-\pi^*$  band,  $\lambda_{\max}$  420 nm corresponding to a yellow coloured solution.

Table VIa

Comparison of experimental and reported wavelengths of maximum absorption ( $\lambda_{\max}$  nm) and molar extinction coefficients ( $\epsilon$  l.mole<sup>-1</sup> cm<sup>-1</sup>) for Pyocyanine

Author	Sample	Solvent	$\lambda_{\max}$ nm. ; (log $\epsilon$ )	$\beta$	p	n- $\pi^*$
	Origin	pH	Spectrophotometric band designation			
Present investigation	Synthetic	50m M Tris pH 8-9	314(4.40) 375(3.77)	238(4.25)		690(3.52)
	Synthetic	0.1M HCl pH 1.0	280(4.62) 387(4.25)	240(4.10)		520(3.34)
Kurachi (1958b)	Biosynthetic	aqueous alkali aq. acid	312(4.5) 379(3.9) 278(4.6) 387(4.3)	238(4.3) 242(4.1)		690(3.61) 520(3.30)
Frank and DeMoss (1959)	Biosynthetic	2M Tris	- - -	-	-	690(3.63)
MacDonald (1963)	Synthetic	0.1M HCl	- - -	-	-	520(3.36)
Corbett (1964)	Synthetic	Methanol 0.1M HCl/50% MeOH	325 367 293 387	-	-	740(-) 530(-)
Zuagg (1964)	Synthetic	aq.alkali pH 7-8 aq. acid pH 2.5	312 379 287 387(4.43)	239 246		690(-) 525(3.70)
MacDonald (1966)	Biosynthetic	CHCl <sub>3</sub>	- - -	-	-	695(-)
Chang and Blackwood (1968)	Synthetic	0.2M HCl pH 1.5	279 390	208		-
Ingladew and Campbell (1969)	Synthetic	aq.alkali pH 7.7	- - -	-	-	690(3.52)
Azuma and Witter (1970)	Synthetic & Biosynthetic	aq.alkali	- - -	-	-	690(3.63)

$\beta$ , p and n- $\pi^*$  relate the type of electron transition to bonding and symmetry in the molecule.

Dash (-) denotes not reported.

Table VIb

Comparison of experimental and reported wavelengths of maximum absorption ( $\lambda_{\max}$ , nm) and molar extinction coefficients ( $\epsilon$ , l. mole<sup>-1</sup> cm<sup>-1</sup>) for 1-Phenazinol and 1-Methoxyphenazine

Author	Sample	Solvent	$\beta$	$\lambda_{\max}$ nm. ( $\log \epsilon$ )	n- $\pi^*$
	Origin	pH		Spectrophotometric band designation	
1-Phenazinol	Present investigation	50mM Tris pH 8.5	260(4.52)	350 <sup>S</sup> 360 <sup>S</sup> 370(3.77)	420(3.34)
		0.1M NaOH/50% MeOH pH 11.0	238(4.30)295(4.61)	364 <sup>S</sup> 375(3.50)	520(3.36)
		0.1M HCl/50% MeOH pH 1.0	268(4.60)	375 <sup>S</sup> 385(4.20)	505(3.2)
1-Methoxyphenazine	Present investigation	aq. alkali pH 10.0	260	350 410	510(-)
		50mM Tris pH 8.5	260(4.65)	343 <sup>S</sup> 353 <sup>S</sup> 362 369(3.9)	425(3.36)
		0.1M HCl/50% MeOH pH 1.0	274(4.6)	375 <sup>S</sup> (4.0)385(4.2)	505(3.2)
1-Methoxyphenazine	Present investigation	0.1M NaOH/50% MeOH pH 11.0	295(4.65)	364 <sup>S</sup> 375(3.5)	530(3.35)
		50mM Tris pH 8.5	260(4.65)	350 <sup>S</sup> 358 <sup>S</sup> 368 <sup>S</sup> (3.86)	410(3.28)
		MeOH	261(4.7)	350 360 366(3.9)	410(3.4)
1-Methoxyphenazine	Corbett (1964)	0.1M HCl 50% MeOH pH 1.0	268	372 383	437(3.2) 481 <sup>S</sup>

S, Absorption observed as shoulder on more intense peak



Figure 6a: Ultra-violet and visible spectrum of Pyocyanine

(80  $\mu\text{g/ml}$ ) in 50 mM Tris, pH 8.5;

(a) 10 mm path

(b) 2 mm path

(c) 2 mm path

Figure 6b: Ultra-violet and visible spectrum of Pyocyanine

(95  $\mu\text{g/ml}$ ) in 0.1M HCl/50% MeOH;

(d) 2 mm path

(e) 2 mm path, 1:2 dilution

Figure 6

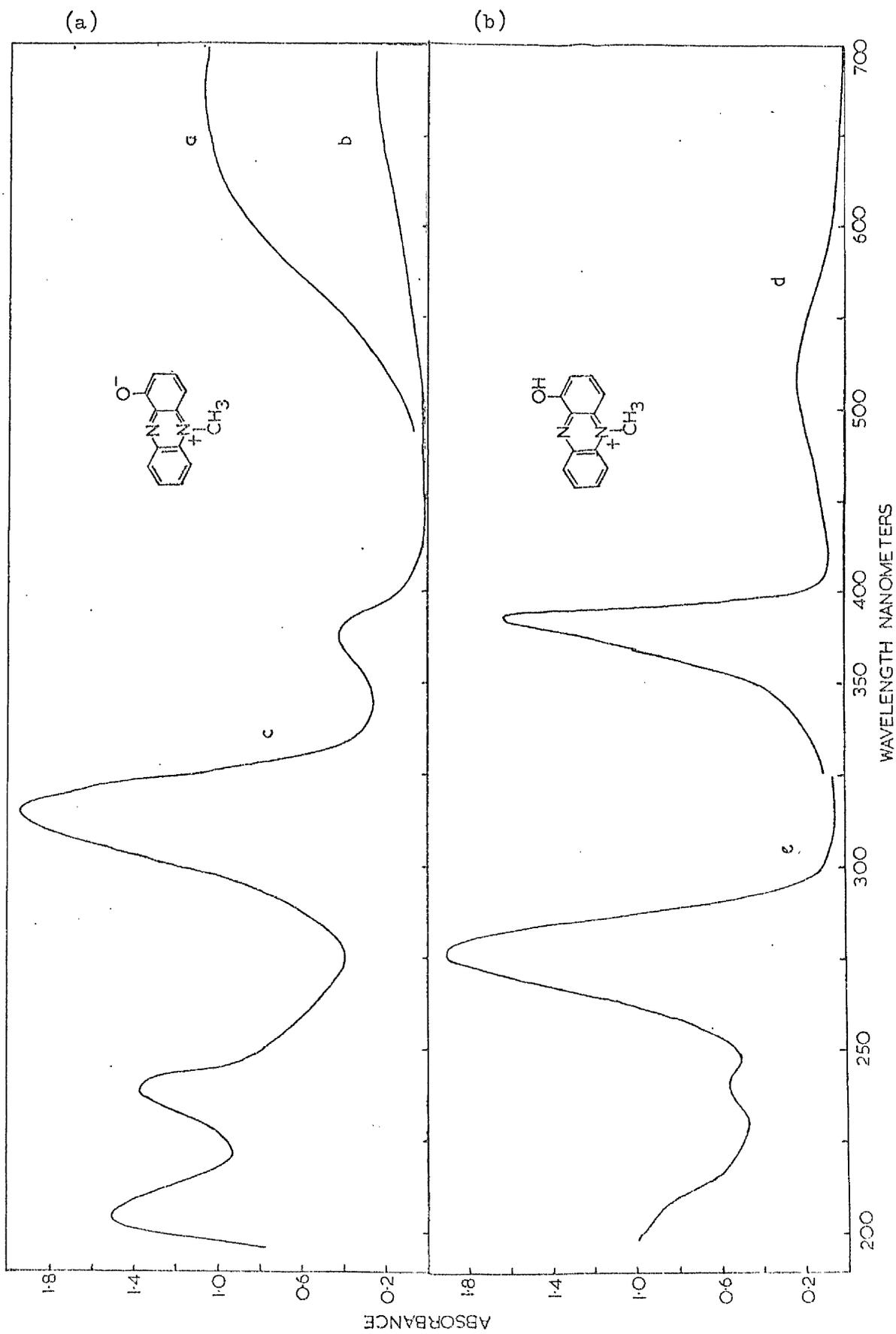




Figure 6c: Ultra-violet and visible spectrum of 1-Phenazinol  
(42  $\mu\text{g/ml}$ ) in 0.1M NaOH/50% MeOH;

(a) 10 mm path

(b) 2 mm path

Figure 6d: Ultra-violet and visible spectrum of 1-Phenazinol  
(20  $\mu\text{g/ml}$ ) in 0.1M HCl/50% MeOH;

(c) 10 mm path

(d) 2 mm path

Figure 6

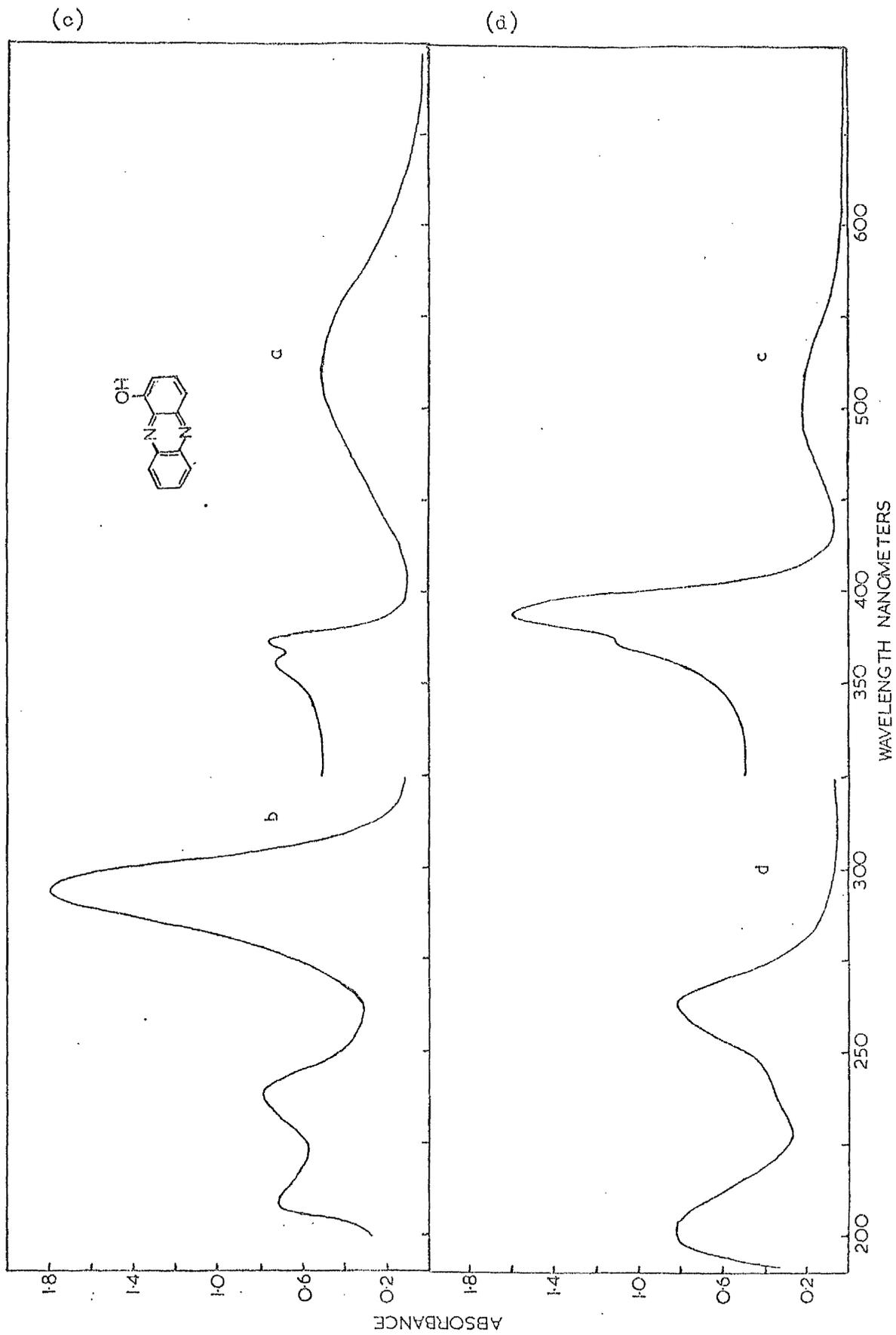
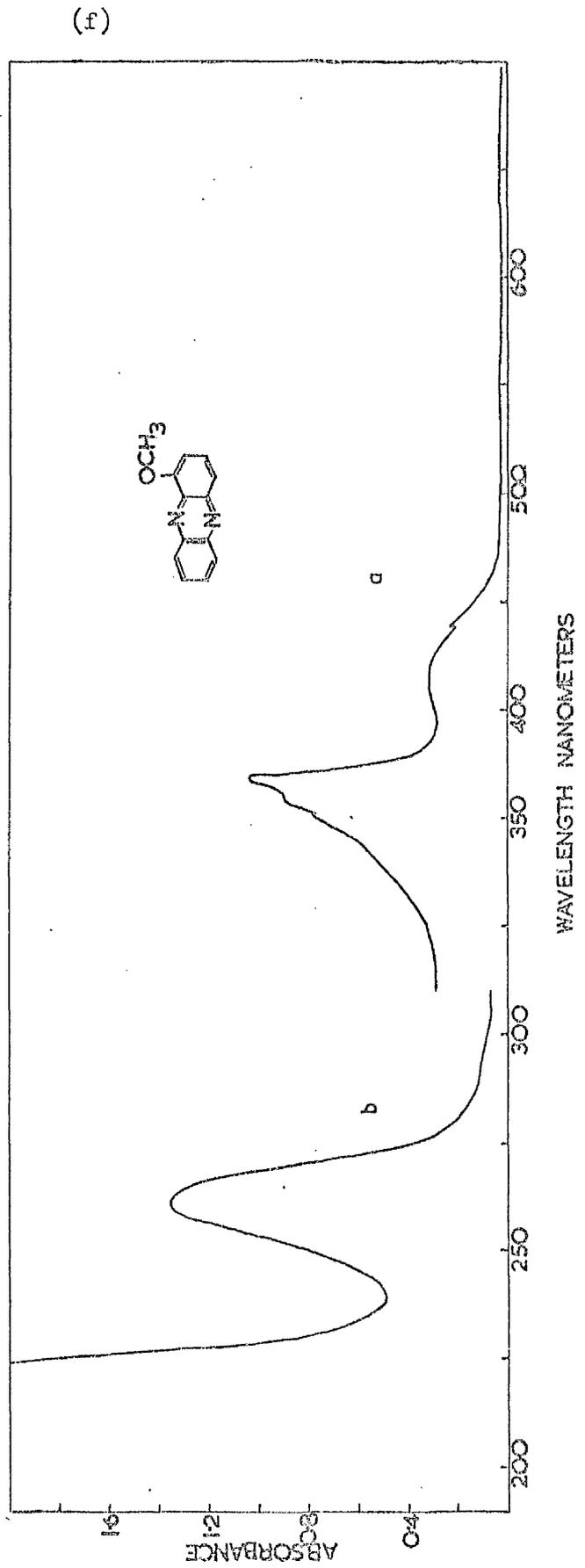
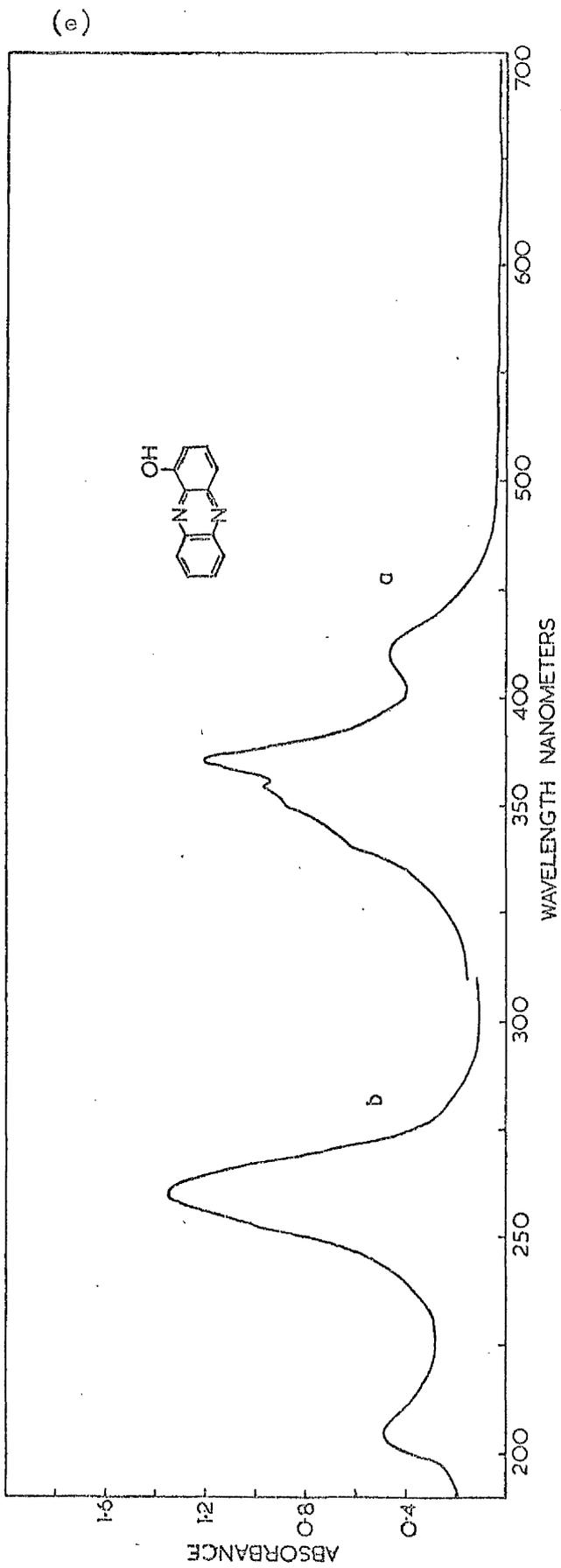




Figure 6e: Ultra-violet and visible spectrum of 1-Phenazinol  
(40  $\mu\text{g}/\text{ml}$ ) in 50 mM Tris, pH 8.5;  
(a) 10 mm path  
(b) 10 mm path, 1:2 dilution

Figure 6f: Ultra-violet and visible spectrum of 1-Methoxyphenazine  
(38  $\mu\text{g}/\text{ml}$ ) in 50 mM Tris, pH 8.5;  
(a) 10 mm path  
(b) 2 mm path

Figure 6



As there is no evidence for the occurrence of 1-methoxyphenazine in P. aeruginosa cultures, this spectrum was not investigated in such detail. Figure 6f shows the absorption peaks of a 0.16 mM 1-methoxyphenazine solution in Tris at pH 8.5. The most intense peak, the  $\beta$  band absorption, has  $\lambda$  max at 260 nm,  $\epsilon = 44,000$ . The complex series of peaks with  $\lambda$  max 368 nm,  $\epsilon = 7,200$  and two shoulders with  $\lambda$  max 368 nm and 350 nm,  $\epsilon = 6,400$  and 5,500 respectively give an ultra-violet spectrum which is similar to that of 1-phenazinol. The visible absorbance has a shift to shorter wavelength giving  $\lambda$  max at 410 nm, although the extinction of the peak is similar to that of 1-phenazinol in Tris.

The 1-methoxyphenazine spectrum in Tris is closely similar to that reported by Corbett using methanol as solvent.

#### D. Infra red absorption spectra of synthesized compounds

As an additional criterion for the purity of synthesized pyocyanine and other phenazines the infra-red spectra from 4000 to 625  $\text{cm}^{-1}$  were recorded using the KBr disc method. This method allowed comparison of the spectral characteristics reported for purified preparations by Corbett (1964).

The infra-red spectrum of pyocyanine is shown in Figure 7a. The region between 4000  $\text{cm}^{-1}$  and 3000  $\text{cm}^{-1}$  is characterised by a sharp peak at 3500  $\text{cm}^{-1}$  and a closely situated broad peak at 3400  $\text{cm}^{-1}$ . Where additional information was required the spectrum was recorded in a solvent which was transparent in the wavenumber region of interest. For example, the spectrum of pyocyanine in carbon tetrachloride demonstrated that the two peaks mentioned above were absent in solution. They correspond to the broad slope



Figure 7a: Infra-red absorption spectrum of Pyocyanine;

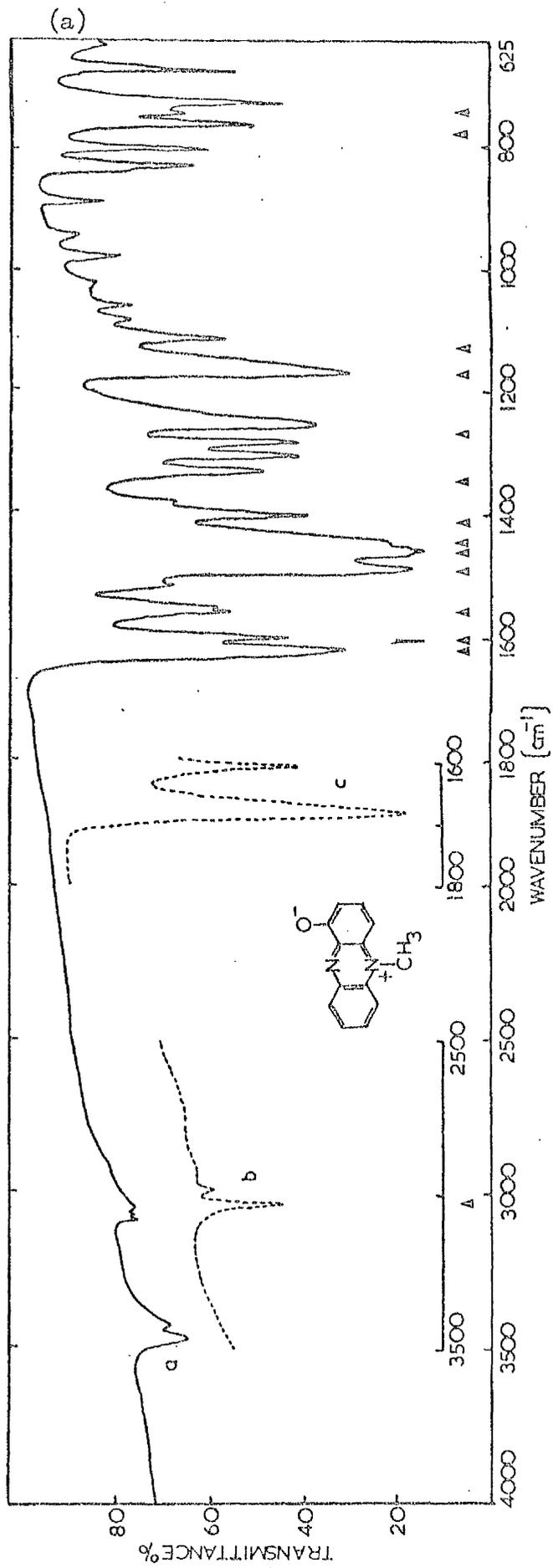
(a) KBr disc, 1 mg/3 mg

(b) 3500-2500  $\text{cm}^{-1}$ , 5 mg/ml in  $\text{CCl}_4$ , 1.0 mm path

(c) 1800-1600  $\text{cm}^{-1}$ , 2 mg/ml in  $\text{CHCl}_3$  1.0 mm

path offset

Figure 1



observed in the solution spectrum over the  $4000\text{ cm}^{-1}$  --  $3500\text{ cm}^{-1}$  region and are due to moisture of crystallisation in the sample.

Both the solid and carbon tetrachloride solution spectra show peaks at  $3050\text{ cm}^{-1}$  and  $2950\text{ cm}^{-1}$ , those in the liquid being much more distinct. Peaks in this region correspond to C - H stretching of aromatic and aliphatic type respectively.

Below  $1650\text{ cm}^{-1}$  is the fingerprint region of the spectrum, a characteristic of the molecule. Major peaks were noted at 1620, 1600, 1553, 1485, 1455, 1440, 1160, 760 and  $736\text{ cm}^{-1}$ .

The offset part of the spectrum at  $1700\text{ cm}^{-1}$  shows the absorptions of pyocyanine in chloroform solution. The form of the molecule in solution gives a strong peak at  $1670\text{ cm}^{-1}$  and a smaller one at  $1650\text{ cm}^{-1}$ .

For ease of comparison the absorption peaks in the region  $3050\text{ cm}^{-1}$  to  $625\text{ cm}^{-1}$ , investigated as a KBr disc preparation by Corbett, which correspond with those found in this investigation are arrowed in the spectrum. The major peaks quoted by Corbett with his assignment of vibration type are presented in Table VIIa and close agreement is observed.

The spectrum of l-phenazinol, given in Figure 7b is separable into two regions : those absorptions occurring above  $3000\text{ cm}^{-1}$ , and those in the fingerprint region between  $1650\text{ cm}^{-1}$  and  $625\text{ cm}^{-1}$ .

The spectrum of the KBr disc sample has a broad absorption between  $3400$  and  $3100\text{ cm}^{-1}$ . This corresponds to the region of (O - H) stretching and in this case it almost completely masks the aromatic C - H absorption at  $3020\text{ cm}^{-1}$ . Using tetrachlorethylene as solvent the l-phenazinol solution

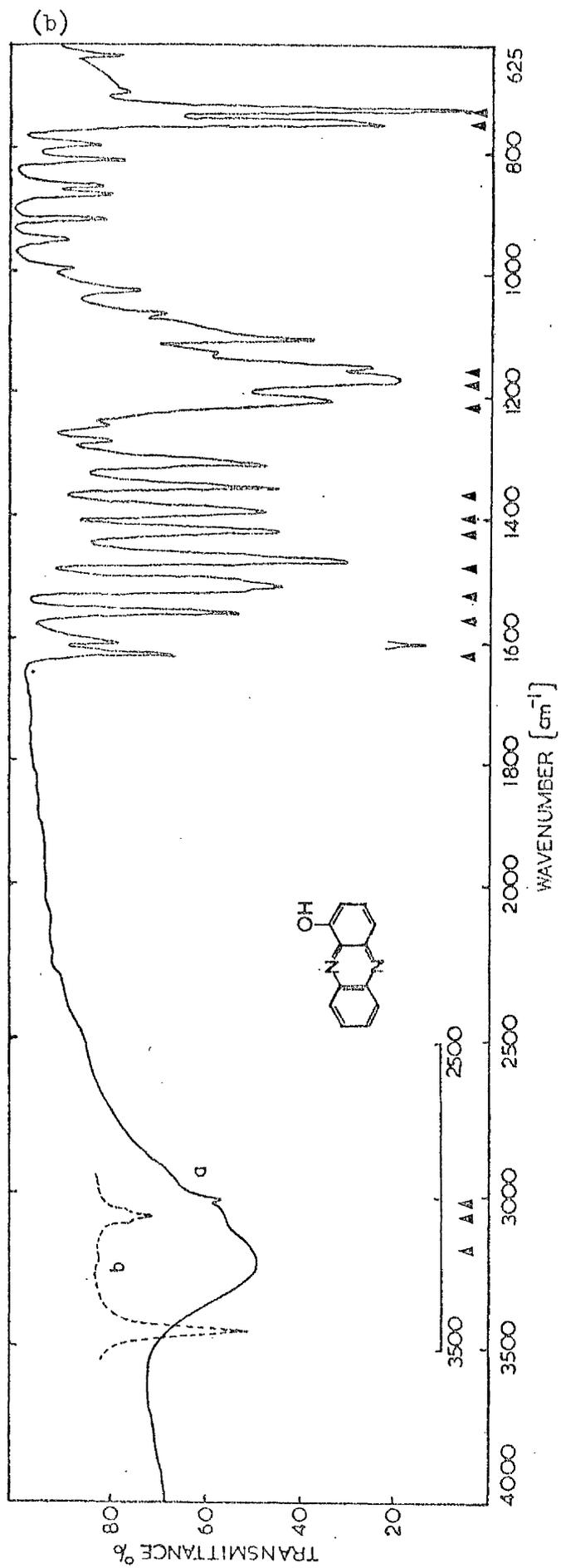


Figure 7b: Infra-red absorption spectrum of 1-Phenazolinol;

(a) KBr disc, 1 mg/3 mg

(b) 3600-2800  $\text{cm}^{-1}$ , 1 mg/ml in  $\text{C}_2\text{Cl}_4$

Figure 7



spectrum, given as an inset between 3500 and 3000  $\text{cm}^{-1}$  shows that the broad absorption is sharpened to a peak at 3400  $\text{cm}^{-1}$ . The absorption at 3060  $\text{cm}^{-1}$  is more clearly resolved and observed to be a set of three closely situated peaks.

The fingerprint region of the spectrum has major absorptions which occur at the following wavenumber values ( $\text{cm}^{-1}$ ): 1623, 1555, 1510, 1470, 1420, 1390, 1350, 1300, 1220, 1170, 1160, 1100, 759 and 736. For the KBr disc preparation the absorptions according to Corbett are presented in Table VIIb and the close correlation observed with those in spectrum is illustrated by the arrowed peaks.

Turning to *l*-methoxyphenazine the solid state spectrum (KBr disc) and solution spectrum in tetrachlorethylene are presented in Figure 7c. Absorptions above 1650  $\text{cm}^{-1}$  occur only in the region 3100  $\text{cm}^{-1}$  to 2800  $\text{cm}^{-1}$  and are observed in the solid phase at wavenumbers ( $\text{cm}^{-1}$ ) 3080, 3040, 3000, 2970, 2950 and 2860. The solution spectrum was recorded to monitor for the possibility of absorptions above these values which were not observed in the solid phase. None was found. The wavenumbers ( $\text{cm}^{-1}$ ) of the aromatic C - H vibration type are 3100, 3040 and 3000 with aliphatic C - H peaks at 2980, 2930 and 2880  $\text{cm}^{-1}$ . The solid state spectrum of *l*-methoxyphenazine has peaks which are in close agreement with those reported by Corbett. The literature values corresponding to those observed in the present investigation are arrowed in the spectrum shown in Figure 7c. The wavenumber values ( $\text{cm}^{-1}$ ) for absorptions below 1650  $\text{cm}^{-1}$  are as follows: 1623, 1603, 1550, 1520, 1480, 1460, 1350, 1290, 1280, 1230, 1210, 1132, 1098, 760, 745 (doublet) and 710.

It will be noted that by selecting the distinct infra-red features



Figure 7c: Infra-red absorption spectrum of 1-Methoxyphenazine;

(a) KBr disc, 1 mg/3 mg

(b) 3500--2800  $\text{cm}^{-1}$ , 2 mg/ml in  $\text{C}_2\text{Cl}_4$

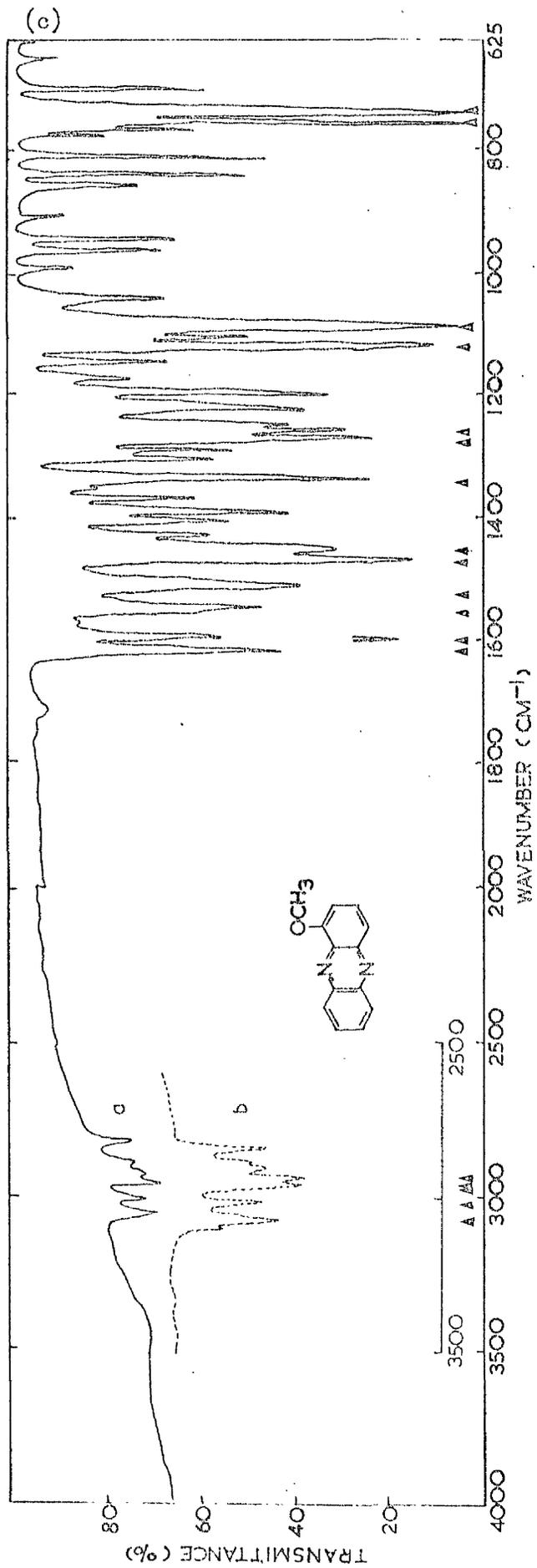


Table VIIa

Characteristic infra-red absorption frequencies of Pyocyanine (from Corbett, 1964)

Mode	Frequency ( $\text{cm}^{-1}$ )
General phenazine ring (aromatic)	3100-3000
Skeletal	1610-1580 ; 1510 ; 1465 ; 1460 ; 1355.
	1621 ; 1548 ; 1320
In plane	1200-950
Out of plane - 4 adjacent	749 ; 740
- 3 adjacent	760
Specific	1630
Fingerprint	1622(S) 1598(S) 1553(S) 1488(S) 1454(S)
	1440(M) 1407(M) 1338(M) 1249(S) 1158(S)
	1105(M)
	760(S) 736(S)

Intensity of peaks in fingerprint region are designated strong (S) or medium (M).

Table VIIb

Characteristic infra-red absorption frequencies of 1-Phenazinol and 1-Methoxyphenazine (from Corbett, 1964)

Mode	Frequency (cm <sup>-1</sup> )
1-Phenazinol	
Lactam : O-H O stretch (Intermolecular bonding solid)	3500-3200 (BROAD)
O-H N stretch (Intramolecular bonding in liquid)	3450
Lactam : N-H stretch	3100
C-O stretch	1220-1150
C-O stretch	1220-1150
Fingerprint : skeletal vibrations	1623(S) ; 1554(M) ; 1510(W) ; 1470(S) ; 1423(M) 1390(S) ; 1350(M) ; 1221(M) ; 1174(M) ; 1160(M)
C-H bending	759(S) ; 736(S)
1-Methoxyphenazine	
Methoxyl : C-H stretch	2980 ; 2920
C-O stretch	1220-1190
Fingerprint : skeletal vibrations	1623(M) ; 1603(M) ; 1554(M) ; 1520(M) ; 1480(S) ; 1458(M) ; 1438(M) ; 1418(M) ; 1349(S) ; 1280(S) ; 1290(S) ; 1236(S) ; 1210(S) ; 1132(S) ; 1098(S) ; 764(S) ; 748(M) ; 742(M) ; 710(M) .
C-H bending	

Intensity of peaks in the fingerprint region are designated strong (S) or medium (M)

of pyocyanine, 1-phenazinol and 1-methoxyphenazine it is possible to confirm the identity of individual samples. Levels of pyocyanine contamination with 1-phenazinol of the order of 10% could be detected in solution spectra at  $3400\text{ cm}^{-1}$ . This aspect is useful for confirming the purity of pyocyanine preparations.

## SECTION II : THE EFFECT OF CULTURAL CONDITIONS

### UPON PYOCYANINE PRODUCTION

#### A. Pyocyanine production by P. aeruginosa using solid and liquid media

During the early part of this investigation P. aeruginosa was grown on nutrient agar or peptone agar, both being media in which readily visible pyocyanine production occurs. Cells harvested from these media were not, however, suitable for the metabolism experiments described in a later section (see page 159) because of their high rate of endogenous respiratory activity. This activity depends upon the utilisation of intracellular reserve material identified as protein and RNA by Gronlund and Campbell (1963). In an attempt to reduce this the cells were grown on a simple defined agar medium containing glucose and ammonium sulphate (Appendix 1 ). Such cells had a reduced rate of endogenous respiration as hoped for, but unexpectedly it was noticed that pyocyanine was not produced by this culture. This was not due to lack of growth which, in fact, was just as abundant on the glucose-ammonium sulphate as on the peptone agar.

To explore these effects more fully it seemed desirable to change from solid to liquid media and initially the Yeast-Tryptone medium (Y-T) of von Tigerstrom and Razzell (1968; Appendix 1. ) was chosen. Cell growth

in Y-T medium invariably gave high opacity cultures (O.D. 490 of between 3.8 and 4.2) but a problem was encountered in consistency of pyocyanine production. For example, early subcultures of newly opened ampoules of NCTC 6750 produced pyocyanine but later subcultures did not. Even early subcultures gave variable yields of the pigment for no obvious reason. A typical series of manipulations and the variable results obtained is illustrated in Figure 8. It will be noted that the amount of pyocyanine, as judged by the colour of the oxidised supernatants, was affected by the length of storage of the stock culture, inoculum size and other unidentified factors.

After these and other unsuccessful attempts to obtain regularity of pyocyanine production in Y-T medium the difficulties were overcome by growing the cultures in two stages as described in a contemporary paper (1969a) by Ingledew and Campbell.

B. Pyocyanine production and determination  
in Resuspension Medium

1. The Resuspension Medium

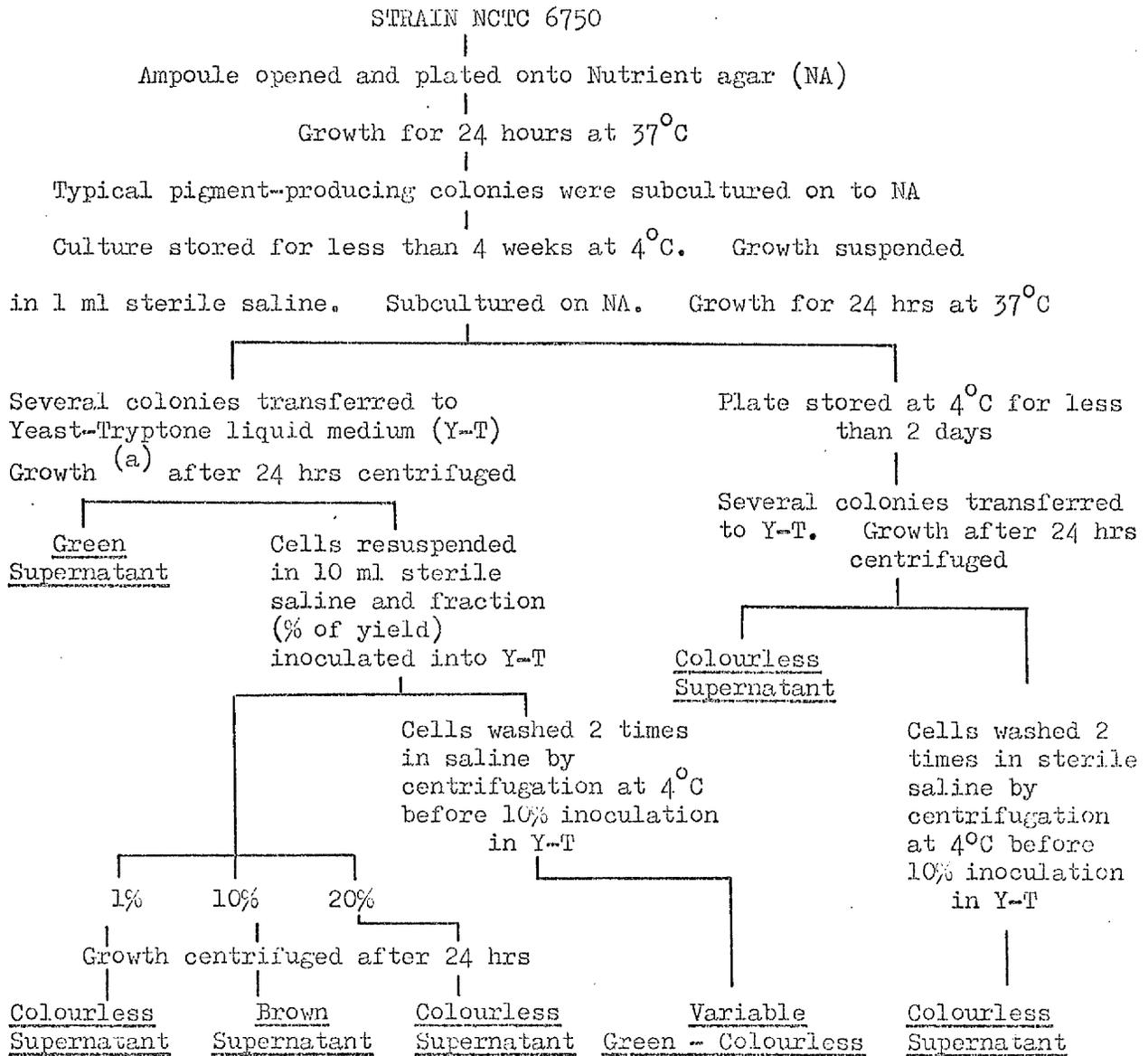
The technique of Ingledew was found to give satisfactorily consistent yields of pyocyanine in liquid medium. The two stage procedure which was evolved for strain ATCC 9027 consists in

- (a) preparation of washed cells from a culture grown to the stationary phase in Y-T medium
- (b) incubating these cells in Resuspension Medium - a defined medium notable for its total lack of phosphate.

Ingledew's technique was therefore applied to the P. aeruginosa

Figure 8

Flow diagram of a typical series of manipulations leading to variation  
in Pyocyanine production



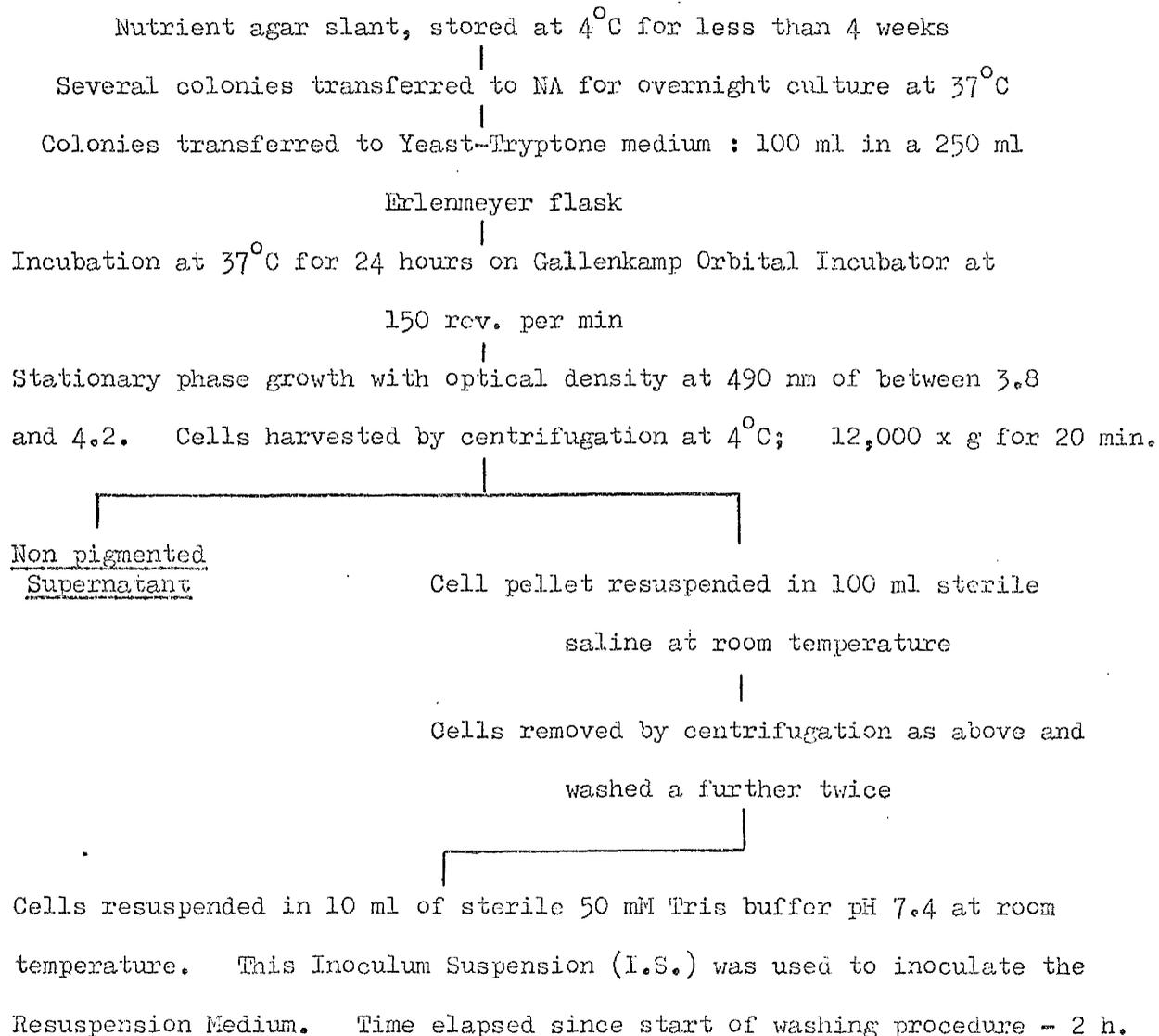
Cells plated on NA. Growth of isolated colonies after 24 hours exhibited characteristic diffusible green pigment.

strain used in this investigation, NCTC 6750, to determine its suitability. However one difficulty was the fact that Resuspension Medium contained 2-ketogluconate which was not commercially available. The initial attempts to reproduce Ingledew's conditions were done with succinate as an alternative carbon source. The formula of this Resuspension Medium is given in Table XI. Figure 9 presents the culture technique for obtaining the Inoculum Suspension from the non-pigment producing, stationary phase Y-T cells. Before describing the results obtained with this medium, the method of measuring pyocyanine in culture supernatants had to be investigated and it is therefore discussed next.

## 2. Choice of method for measuring Pyocyanine in culture supernatants

The determination of pyocyanine concentration present in Resuspension Medium supernatants was made by two methods. The first was based on the exhaustive extraction procedure used by Ingledew and described earlier (page 49). The chloroform extracts of supernatants were re-extracted with 0.1M HCl to leave any contaminating 1-phenazinol in the organic phase. Pyocyanine was recognised as a characteristic red colour in aqueous acid solution. Neutralisation with 1M Tris was followed by chloroform extraction. The blue pyocyanine solution was evaporated to dryness in air and the crystalline pigment estimated gravimetrically.

The second method was based on the more rapid spectrophotometric determination of Kurachi (1958b). The extinction of pyocyanine at the visible  $\lambda$  max of 690 nm ( $E_{690}$ ) gave reliable estimates for fully oxidised, optically transparent culture supernatants.  $E_{690}$  values were calibrated against standard solutions of synthesized pyocyanine. Estimations by this method will be discussed more fully in a later section (see page 90).

Figure 9Flow diagram for the preparation of the inoculum for ResuspensionMedium

The gravimetric method gave estimates for pyocyanine that were within the range  $90\% \pm 5\%$  of the spectrophotometrically-determined value.

3. Consistency of observation of Pyocyanine in Standard Resuspension Medium Supernatant

The yield of pyocyanine reported by Ingledew for duplicate flasks was  $95 \mu\text{g/ml}$  ( $\pm 3\%$ ). The use of a different strain of *P. aeruginosa* and the substitution of disodium succinate for 2-ketogluconate made necessary the manipulation of conditions which will be described later (see page 99). The optimum conditions gave a so-called 'Standard Supernatant' and reliable pyocyanine production. The range of pyocyanine quantities experienced in several experiments are given in Table VIII. The mean of  $80 \mu\text{g/ml}$  with a range for duplicate flasks of  $\pm 5\%$  are in close agreement with the results reported by Ingledew. The 'greater variation in different experiments' described by Ingledew was found in this investigation to give yields within a  $\pm 15\%$  range of the mean.

4. Ultra-violet and visible absorption spectra of Standard Supernatant

The ultra-violet and visible spectrum of the optically clear and fully oxidised supernatant is presented in Figure 10b with that of pure pyocyanine at an equivalent concentration in Tris in Figure 10a for comparison. The quantity of pyocyanine present in this Standard Supernatant was  $70 \mu\text{g/ml}$  ( $\pm 4$ ) and this was used for calculating extinction coefficients.  $E_{690}$  of 1.1 corresponds to  $70 \mu\text{g/ml}$ . Therefore  $210 \text{ mg/ml}$  or  $1\text{M}$  Pyocyanine has  $E_{690} = 3300$ .

It will be noted that the  $\lambda_{\text{max}}$  values in the supernatant spectrum :-

TABLE VIII

Range of Pyocyanine levels estimated in "Standard Supernatant" by  $E_{690}$

Experiment	Flask	
	a	b
1	1.22	1.34
2	1.36	1.42
3	1.28	1.40
4	1.12	1.22

Estimates for duplicate flasks fall within the range  $\pm 5\%$

Levels produced in different experiments fall within range  $\pm 15\%$



Figure 10a: Ultra-violet and visible spectrum of Pyocyanine

(70  $\mu\text{g/ml}$ ) in 50 mM Tris, pH 8.5;

(a) 10 mm path

(b) 2 mm path

(c) 2 mm path

Figure 10b: Ultra-violet and visible spectrum of Standard

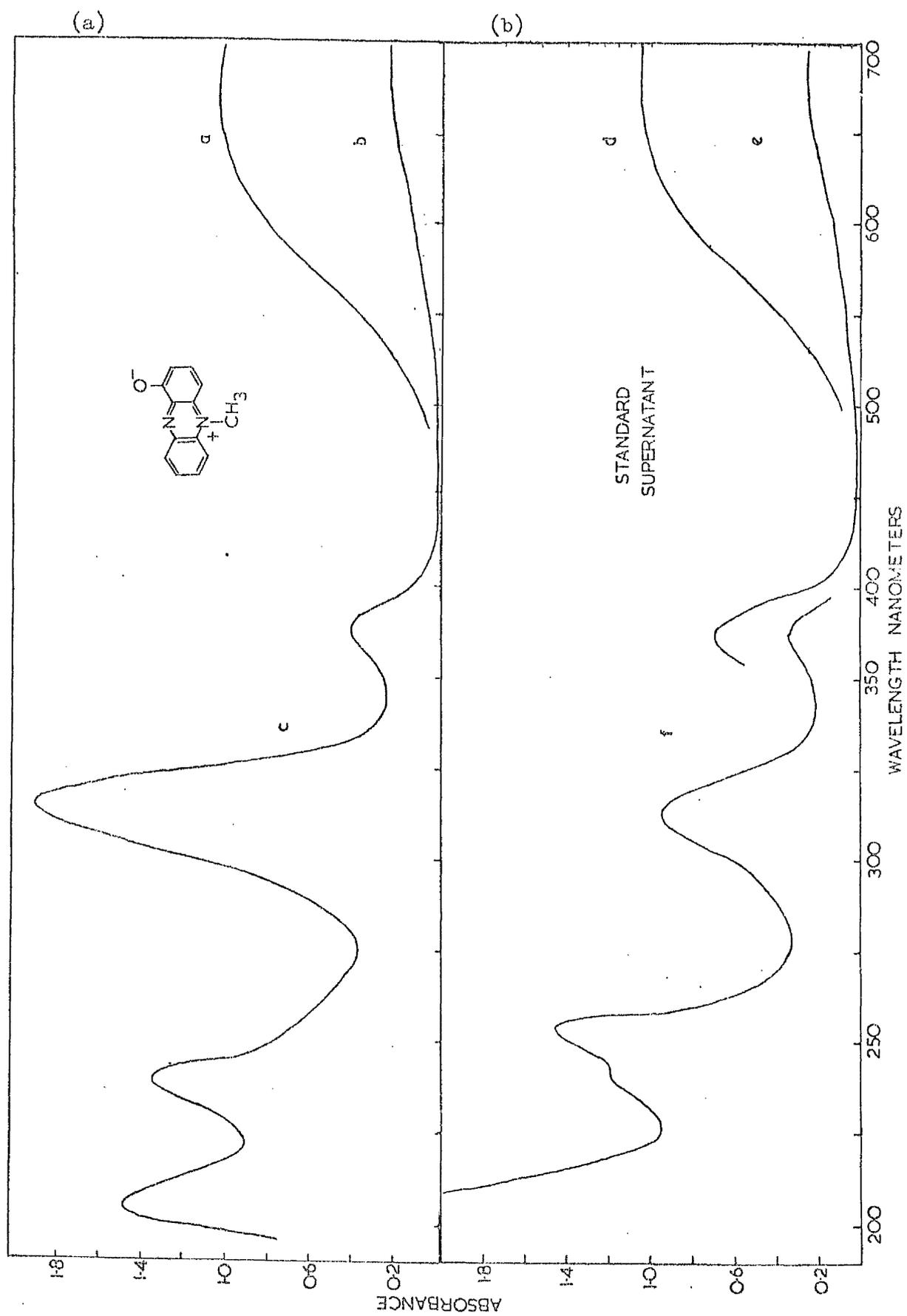
Supernatant;

(d) 10 mm path

(e) 2 mm path

(f) 2 mm path, 1:2 dilution

Figure 10



690 nm, 375 nm, 314 nm and 238 nm correspond to those positions of maximum absorption in pyocyanine. The supernatant spectrum had a new peak at  $\lambda$  max 255 nm. When calculating intensities of these absorptions the cell path length and dilutions must be considered. It was most suitable to estimate  $E_{690}$  with 10 mm path and undiluted sample although it was shown that the 2 mm path gave proportional estimates in the 500 - 700 nm range. 2 mm pathlength cells were used below 450 nm and with supernatants, a 1 in 2 dilution was necessary. The molar extinctions are given in Table IX and for supernatant, are based on the molar concentration of pyocyanine (although all are not ascribable to that molecule) for purposes of comparison.

It will be noted that the absorptions at  $\lambda$  max (nm) 690 and 314 have similar extinctions in both the supernatant and synthesised pyocyanine. The peaks at 238 nm and 375 nm are increased relative to those of pure pyocyanine to an extent of 13,800 and 3,600 molar extinction units. The supernatant spectrum exhibits a new peak with  $\lambda$  max 255 nm,  $\epsilon = 38,400$ . These divergencies are presumably due to substances other than pyocyanine in the culture supernatant.

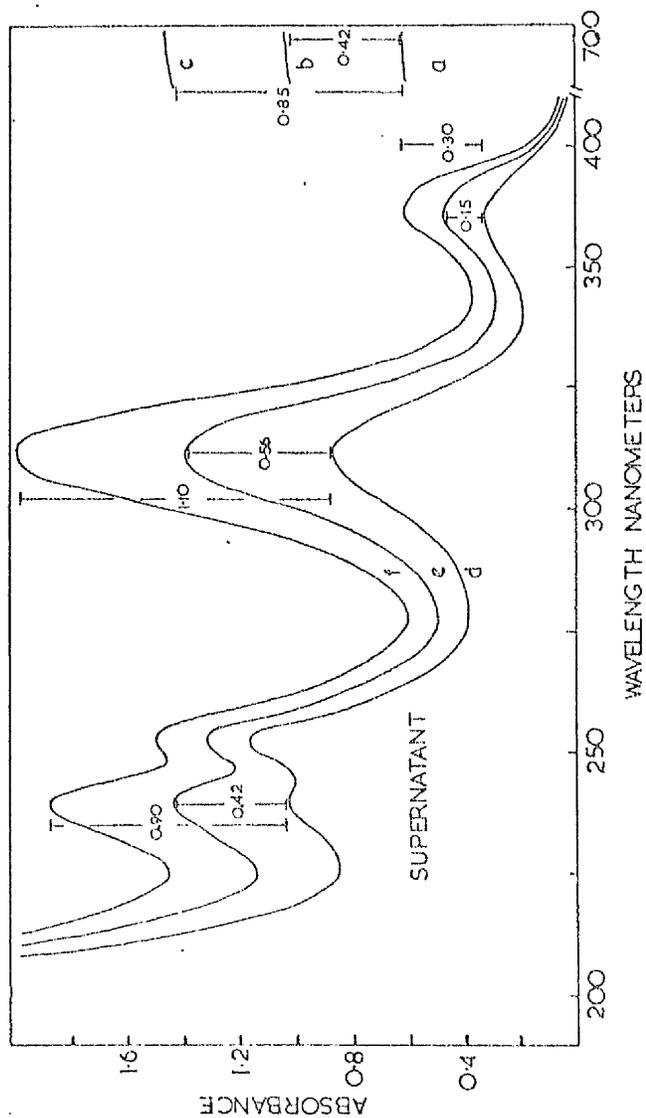
##### 5. Spectrophotometric detection of Pyocyanine added to Standard Supernatant

As an additional validation of the spectrophotometric assay, pyocyanine was added in measured quantities to Standard Supernatant in order to confirm the source of the ultra-violet and visible absorption peaks. Figure 11 illustrates the absorption changes occurring in a 1:2 dilution of supernatant (a), when pyocyanine was added to give a final concentration of 25  $\mu\text{g/ml}$  (b), and 50  $\mu\text{g/ml}$  (c). The spectra above 660 nm were recorded in 10 mm pathlength cells and below 450 nm, 2 mm pathlength cells were used. After

TABLE IX

Absorption  $\lambda_{\max}$  (nm) and extinction coefficients  
for "Standard Supernatant" and synthesized Pyocyanine

Pyocyanine		Standard Supernatant	
$\lambda_{\max}$ nm	$\epsilon$ l.mole <sup>-1</sup> cm <sup>-1</sup>	$\lambda_{\max}$ nm	$\epsilon$ l.mole <sup>-1</sup> cm <sup>-1</sup>
690	3,300	690	3,300
375	5,900	375	9,900
314	25,200	314	26,000
		255	38,400
238	17,800	238	31,600



Ultra-violet and visible spectra of supernatants with added Pyocyanine;

- (a) 10 mm path, 1:2 dilution of Standard Supernatant
- (b) as (a) with 25  $\mu\text{g/ml}$  pyocyanine added
- (c) as (a) with 50  $\mu\text{g/ml}$  pyocyanine added
- (d) 2 mm path, 1:2 dilution of Standard Supernatant
- (e) as (d), with 25  $\mu\text{g/ml}$  pyocyanine added
- (f) as (d), with 50  $\mu\text{g/ml}$  pyocyanine added

arithmetic conversion of absorbance values to the equivalent undiluted sample and 10 mm pathlength, the increases which correspond to the quantities of pyocyanine added are given in Table X. For purposes of comparison the absorption at 314 nm was chosen as 100% extinction and the relative strengths of the absorptions are quoted alongside in the Table X. The extinctions of the peaks for supernatant and pyocyanine solution were calculated in the same way.

The addition of pyocyanine caused absorption increases in the spectrum of the supernatant which were estimated from the absolute absorbance values. By comparing the relative extinctions for pure pyocyanine with the relative intensities of pyocyanine added to supernatant, close agreement is evident at four of the five tabulated  $\lambda$  max values. The exception - the 255 nm absorbance - is not present in pure pyocyanine and therefore the increases in absorbance at this wavelength in supplemented supernatant are not in proportion to the quantity added, viz. absolute increases in absorbance of 1.5 and 1.6 at 255 nm. These increases are comparable with those observed at minimum absorption values and are non-specific contributions.

In these examples the relative intensities of peaks at  $\lambda$  max (nm) 690 and 314 i.e. 15.0 for 25  $\mu\text{g}/\text{ml}$  addition, 15.5 for 50  $\mu\text{g}/\text{ml}$  addition and 12.7 for standard supernatant, are close to that observed in pure pyocyanine (13.1). This was taken as evidence of a satisfactory correlation between the extinctions at  $\lambda$  max 690 and 314 nm and the concentration of pyocyanine either in culture supernatant or in simple solutions of the pure pigment.

The relative absorption at the  $\lambda$  max 375 nm in supernatant (38.0) is greater than the ratio for pure pyocyanine (23.4) indicating a contribution from some other component in the solution at this wavelength.

Table X

Comparison of extinction ratios<sup>a</sup> for absorptions on Pyocyanine and of Standard Supernatant supplemented with known amounts of Synthesized Pyocyanine

Absorption $\lambda$ max (nm)	Absorbance values <sup>b</sup> for				Relative extinctions for	
	Pyocyanine added to Supernatant		Pyocyanine at equivalent ug/ml		Pyocyanine in Tris Supernatant	Standard Supernatant
	25 ug/ml	50 ug/ml	Absolute	Relative		
690	0.84	15.0	1.70	15.5	13.1	12.7
375	1.50	26.8	3.0	27.2	23.4	38.0
314	5.6	100	11.0	100	100	100
255	1.5	27	1.6	14	N.P.	148
238	4.2	75	9.0	81	71	122

a, Major absorption peak (314 nm) arbitrarily assigned the value 100%

b, Absorbance at uniform sample concentration and pathlength proportional to extinction

NP, No peak observed

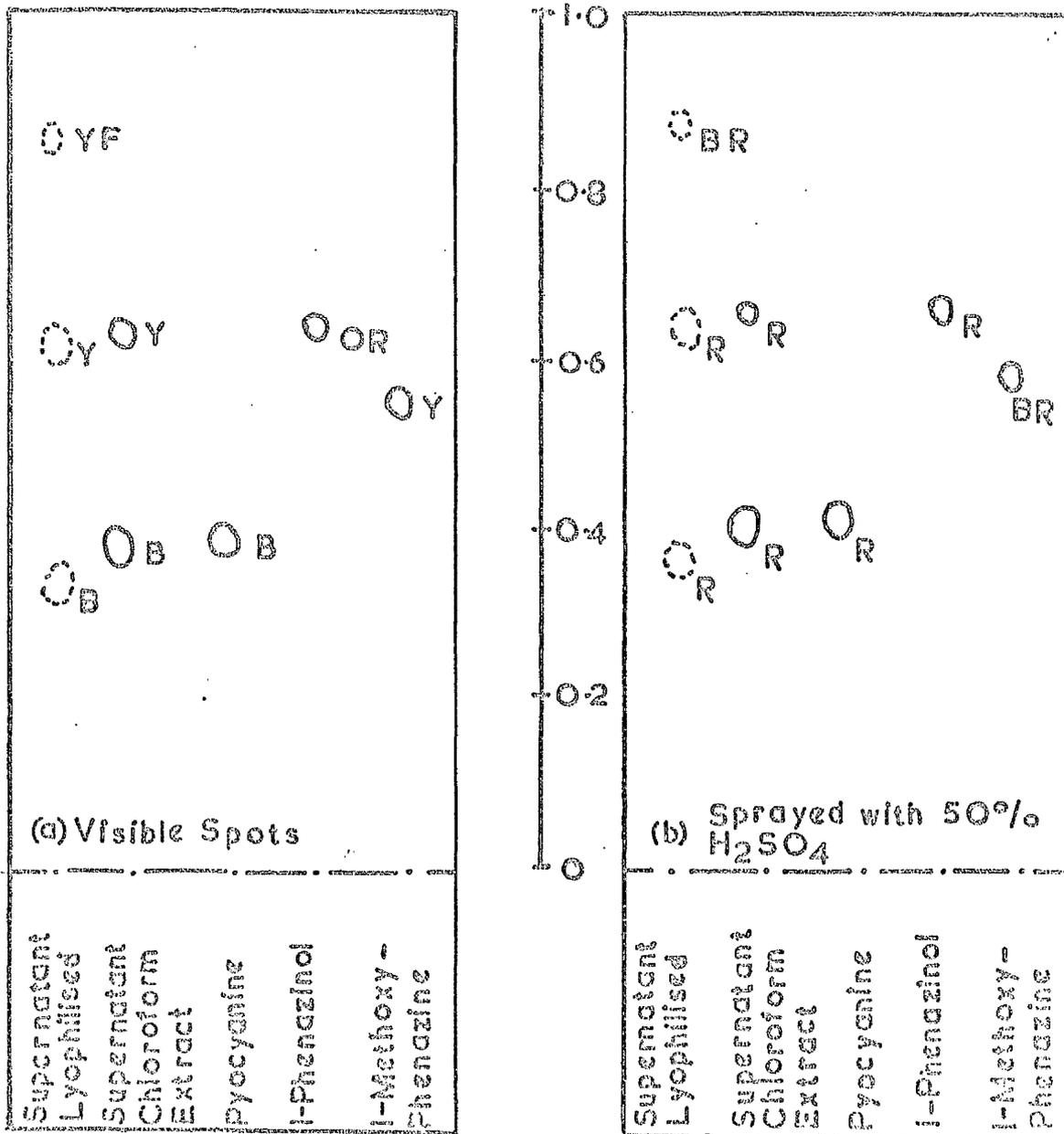
Also the non-pyocyanine absorption at 255 nm overlaps with the pyocyanine peak at 238  $\lambda$  max such that the intensity is greater than for pure pyocyanine. (Supernatant relative intensity at 238 nm is 122 of 71 in pure pyocyanine).

In summary, pyocyanine can be determined without interference in the supernatant at 690 and 314 nm. Peaks at 365 nm and 238 nm have contributions from pyocyanine and other sources. The absorption at 255 nm is not directly related to pyocyanine.

#### 6. Chromatography of culture supernatant preparations

By extracting 5 ml of the standard supernatant with chloroform, a sample was obtained for chromatographic analysis on a Thin Layer Chromatoplate adsorbed with 250  $\mu$  thickness of Silica Gel G 'Merck'. The extract was concentrated five-fold by evaporation and 50  $\mu$ l was applied to the plate using a 10 microlitre pipette. Approximately 50  $\mu$ l of a 5 mg/ml lyophilised supernatant preparation in chloroform was also spotted onto the plate. Authentic samples of Pyocyanine, 1-Phenazinol and 1-Methoxyphenazine (at 1 mg/ml; 50  $\mu$ l aliquots) were applied to the plate as standards. A suitable solvent system was chosen by applying a few drops of standard mixture to the chromatoplate. Solvents of gradually increasing polarity were dropped on to this spot until a circle of 2 cm diameter had developed. The solvent giving the greatest number of concentric rings was the binary mixture of chloroform and 10% methanol. Trial runs indicated adequate resolution of the standard compounds.

The development of plates spotted with supernatant preparations was stopped after the ascending solvent had moved 12 cm on the adsorbent. The dried plates, before and after spraying with 50%  $H_2SO_4$ , appeared as shown



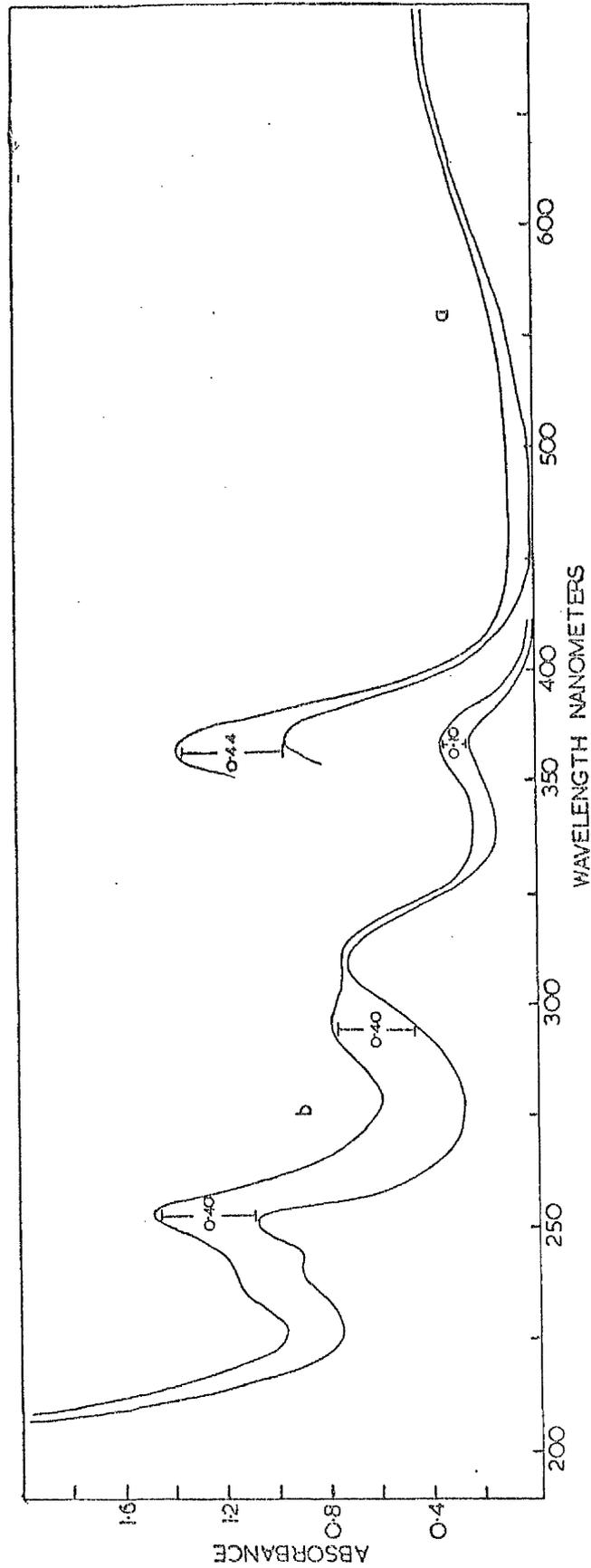
Thin layer chromatograms of supernatant preparations and known phenazines. Colour code : B - blue ; Y - yellow ; OR - orange ; F - fluorescent under UV light ; R - red and BR - brown.

in Figures 12 . The authentic samples were resolved as distinct spots and their identity confirmed by colour changes on acidification :- Pyocyanine,  $R_f$  0.38, blue to red; 1-Phenazinol,  $R_f$  0.64, orange-yellow to red; 1-Methoxyphenazine,  $R_f$  0.55, yellow to brown. The culture extract contained two visible spots, one corresponding in colour and  $R_f$  to pyocyanine and the other similar to 1-Phenazinol. The lyophilised supernatant sample also had two visible components, a faint blue region near pyocyanine  $R_f$  0.3-0.35 and a streaked yellow area  $R_f$  0.6-0.65 approximating to 1-phenazinol. A third and faintly visible spot was detected only under UV light ( $R_f$  0.8-0.85). Since the extraction procedure could have caused degradative changes in the sample, its value for identifying phenazines in the culture was limited. The chromatographic technique was not pursued as its major benefit was in confirming the homogeneity of the synthesized compounds.

#### 7. Spectrophotometric detection of 1-Phenazinol added to Standard Supernatant

The possible contribution of 1-phenazinol to the spectrum of Standard Supernatant was considered because of the chromatographic evidence for the presence of this component. Therefore 1-phenazinol was added to a suitable dilution of standard supernatant to give a final 10  $\mu\text{g}/\text{ml}$ . The effect on the supernatant spectrum is shown in Figure 13 . As with the pyocyanine spectra, 10 mm cells were used between 350 nm and 700 nm and 2 mm cells in the ultra-violet. Increased absorption occurred in the visible region between 400 nm and 500 nm although no peak was evident. The  $\lambda$  max 690 nm of pyocyanine was unaltered in intensity. At the 375 nm peak, which was recorded in both pathlength cells, an increase in intensity from 1.0 to 1.4 was noted in 10 mm cells and 0.25 to 0.35 in 2 mm cells. The absorption

Figure 13



Ultra-violet and visible spectra of supernatant with added l-Phenazinol

(a) 10 mm path, 1:2 dilution of Standard Supernatant with 10 µg/ml

l-Phenazinol  
(b) as (a), 2 mm path

at 314 nm was unaltered by l-phenazinol addition. A new peak appeared at 290 nm corresponding to an absorbance increase at this position of 0.45 to 0.85. The combined peak at 255 nm and 238 nm (shoulder) increased from 1.1 to 1.5 and from 0.9 to 1.2 respectively.

The position of the observed increases at  $\lambda$  max 420, 375 and 260 nm correlate with the  $\lambda$  max values reported for l-phenazinol in Tris (Figure 6e ). The 290 nm absorption only finds an analogy where strongly alkaline pH 11.0 solvent was used to record the spectrum (Figure 6c ). The absorption peaks at 290 nm could result from the interaction with other 'Standard Supernatant' components through the metal chelating property of l-phenazinol (Moos and Rowen, 1953). The presence of l-phenazinol did not lead to erroneous assessment of pyocyanine at  $E_{690}$ .

### C. Effect of changing the composition of Resuspension Medium

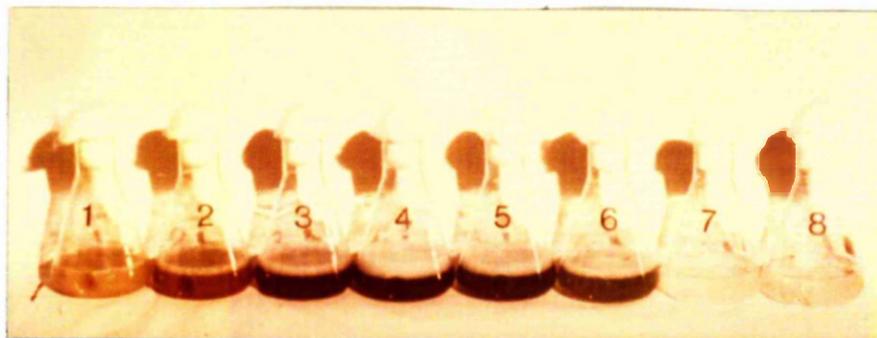
#### 1. Effect of Inoculum size on growth and Pyocyanine production

As the bacterial strain and substrate used here were different to those employed by Ingledew in his original work with Resuspension Medium, it was necessary to establish the conditions for maximum pyocyanine production. To determine the optimum inoculum ratio of stationary phase culture into Resuspension Medium, a series of flasks were set up in duplicate, containing a range of volumes of Inoculum Suspension. After 20 h incubation, when production had reached its maximal level, pyocyanine concentration was determined by  $E_{690}$ . This was recorded on an optically clear supernatant (12,000 x g, 20 min, 4°C) containing a few drops of hydrogen peroxide (30%). The latter caused no change in the spectrum of the oxygenated culture

supernatant and was added to ensure that all pyocyanine was in the oxidised form. The cell pellet was resuspended in saline and cell suspension opacity O.D.<sub>490</sub> determined. This wavelength was chosen because it marks a point of minimum absorption in the pyocyanine spectrum.

A striking result of this experiment was the difference in colour (Plate 1) of the cultures at the end of incubation. With an inoculum ratio of 25 or 50% the flasks showed the blue-green colour characteristic of pyocyanine production. Higher inoculum ratios gave terminal cultures which were brown and very small inocula, e.g. 3%, showed no colour development. The corresponding estimates of pyocyanine and cell suspension opacity are presented in Figure 14 (mean of duplicate flasks).

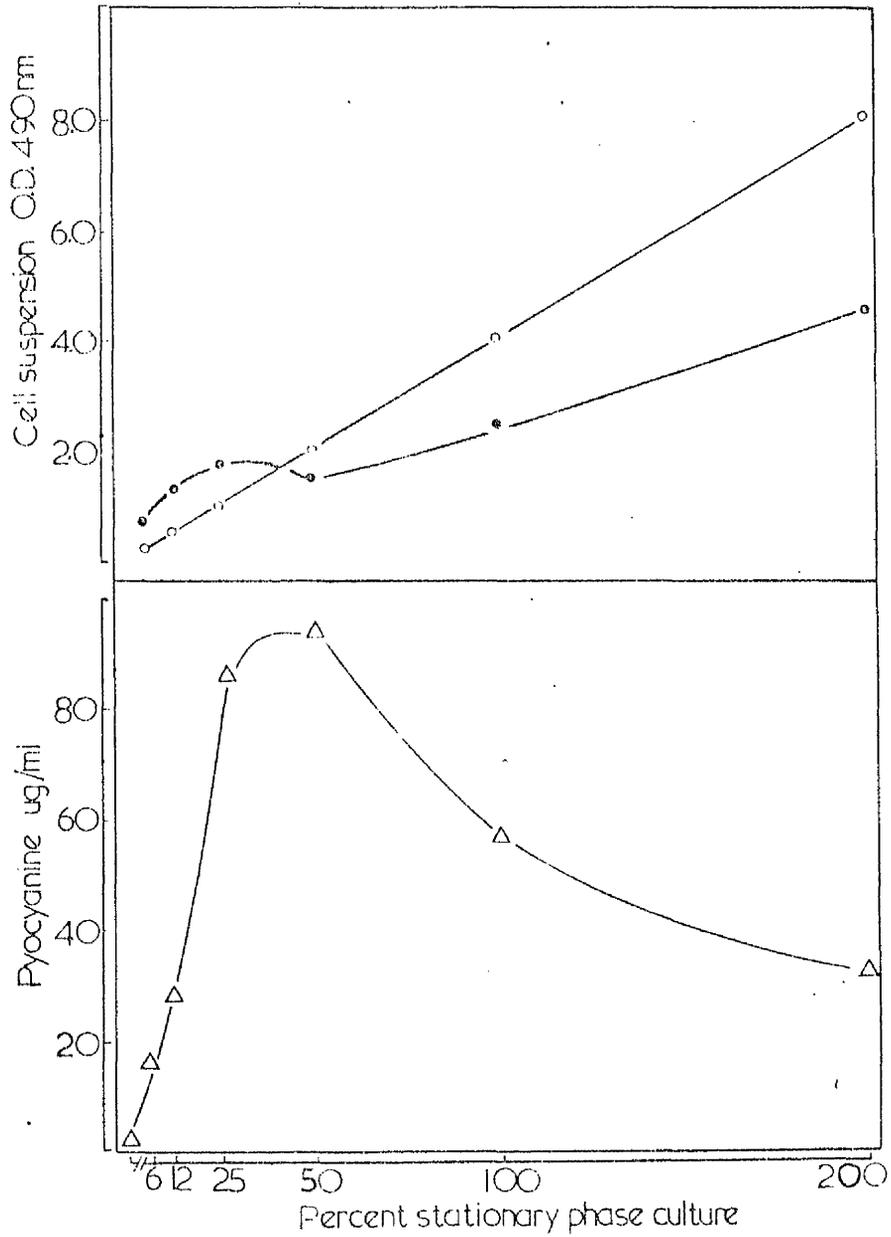
Plate 1



The effect of inoculum size on the colour of P. aeruginosa cultures in Resuspension Medium; 1 - 200%; 2 - 100%; 3 - 50%; 4 - 25%; 5 - 12%; 6 - 6%; 7 - 3%; 8 - 1%

The figure illustrates a distinct maximum in pyocyanine produced by inoculum level between 25 and 50% of stationary phase culture. An inoculum equivalent to 100 of the stationary phase culture gave 60% of optimum pyocyanine yield. 12% inocula gave 30% yield.

Figure 14



Effect of inoculum size on growth and pyocyanine production in Resuspension Medium. Cell suspension opacity at 20h (●) and at 0h (○) are shown in upper half and the pyocyanine produced in Resuspension Medium after 20 h (Δ) in the lower half of the figure.

A correlation between pyocyanine production and the change in cell suspension opacity (initial relative to final) was detected. At 40% inoculum level no net change resulted. Lower inocula gave net increases in opacity and higher ones, net decreases in opacity.

In conclusion, the optimum level of inoculation for Resuspension Medium in order to achieve maximum growth and optimum pyocyanine production was 25%. This inoculum gave, after incubation and centrifugation, that which will be referred to as 'Standard Supernatant'.

## 2. Essential and non-essential components of Resuspension Medium

As there is no satisfactory explanation for the environmental conditions which cause P. aeruginosa to produce pigment, the inclusion of components in the Resuspension Medium was based upon the combined empirical findings of investigations described in the Introduction. Ingledew's medium was distinct in having no phosphate although earlier workers such as Burton et al., (1948) suggested that an optimum minimal level was required.

The necessity for all the components of Ingledew's system does not appear to have been investigated. It was therefore decided to identify the components of Resuspension Medium essential for pyocyanine production. As shown in Figure 8 (page 84 ), the Inoculum Suspension (from Y-T medium) did not produce pyocyanine by transfer to fresh Y-T medium. Thus the stimulus for pyocyanine production must reside in the Resuspension Medium, the components of which have been given in Table XI .

All the components listed have been reported necessary for pyocyanine production by various authors, although the conditions and the strain of organism have varied. Additional components cited by Burton et al., (1948)

Table XI

## Composition of Resuspension Medium

Component	mM
Na <sup>+</sup>	88.0
NH <sub>4</sub> <sup>+</sup>	19.0
K <sup>+</sup>	6.3
Mg <sup>2+</sup>	0.98
Succinate	37.0
Cl <sup>-</sup>	27.0
SO <sub>4</sub> <sup>2-</sup>	7.0
Tris	50.0

The medium was made up as disodium succinate 10g,  
NH<sub>4</sub>Cl 1.0g, KCl 0.47g, Na<sub>2</sub>SO<sub>4</sub> 1.0g and  
MgCl<sub>2</sub> 0.2g in 1 litre of 50 mM Tris

Table XII

Effect of changes in the components of Resuspension Medium on  
Pyocyanine production and growth

Medium	Relative Pyocyanine Production ( $E_{690}$ ) <sup>a</sup>	Relative Cell Opacity ( $E_{490}$ ) <sup>a</sup>
Complete Resuspension Medium	100	100
Without KCl	70-80	120
Without $MgCl_2$	90-100	80
Without Tris	40-50	120
Without $Na_2SO_4$	10-20	70
Without Succinate	10-20	50
Without $NH_4Cl$	10	60
$FeSO_4$ added	90-100	85
$K_2HPO_4$ added	10	140
$FeSO_4 + K_2HPO_4$ added	10	160

a. To serve as standard for comparison the control was arbitrarily assigned the value 100% for Pyocyanine and final opacity level. The 10% reading in the presence of phosphate is not due to pyocyanine.

as being necessary are  $\text{Fe}^{2+}$  and  $\text{PO}_4^{3-}$ . My experiments were therefore of two types: the effect of a) stepwise omission of the components of Resuspension Medium and b) addition of  $\text{Fe}^{2+}$  and/or  $\text{PO}_4^{3-}$ . The latter were added at 40  $\mu\text{M}$  and 40  $\text{mM}$  respectively, the concentrations present in Y-T medium. The effect on the pyocyanine produced under Standard Supernatant conditions was monitored after the deletion or addition of the various components. All flasks were inoculated as for Standard Supernatant production and had a final volume of 100 ml. The cell suspension opacity and  $E_{690}$  of the clear supernatant were monitored as previously described, giving the estimates of growth and pyocyanine production which are recorded in Table XII. The results are expressed relative to the values observed in the ordinary Resuspension Medium control.

The following conclusions were reached:  $\text{MgCl}_2$  and  $\text{KCl}$  are not essential components of the Resuspension Medium. In contrast,  $\text{Na}_2\text{SO}_4$ , succinate and  $\text{NH}_4\text{Cl}$  are all necessary, as is the Tris buffer. Addition of  $\text{Fe}^{2+}$  did not influence pigment production but addition of phosphate completely prevented pyocyanine from being made.

The opacity changes show no correlation with pyocyanine production although increases occurred in all cases. The addition of  $\text{Fe}^{2+}$  and phosphate stimulated growth beyond that in the control.

In conclusion, the essential features of Resuspension Medium appear to be Tris,  $\text{Na}_2\text{SO}_4$ , succinate and  $\text{NH}_4\text{Cl}$  and the absence of phosphate.

### 3. Pyocyanine production and growth in Resuspension Medium with replaced components

The knowledge that the essential components of the Resuspension

Medium were  $\text{NH}_4\text{Cl}$ ,  $\text{Na}_2\text{SO}_4$  and disodium succinate led to speculation as to the acceptability of alternatives for pyocyanine production. The components chosen as substitutes for the  $\text{NH}_4\text{Cl}$  nitrogen source were  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{KNO}_3$  and  $\text{NH}_2\text{OH} \cdot \text{HCl}$ . The  $\text{Na}_2\text{SO}_4$  component was replaced by possible alternative sulphur sources,  $\text{Na}_2\text{SO}_3$  and  $\text{Na}_2\text{S}_2\text{O}_3$ . In place of succinate as a carbon source, substitutes were selected from Krebs cycle intermediates - citrate and glutamate and two other metabolites were tested - glycerol and glucose. All substitutes were added at equivalent mM concentrations to Resuspension Media that were otherwise as normal. Stationary phase culture was used as a 25% inoculum in all flasks and the control was set up to give Standard Supernatant.

The effects of these substitutions are given in Table XIII and are expressed in terms of relative Pyocyanine production and growth. It will be noted that none of the replacements gave better yields of pyocyanine than the control although growth in some cases was substantially promoted. By adding  $\text{NH}_4^+$  and  $\text{SO}_4^{2-}$  as one component 60-65% of the normal level of pyocyanine was produced. This indicates that the  $\text{Na}^+$  is not the essential ion of  $\text{Na}_2\text{SO}_4$ . Neither  $\text{KNO}_3$  nor  $\text{NH}_2\text{OH} \cdot \text{HCl}$  gave good pyocyanine production (25% and 10% respectively) and some cell lysis was observed with the latter. It seemed easier to supply alternative sulphur sources, as  $\text{SO}_3^{2-}$  and  $\text{S}_2\text{O}_3^{2-}$  gave fairly good pyocyanine yields (40-45% and 60-65% respectively). With these alternative sulphur sources, growth was improved relative to that achieved by  $\text{SO}_4^{2-}$  in the control.

The pattern observed with the replacement carbon sources glycerol and glucose is one of reduced levels of pyocyanine (50% and 30%) and proportionate increases in growth (190% and 260%). Similarly for citrate

Table XIII

Effect of replacing essential components of Resuspension Medium on  
Pyocyanine production and growth

Component	Replacement	Relative Pyocyanine produced ( $E_{690}$ ) <sup>a</sup>	Relative cell opacity ( $E_{490}$ ) <sup>a</sup>
$NH_4Cl$	$(NH_4)_2SO_4$	60-65	100
	$KNO_3$	20-25	100
	$NH_2OH.HCl$	10	80
$Na_2SO_4$	$Na_2SO_3$	40-45	110
	$Na_2S_2O_3$	60-65	130
Succinate	Glycerol	45-50	190
	Glucose	25-30	260
	Citrate	65-70	180
	Glutamate	60-55	120

a. To serve as standard for comparison the control was arbitrarily assigned the value 100% for Pyocyanine and final opacity level.

and glutamate, reduced pyocyanine coincided with improved growth. Overall it was evident that the factors promoting growth and those promoting pyocyanine production are quite separate.

4. Spectrophotometric analysis of supernatants from Resuspension Media with replaced components

The supernatants of Resuspension Medium cultures with replaced carbon, nitrogen and sulphur sources were examined by spectrophotometry in the same way as described previously for Standard Supernatant. As is observed in Table XIVa and XIVb where the wavelength positions of the  $\lambda$  max (nm) values are given, there is good agreement with the peaks of Standard Supernatant. The cultures produced pyocyanine in variable amounts although at certain UV absorption peaks there were contributions from other components. The estimation of pyocyanine by  $E_{690}$  was shown to be well founded in the Standard Supernatant case. The extinction ratio  $\frac{E_{690}}{E_{314}}$  for Standard Supernatant and pure pyocyanine falls within the range 12-15%, and this is taken as evidence of spectral correlation at these two peak positions. In all cases quoted in Tables, the correspondence of absorptions at 690 nm and 314 nm with those of pure pyocyanine is upheld. In the case of no pigment production the ratio is not calculated. With Standard Supernatant the "foreign" absorption contributing to the peak at 375 nm was reliably estimated by the ratio  $\frac{E_{375}}{E_{314}}$  (38-42%). The trend of this ratio towards that found in pure pyocyanine (23-27%) gives a measure of the interference with the pure spectrum. These ratios calculated for the spectra in Tables XIV are all greater than 45%. Those closest to the control value of 38-42% are the carbon substitute, citrate and the nitrogen substitute  $(NH_4)_2SO_4$ , and these represent the best replacements for pyocyanine production. The increasing values of this ratio correlate well with reduced quantities of pyocyanine production given in Table XIII.

Table XIVA

Absorbance values for visible and ultra-violet spectra of supernatants  
from Resuspension Media with replaced nitrogen and sulphur sources

Nitrogen or Sulphur Source	Colour	Absorbance values at $\lambda$ max (nm)					$\frac{E_{690}}{E_{314}} \%$ $\frac{E_{275}}{E_{314}} \%$	
		690	375	312	255	238		
Usual	Blue-Green	1.1	3.4	8.2	12	10	13	41
$(\text{NH}_4)_2\text{SO}_4$	Green	0.6	2.1	4.7	8.5	7.4	13	45
$\text{KNO}_3$	Green	0.25	1.3	1.8	3.5	NP	14	72
$\text{NH}_2\text{OH.HCl}$	Clear	0.0	<u>0.3</u>	<u>0.5</u>	<u>2.0</u>	NP	-	-
$\text{Na}_2\text{SO}_3$	Green	0.45	1.8	3.2	11	11	14	56
$\text{Na}_2\text{S}_2\text{O}_3$	Dark Green	0.65	2.0	4.2	9.2	<u>9.2</u>	15.5	47

NP, No peak observed. Underlined absorbances quote background values  
at  $\lambda$  max position where no actual peak was observed. Dash (-)  
indicates no meaningful estimate

Table XIVb

Absorbance values for visible and ultra-violet spectra of supernatants  
from Resuspension Media with replaced carbon sources

Carbon Source	Colour	Absorbance values at $\lambda$ max (nm)					$\frac{E_{690}}{E_{314}}$ %	$\frac{E_{375}}{E_{314}}$ %
		690	375	312	255	238		
Succinate (usual)	Blue-Green	1.3	4.3	10	15	14	13	42
Citrate	Blue-Green	0.9	3.2	7.0	10	10	13	45
Glutamate	Blue	0.8	3.2	6.4	9.6	9.2	12.5	50
Glycerol	Green	0.7	3.0	5.2	11	NP	13.5	58
Glucose	Green	0.3	3.4	2.4	18	NP	12.5	142

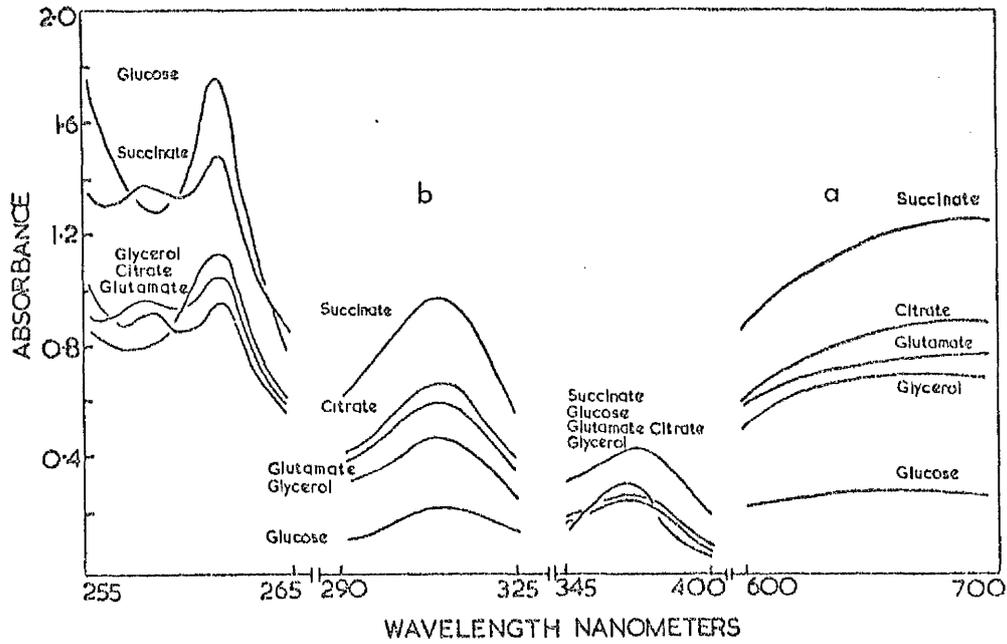
NP, No peak observed

In summary, none of these manipulations in the formula of Resuspension Medium improved pyocyanine production; most of the alternatives were strongly disadvantageous. The trend to reduced levels of pyocyanine is accompanied in the case of carbon substitutes by spectral changes in the supernatant which cause the absorptions at 375 and 314 to change their relative intensity. The spectra illustrating this trend away from the spectrum of pure pyocyanine are shown in Figure 15 . The 10 mm pathlength absorptions at 690 nm are in agreement with the pyocyanine level produced. The absorptions measured at 375 nm, 314 nm, 255 nm and 238 nm in 2 mm cells show the change in the ratio  $\frac{E_{375}}{E_{314}}$  and the disappearance of an observable peak at 238 nm with glucose and glycerol.

5. Growth in Resuspension Medium under atmospheres of  
hydrogen and nitrogen

To observe whether or not oxygen in the Resuspension Medium was necessary for the synthesis of pyocyanine, cultures of the Standard Supernatant type were incubated under hydrogen and nitrogen atmospheres (Table XV ). No pyocyanine production was observed in the supernatants from incubation under hydrogen or nitrogen (with or without added  $H_2O_2$  prior to spectrophotometry). Supernatants grown under hydrogen showed two absorptions at 263 nm and 240 nm due to unidentified substances. The cell suspension opacity fell during incubation, indicating cell lysis. The supernatant from cultures under the nitrogen atmosphere had a yellow fluorescence under UV light and the spectrum showed two minor peaks at 360 nm and 255 nm. The change in cell suspension opacity from 1.0 to 1.2 during the 20 h incubation indicated slight growth.

Figure 15



Ultra-violet and visible spectra of Resuspension Media supernatants with replaced carbon sources; (a) 10 mm path and (b) 2 mm path, 1:2 dilution.

Table XV

Effect of anaerobic incubation on Pycyanine production and growth

Atmosphere	Colour	Pycyanine Produced (E <sub>690</sub> )	Change in Cell Suspension Opacity E <sub>490</sub> (0h - 20h)	Absorbance values at λ <sub>max</sub> (nm)						
Nitrogen	Yellow fluorescence under U.V.	0	1.0 - 1.2	0	0.1	0.26	<u>0.26</u>	0.9	1.2	NP
Hydrogen	None	0	1.0 - 0.5	0	0	<u>0.12</u>	<u>0.18</u>	0.7	-	0.68

NP, No peak observed. Underlined absorbances quote background values at λ<sub>max</sub> position where no actual peak was observed. Dash (-) indicates no meaningful estimate.

#### D. Pyocyanine production in Minimal Resuspension Medium

The combined results of the experiments in Section C indicate that the minimum requirements for pyocyanine production are di-sodium succinate (37 mM),  $\text{NH}_4\text{Cl}$  (19 mM) and  $\text{Na}_2\text{SO}_4$  (7 mM). One might therefore predict that under the standard conditions with the 25% inoculum level, a medium of the above composition should give substantial pyocyanine production. Accordingly, a Minimal Resuspension Medium was made up with these components in 50 mM Tris. To determine the best conditions, the medium was inoculated with a variety of volumes of Inoculum Suspension. The pyocyanine production and growth which resulted are expressed in Table XVI. The colour variations were qualitatively similar to those observed in the complete Resuspension Medium experiment. However, the minimal medium supported at its best only 55% of the pyocyanine produced in Complete Resuspension Medium. This best value occurred at an inoculum level of 25% which is the same as complete medium. This reduced level of Pyocyanine was nevertheless accompanied by improvement in growth reaching an O.D. of 2.2 compared with 1.6 in the control. Higher than optimum inoculum levels in Minimal Medium gave net lysis in agreement with the results shown in Figure 14.

The spectra of supernatants from flasks with various inoculum ratios are shown in Figure 16. The corresponding absorbance values for undiluted sample in 10 mm cells are given in Table XVII. In considering Figure 16 it must be remembered that for control supernatants and those minimal cultures having 10% and 25% inoculum levels, 10 mm pathlength was used between 450 nm and 700 nm. Between 200 nm and 450 nm 1:2 dilutions in 2 mm pathlength gave the spectra recorded.

The lower part of Figure 16 shows the spectra of supernatants from



Figure 16: Ultra-violet and visible spectra of supernatants  
from minimal media with different inoculum sizes;

- (a) 10 mm path
- (b) 2 mm path, 1:2 dilution
- (c) 20 mm path
- (d) 2 mm path

Figure 16

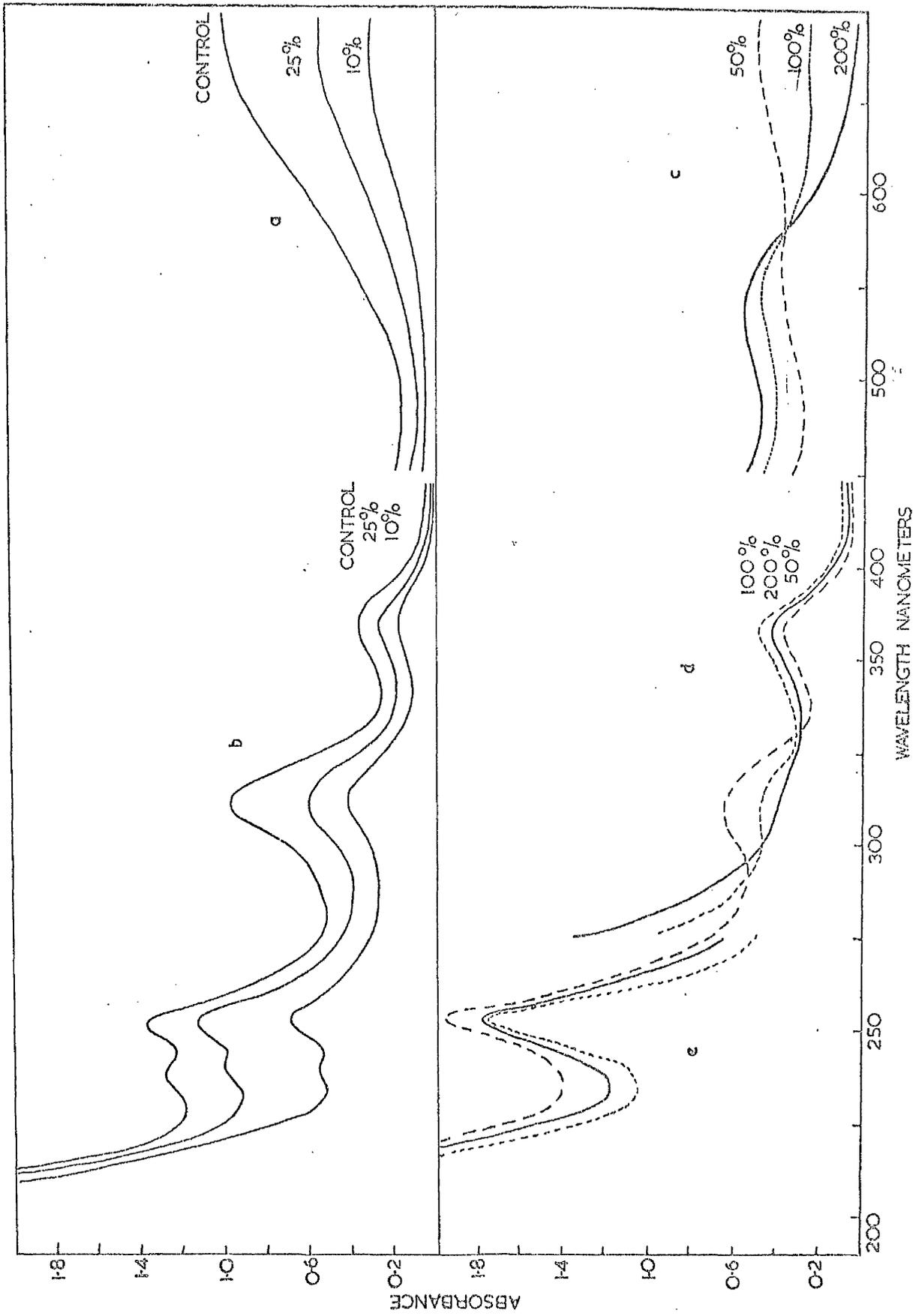


Table XVI

Effect of varying inoculum size in Minimal Resuspension Medium on  
Pyocyanine production and growth

Inoculum % Stationary phase culture	Colour	Relative Pyocyanine production ( $E_{690}$ ) <sup>a</sup>	Change in cell opacity (0h $\rightarrow$ 20h, $E_{490}$ ) <sup>a</sup>	
Control <sup>b</sup>	Blue-Green	100	0.8	1.6
10	Green	30	0.3	1.2
25	Blue-Green	55	0.8	2.2
50	Blue-Grey	25	1.6	1.8
100	Red	13	3.0	2.6
200	Brown	10	6.0	5.2

a, To serve as standard for comparison the control was arbitrarily assigned the value 100% for Pyocyanine level

b, Inoculum of 25% in complete Resuspension Medium

Table XVII

Absorbance values for visible and ultra-violet spectra of supernatants from minimal Resuspension Media with varying inoculum sizes

Inoculum % Stationary Phase Culture	Absorbance values at $\lambda$ max (nm)					$\frac{E_{375nm}}{E_{314nm}}$ %	$\frac{E_{690nm}}{E_{314nm}}$ %		
	690	560	530	365	314			255	238
Control	1.0	NP	NP	3.8	9.5	14	13	40	11.0
10	0.35	NP	NP	1.8	4.2	7	5.5	43	8.4
25	0.55	NP	NP	2.8	6.0	11	10	47	9.2
50	0.25	0.2	NP	2.0	3.4	10	<u>7</u>	59	7.4
100	0.13	NP	0.25	2.2	2.5	18	<u>10.5</u>	88	5.2
200	0.02	NP	0.29	2.5	2.1	18	<u>12</u>	119	1.0

NP, No peak observed. Underlined absorbances quote background values at  $\lambda$  max position where no actual peak was observed.

higher inoculum ratios. The pigment observed in these cases was of much lower intensity and 20 mm pathlength cells were required between 450 and 700 nm. 2 mm pathlength cells were suitable below 450 nm although 1:2 dilution was necessary to record the 255 nm peak of 100% and 200% inoculum ratios.

It will be observed that with 10% and 25% inoculum ratios spectra closely correlated with those of Standard Supernatant at all  $\lambda$  max values. The ratio of extinctions  $\frac{E_{690}}{E_{314}}$  and  $\frac{E_{375}}{E_{314}}$  were closest in these cases to the standard value.

The higher inoculum ratios in Minimal Medium gave distinct visible and ultra-violet spectra. The 50% inoculum level retained the  $\lambda$  max 690 nm although a new peak at 560 nm was observed. The 100% inoculum level caused  $\lambda$  max 690 nm to decrease and the one at 530 nm to predominate. This trend was more pronounced with the 200% inoculum ratio. The measurements of Pyocyanine by  $E_{690}$  in these cases can only express the maximum possible level present.

### SECTION III : BIOCHEMICAL CHARACTERISATION OF

#### P. AERUGINOSA CELLS

##### A. Kinetics of growth and Pyocyanine production

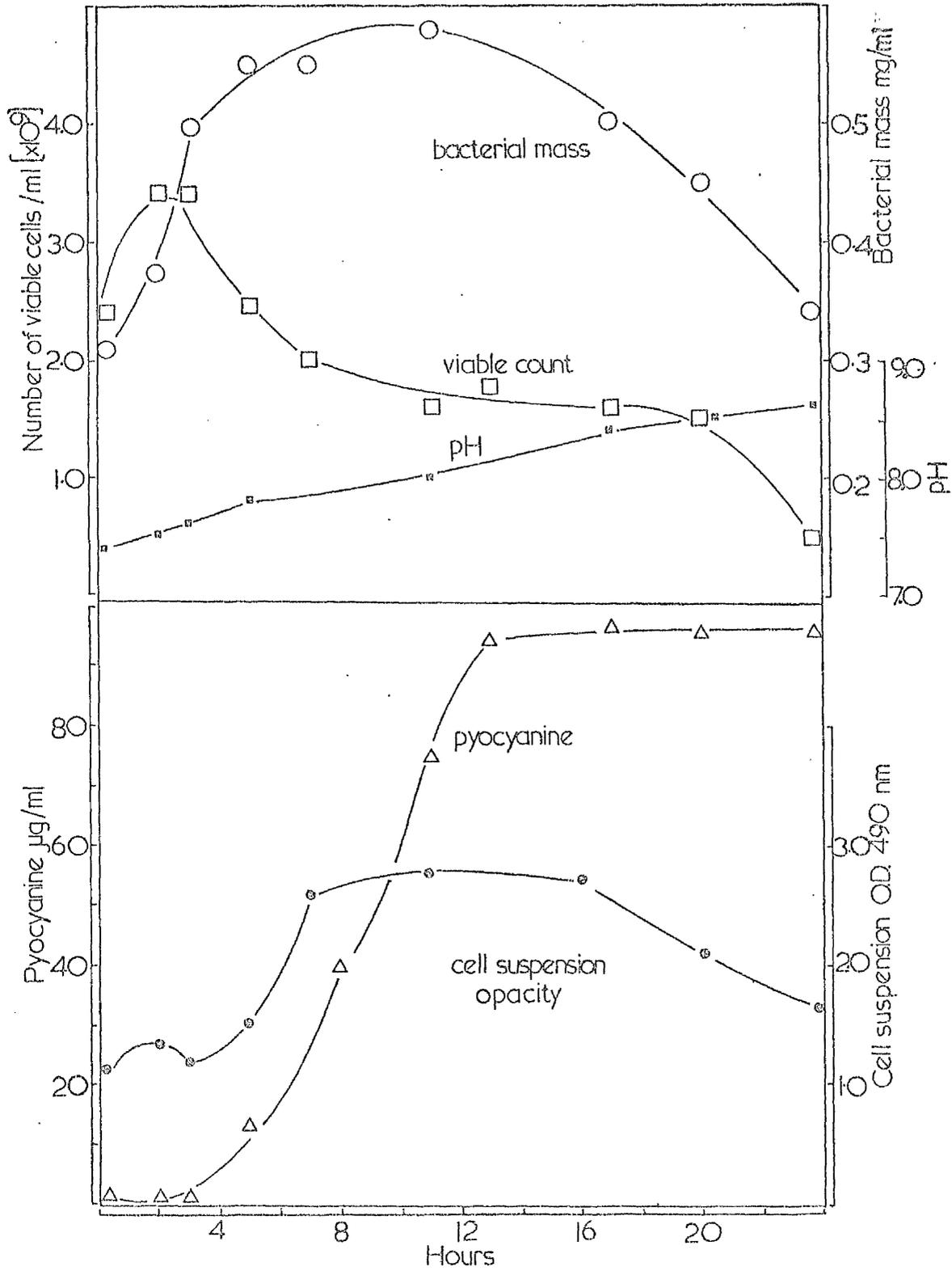
Having established that the use of Resuspension Medium gave reproducible production of pyocyanine, the next step was to compare the growth curve of NCTC 6750, under the defined optimal conditions of 25% inoculum level, with that reported by Ingledew and Campbell (1969a) for strain ATCC 9027. Cultures in Resuspension Medium were therefore set up

under the optimal conditions and samples withdrawn at various times (0h, 2h, 3h, 5h, 7h, 11h, 16h and 20h) for the measurement of cell suspension opacity, total bacterial mass, viable cell count, pH and pyocyanine production. The results (Figure 17 ) show that pyocyanine was first detected at 3h after inoculation of the Resuspension Medium. From that time its level increased linearly until at 13h it reached a plateau level. Thereafter the concentration of pyocyanine remained constant in the supernatant over the remaining period of observation. In other experiments where measurements were made after 48h, pyocyanine concentration had decreased by only 10%.

The cell suspension opacity (O.D.<sub>490</sub> of 1.0) at 0h changed little over the lag interval of pyocyanine production. At 3h the opacity began to increase and the rate paralleled that observed for pyocyanine. The maximum opacity was reached at 7h and it remained constant until pyocyanine concentration was at the plateau level (16h). The decrease after this time was confirmed by the value of 1.8 - 2.0 which was regularly observed at 20h.

The number of viable cells at 0h ( $2.4 \pm 0.4$ )  $\times 10^9$  per ml corresponded to a total bacterial dry mass of 0.31 mg per ml. The most rapid increases in these two parameters occurred over the lag interval from 0h to 3h. Viable count increased from 2.4 to  $3.4 \times 10^9$  cells/ml. This increase in the viable count during the lag interval, in fact, represented the only cell division occurring in the culture. After 3h the viable count decreased within the next three hours to its original 0h count. This point marks the 50% level of pyocyanine production. Further changes occurred only after 20h when the viable count fell to much lower levels.

The decrease which occurred in the viable count after 3h did not cause the bacterial mass to stop increasing. The latter levelled off at a



Kinetics of growth and Pyocyanine production

maximum 0.58 mg/ml after 8h. The decline in bacterial mass at 14h corresponded to decreasing cell suspension opacity and started when pyocyanine production had ceased. At the typical observation time of 20h the bacterial mass had fallen to 0.45 mg/ml. The pH changes over the course of pyocyanine production showed a steady increase from 7.4 at 0h to 8.6 at 20h.

The above results on pyocyanine production cell, suspension opacity and pH show good agreement with those reported by Ingledew with ATCC 9027.

#### B. Changes in the chemical composition of cells and supernatants

There appears to be no published information on the changes in the chemical composition of P. aeruginosa cells during pyocyanine production. It was therefore decided to make a variety of chemical analyses on the cells over the period of incubation in Resuspension Medium. The non-pyocyanine producing cells harvested from the initial growth medium were represented by the 0h sample. Between 0h and 3h there is a phase of cell division and after this 'pyocyanine lag interval' the cells characteristically start to produce the green pigment. Cells at 3h were removed to record the changes that had taken place. When pyocyanine had reached the plateau level (20h) the cells were compared analytically with those at 0h and 3h to see what changes had occurred.

It should be remembered that the "trigger" for pyocyanine production in the Resuspension Medium as suggested by Ingledew, and confirmed here, was the total absence of added phosphate. Therefore in this series of experiments attention was focussed on determining the levels of phosphate-containing macromolecules (DNA, RNA, FL, LPS and ATP) in the pyocyanine-producing cells.

The assay procedure, except where stated otherwise, was carried out on

cell samples removed at 0h, 3h and 20h from the Standard Resuspension Medium. Suitable cell aliquots of different sizes were harvested from duplicate flasks, washed once with saline by centrifugation at  $4^{\circ}\text{C}$  and stored at  $-20^{\circ}\text{C}$  until required for analysis.

The results are expressed in two ways :

- 1) as weight of component per flask, i.e. per 100 ml of culture in Resuspension Medium
- 2) as percent contribution of the component to the total bacterial mass.

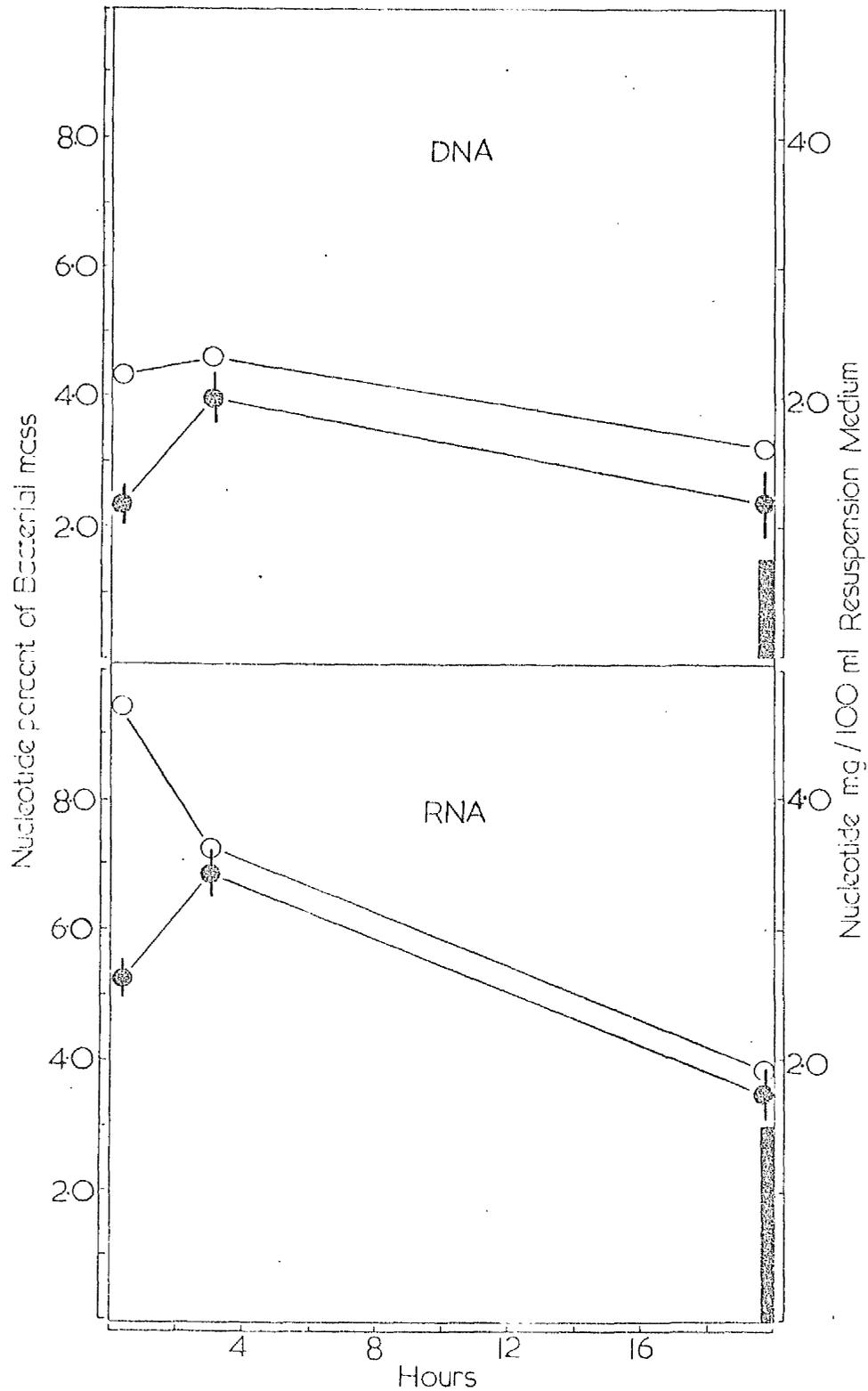
This second method was included because of the 60% increase in bacterial mass during the first three hours.

Some analyses were also done on culture supernatants from the Resuspension Medium. Before analysis, these latter samples were ultrafiltered at  $0^{\circ}\text{C}$  through a UM 05 grade membrane with a molecular weight exclusion limit of 500 (Amicon Corp. Massachusetts). This was done primarily to remove pyocyanine whose colour would otherwise have interfered with the colour reactions of some of the biochemical assays.

a. Nucleic acid

Immediately after the cells had been harvested and washed at  $0^{\circ}\text{C}$  in  $0.25 \text{ N HClO}_4$ , the nucleic acids were extracted. The DNA and RNA spectrophotometric assays were described by Munro and Fleck (1966) and are based on the method of Schneider (1957). Nucleotides were acid hydrolysed and the purine bound deoxyribose and furfural derivative of purine bound ribose were determined by their colour reactions with diphenylamine ( $E_{600}$ ) and with orcinol ( $E_{660}$ ) respectively. Pyrimidine-bound nucleotides do not react under these conditions. Culture volumes of between 5 and 20 ml were used to

Figure 18



Nucleic acid content of cells and supernatant.

●, Values per 100 ml of Resuspension Medium.

O, Percent contribution to cell mass. Columns indicate nucleic acid (mg/100 ml) in culture supernatant. Range of values is expressed as bar through point.

estimate DNA and 2 - 5 ml for RNA. Figure 18 shows the weights of polynucleotides in the cells at the three sampling times. The yields were calculated after calibration of the method using purified samples of DNA and RNA which had been treated in an identical manner. The percentage contribution to the cell was based on the measured total bacterial mass as described for the growth curve.

DNA increased in the first three hours from 1.21 to 2.05 mg per 100 ml, an increase which was sufficient to maintain its level in the cells at 4.2 - 4.3 percent. At 20h, when pyocyanine was on the plateau level, the DNA had returned to its 0h level of 1.25 mg per 100 ml of culture. At this time the supernatant contained 0.75 mg of soluble DNA per 100 ml.

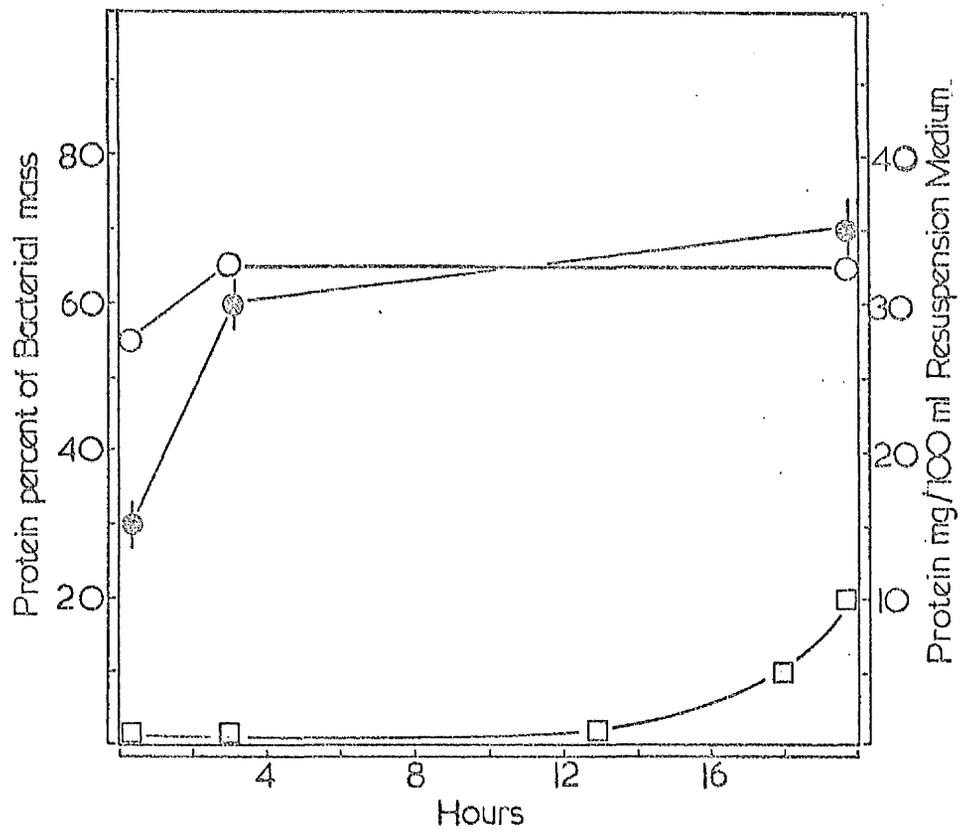
In contrast to DNA, the increase in RNA during the lag interval from 2.65 to 3.48 mg per 100 ml was not quite in proportion to the increase in bacterial mass and the percent value in the cells fell from 9.3 to 7.1 as a result. After twenty hours the cells from 100 ml culture contained the equivalent of 1.85 mg of RNA which represented 3.9 percent of the total bacterial mass. The quantity of soluble RNA in the culture supernatant was 1.5 mg.

b. Protein

Protein was estimated by the method of Lowry et al., (1951) using bovine serum albumin as standard. Samples were prepared by sonicating the cells from 5 ml of Resuspension Medium in 3 ml of Lowry Reagent A at 0°C for 10 min to obtain a clear suspension.

At 0h the cell-associated protein measured 15.4 mg per 100 ml as shown in Figure 19 . Growth during the lag interval gave a protein level

Figure 19



Protein content of cells and supernatant.

●, Values per 100 ml of Resuspension Medium

○, Percent contribution to cell mass

□, Release of protein (mg/100 ml) into the supernatant.

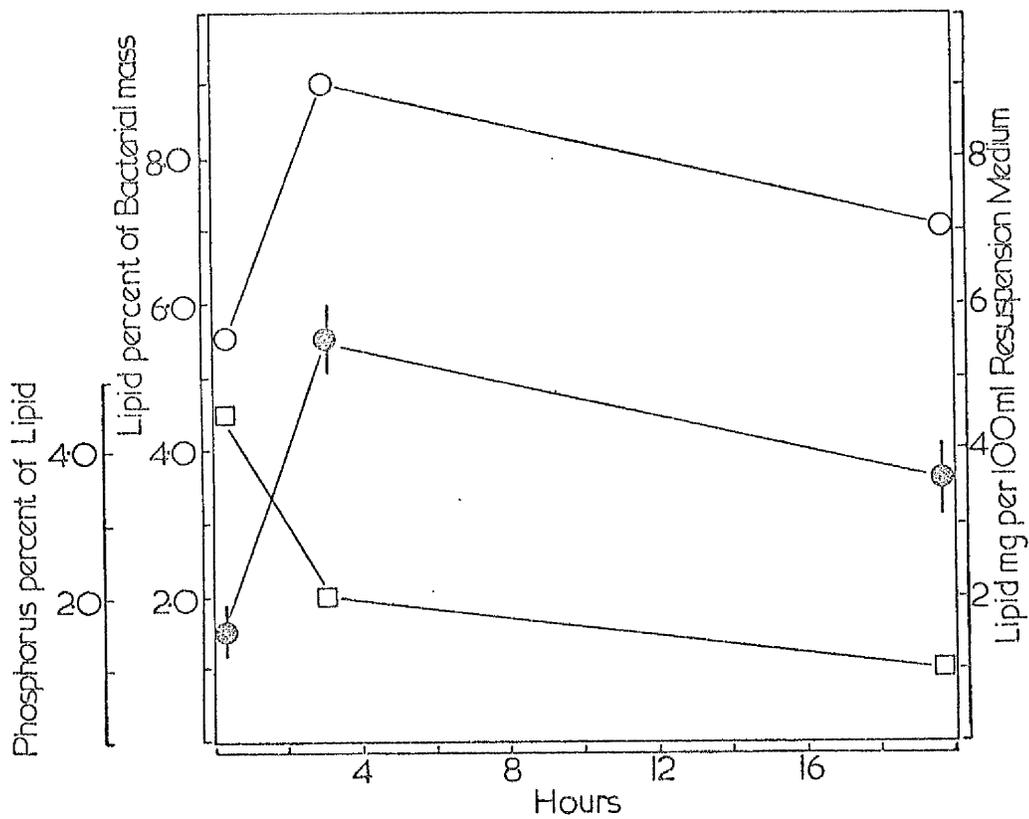
of 31.4 mg per 100 ml at 3h. This level appeared to remain steady through the phase of pyocyanine production and was 34.5 mg per 100 ml at 20h. This synthesis of protein had the effect of increasing the contribution of protein to the cell mass from 55 to 65%. The 20h figure was 67%. The observed increase and retention of cellular protein over the 20h period is thus different to the behaviour of the nucleic acids. Nevertheless, some protein was detectable in the supernatant. Over the first 13h there was less than 1 mg per 100 ml. At 18h, protein had increased to 5 mg per 100 ml and by 20h to 10 mg per 100 ml in the supernatant.

The occurrence of the bulk of protein synthesis during the first 3h correlates with growth as judged by viable count increase during this period. The release of protein into the supernatant after 18h is correlated with nucleic acid release and the decrease in the opacity of the cell suspension in the later stage of incubation in Resuspension Medium.

c. Lipid and Phospholipid

Lipid analyses were done on cells pooled from two flasks (200 ml) and lipids were extracted with chloroform-methanol according to Folch et al., (1957). Figure 20 records the range of increases measured in four separate experiments. The dry weight of extracted lipid was 1.5 mg per 100 ml at 0h and increased to 5.5 mg per 100 ml at 3h. After 20h the value had fallen to between 3.2 and 4.2 mg per 100 ml. The range of estimates was greater at 3h and 20h than for the 0h sample possibly due to the varying degree of extractibility of the lipid. The mean estimates gave the percent of bacterial mass as 5.6 at time of inoculation increasing to 9.0 at 3h. The value at 20h was 7.1%.

Figure 20



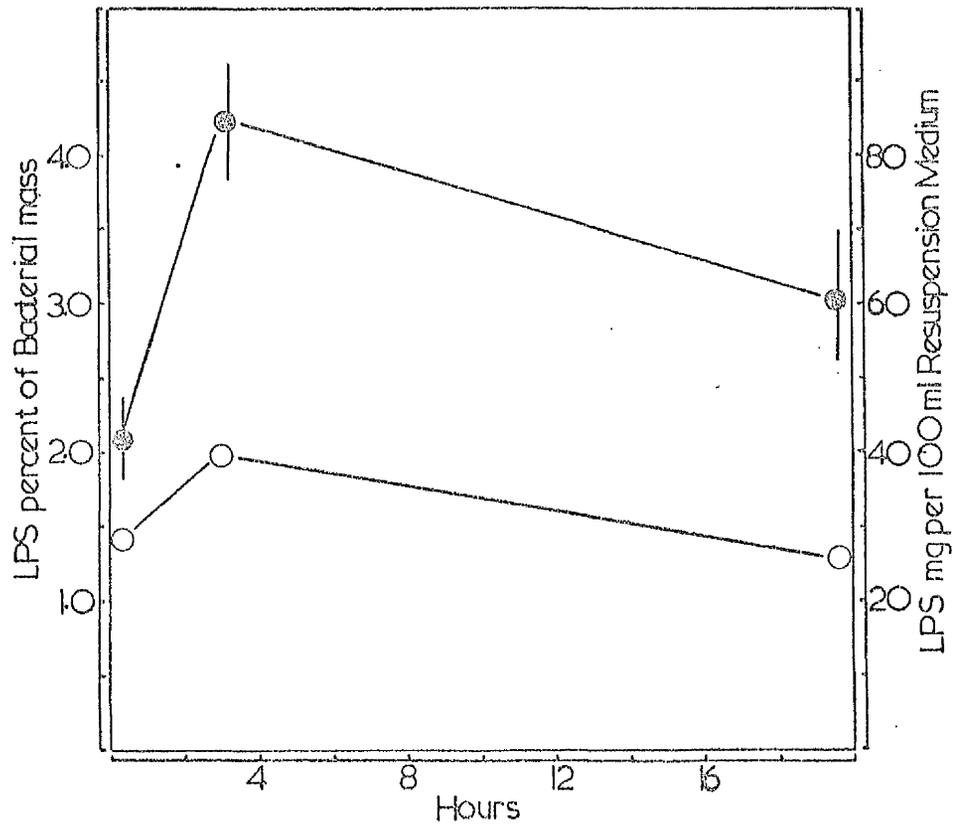
Lipid content of cells.

●, Values per 100 ml of Resuspension Medium

○, Percent contribution to cell mass

□, Level of phosphorus in extracted lipid.

Figure 21



Lipopolysaccharide content of cells.

●, Values per 100 ml of Resuspension Medium

○, Percent contribution to cell mass

Although an increase in total cell lipid was detected, the phospholipid component as estimated by lipid phosphorus analyses (Allen, 1940) did not show a parallel change. The 0h level of 4.1% phospholipid in P. aeruginosa compares well with that reported by Martin and MacLeod (1971). The phospholipid at 3h was 2% of the total lipid and the value fell to only 1% after 20h in Resuspension Medium.

d. Lipopolysaccharide

The cell-associated LPS was extracted from the cells harvested from 1,000 ml of Resuspension Medium using the phenol-water procedure of Westphal et al., (1952). The opalescent pellet of LPS was monitored for nucleic acid contamination by E<sub>260</sub> and was rendered free of this contamination by two cycles of ultracentrifugation.

Amounts of LPS were measured by the Johnson dichromate oxidation method with glucose as standard. The values expressed in Figure 21 show the range of estimates in three extractions. At 0h the level of LPS was 40 µg per 100 ml of culture. This increased to 85 µg per 100 ml during the first 3h, after which it fell to near the initial value. When consideration is given to the changes in bacterial mass, the percent contribution of LPS remained fairly steady; 0h, 0.14%, 3h, 0.2% and 20h, 0.13%. The latter corresponds to 60 µg per 100 ml. It should be remembered that this estimate was made on the cell pellet from Resuspension Medium at 12,000 x g and as such may contain some sedimentable LPS from the supernatant.

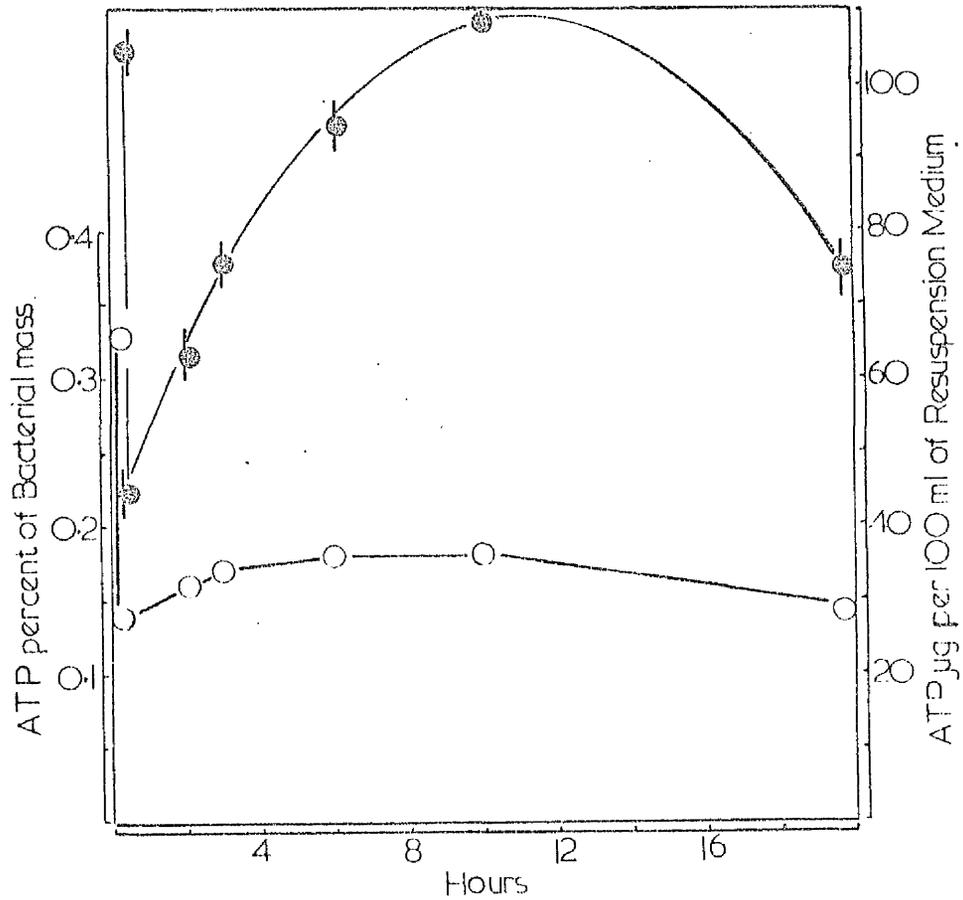
e. Adenosine triphosphate pool

The level of ATP in the cells was thought to be a valuable indicator of cell response to the low phosphate environment of the Resuspension Medium.

The assay procedure employed here is that described by Holms et al., (1972), a modification of the method of Cole et al., (1967) based on that of McLroy (1963) and using firefly lantern extract. The method has been shown by Holms to be a measure of intracellular ATP in bacteria. Samples (4 ml) from duplicate cultures were extracted with perchloric acid, which after neutralisation gave an aqueous ATP solution. This was stored at  $-20^{\circ}\text{C}$  for not more than 48h. The possible instability of ATP at this temperature was allowed for by observing stored standard ATP solutions which had been extracted from complete medium and processed in parallel with the unknown samples. Extracts were also made of cells from Y-T medium to detect the effect of the washing during preparation of the Inoculum Suspension. At the time of the assay, standard ATP solutions in water were used for calibration. The values of ATP in the bacterial extracts were corrected for the slight decay of the stored standards and the determinations at times before and during Resuspension Medium growth are given in Figure 22. In the 48h between extraction and measurement the standards had decayed by less than 5%.

The quantity of ATP in the equivalent cell weight of 1 flask at 0h observed before the cells were washed was 108  $\mu\text{g}$ . At time of inoculation (0h) the level was 45  $\mu\text{g}$ . This increased over the lag interval to 63  $\mu\text{g}$  per 100 ml at 3h, although in terms of percent of bacterial mass this represents a negligible change from 0.14 to 0.16. ATP synthesis continued until it reached a maximum at 10h. At this point it was 109  $\mu\text{g}$  per 100 ml, almost back at its pre-washing level and constituting 0.17% of the cell mass. When the cells had been in the presence of maximum pyocyanine for 6h (i.e. 20h) the ATP was 75  $\mu\text{g}$  per 100 ml and 0.14% of the bacterial mass.

Figure 22



Adenosine triphosphate pool in cells.

●, Values per 100 ml of Resuspension Medium

○, Percent contribution to cell mass.

Dual values at 0h represent levels before (upper points) and after (lower points) the Inoculum Suspension washing procedure.

It was concluded that the ATP remained relatively constant, between 0.14 and 0.17% of the total cell mass, through incubation of P. aeruginosa in the Resuspension Medium.

### C. Electrophoretograms of cell envelope proteins

Acrylamide disc gel electrophoresis was employed to examine the pattern of cell envelope proteins at two phases of the investigation.

In early experiments, envelopes were prepared by the method of Repaske (1958) using EDTA and lysozyme followed by DNA'ase addition. Whole cells were separated and the cell envelopes washed by centrifugation in 50 mM Tris. Preparations were sonicated before use for 10 min at 0°C and 0.1% SDS was added as dissociating agent to permit the proteins to migrate according to molecular size (Reynolds and Tanford, 1970). Electrophoresis was done in separating gels containing 11.7% (w/v) acrylamide (Davis, 1964) and molecular weight estimates were made by running proteins of known dalton value in parallel.

In later experiments pressure cell lysis (Biotec) was used to obtain envelopes in greater yield and at the characteristic time intervals for sampling the Resuspension Medium growth. The preparations were washed as described for the lysozyme preparations. Meanwhile the literature at that time contained a reference to an analysis of E. coli envelope proteins (Starka, 1971) using a more porous 7% acrylamide gel system. It was decided to employ this under the conditions described for 11% gel. The modification described by Grula and Savoy (1971) was also adopted, namely, addition of 0.1%  $\beta$ -mercaptoethanol to improve the separation of the resolved bands.

Figure 23 shows the electrophoretograms of lysozyme preparation of

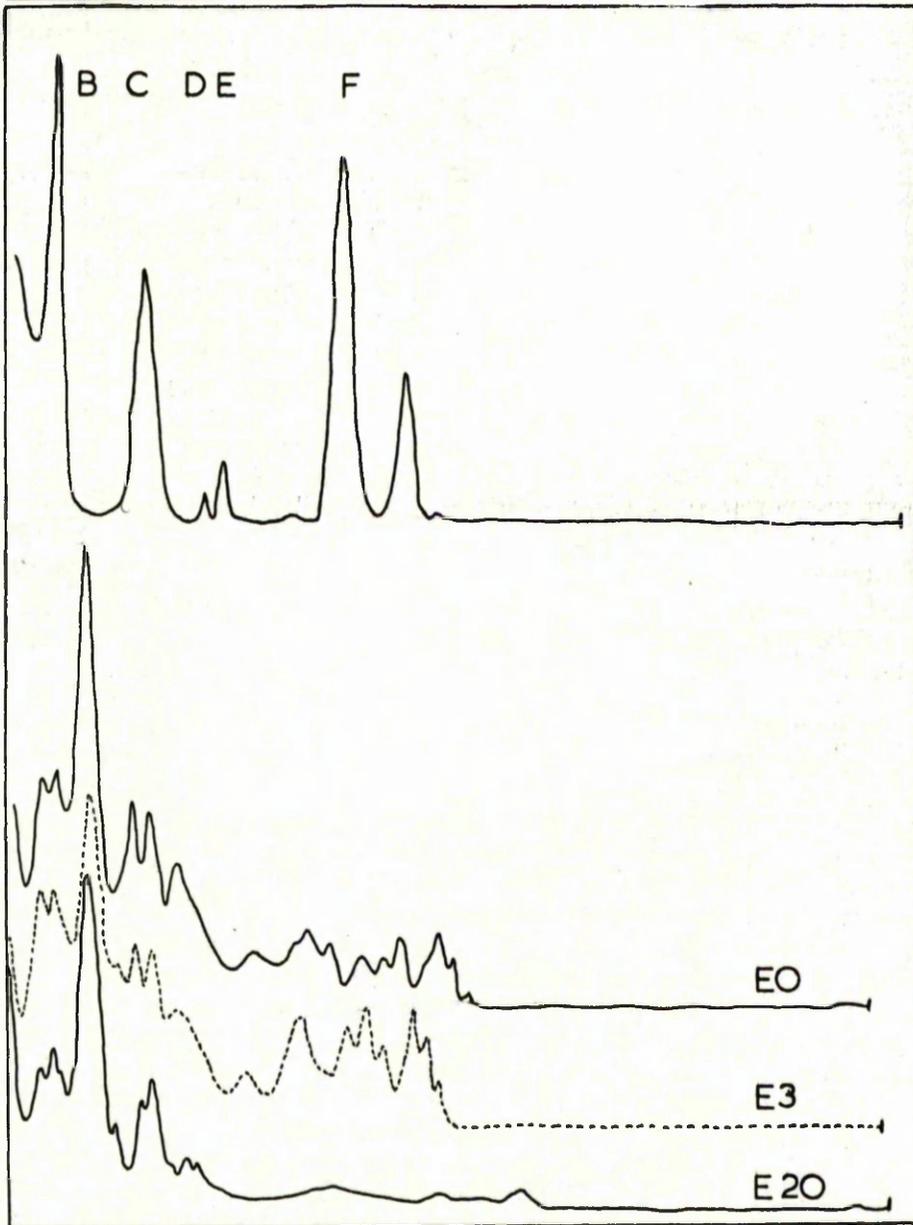


Figure 23: Electrophoretograms of cell envelope proteins in 11.7% acrylamide gels. Cell envelope proteins were prepared using cells removed from Resuspension Medium at zero time (E0), 3h (E3) and 20h (E20). The standard protein mixture described in the text was run in parallel (shown top): A = 12,400 daltons ; B = 17,800 daltons ; C = 25,000 daltons ; D = 45,000 daltons ; E = 45,000 daltons and F = 67,000 daltons.





Figure 24: Electrophoretograms of cell envelope proteins in 7.0% acrylamide gels. Cell envelope proteins were prepared using cells removed from Resuspension Medium at zero time (E0), 3h (E3) and 20h (E20). The standard protein mixture described in the text was run in parallel (shown top): B = 17,800 daltons ; C = 25,000 daltons ; D = 45,000 daltons ; E = 45,000 daltons and F = 67,000 daltons.



cell envelopes run on the 11.7% gel system. The major bands appeared at molecular weight 67,000 and a characteristic band was observed in all samples at 25,000 daltons. In the 0h sample a band at 17,000 daltons was a major component. This was lost after 3h growth of the cells in Resuspension Medium.

Figure 24 gives the resolution achieved with the more porous 7.0% gel, designed to improve separation of higher molecular weight components obtained from the mechanical rupture of the cells. The complex pattern of components in the 17,500 - 25,000 dalton region was essentially the same for 0h, 3h and 20h samples. In all preparations the component at 17,500 remained the same. The complex of peaks at 67,000 daltons was well resolved in the 0h and 3h samples although in the 20h sample it was less well defined.

A comparison of the two methods of preparation indicates that enzymic lysis gave fragments of smaller molecular weight than those obtained by mechanical rupture. The electrophoretic technique was used to detect the possible synthesis of new proteins specific for pyocyanine production. Since the results indicated degradation of components no further experiments were done.

#### D. Electron Micrographs of *P. aeruginosa* cells

Cells harvested at different times from Resuspension Medium were examined in the electron microscope both as thin sections and as negatively stained preparations.

Control cells which had been fixed in Y-T medium appeared as undamaged intact cells having dark stained cytoplasmic granules similar to

Table XVIII

Summary of Electron Micrograph observations on P. aeruginosa cells from Resuspension Medium

Sample	CONTROL	0h	3h	20h
(Plate number)	(1)	(3)	(6)	(10)
Anatomical characteristic				
CHROMATIN	Dispersed	Localised and fibrillar	Dispersed	Both dispersed and fibrillar
POLYSOMES	Dispersed	Localised	Dispersed and less numerous	Dispersed
POLYPHOSPHATE	Present	Absent	Absent	Absent
GENERAL	Normal morphology no leakage	Irregular morphology Some leakage and LPS release	More regular morphology Increased leakage and LPS Release	Normal morphology and circular envelope profiles Appreciable leakage from higher proportion of cells
				Some normal cells persist throughout

those reported as polyphosphate by Carrick and Berk (1971).

The washing procedure for the preparation of Inoculum Suspension resulted in cells of irregular shape and the release of some cellular components. The dark stained granules were absent. After the pyocyanine lag interval the cells were similar to the control although increased leakage was observed. The LPS released from the cell surface gave sectioned profiles similar to those obtained by Shands (1971) in purified LPS preparations isolated by phenol water extraction. Shands interpreted the disc and doughnut shaped appearance as bi-layer formation, each half consisting of core polysaccharide and covalently linked lipid.

At the end of incubation in Resuspension Medium the leakage and LPS release had continued giving a higher proportion of empty cell hulls observed as circular profiles.

The summary Table XVIII compares the major anatomical characteristics of control cells with those at 0h, 3h and 20h as observed in low magnification survey micrographs. It was evident that cell leakage and disintegration of LPS-Lipid-Protein complex was progressive with time of incubation.

All electron micrographs were kindly provided by Dr. J.H. Freer of this department.



Plates 2 - 3. Electron micrographs of thin sectioned whole cells of P. aeruginosa fixed with glutaraldehyde in the Y-T growth medium before washing as outlined in Inoculum Suspension preparation.

Plate 2 shows a low power survey micrograph of undamaged whole cells from a stationary phase culture. X 34,500.

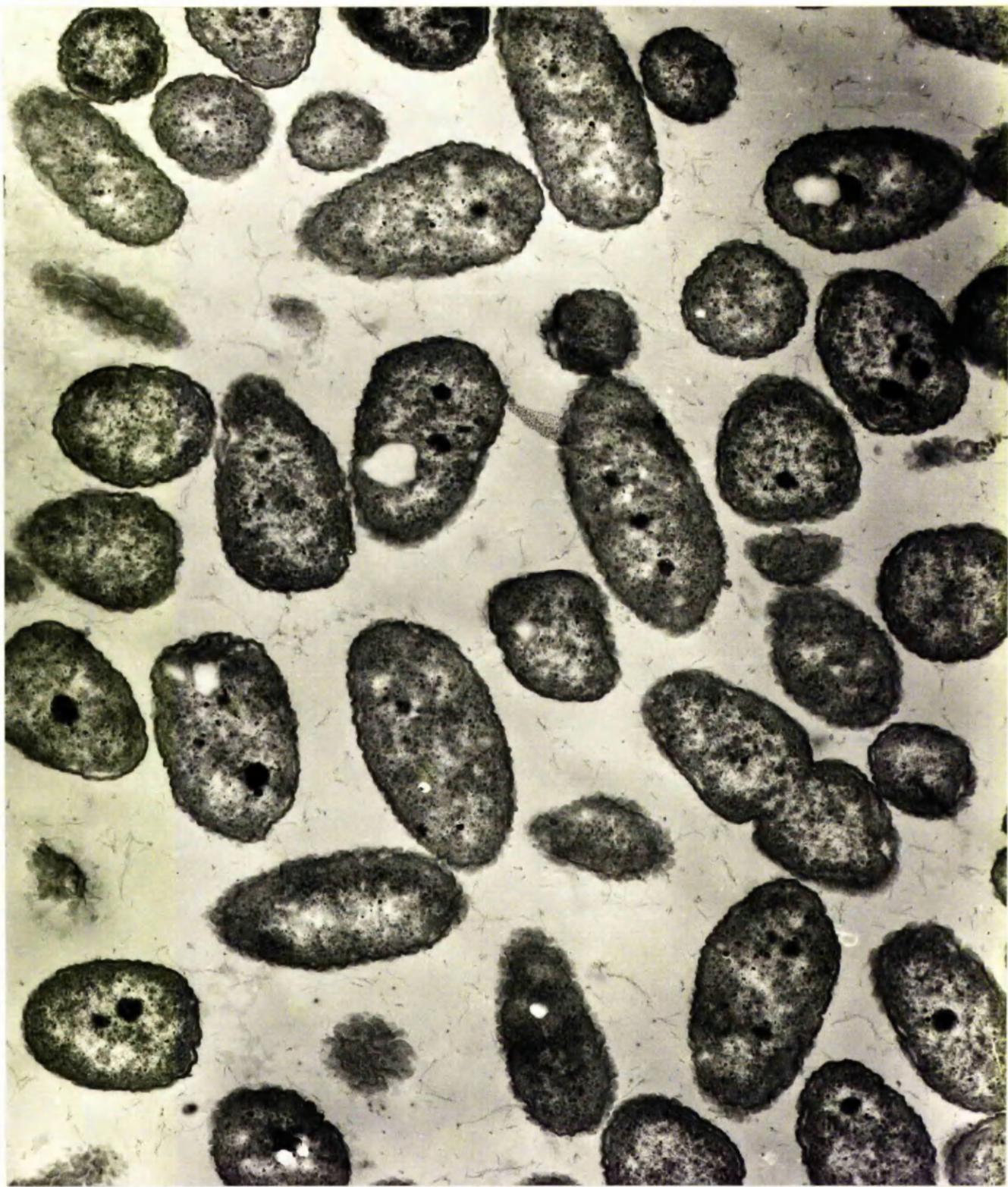
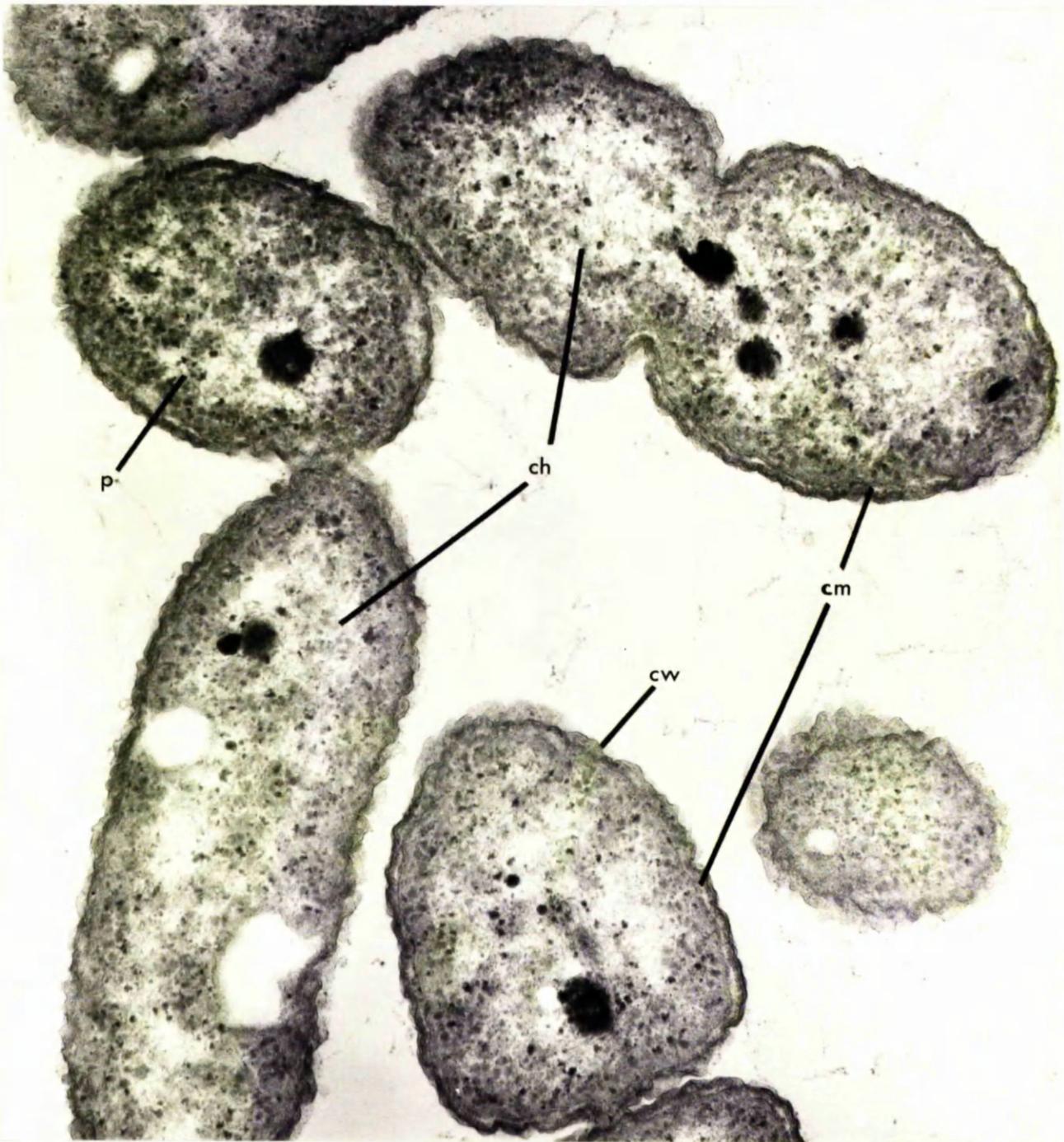




Plate 3 shows the details of control cell structure. Note the characteristic appearance of the chromatin (ch), the cell wall (cw) and the cytoplasmic membrane (cm). The darkly stained areas of the cytoplasm probably represent polysomes (p) and the larger densely stained areas are of unknown composition.

Electron transparent areas in the cytoplasmic region of the cell are often seen to be membrane bound and probably originate from invaginations of the plasma membrane. X 72,600.





Plates 4 - 6. Electron micrographs of thin sectioned whole cells of P. aeruginosa fixed with glutaraldehyde in the Resuspension Medium immediately after inoculation (0h).

Plate 4 is a low power survey micrograph. Note the irregular outline of the cells and the presence of several cells showing extensive leakage. The nuclear material (n) appears more fibrillar and localised than that observed in the control cells. The cytoplasmic polysomes (p) are seen more closely packed than in the control cells. The darkly stained areas noted previously were largely absent. X 34,500.





Plates 5 and 6 show cells from Oh at higher magnification.

Plate 5 shows evidence of LPS peeling from the outer layer of the cell envelope (1) and the presence of some free LPS in the form of sheets (s) and vesicles (v). X 72,600.

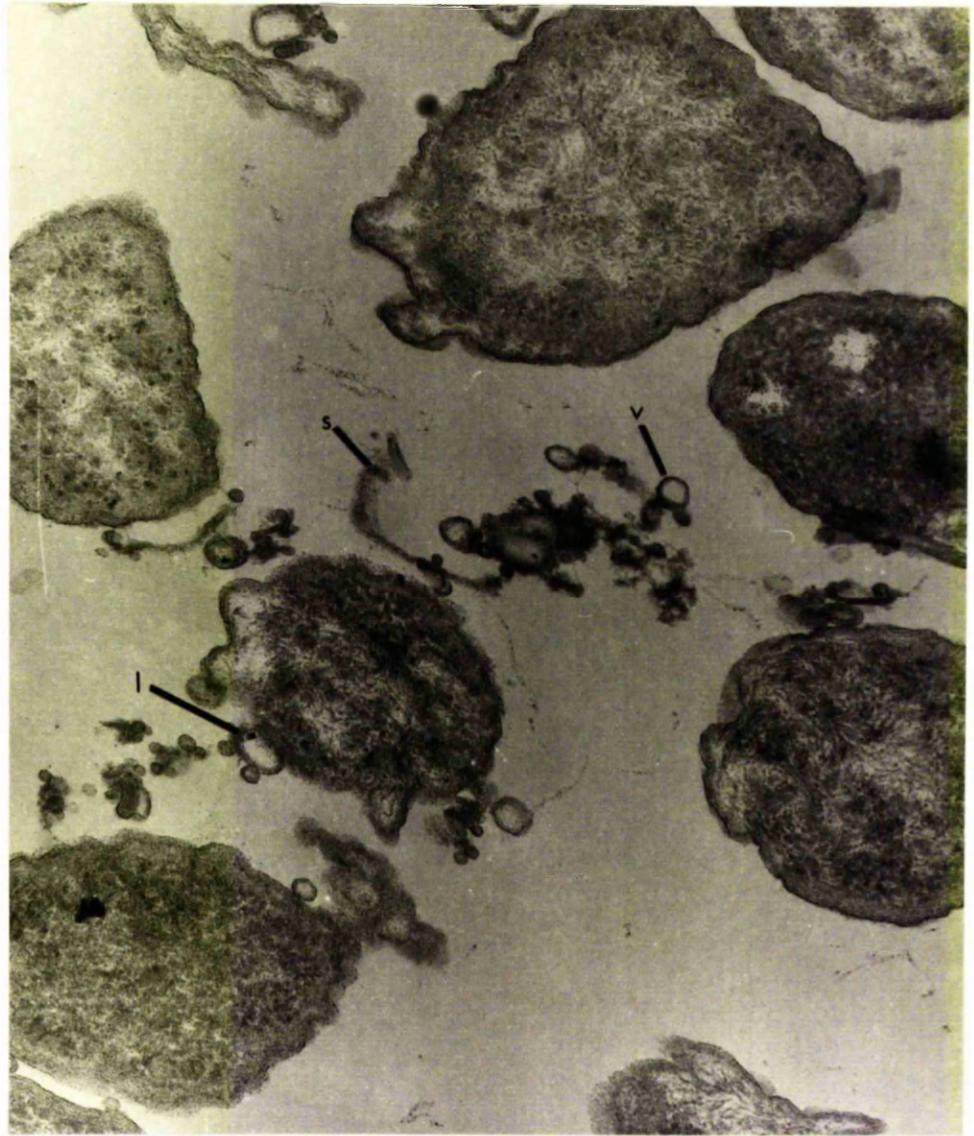
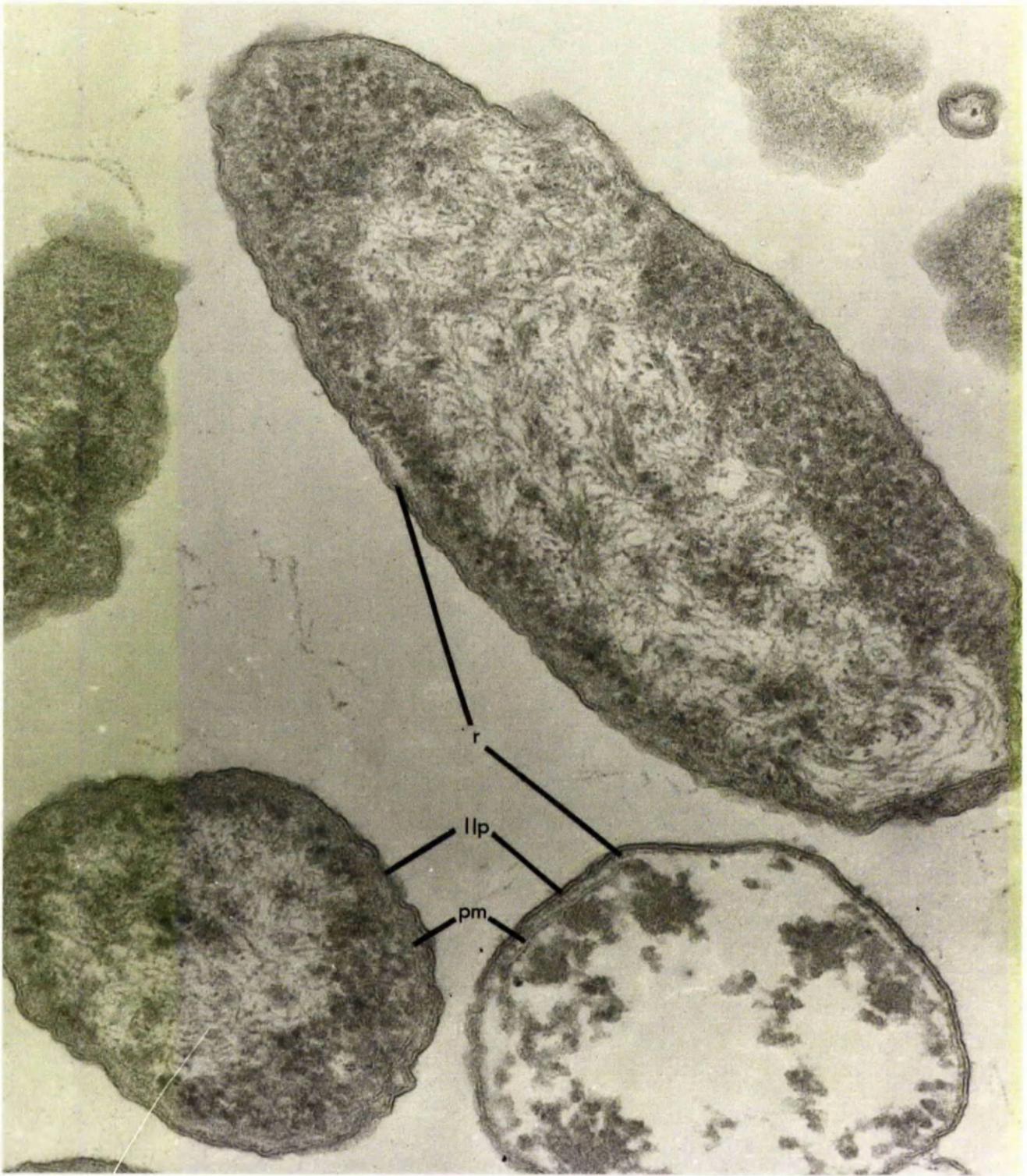




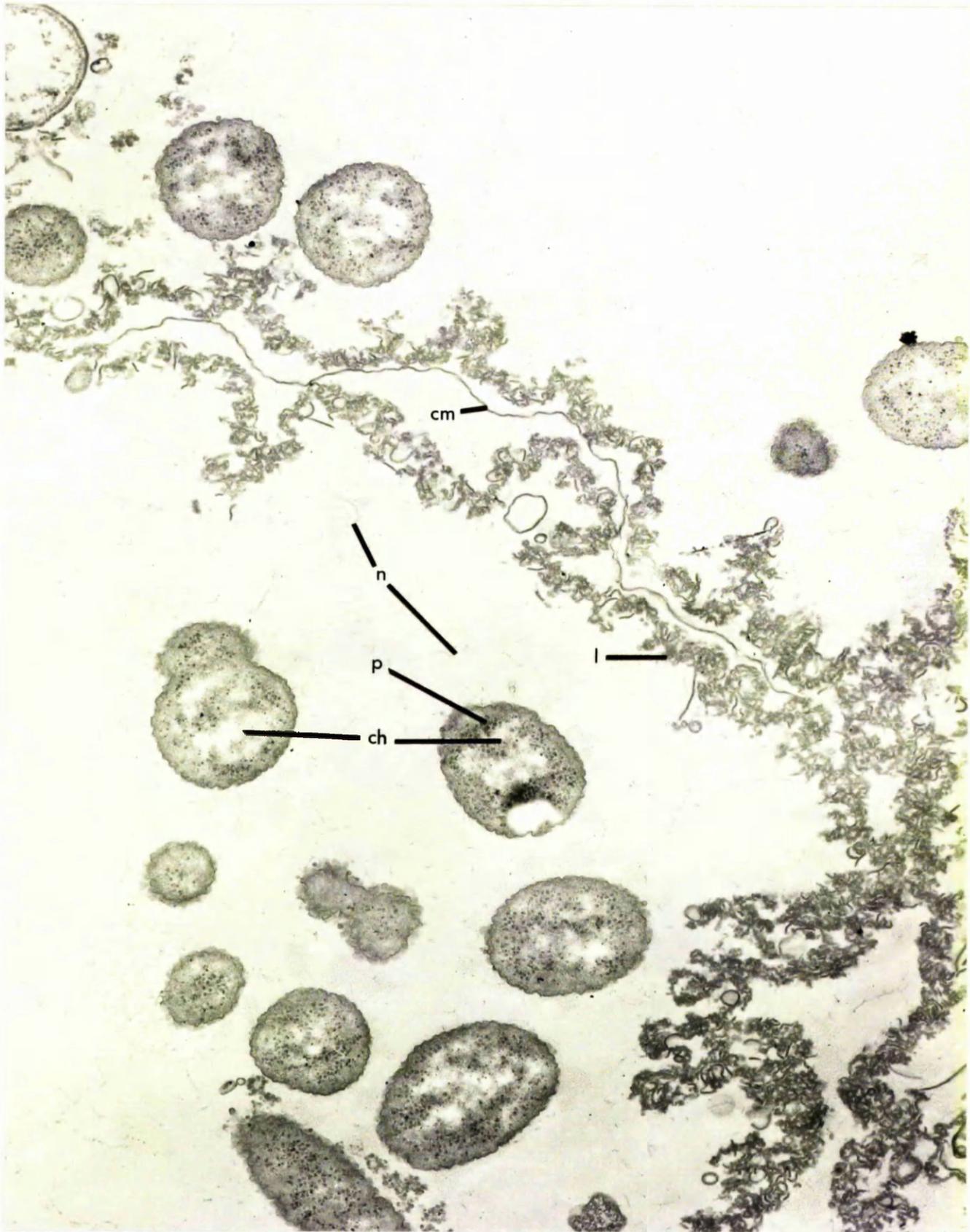
Plate 6 illustrates detail of the cell envelope structure :  
the outer triple layered profile of LPS-Lipid-Protein (llp)  
complex is clearly visible whereas the plasma membrane  
profile (pm) appears discontinuous presumably due to  
fragmentation during lysis. The rigid layer (r) is  
discernable in the cell envelope profile and appears as a  
diffuse band immediately interior to the outer element of  
the wall. X 117,300.

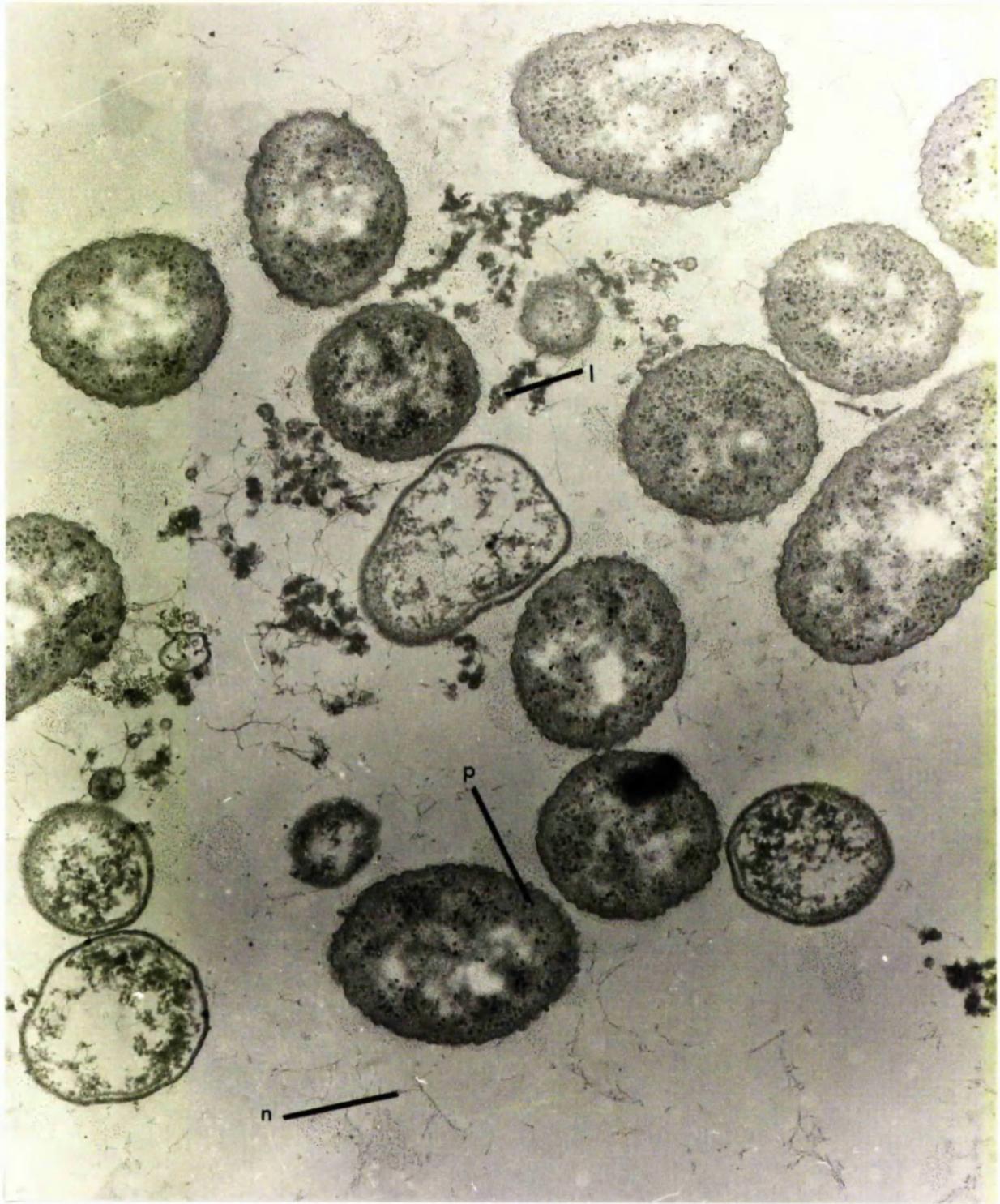




Plates 7 - 10. Electron micrographs of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium at the end of the lag interval (3h) with glutaraldehyde.

Plates 7 and 8 show low power survey micrographs. Note the dispersed appearance of the chromatin (ch) similar to that observed in the control cells (Plate 2). Polyosomes (p) are present as in the control although less numerous and embedded in an amorphous material. Increased disruption is evidenced by the amount of free envelope material present in the preparation - nuclear material (n), cytoplasmic membrane fragments (cm) and free LPS (l). X 34,500 and 42,300 respectively.

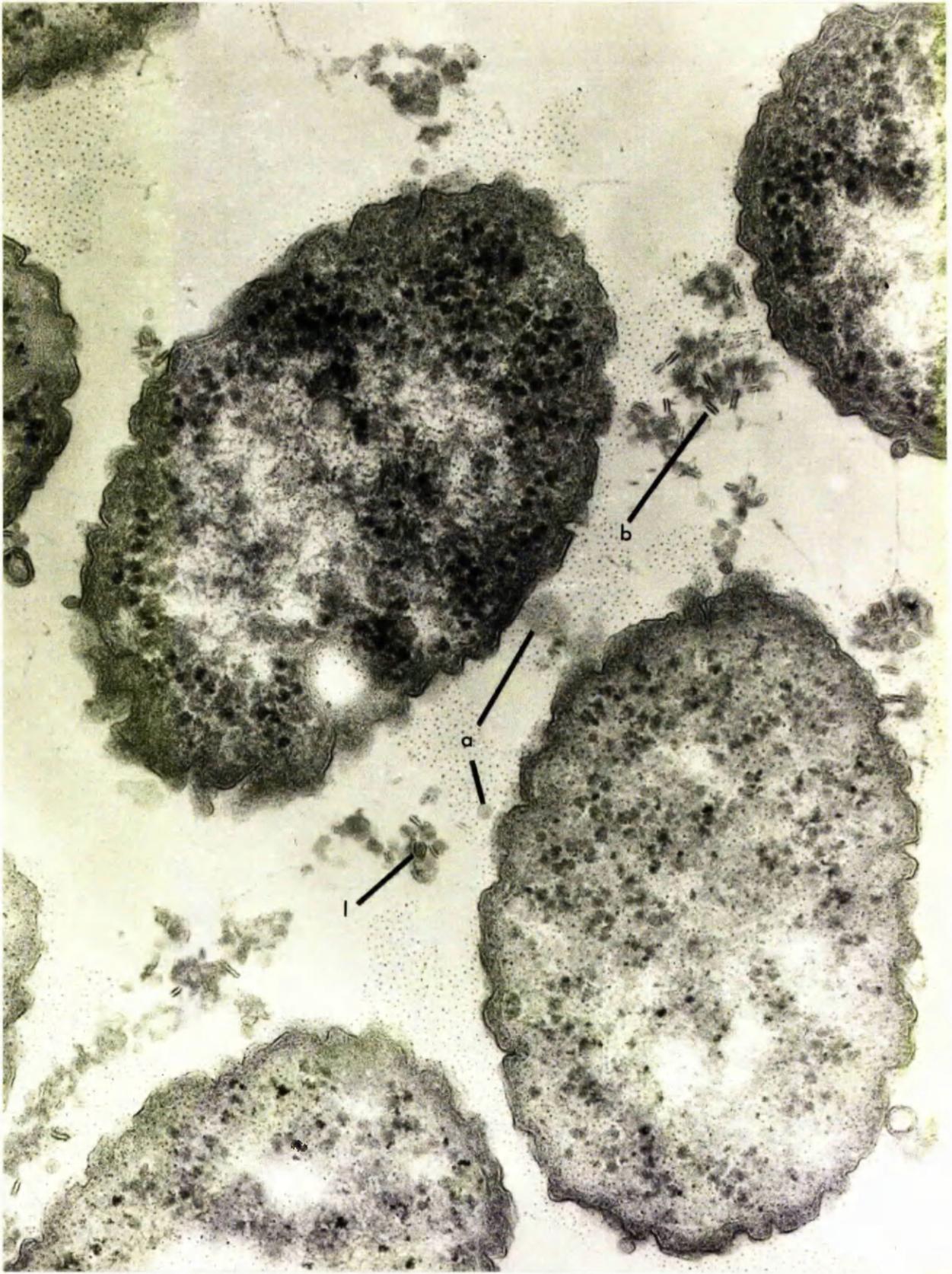


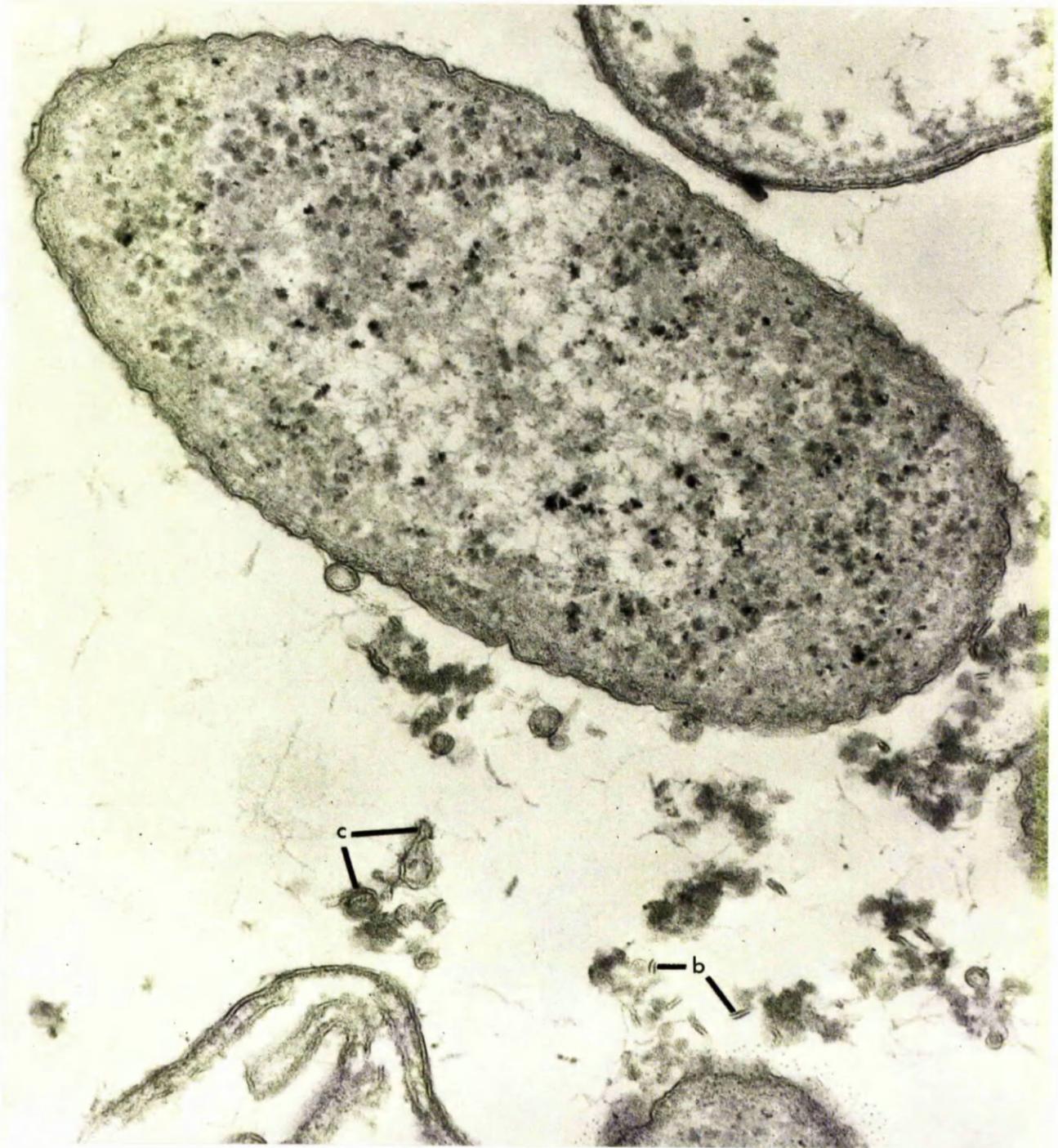




In Plates 9 and 10 note the irregular cell wall profile in comparison to that in the control and Oh samples. The release of LPS vesicles from the surface of the cell envelope and the presence of numerous characteristic LPS complex profiles are observed.

The sections of LPS appear as either flattened discs viewed face on (a) or when these are sectioned at a variety of angles as trilaminar structures (b) or discs and trilaminar profiles viewed at an angle (c). X 117,300.

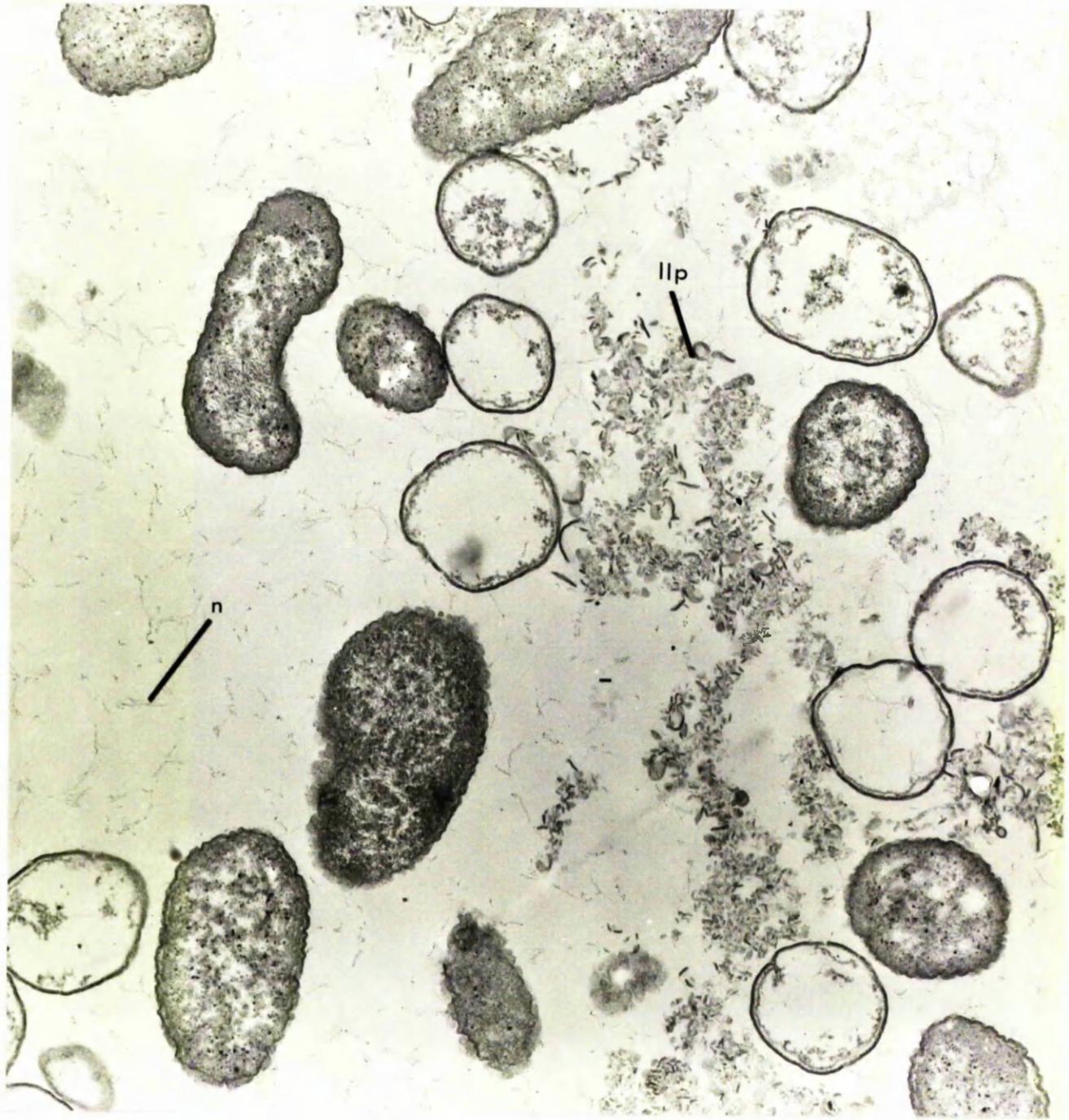






Plates 11, 12 and 13. Electron micrographs of thin sectioned whole cells of P. aeruginosa fixed in Resuspension Medium after twenty hours (20h) with glutaraldehyde.

Plate 11 is a low magnification survey micrograph. Note the empty cell hulls and the presence of released cytoplasmic material -- nuclear material (n) and the LPS-Lipid-Protein (llp) complex. The predominance of circular profiles in the cell envelope of empty cells suggests a loss of rigidity in the cell wall, possibly resulting from the activity of autolytic enzymes. X 34,500.





In Plates 12 and 13 the distinctive nuclear appearance is not the same in every cell, some cells showing the well defined fibrillar chromatin (ch) whereas others show dispersed and ill defined chromatinic areas (ch'). Polysomes (p) are randomly dispersed in the cytoplasm similar to those observed in the control cells. Cell hulls are more frequently observed as is the release of LPS (l) seen in characteristic profile. X 72,600, X 117,300 respectively.







Plate 14. Electron micrograph of negatively stained 3h envelopes of P. aeruginosa after extensive washing in 50 mM Tris X 72,600.

The sheets of material consist of a mixture of LPS-Lipid-Protein complex and fragments of plasma membrane. There is no indication of contamination with cytoplasmic material.



## SECTION IV : INHIBITION OF PYOCYANINE PRODUCTION

### A. Effect of phosphate addition

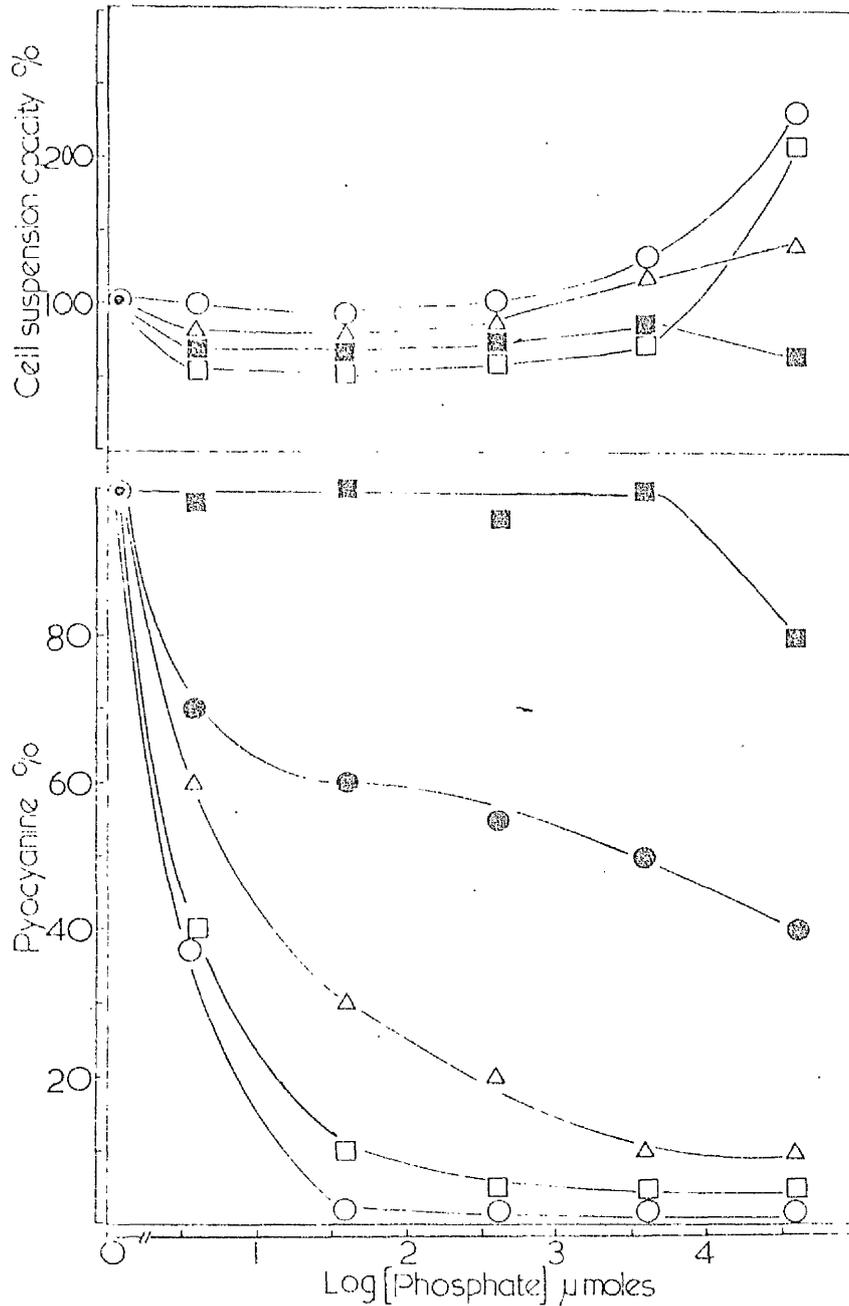
A characteristic feature of the Resuspension Medium is its lack of phosphate. Results described under 'Composition of Resuspension Medium' (see page 103 ) indicated that when phosphate at 40 mM (concentration in Y-T medium) was present then pyocyanine was not produced.

In order to explore this effect further, experiments were done in which various phosphate concentrations were added to Resuspension Medium at various intervals during the 20h incubation period. Equimolar quantities of  $K_2HPO_4$  and  $KH_2PO_4$  were used to give a range of logarithmically spaced concentrations from 40  $\mu$ M to 400 mM. These levels of phosphate expressed as  $\log_{10} [PO_4^{3-}]$ , from 0.602 to 4.602 were added to the Standard Resuspension Medium at the times specified in Figure 25 . The growth medium concentration was represented by 40 mM phosphate. The figure shows the values, at 20h, of the cell suspension opacity and the pyocyanine production as a percent of the control. At the times chosen the quantities of pyocyanine which had already been produced in the supernatant were as follows: 0h, 0%; 3h, 10%; 6h, 20-30%; 10h, 60 - 70%; and at 12h, 90-100%.

By adding 40  $\mu$ M phosphate at 0h and at 3h the pyocyanine production reached only 40% of its normal level. Addition at 6h and 10h resulted in 60% and 70% pyocyanine production respectively. The 10h cells seemed to be inhibited in the production of more pyocyanine by the presence of 40  $\mu$ M phosphate.

It is possible that further synthesis took place followed by

Figure 25



Cell suspension opacity and Pyocyanine production in the presence of added phosphate.  $\circ$ , Phosphate added at 0h;  $\square$ , at 3h;  $\triangle$ , at 6h;  $\bullet$ , at 10h and  $\blacksquare$ , at 12h. The same symbols express the final cell suspension opacity resulting from phosphate addition at the specified time. Values at 10h and 12h coincided. To serve as standard for comparison untreated control was assigned the value 100% for Pyocyanine and final opacity level.

degradation of pyocyanine to give the final levels observed. 12h addition of phosphate at levels below 40 mM did not affect pyocyanine production.

The addition of 400  $\mu$ M phosphate and above, at 0h and 3h resulted in complete inhibition of pyocyanine production. The 400  $\mu$ M concentration of phosphate added at 6h and 10h prevented further pyocyanine synthesis as did 4 mM at 6h. By adding 4 mM at 10h however, the final pyocyanine level was lower than that at 10h and degradation may have occurred.

A similar effect was noted when 40 mM was added at 6h and 10h and the further pyocyanine production was prevented while levels fell from 20%  $\rightarrow$  10% and from 60%  $\rightarrow$  50% respectively. This degradation was observed by treatment of the 12h cells only with high phosphate concentration i.e. 400 mM.

In summary, at early stages in pyocyanine production, 0h, 3h and 6h, the cells were sensitive to 40  $\mu$ M phosphate and this caused what appeared to be instantaneous pyocyanine inhibition. Higher levels of phosphate were necessary to cause the degradation of pyocyanine by older cells.

The differences in 20h cell suspension opacities in the presence of phosphate are shown in the top part of Figure 25 . The results for 10h and 12h cells were identical.

It was noted that concentrations of between 4 mM and 40 mM caused opacity increase for 0h and 6h cells. Cells at 10h and 12h required 400 mM phosphate to give greater opacity than the control. The 3h cells seemed less able to use 40 mM for growth although 400 mM did stimulate the suspension opacity. In general, where increased opacity of the cell suspension was observed it coincided with reduced levels of pyocyanine in the supernatant.

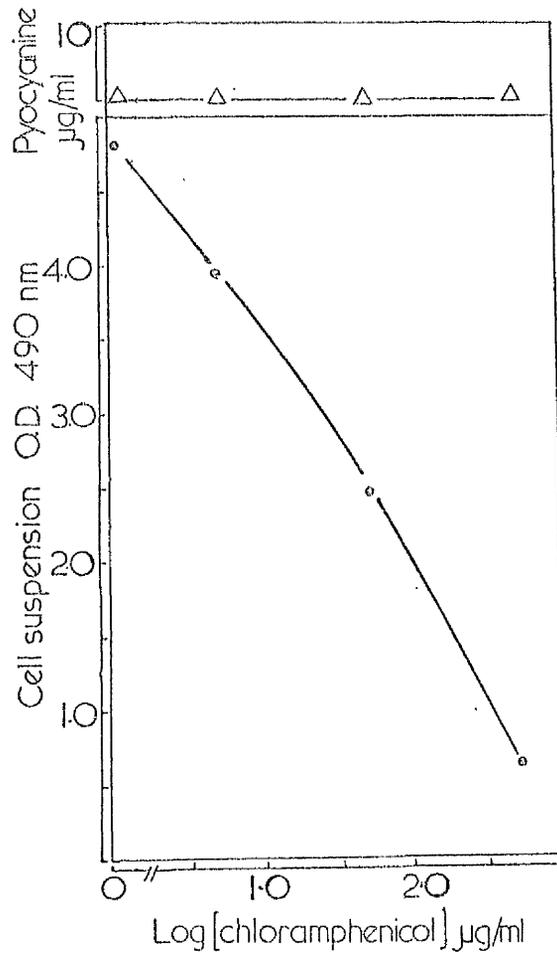
### B. Effect of chloramphenicol addition

The observation that protein synthesis accompanied the growth of cells in the Resuspension Medium during the interval from 0h to 3h suggested a specific requirement for certain enzymes before pyocyanine could be produced.

Chloramphenicol as an accepted inhibitor of protein synthesis was added to the Resuspension Medium to determine if it would affect the production of pyocyanine. Before determining the test levels it was necessary to establish the Minimum Inhibitory Concentration (MIC) of chloramphenicol for this strain of P. aeruginosa. Cells from the Inoculum Suspension were used to give a 25% inoculum in a Resuspension Medium with 40 mM phosphate added, to constitute a full growth medium. Chloramphenicol was tested at 6 µg/ml, 60 µg/ml and 600 µg/ml as shown on the abscissa of Figure 26. The 20h cell suspension opacity values in these flasks and in the control without chloramphenicol are given. 50% reduction in O.D.<sub>490</sub> resulted from the presence of chloramphenicol at 60 µg/ml and this was accepted as MIC. It was noted that when growth in the phosphate-supplemented Resuspension Medium was inhibited, pyocyanine production was not observed.

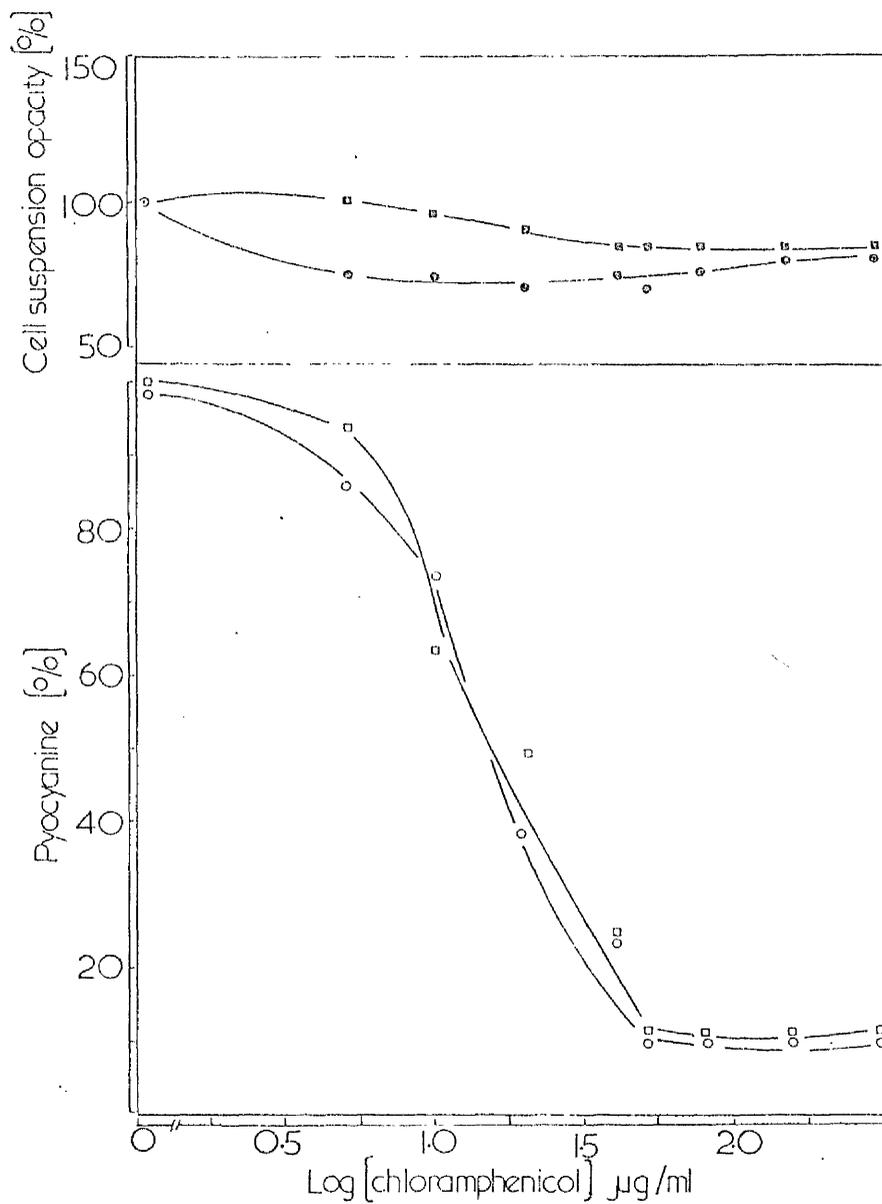
A range of chloramphenicol concentrations close to the MIC of 60 µg/ml were chosen and added to the Standard Resuspension Medium at zero time and at 3h. The effect on pyocyanine production and cell suspension opacity are shown in Figure 27 where chloramphenicol is expressed on a log scale  $60 \mu\text{g/ml} = 1.78$ . From the graph of percent pyocyanine production it is noted there is little difference in the sensitivity of 0h and 3h cells to chloramphenicol. At the lowest concentration tested, 5 µg/ml, the 0h cells gave a final pyocyanine level of 85%. With 3h cells the final level was 95%.

Figure 26



Minimum inhibitory concentration of chloramphenicol for growth in phosphate - supplemented Resuspension Medium.  $\odot$ , represents the final cell suspension opacity resulting from 0h treatment of the cells.  $\Delta$ , represents the Pyocyanine produced by the treated cells.

Figure 27



Cell suspension opacity and Pyocyanine production in the presence of added chloramphenicol.  $\circ$ , Represent the Pyocyanine production resulting from 0h treatment of the cells and  $\square$ , from 3h treatment. The same symbols express the final cell suspension opacity resulting from chloramphenicol treatment. To serve as standard for comparison the control was assigned the value 100% for Pyocyanine and final opacity level.

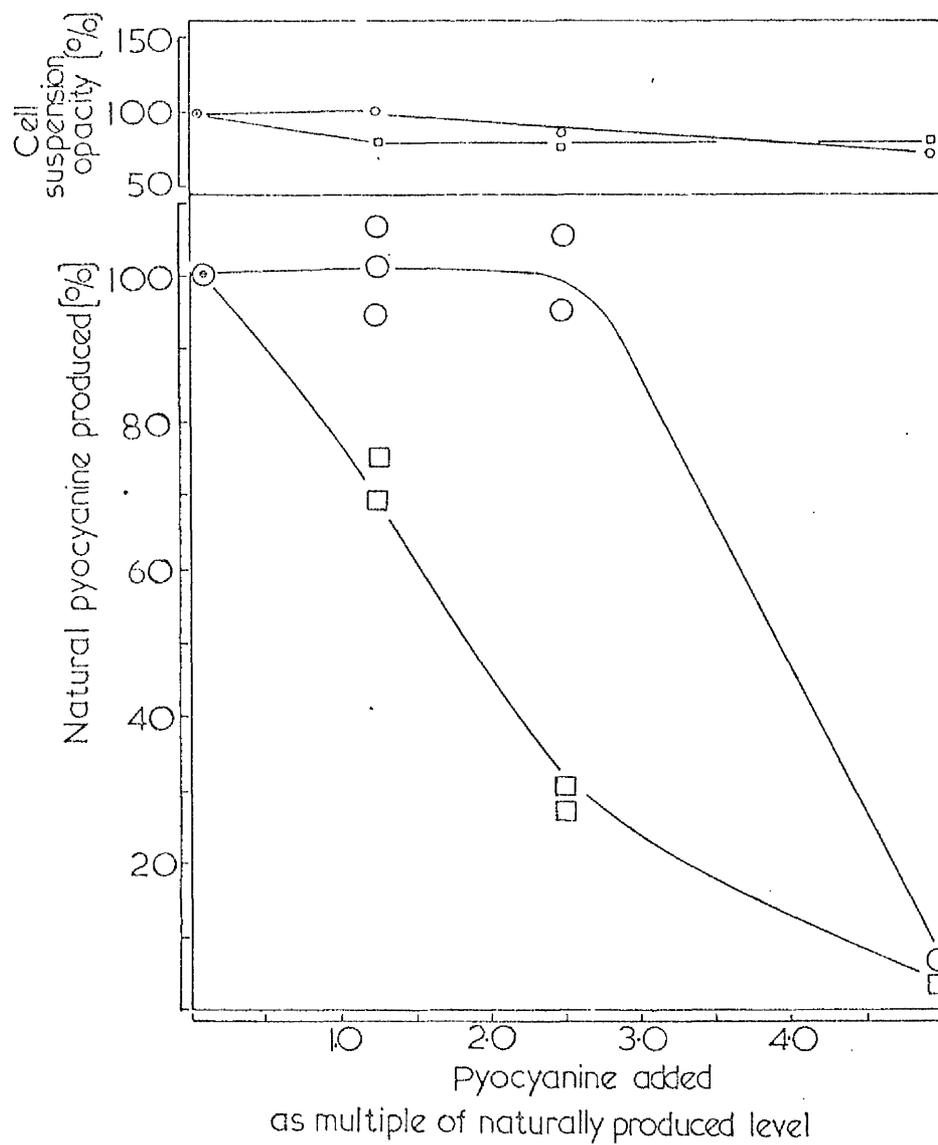
of the control. 20  $\mu\text{g}/\text{ml}$  of chloramphenicol was sufficient to cause 50% reduction in pyocyanine production in both the 3h and 0h cases. The MIC of chloramphenicol caused pyocyanine to fall below the minimum detectable level in these experiments ( $< 10\%$ ). This was effectively complete inhibition of pyocyanine production.

The cell suspension opacity values illustrated as percent of control in the upper part of Figure 27 show that with 0h cells, the opacity fell to 75% as a result of the addition of 10  $\mu\text{g}/\text{ml}$ . It remained at this lowered level with other chloramphenicol concentrations. The opacity of 3h cells was higher at 90% and was only lowered by treatment with the MIC of chloramphenicol to 85%. Although 0h cell opacities fell to lower levels than those at 3h there was no similar divergence in pyocyanine production. Pyocyanine production therefore appeared to be more sensitive to suppression by chloramphenicol than was growth in phosphate-supplemented Resuspension Medium. After the three hour lag interval, during which maximum increase in protein has been shown to occur, chloramphenicol also caused inhibition of pyocyanine production.

#### C. Effect of adding synthesized pyocyanine

To determine whether or not the pyocyanine producing system was subject to feedback inhibition by its own end product, purified pyocyanine was added at 0h and at 3h in multiple units of the quantity normally found in Standard Supernatant. The quantities of pyocyanine produced biologically were estimated by  $E_{690}$  after account had been taken of the amount added. The results are shown in Figure 28. The 1.25 unit and 2.5 unit levels of pyocyanine, when added to 0h cells, did not affect subsequent pyocyanine production by the culture. However when pyocyanine was added at the 5 unit

Figure 28



Cell suspension opacity and Pyocyanine production in the presence of added Pyocyanine. O, Pyocyanine addition at 0h and □, at 3h. To serve as standard for comparison the control was assigned the value 100% for Pyocyanine and final opacity level.

level, pigment production by 0h cells was reduced to a negligible quantity. The assay was performed on suitably diluted samples. By treating 3h cells in a similar way the response observed was quite different. 1.25 units of pyocyanine caused significant reduction in the subsequent production of the pigment to 70% of the control value. 2.5 units of pyocyanine caused even greater depression and only 20 to 30% of the normal amount was found to have been produced. As observed in the case of 5 units added at 0h, the 3h cells were completely inhibited in their pyocyanine production.

Changes in the level of cell suspension opacity were small. The addition of 1.25 units caused 3h cell suspension opacity to fall to 85% whereas the 0h cells had their opacity unaltered. The opacity at other points in the upper part of Figure 28 indicate that values were never lowered by more than 15%. No stimulation of growth was observed.

It can be deduced that both cell types 0h and 3h are sensitive to extracellular pyocyanine. Those cells on the point of producing the pigment are more sensitive to added pyocyanine and respond by producing lower than normal levels.

#### SECTION V : THE EFFECT OF PYOCYANINE ON P. AERUGINOSA

##### A. The endogenous respiratory activity of whole cells

The preliminary observations on cells described in the section 'Pyocyanine Production using Solid and Liquid Media' (see page 82 ) indicated that for cell suspensions of equivalent opacity, the rate of endogenous metabolism measured in the Oxygraph varied with the type of medium used. A summary of the observations made is given in Table XIX. The

Table XIX

Summary of observations on P. aeruginosa cells grown on solid media

Observation	Agar Medium <sup>a</sup>	
	Glucose-ammonium salts	Nutrient-peptone
Growth	+	+
Pyocyanine production	-	+
Endogenous respiration <sup>b</sup> ( $\pm$ ISD)		
(i) Untreated cell suspension	1.0 ( $\pm$ 0.1)	2.0 ( $\pm$ 0.1)
(ii) Pyocyanine treated cell suspension	1.3 ( $\pm$ 0.1)	2.2 ( $\pm$ 0.1)

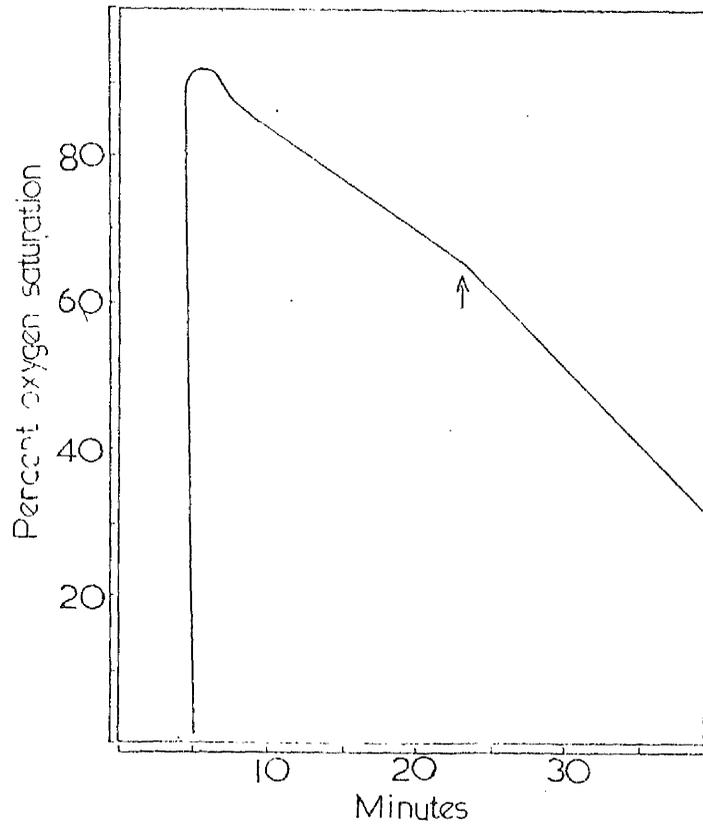
a, Composition in Appendix

b, Relative to arbitrary scale

endogenous activity of cells grown on peptone nutrient agar was double that of cells from glucose-ammonium salts medium. The two media were distinct in that the former supported pyocyanine production and the latter did not.

When the two cell types were treated with 50  $\mu\text{g}$  of pyocyanine, those which had produced the pigment were stimulated in oxygen consumption rate by 10% and those which had not given pigmented growth were also stimulated, but to a greater (30%) extent.

The implication that stimulation of the cells by pyocyanine was related to the production of the pigment led to experiments investigating the part played by pyocyanine in the metabolism of cells during their growth in Resuspension Medium. The cell yield from 1 ml of Resuspension Medium was harvested and washed three times in sterile physiological saline. The suspension at 10 times original concentration was stored at 2°C for 24 h to establish a steady state of endogenous metabolism. Before use, the cell suspension was restored to 37°C and diluted 1:5 with 50 mM Tris buffer, also at 37°C, in the Oxygraph chamber. Figure 29 shows a typical trace of oxygen consumption by 3h cells. When the system had equilibrated, estimates were made over the 10 min steady rate of oxygen consumption. It was shown by calibration of the apparatus that for the suspending buffer solution, the dissolved oxygen at 37°C was the same as that for Ringer's solution. The latter contains 5  $\mu\text{l}$  of oxygen per ml at 100% saturation and ambient barometric pressure. The change of 10% in Figure 29 in the saturation of 5 ml sample therefore means that  $\frac{5 \times 5 \times 10}{100} = 2.5$   $\mu\text{l}$  of oxygen consumed in the 7 min interval studied. This gives a rate per ml of cell suspension equivalent to 21.4  $\mu\text{l}$  of oxygen per hour. The arrow in Figure 29 represents the point of addition of unit level of pyocyanine to this sample of 3h cells.

Figure 29

Oxygraph trace of 3h cell respiratory rate with added Pyocyanine  
(point of addition arrowed)

The effect was a more rapid rate of oxygen consumption. The observed 20% change in 9.5 min represents a rate of 31  $\mu$ l of oxygen consumed per h.

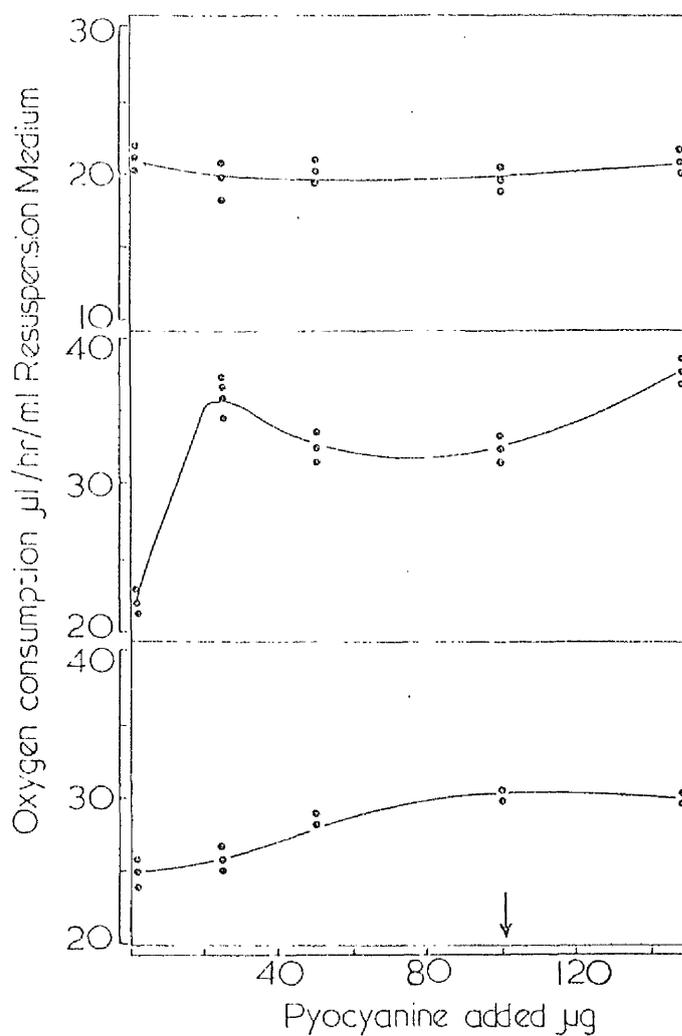
The effect of treating other cell types from the Resuspension Medium, with a variety of pyocyanine concentrations close to unit level, is shown in Figure 30. The 3h result quoted above is shown in the central part of the figure and unit level of pyocyanine is arrowed on the pyocyanine scale. As shown in the top part of the figure, 0h cells were not stimulated above their untreated rate of 21  $\mu$ l of  $O_2$ /h. The addition of pyocyanine up to 1.5 units gave rates which varied between 18 and 22  $\mu$ l of  $O_2$  per h.

In the case of 3h cells 0.25 units (25  $\mu$ g) of pyocyanine caused stimulation of the oxygen consumption from 21 to between 33 and 37  $\mu$ l of  $O_2$ /h. Stimulation was also observed with 0.5 and 1.0 unit level of pyocyanine although rates increased to only 31 - 34  $\mu$ l of  $O_2$ /h. 1.5 units caused a consumption of between 35 and 37  $\mu$ l of  $O_2$ /h which was similar to that observed with 0.25 units.

20h cells also had increased respiratory rates on treatment with pyocyanine. Maximum stimulation from the original of 25  $\mu$ l of  $O_2$ /h to 30  $\mu$ l of  $O_2$ /h was observed with 1.0 and 1.5 units of pyocyanine. 0.25 and 0.50 units gave rates of 26 and 28 respectively.

The overall results show that pyocyanine stimulated the respiration of 3h and 20h but not 0h cells. The larger increases observed with 3h cells than with 20h are in agreement with the preliminary observation of stimulation in cells which had not produced pyocyanine. The marked activity of 0.25 units in the 3h sample may indicate selectivity of the level which is closest to the one which the cells experience as a result of their own production. The 20h cells responded best to unit level of pyocyanine treatment.

Figure 30



Effect of added Pyocyanine on the rate of endogenous respiration of cells from Resuspension Medium (unit level of Pyocyanine arrowed). Cells were removed from Resuspension Medium at 0h, 3h and 20h. The suspension of washed cells was stored for 24h before testing respiratory rate.

The possibility that pyocyanine was being consumed as a substrate in these experiments is unlikely as the 3h response is not linear with concentration. The variation in untreated respiratory rates 0h, 21; 3h, 22 and 20h, 25 ( $\mu\text{l O}_2/\text{h}$ ) could be caused by several factors:

- (a) the rate of consumption of stored metabolites
- (b) response of the cells to washing and cold storage, or
- (c) the availability of exogenous metabolites through cell lysis.

B. Cell envelope bound succino-oxidase

As outlined above, a variety of effects could be responsible for the observed effects of pyocyanine on whole cell endogenous metabolism. Campbell et al., (1962b) demonstrated that both succinate dehydrogenase and succino-oxidase activity was associated with particulate fractions of the cell hull in P. aeruginosa. As the intracellular reserve material is contained in the cell cytoplasm there was an opportunity to investigate the effects of pyocyanine on the enzyme system of the host organism using a single substrate.

Oxygraph experiments as described for whole cell respiration were performed using the cell envelope suspension prepared previously (see page 55 ). The suspension was used undiluted in volumes equivalent to 20 ml of Resuspension Medium cell yield. Substrate (20 mM disodium succinate) was added to give a final 4 mM concentration in a total reaction volume of 5 ml made up with Tris buffer at 37°C.

The rate of oxygen consumption by the cell envelope preparation without added substrate was negligible. In Table XX the rates with added substrate are given for three envelope preparations measured in duplicate. The low rate of activity caused the trace to deviate from linearity below 60%

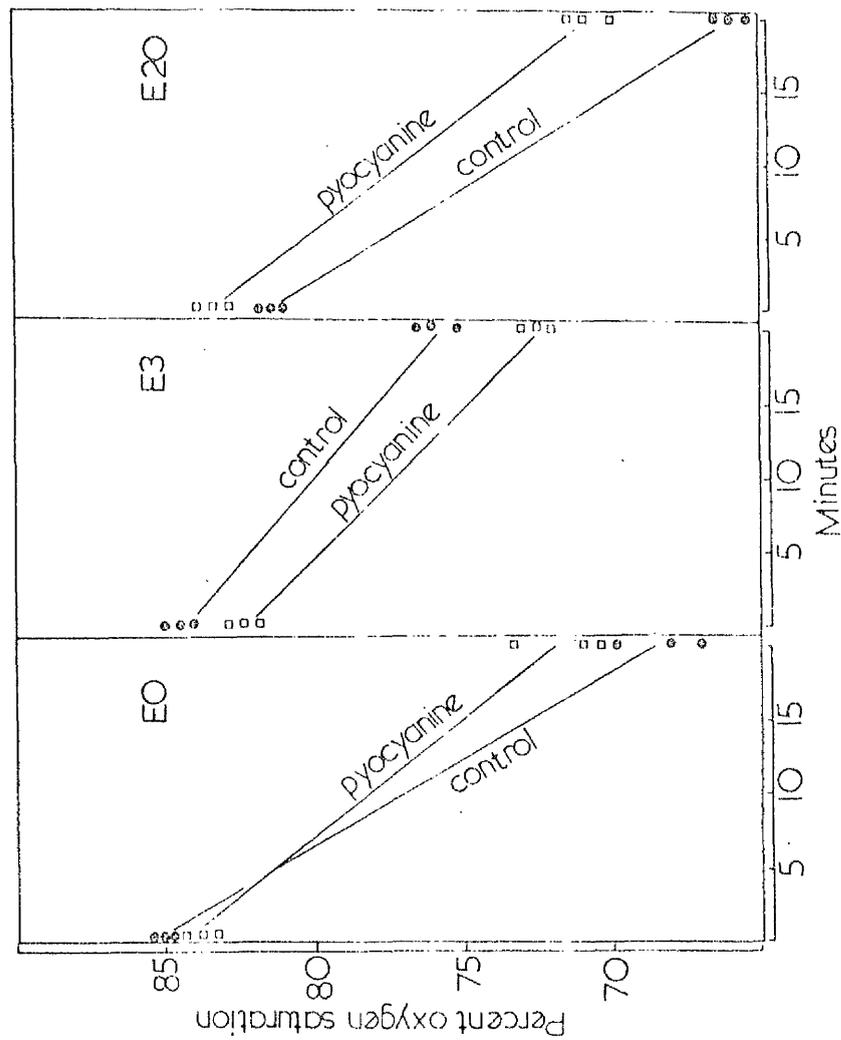
Table XX

Effect of Pyocyanine addition on cell envelope bound succino-oxidase

Cell envelopes from 20 ml of Resuspension Medium	Pyocyanine added ( $\mu\text{g}$ )	$\mu\text{l}$ of oxygen consumed/h <sup>a</sup>		
		Before adding Pyocyanine	After adding Pyocyanine	% Change
0h	250	9.4	8.2	-13
	500	12.0	10.1	-16
	1000	11.8	9.8	-17
3h	250	6.7	7.1	+6
	500	6.6	7.5	+15
	1000	7.2	8.6	+20
20h	250	13.5	13.5	0
	500	12.4	9.2	-25
	1000	13.5	12.0	-8

a, Values expressed are mean of three estimates (within range  $\pm 3\%$ )  
on duplicate envelope preparations

Figure 31



The effect of Pyocyanine addition on cell envelope bound succino-oxidase. Cell envelopes were prepared using cells removed from Resuspension Medium at zero time (EO), 3h (E3) and 20h (E20). Pyocyanine added (500  $\mu\text{g}$ ) was 25% of unit level.

saturation with oxygen and so all rates were measured between 85 and 65% dissolved oxygen. The effect of pyocyanine addition was recorded after standardisation of the rate of untreated succino-oxidase activity. The 0h envelopes (E0) from different batches consumed oxygen at between 9.4 and 14.0  $\mu\text{l}/\text{h}$ . Pyocyanine at a concentration between 250  $\mu\text{g}$  and 1,000  $\mu\text{g}$  (12.5 and 50% of unit pyocyanine level respectively) had a similar inhibitory effect on succino-oxidase ranging from 13 to 15%.

By contrast 3h cell envelopes (E3) showed stimulation on treatment with pyocyanine, 1,000  $\mu\text{g}$  resulting in a 20% increase with 500  $\mu\text{g}$  and 250  $\mu\text{g}$  less effective at 15% and 6% respectively.

Cell envelopes at 20h responded in a similar way to those at 0h consuming oxygen at a slower rate in the presence of added pyocyanine. 500  $\mu\text{g}$  reduced the activity by 25% whereas 100  $\mu\text{g}$  was inhibitory by only 8%. 250  $\mu\text{g}$  had no effect.

Using a standard 500  $\mu\text{g}$  dose (25% of unit level) the effect of pyocyanine was further investigated with one envelope preparation as shown in Figure 31. The slope of control respiration in E0 and E20 envelopes (85  $\rightarrow$  68 and 82  $\rightarrow$  66 respectively) was greater than that in pyocyanine treated samples (84  $\rightarrow$  71 and 84  $\rightarrow$  72) implying inhibition of succino-oxidase. E3 envelopes had control rates of oxygen consumption which decreased the saturation from 84.5 to 76% while pyocyanine stimulated the rate giving 83.5 to 73.5% saturation in the same time.

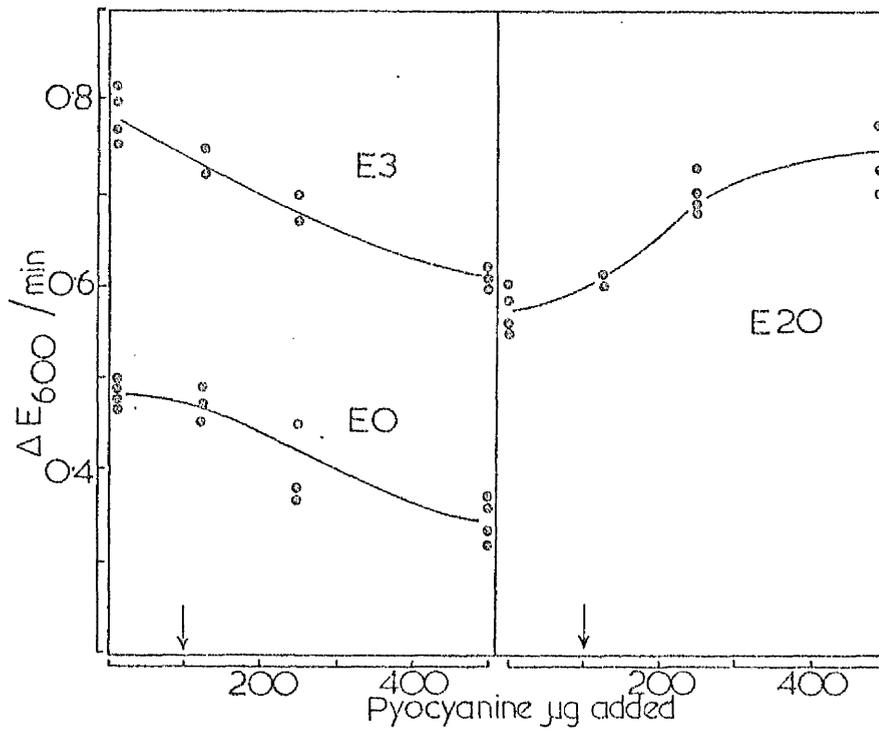
### C. Cell envelope bound succinate dehydrogenase

As described by Norton *et al.*, (1963) the succinate dehydrogenase

(SDH) bound to the cell hull in P. aeruginosa can be assayed by the standard spectrophotometric dye reduction method of Ellis (1959). The complete reaction mixture contained approximately 1% of the yield of cell envelopes from Resuspension Medium and KCN was added to inhibit the cytochrome system. The transfer of electrons from the dehydrogenase to the final 2,6-dichlorophenol-indophenol (DCIP) acceptor was mediated by phenazine methosulphate (PMS). The oxidised form of DCIP absorbed at 600 nm and, on reduction, the colour change from blue to green was monitored as a decrease in  $E_{600}$ . By adding all reagents to the blank except PMS, only DCIP reduction resulting from PMS electron transfer was recorded. Substitution of pyocyanine at equimolar concentration in place of PMS indicated that no reduction in  $E_{600}$  occurred. Pyocyanine was not a suitable substitute or competitor for PMS. The effect of adding equal quantities of pyocyanine to both blank and reaction cuvettes is shown in Figure 32. It was noted that the rate of change of  $E_{600}$  with E0 and E3 envelopes was depressed by pyocyanine at unit level (the lowest tested). Inhibition of SDH activity increased with increasing pyocyanine concentration up to the 5 unit level. In contrast, the E20 envelopes were stimulated by unit level of pyocyanine and, although the effect was not linear with concentration, pyocyanine produced greater SDH activity at the 2 and 5 unit levels.

The proportion of  $\Delta E_{600}$  which resulted from reduction of pyocyanine and from DCIP was not determined. The variation of activity, inhibition and stimulation depending upon the envelope preparation being tested, implied an association of pyocyanine with the system: (enzyme-substrate-PMS). This rules out the non-specific reduction of pyocyanine by PMS and underlines the variable response of enzyme systems in the host to the action of pyocyanine.

Figure 32



Effect of Pyocyanine addition on cell envelope bound succinate dehydrogenase. Cell envelopes were prepared using cells removed from Resuspension Medium at zero time (E0), 3h (E3) and 20h (E20). Unit level of Pyocyanine is arrowed.

DISCUSSION

### A. Determination of Pyocyanine

The first problem encountered in this investigation was to define the conditions for obtaining high and consistent yields of pyocyanine in liquid cultures of the type strain of Pseudomonas aeruginosa. But before this could be done it was necessary to establish a satisfactory assay for the pigment. To provide a standard for this assay, pyocyanine was synthesized chemically and the pure material characterised by chromatography and spectrophotometry. In the latter measurements good agreement was obtained with the max absorptions and extinction coefficients previously reported by Kurachi (1958b), Corbet (1964) and Zuagg (1964).

Because of the possibility of pyocyanine assays being affected by related phenazines, similar spectrophotometric and chromatographic studies were done with l-Phenazinol and l-Methoxyphenazine. Having the three pure compounds available it was then possible to interpret the visible and ultra-violet spectra of P. aeruginosa culture supernatants in terms of known substances. An early conclusion emerging from this work was that pyocyanine was the only pigment produced by the NCTC 6750 type strain of P. aeruginosa grown in phosphate -- deficient medium. The wavelengths 314 and 690 nm were found to be the most suitable for spectrophotometric measurement of pyocyanine because of the intense absorption peaks at these values and because of minimal interference from other substances. However, as pyocyanine itself can change colour with pH and Eh, culture supernatants were routinely adjusted to pH 8.5 and a few drops of hydrogen peroxide added before spectrophotometric estimation.

Although under standard conditions pyocyanine was the major light-absorbing substance found in culture supernatants, the spectrum of the supernatant was not exactly the same as that of pure pyocyanine. In particular, the supernatant showed an absorption at 255 nm which was absent from the spectrum of pyocyanine. The component responsible for this was not identified, but nucleic acid may be suspected since culture supernatants were later shown to contain this product of cell lysis. Another peak at 375 nm is less easy to explain. This wavelength corresponds to one of the peaks of pyocyanine but the absorbance of the supernatant at this wavelength is greater than could be attributed to pyocyanine, as measured at 314 or 690 nm. However, these unidentified peaks were not prominent in the spectrum, nor did they interfere with absorption measurements at 690 or even at 314 nm. In this investigation the 690 nm peak was routinely used for the spectrophotometric assay of pyocyanine.

A further assay question which had to be settled was whether to measure pyocyanine by direct spectrophotometry of culture supernatants as done by Kurachi (1958b) or whether to follow the chloroform extraction procedure of MacDonald (1966). The latter is technically more laborious and also may cause the degradation of pyocyanine to 1-phenazinol (Kurachi, 1959c and Moos and Rowen, 1953). It may also fail to extract all the pyocyanine because the pigment may be bound to other compounds (Swan and Felton, 1957). It was therefore decided to check the validity of the simple spectrophotometric measurement at 690 nm. The method for checking, which does not appear to have been explored previously, consisted in adding known amounts of pyocyanine, which had been measured

gravimetrically, and finding the optical density. This gave a series of absorbance values which obeyed Beer's law within the desired limits and showed that direct spectrophotometric measurement of the  $E_{690}$  of the culture supernatants was a reliable index of pyocyanine concentration.

One final point before leaving the subject of pigment assay is the apparent absence of fluorescin. According to Schoental (1941) this pigment is characteristic of P. aeruginosa and one might therefore expect it to be produced by the type strain. However, no fluorescent substance was detected in cultures or supernatants examined under ultra-violet light. Nor were the intense UV absorption peaks of fluorescin at 240 and 280 nm (Zanker and Peter, 1958) seen in the supernatant spectrum. With another strain (ATCC 9027) King, Campbell and Eagles (1948) stated that fluorescin production required phosphate and iron in the culture medium - components which were deliberately omitted from the "Resuspension Medium" used for pyocyanine production in my work. A separate study would therefore be required to establish whether the type strain is capable of fluorescin synthesis under other cultural conditions or whether special fluorescin-producing strains would have to be used to study this pigment.

#### B. Cultural Conditions for Pyocyanine Production

It was found during the early part of this work that P. aeruginosa NCTC 6750 consistently produced pyocyanine when grown on ordinary nutrient agar. In contrast, when the cells were cultured in a nutritionally similar, but liquid, medium the pigment was produced only erratically. At first it was thought that this might reflect a

spontaneous loss of ability to produce pigment as reported, but not explained, by MacDonald (1966). However, in retrospect it is clear that the factor responsible for preventing pyocyanine production in the nutrient broth used in this investigation is almost certainly the high level of phosphate. (On the other hand this does not explain why pyocyanine is produced on nutrient agar which certainly contains phosphate.)

Eventually, success in obtaining reproducible pyocyanine production in defined liquid medium was achieved by a modification of the two-stage culture technique of Ingledew and Campbell (1969a). This involved growing the cells in shake cultures of a rich liquid medium containing phosphate and, after harvesting and washing, transferring them to 'Resuspension Medium' which was nutritionally complete apart from lacking phosphate. Somewhat similar techniques have been applied in industrial fermentations to produce valuable byproducts of microbial metabolism such as streptomycin, nystatin, chlortetracycline etc. The method however does not seem to have been much used in laboratory-scale cultures except by some recent workers preparing extracellular haemolysins from Escherichia coli (Rennie and Arbuthnott, 1971) and Staphylococcus aureus (Bernheimer and Grushoff, 1967). Although not a two-stage process, the production of diphtheria toxin in a medium of low iron content (Pappenheimer, 1965) may perhaps fit into the same general category.

The Resuspension Medium used in this investigation differed from that of Ingledew and Campbell in having 0.037 M disodium succinate in place of 0.05 M sodium 2-ketogluconate as the carbon source. Despite this change and despite the fact that my work was done with a different

strain of P. aeruginosa, there was a remarkable similarity in the final concentration of pyocyanine produced. My NCTC 6750 strain in the succinate medium yielded 70 - 80 ug/ml whereas Ingledew and Campbell obtained 100 ug/ml with ATCC 9027 in the 2-ketogluconate medium. MacDonald (1966) reported 100 - 300 ug/ml produced by ATCC 9027 in various media.

An important consideration in the use of Resuspension Medium was the level of inoculum added. This was expressed as the percent, by volume, of stationary phase culture in the Growth Medium which was added (in the form of washed cells) to the Resuspension Medium. Best results were obtained with a 25% inoculum, although 50% was almost as good. Very small inocula such as 1% or very large inocula, such as 200% gave much lower yields of pigment. The 25% inoculum was therefore used routinely. It was surprising that in the original work with Resuspension Medium, Ingledew did not report on the effect of inoculum size but simply used a 100% inoculum. The only previous work with a similar observation of the critical effect of inoculum size is that of Grossowicz et al. (1957) using his own isolate of P. aeruginosa. Grossowicz did not express inoculum size relative to his initial growth medium but the yield of pyocyanine was doubled by a four-fold increase in inoculum size. His system differed from that used here since P. aeruginosa synthesized pyocyanine in the presence of 20 mM phosphate buffer.

The next step in the investigation was to see if Resuspension Medium could be improved by further modifications of its formula. The changes were of four types: omission of components, substitution of alternative carbon, nitrogen and sulphur sources, addition of phosphate

and/or iron, and using a hydrogen or nitrogen atmosphere above the medium in place of air. The result of all these permutations can be summarized briefly: none gave as high a level of pyocyanine as ordinary Resuspension Medium containing succinate; many of the alternative media gave more growth but this was not accompanied by better pigment production. Indeed the converse was frequently observed. Addition of small amounts of phosphate either diminished or completely abolished pyocyanine production. In short, production of the pigment in the two-step procedure has an absolute requirement for aerobic conditions, suitable metabolizable sources of carbon, nitrogen and sulphur, and the complete absence of phosphate. Other factors such as Tris,  $Mg^{2+}$  and  $K^+$  have a promoting effect on pigment production but are not absolutely required.

$Fe^{2+}$  does not appear to influence the system except that it promotes growth. These results are in contrast to the 3 mM requirement of strain ATCC 9027 for phosphate, reported by Burton et al. (1948) and with the pigment-promoting action of iron (Kurachi, 1958c) in an unspecified strain of P. aeruginosa.

The replacement experiments with alternative carbon sources merit further consideration. For maximum pigment production, succinate was superior to citrate and glutamate which in turn were better than glycerol. Glucose was the least suitable substrate for pyocyanine production but it gave more growth than any of the alternatives. There was therefore a tendency for an inverse relationship between growth and pyocyanine production with the different carbon sources. The greater pigment production with succinate may arise from the presence of succinate as the carbon source in the first-stage Growth Medium. That is, from considerations of enzyme adaptation, the organism may be

able in the Resuspension Medium to metabolise more rapidly a carbon source which it has recently been using. An alternative reason is found in the catabolite repression i.e. where enzymes required to metabolise certain substrates are repressed by products of succinate metabolism. This has been shown to occur in P. aeruginosa (Clark and Brammar, 1964) and is discussed in a later section.

### C. Characterisation of P. aeruginosa during Pyocyanine Production

Having developed a satisfactory assay for pyocyanine and having explored the variables associated with using Resuspension Medium, the next step was to define the biochemical state of the cells before, during and after pyocyanine production. The two questions which seemed most important were -

- (a) Is synthesis of the pigment accompanied by growth? and
- (b) How do the various phosphate-containing molecules of the cell (ATP, DNA, RNA, Lipid and Lipopolysaccharide) respond to the low phosphate content of the Resuspension Medium?

The question of whether or not pigment production is associated with growth was left unresolved in MacDonald's (1967) review on pyocyanine. The majority of workers including Burton et al. (1948), Blackwood and Neish (1957) MacDonald (1963) and Ingledew and Campbell (1969a) used experimental conditions where pyocyanine production was associated with growing cultures. In contrast, Grossowicz et al. (1957) and Halpern et al. (1962) used washed cell suspensions. The former group, taking O.D. as index of growth, concluded that the pigment was produced by cultures which were just entering the stationary phase. The latter

group believed they were dealing with resting cells as judged by constancy of viable count. Because of the difficulty in resolving this situation, the present study on the kinetics of pyocyanine production used a variety of growth-indicating parameters namely, viable count, O.D., dry weight and cell-associated protein.

The time-course of pyocyanine production fell into three phases. Phase One, which lasted for three hours, was characterised by no production of pigment although the cells exhibited definite metabolic activity. For example the cell-associated protein doubled, cell mass increased by 70% and viable count by 40%. The O.D. of the cell suspension was unchanged. Phase Two was the period of linear increase in pyocyanine content of the supernatant and lasted from three hours to thirteen hours. During this time, while pyocyanine level was steadily increasing, O.D. increased by 180%, cell mass remained almost constant and the viable count decreased to the level at zero time. Protein content was not measured at thirteen hours.

Phase Three, the stationary phase with respect to pigment production, lasted from thirteen hours to twenty-four hours. Here the pyocyanine level remained constant, but the dry weight, viable count and O.D. all declined. This was due to cell lysis which could be detected by the appearance of protein and nucleic acid in culture supernatants at twenty hours.

The pattern of events revealed by this kinetic study is similar to, but more complete than, that described by Ingledew and Campbell (1969a). Both are consistent with the classification of

pyocyanine as a secondary metabolite i.e. a substance which is produced in circumstances where the actively growing cell is unable to sustain balanced growth. It is clear that the controversy emerging from MacDonald's (1967) review is due, at least in part, to the different indices used to assess growth. Mitchell and Moyle (1956a) have shown that the concentration of intracellular constituents during changes in the growth phases do not relate to cell numbers. Schlaechter (1958) demonstrated the close parallel of cell mass and opacity.

As stated above, the critical role of phosphate in inducing pyocyanine synthesis made it of interest to measure changes in the phosphate-containing molecules of the cells incubated in Resuspension Medium. Such studies have not been reported in the literature. Cells were therefore harvested and analysed after zero, three and twenty hours in the Resuspension Medium, these being the times of most interest for possible correlation with pyocyanine production.

As already reported, the cell mass harvested at three hours showed evidence of growth as judged by the increases of 70%, 100% and 40% respectively in dry bacterial mass, total protein and viable count. To this we can now add the facts that DNA and ATP increased in proportion to the cell mass, while total lipid and lipopolysaccharide increased in proportions which were 40% and 140% greater than the cell mass increase. In contrast, RNA increased by only half of the mass increase and phospholipid showed a relative decline. This pattern of events is similar to that which would be expected with cells going from the log to the stationary phase of growth where RNA contribution to the cell mass

decreases and DNA contribution increases (Herbert, 1961). These general changes in the composition of growing cells, described by Herbert correspond to a decreasing rate of bacterial growth and the return of RNA and DNA to the levels typical of resting cells.

The changes in viable count, protein, DNA and RNA during incubation in Resuspension Medium are similar to those observed by Hou, Gronlund and Campbell (1966) in cultures of *P. aeruginosa* which were starved of phosphate. The phosphate required for the cell division and protein synthesis, which these workers observed, was thought to be supplied through the degradation of intracellular reserve material, shown by Gronlund and Campbell (1963) to be RNA. As in the Resuspension Medium, Hou detected a relative increase in DNA content of the cells and a decrease in RNA incubation in medium without phosphate.

During the period from three to twenty hours in Resuspension Medium, during which pyocyanine was produced, the cells show a marked loss of RNA and phospholipid and proportionately smaller losses of DNA, LPS and total lipid. ATP was maintained at a relatively constant level throughout the whole twenty hours of incubation - result which might be expected from the central position of ATP in the phosphorus metabolism of the organism.

In summary, pyocyanine production occurs after the phase of growth and during a period when net synthesis of protein, DNA, lipid ATP and LPS has ceased, and RNA and phospholipid levels show rather marked declines.

#### D. Effects of Phosphate Deprivation

It would be desirable to interrelate all the variables studied according to a unified theory of the effect of phosphate deprivation on microbial metabolism. The status of knowledge in this area of bacterial physiology is fragmentary partly because of its inherent complexity and partly because most studies on biosynthetic pathways involving phosphorus have been done in phosphate-containing media. A leading biochemist once stated to me "Phosphate is always there". (Davidson, personal communication.)

In a recent review Demain (1972) reported on secondary metabolites which are controlled to some extent by phosphate. These include prodigiosin from Serratia marcescens (Jackson Bunting and Morrison, 1963) and streptomycin from Streptomyces griseus (Demain and Inamine, 1970). Demain considered that formation of these secondary metabolites was prevented by feed-back repression in which inorganic phosphate inhibited certain phosphatases. The justification for this view was that since many biosynthetic intermediates of secondary pathways are phosphorylated, whereas the ultimate products are not, phosphatases must participate in biosynthesis. Streptomycin was considered to be a good example. Here biosynthesis was markedly inhibited by phosphate and the penultimate compound streptomycin phosphate was shown to accumulate in phosphate-limited, streptomycin-producing cultures (Millar and Walker, 1970).

Phosphate was found to inhibit prodigiosin biosynthesis (Jackson et al., 1963), and the amount of phosphate which was added

caused proportionate decrease in pigment synthesis. The role of phosphate in this biosynthetic process has not been investigated further.

The problem of generalising on the effect of phosphate deficiency centres on the lack of knowledge concerning phosphate acquisition and metabolism. It is not known how phosphate can regulate biosynthetic pathways or why the organism should respond by producing pyocyanine and other secondary metabolites when confronted with a phosphate-deficient environment.

The regulation of phosphatases is not the only method by which phosphate can exert a decisive effect on biosynthetic pathways. It may be significant that the level of ATP in the Resuspension Medium cells at zero time was 60% lower than had been detected in the Y-T medium cells. The ATP of cells throughout their incubation in Resuspension Medium remained at the same relatively low level. There is a distinct possibility, according to Atkinson (1969), that secondary metabolism can be regulated by energy charge i.e. the activation and inhibition of the enzymes of primary metabolism by the relative levels of ATP, ADP and AMP in the cell. A low-phosphate environment in Resuspension Medium causes ATP to be kept at a reduced level, relative to that in the growth medium.

This type of low energy charge in the cell has been shown to promote chlortetracyclin formation where the biosynthetic process does not involve any known phosphorylated intermediates. Using two strains of Streptomyces aureofaciens which differed in their ability to produce the antibiotic, Janglova, Suchy and Vanek (1969) found that

the ATP level was high during growth and then decreased to a low level at which it was maintained during the fermentation cycle. An inverse relationship existed between the amount of chlortetracyclin produced and the ATP pool levels in the two strains.

#### E. Control Mechanisms in Pyocyanine production

Since pyocyanine is generally considered to be a secondary metabolite i.e. a natural product with no necessary and obvious function in the growth of the producing organism, the remaining observations in this investigation are set within the context of secondary metabolism.

According to Weinberg (1971) the relationship of secondary metabolism to bacterial cell multiplication is as follows: After the period of logarithmic growth and before the onset of the stationary phase, the secondary metabolite is detected in the culture supernatant. Under usual laboratory conditions the interval between logarithmic growth and the establishment of stationary phase conditions is approximately two hours. This time is sufficient for transcription and translation of the synthetases of secondary metabolism i.e. a phenotypic change induced by the environment to exercise control over the biosynthetic pathways and ensure that the cells function most economically.

The secondary metabolic process is characterised by two phases of enzymic activity. The first is the formation of the synthetases which have been repressed during the growth phase. The phenoxazinone synthetase in the biosynthesis of actinomycin (Katz, 1967)

is a classic example. Here, derepression involves protein synthesis and not precursor activation, since it has been observed that inhibitors of protein synthesis block the formation of the enzyme and subsequent synthesis.

The second enzymic process is the activity of the synthetases which involves, for example, packaging of the primary substances such as malonate, acetate and shikimate into the final product.

The control mechanisms governing these phenotypic changes in bacteria include feedback regulations which has two distinct modes of action -

- (a) feedback inhibition, where the end product causes reduction in the activity of one enzyme in the chain of reactions.
- (b) feedback repression, where the control is exercised over the synthesis of the enzymes themselves.

In this investigation it would found that the addition of low concentrations (40uM) of phosphate caused almost immediate inhibition of pyocyanine synthesis. However, with cells which had been producing pyocyanine for several hours, higher levels of phosphate were required for inhibition. The point at issue is whether the reduced activity of the enzymes involved in pyocyanine synthesis is the result of feedback inhibition or repression of phosphatases. The potential biosynthetic precursors of pyocyanine e.g. 5-phosphoshikimate, are phosphorylated whereas pyocyanine itself is not. This could be analagous to the inhibition of streptomycin formation by phosphate described earlier. The situation with pyocyanine agrees with the observation that, as

incubation in Resuspension Medium progresses, increased levels of phosphate are required to inhibit pyocyanine synthesis. In the latter stages of the culture, phosphatase activity would have risen to its maximum and therefore require higher phosphate concentration for inhibition.

To investigate the requirement for protein synthesis in the pyocyanine-producing system, experiments were made with chloramphenicol -- a known inhibitor of protein synthesis. During the three hour period in Resuspension Medium which preceded the appearance of pyocyanine, all the observed increases in the protein content of the cells occurred. Certainly pyocyanine was not produced automatically just because cell growth was prevented, as the addition of growth inhibitory concentrations of the antibiotic to the culture in ordinary nutrient medium with phosphate did not give pyocyanine. In the Resuspension Medium, chloramphenicol inhibited pyocyanine production at levels considerably less than were needed to inhibit growth in a full nutrient medium. Moreover cells at zero time and at three hours in Resuspension Medium were equally sensitive in respect of pigment production.

It appears that by inhibiting the protein synthesis over the period from zero to three hours the formation of the required synthetases for pyocyanine production is prevented. Even after three hours, those cells which have had the necessary enzymes induced or derepressed, are subject to inhibition by chloramphenicol. This implies that continued protein synthesis is necessary after three hours. Previously, the observed inhibition of pyocyanine production by chloramphenicol was complicated by a parallel inhibition of growth (Schneirson et al., 1960).

It was therefore impossible to distinguish the effects of chloramphenicol on growth from those on the pyocyanine biosynthetic system. As it has been demonstrated in this investigation that growth does not accompany pyocyanine production after three hours in the Resuspension Medium, derepression of the enzymes is implied.

By exposing "zero-hour" cells to unit level of synthesized pyocyanine, i.e. that concentration of pyocyanine normally found in Resuspension Medium after twenty hours, the final level of naturally produced pyocyanine was unchanged. Similarly the addition of approximately three units of synthesized pyocyanine had no effect, although five units resulted in complete inhibition. By contrast the "three-hour" cells were inhibited by 30% and 70% when one unit and three units respectively were added. The "three-hour" cells were completely inhibited by five units of synthesized pyocyanine. One possible reason for the difference is altered cell permeability which could result in "zero-hour" cells being insensitive and "three-hour" cells receptive to extracellular pigment. This would also require a situation whereby the synthetic pyocyanine, which was added at zero time, was not available after three hours to act in the same way as the synthetic pyocyanine freshly added at that time. No fixation or inactivation was detected by the visual observation of the pyocyanine colour during the zero to three hour period.

The observations can be considered as an example of feedback inhibition of the pyocyanine biosynthesis by end product. The "three-hour" cells would probably be more susceptible than "zero-hour" ones as the synthetases, on which feedback inhibition was operative, would only

be present after that period of incubation. The surprising inhibitory effect produced by the addition of five units of pyocyanine to the "zero-hour" cells would result if the inactivating process found with lower levels of pyocyanine was unable to affect all of the added pigment. The residual pigment, to which the treated "zero-hour" cells would be exposed after three hours, need only amount to unit level before significant reduction in natural pyocyanine would result.

A typical example of feedback inhibition is found in the production of chloramphenicol by Streptomyces venezualae (Malik and Vining, 1970) here accumulation of the secondary metabolite inhibits its own formation. Malik recognised the complication of the antibiotic being degraded by the producing organism and the fact that different rates of breakdown accompanied different growth rates of the organism in batch culture. This is similar to P. aeruginosa in Resuspension Medium although no breakdown of pyocyanine was observed. The major metabolic activity occurs over the first three hours and the response of "zero" and "three hour" cells to pyocyanine is quite distinct.

The problem of the past history of the culture of P. aeruginosa and the variation in pyocyanine production which results from using different substrates and inoculum ratios in Resuspension Medium can be regarded as examples of catabolite repression, i.e. the inhibition, inactivation or repression of enzymes by catabolism of carbon sources. Depending upon the suitability of a substrate, the organism will exhibit different growth rates, resulting in various degrees of repression of the enzymes involved in the biosynthesis of pyocyanine. The previous history of the organism has been shown to

influence the consumption of endogenous substrate (Gronlund and Campbell, 1966) and, after the log phase growth of the organism on a variety of nutrient media, MacKelvie Gronlund and Campbell (1968) demonstrated that the relative consumption rates of intracellular protein and RNA were dependent upon the previous growth substrate. It is possible that the previous growth conditions and rate of growth of P. aeruginosa on different substrates could influence the enzymes of pyocyanine biosynthesis in a similar way to the -- galactosides repression observed in E. coli (Okinaka and Dobrogosz, 1967).

The part played by endogenous substrates in catabolite repression in P. aeruginosa is potentially of greater significance in that the breakdown of intracellular protein will liberate tryptophan, phenylalanine and tyrosine, primary metabolic branch point products of the pyocyanine biosynthetic route. In this way the liberation of either catabolite repressors or feedback inhibitors could provide a reason for the control exercised by environmental conditions over pyocyanine production.

#### F. Function of Pyocyanine in P. aeruginosa

As already pointed out the function of secondary metabolites in general is obscure. If a definite growth requirement could be demonstrated the product would cease to be classed as a secondary metabolite. The question of the function of pyocyanine production in P. aeruginosa is conveniently discussed under two headings. The first includes those functions affecting the producer cell. The second,

attributes to pyocyanine a function which is common to all secondary metabolites.

Pyocyanine is indeed capable of acting as an accessory metabolite for the producer cell. In this investigation the effect of supplying exogenous pyocyanine to a cell suspension, which was respiring on endogenous substrate only, was found to be related to the stage of pigment production of the cell. Thus cells removed from Resuspension Medium at three hours and which were on the point of producing their own pyocyanine were selectively stimulated in their rate of oxygen consumption when pyocyanine was added at unit level. The comparable cell suspensions prepared at zero time and at twenty hours was insensitive to the addition of pyocyanine. These observations indicate that the cells which are in the metabolic state where they are required to produce pyocyanine, are uniquely able to use the pigment as a respiratory catalyst in the consumption of oxygen. These results contrast with the investigation of Kurachi (1960) into the physiological significance of pyocyanine. The pigment was found to have no effect on the normal respiration, although the type of cells being used were not classified in respect of their stage of natural pyocyanine production.

Analysis of the effect of pyocyanine in respiration was attempted using cell envelope preparations. Campbell et al., (1962) demonstrated the presence of the succino-oxidase system in cell envelope preparations from P. aeruginosa. This system comprises the succinate dehydrogenase, a flavoprotein mediated enzyme, which transfers electrons to cytochrome oxidase which, in turn, uses oxygen as the final acceptor. The activity of pyocyanine in the succino-oxidase reaction depended upon

the type of cell envelopes being tested. Envelope preparations from cells removed from Resuspension Medium at three hours were stimulated in their rate of oxygen consumption whereas those "zero time" and "twenty-hour" cells were inhibited in their ability to transfer electrons to oxygen in the presence of pyocyanine. This activity was not proportional to the concentration of pyocyanine, nor was there any evidence of gross reduction of the pigment. It was therefore concluded that pyocyanine was not acting as a simple 'electron sink' responsible for diverting the electron flow from oxygen.

With the aim of characterising the activity in the succinoxidase preparation, the succinate dehydrogenase was monitored independently. The cytochrome system of the cell envelope preparations was first inactivated by cyanide. An alternative route of electron transfer was provided by phenazine methosulphate (PMS) as carrier and dichlorophenol-indophenol (DCIP) as final acceptor. In the dehydrogenase assay, pyocyanine was unable to act in place of PMS or DCIP, not was it subject to gross reduction. The added pyocyanine was found to stimulate selectively the dehydrogenase enzyme attached to the envelope preparation from "twenty-hour" cells. Cell envelopes from "zero-hour" and "three-hour" cells were inhibited by pyocyanine. Since the activity exhibited by pyocyanine is a function of the enzyme composition of the cells, specific changes in succinate dehydrogenase must occur during incubation in Resuspension Medium. In this way pyocyanine is able to participate in the complex interaction of (succinate dehydrogenase - flavoprotein - phenazine methosulphate).

A possible explanation for the different effects of pyocyanine

on P. aeruginosa cells and cell envelope preparations can be derived from the activity attributed to it in the mitochondrial electron transport chain. Harmann and McBrinn (1963) found that succinate dehydrogenase of mitochondria was inhibited by pyocyanine at two sites in the respiratory chain. One site of action of pyocyanine was the flavoprotein itself -- the stereospecific binding of the substrate was considered to be affected. This resulted in conversion of the enzyme--flavine to a form which was less active. The second site of action was in the cytochrome chain -- at cytochrome b. If the observed effects of pyocyanine in the succino-oxidase of P. aeruginosa are in any way similar, the succino-oxidase reaction would monitor both sites of activity. In the succinate dehydrogenase assay, cytochromes do not participate and the flavine enzyme site would give the observed results.

In summary, pyocyanine is capable of functioning in the respiratory system of P. aeruginosa. The activity is governed by the metabolic state of the organism in relation to the condition of its own pyocyanine-producing enzymes.

If we look now at the broader implications of secondary metabolism, pyocyanine provides a good example of the current view on the general function of this class of compound. Woodruff (1966) has put forward the interesting proposal that secondary metabolism is required by the producing organism so that it can achieve a balanced death. The recent work of Gentry, Smith, Schnute, Weber and Weinberg (1971) has shown that the distortion of pyocyanine formation by added phosphate gives cell suspensions whose longevity is lower than that in the control, where secondary metabolism takes place normally.

In nature, it is difficult to imagine a cultural environment where the organism would have all the required nutrients in unlimited amounts and where continuous multiplication would therefore occur. Thus the artificially contrived Resuspension Medium may resemble the low phosphate milieu encountered in many natural situations.

Finally the antibiotic activity of pyocyanine should not be forgotten for it may provide P. aeruginosa with a selective advantage over other micro-organisms in natural environments where competition for phosphate may be severe.

#### G. Further perspectives

The mechanism of phosphate uptake prior to its incorporation into the metabolic pool of the bacterial cell has received less attention than the uptake of organic nutrients. Only in the case of Staphylococcus aureus (Mitchell and Moyle, 1956b) and Streptococcus faecalis (Harold, Harold and Abrams, 1965) has the accumulation of this ion been investigated in detail. Mitchell found evidence of a highly specific exchange diffusion system which was active in transporting the phosphate in both directions across the membrane osmotic barrier. The proposed carrier had specific binding sites for phosphate and in the absence of nutrient it was active in transporting the ion in both directions. In the presence of substrate, the absorbed phosphate entered the metabolic pathway and efflux of phosphate was prevented. Harold described the problem as being that of distinguishing between transport and metabolism, because phosphate appeared almost immediately in the metabolic ATP pool. The access mechanism was thought to depend on the physiological state of

the cells and the growth substrate. The observed dependence of the phosphate acquisition rate on the substrate was attributed to the ATP content of cells grown on different substrates. Further insight into the physiology of phosphate utilisation may be acquired by the study of the pathology of cells encountering phosphate-deprivation as is the case with *P. aeruginosa* in Resuspension Medium.

The two-stage culture technique of this investigation could well be applied more generally in the study of secondary metabolism. Examples are the study of phosphate regulation of prodigiosin, streptomycin and other pigments and antibiotics. The field of bacterial toxin production is another area which merits more detailed consideration in this context.

In the particular case of pyocyanine production, the two-stage culture method eliminates the difficulties of pigment variation, past history of the inoculum and presents a model non-proliferating system for the study of secondary metabolism. Useful contributions could be made by analysing the fate of labelled phosphate in Resuspension Medium or by isolation of a cell-free, pigment-synthesising system.

REFERENCES

REFERENCES

- ALLEN, R.J.L., (1940). The estimation of Phosphorus. Biochem J., 34, 858-865.
- AMBLER, R.P., (1963). The purification and amino acid composition of Pseudomonas cytochrome c-551. Biochem. J., 89, 341-349.
- ARMSTRONG, A.V. and STEWART-TULL, D.E.S., (1971). The site of activity of extracellular products of Pseudomonas aeruginosa in the electron transport chain of mammalian cell respiration. J. Med. Microbid., 4, 263-270.
- ATKINSON, D.E., (1969). Regulation of enzyme function. Ann. Rev. Microbial., 23, 47-68.
- AZOULAY, E and COUCHOUD-BEAUMONT, P., (1965). Etude de la cytochrome-oxidase de Pseudomonas aeruginosa. Biochim. Biophys. Acta., 110, 301-311.
- BABES, A., (1890). Colouring matters and aromatic products from Bacillus pyocyanicus. J. Chem. Soc., 58, 189-190.
- BENTLEY, R. and SCHLECHTER, L., (1960). oxidation of mono- and disaccharides to aldonic acids by Pseudomonas species. J. Bacteriol., 79, 346-355.
- BERK, R.S., (1963). Nutritional studies on the 'autoplaque' phenomenon in Pseudomonas aeruginosa. J. Bacteriol., 86, 728-734.
- BERK, R.S., (1966). Nutritional requirements for autoplaque production in AP<sup>+</sup> strains of Pseudomonas aeruginosa. Can. J. Microbiol., 12, 371-375.
- BERNHEIMER, A.W. and GRUSHOFF, P., (1967). Cereolysin : Production, purification and partial characterisation. J. gen. Microbiol., 46, 143-150.
- BILLIAR, R.B., KNAPPENBERGER, M. and LITTLE, B., (1970). Xanthine oxidase for calibration of the oxygen electrode apparatus. Analytical Biochem., 36, 101-104.
- BISHOP, D., PAUNDYA, K. and KING, H., (1962). Ubiquinone and vitamin K in bacteria. Biochem. J., 83, 606-614.

- BLACKWOOD, A.C. and NEISH, A.C., (1957). Pyocyanine formation from labelled substrates by Pseudomonas aeruginosa. Can. J. Microbiol., 3, 165-169.
- BRENNER, S. and HORNE, S.W., (1959). A negative staining method for high resolution electron microscopy of viruses. Biochim. Biophys. Acta., 34, 103-110.
- BROWN, M.R.W. and FOSTER, J.H.S., (1970). A simple diagnostic milk medium for Pseudomonas aeruginosa. J. Clin. Pathol., 23, 172-177.
- BROWN, M.R.W. and RICHARDS, R.M.E., (1964). Effect of polysorbate (Tween) 80 on the resistance of Pseudomonas aeruginosa to chemical inactivation. J. Pharm. Pharmac., 16, 51T-55T.
- BURTON, K., (1956). A study on the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J., 62, 315-323.
- BURTON, M.O., CAMPBELL, J.J.R., and EAGLES, B.A., (1948). The mineral requirements for pyocyanine production. Can. J. Research., 26, 15-22.
- BURTON, M.O., EAGLES, B.A. and CAMPBELL, J.J.R., (1947). The amino acid requirements for pyocyanine production. Can. J. Research., 25, 121-128.
- CALTRIDER, P.G. (1967). Pyocyanine. In Antibiotics. I. Mechanism of Action, p.117-121. Ed. by Gottleib, D. and Shaw, P.D., Springer-Verlag, Berlin.
- CAMPBELL, J.J.R., GRONLUND, A.F. and DUNCAN, M.G., (1962a). Endogenous metabolism of Pseudomonas. Ann. N.Y. Acad. Sci., 102, 669-677.
- CAMPBELL, J.J.R., HOGG, L.A. and STRASDINE, G.A., (1962b). Enzyme distribution in Pseudomonas aeruginosa. J. Bacteriol., 83, 1155-1160.
- CAMPBELL, J.J.R., MACQUILLAN, A.M., EAGLES, B.A. and SMITH, R.A., (1957). The inhibition of keto acid oxidation by pyocyanine. Can. J. Microbiol., 3, 313-318.
- CAMPBELL, J.J.R. and SMITH, R.A., (1956). The enzymes of the tricarboxylic acid cycle of Pseudomonas aeruginosa. Can. J. Microbiol., 2, 433-440.

- CARRICK, L.Jr., and BERK, R.S., (1971). Membranous inclusions of Pseudomonas aeruginosa. J. Bacteriol., 106, 250-256.
- CARTER, R.E and RICHARDS, J.H., (1961). The biogenesis of phenazine pigments. J. Am. Chem. Soc., 83, 495-496.
- CASE, E.M. and MCILWAIN, H., (1951). Respiration and phosphorylation in preparations from mammalian brain. Biochem. J., 48, 1-11.
- CAVALLITO, C.J., (1946). Relationship of thiol structures to reaction with antibiotics. J. Biol. Chem., 164, 29-34.
- CHANG, P.C. and BLACKWOOD, A.C., (1968). Simultaneous biosynthesis of pyocyanine, phenazine-1-carboxylic acid oxychlororaphine from labelled substrates by Pseudomonas aeruginosa Mac 436. Can. J. Microbiol., 46, 925-929.
- CLARKE, P.H. and BRAMMER, W.J., (1964). Regulation of bacterial enzyme synthesis by induction and repression. Nature, Lond., 203, 1153-1155.
- CLEMO, G.R. and MCILWAIN, H., (1938). Phenazine series. VII. The pigment of Chromobacterium iodinum, the phenazine di-N-oxide. J. Chem. Soc., 479-483.
- CLUZET, J., (1921). Spectrophotographic study of the pigments of Bacillus pyocyaneus. J. physiol. pathol. gen., 17, 378-382.
- COLE, H.A., WIMPENNY, J.W.T. and HUGHES, D.W., (1967). The ATP pool in Escherichia coli. 1. Measurement of the pool using a modified Luciferase assay. Biochim. Biophys. Acta., 143, 445-453.
- CORBETT, J.F., (1964). The infrared and ultraviolet spectra of hydroxy phenazines. Spectrochim. Acta., 20, 1665-1678.
- COWAN, S.T. and STEEL, K.J., (1965). In "Manual for the Identification of Medical Bacteria". Cambridge University Press.
- CURTIN, J.A. PETERSDORF, R.G. and BENNET, I.L., (1961). Pseudomonas bacteria : a review of ninety-one cases. Ann. Intl. Med., 54, 1077-1107.
- DAVIS, J.B., (1964). Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci., 121, 404-427.

DE LEY, J. and KERSTERS, K., (1964). Oxidation of aliphatic glycols by acetic acid bacteria. Bact. Rev., 28, 164-180.

DEMAIN, A.L., (1972). Cellular and environmental factors affecting the synthesis and excretion of metabolites. J. appl. Chem. Biotechnol., 22, 345-362.

DEMAIN, A.L. and INAMINE, E., (1970). Biochemistry and regulation of streptomycin and mannosidostreptomycinase ( $\alpha$ -D-mannosidase) formation. Bact. Rev., 34, 1-19.

DE MEIO, R.H., KISSIN, M. and BARRON, K.S.G., (1934). Studies on biological oxidations. IV. On the mechanism of the catalytic effect of reversible dyes on cellular respiration. J. Biol. Chem., 107, 579-590.

DE VITO, E. and SANTOME, J.A., (1965). Disc electrophoresis of proteins in the presence of sodium dodecyl sulphate. Experientia, 22, 124-125.

DICKENS, F., (1936). CLXXVI. The metabolism of normal and tumour tissue. XVII. The action of some derivatives of phenazine, quinoline and pyridine on the Pasteur reaction. Biochem. J., 30, 1233-1241.

DICKENS, F. and MCILWAIN, H., (1938). CCX. Phenazine compounds as carriers in the hexose monophosphate system. Biochem. J., 32, 1615-1625.

DOI, O. OGURA, M. and UMEZAWA, H., (1968). Inactivation of kanamycin, neomycin and streptomycin by enzymes obtained in cells of Pseudomonas aeruginosa. Appl. Microbiol., 16, 1276-1281.

EASTABROOK, R.W., (1967). Mitochondrial respiratory control and the polarographic measurement of ADP : O ratio. Methods in Enzymology, X, 41-47. Ed. by Eastabrook, R.W.S. and Pullman, M.E., Academic Press, London.

EHRISMAN, O., (1934). Pyocyanine and bacterial respiration. Zeitschr. Hyg. Infektionskrankh., 116, 209-224.

EHRISMAN, O. and NOETHLING, W., (1936). Absorption spectra of pyocyanin, prodigiosin and violacein. Biochem. Z., 284, 276-282.

ELLS, H.A., (1959). A colourimetric method for the assay of soluble succinic dehydrogenase and pyridine nucleotide linked dehydrogenases. Arch. Biochem. Biophys., 85, 561-562.

EMMERICH, R. and LÖW, O., (1899). Bakteriolytische Enzyme als Ursache der erworbenen Immunität und Heilung von Infektionskrankheiten durch dieselben. Zeitschr. Hyg. Infektionskrankh., 31, 1-12.

EMTNER, N. and DOUDEROFF, M., (1952). Glucose and gluconic acid oxidation of Pseudomonas saccharophila. J. Biol. Chem., 196, 853-862.

ESPINOSA, M., HIDALGO, A. and PORTOLES, A., (1972). Chromogenesis variations in strains of Pseudomonas aeruginosa growing in the presence of chloramphenicol and oxytetracycline. Can. J. Microbiol., 18, 99-103.

FAZEKAS, J.F., COLYER, H., NESIN, S. and HIMIVICH, H.E., (1939). Effect of pyocyanine on cerebral metabolism. Proc. Soc. Exptl. Biol. & Med., 42, 446-448.

FISHER, E. and ALLEN, J.H., (1958). Mechanisms of corneal destruction by Pseudomonas proteases. Amer. J. Ophthalmol., 46, 249-254.

FOLCH, J., LEES, K. and STANLEY, G.H.S., (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

FORDOS, M., (1860). Cited by Jordan (1899). C. r. hebdom. Seanc. Acad. Sci., Paris, 51, 215.

FRANK, L.H. and DE MOSS, R.D., (1959). On the biosynthesis of pyocyanine. J. Bacteriol., 77, 776-782.

FRASCA, J.M. and PARKS, V.R., (1965). A routine technique for double staining ultrathin sections using uranyl and lead salts. J. Cell. Biol., 25, 157-161.

FRIEDHEIM, E.A.H., (1931). Pyocyanine an accessory respiratory enzyme. J. Exptl. Med., 54, 207-221.

FRIEDHEIM, E.A.H., (1934). Effect of pyocyanine on the respiration of some normal tissues and tumours. Biochem. J., 28, 173-179.

FRIEDHEIM, E.A.H. and MICHAELIS, L., (1931). Potentiometric study of pyocyanine. J. Biol. Chem., 91, 355-368.

FUGITA, H., (1957). Influence of antibiotics on the production of pyocyanine and fluorescin by Pseudomonas aeruginosa and the occurrence of non pyocyanic strains from clinical specimens. Die Med. J., VII, (1), 169-174.

GARROD, L.P. and O'GRADY, F., (1968). In "Antibiotics and Chemotherapy" p. 291, Livingstone, London.

GENTRY, M.J., SMITH, D.K., SCHNUTE, S.F., WEBER, S.L. and WEINBERG, E.D., (1971). Pseudomonas culture longevity : control by phosphate. Microbios., 4, 205-215.

GERBER, N.N., (1967). Phenazines, phenoxazinones and dioxopiperazines from Streptomyces thioluteus. J. Org. Chem., 32, 4055-4057.

GERBER, N.N., (1969). New microbial phenazines. J. Heterocyclic. Chem., 6, 297-300.

GESSARD, C., (1882). La pyocyanine, These. C. r. hebd. Seanc. Acad. Sci., Paris, 94, 536-540.

GESSARD, C., (1890). Nouvelles recherches sur le microbe pyocyanique. Annls. Inst. Pasteur, 4, 88-102.

GIBSON, J., (1961). Cytochrome pigments from the green photosynthetic bacterium Chlorobium thiosulphatophilum. Biochem. J., 79, 151-158.

GLAUERT, A.M. and GLAUERT, R.H., (1958). Araldite as an embedding medium for Electron Microscopy. J. biophys. biochem. Cytol., 4, 191-194.

GRONLUND, A.F. and CAMPBELL, J.J.R., (1963). Nitrogenous substrates of endogenous respiration in Pseudomonas aeruginosa. J. Bacteriol., 86, 58-66.

GRONLUND, A.F. and CAMPBELL, J.J.R., (1965). Enzymatic degradation of ribosomes during endogenous respiration of Pseudomonas aeruginosa. J. Bacteriol., 90, 1-7.

GRONLUND, A.F., and CAMPBELL, J.J.R., (1966). Influence of exogenous substrates on the endogenous respiration of Pseudomonas aeruginosa. J. Bacteriol., 91, 1577-158.

GROSSOWICZ, N., HAYAT, P. and HALPERI, Y.S., (1957). Pyocyanine biosynthesis by Pseudomonas aeruginosa. J. Gen. Microbiol., 15, 576-583.

GRULA, E.A. and SAVOY, C.F., (1971). A detergent polyacrylamide gel system for electrophoretic resolution of membrane and wall proteins. Biochem. biophys. Res. Comm., 43, 325-332.

HALPERN, Y.S., TENEH, M. and GROSSOWICZ, N., (1962). Further evidence for the production of pyocyanine by non proliferating suspensions of Pseudomonas aeruginosa. J. Bacteriol., 83, 935-936.

HAMILTON, W.A. and DAWES, E.A., (1960). The nature of the diauxic effect with glucose and organic acids in Pseudomonas aeruginosa. Biochem. J., 76, 70P.

HANSON, A.W., (1968). The crystal structure of Myxin. Acta Cryst., B24, 1084-1096.

HARMAN, J.W. and MACBRINN, M.C., (1963). The effect of phenazine methosulphate pyocyanine and EDTA on mitochondrial succinate dehydrogenase Biochem. Pharmacol., 12, 1265 - 1278.

HAROLD, F.M., HAROLD, R.L. and ABRAMS, A., (1965). A mutant of Streptococcus faecalis defective in phosphate uptake. J. Biol. Chem., 240, 3145-3153.

HAWKER, L.E. and LINTON, A.H., (1971). In "Micro-organisms : function form and environment". p78-97. Edward Arnold (Publishers) Ltd.

HAYES, E.E., WELLS, I.C., KATZMAN, P.A., CAIN, C.K., JACOBS, F.A., THAYER, S.A., DOISY, E.A., GABY, W.L., ROBERTS, E.C., MUIR, R.D., CARROL, C.J., JONES, L.R., and WADE, N.J., (1945). Antibiotic substances produced by Pseudomonas aeruginosa. J. Biol. Chem., 159, 725-750.

HAYNES, W.C., (1951). Pseudomonas aeruginosa - its characterisation and identification. J. gen. Microbiol., 5, 939-950.

HELLINGER, E., (1951). Requirements for pyocyanine production by Pseudomonas aeruginosa (Schroeter) Migula. J. gen. Microbiol., 5, 633-639.

HERBERT, D., (1961). The chemical composition of micro-organisms as a function of their environment. Symp. Soc. Gen. Microbiol., 11, 391-416.

HERBERT, R.B. and HOLLIMAN, F.G., (1969). Pigments of Pseudomonas species Part I. Structure and synthesis of aeruginosin A. Part II. Structure of aeruginosin B. J. Chem. Soc., 2514-2520.

HEYDEMAN, M. and AZOULAY, E., (1963). Extraction and properties of aldehyde dehydrogenase from Pseudomonas aeruginosa. Biochim. Biophys. Acta., 77, 545-553.

HEWITT, L.F., (1950). Oxidation-reduction potentials in bacteriology and biochemistry. 6th Ed. E. & S. Livingstone Ltd., Edinburgh.

HILL, R. and WALKER, D.A., (1959). Pyocyanine and phosphorylation with chloroplasts. Plant. Physiol., 34, 240-245.

HILLEMAN, H., (1938). Beiträge zur Kenntnis des Phenazins. III. Mit Über die Stellung der Methylgruppe um Pyocyanin and Über Versuche zur Synthese von Isopyocyanin. Ber. deut. chem. Ges., 71B, 46.

HOLLIMAN, F.G., (1957). Pigments from a red strain of Pseudomonas aeruginosa. Chem. Ind., Dec 28, 1668.

HOLLSTEIN, V., BURTON, R.A. and WHITE, J.A., (1966). In vivo oxidative coupling of anilines and phenolic anilines. Experimentia, XXII, 210-211.

HOLMS, W.H., HAMILTON, I.D. and ROBERTSON, A.G., (1972). The rate of turnover of the adenosine triphosphate pool of Escherichia coli growing aerobically in simple defined media. Arch. für Microbiol., 83, 95-109.

HORIO, T. and KAMEH, M., (1961). Preparation and properties of three pure crystalline bacterial haeme proteins. Biochim. Biophys. Acta., 48, 266-286.

HOU, C.I., GRONLUND, A.F. and CAMPBELL, J.J.R., (1966). Influence of phosphate starvation on cultures of Pseudomonas aeruginosa. J. Bacteriol., 92, 851-855.

HUGH, R. and LEIFSON, E., (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol., 66, 24-26.

INGLEDEW, W.M. and CAMPBELL, J.J.R., (1969a). A new resuspension medium for pyocyanine production. Can. J. Microbiol., 15, 595-598.

INGLEDEW, W.M. and CAMPBELL, J.J.R., (1969b). Evaluation of shikimic acid as a precursor of pyocyanine. Can. J. Microbiol., 15, 535-541.

INGRAM, J.M. and BLACKWOOD, A.C., (1962). Studies on the biosynthesis of pyocyanine. Can. J. Microbiol., 8, 49-56.

JACKSON, D.A., BUNTING, M.I. and MORRISON, D.A., (1963). Suppression of pigmentation by phosphate in certain mutants of Serratia marcescens. Bact. Proc. 53P.

JANGLOVA, Z., SUCHY, J. and VANEK, Z., (1969). Regulation of biosynthesis of secondary metabolites. VII. Intracellular adenosine 5 - triphosphate concentration in streptomyces aureofaciens. Folia Microbiol. (Prague), 14, 208-210.

JENSEN, K.A. and HOLTEN, C.H., (1949). The dipole moment of pyocyanine. Acta. Chem. Scand., 3, 1446-1447.

JOHNSON, M.J., (1949). A rapid micro-method for the estimation of non volatile organic matter. J. Biol. Chem., 181, 707-711.

JOHNSON, G.G., MORRIS, J.M. and BERK, R.S., (1967). The extracellular protease of Pseudomonas aeruginosa exhibiting elastase activity. Can. J. Microbiol., 13, 711-720.

JORDAN, E.O., (1899). Bacillus pyocyaneus and its pigments. J. Exptl. Med., 4, 627-647.

JUDAH, J.D. and WILLIAMS-ASHMAN, H.G., (1951). The inhibition of oxidative phosphorylation. Biochem. J., 48, 33-41.

KASHKET, E. and BRODIE, A., (1963). Oxidative phosphorylation in fractionated bacterial systems. VIII. Role of particulate and soluble fractions from Escherichia coli. Biochim. Biophys. Acta., 78, 52-65.

KATZ, E., (1967). In "Antibiotics". II. Biosynthesis, p276. Ed. by Gottlieb, D. and Shaw, P.O., Springer-Verlag, Berlin.

KATZ, E. and WEISSBACH, H., (1962). Biosynthesis of the actinomycin chromophore. Enzymatic conversion of 4-methyl-3-hydroxyanthranilate to actinocin. J. Biol. Chem., 237, 882-886.

KELLENBERGER, E., RYTER, A. and SECHAUD, J., (1958). Electron microscope study of DNA containing plasmids. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physical states. J. biophys. biochem cytol., 4, 671-678.

KHARCHENKO, N.S., RYABUSHKO, E.O. and PETRUKHOVA, O.I., (1947). Biological activity of a new antibiotic pyocyanine. Vrachebnoe Delo, 27, 19-24.

KING, E.O., WARD, M.K. and RANEY, D.E., (1954). Two simple media for the demonstration of pyocyanine and fluorescein. J. Lab. Clinical Medicine, 44, 301-307.

KING, J.V., CAMPBELL, J.J.R., and EAGLES, B.A., (1948). The mineral requirements for fluorescein production. Can. J. Research, C26, 514-519.

KLIMOFF, J. (1901). Cited by Schoental, (1941). Zeitschr. Hyg. Infektionskrankh, 37, 120.

KOCH, M.L., (1956). Bacteremia due to bacterial species of the genera Aerobacter, Escherichia, Paracolobactrum, Proteus and Pseudomonas. Antibiotic. Med. Clin. Therapy, 2, 113-121.

KORNBERG, H. and PHIZACKERLEY, P., (1961). Malate oxidation by Pseudomonas spp. Biochem. J., 79, 10P.

KURACHI, M., (1958a). Studies on the biosynthesis of pyocyanine. I. On the cultural conditions for pyocyanine formation. Bull. Inst. Chem. Research, Kyoto Univ., 36, 163-173.

KURACHI, M., (1958b). Studies on the biosynthesis of pyocyanine. II. Isolation and determination of pyocyanine. Bull. Inst. Chem. Research, Kyoto Univ., 36, 174-187.

KURACHI, M., (1958c). Studies on the biosynthesis of pyocyanine. III. On the effect of iron and other heavy metals. Bull. Inst. Chem. Research, Kyoto Univ., 36, 188-199.

KURACHI, M., (1959a). Studies on the biosynthesis of pyocyanine. IV. On the effect of methionine and other promoting factors in peptone. Bull. Inst. Chem. Research, Kyoto Univ., 37, 48-58.

KURACHI, M., (1959b). Studies on the biosynthesis of pyocyanine. V. On the bacterial mutation from the point of view of pigmentation. Bull. Inst. Chem. Research, Kyoto Univ., 37, 59-72.

KURACHI, M. (1959c). Studies on the biosynthesis of pyocyanine. VI. On the degradation of pyocyanine. Bull. Inst. Chem. Research, Kyoto Univ., 37, 73-84.

KURACHI, M., (1959d). Studies on the biosynthesis of pyocyanine. VII. On the effect of anthranilic acid. *Bull. Inst. Chem. Research, Kyoto Univ.*, 37, 85-101.

KURACHI, M., (1960). Biosynthesis of pyocyanine. IX. Effect of pyocyanine on the respiration of bacterial cells of *Pseudomonas aeruginosa*. *Bull. Inst. Chem. Research, Kyoto Univ.*, 38, 364-378.

LANDAU, B.R., HASTINGS, A.B. and ZOTTER, S., (1963). Pyocyanine and metabolic pathways in liver slices in vitro. *Biochim. Biophys. Acta.*, 74, 629-634.

LEDDERHOSE, G., (1888). "Über den blauen Eiter. *Deut. Zeit. Chirurgie*, 28, 201-207.

LIENNERSTRAND, A., (1938). "Über die Konkurrenz zwischen Azetaldehyd und pyocyanin molekularem Sauerstoff im fluoridvergifteten Apozymasesystem. *Naturwissenschaften*, 26, 45-46.

LESSIE, T. and NEIDHARDT, F.C., (1967). Adenosine Triphosphate-linked control of *Pseudomonas aeruginosa* Glucose-6-phosphate dehydrogenase. *J. Bacteriol.*, 93, 1337-1345.

LEVITCH, M.E. and RIETZ, R., (1966). The isolation and characterisation of 2-hydroxyphenazine from *Pseudomonas aureofaciens*. *Biochemistry*, 5, 689-692.

LICHSTEIN, H.C. and SAULE, M.H., (1944). Studies on the effect of sodium azide on microbial growth and respiration. III. The effect of sodium azide on the gas metabolism of *Bacillus subtilis* and *Pseudomonas aeruginosa* and the influence of pyocyanine on the gas exchange of pyocyanine free strain of *P. aeruginosa* in the presence of sodium azide. *J. Bacteriol.*, 47, 239-251.

LIU, P.V., (1966). The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. II. Effects of lecithinase and protease. *J. Infect. Diseases*, 116, 112-116.

LIU, P.V. and MERCER, C.B., (1963). The role of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. *J. Infect. Diseases*, 108, 218-228.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J., (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275.

LUFT, J.H. (1961). Improvements in epoxy resin embedding methods. *J. biophys. biochem. Cytol.*, 9, 409-414.

MACDONALD, J.C., (1963). Biosynthesis of pyocyanine. *Can. J. Microbiol.*, 9, 809-819.

MACDONALD, J.C., (1966). Production of pyocyanine by *Pseudomonas aeruginosa* ATCC 9027. *Can. J. Microbiol.*, 12, 771-774.

MACDONALD, J.C., (1967). Pyocyanine. In Antibiotics. II. Biosynthesis, p.52-65. Ed. by Gottlieb, D. and Shaw, P.D., Springer-Verlag, Berlin.

MACKELVIE, R.M., CAMPBELL, J.J.R. and GRONLUND, A.F., (1968). Survival and intracellular changes of *Pseudomonas aeruginosa* during prolonged starvation. *Can. J. Microbiol.*, 14, 639-645.

MCCOMBIE, H. and SCARBOROUGH, H.A., (1923). Chemical composition of bacterial pigments. I. The isolation of pyocyanine and the preparation of its salts. *J. Chem. Soc.*, 123, 3279-3285.

McELROY, W.D., (1963). Determination of adenosine-5-triphosphate (c). Methods of enzymatic analysis, p. 565-566. Ed. by Bergmeyer, H.U. Weinheim (Bergstr.): Verlag Chemie; Academic Press, New York.

MCILWAIN, H.J., (1937). The phenazine series. VI. Reactions of alkyl phenazonium salts; the phenazyls. *J. Chem. Soc.*, 1701-1711.

MCILWAIN, H., (1950). Properties of preparations from the central nervous system which degrade coenzymes I and II; their connection with carbohydrate metabolism. *Biochem. J.*, 46, 612-619.

MALIK, V.S. and VINING, L.C., (1970). Metabolism of chloramphenicol by the producing organism. *Can. J. Microbiol.*, 16, 173-179.

MARCUS, A. and FEELEY, J., (1962). Effect of phenazine methosulphate and pyocyanine on the L-amino acid oxidase reaction. *Biochim. Biophys. Acta.*, 59, 398-407.

MARTIN, E.L. and MACLEOD, R.A., (1971). Isolation and chemical composition of the cytoplasmic membrane of a gram negative bacterium. *J. Bacteriol.*, 105, 1160-1167.

MICHAELIS, L., (1931). The formation of semiquinones as intermediary reduction products from pyocyanine and some other dyestuffs. *J. Biol. Chem.*, 92, 211-232.

MICHAELIS, L., (1932). Theory of the reversible two-step oxidation. *J. Biol. Chem.*, 96, 703-715.

MIGULA (1895). In "Die natürl Pflanzenfam". 1, p.29. Ed. by Engler and Prantl.

MIGULA (1900). In "Bergeys Manual of determinative bacteriology", (1948). 6th Ed. by Breed, R.S., Murray, E.G.D. and Hitchens, A.P: Williams and Wilkins, Baltimore, Md.

MILLICAN, R.C., (1962). Biosynthesis of pyocyanine. Incorporation of <sup>14</sup>C-shikimic acid. *Biochim. Biophys. Acta.*, 57, 407-409.

MILLAR, A.L. and WALKER, J.B., (1970). Accumulation of streptomycin-phosphate in cultures of streptomycin producers grown on a high phosphate medium. *J. Bacteriol.*, 104, 8-12.

MITCHELL, P. and MOYLE, J., (1956a). Osmotic function and structure in bacteria. *Symp. Soc. gen. Microbiol.*, 6, 150-180.

MITCHELL, P. and MOYLE, J., (1956b). Permeation mechanisms in bacterial membranes. *Discuss. Faraday Soc.*, 21, 258-265.

MOOS, W.S. and ROWEN, J.W., (1953). Identification of a copper complex forming fraction of pyocyanine. *Archs. Biochem. Biophys.*, 43, 88-96.

MUNRO, H.N. and FLECK, A., (1966). The determination of nucleic acids. *Methods in biochemical analysis*, 14, 113-176.

NAKAMURA, S., MAEDA, K. and UMEZAWA, H., (1964). Structure of griseolutin B. *J. Antibiotics Tokyo*, 17, 33-36.

NG, F.M.-W. and DAWES, E.A., (1967). Regulation of enzymes of glucose metabolism by citrate in *Pseudomonas aeruginosa*. *Biochem. J.*, 104, 48P.

NORTON, J.E., BULLIER, G.S. and SOKATCH, J.R., (1963). The oxidation of D-alanine by cell membranes of *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta.*, 78, 136-147.

OKINAKA, R.T. and DOBROGOSZ, W.J., (1967). Catabolite repression and pyruvate metabolism in *Escherichia coli*. J. Bacteriol., 93, 1644-1650.

OWEN JR, C.A., KARLSON, A.G. and ZELLER, E.A., (1951). Enzymology of tubercle bacilli and other mycobacteria. V. Influence of streptomycin and other basic substances on diamine oxidase of various bacteria. J. Bacteriol., 62, 53-62.

PAPPENHEIMER, A.M. Jr., (1965). In "Bacterial and Mycotic Infections of Man", p468, 4th Ed. by Dubos, R.J. and Hirsch J.G., J.B. Lippincott, Philadelphia.

RENNIE, R.P. and ARBUTHNOTT, J.P., (1971). Effects of carbohydrates on haemolysin production by *Escherichia coli*. Infect. Immun., 3, 849-851.

REPASKE, R., (1958). Lysis of gram negative organisms and the role of versene. Biochim. Biophys. Acta., 30, 225-232.

REYNOLDS, J.A. and TANFORD, C., (1970). Binding of dodecyl sulphate to proteins at high binding ratios. Possible implications for the state of proteins at high binding ratios. Proc. Nat. Acad. Sci., 66, 1002-1007.

RINGEN, L.M. and DRAKE, C.H., (1952). A study of the influence of *Pseudomonas aeruginosa* from various natural sources. J. Bacteriol., 64, 841-845.

ROBERTS, I.M., (1970). Reduction of compression artifacts in ultra-thin sections by application of heat. J. Microscopy, 92, 57-61.

ROBINSON, G.L., (1932). The growth of *Bacillus pyocyaneus* in synthetic media. Brit. J. Exptl. Path., 13, 310-314.

RUNNSTROM, J. and MICHAELIS, L., (1935). Correlation of oxidation and phosphorylation in haemolysed blood in the presence of methylene blue and pyocyanine. J. Gen. Physiol., 18, 717-727.

SCHLAECHTER, M. MAALOE, O., and KJELDGAARD, N.O., (1958). Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. gen. Microbiol., 19, 592-606.

SCHNEIDER, W.C., (1957). Determination of nucleic acids in tissues by pentose analysis. In 'Methods in Enzymology' vol. 3, p. 680-684. Ed. by Colowick, S.P. and Kaplan, N.O. Academic Press Inc., New York.

SCHNEIDERSON, S.S., AMSTERDAM, D. and PERLMAN, E., (1960). Inhibition of *Pseudomonas aeruginosa* pigment formation by chloramphenicol and erythromycin. Antibiotics and Chemotherapy, X, (1), 30-33.

SCHROETER, J., (1872). Über einige durch Bakterien gebildete Pigmente. Cohn's Beitr. Biol. Pflanzen, 1, Heft 11, 109-126.

SCHOENTAL, R., (1941). The nature of the antibacterial agents present in *Pseudomonas pyocyanae* cultures. Brit. J. Exptl. Pathol., 22, 137-147.

SELEEN, W.A. and STARK, C.N., (1943). Some characteristics of the green fluorescent pigment producing bacteria. J. Bacteriol., 46, 491-500.

SHANDS, J.W. Jr., (1971). The physical structure of bacterial lipopolysaccharides. In Vol IV of "Microbial Toxins", pp 127-144. Ed. by Weinbaum, G., Kadis, S. and Ajl, S.J. Academic Press, New York.

SHAPIRO, A.L., VINUELA, E. and MAIZEL, J.V. JR., (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun., 28, 815-820.

SHEIKH, N.M. and MACDONALD, J.C., (1964). Biogenesis of the N-methyl group of pyocyanine. Can. J. Microbiol., 10, 861-866.

SHOOTER, R.A., WALKER, K.A., WILLIAMS, V.R., HORGAN, G.M., PARKER, M.T., ASHESHOV, E.H. and BULLIMORE, J.F., (1966). Faecal carriage of *Pseudomonas aeruginosa* in hospital patients: possible spread from patient to patient. Lancet, ii, 1331-1334.

SORESINA, C., (1939). The effect of pyocyanine on the growth of tissue cultures in vitro. Tumori, 12, 306-311.

STANIER, R.Y., PALLERONI, N.J. and DOUDOROFF, M., (1966). The aerobic Pseudomonads : a Taxonomic study. J. gen. Microbiol., 43, 159-271.

STARKA, J., (1971). Cell envelope proteins of dividing and non dividing cells of *E. coli*. Feds Letters, 16, 223-225.

STERN, I.J., WANG, C.H. and GILMOUR, C.M., (1960). Comparative catabolism of carbohydrates in *Pseudomonas* species. J. Bacteriol., 72, 601-611.

- STHEEMAN, A.A., (1927). Die Rolles des Pyocyanins im Stoffwechsel vom *Pseudomonas pyocyanae*. *Biochem. Z.*, 191, 320-336.
- STOKES, J.L., PECK, R.L. and WOODWARD, JR., C.R., (1942). Antimicrobial action of pyocyanine, hemipyocyanine, pyocyanase and tyrothricin. *Proc. Soc. Exptl. Biol.*, 51, 126-130.
- SURREY, A.R., (1955). In Organic synthesis 'Pyocyanine', Vol. III, p.753-756. Wiley, New York.
- SUTHERLAND, J. and WILKINSON, J., (1962). Azurin - a blue bacterial protein *Biochem. J.*, 84, 43P.
- SWAN, G.A. and FELTON, D.G.I., (1957). "Phenazines" p. 174-209. Interscience Publishers Inc., New York.
- TAKEDA, R. and NAKANISHI, I., (1959). Pseudomonas pigments. Production of pyoluteorin by fermentation with *Pseudomonas aeruginosa* T359. *Hakko Kogaku Zasshi*, 38, 9-19.
- TOOHEY, J.E., NELSON, C.D. and KROTHOV, G., (1965). Isolation and identification of two phenazines from a strain of *Pseudomonas aureofaciens*. *Can. J. Bot.*, 43, 1055-1062.
- VENABLE, J.H. and COGHESHALL, R., (1965). A simplified lead citrate stain for use in electron microscopy. *J. Cell. Biol.*, 25, 407-408.
- VON TIGERSTROM, M. and CAMPBELL, J.J.R., (1966). The tricarboxylic acid cycle, the glyoxylate cycle and enzymes of glucose oxidation in *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, 12, 1015-1022.
- VON TIGERSTROM, R.G. and RAZZELL, W.E., (1968). Aldehyde dehydrogenase, I. Purification and properties of the enzyme from *Pseudomonas aeruginosa*. *J. Biol. Chem.*, 243, 2691-2702.
- WAKSMAN, S.A. and WOODRUFF, H.B., (1942). Selective antibiotic action of various substances of microbial origin. *J. Bacteriol.*, 44, 373-384.
- WALKER, M.S. and WALKER, J.B., (1971). Streptomycin biosynthesis. *J. Biol. Chem.*, 246, 7034-7040.

WEIL-MALHERBE, H., (1937). XLIV. Studies on brain metabolism. II. Formation of succinic acid. *Biochem. J.*, 31, 299-312.

WEINBERG, E.D., (1970). Biosynthesis of secondary metabolites : Role of trace metals. *Adv. Microbial Physiol.*, 4, 1-44.

WEINBERG, E.D., (1971). Secondary metabolism : Raison d'etre. *Perspectives in Biology & Medicine*, 14, 565-577.

WESTPHAL, O., LÜDERITZ, O. and BISTER, F., (1952). "Über die Extraction von Bakterien mit phenol-wasser. *Zeitschr. f. Naturforschung*, 7B, 148-155.

WILLIAMS, R.P. and HEARN, W.R., (1967). "Prodigiosin" in Antibiotics, vol. 2, p. 410-432. Biosynthesis. Ed. by Gottlieb, D. and Shaw, P.D. Springer-Verlag, Berlin.

WILSON, G.S., and MILES, A.A., (1964). In "Principles of Bacteriology and Immunology", vol. I, p. 636. Arnold, London.

WOODRUFF, H.B., (1966). The physiology of antibiotic production. *Symp. Soc. gen. Microbiol.*, 16, 22-46.

WREDE, F. and STRACK, E., (1924). "Über das pyocyanine den blauen Fabbstoff des *Bacillus pyocyaneus*. *Zeitschr. f. Physiol. Chem.*, 140, 1-15.

WREDE, F. and STRACK, E., (1925). "Über das pyocyanine den blauen Fabbstoff des *Bacillus pyocyaneus*. *Zeitschr. f. Physiol. Chem.*, 142, 103-319.

WREDE, F. and STRACK, E., (1928). "Über das Pyocyanin den blauen Fabbstoff des *Bacillus pyocyaneus*. III. Die Kinstitution des Hemipyocyanin. *Zeitschr. f. Physiol. Chem.*, 177, 177-186.

WREDE, F. and STRACK, E., (1929a). "Über das Pyocyanin den blauen Fabbstoff des *Bacillus pyocyaneus*. IV. Die Konstitution und Synthese des Pyocyanins. *Zeitschr. f. Physiol. Chem.*, 181, 58-76.

WREDE, F. and STRACK, E., (1929b). Synthesis of pyocyanine and some of its homologues. *Ber. deut. chem. Ges.*, 62B, 2051-2057.

YAMANAKA, T. and OKUNUKI, K., (1964). Comparative biochemistry of cytochrome c. *J. Biol. Chem.*, 239, 1813-1817.

YOUNG, L., (1937). The effect of pyocyanine on the metabolism of cerebral cortex. J. Biol. Chem., 120, 659--675.

ZUAGG, W.S., (1964). Spectrophotometric characteristics and some chemical properties of N-methylphenazinium methyl sulphate and pyocyanine at the semiquinone oxidation level. J. Biol. Chem., 239, 3964--3970.

ZANKER, V. and PETER, W., (1958). Die prototropen Formen des Fluorescins Chemische Berichte, 19, 572--576.

SUMMARY

### SUMMARY

The object of this investigation was to define the conditions under which pyocyanine was produced by Pseudomonas aeruginosa and to determine the role of the pigment in the metabolism of the organism.

The type strain NCTC 6750, does not readily produce pyocyanine in ordinary liquid medium, but reproducible pyocyanine yields were obtained using the two-stage culture technique of Ingledew and Campbell (1969a). This involved initial (non pigmented) growth of the organism in broth followed by transfer of washed, stationary-phase cells to a Resuspension Medium in which pyocyanine was produced. This latter medium was characterised by lack of phosphate, being otherwise nutritionally complete. Results with NCTC 6750 were similar to those previously reported by Ingledew and Campbell (1969a) with ATCC 9027 under somewhat similar conditions.

Pyocyanine in culture supernatants was assayed spectrophotometrically at 690 and 314 nm using synthesized pyocyanine as a standard. By comparing the spectra of culture supernatants with the spectra of known phenazines, pyocyanine was identified as the only pigment present in significant amounts.

A detailed examination of Resuspension Medium was made to identify the components which were essential for pyocyanine production. Absolute requirements were found to be: aerobic conditions, metabolizable sources of carbon, nitrogen and sulphur, and the complete absence of phosphate. These findings are contrary to those of Burton et al., (1948) who specified a requirement for phosphate, and to that of Kurachi (1958c) who considered iron essential. Maximum pyocyanine yields were obtained using succinate,  $\text{NH}_4^+$  and  $\text{SO}_4^{2-}$  as metabolizable components.

A critical variable, in the successful use of Resuspension Medium, was the level of inoculum used.

An attempt was made to resolve the controversy (MacDonald, 1967) concerning proliferation of P. aeruginosa during pyocyanine production. Incubation of the cells in Resuspension Medium was characterised by three phases. During Phase One, from zero to three hours, no pyocyanine was produced. However the cell protein doubled. Increases of 70% and 40% were recorded in cell mass and viable count respectively but cell opacity was unchanged. Phase Two, the period of linear increase in pyocyanine concentration, lasted from three to thirteen hours and involved a 180% increase in cell opacity. During this time, cell mass remained constant but the viable count fell. In Phase Three, pyocyanine production, which had already reached its maximum level, stayed constant until observation ceased at twenty four hours. It was thus clear that the cells were not actively growing at the same time as pyocyanine was being produced.

Because of the critical role of phosphate in the production of pyocyanine, the major phosphorus-containing components of the cell were assayed and a rather complex pattern of changes in DNA, RNA, lipid, phospholipid and lipopolysaccharide was observed. However ATP remained constant throughout.

The effect of phosphate in inhibiting pyocyanine production was analysed by adding graded levels at intervals throughout incubation in Resuspension Medium. "Zero-hour" cells were sensitive to 40 $\mu$ M phosphate and the inhibition was proportional to phosphate concentration. As pyocyanine synthesis progressed however, higher levels of phosphate were required for inhibition.

Chloramphenicol at 20% of its growth-inhibitory concentration, reduced pyocyanine production to one half when added to "zero-hour" or "three-hour" cells. As no growth occurred in the latter it was inferred that

pyocyanine formation required protein synthesis.

The addition of synthesized pyocyanine at levels normally produced by the cells (unit level) had no effect on the "zero-hour" cells. "Three-hour" cells were 30% inhibited. Addition of 2.5 "units" of pyocyanine caused 70% inhibition of "three-hour" cells but still no effect on "zero-hour" cells. These results were interpreted as feedback inhibition acting on cells of different permeability.

Although pyocyanine is generally classified as a secondary metabolite, it appears to perform a function in the respiration of *P. aeruginosa*. The endogenous oxygen consumption and the succino-oxidase in isolated cell envelopes of "three-hour" cells was selectively stimulated. Pyocyanine also stimulated the succinate dehydrogenase of cell envelope preparations from "twenty-hour" cells but inhibited the enzyme in "zero-hour" and "three-hour" cell envelopes.

In conclusion, there is a clear need for further general investigations of the acquisition, transport and metabolism of phosphate in micro-organisms. The use of two-stage growth procedures may have further application in the study of other pigments, and of antibiotics and toxins. The production of pyocyanine in Resuspension Medium presents a useful model system for the investigation of secondary metabolism.

APPENDICES

## APPENDIX 1

## Preparation of media

Trypticase Soy Agar (TSA ; B.B.L., Bioquest)

1.5 g	Pancreatic digest of casein
0.5 g	Soy peptone
0.5 g	Sodium chloride
1.5 g	Agar

Dissolve in one litre of distilled water.

Autoclave for 15 min at 15 psi. Pour into plastic petri dishes.

Glucose-ammonium salts agar

Except where otherwise stated 'Analar' grade chemicals were used.

2.0 g	Glucose
20.0 g	$\text{NH}_4\text{Cl}$
4.0 g	$\text{NH}_4\text{NO}_3$
8.0 g	$\text{Na}_2\text{SO}_4$
12.0 g	$\text{K}_2\text{HPO}_4$
4.0 g	$\text{KH}_2\text{PO}_4$
0.4 g	$\text{MgSO}_4$
20.0 g	Agar (Difco)

Dissolve in one litre of distilled water and autoclave (15 min at 15 psi). Pour into plastic petri dishes.

## APPENDIX 1 (Continued)

Yeast-Tryptone liquid medium (after Von Tigerstrom and Razzell, 1968)

3.0 g	$(\text{NH}_4)_2\text{HPO}_4$
4.0 g	$\text{K}_2\text{HPO}_4$
1.0 g	Yeast extract (Difco)
1.0 g	Tryptone (Difco)
2.0 g	Disodium succinate

Dissolve in 900 ml of distilled water and adjust to pH 7.4 with  $\text{NaOH}$  before autoclaving (15 min at 15 psi).

5 mg	$\text{FeSO}_4$
0.5 g	$\text{MgSO}_4$

Dissolve in 20 ml of distilled water and sterilise by membrane filtration using a Millipore (Millipore, London) filter with pore size 0.22  $\mu$ . Using membrane filtration, sterilise 3 ml of 70% aqueous ethanol.

Add the filter sterilised components to the previously autoclaved and cooled medium. Make up to one litre with sterile distilled water and dispense in 100 ml aliquots into 250 ml Erlenmeyer flasks.

## APPENDIX 2

## Preparation of Buffers

Tris buffer

0.05 M Tris buffer pH 7.4

6.05 g Tris (hydroxymethyl) aminomethane

(Puriss. A.R., Koch-Light, Colnbrook).

Dissolve in 1900 ml of distilled water. Add concentrated hydrochloric acid till pH = 7.4. Make up to one litre with distilled water.

Veronal acetate buffer

Basic solution :

19.43 g	Sodium acetate
29.43 g	Sodium 5,5-diethylbarbiturate
34.0 g	NaCl

Make up to one litre with distilled water. To 5 ml of basic mixture add

7 ml	0.1 N HCl
13 ml	distilled water
0.25 ml	1 M Ca Cl <sub>2</sub> (111g per litre)

The solution of volume 25.25 ml has pH 6.1.

## APPENDIX 2 (Continued)

Tris-glycine (Electrophoresis buffer)

0.05 M Tris-glycine pH 8.3.

6.0 g Tris (hydroxymethyl) aminomethane

28.8 g G-glycine

Dissolve in one litre of distilled water. For use, dilute above buffer 1 in 10 and make 0.1% with respect to sodium dodecylsulphate.

## APPENDIX 3

## SDS Disc-gel Electrophoresis

## Stock Solutions:

## A. Separation gel buffer

1N HCl	48 ml
Tris	36.6 g
TEMED (Tetramethylethylenediamine; B.D.H. Poole, Dorset)	0.23 ml
Distilled water to 100 ml	
pH 8.3	

## B. Stacking gel buffer

1N HCl	48 ml
Tris	5.98 g
TEMED	0.46 ml
Distilled water to 100 ml	
pH 6.7	

## C. Separation gel

Acrylamide	46.67 g
BIS (N,N'-methylenebisacrylamide Puriss, A.R., Koch-Light, Colnbrook)	0.612 g
Distilled water to 100 ml	

## D. Stacking gel

Acrylamide	16 g
BIS	4 g
Distilled water to 100 ml	

## APPENDIX 3 (Continued)

E. Stacking gel catalyst

Riboflavin 4 mg  
Distilled water to 100 ml

F. Separation gel catalyst

Ammonium persulphate 0.14 g  
Distilled water to 100 ml

G. 40% Sucrose in distilled water.

H. 20% Sodium dodecyl sulphate (SDS) (Koch-Light) in distilled water.

Stock solutions A, B, E and F were stored at 4°C in brown glass bottles. Solutions C, D and G were freshly made before each experiment.

<u>Preparation of separating gels</u>	<u>11.7%</u>	<u>7.0%</u>
Solution A	2.5 ml	2.5 ml
Solution C	5.0 ml	3.3 ml
Distilled water	2.4 ml	4.1 ml
Solution F	10 ml	10 ml
Solution H	0.1 ml	0.1 ml

Mix well. Pipette 1.0 ml volumes into disc-gel tubes which have one end covered with parafilm. Carefully layer each column with water. Place gels in dark to set (approximately 30 min).

APPENDIX 3 (Continued)Preparation of stacking gell

Solution B	1 ml
Solution D	2 ml
Solution E	1 ml
Solution G	4 ml
Solution H	0.05 ml

Mix well. Pipette 0.2 ml onto the top of each separating gel. Carefully layer each with water to provide a flat surface at the top of the gel on setting. Photopolymerise the gels for 15 min.

Fixative and Stain

Amido Black (Gurr, London)	1 g
Acetic acid	10 ml
Methanol	50 ml
Distilled water	40 ml

The solution was filtered and stored in a capped bottle.

Tracking dye

Progress of electrophoresis is followed by the addition of 5 drops of 1% bromothymol blue to the upper reservoir.

APPENDIX 4Preparation of Reagents

Reagent for Folin test for protein (from Lowry et al., 1951)

Reagent A: 2% Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in  
0.1N sodium hydroxide

Reagent B: 1% copper sulphate ( $\text{Cu SO}_4 \cdot 5\text{H}_2\text{O}$ )  
in distilled water  
2% aqueous solution of  
sodium potassium tartrate  
On the day of use mix equal volumes  
of the above two solutions

Reagent C: To 50 ml of reagent A add 1 ml of  
reagent B. Discard after one day.

Reagent D: Folin-Ciocalteu reagent  
(B.D.H., Poole, Dorset)

Johnson Reagent for total carbon

Dissolve 5.0 g of sodium dichromate ( $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ ) in 20 ml  
of distilled water. Dilute to one litre with 95% sulphuric acid.  
Store in a brown glass bottle protected from the light.

Reagents for DNA and RNA determination (from Munro and Fleck, 1966)

Diphenylamine reagent for DNA: 1.5% diphenylamine in freshly distilled

APPENDIX 4 (Continued)

glacial acetic acid. Add 1.5 ml of concentrated sulphuric acid.

On day of use, add 0.1 ml of 1.6%, w/v, aqueous acetaldehyde to 20 ml of the above solution.

Orcinol reagent for RNA: the stock solution of acidic ferric chloride contains 330 mg of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  dissolved in one litre of concentrated hydrochloric acid. Prepare a 10%, w/v, solution of orcinol in absolute ethanol. Add 0.3 ml orcinol solution to 3.0 ml of stock solution.

Reagents for Phosphorus analysis (from Allen, 1940)

Molybdate reagent: dissolve 8.3 g of ammonium molybdate in 100 ml of distilled water.

Perchloric acid: add 60 ml of concentrated perchloric acid to 40 ml of distilled water.

Amino-naphthol-sulphonic acid reagents:

Add 2.0 g of 1-amino-2-naphthol-4-sulphonic acid to 97 ml of 20% sodium bisulphite. Shake until dissolved. Add 2.7 ml of 20% sodium sulphite. This solution can be used for up to ten days and should be kept in a stoppered bottle in the dark.

Standard phosphorus solution

Dissolve 1.0967 g of oven dried potassium dihydrogen phosphate in 250 ml of distilled water. This solution contains 1 mg per ml of phosphorus.

