

A STUDY OF THE FACTORS

AFFECTING PIGMENT PRODUCTION

BY PSEUDOMONAS AERUGINOSA.

A Thesis

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by

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A C K N O W L E D G E M E N T S .

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- (4) His wife for her encouragement and assistance in the cleaning of some of the glassware.

S U M M A R Y.

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In order to study the conditions for optimum production of red pigments some means of determining these quantitatively had to be developed. A good separation of pigments A and B has been achieved by means of paper chromatography, this forming the basis for their quantitative evaluation.

Pigment production by these organisms is variable, whole batches of cultures often producing none, or only little, of the red pigments. Subculturing the bacteria on agar-slopes resulted in a marked decrease in red pigmentation, which could, however, be restored to its original value by passing the organisms through certain media. Because of this variability it was found necessary to make dried pellets of the *Id* strain of *Pseudomonas aeruginosa* in order to standardise as far as possible the bacteria to be able to compare various findings at different times.

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relative amounts of the two individual pigments produced in cultures of these organisms have been shown to depend largely on the oxygenation conditions, the formation of pigment A being favoured by an increased supply of air, or by the inclusion of a chemical source of oxygen into the medium, the yield of pigment B being hardly affected under these conditions. Microaerophilic cultures, on the other hand, resulted in the production of pigment B only.

The possibility that pigments A and B are interconvertible when added separately to an actively growing culture, grown under oxygenation conditions which favour their individual formation, was investigated. Some of the findings indicate that the organisms can convert pigment A to pigment B when employing a medium lacking glycine.

Growth and red pigment production was found to depend very much on the composition of the medium in which the organisms of the Li strain of *Pseudomonas aeruginosa* grow. The ions, Mg, SO₄, K, PO₄ and Fe have been found essential for good red pigment formation, the various amino acids and carbon sources present in the medium greatly affecting the yields of pigments A and B.

The pH changes which take place in cultures where red pigment production is good are similar, their pH curves all showing two or three depressions which appear to be due to the formation of a number of products, including the precursors of pigment A and B and the red pigments themselves. The production of pigment A always takes place between a pH of 8.0 and 8.5, pigment B being present in maximum amounts only when the cultures have reached their highest pH, a stage when the respiration rate of the culture has dropped to about zero.

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Pseudomonas aeruginosa has been studied and
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INTRODUCTION.

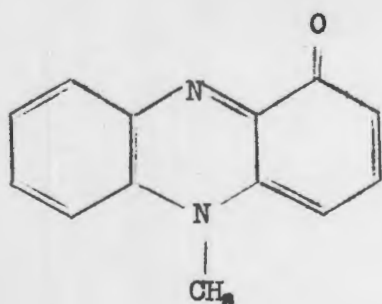
Gessard (1882) established that the blue-green discoloration of purulent secretions and of pus was due to the presence of "*Bacillus pyocyaneus*", which colours the pus blue, due to the formation of the blue pigment pyocyanine. The name given to these organisms was later changed to *Pseudomonas pyocyanea*, and its official name to-day is *Pseudomonas aeruginosa*. These are slender Gram-negative, rod like organisms, about 1 - 3 μ by about 0.5 μ in size. Terminal flagella give them a rapid motility. In cultures these bacteria may occur in clumps, or in long or short chains. They are aerobic, and grow poorly under anaerobic conditions. The optimum temperature of growth is 37°C, but the organisms can grow at room temperature. Little is known about the chemical composition of *Pseudomonas aeruginosa*. These bacteria have according to Nicolle and Alilaire¹ a free water content of 74.99% and a nitrogen content of 9.79%, based on the dry weight of the organisms. Wheeler² found their ash content to be 9.04%. The chemical composition of bacteria in general, however, will vary according to the composition of the medium in which they grow, the age of the organisms, and several other factors.

A great many bacteria, including some species of *Pseudomonas*, produce a variety of pigments, those which have been isolated may be classified as follows³ :

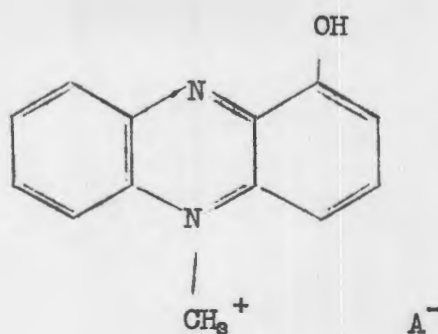
Pyrrole derivatives
 Phenazines
 Carotenoids
 α -naphthoquinone
 Miscellaneous.

Up to now several phenazine pigments have been isolated from cultures of *Pseudomonas*, and the structure of some have been clearly established.

Pseudomonas aeruginosa produces among other pigments the blue pyocyanine which was the first phenazine derivative to be found in nature. Wrede and Strack⁴ isolated this pigment from broth cultures of *Pseudomonas aeruginosa*, and Schoental⁵ from agar cultures. Wrede and Strack⁴ determined the structure of pyocyanine (I) and confirmed it by synthesis. Pyocyanine is freely soluble in chloroform under alkaline conditions, and it may be extracted from cultures with this solvent. Under acid conditions its colour changes from blue to red, being then no longer soluble in chloroform, but is extracted into the acid aqueous layer. The salt possesses the structure shown in II.



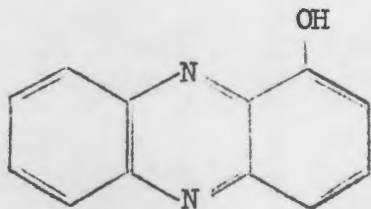
I



II

The absorption spectrum was determined by Nitzsche⁸ in 1944, and later confirmed by Mirvish and Holliman⁷ in 1950.

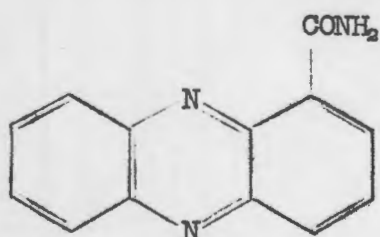
Older cultures of *Pseudomonas aeruginosa*, which initially produce pyocyanine, contain a second pigment known as hemipyocyanine (1 - hydroxyphenazine) (III).



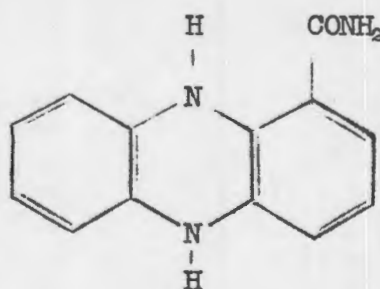
III

Hemipyocyanine was isolated by Fordos⁸ and later by Schoental⁵, and it was prepared from pyocyanine by Wrede and Strack⁴.

*Pseudomonas chlororaphis*⁹ produces the green pigment chlororaphin, which on exposure to air is quickly converted to the yellow oxychlororaphin. The structure of chlororaphin was investigated by Kögl and Postowsky¹⁰, Kögl and Tönnis¹¹, and by McIlwain¹². Chlororaphin is composed of one molecule of the amide of phenazine-1-carboxylic acid (IV) and one molecule of dihydrophenazine-1-carboxylamide (V).

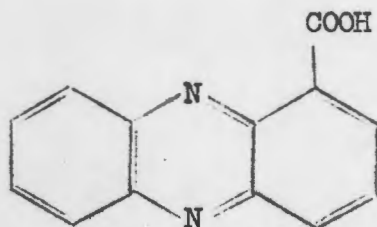


IV



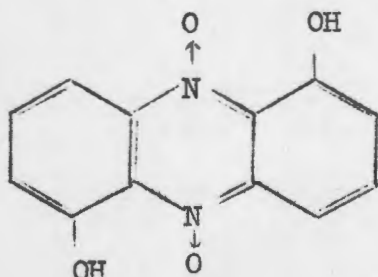
V

W.C. Hayes¹³ and his co-workers, as well as Kluyver and his co-workers, reported on the main pigment produced by *Pseudomonas aureofaciens*; they isolated phenazine alpha-carboxylic acid (VI) from cultures of these bacteria.



VI

Another phenazine pigment, the purple iodinin, is formed by *Chromobacterium iodinum*. The pigment iodinin was shown by Clemo and McIlwain¹⁴ to be the di-N-oxide of a dihydroxyphenazine. The possibility that it was 1:2-dihydroxyphenazine was eliminated by Hegedüs¹⁵. The synthesis of 1:3-dihydroxyphenazine by Clemo and Daghish¹⁶ completed the investigation of the four possible isomers containing two hydroxy-groups in the same ring. It has since been shown by Clemo and Daghish¹⁷ that the two hydroxyl groups in iodinin (VII) occupy the 1:5-positions:



VII

A number of pigments other than phenazine derivatives are produced by *Pseudomonas*; these include a lyochrome from *Pseudomonas fluorescens*¹⁸, and the blue pigment indigoidine from *Pseudomonas indigofera*¹⁹.

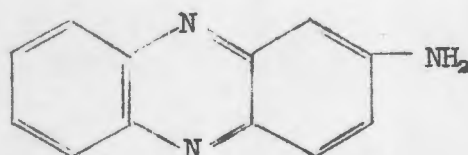
Further investigations carried out on the pigments produced by *Pseudomonas aeruginosa* were discussed by J.A. Rivera in the Symposium on Bacterial Pigments¹³. He obtained excellent pigmentation with some 350 strains. Various pigments could be detected by paper chromatography, but he has not reported on their isolation or structure.

In 1948 Don and van den Ende²⁰ isolated the L-strain of *Pseudomonas aeruginosa* from the urine of a patient suffering from cystitis following partial resection of the bladder. It produced on Hartley's agar a yellow fluorescent pigment which changed after a few days of incubation at 37°C or at room temperature to a clear red, which persisted for several months. They found that the red pigment produced by their isolated strain appeared to consist, at least in part, of a substance differing from pyocyanine in solubility and reaction to change of pH. It differed also from the brown discoloration which occurred on prolonged storage of the usual pyocyanine producing strains.

The red strains appear to be relatively rare. They have been studied by Gessard²¹ and by Meader²². Meader considers

three pigments, viz. pyocyanine, fluorescin, and pyorubin to be present in all strains but to varying amounts. He considers that pyorubin is present in the vast majority of strains, though usually masked by the pyocyanine, and only shows up when the pyocyanine is bleached by oxidation. The pigments appear to be derived originally from leuco-bases. - Gaby²³ described in his work three strains but did not include a red strain in his series.

Professor F.G. Holliman²⁴ of this University has succeeded in isolating small amounts of two red pigments from the L strain of Don and van den Ende²⁰ and is at present investigating their structure. The isolated pigments are crystalline and water soluble; both appear by their absorption spectrum to be related to 2-aminophenazine (VIII).



VIII

He has also studied some of the conditions which will permit optimum production of red pigments in these strains with a view to find a large scale method so that larger amounts of the red pigments can be isolated for their structural investigations. This work has been continued by the author and a report of his findings will be given in this thesis.

Pigmentation by micro-organisms is governed by a variety of factors. Reid²⁵ showed that the principal element essential for the production of pigments by bacteria is nitrogen. The literature on this subject in general is so large that it is impossible to give an extensive review. Only brief mention of some of the studies, as applied especially to the pigment production in *Pseudomonas aeruginosa*, will therefore be given. The supply of certain mineral salts, the carbon and nitrogen sources, the pH of the medium, temperature of incubation, and the oxygen supply are all factors which may at times govern pigment production by bacteria.

Dernby²⁶ reported on the pH requirements for the growth of *Pseudomonas aeruginosa*, 5.6 being the minimum pH, 8.0 the maximum pH, and a pH of 6.6 - 7.0 being the optimum for growth. Stearn and Stearn²⁷ found that best pigment production in *Pseudomonas aeruginosa* occurs between a pH of 6.8 and 7.3.

Little has been reported on the effect of various oxygenation conditions on pigment production in *Pseudomonas aeruginosa*. The author has given much attention to this section in his studies on the behaviour of these organisms.

The composition of the medium has a marked effect on growth and pigment formation in *Pseudomonas aeruginosa*. Robinson²⁸ considered phosphate and probably magnesium as essential

constituents of the media. Available sources of nitrogen for growth were ammonium salts, nitrates, and amino acids; available carbon and energy sources include acetate, lactate, citrate, glycerol, dextrose, amino acids, ethanol, and n-butanol. Robinson worked out the optimum ranges of concentrations of several constituents in his media, and he found that these tended to be rather more limited for pigmentary activity and anaerobic growth than for aerobic growth. - Webster and Bernheim²⁹ showed in 1936 that *Pseudomonas aeruginosa* can break down various amino acids to unidentified products, CO₂ and NH₃; the amino acids include: glycine, alanine, valine, leucine, iso-leucine, and phenylalanine. - Pandalai and Rao³⁰ studied the nutritional requirements of ten strains of *Pseudomonas aeruginosa*. They showed that phosphate was essential for growth of the organisms, and that sulphur in the form of SO₄⁻⁻⁻ and S⁻⁻⁻ improved pigment formation. Lactate was found to be essential for pyocyanine production. - Ph. Lasseur³¹ and his co-workers found that there was no fluorescence with *Pseudomonas aeruginosa*, variety pyocyanogenes, in the absence of Mg, and a maximum amount with 100 - 500 mg. of MgSO₄ per 100 ml. of the employed medium. - Di Maggio³² again showed that *Pseudomonas aeruginosa* retained its capacity for reproduction, motility, pigment production, and fermentations in media lacking Na, Mg, Ca, and K ions. Pigment production took place in synthetic media containing glucose or mannitol as sole carbon source. Peptone had an inhibitory

effect on pigment production.

Burton, Campbell, and Eagles³³ found that amino acids can replace peptone in media used for the production of pyocyanine by *Pseudomonas aeruginosa*. The addition of L-leucine to a medium containing glycine or DL-alanine increased pyocyanine formation whereas DL-phenylalanine under similar conditions was an inhibitor. Uniformly good pyocyanine yields were obtained with 5 different strains of *Pseudomonas aeruginosa* in a medium containing 0.4% glycine or DL-alanine, 0.8% L-leucine, 1% glycerol, and a salt mixture containing K, Fe, Mg, Cl, phosphate and sulphate. Media with acid hydrolysed casein were also found to be satisfactory. These workers also studied the mineral requirements for pyocyanine production³⁴. Growth and pyocyanine formation by

Pseudomonas aeruginosa was studied in a medium containing 1% glycerol, 0.6% glycine, 0.6% L-leucine and various salts. The optimal salt concentrations were 0.03% K_2HPO_4 , 0.05 - 0.5% $MgSO_4 \cdot 7H_2O$, and 0.0005% $FeSO_4 \cdot 7H_2O$. All five of the above ions were necessary for pyocyanine formation. The presence of various other ions in the medium either inhibited growth or pyocyanine production. - King, Campbell, and Eagles³⁵ found that the following salt mixture gave the highest yields of fluorescin with *Pseudomonas aeruginosa* in a 1% glycerol medium: 0.01% $MgCl_2$, 0.06% K_2HPO_4 , 0.005% K_2SO_4 , and 0.05 p.p.m. of $FeCl_3$. The formation of pyocyanine

was also studied and found to be dependent mainly on the concentrations of SO_4^{--} and Mg^{++} , whereas Fe^{+++} was the most critical element for fluorescin production.

Totter and Moseley³⁶ studied the influence of the concentration of iron on the production of fluorescin by *Pseudomonas aeruginosa*. At a final pH between 6 and 8.5 the production of fluorescin by these organisms is related inversely to the concentration of iron in the medium. - The studies of Hellinger³⁷ on the pyocyanine production by a strain of *Pseudomonas aeruginosa*, grown in static cultures, indicated that Mg, PO_4 , SO_4 , and NH_4 ions should be added at the time of inoculation if maximum yields of pyocyanine were to be obtained. The carbon sources preferred were glucose, ethanol or glycerol. The presence of Ca, Na, K or Fe ions was not required for maximum yields of the pigment.

Holliman²⁴ in his investigations found that the following medium gave good red pigment production by the *Id* and *Idi* strains of *Pseudomonas aeruginosa*²⁰ when the cultures were incubated sloped and static at 37°C: 0.1% L-leucine, 0.5% glycine, 0.5% mannitol, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03% KH_2PO_4 , and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (from a stock solution of 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 0.02N H_2SO_4). The pH of this medium is adjusted to 7.0 with KOH.

The reports from various workers on the influence of certain components of their media on the pigment production by

Pseudomonas aeruginosa in general are not in good agreement. The requirements for optimum growth and pigment production by the individual strains of these organisms must thus be carefully studied.

BRIEF OUTLINE OF THE PROBLEM.

There are many strains and variants of *Pseudomonas aeruginosa*, and of the range of pigments they produce, two red pigments from the *Li* and *Li¹* strains²⁰ are formed in relatively high concentration. The problem under investigation involves:

- (1) The isolation of the two red pigments.
- (2) The elucidation of the structure of these pigments.
- (3) Observations in the red pigment production.
- (4) The biological sequence in the formation of these two pigments.

The work on the isolation of the two red pigments, referred to in this thesis as "pigment A" and "pigment B", and the elucidation of their structure has been started by Holliman²⁴ and the investigations are still in progress. The more important observations in the red pigment production by the *Li* and *Li¹* strains of *Pseudomonas aeruginosa* include:

- (a) Variability of the organisms.
- (b) Sensitivity of the organisms to:
 - (i) oxygenation conditions;
 - (ii) the composition of the medium.

- (c) pH changes during growth and pigment formation.
- (d) Respiration rates of the organisms during growth and pigment formation.

Many private communications from Professor Holliman on his earlier investigations have been of great help to the author.

SECTION A.TECHNIQUES USEDPART 1: Cleaning and sterilizing of apparatus.

All glassware was brushed using Teepol, rinsed at least ten times with tap water, and then thoroughly rinsed with distilled water. After drying, all openings were plugged with cotton wool.

Sterilization of all apparatus by dry heat was carried out at a temperature of 180°C for at least one hour, and by moist heat under a pressure of 15 - 18 lbs. for 20 minutes or longer. All the usual precautions regarding the manipulation and packing of the autoclave were strictly observed.

The cleaning of the glassware, the preparation of all media, the sterilizing of all apparatus and media, as well as all inoculations were carried out by the author. He was thus solely responsible for all operations, and he could ensure, within reason, that no foreign material or unwanted micro-organisms were introduced into the cultures, which would have spoilt the experiments and would have made the observations and findings unreliable. Much time was therefore spent on the preparation of the many experiments.

PART 2: Methods of inoculation.

Porter³⁸ and other workers reported that the size and age of the inoculum appear to have a considerable effect on the

adjustment of an organism to a fresh medium. The author in his work has thus attempted to standardise the inoculum as far as possible. For all quantitative work the "parent culture" originated from a pellet* of the dried organisms of the Li strain of *Pseudomonas aeruginosa*. One to two pellets were aseptically transferred to a boiling tube containing 15 ml. of the "G.L.M."** liquid medium, shaken, then closed with a cotton wool plug and incubated sloped at 37°C. A loop-full at a time of the surface growth of this culture, which took 24 - 48 hours to develop, was used to inoculate further boiling tubes containing 15 ml. of the G.L.M. medium, which were closed with a cotton wool plug and incubated sloped at 37°C. These cultures are in this thesis referred to as "parent cultures". A loop-full of the surface growth of one of these parent cultures, after 24 hours of incubation, was used as an inoculum for a medium, which, in this thesis, is then said to be "inoculated".

It is found that the Li strain of *Pseudomonas aeruginosa* forms a pellicle on the surface of the G.L.M. liquid medium, which on shaking results in clumping and it is therefore difficult to obtain a uniform inoculum from liquid cultures of these organisms. If, however, after shaking the parent culture, the clumps are allowed to settle to the bottom of the tube, it is possible to withdraw a portion from the centre

* Making of pellets, page 36.

** Composition of the G.L.M. medium, page 22.

of the culture which then contains the organisms in a finely dispersed form. This dispersion is in this thesis referred to as the "starter" of the *L1* strain of *Pseudomonas aeruginosa*, which is used in experiments where comparative results are required. Equal volumes of the "starter" were used for all inoculations in a series of experiments.

For other sets of experiments, where the inoculum had to be standardised even more carefully, a large quantity of the medium was inoculated with a known volume of the "starter", shaken, and several portions of this were aseptically transferred to sterile culture vessels, each culture then being allowed to develop under specified conditions.

A modified method of inoculation was used whenever a new medium was tested for its suitability for growth and red pigment formation. The surface growth of the "parent culture" was used as an inoculum for the new medium and the surface growth of this sub-culture was then used to inoculate all the culture vessels containing the same medium under test. In this way the transfer of unwanted material from culture to culture was minimized.

PART 3: Measurements of respiration rates.

Respiration rates were used as a measure of viable cells on account of the clumping of the organisms in liquid cultures prohibiting the use of the dilution-plate counting methods.

The respiration rate of the organisms at 37°C was determined by the manometric technique, using the Warburg Manometer. 2 ml. of the culture under examination were used for each determination, placing into the centre well of the flask a small roll of filter paper moistened with 0.2 ml. 10% KOH, so increasing the surface area for the absorption of CO₂. The respiration rate is expressed in μ l of O₂ per ml. of culture per hour.

PART 4: Method employed for the quantitative estimation of pigments "A" and "B".

The Li and Idi strains of *Pseudomonas aeruginosa* produce red pigments under favourable conditions in suitable media. In order to study the conditions for optimum production of red pigments some means of determining these quantitatively had to be developed. At the beginning of the biological work the red colour intensity was judged visually, primarily to see whether any red pigments were produced or not. Later the colour intensity was estimated colorimetrically on filtered cultures. Obvious shortcomings of this method are, that

- (i) the cultures contain a mixture of pigments, and hence the determination of individual pigments is impossible;
- (ii) during filtration pigments are adsorbed on to the filter pad;
- (iii) only approximate relative values of total red pigment concentration can be obtained as no suitable standard can be used.

The red pigments could not be extracted directly and individually from the cultures with organic solvents. A suitable method had

thus to be devised.

A good separation of pigments A and B has been achieved by means of paper chromatography. It was soon recognised that this method had immense possibilities for the quantitative determination of these two red pigments. The upper layer of a 5 : 1 : 4 mixture of n-butanol, acetic acid and water was found to be a good solvent for one-dimensional separation in downward development, using a reasonably airtight glass container with a suspended trough to hold the developing solvent.

For the qualitative work strips of paper varying from 2 to 3 inches in width and 14 - 16 inches in length were used. A small quantity of the culture was absorbed on to the paper as a spot and after drying further quantities were applied. Location of the pigments was achieved by comparing R_F values with pigments A and B which were run alongside the unknown on the same strip of paper. In much of the qualitative work the R_F value of some of the pigments which were studied was not used, but reference was made to the distance moved by pigments A and B, the position then being specified as R_A and R_B respectively.

Pigment production by *Pseudomonas aeruginosa* is variable, whole batches of cultures often producing no or only little of the two main pigments. All experiments on pigment formation were thus done by growing at least 5 cultures, and then combining these before determining the two main pigments

quantitatively by paper chromatography.

For quantitative work 2 to 3 ml. of the combined cultures were absorbed as a band in 3 or more applications on to a thick absorbing paper. Sheets of 3 mm. paper* varying from 6 - 9 inches in width and 14 - 16 inches in length were successfully used. Pigment A moves rapidly under these conditions having an R_F value of 0.39 at 20°C, whereas pigment B has an R_F value of only 0.10 at 20°C, provided the freshly prepared solvent is used. Other pigments, being present in the red cultures in small but varying amounts and having R_F values which differ sufficiently from those of pigments A and B, did not interfere in most of the determinations with the separation of the two main pigments. Occasionally the "A" band had to be cut off when it approached the bottom of the sheet so allowing the "B" band to separate better from other pigments. Location of the pigments was easily achieved by comparing R_F values with pigments A and B run alongside on separate strips with the unknown. - After separation had been achieved the chromatogram was removed from the glass tank and dried at room temperature. The bands of pigments A and B were carefully cut out from the chromatogram, cut into small pieces and the pigments were then eluted with a known volume of 1N HCl. The clarified solution was determined photometrically by comparison with standard solutions of the pure pigments (isolated by Holliman²⁴) in 1N HCl.

* Whatman.

The method for the quantitative determination of pigments A and B was tested in several ways. Known amounts of the pigments were applied on to 3 MM. paper and then the percentage recovery was determined. In the one series of the tests the pure pigments were dissolved in distilled water, in another series they were dissolved in the G.L.M. medium in order to see whether any constituents of the medium do interfere with the separation of the two pigments, and in yet another series the pigments were added to a culture in which the concentrations of pigments A and B had been previously determined by the described method, again to see whether any of the other products formed do interfere with the method. The concentration of the pigments was varied to extremes in order to establish what percentage of recovery might be expected. The highest concentrations of the two pigments used in the tests are of the order of the concentrations in which they are found in cultures which gave good red pigmentation. The following table summarizes the results of the tests:-

| Concentration of pigment A in mg. per litre solution: | Concentration of pigment B in mg. per litre solution: | Pigments dissolved in: | Number of applications on to 3 MM. paper: | Total number of ml. solution applied: | % Recovery | |
|---|---|------------------------|---|---------------------------------------|------------|------------|
| | | | | | Pigment A: | Pigment B: |
| 20.0 | NIL | H ₂ O | 3 | 2.05 | 94.5 | - |
| 20.0 | NIL | H ₂ O | 3 | 1.98 | 91.5 | - |
| 2.6 | NIL | H ₂ O | 6 | 4.00 | 80.8 | - |
| 2.6 | NIL | H ₂ O | 6 | 4.10 | 82.1 | - |
| NIL | 42.0 | H ₂ O | 3 | 1.85 | - | 90.5 |
| NIL | 42.0 | H ₂ O | 3 | 1.96 | - | 91.2 |
| NIL | 3.1 | H ₂ O | 6 | 3.88 | - | 83.9 |
| NIL | 3.1 | H ₂ O | 6 | 4.05 | - | 80.0 |
| 19.5 | 36.5 | H ₂ O | 3 | 1.97 | 90.7 | 90.4 |
| 19.5 | 36.5 | H ₂ O | 3 | 1.88 | 88.5 | 88.2 |
| 19.5 | 36.5 | G. I. M. medium | 2 | 1.32 | 86.8 | 87.8 |
| 19.5 | 36.5 | G. I. M. medium | 3 | 1.90 | 90.0 | 91.3 |
| 19.1 | 39.3 | Red culture | 4 | 2.08 | 86.0 | 87.3 |
| 19.1 | 39.3 | Red culture | 4 | 2.11 | 85.2 | 89.5 |

* Equal volumes of a mixture of pigments A and B in water, containing 19.5 mg. of A and 36.5 mg. of B respectively, and of a red culture in which the concentration of A and B were found by the described method to be 18.7 mg. and 42.1 mg. respectively, were mixed.

* *

The duplicate values obtained in the many determinations of pigments A and B in the cultures throughout the whole investigation were always in good agreement, the values found rarely differed by more than 5%. The method, although not very accurate for lower concentrations, yet reproducible in all cases, was therefore considered satisfactory for the biological work.

The standard solution of pigment B in 1 N HCl is not stable, whereas that of pigment A was found to keep well. After storing the standard solution of B for 6 months in the refrigerator its concentration decreased by 25%.

SECTION B.

THE BEHAVIOUR OF THE ORGANISMS.

PART 1: Effect of subculturing and recovery of red pigment production.

(1) Introduction.

Holliman²⁴ established that good red pigment formation in *Pseudomonas aeruginosa*, strains Ii and Iii, takes place when using a medium of the following composition*: 0.1% L-leucine, 0.5% glycine, 0.5% mannitol, 0.1% $MgSO_4 \cdot 7H_2O$, 0.03% KH_2PO_4 , and 0.001% $FeSO_4 \cdot 7H_2O$ (from a stock solution of 1% $FeSO_4 \cdot 7H_2O$ in 0.02 N H_2SO_4) in water. The pH is adjusted to 7.0 - 7.1 with 1 N KOH.

This medium has been used by the author in most of his experimental work. It will in this thesis be referred to as the "Glycine-Leucine-Mannitol" medium and will be abbreviated with the letters "G.L.M.". For all slopes the G.L.M. medium with the inclusion of 2% Bacto-Agar was used.

(ii) The effect of subculturing on G.L.M. slopes.

G.L.M. slopes, inoculated with the Ii strain of *Pseudomonas aeruginosa*, turn dark purplish-

* The effect of various constituents of a medium on pigment formation by *Pseudomonas aeruginosa* in general has been briefly outlined in the introduction of this thesis. Prof. Holliman²⁴ experimented with various media and found his above mentioned medium most suitable for red pigment production by the Ii and Iii strains of *Pseudomonas aeruginosa*.

red in a short time, occasionally requiring only 4 days to develop their maximum red colour. The following are the average, normal colour changes which take place when the slopes are incubated at 37°C:

- After 1 day: very pale yellow with a green tinge. The pigments are only on or just below the surface of the agar.
- After 2 days: bright fluorescing yellow. The colour has spread throughout the solid medium.
- After 3 days: still bright fluorescing yellow, but $\frac{1}{2}$ " - 1" of the tip of the solid medium is turning distinctly pink to red.
- After 4 days: orange-red throughout, with the possible exception of the centre portion of the medium.
- After 5 days: fairly dark purplish red throughout the medium.
- After 6 - 7 days: dark purplish red; no further colour changes appear to take place after this. Even 2 months old slopes are very dark purplish red.

Not all slopes of the *Id* or *Idi* strains turn dark red. An occasional slope has a marked brown or even olive green tinge. Some of the slopes have only a slight red pigmentation. It was repeatedly observed that after several subcultures from slope to slope the amount of red pigment produced was small as compared with the original slope, some developing only a very pale pink colour even after prolonged incubation at 37°C. This was further investigated.

Four slopes at a time were inoculated with the *Id* strain of *Pseudomonas aeruginosa*. Successive subcultures were made at 24 hourly intervals and they were incubated

at 37°C until no further change in colour took place. Only little difference in red pigmentation could be observed up to the 3rd subcultures. Thereafter the formation of red colour showed a gradual decrease, often accompanied by the development of a brownish or olive green tinge. It was repeatedly observed that after 10 - 12 subcultures the amount of red pigment produced was very small, and further subculturing had little or no effect.

(iii) The effect of subculturing in the liquid G.L.M. medium.

On inoculating boiling tubes, containing 15 ml. of the G.L.M. medium, with the Id strain of *Pseudomonas aeruginosa*, plugging these with cotton wool, and incubating them sloped and undisturbed at 37°C, dark red pigmentation results in 6 - 9 days. The following are the average, normal colour changes which occur:-

- After 1 day: very pale yellow with a green tinge; the pigments are just below the surface of the culture.
- After 2 days: bright fluorescing yellow. The colour has diffused throughout the culture. Surface growth is very heavy, the remainder of the culture being only hazy.
- After 3 days: Still bright yellow throughout, turning olive green or brownish at the tip nearest the plugged end of the tube.
- After 4 days: Part of the surface growth starts to settle. The colour on the surface of the culture is dirty olive green or brownish green, the remainder being deep yellow.
- After 5 days: The tip nearest the plugged end of the tube turns distinctly red. There are otherwise no further visual changes.

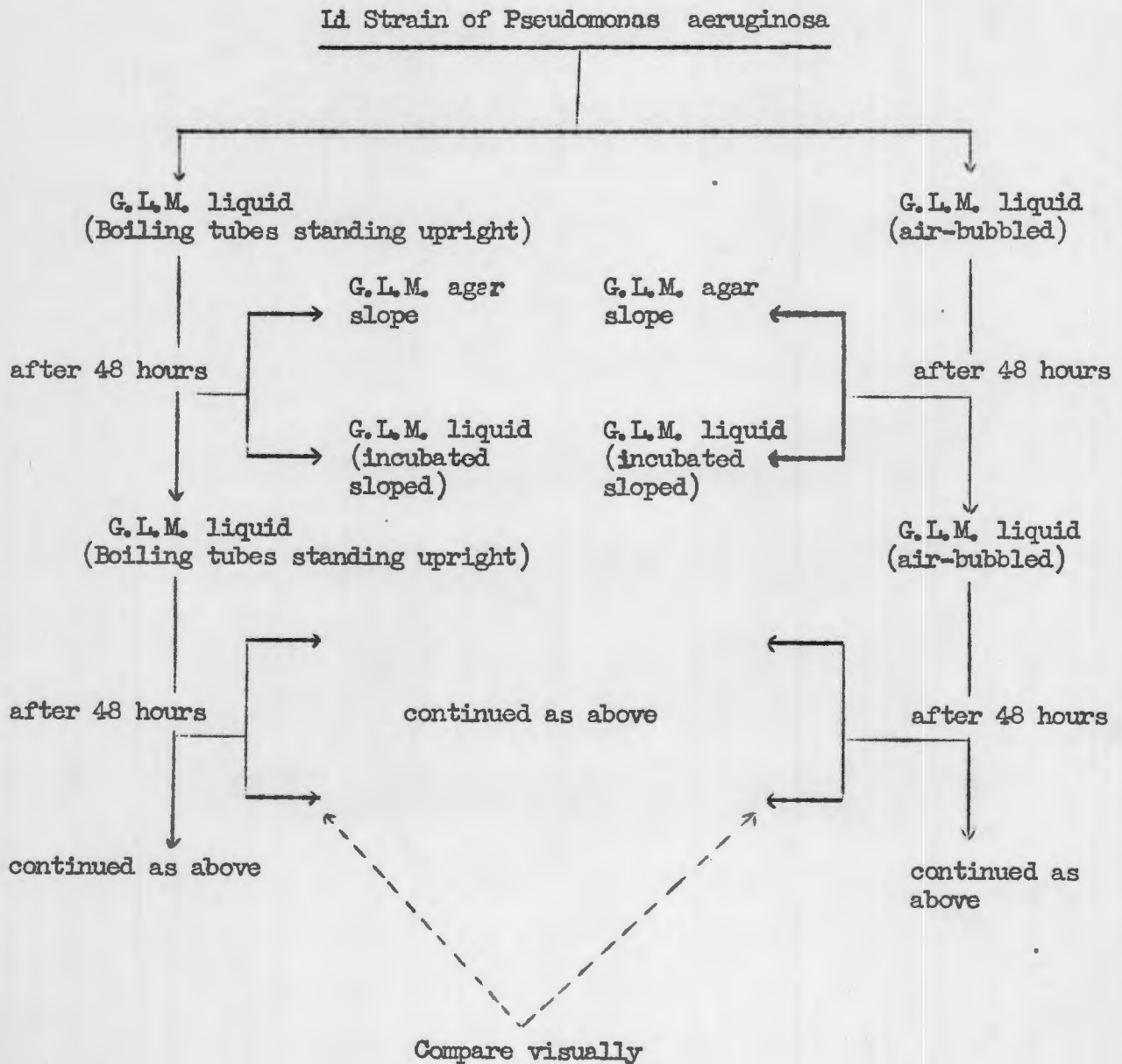
- After 6 days: The surface of the whole culture is orange red, the remainder being yellowish to brown.
- After 7 days: The culture is purplish red throughout. There is no more surface growth.
- After 8 days: The culture has cleared and turned deep purplish red in colour throughout.

No further changes appear to take place, unless the culture is disturbed.

The colour changes in the case of air-bubbled cultures are different, turning yellow after 24 - 48 hours and usually remaining yellow to yellow-brown for a further 48 - 72 hours, after which a pale to dark red coloration is obtained.

The effect of subculturing the organism in the G.L.M. liquid medium under certain conditions was investigated as subculturing on slopes had a marked effect on red pigment formation. Four boiling tubes, containing 15 ml. of G.L.M. medium, were inoculated with the Li strain of *Pseudomonas aeruginosa*. Two of these were plugged with cotton wool and kept standing upright, and through the other two a slow but constant stream of air was bubbled. All tubes were incubated at 37°C for 48 hours after which they were separately subcultured into liquid G.L.M. media. At the same time two G.L.M. slopes and two boiling tubes each containing 15 ml. of the G.L.M. medium were inoculated, incubated at 37°C, and the red colour was then compared visually after maximum red pigmentation had developed. The boiling tube was in this case kept sloped. The subculturing was repeated 24 times, i.e. for over 1½ months, after

which pellets of the organisms were made, kept over P_2O_5 , and later, when a method for the determination of the two main pigments was found, the concentrations of pigments A and B were quantitatively estimated and compared with those of the original pellets, which were kept under identical conditions.



Observations after 24 successive subcultures:

(a) Visual changes:

The effect of subculturing in the liquid G.L.M. medium was not nearly as pronounced as was the case in the subculturing of the slopes. The red pigment production was still quite good even after the 24 subcultures.

There was a gradual decrease in red pigmentation in the slopes which were made from the static subcultures. After the 24 subcultures the slopes never developed a purplish red colour, but they turned pale orange red to brownish in shade. The slopes of the bubbled subcultures always resulted in dark **purplish red** pigmentation, and in general the red pigments developed faster than in the slopes of the static subcultures.

A gradual decrease in red pigmentation could be observed in the sloped liquid G.L.M. cultures which were obtained from the successive subcultures. Those from the bubbled subcultures always developed a purplish red colour, whereas those from the static ones had eventually a brownish tinge. The time required for red pigmentation was about the same in both cases.

(b) Changes in the concentration of the red pigments:

The pellets of the original *Id* strain (not subcultured), and those made from the organisms of the static and bubbled cultures after 24 successive subcultures were placed each separately into 5 boiling tubes containing 15 ml. of the liquid G.L.M. medium.

After 24 hours incubation at 37°C fresh tubes containing the medium were inoculated with a loopful of the surface growth of each of the cultures and kept sloped. Incubation was continued until maximum red pigmentation had developed. The contents of the 5 boiling tubes of each batch of cultures was combined and pigments A and B were then determined quantitatively. The following table summarizes the results:-

| Origin of Pellets: | mg. of pigment A /litre culture | mg. of pigment B /litre culture |
|---------------------|------------------------------------|------------------------------------|
| Original Id | 6 | 42 |
| Static subcultures | 2 | 24 |
| Bubbled subcultures | 0 | 16 |

Although the subculturing of the organisms in the G.L.M. liquid medium resulted in a decrease of red pigmentation, it was not nearly as marked as the subculturing on the G.L.M. slopes. Further experiments were thus carried out with the view of finding a method to restore the ability to produce red pigments, after this ability had been lost by successive subculturing of the organisms on G.L.M. agar slopes.

(iv) Restoration of ability to produce red pigments.

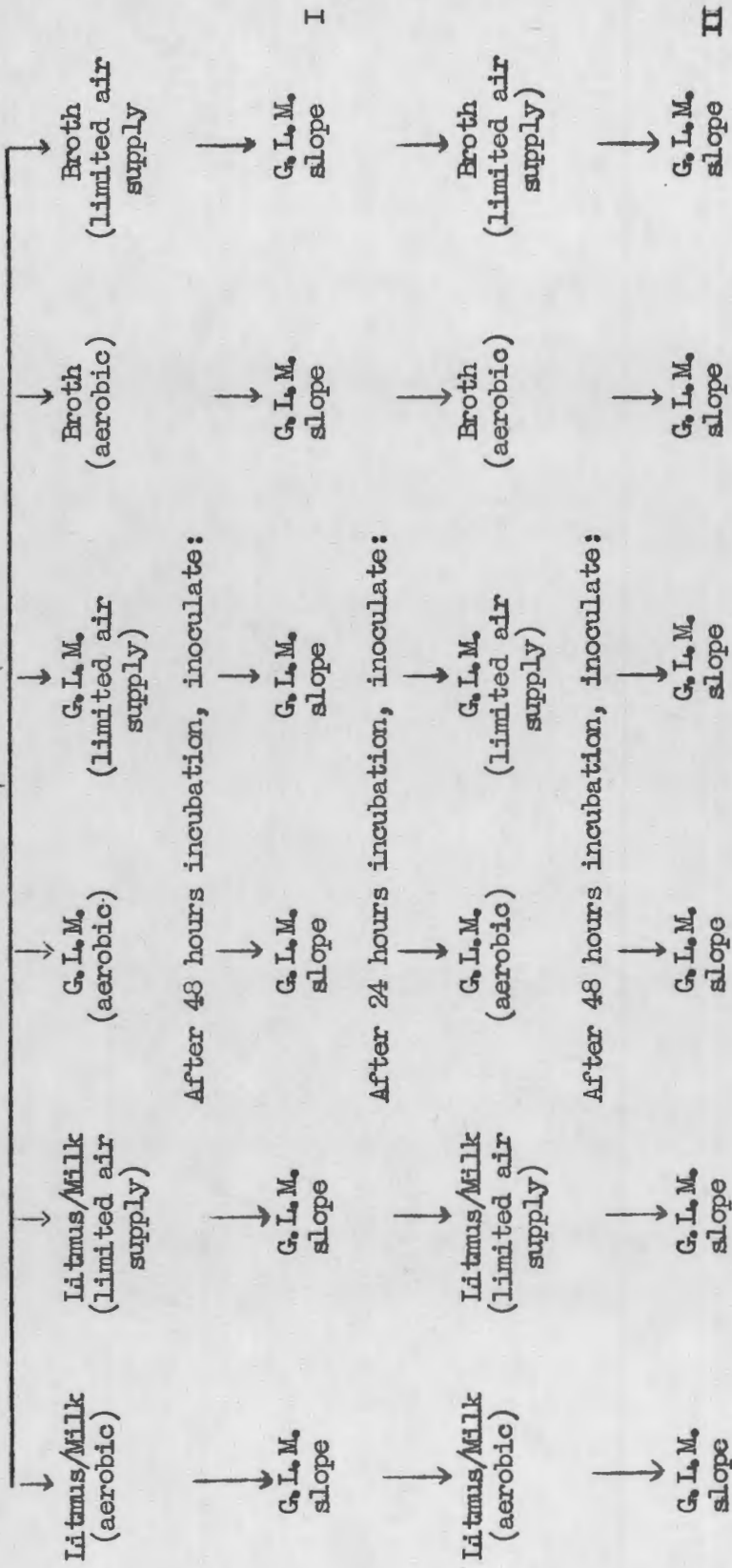
A series of experiments was carried out to find a method by which the ability of the bacteria to produce the red pigments could be restored to its original value.

It has been observed by Dr. Don³⁹ of this University that passing the bacteria through Litmus/Milk* in many cases improved the production of red pigments, and an investigation of this observation was made in the following way. The organisms, obtained from the slopes of subcultures where red pigmentation was very poor, were passed in parallel experiments through Litmus/Milk, the G.L.M. liquid medium, and Hartley's Beef Digest** Broth. The air supply was varied to the extreme, half of the bottles in each case being closed tightly with a screw cap (i.e. limited air supply), the other half being plugged with cotton wool (i.e. aerobic conditions). The bottles were incubated standing upright at 37°C.

* A medium of Litmus/Milk is obtained by dissolving 105 g. of Bacto-Litmus-Milk powder (Difco-Laboratories) in 1000 ml. of distilled water.

** Hartley's Broth is a triptic digest of beef heart.

With each of 6 slopes, poor red pigment producers, inoculate:



All slopes were examined visually after no further changes in colour had taken place, and only those slopes which were now dark red in colour were counted as having been restored to good red pigment producers. As a control the original 6 pale red slopes were further subcultured from slope to slope. These all remained poor in red pigment formation. This experiment was repeated twice. The following tables summarize the findings of the experiments:

(a) Passing the organisms only once through the media, i.e. slopes I:

| Medium: | Number of slopes which improved in red pigment production: | | | |
|-----------------------------|--|---------|---------|-------------------|
| | Max. 12 | Max. 12 | Max. 12 | Total: Max. 36 |
| Litmus/Milk | 1 | 0 | 4 | 5 |
| G.L.M. | 8 | 5 | 8 | 21 |
| Hartley's Beef Digest Broth | 3 | 2 | 4 | 9 |

It made only little difference to the result whether the organisms were passed through the medium aerobically or with a limited air supply, viz. of the total of 35 "improved" slopes the bacteria of 22 passed through the media where the air supply was limited. The red colour intensity in all improved slopes was, however, about the same.

(b) Passing the organisms twice through the media, i.e. slopes II:

| Medium: | Number of slopes which improved in red pigment production: | | | |
|-----------------------------|--|---------|---------|-------------------|
| | Max. 12 | Max. 12 | Max. 12 | Total: Max. 36 |
| Litmus/Milk | 5 | 4 | 5 | 14 |
| G.L.M. | 9 | 11 | 10 | 30 |
| Hartley's Beef Digest Broth | 4 | 5 | 5 | 14 |

The difference in result when passing the organisms aerobically or with a limited air supply through the media was in this case more pronounced, viz. of the total of 58 "improved" slopes the bacteria of only 16 passed through the media where the air supply was limited, but again the red colour intensity in all improved slopes was about the same.

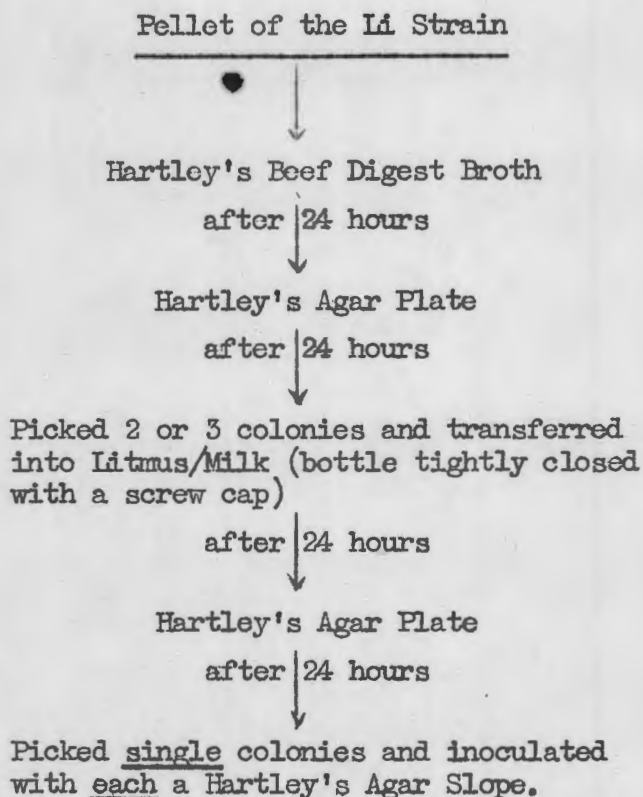
Passing the organisms for a third time through the media had little or no added effect.

The ability of the bacteria to produce the red pigments could thus in many cases be restored. Advantage was taken of this in the making of the pellets of the organisms (see page 36).

(v) "Maytham's 'Yellow' Slope".

In the beginning of all the biological investigations Mr. Maytham of the Bacteriology Department of this University supplied

the author with the slopes of the Li strain of *Pseudomonas aeruginosa* for his experimental work. Maythan prepared the slopes in the following way:



Maythan supplied the author on one occasion with two slopes of the Li strain, one of which turned red in colour as was expected, the other, however, remaining yellow for 5 days, the colour then changing to an olive green to yellow shade. No red pigments were produced, not even on prolonged incubation at 37°C. - This yellow slope was subcultured for 8 weeks but not one of the subcultures resulted in red pigmentation. The organisms of Maythan's yellow slope were then passed several times through Litmus/Milk, the G.L.M.

medium, and Hartley's Beef Digest Broth as was done in the experiments for the restoration of the ability to produce red pigments. Again no red pigmentation resulted. - A large number of boiling tubes, containing 15 ml. of the G.L.M. medium, were inoculated with the bacteria of Mythar's yellow slope; approximately one out of every six cultures developed a trace of red coloration, this being due to the presence of small amounts of pigment B and/or of another red pigment which has an R_A^* of 0.14 when using the n-butanol-acetic acid-water solvent. Attempts were made to oxidise the filtrate of those cultures which turned only yellow in colour, air and H_2O_2 being used at various pH's. No red colour resulted, whereas the later yellow pigments of the normal red cultures could in each case be easily oxidised to red at pH's higher than 8.0^{**}. - In picking single colonies Mythar must have selected a yellow variant. - In later experiments the liquid G.L.M. medium, shallow and large in surface area, was inoculated with the organisms of the "yellow" slope and incubated aerobically at 37°C; a fairly dark red colour developed after 5 days. Both pigments A and B were found to be present, viz. 4.0 mg. and 9.5 mg. per litre culture respectively. Six G.L.M. agar slopes were inoculated with the organisms of this culture; not one of these turned red, all developing the usual yellow colour only.

* Definition of R_A and R_B , page 17.

** The ease of oxidation with air or H_2O_2 of the yellow cultures or of their filtrates at various pH's will be discussed more fully in Section B, part 3, p. 81.

The main differences in behaviour between Maytham's yellow strain and the normal red strain are listed below:

| Maytham's Yellow Strain: | The Red Strain: |
|--|---|
| (i) Gelatinous growth on G.L.M. agar slopes which clears completely after 2-3 days of incubation at 37°C | (i) Firm growth on G.L.M. agar slopes which clears only after one to two weeks of incubation at 37°C |
| (ii) No metallic effect on G.L.M. agar slopes | (ii) Very often a metallic effect on G.L.M. agar slopes. |
| (iii) On G.L.M. agar slopes only a 'dirty' yellow colour is produced. | (iii) On G.L.M. agar slopes a deep purplish red colour is produced. |
| (iv) On drying the organisms on G.L.M. agar plates "scales" are formed. | (iv) On drying the organisms on G.L.M. agar plates a "powder" is formed. |
| (v) In a sloped liquid G.L.M. medium only a dirty yellow colour (occasionally a very pale red colour) is produced. | (v) In a sloped G.L.M. medium a dark purplish red colour is formed. |
| (vi) No clearing of the cultures in the liquid G.L.M. medium takes place at all. | (vi) Clearing of the cultures in the liquid G.L.M. medium takes place prior to deep red pigmentation. |
| (vii) Bubbled G.L.M. cultures produce heavy white growth only. | (vii) Bubbled G.L.M. cultures may produce occasionally heavy white growth only, but normally result in pale to dark red pigmentation. |
| (viii) Under microaerophilic conditions in the G.L.M. medium a very pale pink may result | (viii) Under microaerophilic conditions in the G.L.M. medium a fairly dark red colour results in the majority of cultures. |

| Maytham's Yellow Strain: | The Red Strain: |
|--|---|
| (ix) The yellow pigments produced in the liquid G.L.M. medium cannot be oxidised to red. | (ix) Most of the yellow pigments produced in the liquid G.L.M. medium can be oxidised to red. |

The results of further investigations on Maytham's yellow strain will be reported on in later sections of this thesis.

(vi) The making of pellets of the Ii strain of Pseudomonas aeruginosa.

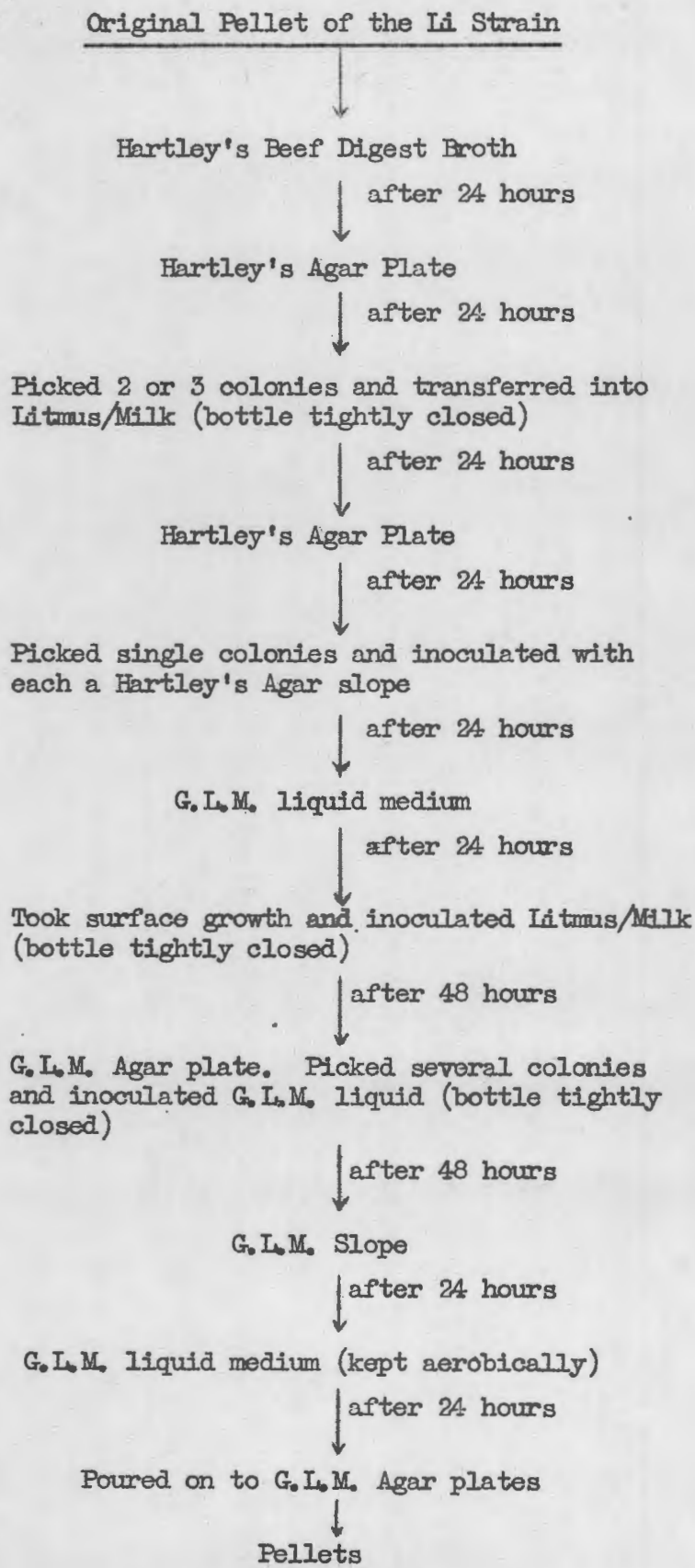
It was found necessary to produce pellets from the Ii strain of Pseudomonas aeruginosa which was specially subcultured in order to produce the optimum amount of red pigments, as:

- (a) the slopes supplied by the Bacteriology Department of this University gave inconsistent, occasionally even poor, or no red pigmentation*;
- (b) to standardise as far as possible the bacteria to be able to compare various findings at different times;
- (c) to produce as much as possible of each of the pigments on a large scale, so facilitating their isolation by Professor F.G. Holliman of this University.

In this full advantage was taken of the findings of Section B, Part 1 (iv) of this thesis. The pellets were thus obtained from the following subcultures:

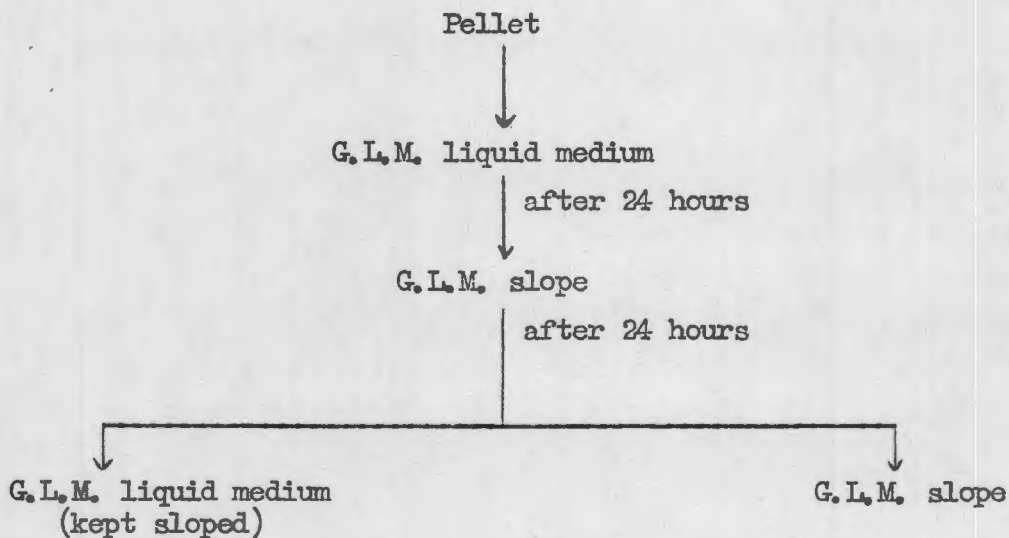
* Maytham's yellow slope produced no red pigments (Section B, Part I (v)).

Carried out by the Bacteriology Department
of this University



(The pellets were prepared by the Bacteriology Department of this University).*

Five separate batches of pellets were made and kept over P_2O_5 . They were tested in the following way:



Both, the final G.L.M. liquid cultures and the slopes developed after a week of incubation at $37^{\circ}C$ a dark purplish red colour, being approximately equally intense for all five batches of pellets. No method for the quantitative determination of pigments A and B was available at that time. - The pellets have kept so far very well for a period of over $1\frac{1}{2}$ years.

* The method was developed by Lord Stamp⁴⁰ in 1947.

SECTION B.PART 2: The effect of oxygenation.(a) On red pigment production in general.(i) Introduction.

The Li strain of *Pseudomonas aeruginosa* grows best under aerobic conditions, poorly microaerophilically, and there is only slight growth under conditions where the air supply is strictly limited to a minimum (i.e. almost anaerobic conditions). Red pigment production occurs best in aerobic cultures, but can also take place microaerophilically, and no red pigments are formed under conditions where the air supply is strictly limited to a small volume.

For the purpose of this thesis the meaning of some of the terms used are:

"Aerobic" cultures are those which are in contact with air, i.e. with free oxygen. The air supply may be varied by means of:

- (i) slow diffusion of air through the cotton wool plug used for closing the culture vessel;
- (ii) passing air over the surface of the culture;
- (iii) bubbling a fine stream of air through the culture;
- (iv) disturbing the surface of the culture continuously by the mechanical shaking of the culture vessel which is sealed with a cotton wool plug.

The organisms in "microaerophilic" cultures grow in a medium not totally devoid of oxygen; these cultures are covered with a $\frac{1}{2}$ - $\frac{3}{4}$ inch layer of autoclaved medicinal liquid paraffin, so

allowing a very slow diffusion of oxygen into the culture.

A culture which is allowed to grow in a "limited" amount of oxygen is one where the oxygen supply is strictly limited to that amount of oxygen which the medium and the culture vessel, sealed with a rubber stopper, contains.

A "static" culture is one which is never disturbed during its period of incubation.

It has been conclusively established that optimum red pigmentation takes place in shallow static aerobic cultures where the culture vessel is sealed with a cotton wool plug when using the G.L.M. medium. The amount of pigment A produced under these conditions varies and rarely exceeds 7 mg. per litre culture and it is occasionally completely absent, on one occasion 20.5 mg. of pigment A were formed. The concentration of pigment B is about the same in each case, varying on the average from 40 mg. to 46 mg. per litre culture. The highest concentration of pigment A is produced when including a chemical source of oxygen into the G.L.M. medium. The amount of pigment A produced in G.L.M. media containing $\frac{1}{2}\%$ KNO_3 varies from 14 mg. to 42 mg. per litre culture, and that of pigment B again on the average from 40 mg. to 46 mg. per litre culture. Sometimes, however, a whole batch of cultures fails to give red pigmentation.

In most cultures small but varying amounts of other red pigments

are present; the one which occurs most frequently has an R_A^* value of 0.14 when using the n-butanol-acetic acid-water solvent.

Another red pigment which is occasionally found has an R_A value of 0.52.

Various oxygenation methods have been studied in order to find the optimum conditions for red pigment formation, so that larger quantities of these may be obtained on as large a scale as possible, so greatly facilitating Professor Holliman's investigations on the structure of the pigments. The oxygenation conditions studied were:

I. Aerobic

(a) Static cultures:

- (i) shallow;
- (ii) deep;
- (iii) disturbed shallow.

(b) Air-bubbled cultures:

- (i) slow;
- (ii) fast.

(c) Variable.

(d) Shaking cultures.

II. Microaerophilic.

III. Interchanging of aerobic and microaerophilic conditions.

IV. Limited air supply.

V. Use of a chemical source of oxygen.

- (a) Aerobically.
- (b) Microaerophilically.
- (c) With a limited air supply.

* Definition of R_A and R_B , page 17.

(ii) Pigment production under aerobic conditions.

(a) Static cultures:

Professor F.G. Holliman in his work has observed that good red pigment production took place when using his G.L.M. medium, and growing the cultures aerobically and undisturbed. His main object was to produce the red pigments on a large scale so that their isolation and elucidation of their structure could be facilitated. Professor Holliman used in his large scale method white square Gin bottles, with a capacity of approximately 750 ml., each containing 200 ml. of his G.L.M. medium*, and incubating the cultures of the Li strain at 37°C with the cotton wool plugged bottles lying on their sides. This culture has a large surface area, and it is only approximately 2 cm. deep. No better large scale method for red pigment production has been developed up to now. Many attempts were made, but without much success.

Professor Holliman's observations were further investigated. Eighteen boiling tubes, each containing 15 ml. of the G.L.M. medium, were inoculated with the Li strain of *Pseudomonas aeruginosa*, a third of them being incubated standing upright (cultures deep and surface area small) and the remainder being kept sloped, thus increasing the surface area, and decreasing the depth of the culture. One half of the sloped cultures were

* Since the author's findings on the effect of KNO_3 in the medium, Professor Holliman has included $\frac{1}{2}\%$ KNO_3 into his G.L.M. medium (for composition see page 22) as this modified medium gives an increased amount of pigment A without affecting the yield of pigment B.

once daily disturbed by brief shaking. All tubes were plugged with cotton wool and incubated at 37°C. The undisturbed sloped cultures took 7 - 8 days to turn dark purplish red in colour, the disturbed sloped ones developed a red coloration in 5 - 7 days, whereas the 'upright' cultures took from 12 - 18 days, their eventual colour having a distinct brown tinge. The contents of the 6 corresponding tubes were combined and the concentration of pigments A and B was determined quantitatively. The following table summarizes the results of this experiment:-

| Oxygenation conditions | Average number of days required for red pigmentation: | Pigment A in mg. per litre of culture: | Pigment B in mg. per litre of culture: |
|----------------------------|---|--|--|
| Upright culture | 15 | 0 | 29 |
| Undisturbed sloped culture | 8 | 3 | 42 |
| Disturbed sloped culture | 6 | 13 | 27 |

The concentration of pigments A and B produced in disturbed sloped cultures varies considerably, however; in another series of experiments these yielded only 2.0 mg. and 15.5 mg. per litre culture of pigments A and B respectively. The amounts of the red pigments produced in static cultures is reasonably constant.

Almost complete clearing of the undisturbed cultures always takes place prior to dark red pigmentation, being probably due to the lysing of the organisms. This effect, which has been previously

observed by Holliman²⁴, appears to be related to red pigment formation. Disturbed cultures do not clear and pigment production in these is never good.

(b) Bubbled cultures.

The possibility that good red pigment production might take place in well aerated cultures was studied. Twelve boiling tubes at the time, each containing 15 ml. of the G.L.M. medium, were inoculated with the *Ld* strain of *Pseudomonas aeruginosa*, and to each 1 drop of tri-n-butyl phosphate* were added in order to prevent excessive frothing. A constant stream of air was passed via cotton wool through all cultures, through 6 at a rate of approximately 15 ml./minute, and through the remainder at a rate of about 60 ml./minute. All cultures were incubated at 37°C. - At the beginning the red colour intensity was judged visually only.

Red pigment formation was found to be extremely variable under the above conditions, rarely being really satisfactory. The slow bubbled cultures gave, in most cases, a darker red colour than the fast bubbled ones, though the reverse was observed occasionally. Red pigmentation was achieved usually more rapidly by the former method ((a) above), requiring on the average only six days. Quite a number of the cultures produced no red pigments at all, remaining

* 100 ml. of tri-n-butyl phosphate were prepared by the method described in "Organic Synthesis", Vol. XVI, page 9.

white due to the heavy growth of the organisms. Out of a total of 33 bubbled cultures, 8 developed no colour at all, 11 turned to a very pale red colour only, the remainder being fairly dark purplish red. Clearing of the bubbled cultures never took place. Owing to the great variability in red pigment formation by the bubbling methods, the contents of all boiling tubes were combined in order to obtain an average value; the mixture was found to contain 10 mg. of pigment A and 20 mg. of pigment B per litre culture, and there were small quantities of two other red pigments present, one having an R_A^* of 0.14 and the other of 0.52 when using the n-butanol-acetic acid-water solvent.

(c) Variable aeration.

Aeration by means of slow or fast regular air-bubbling through cultures of *Pseudomonas aeruginosa* favoured the growth of the organisms, but not red pigment formation. In static cultures, on the other hand, growth was moderate and red pigmentation good. The possibility that variable oxygenation conditions might favour both growth and red pigment production was thus investigated.

Conical flasks of 300 ml. capacity, each containing 100 ml. of the G.L.M. medium, were employed. They were all inoculated with an equal volume of a "starter"** of the L1 strain of

* Definition of R_A and R_B , page 17.

** Meaning of "starter", page 15.

Pseudomonas aeruginosa, and were then incubated at 37°C. The air supply was varied for each culture, viz.:

- (i) constant rate of air bubbling throughout, - as a control;
- (ii) plugged with cotton wool and kept static, - as a control;
- (iii) for the first 24 hours rapid air bubbling, then decreasing the rate at 24 hourly intervals;
- (iv) plugged with cotton wool for 24 hours, then air bubbling first slowly, then increasing the rate of bubbling at 24 hourly intervals;
- (v) passing a constant supply of air over the surface of an otherwise static culture;
- (vi) for the first 24 hours rapid air-bubbling, thereafter passing a constant supply of air over the surface of an otherwise static culture;
- (vii) intermittent aeration of an otherwise static culture.

The cultures were incubated until no further colour changes could be observed. The method for the determination of pigments A and B had at this time not been developed yet, no quantitative results are therefore available. Methods (i) and (ii) have been discussed previously, the latter giving always good red pigment formation, provided the culture is shallow. All the other methods resulted in only poor to fair red pigmentation. The following observations were made:

- (i) fairly dark purplish red after 5 days;
- (ii) dark purplish red after 5 days;
- (iii) fairly dark orange red after 6 days;
- (iv) pale pink after 6 days;

- (v) pale orange red after 5 days;
- (vi) fairly dark purplish red after 9 days;
- (vii) pale orange red after 8 days.

None of the methods, in which the air supply was varied, was found to be suitable for good red pigment formation. Growth of the organisms is always good in highly aerated cultures, red pigment production, on the other hand, is generally unsatisfactory. Complete clearing of the cultures, which appears to be related to deep red pigmentation, does not take place. - There is a variation in the supply of air in the shallow static cultures as well, and here red pigment production is best. The air supply is high at the beginning, then decreasing rapidly as the organisms thrive. Only a low concentration of oxygen is thereafter maintained, due to the slow diffusion of air through the cotton wool plug. Lysis of the organisms then allows the oxidation by air of the yellow precursors to the red pigments. A variation in the supply of air thus appears to be necessary for good red pigment formation, the concentration of oxygen, however, being very low on the whole. This was not the case in the above cultures where the air supply was varied, resulting in poor red pigmentation.

(d) Shaking cultures.

Red pigment formation by the *L1* strain of *Pseudomonas aeruginosa* under highly aerobic conditions was studied more out of interest. It was suspected

that red pigmentation would be poor by this method because of previous observations, yet the behaviour of micro-organisms under extreme conditions is often unpredictable, and such conditions should thus be investigated.

Four conical flasks of 1000 ml. capacity each containing 100 ml. of the G.L.M. medium, were used in this method in order to avoid excessive splashing. Three drops of tri-n-butyl phosphate were added after inoculation, and the flasks were then fastened on to the platform of a shaking machine. All flasks were closed with a cotton wool plug and the cultures were incubated at 37°C. The rate of shaking was adjusted to a speed at which the surface of the culture was just disturbed so achieving very good aeration. Red pigmentation under these conditions took only a short time, varying from 36 - 72 hours. The following are the average colour changes which were observed:

- After 12 hours: white, due to heavy growth;
- After 24 hours: yellow with a green tinge;
- After 36 hours: buff to reddish brown in colour;
- After 48 hours: pale purplish red.

Red pigmentation was, on the whole, poor, pigment A being absent or present in traces only in most cases, and the range in the concentration of pigment B being from 3 - 8.5 mg. per litre of culture. Occasionally no colour was produced. Clearing of the cultures never took place. - Slight agitation or aeration

favours growth of the organisms in the liquid G.L.M. medium, since it aids in breaking up the clumps of cells. Vigorous shaking, however, may have led to a denaturation of the cellular proteins or to cellular disintegration.

(e) Red pigment production under microaerophilic conditions.

The Li strain of *Pseudomonas aeruginosa* is capable of growing under microaerophilic conditions. The growth is poor when using the G.L.M. medium as such, but is quite heavy when including KNO_3 into the medium.

Previous observations have indicated that pigment production was generally good in cultures where the growth of the organisms was only moderate; it was therefore felt that pigment formation under microaerophilic conditions should be studied.

100 ml. of the G.L.M. medium were placed into a 300 ml. conical flask, inoculated with the Li strain of *Pseudomonas aeruginosa*, and the surface was then covered with a $\frac{1}{2}$ inch layer of medicinal liquid paraffin (S.G. = 0.85), which had been autoclaved previously. The culture was incubated at $37^\circ C$. Hardly any growth took place during the first 2 days, after which the culture became hazy. On the 11th day a greenish yellow colour developed, which changed gradually to a golden yellow. After 25 days the colour changed to a pale orange red. Clearing of the culture now took place and a fairly dark purplish red pigmentation

developed, - altogether 33 days after the inoculation of the medium. Similar observations were made when this experiment was repeated later. The length of time taken for red pigmentation varied considerably, viz. from 26 - 42 days. Occasionally only yellow pigmented cultures resulted; their filtrate could be oxidised with air or H_2O_2 to a red pigment, having the same R_{F} as that of pigment B. The red colour intensity of the cultures varied greatly, pigment A always being absent, and the concentration of pigment B lying between 10.5 mg. to 32 mg. per litre of culture.

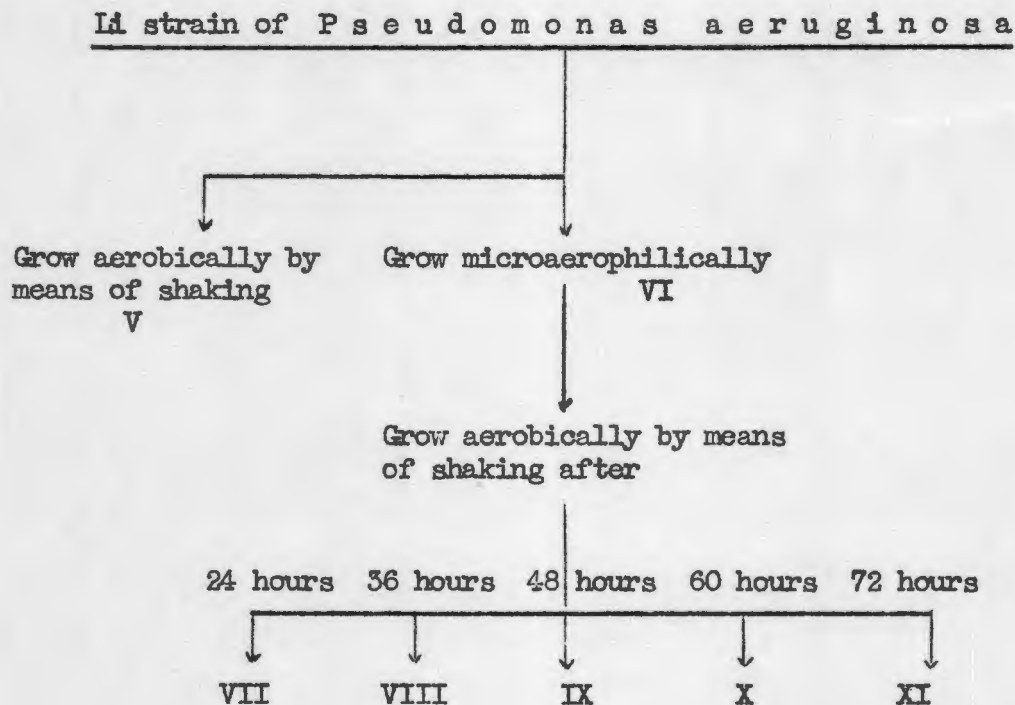
(f) Interchanging of aerobic and microaerophilic conditions.

In the experiments on the restoration of the ability to produce red pigments (page 28) some difference in the behaviour of the organisms was observed when passing these through certain media aerobically or through the same media where the air supply was limited. The interchanging of almost extreme conditions was therefore investigated in order to establish whether these affect red pigment production.

Two separate 1-litre conical flasks, each containing 200 ml. of G.L.M. medium, were inoculated with equal volumes of a "starter"* of the Li strain of *Pseudomonas aeruginosa* and incubated at 37°C. One culture was grown aerobically by means of shaking, the other was kept microaerophilically by being covered with a $\frac{1}{2}$ inch layer of sterilized medicinal liquid

* Meaning of "starter", page 15.

stage of growth is preceded by a microaerophilic one. - The aim of the next experiment was to establish the optimum length of time for the microaerophilic stage which must precede the aerobic one in order that best red pigment production might result. 100 ml. portions of a microaerophilic culture were withdrawn at 12 hourly intervals and these were then incubated aerobically by means of shaking:



All samples were incubated until maximum red pigmentation had developed; the colours were compared visually only:

- Culture V: pale purplish red after 3 days;
- Culture VI: fairly dark purplish red after 29 days;
- Culture VII: pale orange red after 2 days of aerobic growth;
- Culture VIII: fairly dark orange red after 2 days of aerobic growth;
- Culture IX: fairly dark purplish red after 2 days of aerobic growth;

Culture X: fairly dark purplish red after 1 day of aerobic growth;

Culture XI: fairly dark purplish red after 1 day of aerobic growth.

Culture IX, which had a microaerophilic stage of 48 hours, developed the darkest red colour. Although red pigment production is slightly improved when aerobic growth is preceded by a microaerophilic stage of growth, it is never really satisfactory. Best red pigment formation still takes place in static shallow non-bubbled aerobic cultures.

(g) Cultures with limited air supply.

It had already been established that the conditions for optimum growth were by no means the best conditions for red pigment formation. Red pigmentation is generally poor in cultures where growth is good. The possibility that red pigmentation might take place under conditions where the air supply is strictly limited, - conditions under which growth is very poor - was therefore investigated.

100 ml. of the G.I.M. medium were transferred into a sterilized conical flask with a capacity of just over 100 ml. and inoculated with a known volume of a "starter"* of the Li strain of *Pseudomonas aeruginosa*. Sufficient autoclaved liquid paraffin was poured on to the surface of the culture to almost fill the flask, which was then tightly sealed with a

* Meaning of "starter", page 15.

rubber stopper, (culture I). Further 100 ml. portions of the G.L.M. medium were transferred into sterilized flasks of 150 ml. (culture II), 250 ml. (culture III), 500 ml. (culture IV), and 1000 ml. (culture V). All flasks were inoculated with the same volume of the above starter of the *Id* strain, sealed with a rubber stopper, and incubated at 37°C. The following table summarizes the results:

| Culture: | Total air supply: | Growth: | Colour after 1 week: | Colour after 3 months: |
|----------|-------------------|-----------|----------------------|------------------------|
| I | about nil | very poor | white | white |
| II | 50 ml. | poor | white | white |
| III | 150 ml. | poor | white | white |
| IV | 400 ml. | fair | white | off white |
| V | 900 ml. | good | yellow green | yellow brown |

The pH of the filtrate of culture V was raised with KOH from 7.4 to 8.5, at which it could now be oxidised with air or H_2O_2 to a fairly dark purplish red, the colour being chiefly due to the presence of pigment B, - pigment A being absent as shown by paper chromatography. Red pigment formation under almost anaerobic conditions or with a small but limited amount of air could thus not be achieved by this method. The yellow precursor of pigment B could, however, be formed when a little air was supplied.

(h) The use of a chemical source of oxygen.

(i) Inclusion of KNO_3 into the G.L.M. medium and incubating the cultures aerobically:

Robinson²⁸ in 1932 showed that the following medium supported

growth of *Pseudomonas aeruginosa* at pH 7.6 under anaerobic conditions: sodium citrate, 1.0 g.; sodium nitrate, 0.5 g.; $MgSO_4$, 0.05 g.; phosphate buffer (0.05 M), to 100 ml. He showed thus that NO_3^- can act as a chemical source of oxygen. - The enzyme nitratase was first observed in *Escherichia coli* by Green, Stickland, and Tarr in 1934⁴¹. It has since been shown that the majority of bacteria can reduce nitrates to nitrites. Collins⁴² showed that aeration, the depth of the medium, and the shape of the flask all had a marked effect on the formation of nitrate reducing enzymes by *Pseudomonas aeruginosa*.

It was observed that the inclusion of a chemical source of oxygen into the G.L.M. medium greatly affected red pigment formation by the Li strain of *Pseudomonas aeruginosa*. Twelve boiling tubes, 6 of which contained 15 ml. of the G.L.M. medium, and the remainder each containing 15 ml. of the G.L.M. medium with inclusion of 0.5% of KNO_3 , were inoculated with the Li strain of *Pseudomonas aeruginosa*, plugged with cotton wool and then incubated sloped at 37°C. Dark red pigmentation resulted in the first batch of 6 cultures after 6 - 9 days, the remainder requiring only 4 - 6 days. The following are the average changes which take place in the cultures which are supplied with KNO_3 :

After 1 day: bright greenish-yellow throughout; surface growth heavy; slight evolution of gas.

- After 2 days: yellow with a brown tinge throughout;
- After 3 days: olive green colour on the surface of the culture, the remainder being yellow brown with the exception of the tip of the culture nearest the plugged end of the tube which is red;
- After 4 days: fairly dark orange red throughout;
- After 5 days: partial clearing of the culture; the shade of red is more purplish;
- After 6 days: the culture has cleared and is dark purplish red in colour.

Qualitative examination of the red cultures by means of paper chromatography showed that a number of pigments were produced, pigments A and B being again the main ones in the majority of cultures. After combining the contents of each batch of 6 cultures, pigments A and B were determined quantitatively. The following table summarizes the results of three separate sets of experiments:

| Medium | mg. of pigment A per litre of culture: | mg. of pigment B per litre of culture: |
|---|--|--|
| G. L. M. | 3 | 40 |
| G. L. M. + $\frac{1}{2}\%$ KNO ₃ | 20 | 41 |
| G. L. M. | 0 | 46 |
| G. L. M. + $\frac{1}{2}\%$ KNO ₃ | 42.5 | 46.5 |
| G. L. M. | 6 | 41 |
| G. L. M. + $\frac{1}{2}\%$ KNO ₃ | 28.5 | 42.5 |

The inclusion of 0.5% KNO₃ into the G. L. M. medium results in a marked increase in concentration of pigment A. It must be

pointed out, however, that occasionally whole batches of cultures during several years of investigation resulted in poor red pigment formation.

Further experiments were conducted to determine the amount of KNO_3 to be included into the G.L.M. medium for the optimum production of both pigments A and B. 5 Boiling tubes for each of the concentrations of KNO_3 listed in the table below, containing 15 ml. of the medium, were inoculated with equal volumes of a "starter"* of the Li strain of *Pseudomonas aeruginosa*, plugged with cotton wool, and incubated sloped at 37°C . The following results were obtained:

| % of KNO_3 in the G.L.M. medium: | mg. of pigment A per litre of culture: | mg. of pigment B per litre of culture: |
|---|--|--|
| 0 | 7 | 44 |
| 0.5 | 14.5 | 42 |
| 1.0 | 16.5 | 33 |
| 1.5 | 8 | 20 |
| 2.0 | 5.5 | 12.5 |

Best red pigment production therefore takes place when using the G.L.M. medium with the inclusion of 0.5% KNO_3 . The culture containing 1% of KNO_3 required only 4 days for red pigmentation, the remainder taking 6 days. With the increase in the concentration of KNO_3 there was also an increase in the amount of another red

* Meaning of "starter", page 15.

pigment, having an R_A^* of 0.52 when using the n-butanol-acetic acid-water solvent. In all cultures the red pigment with an R_A of 0.14 was present. Occasionally a very slow moving pigment with an R_A of 0.06, which showed up as a mauve spot on the chromatogram, was observed.

Red pigment production in bubbled cultures and shaking cultures was generally poorer when the G.L.M. medium contained 0.5% of KNO_3 . In shaking cultures pigment A was always absent.

(ii) Inclusion of KNO_3 into the G.L.M. medium and incubating the cultures microaerophilically.

The inclusion of KNO_3 into the G.L.M. medium had a marked effect on red pigment formation by *Pseudomonas aeruginosa* when the cultures were incubated aerobically. Under these conditions more of pigment A was produced than in cultures grown in the G.L.M. medium as such. The effect on pigment production by growing the organisms microaerophilically in the G.L.M. medium containing KNO_3 was thus investigated.

Growth of the organisms in the G.L.M. medium containing 0.5% of KNO_3 is good, and is accompanied with the evolution of nitrogen gas during the 2nd and part of the 3rd day of incubation at 37°C. Red pigmentation takes place after 18 - 30 days, the cultures containing from 8 mg. to 18 mg. of pigment B per litre, pigment A being absent in almost all cultures, and when present then only in

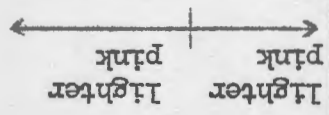
* Definition of R_A and R_B , page 17.

traces. Small quantities of other red pigments are frequently present. Occasionally only pale yellow cultures are produced, the filtrates of which can be oxidised with air or H_2O_2 to red.

An experiment was carried out to determine the amount of KNO_3 to be included into the G.L.M. medium for optimum red pigment formation under microaerophilic conditions. 8 Conical flasks of 100 ml. capacity, each containing 40 ml. of the medium, were inoculated with equal volumes of a "starter"* of the Li strain of *Pseudomonas aeruginosa*. Liquid paraffin was poured on to the surface \ddagger of the cultures which were then incubated at 37°C. The following table summarizes the results:

* Meaning of "starter", page 15.

| % KNO ₃ in the G. I. M. medium: | Growth after: | | | Nitrogen gas evolved | Pigmentation of the cultures: | Days required for pigment formation: |
|--|---------------|---------|---------|----------------------|-------------------------------|--------------------------------------|
| | 24 hrs. | 48 hrs. | 72 hrs. | | | |
| 0 | | poor | poor | - | | 32 |
| 0.50 | good | | | 24 - 60 hrs. | pale purplish red | 18 |
| 0.75 | good | | | 24 - 60 hrs. | pale pink | 3 |
| 1.0 | good | | | 24 - 60 hrs. | pink | 3 |
| 1.25 | fair | good | | 30 - 66 hrs. | pink | 3 |
| 1.50 | fair | good | | 30 - 66 hrs. | darkest pink | 3 |
| 2.0 | poor | good | | after 48 hrs. | pale pink | 4 |
| 3.0 | | poor | good | after 48 hrs. | very pale pink | 4 |



Red pigment production on the whole was poor, the medium containing 1.5% of KNO_3 resulting in darkest red pigmentation.

(iii) Inclusion of KNO_3 into the G.L.M. medium and incubating the cultures with a limited air supply.

It had already been shown that no red pigmentation resulted in cultures which were grown with a very limited air supply (see page 53) when using the G.L.M. medium as such. The inclusion of KNO_3 into the medium, which generally affects red pigment formation, and then growing the cultures with a restricted air supply, was thus investigated.

100 ml. of the G.L.M. medium containing 0.5% KNO_3 were transferred into a sterilized conical flask with a capacity of just over 100 ml. and inoculated with a known volume of a "starter" of the *Id* strain of *Pseudomonas aeruginosa*. Sufficient autoclaved medicinal liquid paraffin was poured on to the surface of the culture almost to fill the flask, which was then tightly sealed with a rubber stopper (culture I). Further 100 ml. portions of the G.L.M. medium containing 0.5% KNO_3 were transferred into sterilized flasks of 150 ml. (culture II), 250 ml. (culture III), 500 ml. (culture IV), and 1000 ml. (culture V) capacity; no liquid paraffin was poured on to the surface of the medium in these cases. All flasks were inoculated with equal volumes of the above starter of the *Id* strain, sealed with a rubber stopper, and the cultures were then incubated at 37°C . All cultures turned yellow in colour after 2 days; no further visual colour changes took place even after

3 months of incubation. The filtrate of all the above yellow cultures could be oxidised with air or H_2O_2 to red; the pigment formed was mainly B, pigment A being completely absent. The chromatogram also showed the presence of small quantities of two other pigments, viz. (a) a red pigment with an R_A^* of 0.14, and (b) a mauve pigment with an R_A of 0.06, when using the butanol-acetic acid-water solvent.

The above experiment was repeated using the G.L.M. medium with the inclusion of 1.5% KNO_3 , an amount which resulted in optimum red pigmentation under microaerophilic conditions (see page 59). All cultures turned pale pink after 4 days, the colour being due mainly to the presence of pigment B; pigment A was absent again as shown by paper chromatography.

Conclusions

Oxygenation conditions play an important part in red pigment production in cultures of the Id strain of *Pseudomonas aeruginosa* when using the G.L.M. medium. Optimum red pigmentation takes place in shallow static aerobic cultures, where the vessel has been plugged with cotton wool. Under these conditions up to 7 mg. of pigment A and up to 46 mg. of pigment B per litre of culture is formed when using the G.L.M. medium as such, and up to 42 mg. of pigment A and up to 46 mg. of pigment B per litre of culture are produced when using the G.L.M. medium with the inclusion of 0.5% of KNO_3 . Even so, red pigment formation is variable, whole batches of cultures occasionally failing to produce

* Definition of R_A and R_B , page 17.

any red pigments at all.

It may also be concluded that the relative amounts of pigments A and B produced appear to depend on oxygenation conditions, the formation of pigment A being favoured by an increased air supply, although excessive or no aeration is unfavourable to the formation of both pigments. The following table summarizes the average results obtained during the course of investigation, and it shows at the same time the effect of oxygenation on the formation of the two pigments:

| Medium | Oxygenation Conditions | Pigment A in mg./ litre culture | Pigment B in mg./ litre culture |
|----------------------------------|--|--|--|
| G. L. M. | Undisturbed, static, shallow culture, plugged with cotton wool | 3.5 | 43 |
| G. L. M. | Occasionally disturbed, but otherwise static, shallow culture, plugged with cotton wool | 13 | 27 |
| G. L. M. | Air-bubbled cultures | 10 | 20 |
| G. L. M. | Shaking cultures | trace | 6 |
| G. L. M. + 0.5% KNO ₃ | Undisturbed, static, shallow culture, plugged with cotton wool | 14.5 | 42 |
| G. L. M. + 1.5% KNO ₃ | " " " " " " | 8 | 20 |
| G. L. M. + 2.0% KNO ₃ | " " " " " " | 5.5 | 12.5 |
| G. L. M. | Microaerophilic culture | 0 | 21 |
| G. L. M. + 0.5% KNO ₃ | " " | 0 | 13 |
| G. L. M. | Restricted air supply (see page 53) | 0 | 0 |
| G. L. M. + 0.5% KNO ₃ | Restricted air supply (see page 60) | 0 | 0 |
| G. L. M. + 1.5% KNO ₃ | Restricted air supply (see page 61) | 0 | trace |

It is interesting to note that in microaerophilic cultures the yield of pigment B is decreased by the addition of 0.5% KNO_3 to the G.L.M. medium, little or no pigment A being formed, whereas under aerobic conditions, the addition of KNO_3 increases the yield of pigment A without greatly affecting the yield of pigment B.

The effect of oxygenation on the formation of pigments A and B was further investigated and this will be discussed in the following section.

SECTION B.PART 2: The effect of oxygenation.(b) On the formation of pigments A and B in particular.

Best red pigment production in general occurs in static cultures where the tubes have been plugged with cotton wool. Cultures grown under these conditions use up oxygen in the tubes which is slowly replaced by air so that the oxygen in the space above the culture becomes progressively diluted by nitrogen.

It has also been observed that oxygenation conditions can affect the relative amounts of pigments A and B formed in a culture of the Li strain of *Pseudomonas aeruginosa*. Thus the inclusion of 0.5% KNO_3 in the G.L.M. medium resulted in a marked increase in the yield of pigment A (see p. 53a). It was found, too, that for static, shallow cultures grown in boiling tubes using the G.L.M. medium as such, the effect of replacing the loose cotton wool plug by a tightly fitting one was to reduce the yield of pigment A very considerably. In neither of these cases was the yield of pigment B greatly affected, as was shown by the following experiment.

10 Boiling tubes at a time, each containing 15 ml. of the G.L.M. medium, were inoculated with equal volumes of the same "starter"* of the Li strain of *Pseudomonas aeruginosa*. 5 of these were plugged with an $\frac{1}{2}$ - $\frac{3}{4}$ inch layer of cotton wool

* Meaning of "starter", page 15.

very loosely *, the remainder being tightly plugged with an 1 - 1½ inch layer of the same quality of cotton wool. All tubes were incubated sloped at 37°C. When maximum red pigmentation had developed, the contents of the 5 cultures for both sets of conditions were combined and the concentration of pigments A and B were determined quantitatively. The following are the results obtained:

| Cotton wool plug fitted: | Concentration of Pigment A in mg. per litre culture: | Concentration of Pigment B in mg. per litre culture: |
|--------------------------|--|--|
| Short and loose | 14.5 | 38 |
| Long and tight | 0 | 41 |
| Short and loose | 12 | 39 |
| Long and tight | 1.5 | 37 |
| Short and loose | 14 | 42 |
| Long and tight | trace | 44 |

Another experiment was carried out to investigate whether the oxygenation conditions affect the formation of pigments A and B. 200 ml. of the G.L.M. medium were transferred into a square gin bottle with a capacity of 755 ml. It was plugged with cotton wool, autoclaved, and after cooling it was inoculated with the Ii strain of *Pseudomonas aeruginosa*. The bottle was placed on its side and incubated at 37°C. 5 ml. of this culture were withdrawn aseptically from time to time and placed in a small petri-dish with a capacity of approximately 8 ml. The lid was fitted

* All boiling tubes are as a rule plugged fairly tightly with a 1 - 1½" layer of cotton wool in order to avoid contamination.

in such a way, being lifted at the one side by means of a glass rod, that air could readily enter it, thus resulting in a shallow, static, well aerated culture with a large surface area. All cultures in the petri-dishes were incubated at 37°C. The pH was measured at the time when each sample was withdrawn from the bulk culture in the gin bottle, and again after red pigmentation had fully developed in each of the cultures in the petri-dishes, which took only 24 - 48 hours in each case. The concentration of pigments A and B was then determined, and compared with that of the bulk culture which was also incubated until red pigmentation had taken place. The growth of the organisms in the cultures in the petri-dishes was very heavy. The following table summarizes the results of this experiment.

| Sample removed at pH: | Final pH of the culture in the petri-dish: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|--|--|-------------------------------------|-------------------------------------|
| 7.1 | 9.05 | 11 | 24 |
| 7.5 | 9.1 | 6.5 | 33 |
| 7.9 | 9.1 | 3.5 | 38 |
| 8.2 | 9.15 | 1.5 | 42 |
| 8.7 | 9.15 | 0 | 40 |
| (Bulk culture in gin bottle as a control: 7.1 | 9.1 | 0 | 46 |

Similar results were obtained when this experiment was repeated. The significance of the pH of the culture in relation to red pigment

production will be fully discussed in the next section of this thesis. One observation is clear, however, viz. that from the outset well aerated shallow static cultures produce more of pigment A than poorly aerated ones, and that this appears at least in some cases to take place at the expense of pigment B, there then being the following approximate relationship:

$$[A] \approx \frac{[B_S] - [B_a]}{2} ,$$

where $[A]$ = concentration of pigment A in mg. per litre culture;

$[B_S]$ = concentration of pigment B in mg. per litre of an aerobic static shallow culture (e.g. the bulk culture in the gin bottle);

$[B_a]$ = concentration of pigment B in mg. per litre of a well aerated culture, (e.g. the cultures in the petri-dishes).

The following examples of the above experiment illustrate this approximate relationship:

- (i) Sample removed from the bulk culture in the gin bottle at pH = 7.1, then grown highly aerobically:-

$$\begin{aligned} [A] &\approx \frac{46 - 24}{2} \\ &= 11 \end{aligned}$$

($[A]$ actually found = 11)

- (ii) Sample removed from the bulk culture in the gin bottle at pH = 7.9, then grown highly aerobically:-

$$\begin{aligned} [A] &\approx \frac{46 - 38}{2} \\ &= 4 \end{aligned}$$

([A] actually found = 3.5)

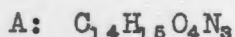
The average concentrations of pigments A and B in bubbled cultures are 10 mg. and 20 mg. per litre of culture respectively. The above mentioned relationship holds here again reasonably well, remembering that the average concentration of pigment B produced in static shallow cultures when using the G.L.M. medium is 43 mg. per litre of culture, pigment A being present in most of these cultures in only very small quantities.

$$[A] \approx \frac{43 - 20}{2}$$

$$= 11.5$$

(Average [A] found in bubbled cultures = 10).

It must be pointed out, however, that the organisms cannot utilize pigment B, but they can definitely utilize pigment A, thereby not producing more of pigment B in its place (see Section E). Probably not much importance should be attached to the above mentioned approximate relationship. It does not apply, for instance, to the results obtained in the experiment on page 65. The above mentioned relationship appears to indicate that 2 mg. of pigment B \equiv 1 mg. of pigment A (presumably with the formation of unidentified by-products). The significance of this relationship is not as yet understood and might well be fortuitous. - The probable molecular formulae of the pigments* are:



* Molecular formulae of the pigments supplied in a private communication by Holliman²⁴.

The experiment on varying the oxygenation conditions by withdrawing samples from a bulk culture of *Pseudomonas aeruginosa* at different pH's (see page 65) was carried out concurrently with one in which the G.L.M. medium contained 0.5% KNO_3 . The added effect of a chemical source of oxygen could thus be studied. The following table summarizes the results on the pigment production under these conditions:

| Sample removed at pH: | Final pH of the culture in the petri-dish: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---|--|-------------------------------------|-------------------------------------|
| 7.1 | 9.15 | 28 | 34 |
| 7.65 | 9.2 | 24 | 40 |
| 8.25 | 9.2 | 20 | 43 |
| 8.5 | 9.3 | 22 | 40 |
| 8.8 | 9.2 | 20 | 42 |
| (Bulk culture in gin bottle as control, 7.1 | 9.2 | 22 | 41 |

It is of special interest that the concentration of pigment A in this culture is hardly affected by the presence of much free oxygen from the air (compare results in the absence of KNO_3 , page 66). A chemical source of oxygen seems to be more effective than free oxygen for the maximum production of pigment A.

The formation of pigment A is thus largely dependent on the oxygenation conditions, whereas the production of pigment B appears to be less affected under similar conditions.

SECTION B.PART 3: The pH and colour changes during the growth of the organisms.(1) Introduction.

The course of all biological processes is greatly influenced by the pH of the fluid in which they take place. Growth and the production of many substances, such as enzymes, are all to a greater or lesser extent dependent upon the pH of the medium in which the organisms grow. The optimum pH for growth of *Pseudomonas aeruginosa* lies according to Derby²⁶ between a pH of 6.6 and 7.0. In this work it was found that the G.L.M. medium with a minimum pH of 5.5 and a maximum pH of 8.4 still supported the growth of the Li strain of *Pseudomonas aeruginosa*, and that good growth of these organisms was achieved by adjusting the pH of the G.L.M. medium to 6.8 - 7.2. - A careful study of the pH changes which take place during the growth period of these bacteria, and its relation to red pigmentation was made.

The electrometric method of measuring the pH was employed throughout; this method was found to be reliable and the only one available, as the cultures were coloured, at least at some stage of growth. All measurements were made on 5 ml. samples of the cultures under investigation, using the "PYE" direct reading pH meter (Model 11081) with a glass electrode.

The first experiment was designed just to follow the normal pH changes which take place in a shallow static culture of the Ii strain of *Pseudomonas aeruginosa* in the G.L.M. medium*, which was incubated aerobically at 37°C. The colour changes were noted at the same time. Later experiments were conducted to observe the pH changes in cultures grown under various oxygenation conditions, and determining by paper chromatography which red pigments were formed at these pH's.

(ii) The pH and colour changes in the G.L.M. medium.

200 ml. of the G.L.M. medium in a white square gin bottle of approximately $\frac{3}{4}$ litre capacity, at a time, were inoculated with the Ii strain of *Pseudomonas aeruginosa*. The bottle was closed with a loose cotton wool plug** and incubated lying on its side at 37°C. 5 ml. samples of this bulk culture were aseptically withdrawn at intervals from about the middle of the culture, and their pH's were measured, noting the colour changes at the same time and determining by paper chromatography which of the red pigments was present. The following table summarizes the average results of this experiment (for the graph see fig. 1):

* Composition of the G.L.M. medium, page 22.

** With a loose cotton wool plug more of pigment A is produced; page 65.

pH

C O L O U R

| | | | | |
|-----------------|-------------------|--------------------------------|------------|--------------|
| TURBID WHITE | YELLOW 'GREEN' | OLIVE GREEN or YELLOW BROWN | ORANGE RED | PURPLISH RED |
|-----------------|-------------------|--------------------------------|------------|--------------|

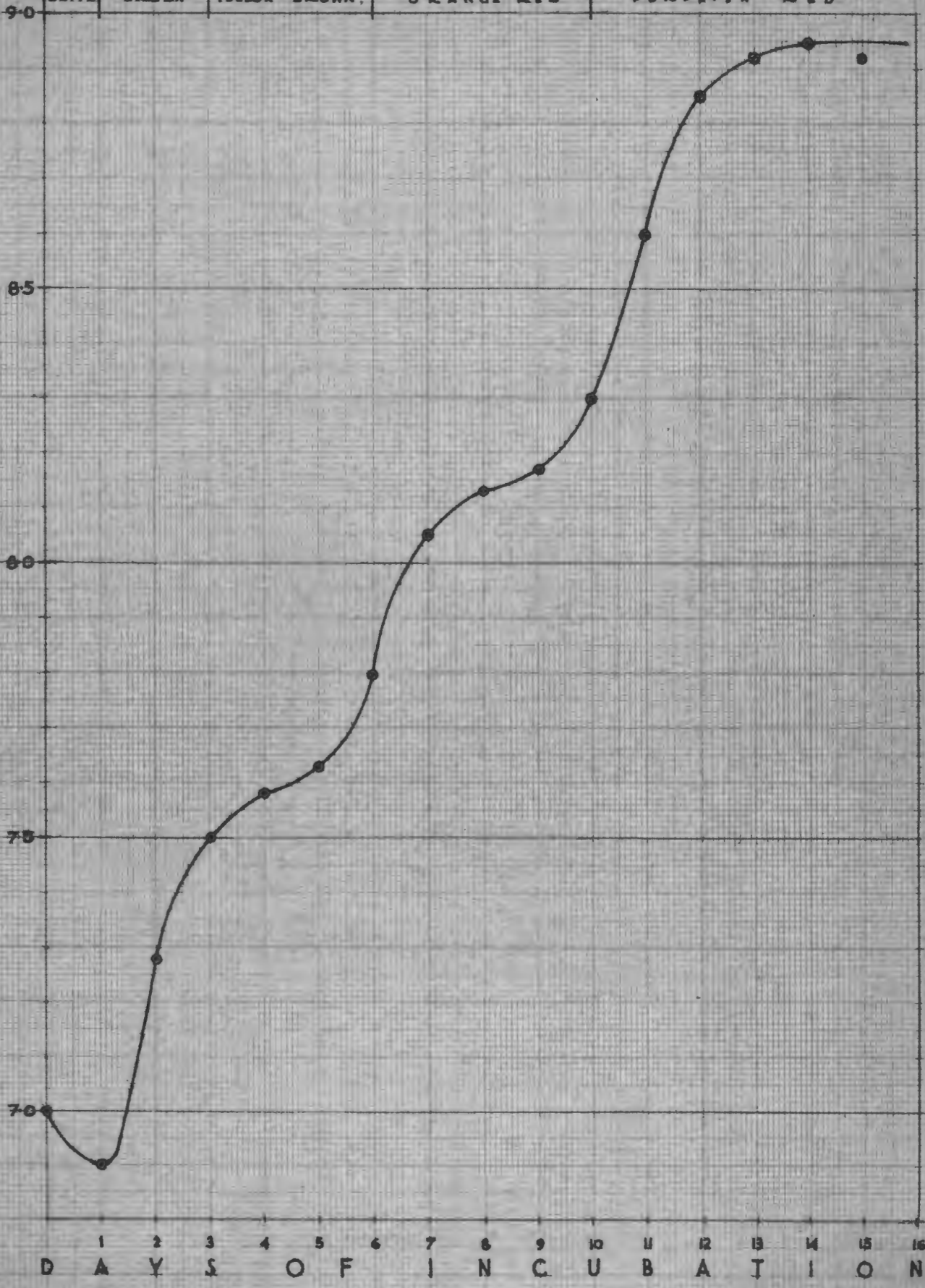


FIG 1

| Days of Incubation: | Colour of the culture: | Red pigments present: | pH of the sample with-drawn: |
|---------------------|--|----------------------------|------------------------------|
| 0 | - | - | 7.0 |
| 1 | Turbid white | - | 6.90 |
| 2 | Yellow-green | - | 7.28 |
| 3 | Yellow-green | - | 7.50 |
| 4 | Olive-green or yellow brown | - | 7.58 |
| 5 | Olive-green or yellow brown | - | 7.63 |
| 6 | Olive-green or yellow brown, tip of culture red | - | 7.80 |
| 7 | Surface of culture orange red, remainder brownish | Trace of A | 8.05 |
| 8 | Pale orange red | A | 8.13 |
| 9 | Darker orange red | A and trace of B | 8.17 |
| 10 | Orange to purplish red | A and B | 8.30 |
| 11 | Purplish red | A and much of pigment B | 8.60 |
| 12 | Dark purplish red | | 8.85 |
| 13 | | | 8.92 |
| 14 | | | 8.95 |
| 15 | | | 8.92 |
| 20 | | | 8.90 |

Pigment A is therefore present before pigment B, and when present it can be detected by means of paper chromatography when the culture has reached a pH of 8.0 - 8.3, there then being a distinct depression in the pH curve, which is more pronounced when pigment A is produced in larger quantities. When pigment A is absent, then the first red, being purplish in shade, due to the presence of pigment B, becomes visible when the culture has reached a pH of 8.6 - 8.9, there then

being no depression in the "normal" pH curve between the values of 8.0 and 8.3. A few deviations from this usual behaviour have been observed occasionally, pigment B then being produced at a lower pH, viz. 8.1 upwards. - The significance of the depression of the pH, as seen from the pH curve (fig. 1), viz. between the values of 7.4 and 7.8, has not been established; it might, however, be due to the formation of a precursor of the pigment or to metabolic changes which accompany the production of the pigment.

(iii) The pH and colour changes in the G.L.M. medium with the inclusion of 0.5% KNO₃.

The *Li* strain of *Pseudomonas aeruginosa* usually produces more of pigment A in the G.L.M. + 0.5% KNO₃ medium than in the G.L.M. medium as such, and red pigmentation takes less time. An experiment to investigate the pH and colour changes in cultures when using the G.L.M. + 0.5% KNO₃ medium was thus desirable for the purpose of comparison.

The experiment, described under (ii) above, was repeated several times, using in this series the G.L.M. + 0.5% KNO₃ medium. - The following table summarizes the average results of this series of experiments (for the graph see fig. 2):

pH

OLIVE GREEN
OR
YELLOW
ORANGE RED

C O L O U R

PURPLISH RED

9.0

8.5

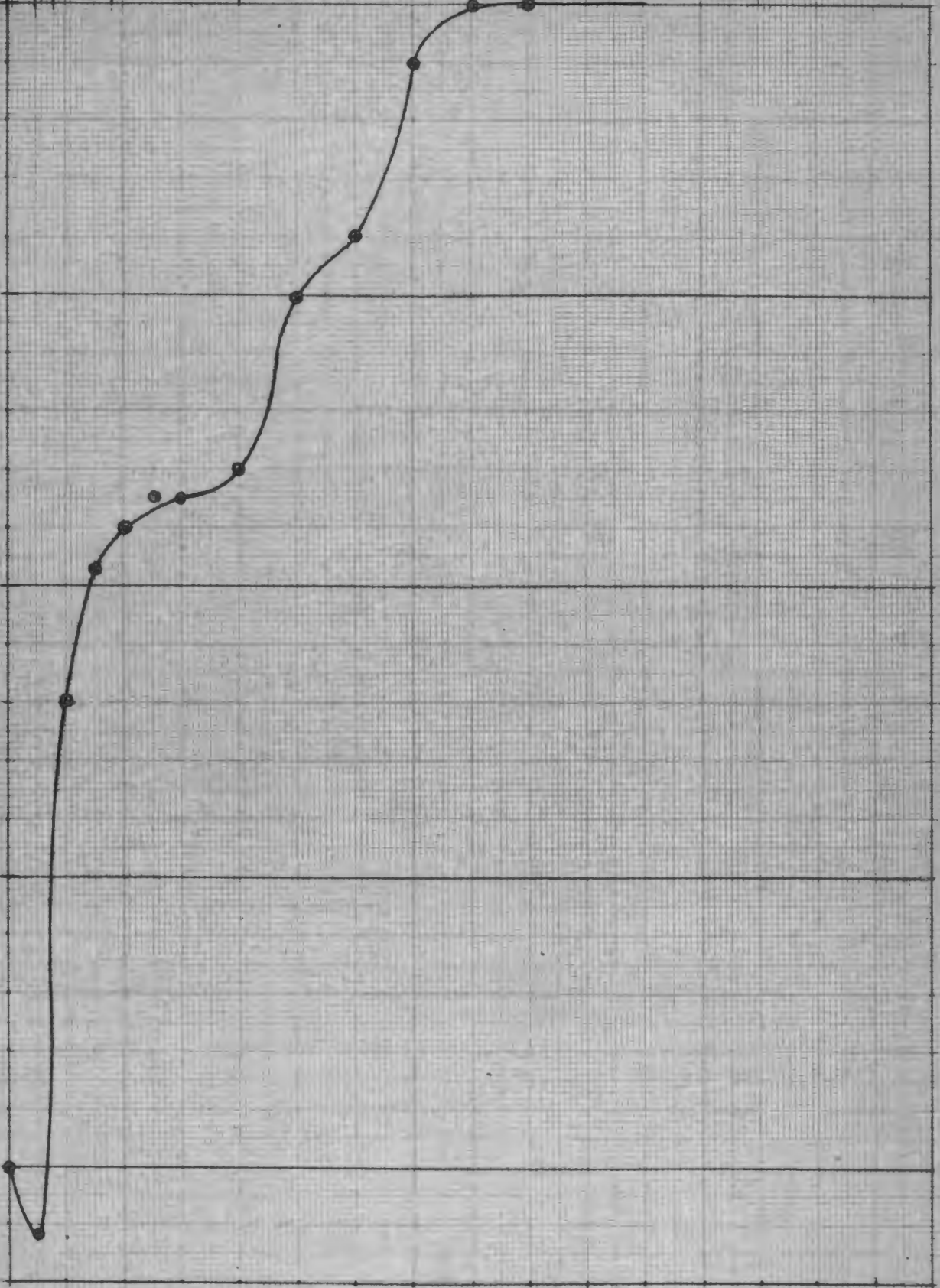
8.0

7.5

7.0

D A Y S O F I N C U B A T I O N

F I G 2



| Days of Incubation: | Colour of the culture: | Red pigments present: | pH of the sample with-drawn: |
|---------------------|---|-----------------------|------------------------------|
| 0 | - | - | 7.0 |
| 1/2 | Turbid white | - | 6.88 |
| 1 | Yellow green | - | 7.80 |
| 1 1/2 | Olive-green or yellow brown | - | 8.03 |
| 2 | Olive-green or yellow brown, tip of culture red | Trace of A | 8.10 |
| 2 1/2 | Surface of culture orange red | A and trace B | 8.15 |
| 3 | Orange red | A and trace B | 8.15 |
| 4 | Orange to purplish red | A and B | 8.20 |
| 5 | } Purplish red | } A and much of B | 8.50 |
| 6 | | | 8.60 |
| 7 | | | 8.90 |
| 8 | | | 9.0 |
| 9 | | | 9.0 |
| 10 | | | 9.05 |
| 15 | | | 9.0 |

Pigment A appears in these cultures also only when a pH of at least 8.0 has been reached, pigment B being completely absent at this stage. There is again a marked depression in the pH of the cultures (as shown in Fig. 2) between the values of pH 8.0 and 8.2. The second depression, lying between pH's of 8.4 and 8.7, is not always present.

(iv) Possible significance of the pH in relation to pigment formation.

Red pigment production by the Id strain of

Pseudomonas aeruginosa occurs best in the G.L.M. and the G.L.M. + 0.5% KNO_3 media when the shallow static cultures are incubated aerobically, the culture vessel being plugged with cotton wool. The pH and colour changes in these cultures (as described in (ii) and (iii) above) might therefore be more significant than those observed in cultures where pigment production is fair to poor. Further experiments were thus carried out on these static aerobic cultures to investigate the possible relationship of these changes.

Attempts have been made to oxidise with air or H_2O_2 the yellow cultures after they had reached a pH of 7.3 to 8.0 and then raising their pH artificially with KOH to 8.5 - 9.5. Pigment A was absent in all cases, pigment B being formed in varying quantities as was shown by paper chromatography. - The oxidation with air or H_2O_2 of yellow brown to pale orange cultures, having developed a natural pH of 8.15 or higher, resulted in most cases in the formation of both pigments A and B; this was again shown by paper chromatography.

The formation of the yellow "precursor" of pigment A appears to take place at a particular narrow pH range, provided the oxygenation conditions are suitable*, whereas that of pigment B is formed over a

* Page 64.

wider pH range, the oxygenation conditions not being as important. The actual formation of the yellow precursor of pigment A appears to depress slightly the pH of the culture. - The results of the following experiments seem to substantiate some of the above mentioned points, at least to some extent:

- (a) Examination of the summarized results on p. 66 shows that the concentration of pigment A decreases with an increase in pH of the culture whereas that of pigment B increases.
- (b) On inoculating in parallel experiments the G. L. M. and the G. L. M. + 0.5% KNO₃ media separately at various initial pH's, the concentration of pigment A varied within the usual limits, whereas that of pigment B decreased considerably in the cultures which were started at a raised pH:

| Medium: | Inoculated at pH: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|----------------------------------|-------------------|-------------------------------------|-------------------------------------|
| G. L. M. | 7.0 | 7 | 44 |
| G. L. M. | 8.1 | 4.5 | 30 |
| G. L. M. + 0.5% KNO ₃ | 7.0 | 14.5 | 42 |
| G. L. M. + 0.5% KNO ₃ | 8.0 | 21 | 25 |

The inoculation of the media at higher pH's resulted in poor growth.

- (c) Microaerophilic cultures result usually in the formation of pigment B only*, the red colour then appearing when the cultures have a pH of 8.6 or higher. In one microaerophilic culture, when using the G. L. M. + 0.5% KNO₃ medium, small amounts of pigment A were produced as well, this being formed at a pH of 8.25, the red colour due to pigment B appearing only at a pH of 8.7.

* page 49.

The conditions under which the yellow "precursors" of the pigments can be oxidised within a static culture will be discussed in Part 4 of this section. It might be mentioned here that the oxidation of the yellow "precursor" of pigment B with the air present in the culture vessel occurs only then when the respiration rate of the organisms has reached a low level. The yellow "precursor" of pigment A can, however, be oxidised whilst the respiration rate of the organisms is still high.

(iv) A quantitative study of the pigment formation at various pH's.

The previous experiments have shown that pigment A begins to be formed at pH's of 8.0 - 8.3, and that pigment B is generally only present in appreciable quantities at pH's above 8.6. The observation was studied quantitatively using the G.L.M. + 0.5% KNO₃ medium, because in this the *Id* strain of *Pseudomonas aeruginosa* produces more of pigment A without much affecting the concentration of pigment B.

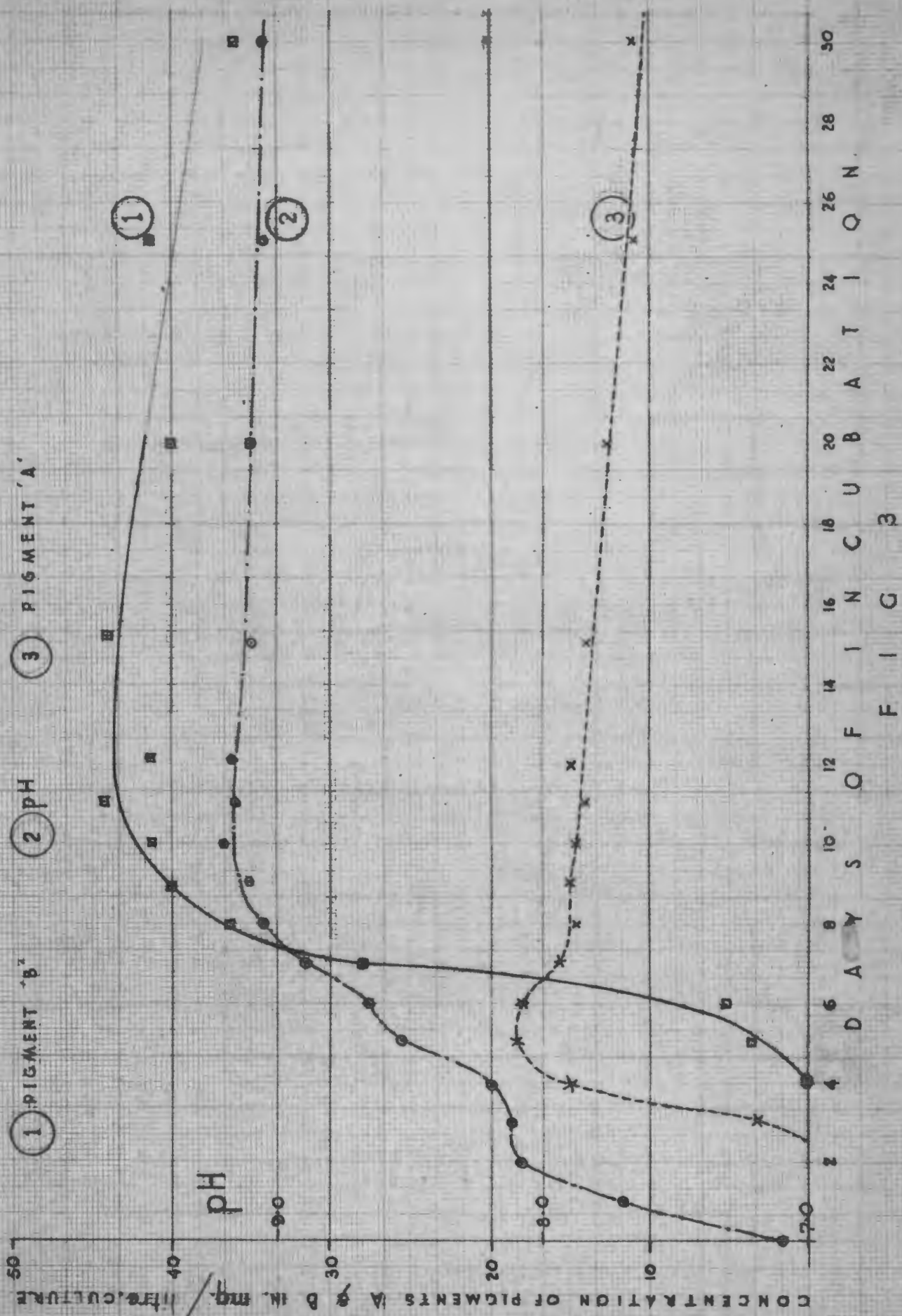
200 ml. of the G.L.M. + 0.5% KNO₃ medium were transferred to a square gin bottle with a capacity of approximately 750 ml. After autoclaving and subsequent cooling, this medium was inoculated with the *Id* strain of *Pseudomonas aeruginosa* and the culture vessel was plugged with cotton wool and incubated lying on its side at 37°C. 5 ml. portions of this parent culture were withdrawn at intervals, and the pH as well as the concentrations of pigments A and B were then determined. One drop of this sample was added to a boiling tube containing 15 ml. of the G.L.M. + 0.5%

KNO_3 medium to test whether the organisms were still viable in this medium. The following table summarizes the results of this investigation:

| Withdrawal of sample after: | pH of the sample: | Pigment A in mg. per litre culture: | Pigment B in mg. per litre culture: |
|-----------------------------|-------------------|-------------------------------------|-------------------------------------|
| Inoculation | 7.05 | - | - |
| 1 day | 7.70 | absent | absent |
| 2 days | 8.08 | " | " |
| 3 " | 8.12 | 4.5 | " |
| 4 " | 8.20 | 15.0 | trace |
| 5 " | 8.53 | 18.5 | 3.5 |
| 6 " | 8.65 | 18.0 | 5.5 |
| 7 " | 8.90 | 15.5 | 28 |
| 8 " | 9.05 | 14.5 | 36 |
| 9 " | 9.10 | 15 | 40 |
| 10 " * | 9.20 | 14.5 | 41 |
| 11 " | 9.15 | 14 | 44 |
| 12 " | 9.18 | 15 | 41 |
| 15 " | 9.10 | 14 | 44 |
| 20 " | 9.10 | 12.5 | 40 |
| 25 " | 9.05 | 11 | 41 |
| 30 " | 9.05 | 11 | 36 |

* Organisms dead.

Figure 3 shows the plotted results. - Pigment A was present in the culture in appreciable quantities before pigment B was formed, and its production took place between pH 8.1 and 8.5. Whilst the pH of the culture was still rising due to further growth of the organisms, the concentration of pigment A decreased first fairly



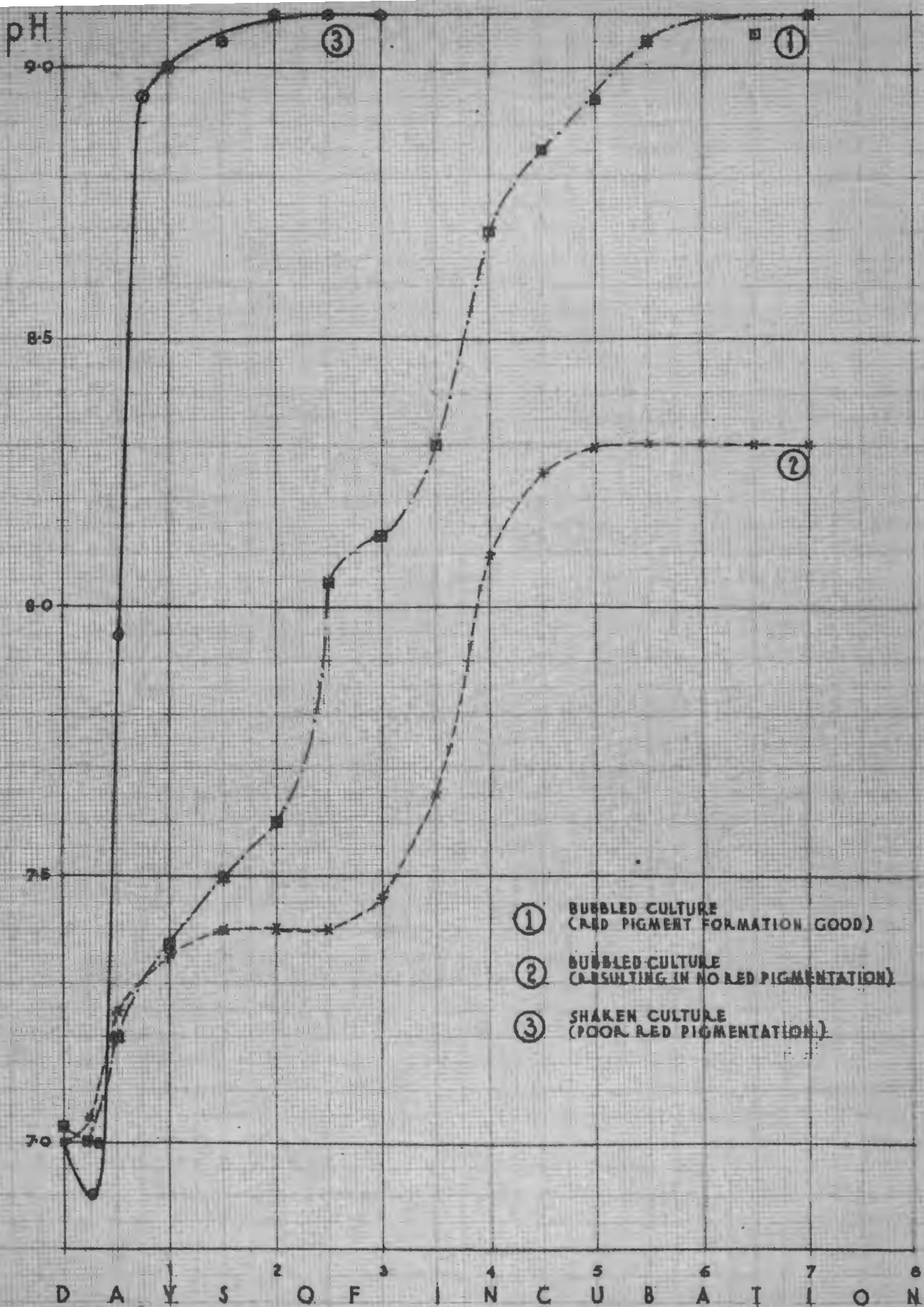
sharply, then only slightly. The formation of pigment B occurred between pH 8.2 and 9.2, reaching a maximum concentration after the culture had reached its highest pH; - the organisms were found to be dead at this pH as was shown by inoculation tests, no growth taking place in the fresh G.L.M. + 0.5% KNO₃ medium. The concentration of pigment B then also dropped slightly on further incubation of the culture, which had a brownish tinge after 30 days.

It must be pointed out that Professor Holliman²⁴ established that both pigments A and B are degraded in the presence of alkali, pigment B more easily than pigment A. This may explain the slow decrease in the concentration of the pigments but not the rapid one of pigment A after 6 days of incubation.

(v) pH changes in cultures grown under various oxygenation conditions.

(a) Aerobic cultures:

The pH changes in cultures of *Pseudomonas aeruginosa* grown under various aerobic conditions were also studied. In cultures where red pigment formation was fair to good (judged visually) the pH changes were very much the same as shown in fig. 1 and 2, but whenever red pigmentation was poor or completely absent, then the pH changes followed a different course. The three graphs in figure 4 illustrate this point. This observation seems to indicate that the pH changes in a culture are to some extent due to the actual formation of pigments A and B, and their precursors.



- ① BUBBLED CULTURE (RED PIGMENT FORMATION GOOD)
- ② BUBBLED CULTURE (RESULTING IN NO RED PIGMENTATION)
- ③ SHAKEN CULTURE (POOR RED PIGMENTATION)

F I G 4

Figure 5 shows the pH changes which take place in the liquid G.L.M. medium which has been inoculated with the organisms from Maytham's slope*. The culture was incubated at 37°C, being kept static and aerobic all the time. No red pigments were produced by this culture and the graph in fig. 5 shows no depressions.

The pH changes of many cultures have been studied and similar observations were made. In all cases 5 ml. portions of the bulk culture were withdrawn at intervals and the pH was measured on these.

(b) Microaerophilic cultures.

Previous experiments have shown that red pigment production is fair in cultures grown microaerophilically, pigment A being absent with one exception. The changes in pH of cultures grown under these conditions were therefore investigated with a view to establishing whether pH changes in a culture of *Pseudomonas aeruginosa* are related to the formation of pigments A and B.

The pH's of the cultures were measured on 5 ml. samples which were aseptically withdrawn at intervals from the bulk cultures. Both cultures, using in one the G.L.M. medium and in the other the G.L.M. + 0.5% KNO₃ medium, turned red after a maximum pH had been reached and after the organisms were no longer viable, as was shown by inoculation tests. Pigment A was absent in both cultures.

The graphs in figure 6 summarize the results of these investigations. The curves appear rather drawn out, due to the long time

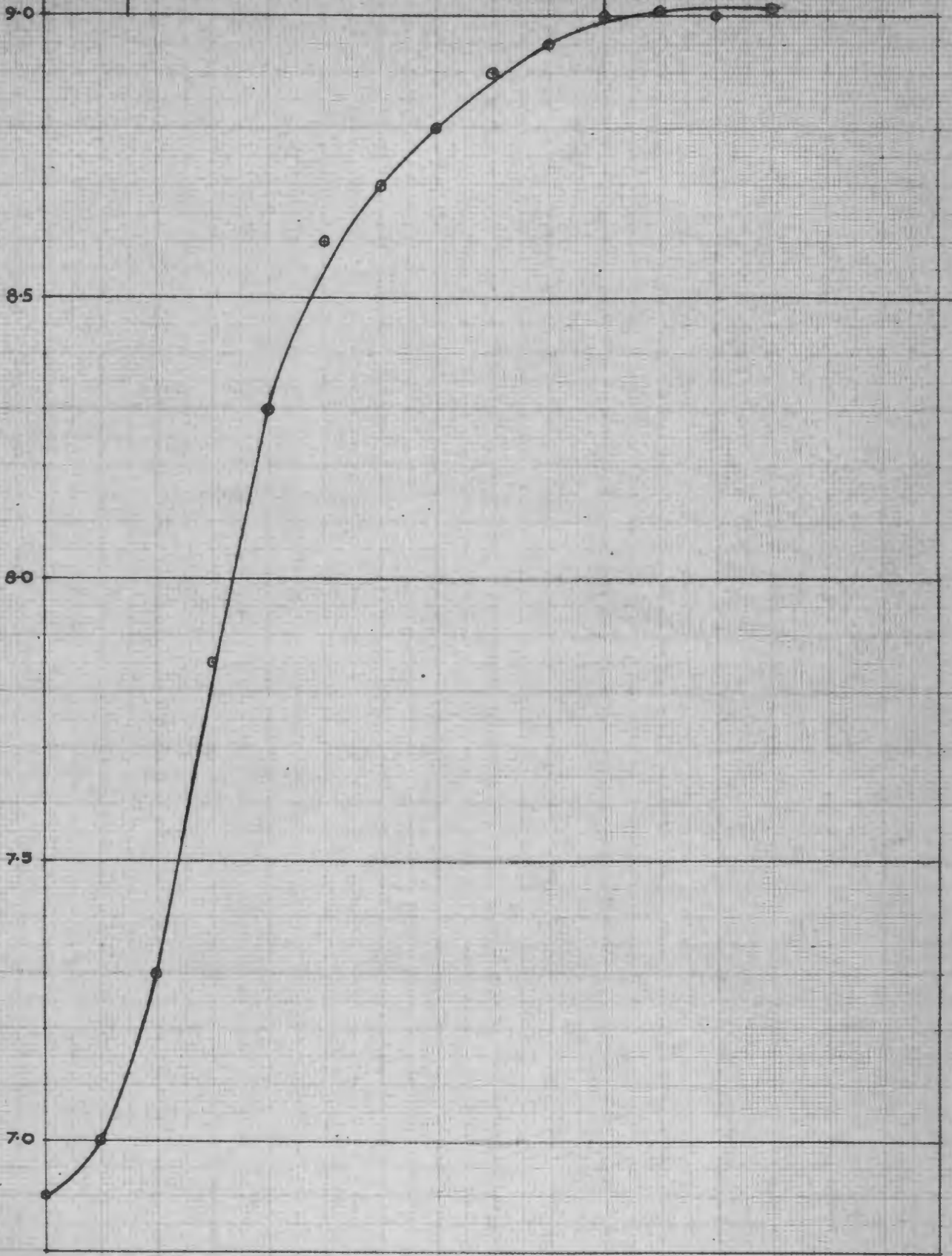
* Maytham's slope, page 32.

pH

TURBID WHITE

C O L O U R
Y E L L O W

B U F F



D A Y S O F I N C U B A T I O N

taken by these cultures to turn red. In aerobic cultures, where red pigment production is good, red pigmentation results in about $\frac{1}{3}$ of the time of that taken by cultures incubated microaerophilically. If this fact is borne in mind then a close resemblance of graph 1 in fig. 6 and that given in figure 2 may be seen.

(vi) The ease of oxidation of a yellow culture at various pH's.

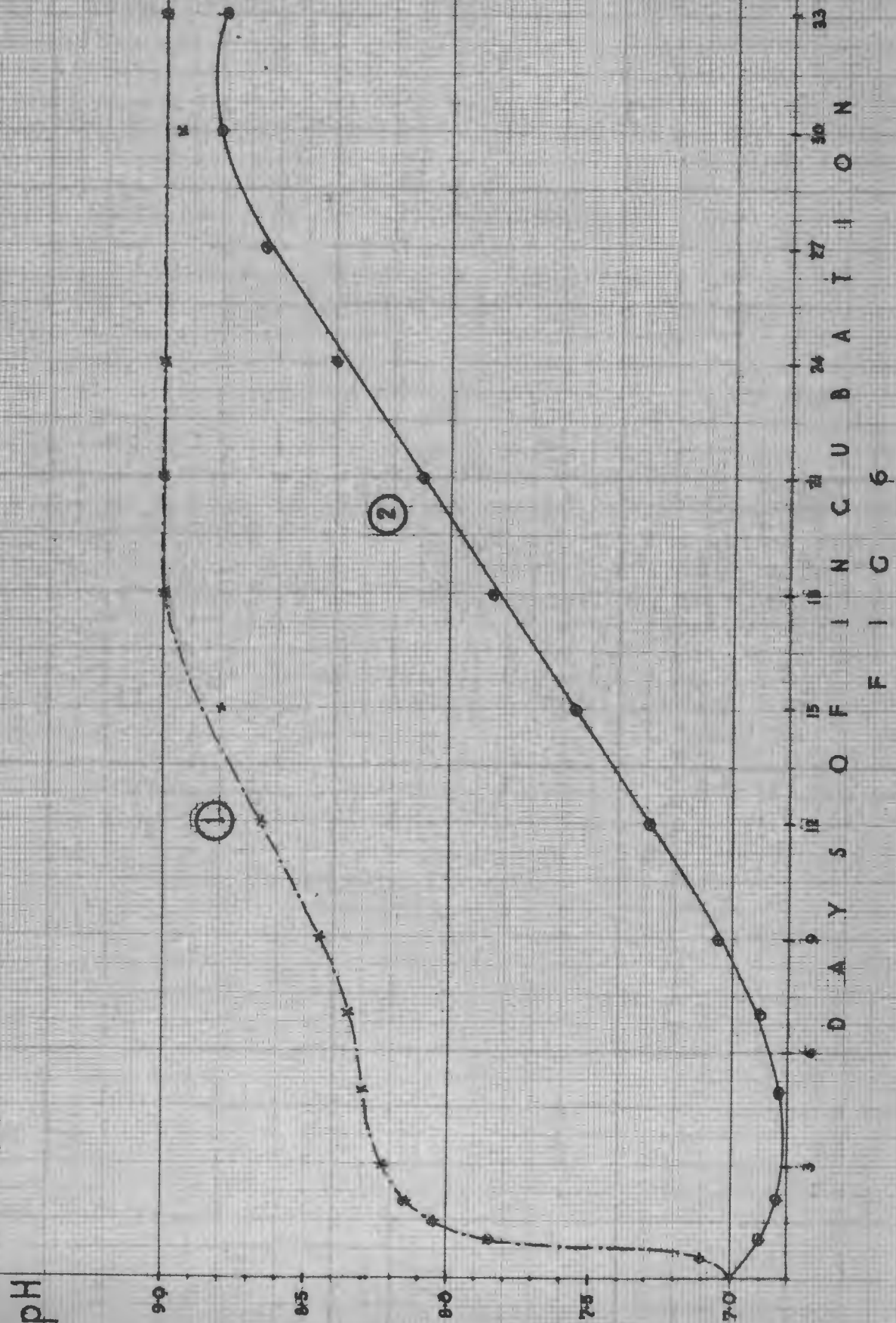
It had been observed that some cultures which had reached the yellow stage turned red more quickly than others when treated with a dilute solution of H_2O_2 . This observation was further investigated.

Eight 5 ml. portions of a culture of the Id strain of *Pseudomonas aeruginosa*, which had turned yellow and had a pH of 7.55, were pipetted into petri-dishes. One of the samples was left at its natural pH, and the pH of the remaining seven was adjusted to higher values using ammonia. 0.2 ml. of a 3% solution of H_2O_2 were added to each sample and the time taken to develop a red colour was recorded. The following were the results:

| pH of sample: | Time taken to develop red colour: |
|---------------|-----------------------------------|
| 7.55 | 30 hours |
| 7.75 | 30 minutes |
| 7.88 | 20 minutes |
| 7.95 | } 2 minutes |
| 8.15 | |
| 8.30 | |
| 8.60 | |
| 9.25 | |

① G.L.M. + 0.5% KNO₃.

② G.L.M.



D A Y S O F I N C U B A T I O N

F I C 6

The oxidation of the yellow cultures to red therefore takes place most rapidly at higher pH's.

(vii) Conclusions.

The pH changes which take place in various cultures where red pigment production is good are similar, their pH curves all showing 2 - 3 depressions which appear to be due to the formation of some products, including the precursors of pigments A and B and the pigments themselves. Cultures in which red pigmentation is poor or absent show different pH changes from those which result in good red pigment formation. The production of pigment A always takes place between a pH of 8.0 and 8.5, pigment B being present in maximum amounts only when the cultures have reached their highest pH.

SECTION B.PART 4: Respiration rates of the organisms in relation to pigment formation.(1) Static aerobic cultures.

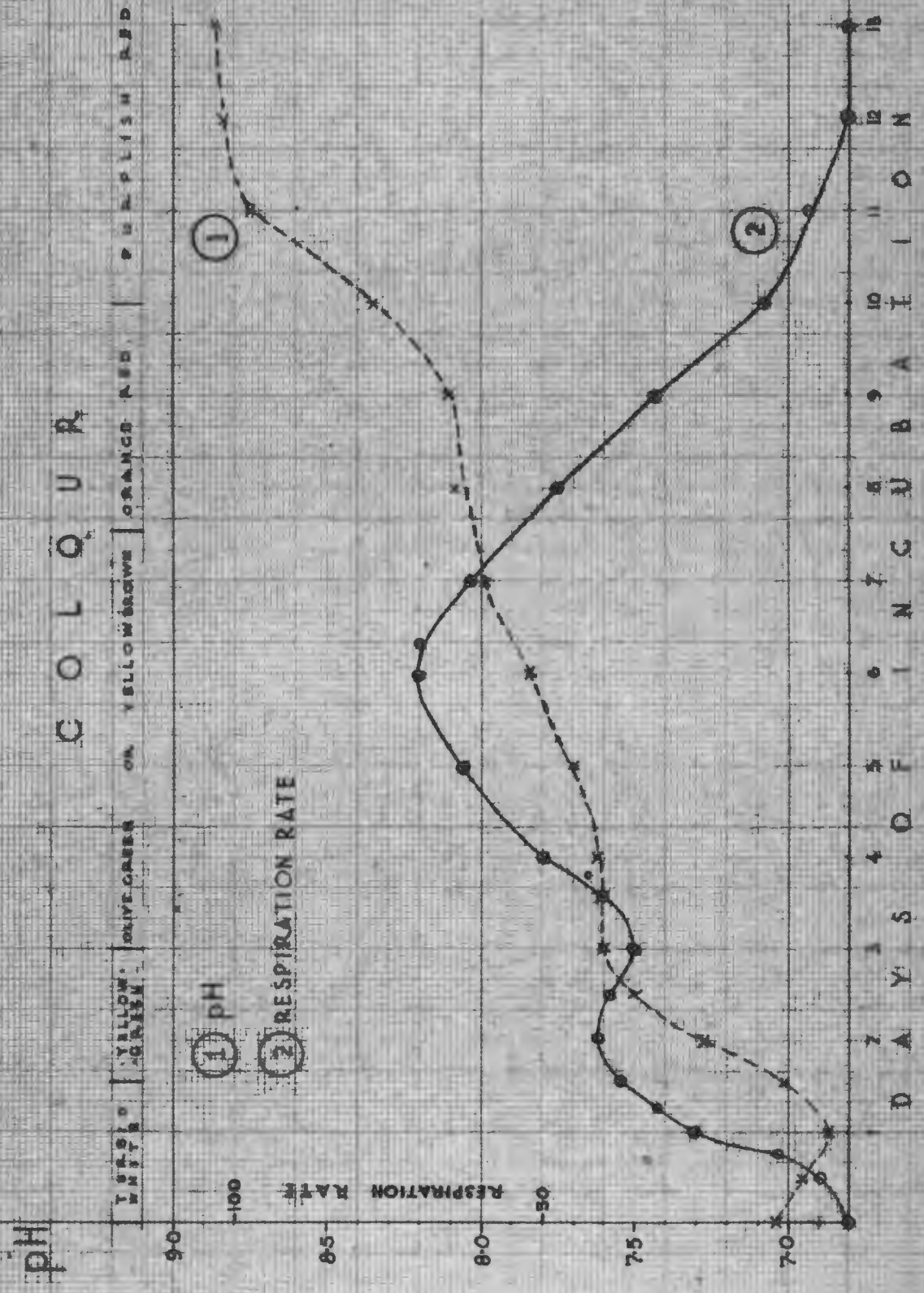
Respiration rates* were used as a measure of viable cells on account of the clumping of the organisms in liquid cultures, which prevents a uniform distribution of viable cells in the "Dilution" and the "Plating" methods usually employed for the counting of living bacteria. The respiration rate of the cultures is always expressed in $\mu\text{l.}$ of O_2 per ml. of culture per hour in this thesis. The respiration rate of the organisms was first determined on aerobic static cultures as these usually resulted in good red pigmentation.

200 ml. of the G.L.M. medium in a square white gin bottle with a capacity of approximately 750 ml. were inoculated with the Id strain of *Pseudomonas aeruginosa*, after which the bottle was plugged with cotton wool and incubated lying on its side at 37°C . 2 ml. portions of this parent culture were withdrawn at intervals and the respiration rate was determined at 37°C , using an oscillation rate of 80 per minute. 5 ml. portions of the same culture were withdrawn at the same time and the pH was measured. All colour changes were noted. The plotted results of this experiment are given in fig. 7.

* Technique of measuring respiration rates, page 15.

STATIC AEROBIC CULTURE OF *Pseudomonas aeruginosa* IN THE G.L.M. MEDIUM

(RESPIRATION RATE, IN $\mu\text{L O}_2/\text{ML CULTURE}/\text{HOUR}$)



D A Y S O F I N C U B A T I O N

The first red colour, being due to the presence of pigment A, appears when the respiration rate of the culture is beginning to fall off after 7 days of incubation. The pH curve flattens out at this point. Pigment B is formed in appreciable quantities when the respiration rate of the culture has dropped to approximately $15 \mu\text{l O}_2$ /ml. culture/hour, reaching its maximum concentration only after the respiration rate is zero. - The pale red culture after 8 days of incubation containing pigment A, also contains the yellow precursor of pigment B*, which is oxidised only when the respiration rate of the culture has dropped to a low value, i.e. when the bacteria require only traces of free oxygen. The yellow precursor of pigment A can therefore be oxidised more readily than that of pigment B.

The experiment was repeated using the G.L.M. + 0.5% KNO_3 medium. The plotted results are given in fig. 8. Very similar observations to those made in the above mentioned experiment were made in this case, pigment A appearing again when the respiration rate of the culture is beginning to fall off. Although the yellow precursor of pigment B* is present at this stage of growth, pigment B itself is formed only when the respiration rate of the culture has dropped to about zero.

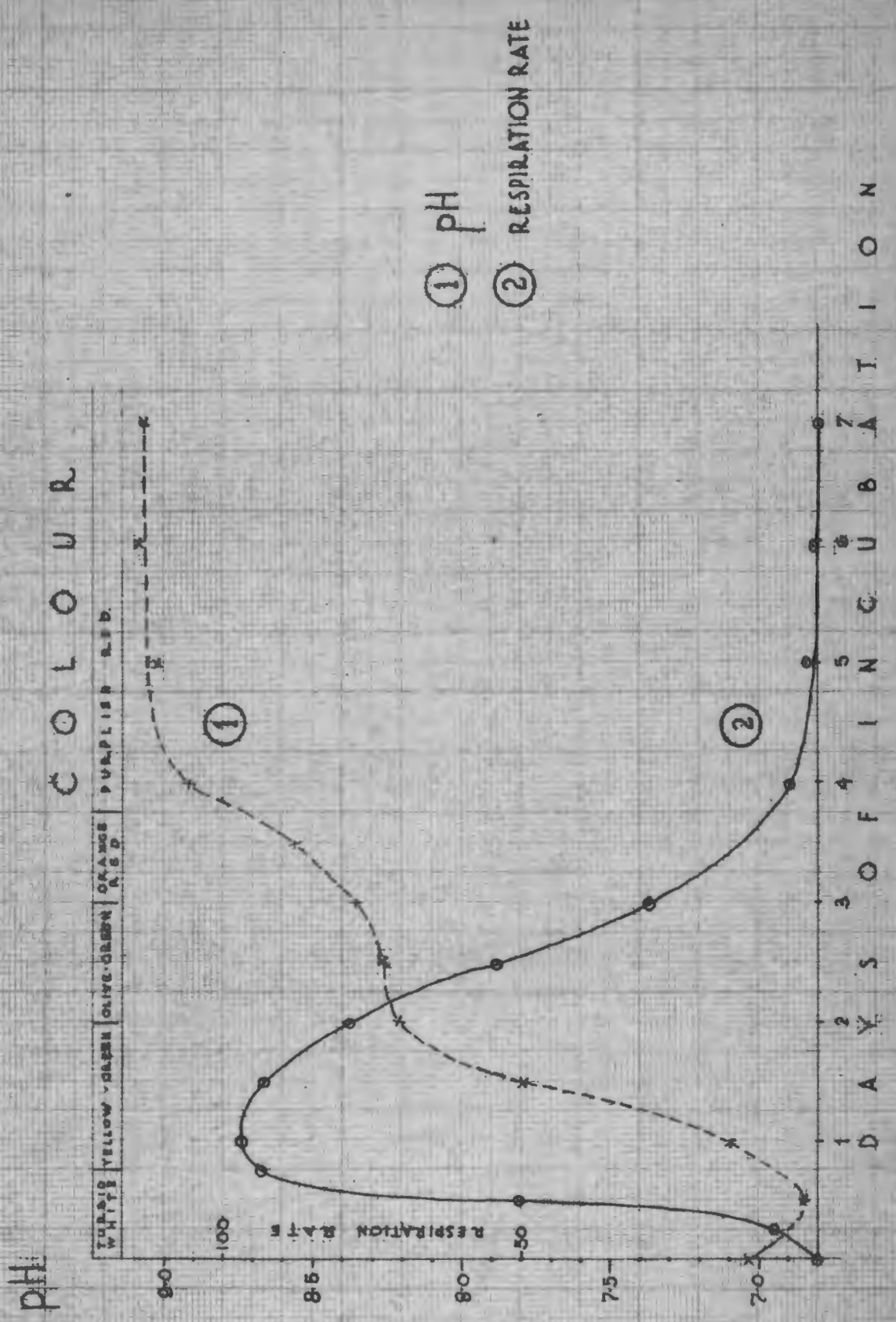
(11) Bubbled cultures.

The respiration rate curves of bubbled cultures of the Li strain of *Pseudomonas aeruginosa*, grown in the G.L.M. medium, had a similar shape to that of the static cultures

* In exposing the culture to much air or by treating it with dilute H_2O_2 , pigment B is formed which can be detected by paper chromatography.

STATIC AEROBIC CULTURE OF *Pseudomonas aeruginosa* IN THE G.L.M + 0.5% KNO_3 MEDIUM

(RESPIRATION RATE IN $\mu l O_2 / ml$ CULTURE / HOUR)



when using the G.L.M. + 0.5% KNO₃ medium (fig. 8). The faster the rate of air-bubbling through these cultures the higher was their respiration rate, which reached a maximum after 40 - 48 hours of incubation, the rate then falling off.

3 conical flasks of 300 ml. capacity, each containing 150 ml. of the G.L.M. medium, were inoculated with equal volumes of a "starter"* of the Li strain of *Pseudomonas aeruginosa*. To each were added 3 drops of tri-n-butyl phosphate in order to avoid excessive frothing. Bubbling tubes were fitted into each culture vessel and the rate of air-bubbling through the culture was varied in each case, viz. 100 ml., 200 ml., and 300 ml. of air per minute respectively. All cultures were incubated at 37°C. The respiration rate was determined on 2 ml. samples of each at intervals. The plotted results are given in fig. 9.

All three aerated cultures developed after 6 days of incubation an evenly dark purplish red colour, which remained unaltered (judged visually), with the exception of the fastest air-bubbled culture where it faded visibly after a further 3 days of incubation. It was shown by paper chromatography that this was mainly due to the disappearance of pigment A. It has already been mentioned that the bacteria can destroy pigment A**, and since some organisms of this highly aerated culture were still living even after 12 days

* Meaning of "starter", page 15.

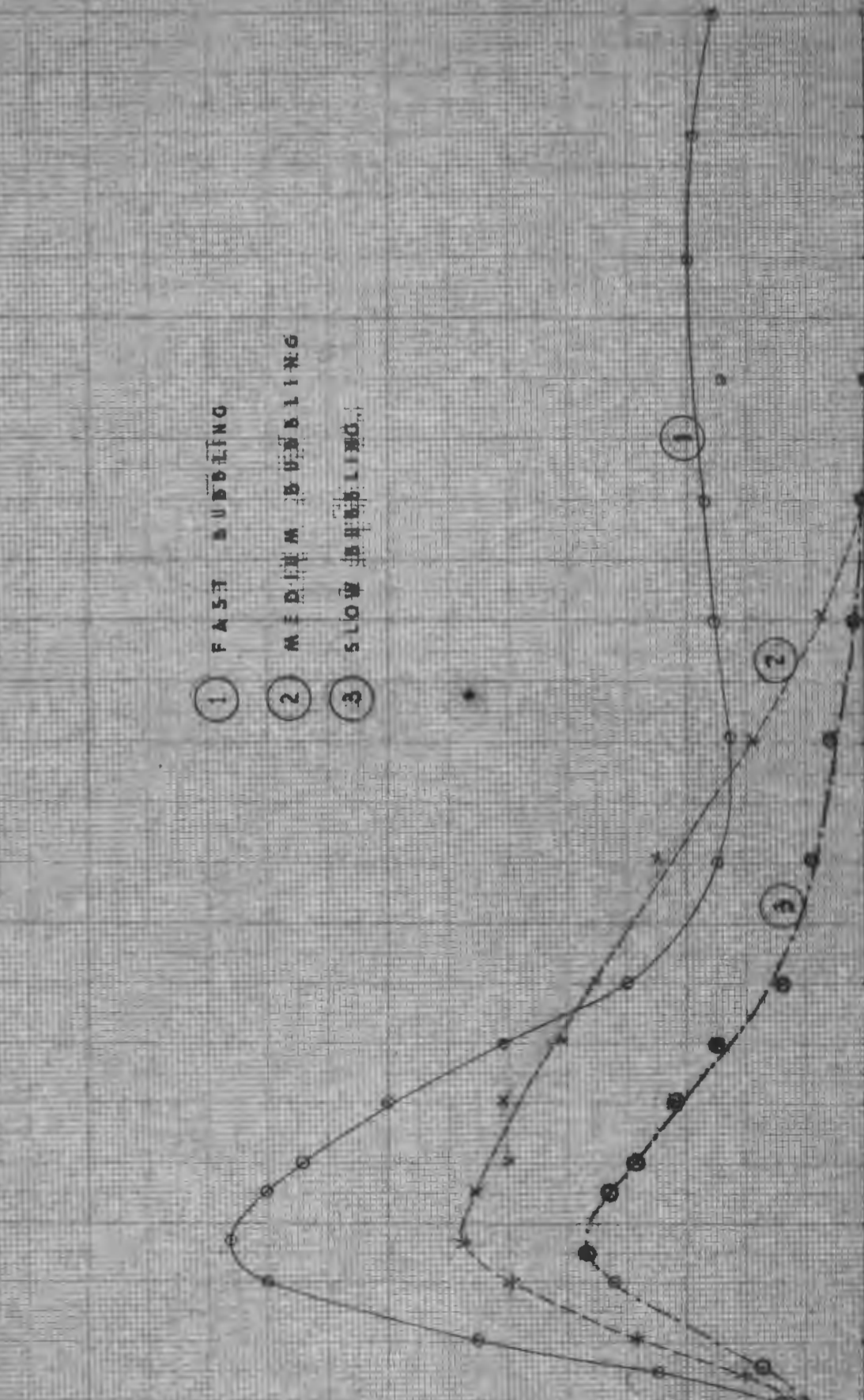
** Evidence for this is given in Section E.

RESPIRATOR RATE IN ml O₂ / ml CULTURE / HOUR

- ① FAST BUBBLING
- ② MEDIUM BUBBLING
- ③ SLOW BUBBLING

1 2 3 4 5 6 7 8 9 10 11 12

D A Y S O F I N C U B A T I O N



of incubation, the above observation might be explained. - Unfortunately the method for the quantitative determination of pigments A and B had not been fully developed when this experiment was carried out.

(iii) Possible relationship between the respiration rate of a culture and red pigmentation.

Some of the experiments which were carried out during the early period of the work, where no method for the quantitative determination of the two red pigments was available, showed that the total red colour intensity (compared colorimetrically on the filtered culture) of static aerobic cultures was approximately proportional to the area below the respiration rate curves of the cultures examined. The relationship did not, however, apply to all the cultures which were incubated under the same conditions.

(iv) Conclusion.

Pigment A appears in cultures of *Pseudomonas aeruginosa* when the respiration rate of the culture is beginning to fall off, pigment B being formed in appreciable quantities when the respiration rate of the culture has dropped to about zero.

SECTION B.PART 5: Nutritional requirements for growth and optimum production of red pigments.(i) Introduction.

For continued life and pigment production

Pseudomonas aeruginosa in general is dependent upon the presence of water, certain inorganic salts, a carbon and nitrogen source, and to some extent on gaseous oxygen. The fundamental problem in this section is the development of a medium of known composition which will permit satisfactory growth and optimum red pigment formation in the *Id* strain of *Pseudomonas aeruginosa*. For this purpose only synthetic media* have been employed.

A number of references to the nutritional requirements of other strains of *Pseudomonas aeruginosa* may be found in the introduction of this thesis. The literature on this subject in general is so large that it is impossible to give an extensive review.

In the experiments of this section 5 boiling tubes (6" x 1"), each containing 15 ml. of the medium under test, were inoculated with the *Id* strain of *Pseudomonas aeruginosa*,

* Synthetic media are those which contain only constituents of known composition and no proteins, broth or similar compounds.

plugged with cotton wool and incubated sloped at 37°C. At the end of the experiment the contents of the 5 boiling tubes were combined and the concentration of pigments A and B were determined on this.

(ii) The inorganic requirements.

(a) Phosphorus.

Robinson²⁸ reported that phosphate and probably magnesium were essential inorganic constituents of the media in which *Pseudomonas aeruginosa* would grow. It may be assumed that all bacteria require phosphorus.

In order to prove that phosphorus is essential for the growth of the organisms, the KH_2PO_4 was omitted from the G.L.M. medium* and it was replaced by a known weight of K_2SO_4 , keeping the $[\text{K}^+]$ constant; the slight change in the $[\text{SO}_4^{--}]$ has little effect on the organisms as will be shown in a later experiment. The pH of the medium was adjusted to 7.0 using KOH.

No growth took place in the medium lacking phosphorus, not even after prolonged incubation. The importance of phosphorus is thus indicated.

(b) Magnesium.

Of the workers who have investigated the effect of Mg^{++} on the growth and pigment production of *Pseudomonas aeruginosa*, the majority have found this constituent to be essential. Pandalai and Rao³⁰, on the other hand, have reported that Mg^{++} is not necessary for either growth or pigmentation.

An experiment was carried out to study the effect of the

* Composition of the G.L.M. medium, page 22.

absence of magnesium in a medium on the organisms, using the G. L. M. medium in which a known weight of K_2SO_4 was used instead of $MgSO_4$ (I), keeping the $[SO_4^{--}]$ constant. The G. L. M. medium (II), and the G. L. M. medium to which an equal amount of K_2SO_4 was added as above (III) were used as controls. The pH of all media was adjusted to 7.0. The following table summarizes the results:

| Medium: | Growth: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---------|----------------|-------------------------------------|-------------------------------------|
| I | Extremely poor | absent | 2 |
| II | Good | 8 | 41 |
| III | Good | 6 | 37 |

The results show that magnesium is essential for satisfactory growth of the *Li* strain of *Pseudomonas aeruginosa*. Although growth is extremely poor in media devoid of magnesium small amounts of pigment B are formed, pigment A being absent.

(c) Potassium.

⁴⁴Rahn found that *Pseudomonas aeruginosa* requires no potassium for growth. Burton, Campbell and Eagles³⁴ in their work established that potassium was necessary for pyocyanine formation by the organisms.

An experiment was carried out to investigate whether potassium

is necessary for growth and red pigment formation by the Ii strain. The G.L.M. medium in which the KH_2PO_4 was replaced by a known weight of NaH_2PO_4 to keep the $[\text{PO}_4^{---}]$ constant (IV), was used in this investigation. The G.L.M. medium (V), and the G.L.M. medium to which an equal amount of NaH_2PO_4 had been added as above (VI), were used as controls. The concentration of pigments A and B was determined on the combined cultures of each medium, the results obtained being:

| Medium: | Growth: | Final colour: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---------|---------|---------------|-------------------------------------|-------------------------------------|
| IV | Fair | Turbid white | absent | absent |
| V | Good | Purplish red | 8 | 41 |
| VI | Good | Purplish red | 7 | 39 |

Growth of the organisms in the absence of potassium is not as good as if it was present in the medium. It is very interesting to note that no yellow or red pigments are produced at all when potassium is omitted from the G.L.M. medium. The function of this element appears to be catalytic, the exact role being still unknown.

(d) Sulphur.

Pandalai and Rao³⁰ showed that sulphur (SO_4^{--}) improved pyocyanine formation by *Pseudomonas aeruginosa*, - the results of Hellinger³⁷ indicate that SO_4^{--} should be added at the time of inoculation if maximum yields of pyocyanine are to be

obtained, - Burton, Campbell and Eagles³⁴ found that sulphur (SO_4^{--}) is necessary for pyocyanine formation, - and King, Campbell and Eagles³⁵ showed that the formation of pyocyanine is dependent mainly on the concentrations of SO_4^{--} and Mg^{++} . The effect of sulphur on red pigment production by the Li strain of *Pseudomonas aeruginosa* was studied, more so because the main pigment, pigment B, contains sulphur*.

The G.L.M. medium was employed, but the amount of MgSO_4 was varied; the $[\text{Mg}^{++}]$ was kept constant by the addition of MgCl_2 . FeCl_2 was used instead of FeSO_4 . The pH of all media was adjusted to 7.0 using KOH. The following were the results:

| % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: | Growth: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---|---------|-------------------------------------|-------------------------------------|
| 0.1 | } Good | 13 | 46 |
| 0.075 | | 14 | 46 |
| 0.05 | | 14 | 44 |
| 0.025 | | 12 | 38 |
| 0.01 | | 10 | 35 |
| 0.005 | Fair | 6 | } absent |
| 0.0005 | Poor | 2 | |
| 0.0 | Poor | absent | |

(The percentage of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in the G.L.M. medium is 0.1).

* Probable molecular formula of pigment B: $\text{C}_{15}\text{H}_{15}\text{O}_6\text{N}_3\text{S}$. (Holliman²⁴).

Red pigment production is about the same when using the G.L.M. medium containing 0.05 - 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; it decreases slightly when only 0.01% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ is present, and with still smaller amounts pigment A only is produced. Growth of the organisms is poor when SO_4^{--} is absent and neither pigment A nor pigment B is formed.

Another experiment was carried out to study the effect of sulphur on red pigment production by the *Li* strain of *Pseudomonas aeruginosa*. MgSO_4 was omitted altogether from the G.L.M. medium and it was replaced by MgCl_2 to keep the $[\text{Mg}^{++}]$ constant. FeCl_2 was used instead of FeSO_4 . The medium was saturated at room temperature with L-cystine* as source of sulphur, and its pH was adjusted to 7.0 with KOH (VII). The G.L.M. medium (VIII), the G.L.M. medium where the MgSO_4 and FeSO_4 were replaced by MgCl_2 and FeCl_2 respectively (IX), and the G.L.M. medium saturated at room temperature with L-cystine (X) were used as controls. The following results were obtained:

| Medium: | Growth: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---------|---------|--|--|
| VII | Good | 6 | 12 |
| VIII | Good | 13 | 46 |
| IX | Poor | absent | absent |
| X | Good | 6 | 8 |

* Solubility of L-cystine, 0.011 g. at 25°C.

The concentration of sulphur expressed as $MgSO_4 \cdot 7H_2O$ in the medium which has been saturated at room temperature with L-cystine as the sole source of sulphur is 0.022%. It can be seen from the previous experiment that the medium containing 0.025% of $MgSO_4 \cdot 7H_2O$ resulted in a yield of 12 mg. pigment A and 38 mg. pigment B. The low yield of red pigments in the medium saturated at room temperature with L-cystine was unexpected.

Both experiments indicate that sulphur is necessary for good growth and red pigment formation by the Li strain of *Pseudomonas aeruginosa*.

(e) Iron.

The function of iron in microorganisms is essentially catalytic being necessary in enzymic systems. Waring and Werkman⁴⁵ have studied the iron requirements of several bacteria and found that *Pseudomonas aeruginosa* requires this element for maximal growth. Burton, Campbell and Eagles³⁴ considered iron ions necessary for pyocyanine formation, and Hellinger³⁷ reported that the presence of Fe ions was not required for maximum yields of pyocyanine. An experiment was therefore carried out to investigate what the effect on red pigment formation by the Li strain would be if iron ions are omitted from the medium in which they grow. The G.L.M. medium with the exclusion of $FeSO_4$ (XI) was employed for this purpose, using the G.L.M. medium as such (XII) as a control. The following results were obtained:

| Medium: | Growth: | Final colour: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---------|---------|---|-------------------------------------|-------------------------------------|
| XI | Good | Pale orange red with green fluorescence | 4 | 17 |
| XII | Good | Dark purplish red | 8 | 41 |

When determining the concentration of pigments A and B* it was noticed that the combined cultures lacking iron ions still contained considerable amounts of one or more yellow pigments, which on oxidation with H_2O_2 turned red. On keeping part of this combined culture in a flask, plugged with cotton wool, for another 3 weeks in a refrigerator it turned dark purplish red. The concentration of pigments A and B was determined on the H_2O_2 -oxidised culture and on the culture kept in the refrigerator, and the results were compared with those originally obtained, i.e. 12 days after inoculating the medium lacking iron ions. The following table summarizes these results:

| Culture: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|--|-------------------------------------|-------------------------------------|
| 12 days after inoculation | 4 | 17 |
| 12 days after inoculation, then oxidised with H_2O_2 | 9 | 36 |
| 12 days after inoculation, then kept aerobically for 3 weeks in the refrigerator | 6 | 38 |
| (G.L.M. 12 days after inoculation) | 8 | 41 |

* Determination of pigments A and B is always carried out on the combined cultures 12 days after inoculating the medium under test.

The catalytic effect of iron ions in the oxidation of the yellow precursors of pigments A and B to the red pigments themselves is thus indicated. It can be seen that the oxidation of the yellow precursors by gaseous oxygen is greatly assisted by iron ions.

(f) Ammonium salts.

A number of early workers, including Koser and Rettger⁴⁶ found that *Pseudomonas aeruginosa* would grow in simple media containing the usual inorganic salts with ammonium chloride or phosphate as a source of nitrogen. Robinson²⁸ included ammonium salts in his list of available nitrogen sources for the growth of these organisms, finding the optimal range of NH_4Cl for aerobic growth to be 0.05 - 0.25%, and for pigmentary activity 0.10%. Burton, Campbell and Eagles³⁴ found that NH_4^+ inhibits pyocyanine formation, whereas Hellinger³⁷ reported that NH_4^+ should be present if maximum yields of pyocyanine are to be obtained. - The effect of NH_4^+ on red pigment production by the Li strain of *Pseudomonas aeruginosa* was thus investigated. The following basic medium was used:

0.15% NH_4Cl
 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 0.03% KH_2PO_4
 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

To this medium various carbon sources* were added separately, viz.

* Pinghui Liu⁴⁷ found that *Pseudomonas aeruginosa* is able to utilize glucose, fructose, galactose, mannitol, trehalose, or glycerol as the sole source of carbon for growth. Many more compounds have been reported by various workers to be suitable as sole carbon source for the growth of these bacteria.

| | | |
|--------------|-------|----------------|
| Medium XIII: | 0.5% | mannitol |
| " | XIV: | 0.5% D-glucose |
| " | XV: | 0.5% galactose |
| " | XVI: | 0.5% fructose |
| " | XVII: | 2% glycerol |

The pH of all media was adjusted to 7.0.

None of the media (XIII - XVII) resulted in red pigmentation, although growth was satisfactory in all but medium XV, where growth was poor. All cultures turned turbid white only. The difference between medium XIII and the G.L.M. medium is that the amino acids in the latter have been replaced by ammonium chloride. - The effect on red pigment production by varying the carbon sources in a medium containing amino acids will be discussed in the following sub-section.

(g) Nitrates

The effect on red pigment formation by the inclusion of a nitrate into the G.L.M. medium has been discussed fully in Section B, Part 2 (page 53A).

(iii) The organic requirements.

(a) Amino acids.

Professor Holliman²⁴ in his work has shown that the Id strain of *Pseudomonas aeruginosa* can utilize glycine and L-leucine and that these organisms produce the red pigments when in addition a suitable carbon source and certain inorganic salts are present in the medium. His G.L.M. medium

with the inclusion of 0.5% KNO_3 , is up to now the best medium for red pigment production. Only a few media were tested to investigate how some of the amino acids affect red pigment formation by these bacteria.

A basic medium was used, of composition

| | |
|--------|---|
| 0.5% | mannitol |
| 0.1% | $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ |
| 0.03% | KH_2PO_4 |
| 0.001% | $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ |

To this were added the amino acids, under investigation, as follows:

| | |
|---------------|--|
| Medium XVIII: | 0.5% glycine |
| " XIX: | 0.5% L-leucine |
| " XX: | 0.5% glycine and 0.1% L-leucine |
| " XXI: | 0.5% glycine and 0.1% L-phenylalanine |
| " XXII: | 0.5% glycine, 0.1% L-leucine, then saturate the medium at room temperature with L-cystine. |
| " XXIII: | As for medium XXII, but replace MgSO_4 and FeSO_4 by MgCl_2 and FeCl_2 in the basic medium. The L-cystine is used here as a source of sulphur. |

The pH of all media was adjusted to 7.0.

The cultures of all media, with the exception of XIX and XXI showed normal colour changes*. Culture XIX gave no colour production at all whilst those exhibited by culture XXI were:

| | |
|--------------|--|
| After 1 day: | Pale yellow brown |
| " 2 days: | Fairly dark yellow brown |
| " 3 " | : Fairly dark yellow brown, the tip nearest the cotton wool plug turning dark brown. |

* Normal colour changes, p. 24.

After 4 - 6 days: the culture is dark brown throughout
 " 7 - 8 " : dark purplish red with distinct brown
 tinge
 " 9 days : dark purplish red; the culture has
 cleared.

The culture was found to contain in addition to pigments A and B, a red pigment having an R_A of 0.14 with the butanol-acetic acid-water solvent, and a blue pigment which remained behind as a spot when using the same solvent.

The following are the summarized results of all the above media:

| Medium: | Growth: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---------|---------|--|--|
| XVIII | } Good | 9.5 | 35 |
| XIX | | absent | absent |
| XX | | 7 | 44 |
| XXI | | 4 | 54 |
| XXII | | 6 | 8 |
| XXIII | | 6 | 12 |

Red pigment formation does not take place when adding L-leucine** alone to the basic medium, but is very satisfactory when the medium contains glycine as the sole source of nitrogen. The medium containing glycine and L-phenylalanine* (instead of L-leucine) resulted in the highest yield of pigment B yet obtained, viz. 54 mg. per

* DL-phenylalanine was not available.

** Burton, Campbell and Eagles³³ reported that no pyocyanine is formed when leucine is employed as the sole source of nitrogen.

litre culture. Burton, Campbell and Eagles³⁸ found in their experiment that pyocyanine formation was inhibited when substituting DL-phenylalanine for L-leucine in their medium. - The inclusion of L-cystine in the G.L.M. medium resulted in poor red pigmentation.

(b) Carbon sources.

Bacteria in general vary greatly in their carbon requirements. Only a few carbon sources have been included in the following basic medium to study how they may affect red pigment formation by the Li strain of *Pseudomonas aeruginosa*:

0.1% L-leucine
 0.5% glycine
 0.1% $MgSO_4 \cdot 7H_2O$
 0.03% KH_2PO_4
 0.001% $FeSO_4 \cdot 7H_2O$

The media tested contained*

Medium XXIV: 0.5% mannitol
 " XXV: 0.5% dextrose
 " XXVI: 0.5% galactose
 " XXVII: 0.5% fructose
 " XXVIII: 2.0% glycerol.

The pH of all the media was adjusted to 7.0 using KOH.

The results obtained were:

* These carbon sources are the same as used in media XIII to XVII.

| Medium: | Growth: | mg. of pigment A per litre culture: | mg. of pigment B per litre culture: |
|---------|---------|--|--|
| XXIV | } Good | 6 | 42 |
| XXV | | 3 | 30 |
| XXVI | Fair | absent | 4 |
| XXVII | } Good | absent | 32 |
| XXVIII | | 84 | 24 |

The results clearly indicate the varying effect of a number of carbon sources on red pigment production by *Pseudomonas aeruginosa*. The medium containing galactose resulted in poor red pigmentation, whereas that containing glycerol gave an extremely high yield of pigment A, viz. 84 mg. per litre culture, by far the highest yield yet obtained. The culture of medium XXVIII was found to contain three red pigments in addition to pigments A and B, these having an R_A of 0.14, 0.36, and 0.78 respectively when using the butanol-acetic acid-water solvent. The culture had a very slimy consistency.

(iv) Conclusions

PO_4^{---} , Mg^{++} , and sulphur (SO_4^{--}) are essential for satisfactory growth of the Li strain of *Pseudomonas aeruginosa*. In addition K^+ , Fe ions and glycine are required for good red pigment formation, K^+ being absolutely essential for red pigmentation. NH_4^+ is not a suitable nitrogen source for the

production of the red pigments, whereas various amino acids and carbon sources greatly affect the yields of pigments A and B.

It would be interesting to study the effect of many other inorganic salts and organic compounds on red pigment formation by the Li strain of *Pseudomonas aeruginosa*.

(v) Brief discussion.

Many of the findings in this section are similar to those obtained by Burton, Campbell and Eagles^{33,34}, who studied the nutritional requirements of some strains of *Pseudomonas aeruginosa* which produce pyocyanine. These workers found that PO_4 , Mg, SO_4 , K and Fe ions were necessary for pyocyanine formation, that NH_4^+ inhibits the production of this pigment, and that no pyocyanine is formed when leucine is employed as the sole source of nitrogen. The addition of L-leucine to a medium containing glycine increased pyocyanine formation, whereas DL-phenylalanine under similar conditions was an inhibitor.

The production of red pigments by the Li strain of *Pseudomonas aeruginosa* was found to depend on almost the same conditions as those given above, except that phenylalanine did not inhibit red pigment formation. The yield of pigments A and B was found to be affected by the carbon source used in the medium. - The optimal salt concentrations for best red pigmentation were not determined.

SECTION C.

Observations in the behaviour of some variants of the Id strain of
Pseudomonas aeruginosa.

(i) Introduction

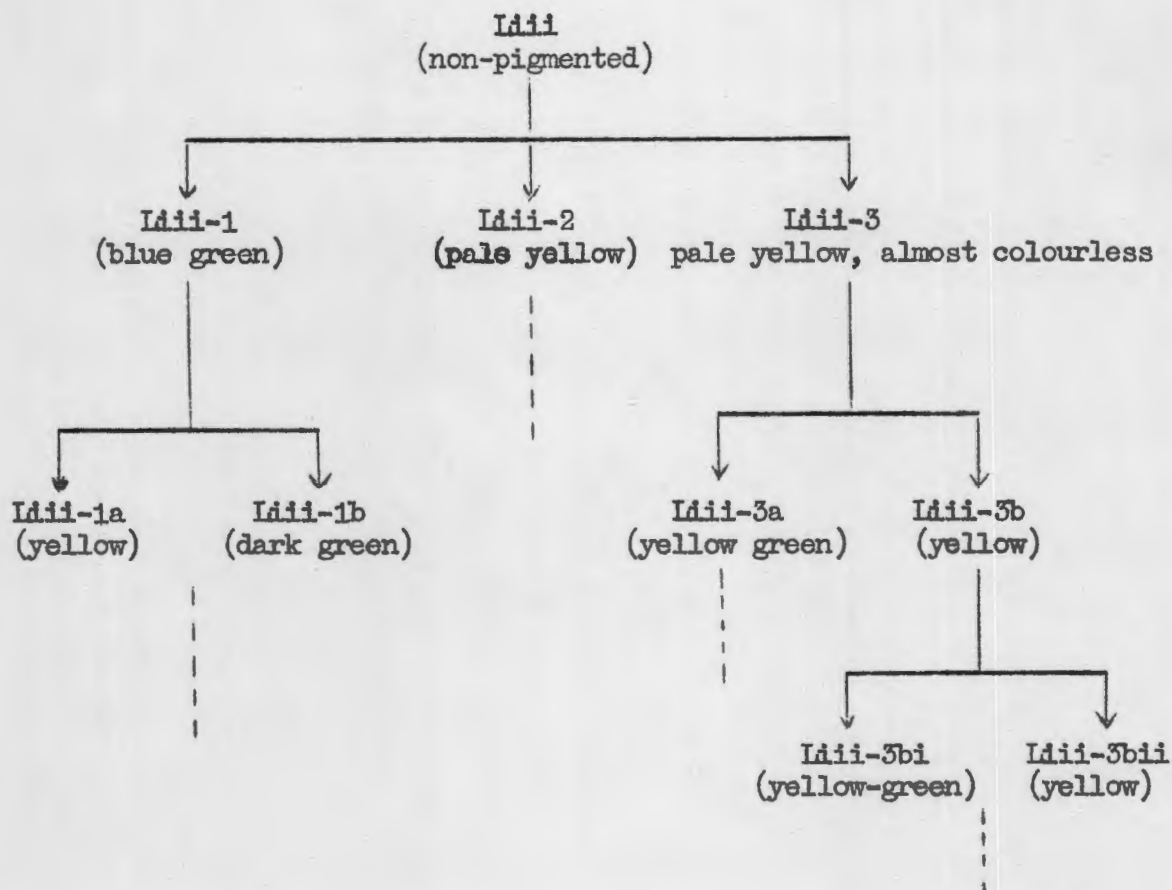
Don and van den Ende²⁰ isolated in 1948 the L-strain of Pseudomonas aeruginosa. When first isolated the strain was non-metallic (Lii), but later a metallic variant (Id) was isolated. These two variants produce red pigments when grown in a suitable medium and under favourable conditions. Most of the work in this thesis has been done on the Id strain of these organisms. A third spontaneous colourless variant (Liii) produced on Hartley's agar three types of colony:

Liii - 1 : a very deep blue-green colony.

Liii - 2 : a pale yellow colony.

Liii - 3 : a very pale yellow, almost colourless colony.

Altogether 21 variants were obtained from the Liii - 1, 2 and 3 variants. The scheme below illustrates the origin of some of the strains referred to in this section.



The dark green Ldii-1b variant was chosen for the purpose of comparing its behaviour with that of the Li strain of *Pseudomonas aeruginosa*.

(ii) The behaviour of the Ldii-1b variant on agar slopes.

6 G.L.M. agar slopes were inoculated with the organisms of the Ldii-1b variant from a slope obtained from the Bacteriology Department of this University. All slopes were incubated at 37°C. The following were the average colour changes that took place:

After 1 day: pale yellow.
 " 2 days: pale yellow green.
 " 3 " : pale yellow green, the tip of the slope turning dark green.

After 4 - 6 days: fairly dark green throughout.

" 7 - 8 " : olive green.

" 9 - 12 " : brownish-green (a dirty looking colour).

An interesting point was that green pigmentation started at the tip of the slope, then later spreading throughout the medium. In the Id strain of *Pseudomonas aeruginosa* red pigmentation also started at the tips of the slopes (page 23).

The effect of subculturing* the organisms of the Liii-1b variant on G.L.M. agar slopes resulted in a gradual decrease in green pigmentation, the 6th subculture being distinctly brown in colour. Passing the organisms of this last subculture once through Litmus/Milk, the culture bottle being tightly closed with a screw cap, resulted in all cases in a marked improvement of green pigment formation. Passing the organisms through the G.L.M. medium or through Hartley's Beef Digest Broth did not restore the ability to produce the green pigment(s)**.

(iii) The behaviour of the Liii-1b variant in the liquid G.L.M. medium.

(a) Under aerobic conditions.

6 Boiling tubes, each containing 15 ml. of the G.L.M. medium, were inoculated with the Liii-1b variant of *Pseudomonas aeruginosa*, closed with a cotton wool plug and incubated sloped and undisturbed at 37°C. The colour changes which took place

* Subculturing the organisms of the Id strain, page 22.

** Restoration of the ability to produce red pigments, page 28.

are listed below:

- After 1 day: the culture was very pale yellow, the surface being pale blue in colour.
- " 2 days: blue-green colouration on the surface of the culture, the remainder being pale yellow brown.
- " 3 - 4 " : fairly dark green throughout.
- " 5 - 12 " : olive green.

When using the G.L.M. + 0.5% KNO₃ medium instead of the G.L.M. medium, green pigmentation was poorer (judged visually), the cultures turning more brownish.

The effect of sub-culturing the organisms of the *Idii-1b* variant in the liquid G.L.M. medium, as well as the effect of various oxygenation conditions on their green pigment formation, was not studied.

(b) Under microaerophilic conditions.

The organisms of the *Id* strain of *Pseudomonas aeruginosa* were capable of producing only one of the main red pigments under microaerophilic conditions. An experiment was carried out to find out whether one of its variants, producing green pigment(s) under aerobic conditions, could form these microaerophilically.

Two conical flasks of 500 ml. capacity, each containing 100 ml. of the G.L.M. medium, were inoculated with the organisms of the *Idii-1b* variant of *Pseudomonas aeruginosa*.

The surface of the cultures was then covered with a $\frac{1}{2}$ to $\frac{3}{4}$ inch layer of autoclaved medicinal liquid paraffin. The cultures developed a pale yellow colour after 6 days of incubation at 37°C , which darkened only slightly after a further 55 days had elapsed. No green pigmentation resulted even after 3 months of incubation.

The experiment was repeated using the G.L.M. + 0.5% KNO_3 medium instead of the G.L.M. medium; again no green pigmentation resulted.

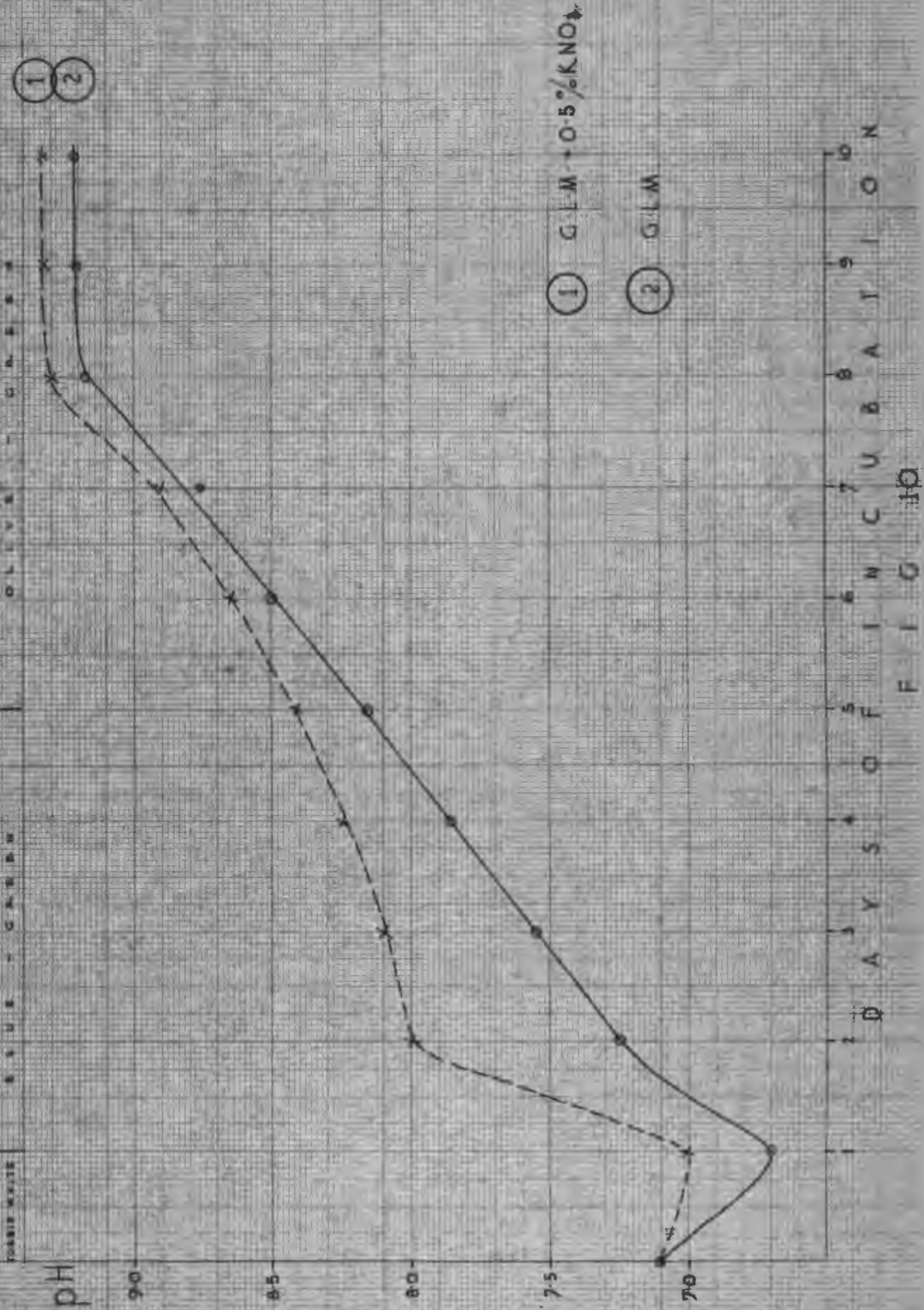
(iv) pH changes in the G.L.M. medium of this variant.

The pH changes were determined in cultures of the Iiii-1b variant of *Pseudomonas aeruginosa*, using in one experiment the G.L.M. medium and in the other the G.L.M. + 0.5% KNO_3 medium. - 200 ml. of each of the media in a white square gin bottle of approximately 750 ml. capacity were inoculated with the organisms of this variant of the Ii strain. The culture vessels were closed with cotton wool plugs and incubated lying on their side at 37°C . 5 ml. samples were aseptically withdrawn at intervals and the pH was determined. Fig. 10 summarizes the results of this investigation. The bacteria were found to be dead when the culture reached its maximum pH, no growth taking place when one drop of the culture was added to 15 ml. of a fresh G.L.M. medium. - There are no marked depressions in the pH curve; the cultures turned more olive green in shade as the pH rose.

(v) Pigments formed by the Iiii-1b variant.

Two main pigments are produced when the organisms of the

C O L L O I D



Iiii-1b variant of *Pseudomonas aeruginosa* are grown aerobically in the G.L.M. medium, and a third one, having an R_F of 0.30 (using the butanol-acetic acid-water solvent) occurs only in traces. A fourth pigment is formed by these bacteria when they are grown microaerophilically in the G.L.M. medium.

Traces of three of the pigments (fig. 11: 1, 2 and 3), which appear to be chemically related, have so far been isolated by paper chromatography*, and their absorption spectra were determined on unknown concentrations of these pigments in 1 N HCl, using the "Beckman" spectrophotometer. The absorption curves are given in fig. 11. The table below gives just a few data regarding these pigments:

| Colour in acid solution: | Colour in alkaline solution: | R_F (using the n-butanol-acetic acid-water solvent: | Absorption maxima (λ , $m\mu$): (pigments in 1 N HCl) |
|--------------------------|------------------------------|---|---|
| Red | Blue | 0.55 | 520 387 277 |
| Pink | Yellow-green | 0.05 | 545 395 295 |
| Mauve | Green | 0.12 | 560 400 300 |

The first pigment listed above has identical properties to those

*

3 MM. paper and the butanol-acetic acid-water solvent were used. The bands of the pigments were cut out of the chromatogram and eluted with a small volume of 1 N HCl.

- ① PYOCYANINE
 - ② MAUVE BAND (a)
 - ③ MAUVE BAND (b)
- (ALL PIGMENTS IN HCl)

LOG E + 2

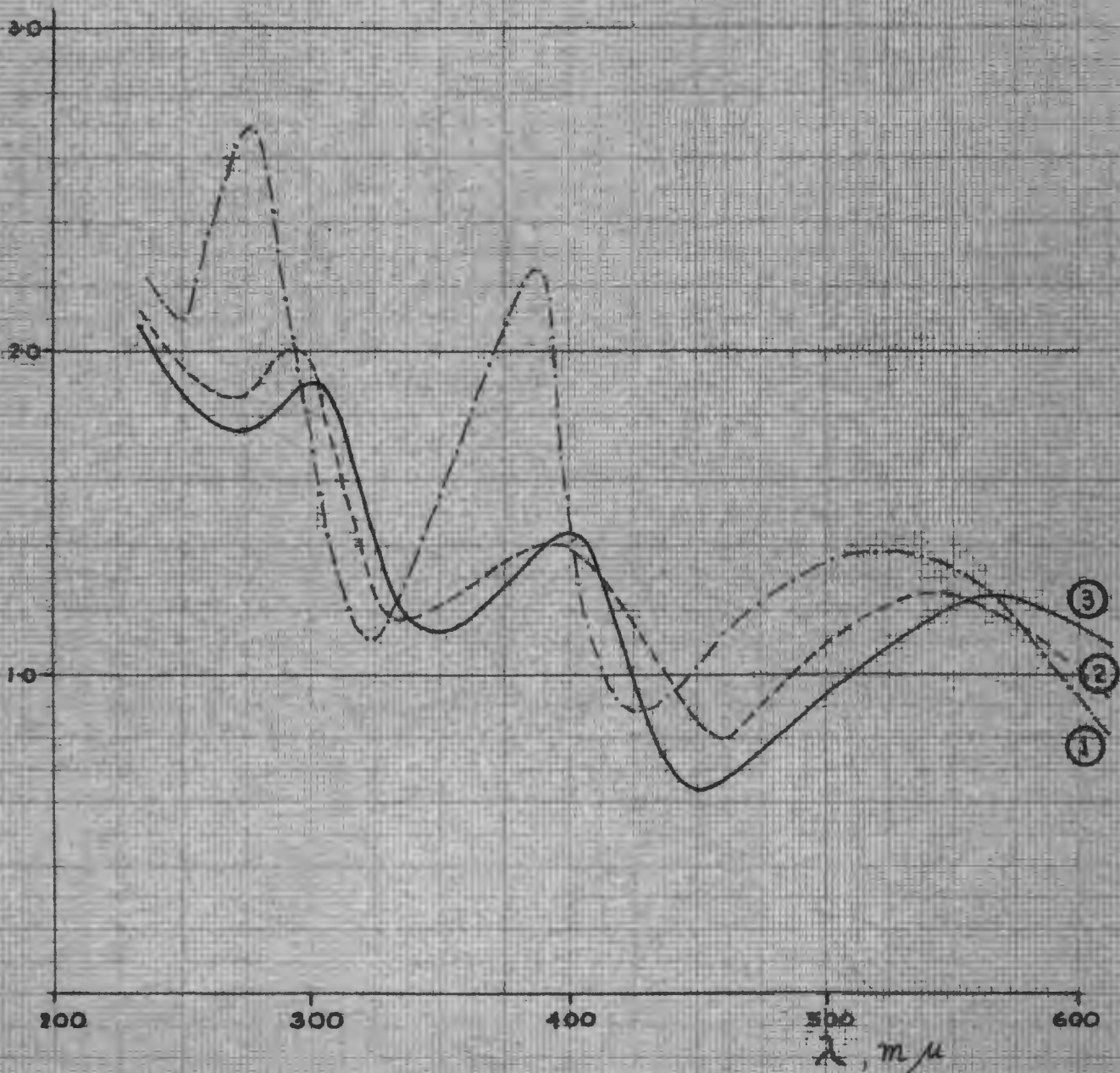


FIG 11

of pyocyanine; - the absorption maxima compare very well with those obtained by Mirvish and Holliman⁷ when they determined the absorption spectrum of pyocyanine in N/1000 HCl. The Liii-1b variant of *Pseudomonas aeruginosa* produced in the G.L.M. medium under aerobic conditions only 25 mg. of pyocyanine per litre of culture* (determined by paper chromatography).

An interesting point to note is that the Liii-1b variant also produces two main pigments as is the case with the Id strain. The absorption spectra of the various pigments suggest that they all are phenazines.

(vi) A brief study of other variants of the L-strain of *Pseudomonas aeruginosa*.

The following variants of *Pseudomonas aeruginosa*** were examined:

- Liii - 1a;
- Liii - 2 ;
- Liii - 3b;
- Liii - 3bi.

Slopes of these organisms were supplied by the Bacteriology Department of this University. Most of the experiments which were carried out with the Liii-1b variant were repeated with the above listed variants. The sub-culturing of these organisms on G.L.M. agar slopes and in the G.L.M. liquid medium resulted in each case

* ³⁷ Hellinger reported a yield of 230 mg. of pyocyanine per litre of the medium she used, and Grosser and Friedrich⁴³ 170 mg. per litre of their medium.

** Origin of the variants, pages 102 and 103.

in the formation of the original colour, there being no marked decrease in its intensity. The behaviour of the bacteria under microaerophilic conditions gave, however, unexpected results.

Four conical flasks of 500 ml. capacity each containing 100 ml. of the G.L.M. medium, were inoculated separately with the organisms of the four variants of *Pseudomonas aeruginosa*. The surface of the cultures was covered with $\frac{1}{2}$ to $\frac{3}{4}$ inch layer of liquid paraffin; all were incubated at 37°C. The following table summarizes the results:

| Variant: | Final colour of the aerobic cultures: | Final colour of the micro-aerophilic cultures: | Days required to develop colour under microaerophilic conditions: |
|----------|---------------------------------------|--|---|
| Liii-1a | Yellow | Yellow | 90 |
| Liii-2 | Pale yellow | Pale red | 55 |
| Liii-3b | Yellow | Blue green | 8 |
| Liii-3bi | Yellow-green | Red | 25 |

The culture of the Liii-2 variant was found to contain 3 mg. of pigment B, and that of the Liii-3bi variant 7.5 mg. of the same pigment, pigment A being absent in these cultures, as it is in the case of the Li strain under microaerophilic conditions.

SECTION D.Possible sequence in the red pigment formation.(1) Introduction.

Cultures of the Li strain of *Pseudomonas aeruginosa* go through various colour changes*, turning purplish red eventually, due mainly to the formation of pigments A and B. A number of yellow shades can be observed during the growth period of these organisms, ranging from a pale greenish yellow to a yellow with a distinct brownish tinge. Investigations were carried out to determine the number of yellow pigments produced, the stage at which they occur, and whether there is a definite sequence of yellow pigments leading to the formation of the red pigments.

At the beginning of this investigation a number of qualitative tests were applied to portions of cultures at various stages in order to establish whether there was a difference in the chemical behaviour of the yellow pigments formed. An obvious shortcoming of this approach was that the cultures might contain a mixture of yellow pigments, the proportion of the individual ones present

* Colour changes in the G.L.M. medium, p. 24.

affecting the tests. - The possible sequence in the formation of red pigments was later studied by paper chromatography.

(ii) Qualitative tests.

16 Boiling tubes at a time, each containing 15 ml. of the G.L.M. medium, were inoculated with the Li strain of *Pseudomonas aeruginosa*, plugged loosely with cotton wool, and the cultures were then incubated sloped at 37°C. Three cultures at random were examined after the first yellow colour had developed, and further batches of three cultures were tested whenever a change in the colour could be observed visually in the remaining samples. The first yellow shade is in this section referred to as "Yellow I", the second one as "Yellow II", etc. Tests, such as the addition of a mineral acid, a base, and a dilute solution of hydrogen peroxide to the culture, and the measurement of its pH, were carried out. This was done because earlier tests had shown that yellow cultures examined at different stages of growth behaved differently when treated with the above reagents. The ease of oxidising a yellow culture to red, for instance, was found to depend largely on its pH*. - The following table summarizes the results of the tests:

* Oxidation of a yellow culture at different pH's, p. 81.

| | Yellow I: | Yellow II: | Yellow III: |
|---|--|---|--|
| Colour of culture | Pale greenish yellow at the surface only. | Darker greenish yellow distributed throughout. | Pale olive green at the surface, remainder brownish yellow. |
| Days of incubation | $\frac{3}{4}$ - $1\frac{1}{2}$ | $1\frac{1}{2}$ - 4 | 3 - 6 |
| pH of culture | 7.2 - 7.4 | 7.3 - 7.8 | 7.7 - 8.1 |
| Acidify with HCl, then make alkaline with KOH. | Colour fades to turbid white. Pale greenish yellow. | Greenish yellow darkens Greenish yellow lightens. | Golden yellow, culture turns more turbid. Bright yellow, culture clearer again. |
| Addition of H_2O_2 to the culture at its natural pH. | | Pale yellow, turning pink when the solution is acidified. | Dark pink (pigments A and B present). |
| Addition of H_2O_2 to the culture made more alkaline with KOH | Colour fades to turbid white. | Pale pink develops rapidly. (Pigment B, but never pigment A, is present). | Bright red colour develops rapidly. (Pigments A and B are present). |
| Addition of H_2O_2 to the acidified culture. | | Colour fades to turbid white. | Pale yellow. |

(Cultures treated with H_2O_2 were left standing for 30 minutes).

The tests were carried out on unfiltered cultures, because at least one yellow pigment of a 3 - 5 days old culture is adsorbed on to the filter pad during filtration. Part of the adsorbed material can be eluted from the pad with a buffer solution of pH 4, resulting in a yellow solution which turns pink when it is exposed to the air for 24 hours; its colour in acid is yellow.

Of the three "yellows" listed in the above table, yellow II is likely to be a mixture of yellow pigments, because only part of yellow II results in the formation of pigment B when it is oxidised with H_2O_2 , as was shown by paper chromatography (p. 119). Yellow III is definitely a mixture of at least two yellow pigments because it yields on oxidation both pigments A and B (p.119). No suitable solvent has been found to extract any one part of yellow II and III from cultures. Insufficient information has been obtained from the above tests to even suggest a possible sequence in the formation of the two red pigments.

The cultures obtained from the organisms of Maytham's yellow slope* very seldom result in the formation of any red pigments, yellow I only being formed as the above tests have shown. These bacteria probably lack the ability to produce the necessary enzymes which are required for the next step in the formation of the precursors of the two red pigments.

* Maytham's yellow slope, p. 32.

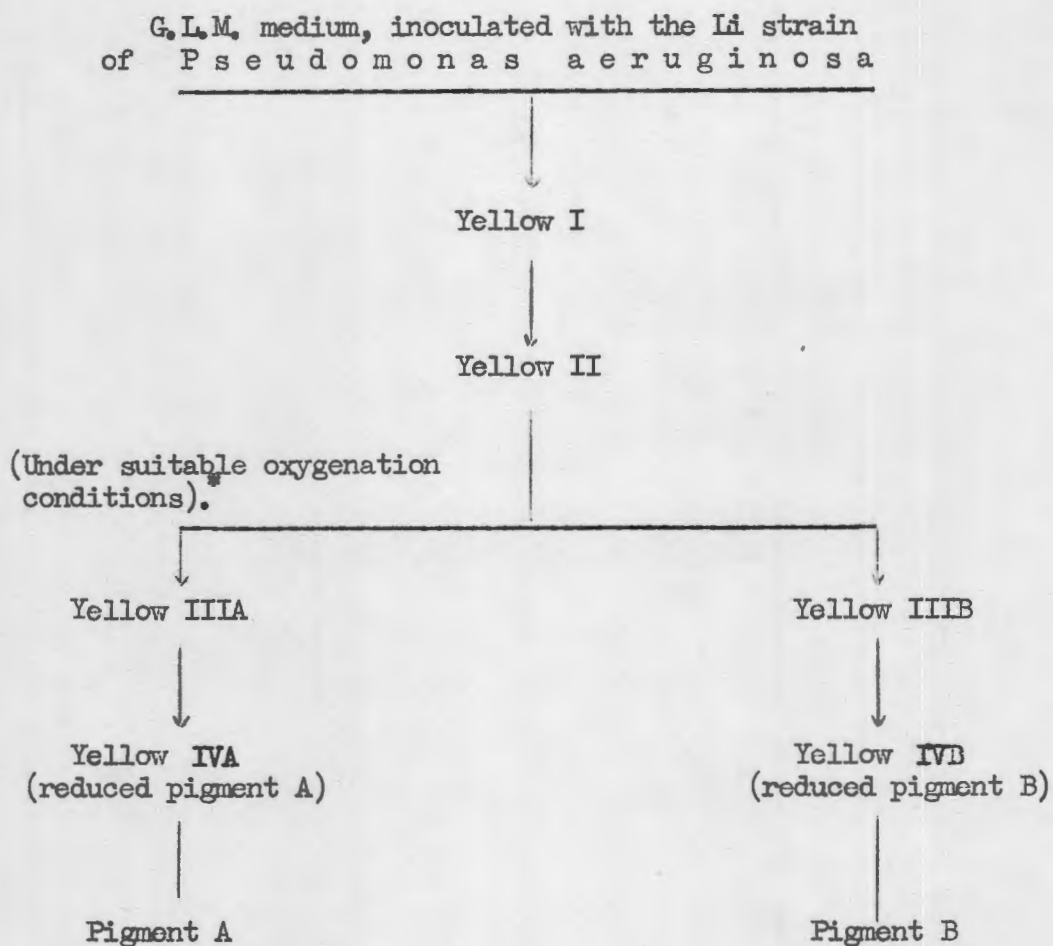
(iii) The oxidation of yellow cultures.

The oxidation with H_2O_2 of yellow cultures of the Id strain of *Pseudomonas aeruginosa* results in most cases in red pigmentation, the red colour intensity increasing as the cultures grow older. This observation was studied quantitatively as it might have thrown some light on the problem under investigation. Two 5 ml. portions of a bulk culture (200 ml. of the G.L.M. medium in a $\frac{3}{4}$ litre square gin bottle) of the organisms were withdrawn at intervals; the pH was measured and then adjusted to approximately 9, using KOH, so facilitating a rapid oxidation of the yellow precursors of the two red pigments. To one sample was added 0.5 ml. of a 3% H_2O_2 solution and to the other 0.5 ml. distilled water. The samples were allowed to stand for 2 hours, after which pigments A and B were determined quantitatively, making allowance for the dilution due to the addition of H_2O_2 and H_2O respectively. The following were the results:

| Sample removed after: | Colour of the culture: | pH of the culture: | mg. of pigment A per litre of sample treated with H ₂ O: | mg. of pigment B per litre of sample treated with H ₂ O: | mg. of pigment A per litre of sample oxidised with H ₂ O ₂ : | mg. of pigment B per litre of sample oxidised with H ₂ O ₂ : |
|------------------------------|--|--------------------|---|---|--|--|
| 1 day | Turbid white | 6.95 | - | - | - | - |
| 2 days | Pale greenish yellow | 7.3 | - | - | - | - |
| 3 " | Darker greenish yellow | 7.5 | - | trace | - | 6 |
| 5 " | Yellow with brown tinge | 7.7 | - | trace | - | 10 |
| 7 " | Surface of the culture is orange red, remainder yellow brown | 8.15 | 4 | 2 | 6 | 18 |
| 9 " | Orange red throughout | 8.35 | 8 | 9 | 7 | 26 |
| 11 " | } Purplish red | 8.75 | 8 | 12 | 7 | 35 |
| 13 " | | 8.90 | 6 | 18 | 7 | 34 |
| 15 " | | 8.95 | 7 | 38 | 6 | 37 |
| (Bulk culture after 15 days) | | 8.95 | | | | |

Pigment A: 9 mg.; pigment B: 40 mg.

The H_2O_2 -oxidation of the culture at various stages of growth indicates that the precursor of pigment B is produced continuously from the 3rd day of incubation onwards, i.e. from that time when yellow II ("darker greenish yellow") is present. The concentration of pigment B increases steadily as the culture grows older, indicating that yellow II is converted continuously into the yellow precursor of pigment B (yellow IIIB). Yellow III ("yellow brown") must contain the yellow precursor of pigment A (yellow IIIA) as well, as it gives rise to both pigments A and B when it is oxidised at a pH of 8.15 or higher. Only little of yellow IIIA is produced, being formed at a certain stage of growth only, viz. during the 7th and 9th days of incubation when a pH of 8.15 - 8.35 has been reached (compare with the summarised results on p. 78). - The following may thus be a possible sequence in the production of the two red pigments:



The later precursors of pigment A may have been formed from yellow I; there is, however, insufficient evidence for this.

(iv) A study of the sequence in red pigment formation employing paper chromatography:

A good separation of pigments A and B has been achieved by means of paper chromatography, using as solvent the upper layer of a 5:1:4 mixture of n-butanol, acetic acid and water. The yellow pigments, produced during the various stages of growth by the *Id*

* Oxygenation conditions for the formation of pigments A and B, Section B, part 2(b).

strain of *Pseudomonas aeruginosa* in the G.L.M. medium, did not separate well with the above solvent. The top layer of a 4:1 n-butanol-HCl mixture, saturated with water at room temperature, gave a reasonably good and fast separation of the yellow pigments produced by these organisms. This solvent and 3 MM. paper were used throughout all the investigations discussed in this and the following sub-section.

Up to 20 boiling tubes at a time, each containing 15 ml. of the G.L.M. medium, were inoculated with the organisms, and the cultures were incubated sloped and aerobically at 37°C. Approximately 0.2 ml. of the yellow culture, examined at various stages of growth, was absorbed in several applications on to the paper as a spot and the location of the pigments was achieved by comparing R_F values with pigments A and B which were run alongside on the same strip of paper.

The cultures, previously said to contain yellow I, resulted in one yellow spot only, having an R_F of 0.15; under ultra-violet light a white-blue spot with an R_F of 0.04 was visible. The yellow II cultures gave in addition to these spots two further yellow spots with R_F 's of 0.24 and 0.31 respectively, the latter being present in small quantities only. The yellow III cultures were found to contain large amounts of the yellow pigment having an R_F of 0.31, and only small quantities of the other pigments mentioned above; it also contained two additional yellow pigments

with R_F 's of 0.45 and 0.40 respectively. A number of other yellow pigments have been observed on occasions and these will be referred to at a later stage. - The following table summarizes the findings of the experiment:

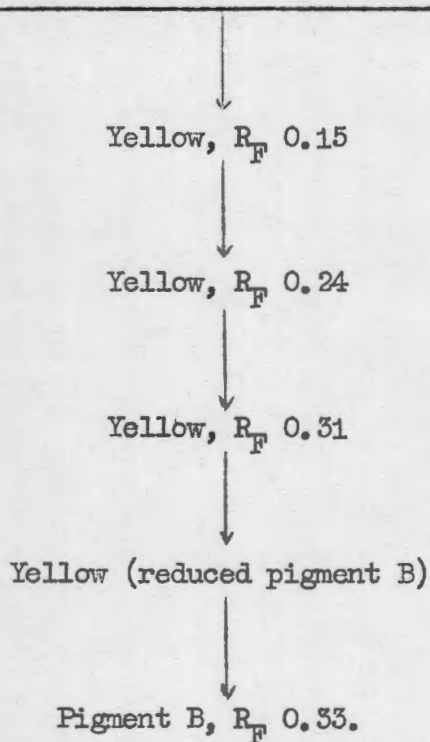
| Yellow stages of the culture: | R_F of the yellow pigments present: |
|-------------------------------|---|
| Yellow I | 0.15 |
| Yellow II | 0.15; 0.24; 0.31 (trace). |
| Yellow III | } 0.15 and 0.24 (trace); } 0.31 and 0.45 (large amounts), } and 0.40 (small amounts). |

The yellow spots were carefully cut out from the chromatogram and the pigments were separately eluted with a small volume of a buffer solution (pH 9.1); one drop of H_2O_2 was added to each solution, then allowing these to stand for 1 hour, after which they were absorbed in several applications on to 3 MM. paper as a spot. The n-butanol-HCl mixture was employed as solvent. - The yellow pigments with an R_F of 0.40 and 0.31 yielded after the H_2O_2 -oxidation pigments A and B respectively; the yellow pigments with R_F 0.15 and 0.24 turned colourless, and the one with an R_F of 0.45 gave a red pigment with an R_F of 0.46.

Microaerophilic cultures, which result in the majority of cases in the formation of pigment B only, were also examined, and it was shown that these produced yellow pigments with R_F 's of 0.15, 0.24 and 0.31 respectively, and occasionally one or two other yellow

pigments. - A possible sequence in the formation of pigment B may therefore be:

G.L.M. medium, inoculated with the *Ld* strain
of *Pseudomonas aeruginosa*

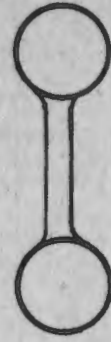
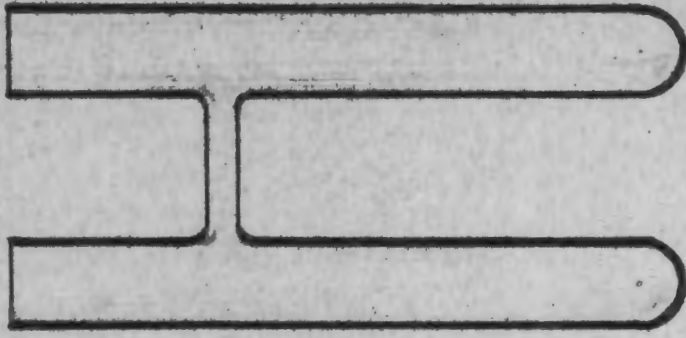


There was at this stage insufficient information on hand to suggest a possible sequence in the formation of pigment A. It appears from the results obtained thus far that the two yellow pigments with R_F 's of 0.15 and 0.24 are common precursors to both pigment A and B, but there is no proof for this as yet.

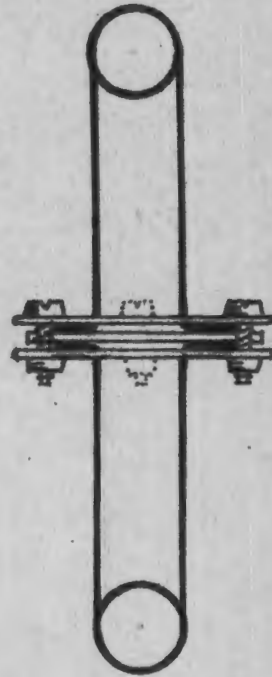
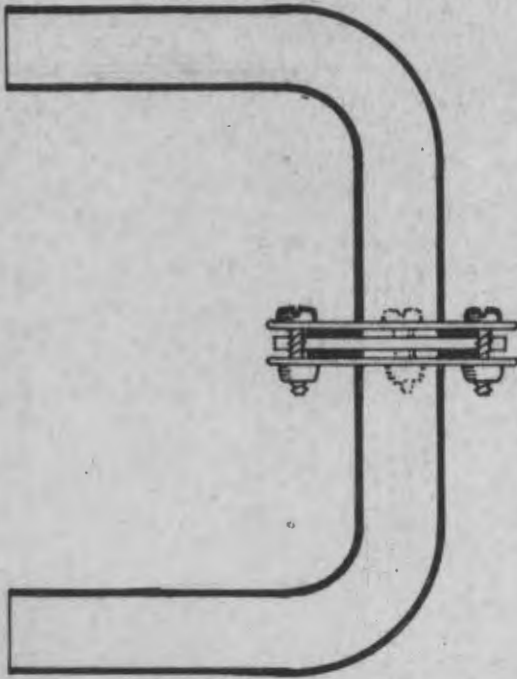
Yellow cultures, obtained from the organisms of Maytham's yellow slope*, were found to contain only two yellow pigments having an R_F of 0.15 and 0.24 respectively, the latter being no longer present in older cultures. - If one or both of these yellow pigments are common precursors of pigments A and B, the organisms (the Li strain, producing normally red pigments) should then be able to convert Maytham's yellow to pigments A and B, provided that the correct oxygenation conditions are applied. An experiment was carried out to investigate this possibility:- 200 ml. of the G.L.M. medium were inoculated with 1 ml. of a starter of the Li strain of *Pseudomonas aeruginosa* and the mixture was shaken thoroughly to ensure an even distribution of the organisms throughout the whole medium. 13 ml. portions of this culture were aseptically transferred to each limb of 5 pairs of inter-connected sterilized boiling tubes (Fig. 12, A). - Pairs of inter-connected tubes were used in this experiment so that the oxygenation conditions** in each limb of a pair of cultures might be the same. - To one limb of the first pair of tubes was added 2 ml. of distilled water and to the other 2 ml. of the filtrate of Maytham's yellow culture, known to contain the two yellow pigments with R_F 's of 0.15 and 0.24 respectively. The openings of all pairs of tubes were plugged loosely with cotton wool, and the cultures were incubated sloped at 37°C. After 24 hours of growth 2 ml. of distilled water were

* Maytham's yellow slope, p. 32.

** Effect of oxygenation conditions on red pigment formation, Section B, part 2(b).



·A·

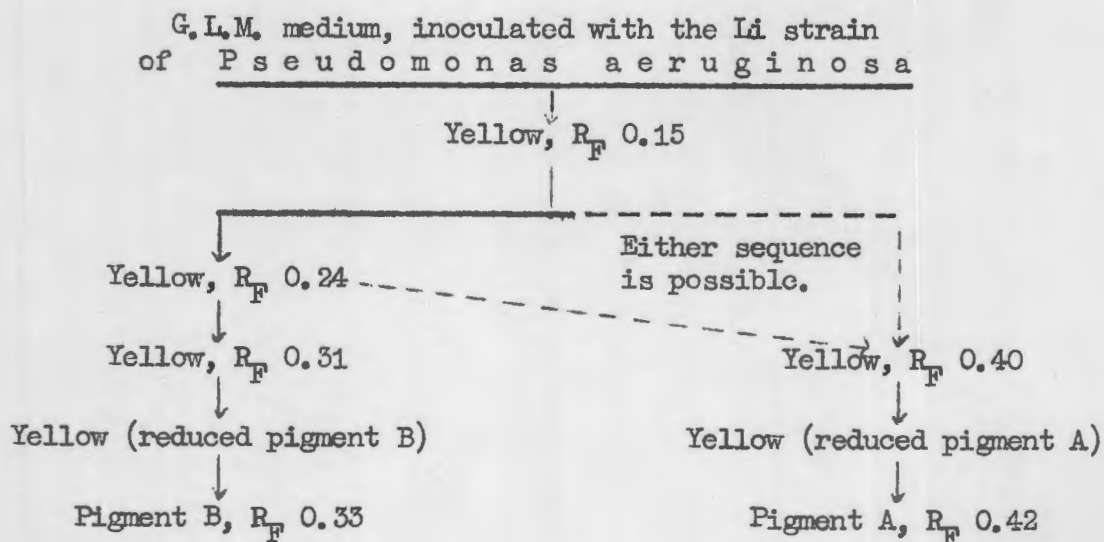


·B·

added to the culture in one limb of the second pair of inter-connected tubes, adding to the other 2 ml. of the same filtrate of Maytham's yellow culture which has been used previously and which had been stored in the refrigerator. All pairs of tubes were then shaken so that the contents of each was disturbed to about the same extent. This was repeated 2, 3, and 5 days after inoculation, Maytham's yellow having thus been added to the actively growing cultures at various stages. All cultures were incubated for 13 days after which no further colour changes could be observed, and pigments A and B were then determined on each. The results of the experiment are summarized in the table given below:

| H ₂ O or Maytham's yellow added to the culture after: | Colour of the culture at the time when H ₂ O or Maytham's yellow was added: | mg. of pigment A per litre of culture to which H ₂ O was added: | mg. of pigment B per litre of culture to which H ₂ O was added: | mg. of pigment A per litre of culture to which Maytham's yellow was added: | mg. of pigment B per litre of culture to which Maytham's yellow was added: |
|--|--|--|--|--|--|
| Inoculation | - | 1.5 | 13.5 | 2 | 13.5 |
| 1 day | Pale yellow throughout | 1.5 | 13 | 4 | 13 |
| 2 days | Yellow with brown tinge | 1 | 13 | 4 | 14 |
| 3 days | Surface of the culture is orange red, remainder yellow brown. | 1.5 | 13.5 | 5.5 | 15 |
| 5 days | Orange to purplish red throughout | 1 | 13 | trace | 17.5 |

The results show that red pigmentation in general is poor. Previous experiments have already shown that red pigment production in disturbed cultures is variable and never as good as in static ones (see page 43). The figures obtained indicate that pigment A is formed from Maytham's yellow when this is added during the early stages of growth, viz. 1 - 3 days after inoculation, whereas pigment B is produced when it is added later, the increase in its concentration being not as marked as that of pigment A. The results of the experiment indicate that both pigments A and B can be formed from Maytham's yellow, containing the two yellow pigments with R_F 's of 0.15 and 0.24 respectively, and since no suitable method is available for the separation of these two yellow pigments in bulk, it is impossible to determine whether the yellow pigment (R_F , 0.15) alone, or both yellow pigments are common precursors of the two red pigments. There are therefore two possible sequences in the formation of pigment A as is indicated by the following scheme:



A further experiment was carried out to investigate whether Maytham's yellow could be converted to pigments A and B with the aid of the red pigment producing organisms of the Li strain of *Pseudomonas aeruginosa*. The apparatus shown in Fig. 12, B was employed for this purpose, keeping cultures in the two limbs separated in the one case by means of a glass plate and in the other by the use of a cellophane membrane, which might allow the diffusion of some of the products of the organisms to take place from one limb of the apparatus to the other. - 50 ml. of the G.L.M. medium were inoculated with 0.5 ml. of a starter of the organisms from Maytham's yellow slope and the mixture was shaken thoroughly to ensure an even distribution of the bacteria throughout the whole medium. 20 ml. portions of this culture were aseptically transferred to one limb of each of the two pieces of apparatus, then placing into the other limbs 20 ml. of a culture of the Li strain of *Pseudomonas aeruginosa*, prepared by mixing thoroughly 0.5 ml. of a starter of these organisms with 50 ml. of the G.L.M. medium. The four openings were plugged loosely with cotton wool, and the cultures were incubated standing upright at 37°C. - If Maytham's yellow pigments can diffuse through the cellophane membrane then the bacteria of the Li strain should, according to the results of the previous experiment, convert them to the red pigments, resulting in an increased concentration of either pigment A or pigment B, or both.

No further colour changes could be observed in any of the cultures after 24 days of incubation. Maytham's culture in the limb ending with the glass partition was only pale orange red, and that in the adjoining one dark purplish red. The cultures separated by the cellophane membrane were about equally dark red, Maytham's culture having an orange tinge due to the presence of a yellow pigment with an R_F of 0.15, and the other a purplish tinge, yellow pigments being absent. It was shown by means of paper chromatography that the yellow pigment with an R_F of 0.15 was not able to diffuse through the cellophane membrane, - at least not in appreciable quantities, - whereas pigment B could. - The cultures of the two limbs of each apparatus were combined, water was added to make up for the loss in volume due to evaporation, and the concentration of pigments A and B was determined. Pigment A was absent in both cases, and the concentration of pigment B was found to be 17 mg. per litre in the combined cultures which were originally separated by a glass plate, and 16 mg. per litre in the other culture. The yellow band, which was present in both chromatograms, was cut out and the yellow pigment was eluted in each case with 15 ml. of distilled water, and the clarified solutions were compared photometrically. Both combined cultures were found to contain about the same amount of the yellow pigment.

The results show that this experiment has failed to give further evidence for the possible conversion of Maytham's yellow to pigments A and B. - The yellow pigment with an R_F of 0.24 was absent in all

cultures; it has been observed previously (p.121) that older cultures of Maytham's strain did not contain this yellow pigment either.

(v) Yellow pigments present in cultures only occasionally.

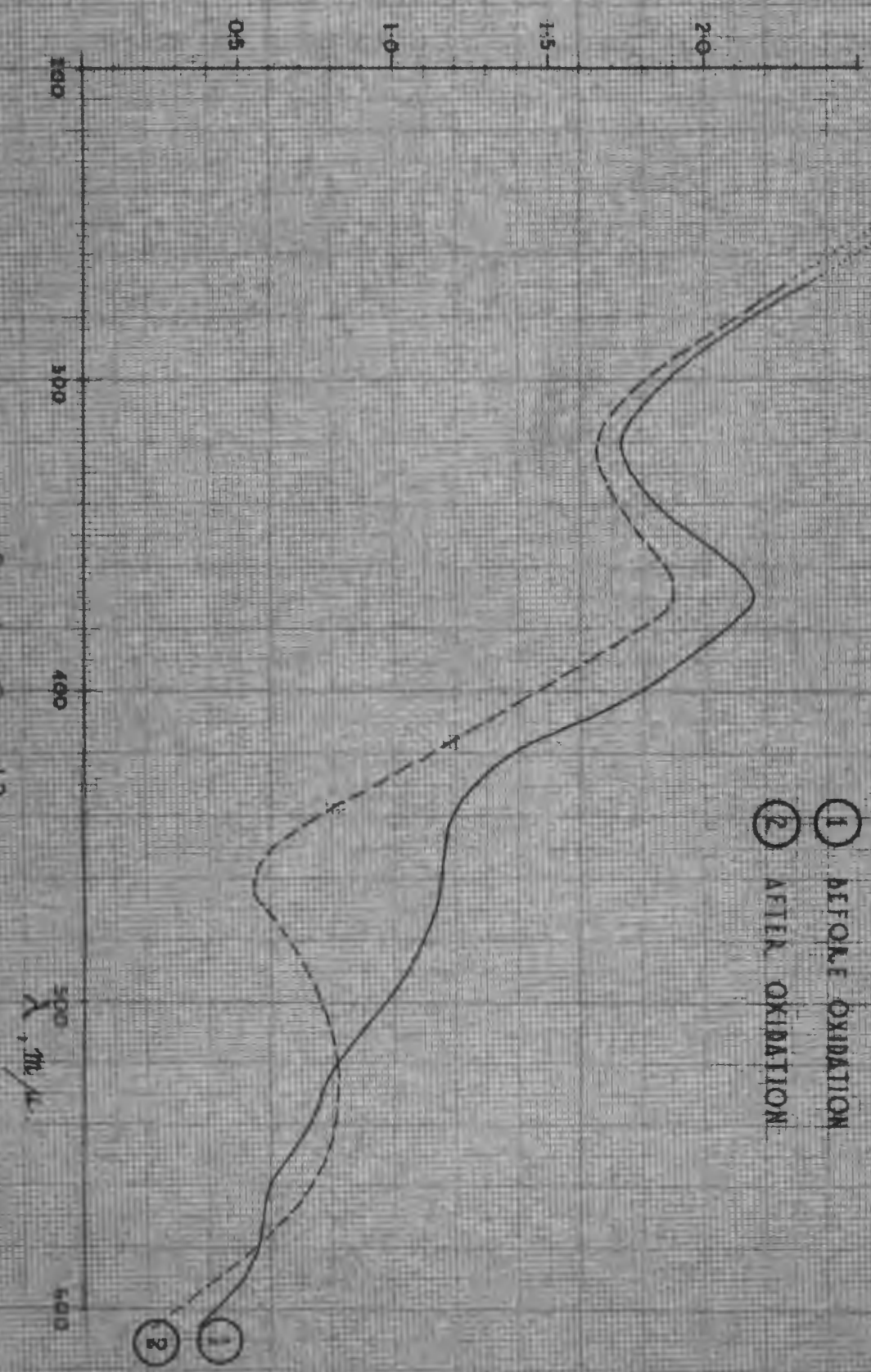
The yellow pigments with R_F 's of 0.15, 0.24, 0.31, 0.40 and 0.45 respectively are always present, at least at some stage, in the cultures of the Li strain of *Pseudomonas aeruginosa*, provided both pigments A and B are formed eventually. The possible role of the yellow pigment, having an R_F of 0.45, in the formation of the two red pigments is not understood. It may precede the yellow pigment (R_F 0.31) in the formation of pigment B, since it occurs together with the latter in large amounts at the same stage of growth*; it is absent, however, in microaerophilic cultures which yield in most cases pigment B only.

Yellow pigments, having R_F 's of 0.35, 0.49, and 0.85 respectively, are occasionally found in yellow cultures of the Li strain, the latter occurring, when present, in large quantities. It was possible to elute small amounts of this yellow pigment (R_F .85) from a chromatogram with a buffer solution of pH 9.15 and to determine its absorption spectrum before and after H_2O_2 -oxidation at the same pH (fig. 13). The oxidation resulted in the formation of a red water soluble pigment, which changed its colour under acid conditions to yellow, being then no longer soluble in water but in n-butanol.

* Yellow pigments present in cultures, page 119.

YELLOW PIGMENT (K_f .85)
IN BUFFER SOLUTION OF PH 9.15.

- ① BEFORE OXIDATION
- ② AFTER OXIDATION



F I C 13

(vi) Conclusion.

A definite sequence in the formation of pigments A and B could not be established. The experimental data indicate that the two red pigments have at least one precursor, a yellow pigment, in common.

SECTION E.Attempted interconversion of pigments "A" and "B".

Oxygenation conditions* play an important part in red pigment formation by the *Ld* strain of *Pseudomonas aeruginosa*, optimum red pigmentation taking place in shallow static aerobic cultures. The relative amounts of pigments A and B produced have been shown to depend largely on oxygenation conditions, the formation of pigment A being favoured by an increased air supply or by the inclusion of a chemical source of oxygen into the medium, the yield of pigment B being hardly affected under these conditions. Microaerophilic cultures, on the other hand, produce in most cases pigment B only. The possibility that pigments A and B are interconvertible when added separately to a culture of the *Ld* strain grown under oxygenation conditions which favour their individual formation was investigated. - Interconnected boiling tubes (fig. 12, A) were used in some of the experiments in order to standardise as far as possible the oxygenation conditions of two cultures under test.

150 ml. of the G.L.M. medium were inoculated with 1.5 ml. of a starter of the *Ld* strain of *Pseudomonas aeruginosa* and then shaken to ensure an even distribution of the organisms throughout the medium. 12 ml. portions of this culture were aseptically

* Oxygenation conditions, Section B, part 2.

transferred to each limb of four pairs of interconnected boiling tubes, then adding to one limb of the first two pairs 3 ml. of an aqueous solution of pigment A (4.0 mg./100 ml.), to one limb of the remaining two pairs 3 ml. of an aqueous solution of pigment B (3.7 mg./100 ml.), and, as a control, 3 ml. of autoclaved distilled water to the other limb of each pair of the connected tubes. The open ends of 2 pairs of tubes were plugged loosely with cotton wool, and the remainder tightly. - The oxygenation conditions of the first 2 sets of cultures favour the formation of pigment A, but those of the remaining ones do not. - All the cultures were incubated sloped at 37°C. They turned yellow after 24 hours of growth, the red pigments, which were added to half of the cultures, being reduced. - It was shown in a separate experiment that cultures, containing reduced pigment A and B, turned red within 2 minutes of passing a stream of air through them. - The concentration of pigments A and B was determined in each case after 12 days, when no further colour changes in the cultures could be observed. The table given below summarizes the findings:

| Culture with an initial concentration of: (Pigments A and B expressed in mg./litre culture) | Cotton wool plug fitted: | Pigment A in mg. per litre of culture: | Pigment B in mg. per litre of culture: |
|--|--------------------------|--|--|
| 8 mg. pigment A. | } Loose | 10.5 | 30 |
| Control to which H ₂ O was added | | 16 | 27 |
| 8 mg. pigment A | } Tight | 2 | 28 |
| Control to which H ₂ O was added. | | 4 | 31 |
| 7.4 mg. pigment B | } Loose | 12 | 34 |
| Control to which H ₂ O was added. | | 14 | 29 |
| 7.4 mg. pigment B | } Tight | 2 | 35 |
| Control to which H ₂ O was added. | | 2 | 27 |

These results indicate that pigment B is neither used by the bacteria nor is it converted to pigment A. - Cultures, to which pigment A had been added, contain even less of this pigment than the control cultures, the concentration of pigment B being hardly affected. It can be concluded that the organisms can use up pigment A, but why its concentration in the control should be higher than in the culture, to which it had been added, is not clear.

An experiment, in which pigment A was added to an actively growing culture at various times, was carried out to determine at what stage of growth pigment A is used by the bacteria, and whether the concentration of pigment B is increased in any of these cultures. - Seven pairs of interconnected boiling tubes were used in this experiment,

transferring into each limb 12 ml. from a bulk culture, freshly prepared by shaking 200 ml. of the G.L.M. medium with 2 ml. of a starter of the Li strain of *Pseudomonas aeruginosa*. To the culture in the one limb of the first pair of tubes was added 3 ml. of an aqueous solution of pigment A (4.0 mg./100 ml.) and to the other, as a control, 3 ml. of autoclaved distilled water. The addition of pigment A and water to one pair of cultures at a time was repeated daily up to the sixth day of incubation, shaking all the cultures at the time of addition to about the same extent. - All tubes were plugged loosely with cotton wool, and the cultures were incubated sloped at 37°C. The concentration of pigments A and B was determined after no further colour changes could be observed in any of the cultures. The results are tabulated below, referring to the cultures to which water had been added as "control", and to the others as "sample":

| Addition of pigment A after: | Colour of the culture at the time of the addition: | Pigment A in mg. per litre culture: | | Pigment B in mg. per litre culture: | |
|------------------------------|--|-------------------------------------|---------|-------------------------------------|---------|
| | | Control: | Sample: | Control: | Sample: |
| Inoculation | - | 9.5 | 8 | 28 | 24 |
| 1 day | Bright yellow | 10 | 2.5 | 27 | 24 |
| 2 days | Greenish yellow | 14 | 15 | 29 | 30 |
| 3 days | Brownish yellow | 12 | 16 | 29 | 32 |
| 4 days | Orange red | 15 | 18 | 26 | 26 |
| 5 days | Orange to purplish red | 17.5 | 15 | 24 | 24 |
| 6 days | Purplish red | 15 | 17 | 26 | 25 |

(Some of the organisms of the 6 day old culture were still viable as was shown by inoculation tests).

The amount of pigment A present in the "sample" cultures, due only to the addition of this pigment at various times, is equivalent to 8 mg./litre. The results of this experiment show thus that the bacteria are using pigment A during all stages of growth, especially so during the earlier ones, there being no increase in the concentration of pigment B because of this. There is thus no evidence that pigment A can be converted into pigment B.

Microaerophilic cultures of the Li strain in the G.L.M. medium result in the majority of cases in the formation of pigment B only, the oxygenation conditions being unsuitable for the production of pigment A. An experiment was carried out to investigate whether the organisms can interconvert the two red pigments under these conditions. - 12 ml. portions of a freshly prepared bulk culture of the Li strain of *Pseudomonas aeruginosa* in the G.L.M. medium were transferred into three 100 ml. conical flasks, adding to one 3 ml. of an aqueous solution of pigment A (4.0 mg./100 ml.), to another 3 ml. of an aqueous solution of pigment B (3.7 mg./100 ml.), and to the remaining one flask 3 ml. of autoclaved distilled water. The surface of all cultures was covered with a layer of medicinal liquid paraffin, and the flasks were closed with cotton wool. All cultures were incubated at 37°C, and the concentration of pigments A and B was determined at the end of the experiment. The following

results were obtained:

| Culture with an initial concentration of: (Pigments A and B expressed in mg./litre | Pigment A in mg. per litre culture: | Pigment B in mg. per litre culture: |
|---|-------------------------------------|-------------------------------------|
| 8.0 mg. of pigment A | } absent | 11 |
| 7.4 mg. of pigment B | | 16 |
| Control to which H ₂ O was added | | 10 |

These results confirm some of the findings of the previous experiments, namely, that the bacteria cannot use pigment B, but that they can break down pigment A without converting it into pigment B.

The organisms of the Ld strain of *Pseudomonas aeruginosa* grow well in the G.L.M. medium from which glycine has been omitted, but these cultures do not result in red pigmentation*. This medium was thus chosen to investigate further the possible interconversion of pigments A and B. - 12 ml. portions of the G.L.M. medium lacking glycine were transferred aseptically into 15 boiling tubes, adding to five 3 ml. of an aqueous solution of pigment A (4.0 mg./100 ml.), to another five 3 ml. of an aqueous

* Omission of glycine from the G.L.M. medium, page 98.

solution of pigment B (3.7 mg./100 ml.), and to the remaining five tubes 3 ml. of distilled water. All media were inoculated with the Li strain of the organisms, plugged loosely with cotton wool, and incubated sloped at 37°C.

None of the cultures turned red, not even after 12 days of incubation. The ten cultures, to which either pigment A or pigment B had been added, were after this time pale yellow in colour, the remainder being off white. - The 5 cultures of each set were combined, their pH was measured, and found to be only 7.3. KOH was added to raise their pH to 9*, and 1 ml. of a 3% H₂O₂ solution was added to each of the 3 cultures, allowing them to stand for 2 hours. The cultures, to which pigment A and B respectively had been added originally, turned red, the other remaining off white. The concentration of pigments A and B was determined on each set of combined cultures, the results being:

| Culture with an initial concentration of: (Pigments A and B expressed in mg./litre) | Pigment A in mg. per litre culture: | Pigment B in mg. per litre culture: |
|--|--|--|
| 8.0 mg. of pigment A | 2 | 3.5 |
| 7.4 mg. of pigment B | absent | 4.5 |
| Control to which H ₂ O was added | absent | absent |

The results of this experiment indicate quite strongly that

* The effect of pH on the ease of oxidation of a yellow culture, page 81.

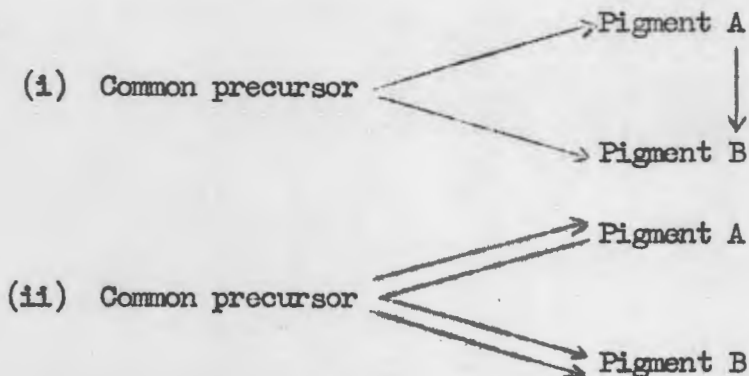
pigment A can be converted into pigment B by the bacteria when the G.L.M. medium is lacking glycine, whereas the reverse appears impossible.

GENERAL DISCUSSION.

Some of the evidence given in section D indicates that pigments A and B have a common precursor. The experiment in which Maytham's yellow, containing two yellow pigments, was added to an actively growing culture at different times has shown quite clearly that it may be converted into pigment A in maximum quantities when added at a stage when the surface of the culture is developing an orange colour, - a stage when pigment A is formed naturally. Pigment B may be formed from Maytham's yellow when this is added to the culture at a later stage of growth (p. 121 - 123). A common precursor is thus clearly indicated.

The quantitative study of red pigment formation at various pH's (p. 77) has shown that pigment A is present in the culture in appreciable quantities before pigment B is formed. With an increase in the pH of the culture the concentration of pigment A decreased first sharply, then slightly. Figure 3, giving the plotted results of this experiment, shows that the flattening of the pigment A curve coincides with a marked increase in the concentration of pigment B, the concentration of pigment A dropping sharply thereafter, indicating the possible conversion of pigment A into pigment B. The results of this experiment also point to the possibility of a common precursor being formed by cultures of the *Id* strain of *Pseudomonas aeruginosa*, which is converted into either pigment A or B, depending on definite conditions. The flattening of the pigment A curve indicates that the conditions for its production from the common precursor are no longer suitable, but are favourable for the formation of pigment B, thus giving rise to a

marked increase in its concentration. The following are possible ways in which the above conversion might take place:



The common precursor appears to be formed up to a stage when the culture has reached a pH of approximately 8, there being no red pigments present at this time (summarised results, p. 78). Between a pH of 8.0 and 8.5 this precursor is converted mainly into pigment A, only small amounts of pigment B being formed. The rate of the conversion of this common precursor into pigment A is in this pH range (8.0 - 8.5) greater than the rate of its conversion into pigment B. The formation of pigment A at higher pH's appears not possible, whilst the production of pigment B occurs best under these conditions.

The conversion of a common precursor into either of the two red pigments thus appears to be dependent on pH. The following might substantiate this suggestion:

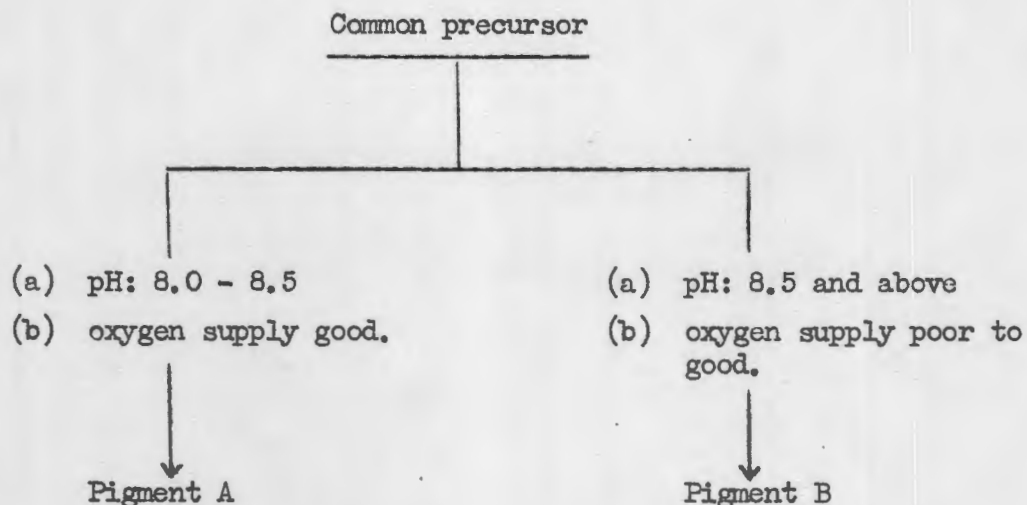
- (a) All the experiments given in section B, part 3, show that pigment A is produced between a pH range of 8.0 - 8.5.
- (b) A culture which has reached a natural pH of 7.5 - 7.7, and which is then raised artificially to a pH of 8.5 - 9.0

(i.e. beyond the pH range suitable for the formation of pigment A) and subsequently oxidised, results in the production of pigment B only (p. 115).

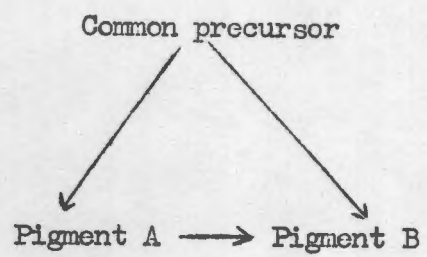
- (c) A culture which has reached a natural pH of 8.35 (i.e. a suitable pH for the formation of pigment A), and which is then raised artificially to a pH of 9 and subsequently oxidised, yields no more of pigment A than the control culture which developed a natural pH of 9 (p. 115).

The importance of pH is thus indicated. It must be pointed out, however, that the common precursor can be produced at pH's greater than 8 as is clearly indicated in the experiment listed on page 76. Here media with initial pH's of 8.0 and 8.1 respectively were inoculated with the organisms of the Id strain of *Pseudomonas aeruginosa*; the cultures resulted in the formation of the usual amount of pigment A, the concentration of pigment B being much less than in cultures starting with a pH of 7.0. These results show that the common precursor is produced almost continuously.

Oxygenation conditions appear also to affect the conversion of a common precursor to the two red pigments, aerated cultures, or cultures supplied with a chemical source of oxygen, resulting in an increased concentration of pigment A, whereas microaerophilic cultures (oxygen supply very poor) result in the majority of cases in the formation of pigment B only. This is further substantiated by the results obtained from the experiments listed in section B, part 2b. - The following scheme is therefore suggested as a summary of the conditions under which pigments A and B appear to be formed from a common precursor:



Although the majority of experiments on the possible conversion of pigment A into pigment B show that the organisms can utilise pigment A but gave no indication of its conversion to pigment B, in the absence of glycine from the G.L.M. medium evidence for the conversion of A to B was obtained (p. 133 - 135). The pH of the cultures to which glycine was not added never exceeded 7.3. This indicates that pigment A is converted into pigment B via a "direct" route and not via the common precursor route, since the cultures, of which the pH was raised artificially to 9 and then subsequently oxidised, resulted in the formation of both pigments A and B. The later precursor of pigment A must thus have been present. If the common precursor had been present it should have yielded at pH 9 pigment B only. The scheme below thus appears applicable:



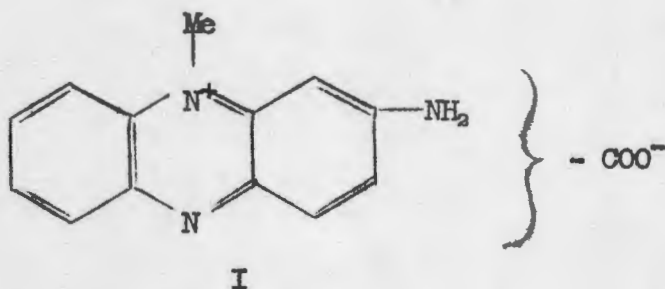
A P P E N D I X.Structural investigations on pigments A and B.

The following information has been supplied by Professor F.G. Holliman²⁴ in a private communication:

(a) Pigment A.

The analyses on the crystalline pigment A indicate a formula $C_{14}H_{11}O_2N_3, 2H_2O$. The absorption spectrum of 2-aminophenazine, in acid solution, is almost identical with that of the pigment. Thermal decomposition of pigment A gives a small amount of sublimate, which, by paper chromatography, has been shown to consist of two products. The behaviour of these substances suggested that they were 2-aminophenazine and the anhydronium base of its methyl quaternary salt. The colour of 2-aminophenazine is red in acid and yellowish in alkali, whereas that of pigment A remains red under similar conditions, which suggests the presence of a quaternary salt group in pigment A.

2-aminophenazine methiodide was found to travel at the same rate, on paper chromatography, as one of the decomposition products of pigment A. Pigment A, however, shows little movement on paper electrophoresis at pH 7, suggesting that it cannot carry a net positive charge as would be required for a methosalt of 2-aminophenazine. The infrared spectrum of the pigment shows the presence of strong bands in regions where the carboxy anion of betaines shows absorption. From this information and from the formula deduced from analyses it seemed likely that the pigment would be a 2-amino-?-carboxy-9-methyl-phenazinium betaine (I).

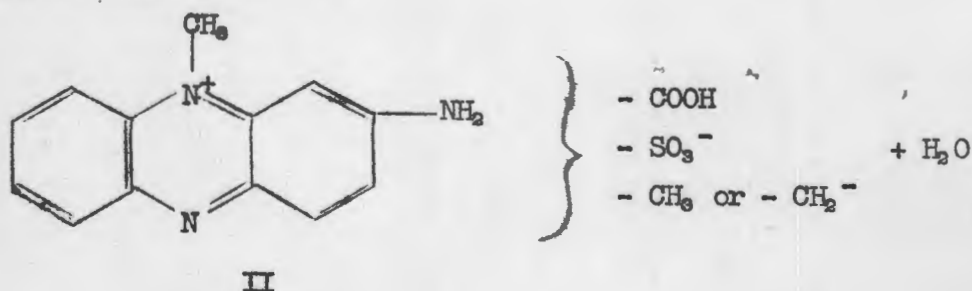


Further investigations are in progress but these are handicapped by the small quantities of pigment available.

(b) Pigment B.

Pigment B, although present in the original culture of the *Li* strain of *Pseudomonas aeruginosa* in greater concentration than pigment A is considerably more unstable, and only small quantities are eventually isolated. Crystallisation is also more difficult than in the case of Pigment A.

The analyses on pigment B indicate an empirical formula of $C_{15}H_{15}O_3N_3S$. The absorption spectrum of the pigment shows that it is very closely related to pigment A as far as the chromophoric-group is concerned. Electrophoretic studies suggest the presence of a strongly ionised acidic group; this is further borne out by the fact that aqueous solutions of the pigment have a low pH. On the basis of the structure of pigment A suggested above, the acidic nature of B, the presence of sulphur and the analytical results, the following formula (II) is suggested:



Pigment B yields no volatile products on thermal degradation. -
Further studies on the structure of the pigment are in progress.

(c) General.

A preliminary publication on these pigments is at present being prepared by Professor Holliman²⁴ and will shortly be submitted. The full details of the isolation of both pigments A and B will be prepared as the first paper on this investigation and will shortly be published.

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