THE RICE INSTITUTE

STUDIES ON PIGMENTATION AND METABOLISM IN *SERRATIA MARCESCENS*

by

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A THESIS

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Abbreviations Used in This Thesis

RNA = Ribonucleic acid

DNA = Deoxyribonucleic acid

TCA = Trichloroacetic acid

Tris = Trishydroxy (aminoethane) (buffer)

Versene = Ethylenediamine tetraacetic acid

ACKNOWLEDGMENTS

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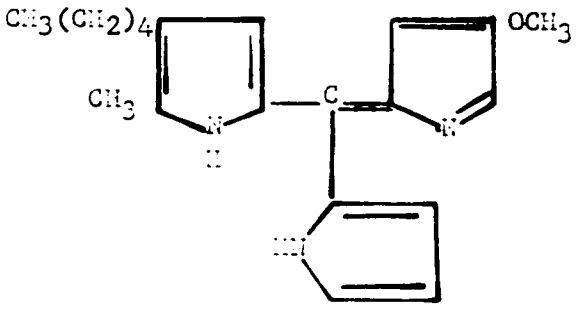
II. INTRODUCTION

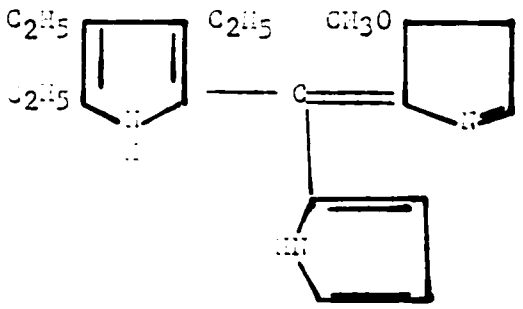
The nutritional requirements for pigment production in Serratia marcescens have been investigated by a number of workers. Hefferan (1903 a,b) listed various media employed to promote pigment production in a variety of bacteria, including Serratia. Kuntz (1900) found that magnesium, sulfate, ammonia and a carbon source were necessary for pigment production. Samkow (1903) verified this requirement of magnesium, not only for pigmentation, but also for optimum growth. Bortels (1927) made an extensive study of the various metals needed for pigment production in microorganisms and found that iron and magnesium were important for growth and pigment production by Serratia. The requirement for iron was verified by Waring and Werkman (1943). Bunting (1942), Bunting et al (1949), and Labrum and Bunting (1953) studied the effects of various amino acids and buffer systems with particular reference to their ability to induce pigment production in Serratia marcescens. They found that media supplemented with peptone or other amino acids induced pigmentation while high levels of phosphate inhibited pigmentation. The influence of amino acids was verified by Weinberg (1951); Linnane and Still (1953) found that unsaturated fatty acids stimulated pigmentation. Antibiotics (Weinberg, 1951; Weil, 1952) and other compounds upon pigment production (Poe and Hawkins, 1949, 1952; Bequet, 1948, 1952) were found to inhibit pigment production in this bacterium.

Chemical analyses of the pigment were carried out by Wrede and his co-workers (1929 through 1934) upon an extracted pigment obtained by the digestion of the cells in sodium hydroxide. These workers found

that the pigment had the empirical formula of $C_{20}H_{25}ON_3$ and proposed that its structure was that of a tripyrrylmethene as follows:



Raudnitz (1933), working concurrently with Wrede and his co-workers, agreed with the tripyrrole configuration of the pigment but felt that the distribution of the side groups should be located differently as follows:



Studies on the isolation and characterization of the pigments of Serratia marcescens have been made by Ehrismann and Noethling (1936) and they published spectral curves of the isolated pigment. Weiss (1949) extracted the pigment with butyl alcohol and chromatographed the resultant extracts on celite columns. He published several spectral curves of the isolated pigment fractions, but, due to incomplete separation of the pigment components from other contaminants, his curves did not show the characteristic sharp maxima.

A study of the biosynthesis of the pigment was made by Hubbard and Rimington (1950) using N^{15} and C^{14} labeled glycine and C^{14}-acetate.

These workers demonstrated that in the newly formed prodigiosin the distribution of glycine and acetate was similar to the distribution of isotopes in porphyrins formed by duck erythrocytes as shown by Wittenberg and Shemin (1950) and Shemin et al (1955). Hubbard and Rimington (1950) compared the spectra of the extracted pigment with a synthetic tripyrrole methane, and they concluded that the structural formula as given by Wrede and Rothhaas (1934) was correct. They also assumed that the isolated pigment represented a single component.

Earlier work on the pigment by Samkow (1903) suggested that it contained metals. Bortels (1927) attributed this binding to contamination of the pigment during the extraction process. The nature of the metal-pigment combination has not been investigated since that time and remains to be elucidated.

The study of bacterial metabolism has been especially useful in establishing biosynthetic pathways for amino acids (McElroy and Glass, 1955), nucleic acids, and the utilization of a large variety of compounds (Fisher, 1952; Roberts et al, 1955) such as the various sugars and other natural metabolites. Bacteria are especially useful in studies in pigment biosynthesis, since they possess few of the side reactions which occur in higher organisms. Ultraviolet mutants lacking some part of a sequential series of enzymatic reactions, such as white, orange, and other mutants produced in Serratia (Rizki, 1954) are useful tools in the investigation of the biosynthetic sequences and at present are being actively investigated as a study of the biosynthesis of prodigiosin. It is noteworthy that mutants produced by gamma irradiation of Serratia have not been used in such studies.

As one means of studying the metabolic patterns in microorganisms, particularly as they pertain to biochemical mutants, the utilization and distribution of radioactive phosphorus (P^{32}) (Roberts and Roberts, 1950; Cowie and Walton, in press), sulfur (S^{35}) (Lampen, et al, 1947; Roberts et al, 1955), and various carbon (C^{14}) labeled compounds (Roberts et al, 1955) have been studied in E. coli. Abelson, et al (1952) studied the role of radioactive carbon dioxide ($C^{14}O_2$) in protein synthesis in E. coli. McLean et al (1947 through 1955) studied the effects of various amino acids, organic acids, nucleic acid derivitives and other compounds on the incorporation of radioactive carbon dioxide ($C^{14}O_2$) into Serratia marcescens, and they found that the addition of most of these compounds to the medium resulted in a decreased incorporation of $C^{14}O_2$.

From the metabolic point of view, the pigment of this organism is of special interest since it is the only known naturally occurring tripyrrole compound. This pigment becomes especially significant in view of the hypothesis of Turner (1940) and Shemin et al (1955) that a tripyrrole is intermediate in the biosynthesis of porphyrins which are the precursors for hemoglobin and a number of the porphyrin enzymes. Corwin (1936) demonstrated that tripyrrole methanes can be, and in some cases are, intermediates in the formation of dipyrroles. This may be the mechanism whereby tetrapyrroles are formed. The work of Shemin et al (1955) demonstrated that a succinate-glycine cycle is important in this synthesis. The mechanism may be abbreviated in this manner:

succinate + glycine --→ amino levulinic acid --→ monopyrrole

prodigiosin ←-- tripyrrole ←→ porphyrin

THE PROBLEM

Two aspects of the Serratia marcescens organism have been studied and are reported in this thesis; (1) deals with the pigment, prodigiosin, and (2) is concerned with the metabolism of this bacterium.

I. The nature of the constituents of prodigiosin and the changes in pigmentation in various mutants produced by gamma ray and ultraviolet radiation were investigated.

II. The overall metabolism of inorganic-P^{32} and $C^{14}O_2$ by the wild-type and mutant organisms were investigated. The nature of the products formed from these radioactive substrates were identified and their relative rate of formation, in respect to the growth curve of the organisms, was studied.

III. METHODS

I. Mutant Production and Pigment Analysis.

A. Growth and Isolation of Mutants.

The organism used in these studies was Serratia marcescens, also known as Bacillus prodigiosus (Bergey's Manual, 1948), and Chromobacterium prodigiosus (Topley and Wilson, 1955). The organism is a small gram negative saprophytic cocco-bacillus measuring 0.7 to 1.0μ by 0.5μ. These organisms produce a deep red pigment on certain media at temperatures below 35°C. This pigment is called prodigiosin (Wrede and Hettche, 1929), after the species designation 'prodigiosus'.

For the growth and isolation of normal and mutant colonies, the following media were employed. The medium, designated minimal (SM) is the medium of Labrum and Bunting, (1949) and was made up as follows:

Glycerol	10.0 grams
Ammonium citrate	5.0 "
Magnesium sulfate	0.3 "
Sodium chloride	0.3 "
Ferric ammonium citrate	0.05 "
Potassium phosphate (dibasic)	10.0 "
Water	1000 ml.
Adjusted to pH 7.0 - 7.4	

The medium designated as complete (SCM) has the same composition as the above medium with the exception that 1.0 grams of yeast extract and 2.0 grams of casein hydrolysate were added, per liter. Other media used are designated as SYM and SHM, and they have the same composition as the SMM medium except that either yeast or casein hydrolysate, respectively, were added. To obtain a solid medium, 2 per cent agar was added. The terms minimal and complete refer to the carbon and

nitrogen content of the medium. A minimal medium contains a limited carbon and nitrogen source sufficient to permit the organisms to grow, while a complete medium contains a wide variety of both carbon and nitrogen. Other media used during the course of this work will be described in the sections dealing with the metabolism of the organism.

Viable counts of the organisms were obtained by growing the organisms in one of the above mentioned media for a given period of time, after which serial dilutions of the broth culture were made in sterile saline, and 0.5 ml. aliquots were plated on petri dishes with minimal agar. The plates were made by spreading the organisms evenly over the surface of the agar with a bent glass rod which had been sterilized before use. It was found that the addition of 0.066 per cent methyl red (Knaysi, 1951) to the medium aided in decreasing the motility of the organisms, thereby facilitating counting of the organisms. However, when color mutants were grown, this concentration of methyl red obscured the results and could not be used. Total counts of the organisms were made in a Petroff-Hausser bacterial counting chamber after the addition of a dilute solution of crystal violet to a saline suspension of the organisms.

To prepare mutants from the wild-type strain, a 10^{-5} dilution was made from a twenty-four hour culture. Five milliliters of this suspension were transferred to a small petri dish and were exposed to irradiation by being placed 12 inches from an ultraviolet light source for periods of 15, 30, or 45 seconds. The suspensions were then plated on agar plates and incubated for 24 to 48 hours at room temperature. All of these procedures were carried out in a darkened room to prevent

photo-reactivation of the organisms (Gates, 1931).

A 24 hour culture also was exposed to gamma radiation from a radio-sodium (Na^{22}) source by placing a test tube containing a 10 ml. suspension against a vial containing 1.5 millicuries of Na^{22}. These were placed in a lead pig for a period of 14 hours. At the end of this exposure, the organisms were diluted a thousand fold with saline and plated on both minimal and complete agar media. From both the ultra-violet treated and the gamma irradiated cells, mutants were isolated by subculturing poorly growing and differently colored colonies. The mutants were classified by type-reactions in the various media as a test for acid and gas production from various sugars, by the production of acetyl methyl carbinol (Voges Proskauer), by gram staining ability, and by morphological description (Topley and Wilson, 1955). Auxano-graphs were made on some of the organisms to determine whether pigment biosynthesis could be affected by various additives. This was accom-plished by seeding an agar plate with the organisms to be tested, mark-ing off the plate into sectors, and adding various supplements in minute amounts to separate sectors. The effect on growth and pigmentation was noted. The supplements used were amino acids, casein hydrolysate, yeast and liver extracts, nucleic acid derivitives and vitamins. The minimal medium was also supplemented with various amino acids, vitamins, nucleic acid intermediates and cofactors, singly and in various combin-ations, and the influence of these additives was observed on the forma-tion of new colonies and on their pigmentation.

B. Pigment Extraction and Analyses.

Pigment from the organisms was extracted by different methods to

determine the one giving the best results. The first method used was basically that of Wrede and Hettche (1929), using NaOH to digest the organisms followed by petroleum ether extraction of the pigment. The extraction method of Hubbard and Rimington (1950) was also tested but it was discontinued because the acids used in this procedure altered the mobility of the pigment during later chromatographic analysis. The method of Weiss (1949), using n-butyl alcohol, was also abandoned as it extracted many interfering substances and the resulting pigment contained impurities.

The following method was developed for routine extractions of the pigment. The cells were washed from the surface of the agar with minimal quantities of distilled water and the resulting suspension was then mixed with about 10 volumes of acetone and shaken overnight in a mechanical shaker. The solution was then centrifuged and the cell debris was re-extracted with small quantities of acetone. The acetone extracts were pooled and an equal volume of petroleum ether was added. The pigment was transferred to the petroleum ether phase and the acetone removed by the addition of several volumes of distilled water. At the interface of the aqueous acetone and the petroleum ether the blue pigment precipitated out. This pigment was removed and extracted with chloroform. The petroleum ether and chloroform solutions were then concentrated <u>in vacuo</u> at 30-40° C. The concentrated pigment was then dissolved in chloroform and extracted three times with 3 per cent aqueous ethylenediamine tetraacetate at a pH of 7.6 to 8.0 to remove any di- and trivalent metals. A dry pigment was obtained from chloroform solution by evaporation of the solvent.

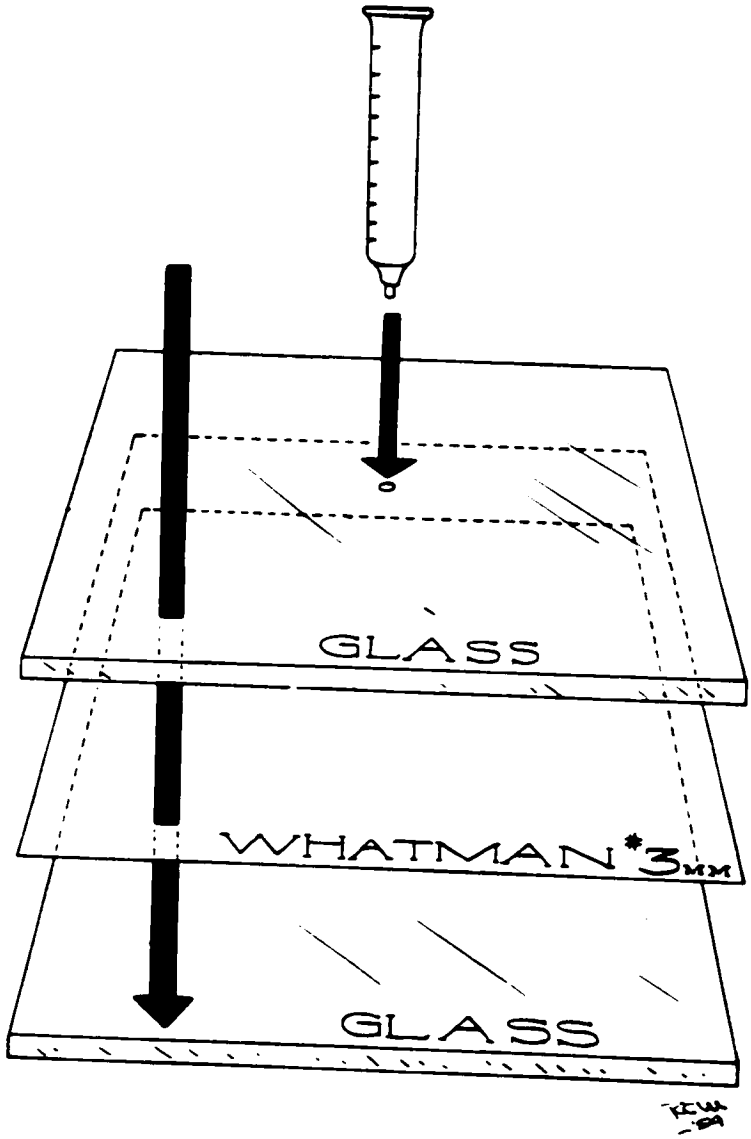
Both ascending and descending methods of paper chromatography were tested to separate the pigment into its various components. However, since the pigment decomposed on the paper due to prolonged exposure to air and difficulty was experienced in obtaining reproducible results, the method of Rappoport et al (1955) (Figure 1) was used for pigment chromatography. In this method (Figure 1) two 1/4 inch glass plates (13 x 13 inches) were used, one of which has a 1/4 inch hole drilled at its center. The material to be chromatographed was placed in a ring about the center of a piece of Whatmann #3 MM paper (13 x 13 inches), permitted to dry, and placed between the two glass plates. A 10 ml. syringe barrel, containing a small amount of powdered paper to retard solvent flow, was fitted tightly into the hole of the top plate. About 200 pounds of lead brick were placed on the plates and the solvent was added through the syringe. This method permitted use of volatile solvents and there was no exposure of the material on the paper to the atmosphere. The chromatograms developed quickly since the solvent moved about 6 to 7 inches in 20 minutes.

Various solvent systems were tested for the separation of the pigment components. Initially, they were separated by using a solvent mixture consisting of 30 parts of heptane, 20 parts of n-butanol, and 2 parts of dichloroethane. To improve the separation of the pigment components, this solution was diluted with two volumes of petroleum ether (b.p. 30-60°C).

Various mixtures of ethyl ether and petroleum ether were also tested but it was found necessary to treat the paper with ammonia fumes after the pigment was spotted on the paper in order to effect proper

Figure 1. Glass plate chromatographic apparatus.

(After Rappoport et al, 1955)


GLASS
WHATMAN #3 MM
GLASS

separation of the various components.

For large scale separation of pigments, columns packed with Whatmann ashless powdered paper were used. The pigment was dissolved in a small amount of chloroform and mixed with a small amount of the powdered paper. The chloroform was then evaporated and the dry powder was transferred to the top of the column. Separation was accomplished by the application of 20 to 30 ml. of 25 per cent ethanol to remove fatty contaminants, followed by 85 per cent ethanol which eluted the main portion of the red pigment, and finally, the remaining red was separated from the blue component with 95 per cent ethanol. The column was then extruded and the blue pigment which remained at the top of the column was removed from the powdered paper with chloroform.

The pigment components eluted from paper chromatograms were weighed and used to obtain standard curves relating optical density to concentration of pigment. A time-growth study was then initiated to determine whether the pigments components varied with the age of the organisms. The organisms were cultured for varying periods up to 34 days. The pigment was extracted, chromatographed, and the optical density for the whole pigment, and the different fractions was determined at 535 mμ. A wet weight of the organisms was determined at each time-study and dry weight determined by drying the organisms to a constant weight at 60°C. A viable count was also made on the culture during this growth study.

The fractions obtained both by paper and column chromatography were analysed in the ultraviolet, visible, and infrared spectra. Since the pigment showed changes in color with changes in different hydrogen ion concentrations, spectral curves were obtained in basic, acidic, and

neutral alcohol solutions. Infrared curves of the pigments were obtained through the curtesy of Dr. Daniel Edwards of the Veterans Hospital (Houston). A solution containing 3 per cent pigment in anhydrous chloroform was prepared and the infrared spectrum was determined on a double beam Perkin-Elmer recording spectrophotometer.

For analytical purposes, the pigment was further purified by preparing its hydrochloride salt. The pigment was dissolved in a small amount of anhydrous dry petroleum ether and dry hydrochloric acid gas was passed through this solution by means of a capillary tube, until all of the pigment precipitated as the hydrochloride salt. This precipitate was recovered by centrifugation and further purified by washing several times with fresh petroleum ether. Initially, to dissolve the blue pigment, it was necessary to add a few drops of chloroform to the petroleum ether. Portions of the hydrochloride salts of the pigments were sent to Huffman Analytical Laboratories (Wheatridge, Colorado) for carbon, hydrogen, nitrogen, chloride, and molecular weight determinations.

Chloride determinations were made locally on the hydrochloride salts by a modification of the procedure of Schales and Schales (1941). The pigments were first digested in 30 per cent hydrogen peroxide and the liberated chloride ion determined.

Nitrogens were determined in this laboratory by the method of Ma and Zuazaga (1942). This is a standard micro-Kjeldahl method using selinium as a digestion catalyst. Pyridoxine hydrochloride was used to check the accuracy of both the chloride and nitrogen techniques. Molecular weight determinations were made by means of the Rast's

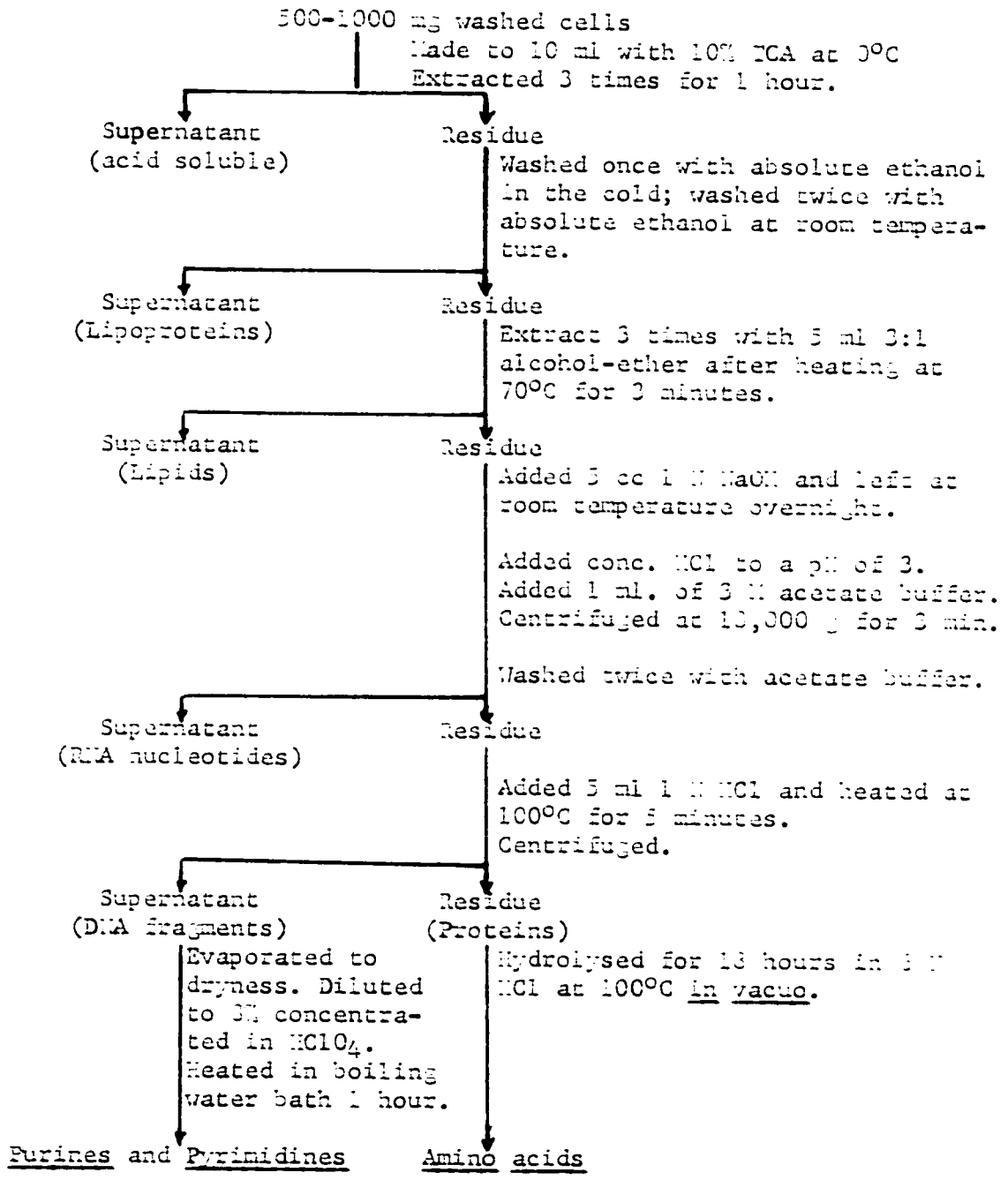
cryoscopic method as outlined in the text book by Shriner (1950). The determinations were accomplished by the use of camphor, alpha-brom camphor, and bromoform. These methods were checked using pure benzoic acid.

Due to the presence of an ash from the pigments not treated with ethylene diamine tetraacetic acid, a study was made using calcium (Ca^{45}) and iron (Fe^{59}) isotopes to determine whether or not these metals were an integral part of the pigment molecule. The wild-type and mutant organisms were grown on a thin layer of minimal broth in the presence of the calcium and iron isotopes. The organisms were cultured for a period of 3 days, after which time they were harvested by centrifugation and washed several times with distilled water. The pigment was then extracted and analysed by the procedures previously described and the radio-calcium determined by counting with a d-h tube. Similarly, the $iron^{59}$ containing pigment was isolated and counted in a deep-well scintillation counter. The pigments were then chromatographed and the individual components were eluted and counted, to determine the distribution of the isotope. Radioautographs of the chromatograms were made.

II. Metabolism

A. Distribution of P^{32} and Nucleic Acid Composition.

The organisms were grown at room temperature (29°C) in a "minimal" broth in which the phosphorus concentration was reduced to 1 gram per liter. Phosphorus 32 (1.2×10^6 cpm) was added to 70 cc of this medium. At the end of 24 hours, the cells were fractionated by the procedure on the following page (Schneider, 1945; Schmidt and Tannhauser, 1945 as modified by Katchman and Fetty, 1955).



The radioactive phosphorus was determined in the above fractions by means of a G-M tube. The ultraviolet determination of the RNA nucleotides and DNA concentrations was made by the method of Tsuboi (1950). The solution was read in a Beckman DU spectrophotometer at 260 mμ. The extinction coefficient for a 1 per cent solution of RNA is 310 and that

of DNA is 320. The amount of material in the sample was determined by the following equation:

$$\frac{\text{O.D.}}{E_{1\%}} = \%\ \text{nucleic acid}$$

where O.D. is the optical density at 260 mμ of the unknown, and $E_{1\%}$ is the extinction coefficient of a 1 per cent solution.

The RNA nucleotides were chromatographed directly, while the DNA was hydrolysed to free the bases before chromatography (Wyatt, 1951).

It was found that 20 to 30 ml. applied in two applications along a line at the origin of the paper chromatograph, gave the best resolution of the free purine and pyrimidine bases. Descending chromatograms were developed on #3 MM Whatman paper. For chromatography of the nucleotides, ribonucleic acid (RNA), and the free bases from the deoxyribonucleic acid hydrolysate, a solution was employed which was composed of a 65 per cent aqueous isopropanol, adjusted to a final concentration of 2N with respect to HCl (Wyatt, 1951).

Following chromatography, the papers were dried and the spots located by means of a short wave (260 mμ) ultraviolet light. The spots were marked and subsequently eluted overnight with 0.1 N HCl. The eluates were analysed by means of the Beckman DU spectrophotometer at the wave length of maximum absorption of the particular compound. Readings were also taken at 250, 260, 280, and 290 mμ to test the purity of the eluted compound. Protein contamination would have been detected by a high absorbancy at these wave lengths (Visher, 1948).

The molar ratios of the compounds were determined and compared to those reported by Elson and Chargaff (1955).

D. $C^{14}O_2$ Metabolism

The method of fractionation described above was also used in studies concerned with $C^{14}O_2$ incorporation by Serratia marcescens. The media used in these studies were as follows:

a) Glycine Medium

Glycine	5.0 gms
NH_4Cl	2.0 "
"Tris" buffer	10.0 "
$MgSO_4$	0.5 "
K_2HPO_4	0.5 "
Ferric ammonium citrate	0.05 "

Distilled water to make 1000 ml adjusted to pH 7.4

b) Acetate Medium

Same as above with 5 grams of acetate replacing the glycine.

c) Minimal Glycerol Medium

Glycerol	3.0 gms
Ammonium chloride	1.0 "
"Tris" buffer	5.0 "
$MgSO_4$	0.5 "
K_2HPO_4	0.05 "
$FeSO_4(NH_4)_2SO_4$	0.07 "

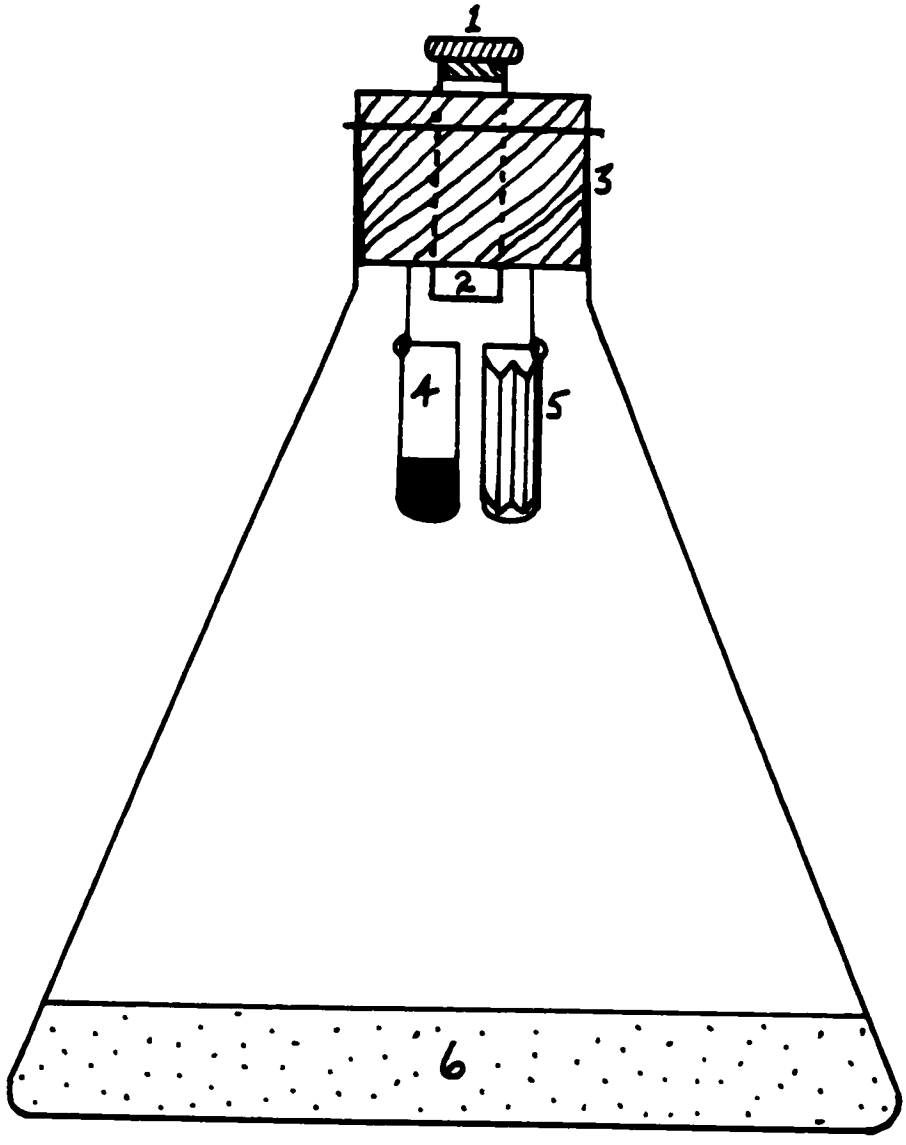
Distilled water to make 1000 ml adjusted to pH 7.4

d) A complete glycerol medium was prepared the same as the glycerol "minimal" with the addition of 0.1 gram of casein hydrolysate and 0.2 gram of yeast extract.

The organisms were grown at room temperature (29°C) in duplicate in 1000 ml. erlenmeyer flasks fitted with rubber stoppers, containing 100 ml. of medium (Figure 2). The $C^{14}O_2$ was liberated in the flasks by suspending a vial containing radioactive $BaC^{14}O_3$ which was decomposed by introducing lactic acid into the vial. This arrangement is shown in Figure 2. A second vial, containing a piece of folded filter paper, was suspended from the rubber stopper for the collection of the excess CO_2 at the end of the experiment. After growing the organisms for 24

Figure 2. Apparatus for the incubation of Serratia marcescens in presence of radioactive carbon dioxide.

A 1000 ml. Erlenmeyer flask was fitted with (1) a serum bottle stopper, which capped (2) a glass tube projecting through (3) a rubber stopper. Two vials were suspended in the flask, one (4) containing barium carbonate into which lactic acid is injected to liberate the $^{14}CO_2$, and one (5) containing a piece of folded filter paper into which is injected 1 ml. 2 N potassium hydroxide at the termination of the incubation period to collect the unreacted CO_2. The medium is labeled "M".


1
3
2
4
5
6

hours, the flasks were cooled and 0.5 cc of 2N KOH was injected into the vial containing the folded paper to collect the residual carbon dioxide. Sufficient 60% TCA was added to the medium to give a final concentration of 10 per cent. The flasks were stored in an ice box overnight to permit complete diffusion of the CO_2 into the collecting vial. The organisms were harvested by centrifugation, washed with distilled water and fractionated as outlined above. The acid soluble components from the supernatant of the organisms grown in the minimal medium was further fractionated by a 48 hour extraction with ether to recover the organic acids. The ether extract was then concentrated and chromatographed for organic acid by the method of Roberts, Cowie, et al (1955) and Moses (1955). Radioautograms were made from these chromatograms.

Radioactivity of the various fractions was determined by counting aliquots of the material under an ultra-thin window, gas flow G-M tube (Nuclear). The CO_2 collected in the alkali trap was precipitated with barium acetate and plancheted on aluminum discs for counting.

A time study was initiated to ascertain the distribution of the $C^{14}O_2$ in the various components during growth. The points chosen were 1 hour (lag phase), 4 hours (log phase), 8 hours (end of log phase) and 24 hours (growth plateau). The organisms were cultivated in minimal glycerol medium in a culture flask similar to the one previously described (Figure 2). Oxygen was supplied, by means of a hypodermic needle inserted into the rubber stopper, under slight positive pressure. The organisms were inoculated into 300 ml. of the medium in 1000 ml. Erlenmeyer flasks which contained 0.13 mg. of $NaHC^{14}O_3$ ($1x10^7$ cpm).

The flasks were then placed on a shaker for the allotted period of time at room temperature (29°C), after which the organisms were harvested and fractionated by the methods outlined above. In addition, the protein fraction in these studies was hydrolysed, and the amino acids were chromatographed on paper by the use of the solvent mixtures n-butanol: acetic acid: water, 4:1:5 (Thompson, 1951) in the first dimension and methanol: n-butanol: diethylamine: water, 10:10:2:5 (Hardy et al, 1955) in the second dimension. The quantity and the specific activity of each amino acid was determined. The lipoproteins and peptides obtained in the alcohol soluble fractions were also hydrolysed and chromatographed on paper. Autoradiograms were made from each of the above chromatograms.

The amino acids concentrations were determined according to the method of Rafatalin (1948). The imino acids (proline and histidine) were analysed by the method of Troll and Keith (1953).

IV. RESULTS

I. MUTANT FORMATION AND IDENTIFICATION OF STRAINS.

Several mutants were obtained from irradiation of the wild-type organisms. Many of these were unstable and reverted to the wild-type strain. Stable mutants which did not revert spontaneously to the wild-type even after repeated subculturings are listed in Table I.

It was found that growing one of the orange mutants next to one of the white mutants caused the edge of the white colony adjacent to the orange to turn red. The degree of induced pigmentation in the white colony was dependent on the proximity of the two colonies and the length of time they were permitted to grow together. This phenomenon has been designated as "syntrophic pigmentation", (Lederberg, 1946) which suggests that one deficient organism is supplied an essential nutrient by another strain growing near by. Subsequent subculturing of the red organisms, obtained by induction from the orange mutants as described above, resulted in growth of the original white colonies. This phenomena is illustrated in Figure 3. This reaction is strain specific. Although several orange varients would induce pigmentation in the white varients, when both were produced from the parent Nima strain of Serratia marcescens, these same orange varients would not react with white varients produced from three other strains of S. marcescens, S, Ny, or Nim. The parent red Nima strain does not have the ability to induce pigmentation in white varients. A similar phenomenon has since been reported by Rizki (1954). All attempts to isolate the factor(s) responsible for the induction of the red pigment in the white

Table I

Isolated Stable Mutants of Serratia marcescens

Mutant	Irradiation	Characteristics
1. orange	Na^{22} gamma	grows well on both minimal and complete medium.
2. orange	U.V. 30 seconds 12"	
3. white	U.V.	yellow hue on yeast medium, pink edge on casein hydrolysate medium.
4. white	U.V.	white on all media. Fed by orange strains.
5. yellow	U.V.	grows well on all media.
6. stripe	U.V.	gives white, red and splotched colonies on complete medium.
7. rose	U.V.	rough colony-grows poorly on minimal medium.
8. brown	U.V.	grows poorly on SIM Dark brown colonies on complete medium.

Table 2

Results from Biochemical Tests on Serratia Strains

Test	(Gamma)Orange	White	Wild-type red
Gram stain	gram negative	gram negative	gram negative
Morphology	0.87μ Short Rod w/chains	same	same
Motility	+	+	+
Glucose	acid-gas	acid gas	acid gas
Lactose	-	-	-
Sucrose	-	-	-
Mannitol	acid	acid	acid
Maltose	acid	acid	acid
Methyl Red	+	+	+
Voges Proskauer	+	+	+
Indole	-	-	-
Nitrate	+	+	+
Starch	±	±	±
Urease	+	+	+
Gelatin	+ no pellicle	+ no pellicle	+ no pellicle
Citrate	+	+	+
Kliegler's	acid-No H_2S	acid-No H_2S	acid-No H_2S
Litmus milk	acid peptidization	acid peptidization	acid peptidization.

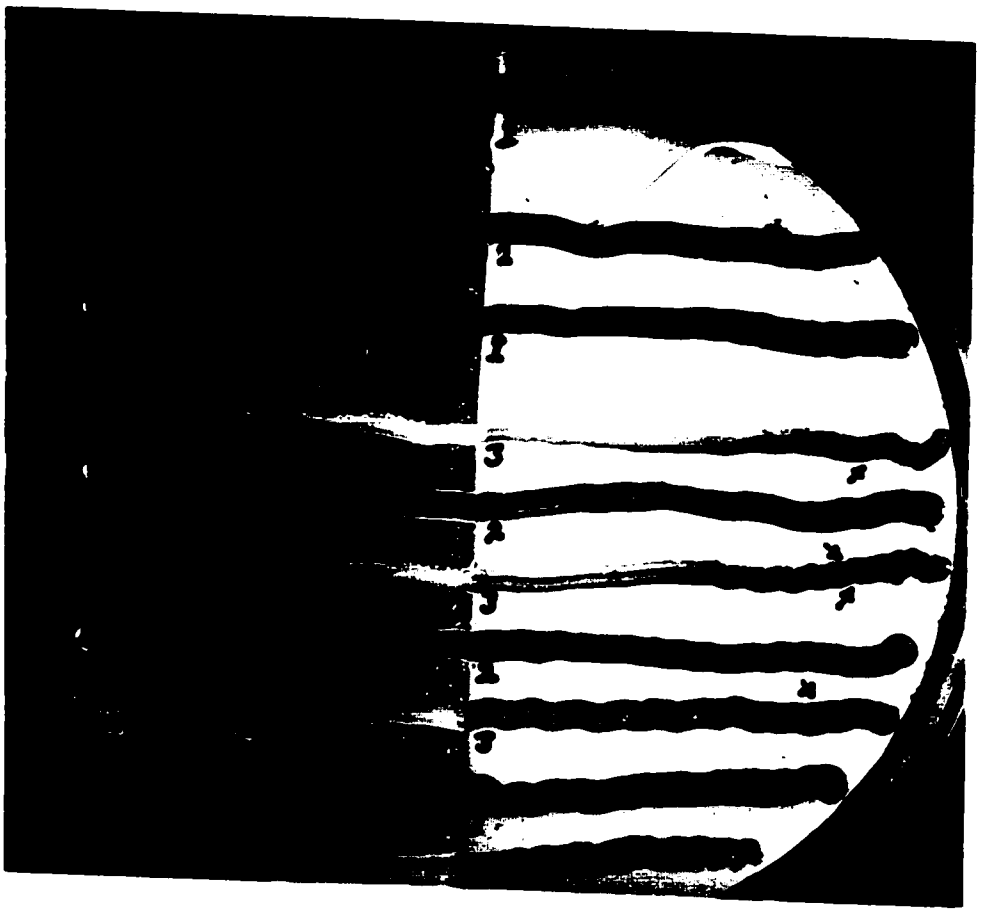
Figure 3. Symbiophic pigmentation

1. Red colonies (Wild-type)

2. Orange colonies (Mutant)

3. White colonies (Mutant)

--→ Indication of induced pigmentation (Red) in White (3) colonies growing near orange (2) colonies.



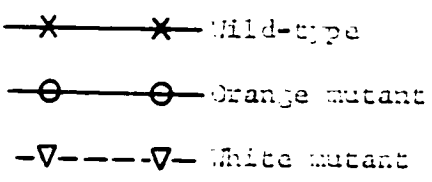
mutant were unsuccessful.

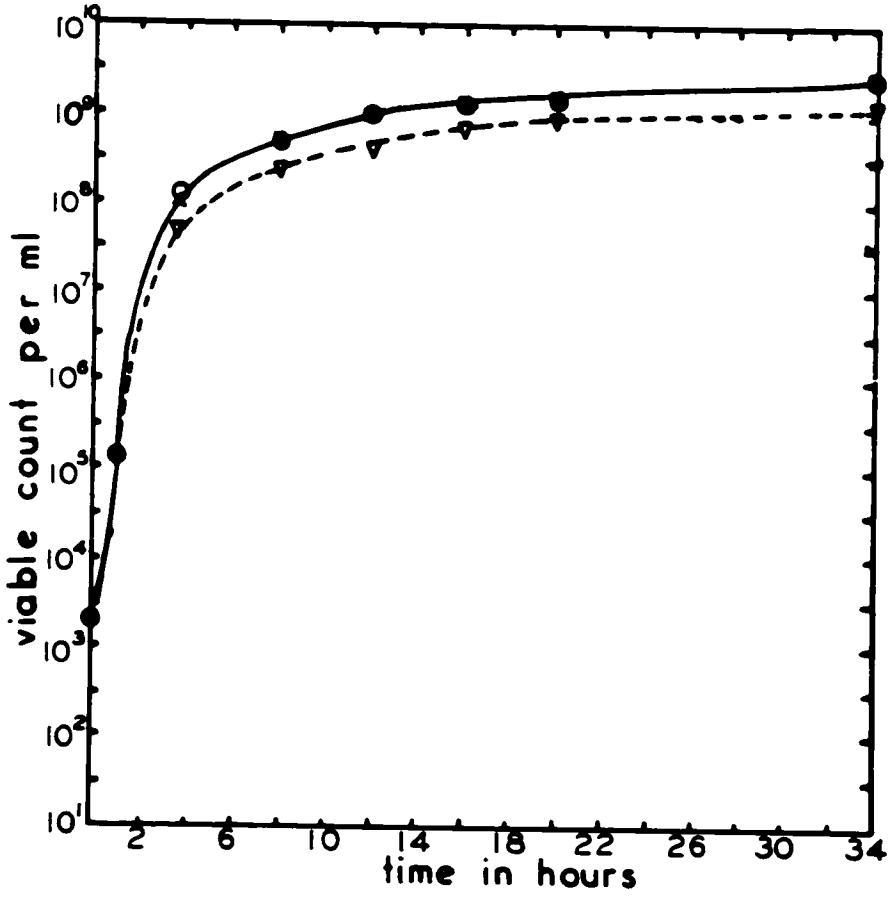
The organisms were tested by Gram staining and were then examined microscopically to determine whether they were all of the genus, Serratia. All the mutants proved to be very similar to the parent organisms, although they differed in pigmentation. Since the white and the orange mutants had displayed an interesting relationship in pigment formation (syntrophic pigmentation), their morphology and biochemical reactions were examined in more detail (Topley and Wilson, 1955). These results are summarized in Table 2. They were found to be identical to the parent strain. In addition to these tests, growth experiments were conducted on the three strains at both 30° and at 37° C in order to determine whether the presence or absence of pigment affected the rate of growth of the organisms. Figure 4 shows the growth curve resulting from this study. Both viable and total counts were determined. This study demonstrated that there was very little difference between the total and the viable counts in the three strains. Organisms grown at 37° C had a slightly higher final concentration of cells at 24 hours and reached the maximum earlier than those grown at 30° C. It was noted that these organisms will pigment only below 35° C as reported by von Eisler and Portheim (1914) and Giolitti (1949). The white mutant consistently showed a lower growth rate than the pigmented organisms (Figure 4).

Supplementing the media with various amino acids, nucleic acid derivitives, vitamins, and cofactors, either singly or in combination, had no effect on the pigmentation of the mutant organisms.

Samples of the isolated pigments were sent to Huffman Analytical

Figure 4. Growth curves of wild-type, orange mutant and white mutant grown at 30°C.




viable count per ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
2
6
10
14
18
22
26
30
34
time in hours

Laboratories (Wheatridge, Colorado) for carbon, hydrogen, nitrogen, and molecular weight analysis. The analysis from the Huffman laboratory indicated a large amount of ash in the isolated pigments. Consequently, all pigments used for analytical work were first washed thoroughly with ethylenediamine tetraacetic acid to remove any metals which might be retained by the pigment. The thiocyanate test was used as a criteria for the absence of iron, and ashing a portion of the pigment in a platinum crucible was used as a test for the absence of heavy metals. A spectral analysis by Dr. J. Rae of the Shell Development Company (Houston, Texas) confirmed the hypothesis that many metals were present initially in the acetone extracted pigment, with calcium, iron, and magnesium as the major contaminents.

II. PIGMENT ANALYSIS.

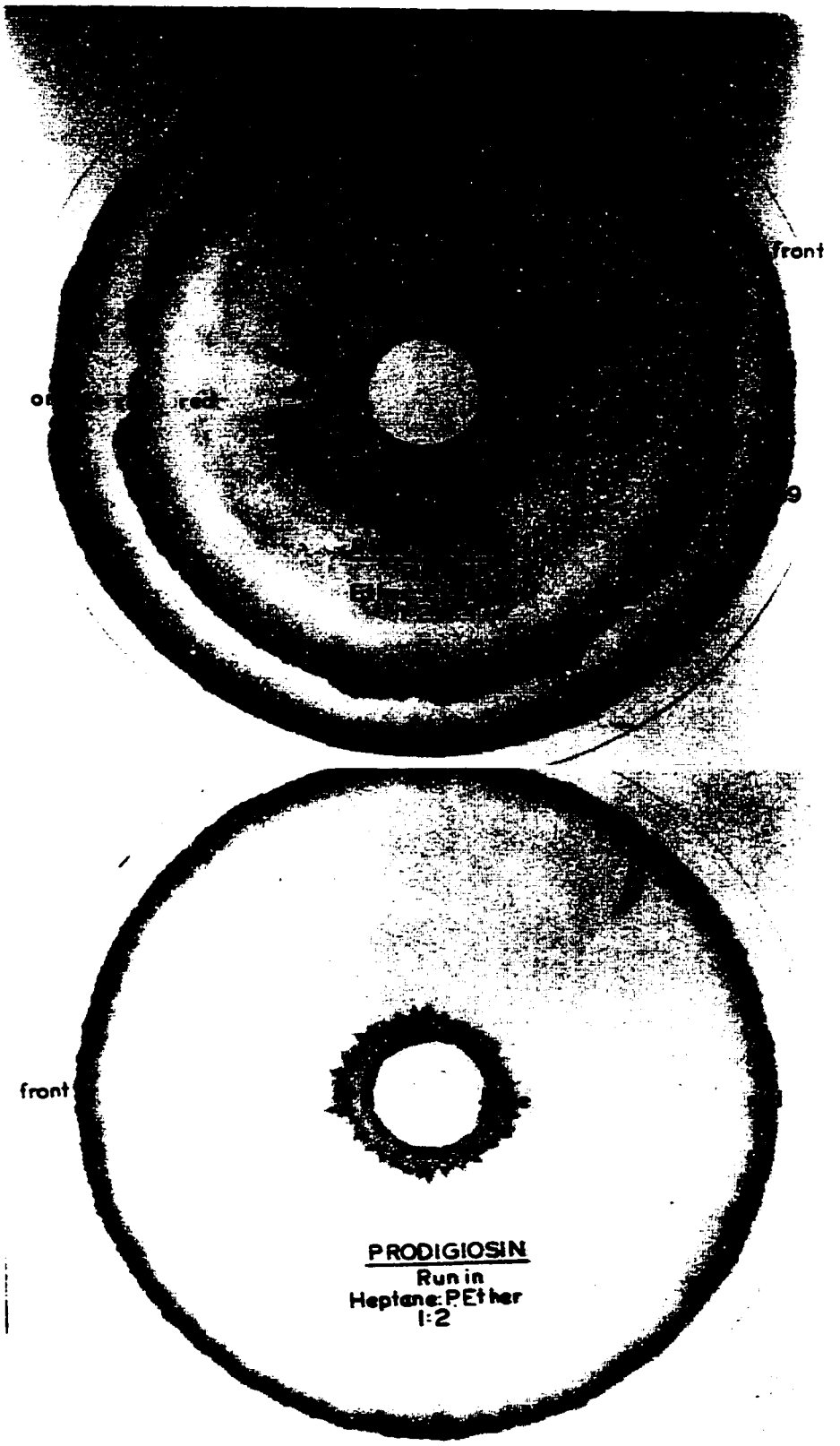
A. Chromatography

In an attempt to characterize the nature of the pigments in the red and orange organisms, the pigment was extracted with acetone and analysed chromatographically and spectrophotometrically.

Chromatography by the ascending, descending, and the plate glass method of Rappoport, et al (1955) gave similar results. Since it was difficult to maintain a saturated atmosphere with volatile solvents in the ordinary chromatographic chamber, it was found expedient to use the plate glass method throughout these investigations. The starred appearance of the slow moving components (Figure 5) seemed to be due to an unequal distribution of the weight on the glass. A number of solvent systems were developed to effect the type of separation necessary for

Figure 5. Circular paper chromatogram of acetone extracted pigment from wild-type Serratia marcescens run in ether:petroleum ether 1:2.

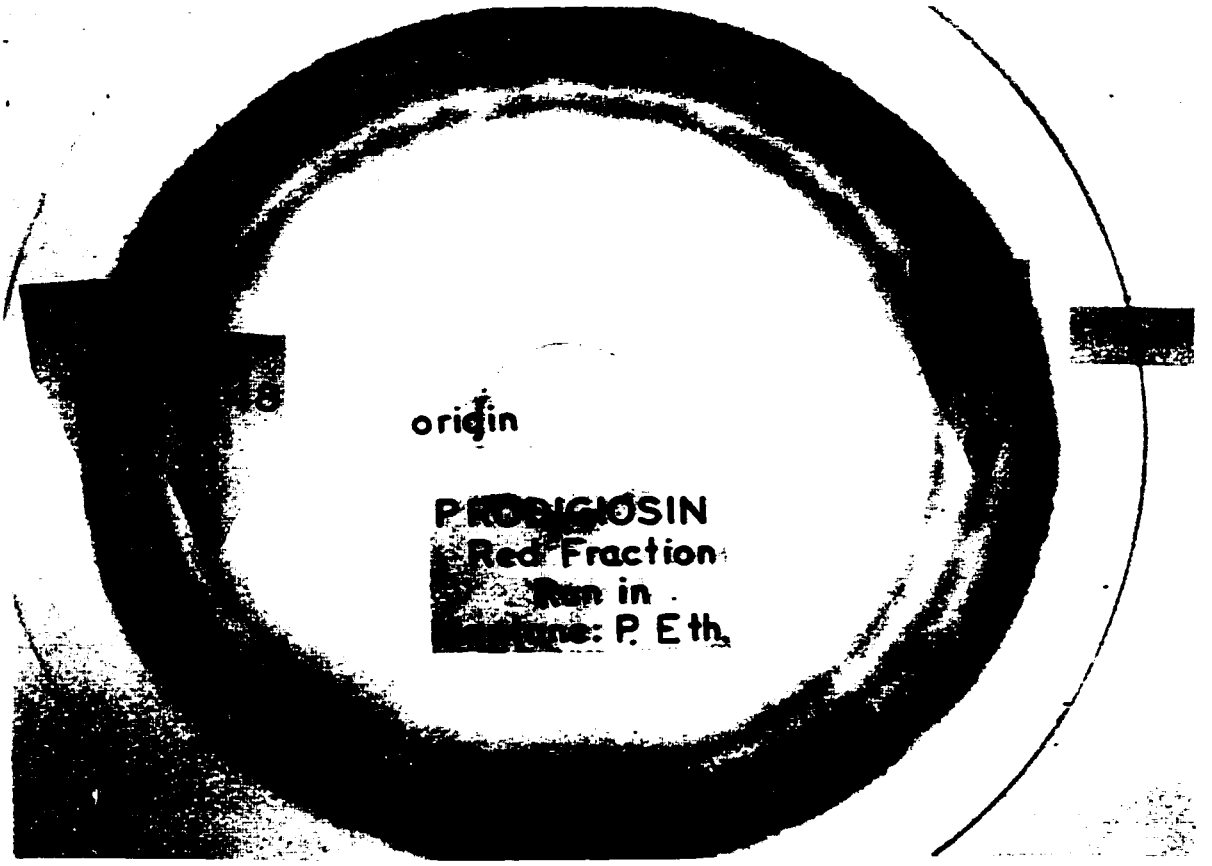
Figure 6. Acetone extracted pigment separated into blue and combined "red" fractions, run in heptane: petroleum ether solvent 1:2. (See text for solvent mixture.)


front
front
PRODIGIOSIN
Run in
Heptane: PEther
1:2

the analysis. A chromatogram of the whole pigment with an ether-petroleum ether solvent (1:2) is shown in Figure 5. The pigment separated into a blue band with an Rf. of 0.18; two red bands, Rf. 0.48, and Rf. 0.70; and an orange band at Rf. 0.39. The presence of more than one band was surprising in view of the fact that the work of Wrede and Hettche (1929), as well as that of Hubbard and Rimington (1950) indicated that the pigment was a single substance and was so stated by the latter workers. A heptane-petroleum ether solvent (1:2) allowed the separation of the red pigments from the blue fraction, as is shown in Figure 6. Subsequent elution and re-chromatography of the red and blue bands of Figure 6 established that the red was distinctly separated from the blue and that the blue component was not a decomposition product of the original red pigment, since no blue component occurred in the re-chromatographed red pigment. These re-chromatographed red and blue fractions are shown in Figures 7 and 8. Figure 7 shows that the red components are separated from the blue pigment, and this chromatogram also illustrates that the components present in the first chromatograph (Figure 5), exclusive of the blue, can be obtained from the isolated red pigment. These pigments were found to be very light sensitive. Prolonged manipulation of the orange and the minor red components in light caused destruction of these compounds rapidly. The main red component at Rf. 0.70 was more stable, while the blue component decomposed only after prolonged exposure to light. The light-sensitivity of the orange Rf. 0.39 and "minor" red components (Rf. 0.48) precluded attempts to collect sufficient quantities of these fractions for more detailed study.

Figure 7. Combined red fraction eluted from chromatograms such as that shown in Figure 6 and rechromatographed in heptane: petroleum ether 1:1.5. 'R1 on 1 refers to the paper chromatogram shown in Figure 5.

Figure 8. Blue fraction eluted from paper chromatograms and rechromatographed with ether:petroleum ether 1:1.


origin
PRODIGIOSIN
Red Fraction
Run in
Ether: P. Eth.


PRODIGIOSIN
Blue Fraction
Run in
Ether: Pet. Ether

The pigment extracted with acetone from the orange mutant was analysed by paper chromatography and gave a chromatogram as shown in Figure 9. The amount of pigment extracted from the orange mutant when compared to the pigment obtained from the wild-type (red) organism (based on cell weight) was in ratio of 1 to 4.

Since the pigments of Serratia marcescens characteristically respond to different pH ranges, (Kefferan, 1903, Bequet, 1928), the isolated components from the wild-type and mutant pigment were tested with acid and alkaline solutions and the observed color changes are tabulated in Table 3.

Pigments extracted from the orange mutant and the wild-type (parent red) and pigments obtained by growing the orange and white cells together (labeled "fed"), as well as an extract of the white strain, were chromatographed by placing the pigments in different sectors of a circle around the center of the paper. The resulting chromatogram is shown in Figure 10. Figure 10 indicates that none of the pigments were present in the white organism. It will be noted that the band near the origin of the "fed" organisms had an intermediate Rf. value between the blue of the wild-type and the orange pigment from the orange mutant. Subsequent elution and spectral analyses of this band (Figure 11) established that it consisted of a mixture of both the blue (a 545 mμ peak) and the orange pigments, (a 500 mμ peak). This is evident when Figures 13a and 14 are compared.

D. Fluorescence.

All of the pigments were examined under ultraviolet light to check

Table 3

The Effect of pH on Pigments of Serratia

Pigment or components	Color in acid solution	Color in basic solution	Properties
Wild-type red	Violet	Brownish yellow	Blue component unstable in alkali; red stable
Blue fraction	Blue black	Muddy brown	Unstable in alkali
Red fraction Rf. 0.70	Wine red	Yellow	Stable at any pH
Orange fraction Rf. 0.39	Rose	Light yellow	Stable at any pH
Mutant orange pigment	Rose	Light yellow	Unstable in alkali

Figure 9. Acetone extracted pigment from orange mutant of Serratia marcescens chromatographed with ether:petroleum ether 1:1.

Figure 10. Acetone extracted pigments of wild-type, mutant orange, mutant white, and pigment from white mutant induced by growing the white and orange organisms together (labeled "Red White"). "WF on 1" refers to the first paper chromatogram shown in Figure 9.

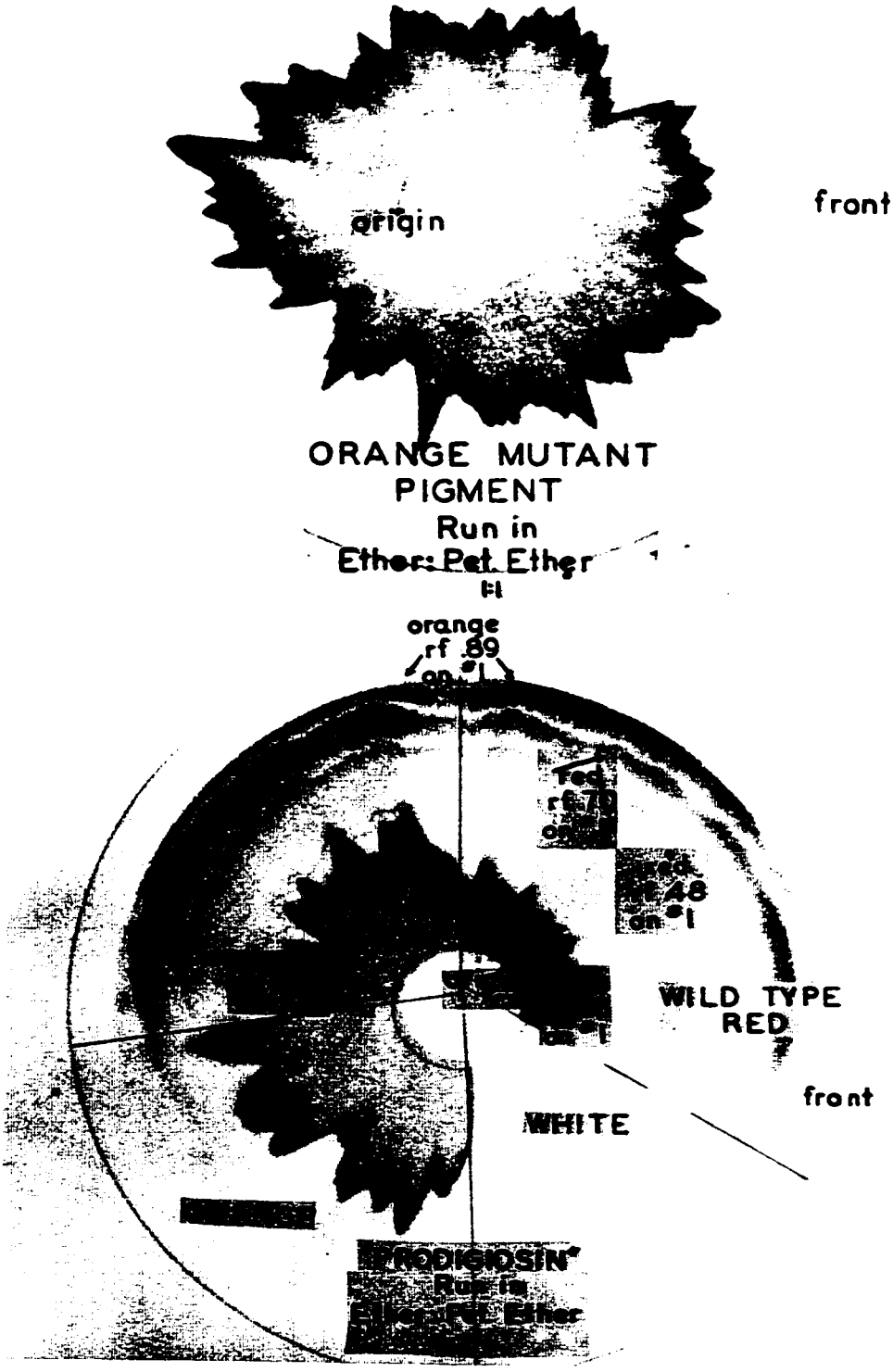
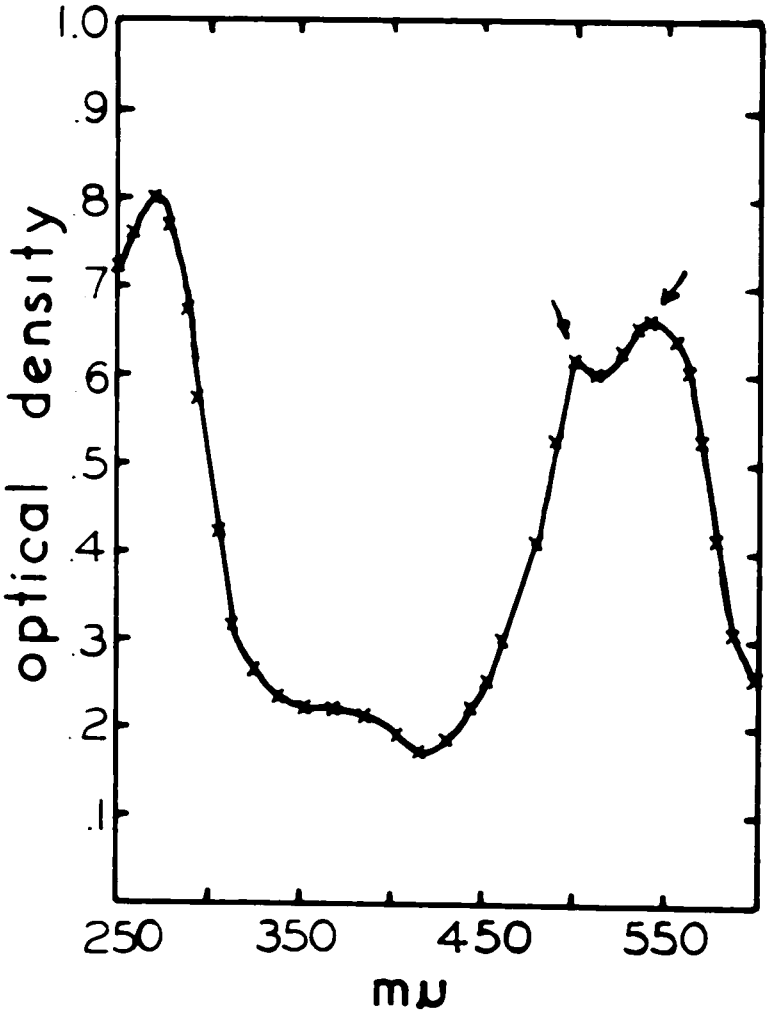

origin
front
ORANGE MUTANT
PIGMENT
Run in
Ether: Pet. Ether
F1
orange
rf .89
WILD TYPE
RED
front
WHITE
PRODIGIOSIN
Run in

Figure 11. Spectral curve of slow moving component eluted from chromatograph of synthetically fed white organisms showing the maxima for both the orange mutant pigment (500 mμ) and the blue component as found in the wild-type organism (540 mμ). (Compare curves on Figures 10a and 11)

The spectral curve obtained in acid alcohol, pH 1.

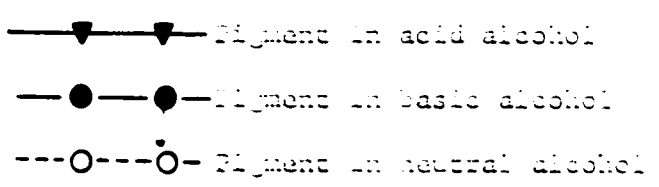

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
optical density
250
350
450
550
mμ

fluorescence. Only the orange component from the wild-type and from the orange mutant exhibited any fluorescence. Some fluorescence was noted in the red component after treatment with ethylenediamine tetra-acetate (Versene) which removed metal components.

3. Spectrophotometry.

The whole pigment of the wild-type strain and its individual components were analysed spectrophotometrically in the ultraviolet, visible, and the infra red regions. The curve obtained for the acetone extract of the whole pigment gave a similar curve (Figure 12a) to the one published by Hubbard and Rimington (1950) for prodigiosin. In Figure 12a the variations in the absorption spectrum of the whole pigment when dissolved in alcoholic, acid, neutral, and basic solutions are shown. Table 4 shows the maxima and minima absorptions of this pigment as compared to that reported by Hubbard and Rimington (1950). It is noteworthy that a distinct shoulder on the acid curve (Figure 12a) occurred at about 500 mμ. This shoulder was attributed by Hubbard and Rimington to an anionic form of the pigment in the acid solution. The present results do not confirm this view. Figure 12b is a plot of the spectral curves of the orange component in acid, base, and neutral solutions. The maximum absorption of this component in acid solution is at 500 mμ. The red component, as shown in Figure 13b, had no shoulder at this wavelength. This may be taken as good evidence that the orange component is responsible for the shoulder seen in the whole pigment at 500 mμ. The various spectral curves for the blue component are shown in Figure 13a. Recombination of the various fractions resulted in an absorption curve indistinguishable

Figures 12a and b. Spectral curves of the whole pigment (a) and the orange component (b) having an R.f. of 0.39 on Figure 5. Arrow depicts shoulder occurring at approximately 500 mμ in whole pigment.



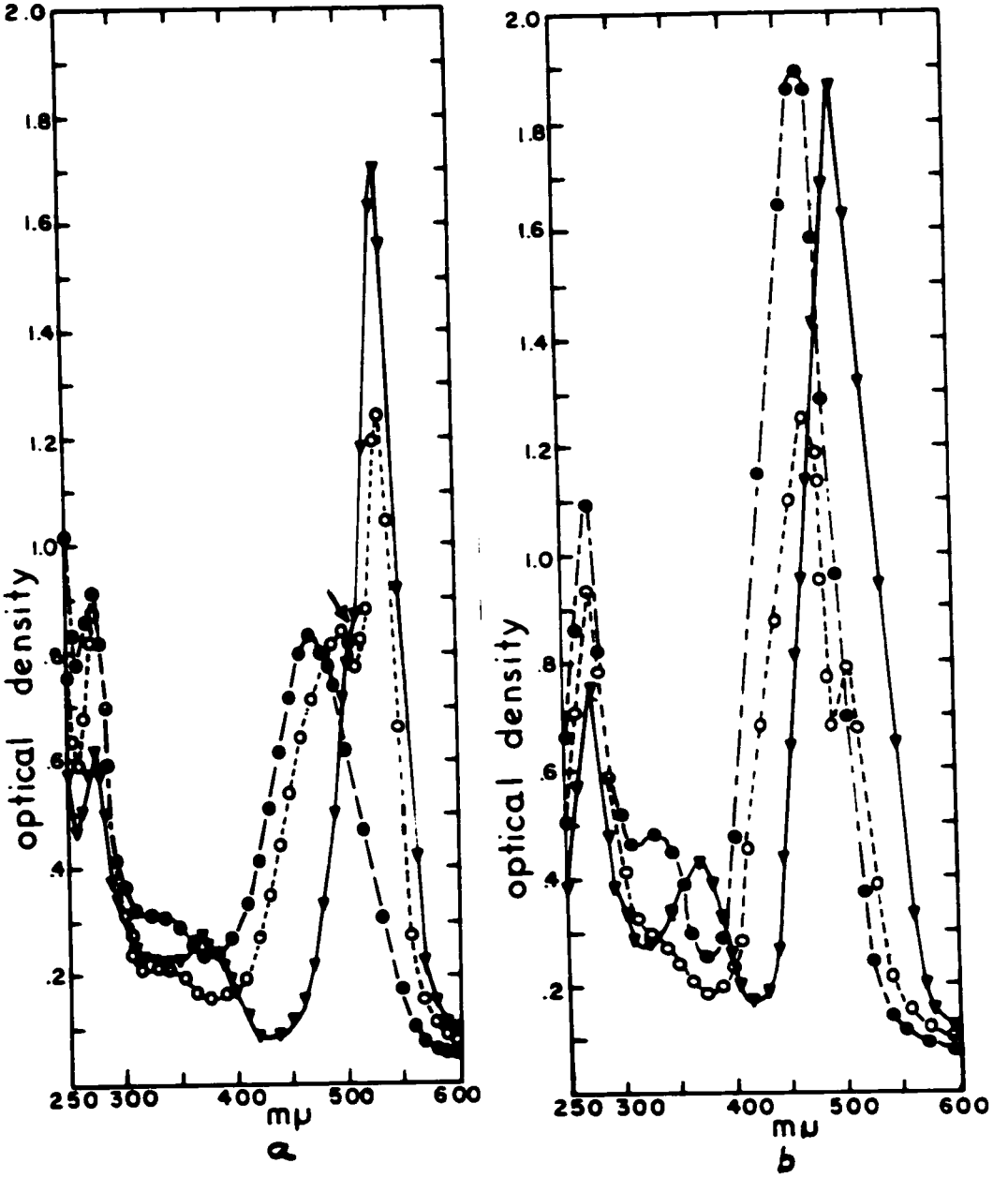
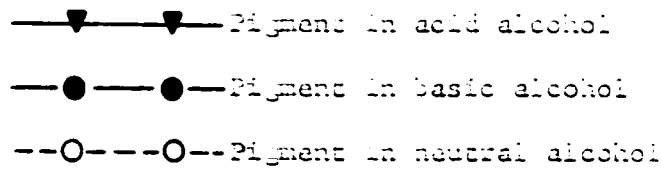

2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
optical density
250 300 400 500 600
mμ
a
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
optical density
250 300 400 500 600
mμ
b

Figure 13a and b. Spectral curves of pigment fractions blue (a) at Rf. 0.10 and red (b) at Rf. 0.70 as shown in Figure 5.



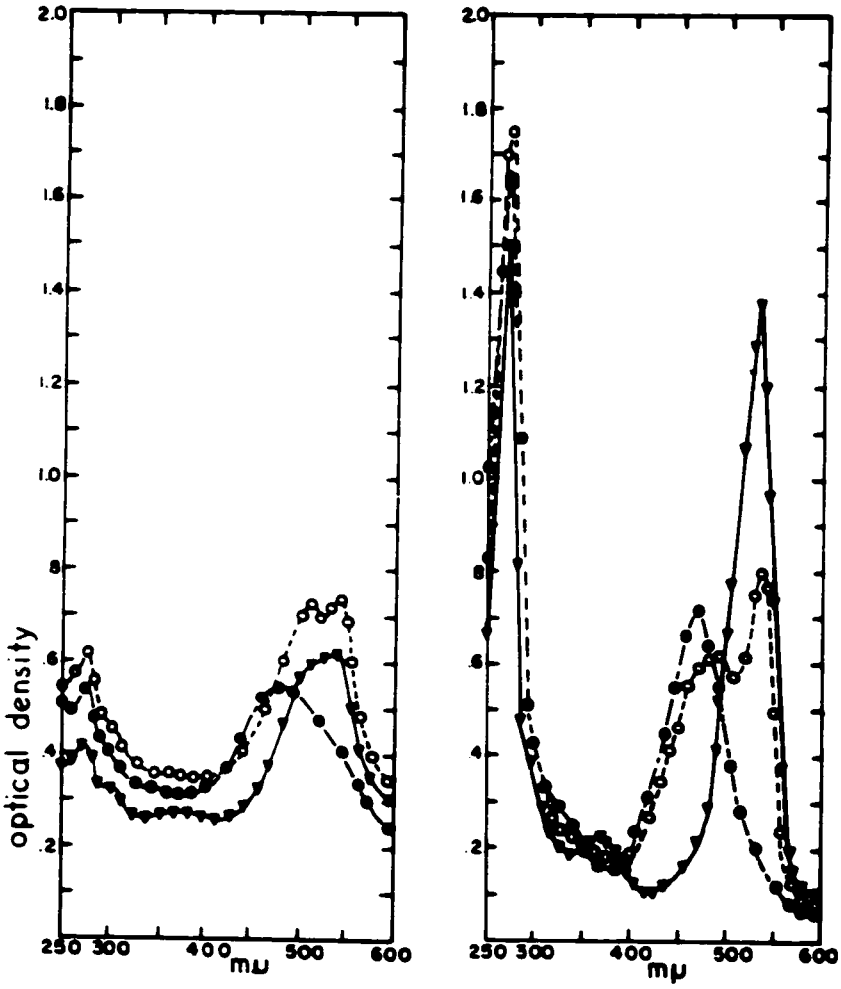

optical density
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
250 300 400 500 600
mμ
a
b

Table 4

Comparison of Spectral Properties of Prodigiosin

Points for comparison	Data from	
	Hubbard and Rimington (1950) in mμ	This investigation in mμ
Acid curve:		
maxima	540, 270	535, 275
minima	425, 335, 245	420, 330, 260
Alkaline curve		
maxima	470, 270	470, 275
minima	380, 250	380, 260
Isosbestic point	495	495
O.D. ratio of acid maximum to alkaline maximum	2.5	2.4

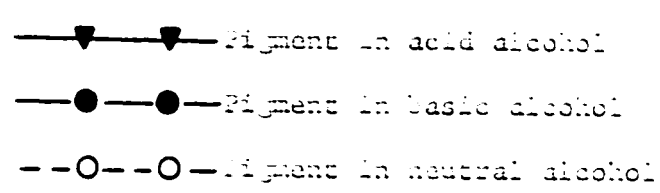
from that of the whole pigment.

Figure 14 shows the various spectral curves of the pigment extracted from the orange mutant. Dilution of this pigment to the extent that the ultraviolet maximum could be plotted resulted in a complete loss of the visible maximum which occurred at 500 mμ. While the curves for this pigment exhibited some differences from the wild-type pigment, the general shape of the curves strongly suggests that the basic structure of both pigments is very similar.

The infrared spectra of the red component, the blue component, and the whole pigment from the wild-type strain are shown in Figure 15. Due to the instability of the minor red and the orange fractions of the wild-type organism, the pigment components could be separated only as the red and the blue, and therefore, only these fractions were analysed. A comparison of the absorption curves of these components shows that they are similar in many respects, which suggests a definite structural relationship between them. However, some differences can be seen and these differences are additive. That is to say, when a peak in absorption curve appears in either of the fractions and not in the other, this peak is evident also in the whole pigment. Figure 16 shows the absorption curve of the orange mutant pigment compared to the absorption curve of the whole pigment of the wild-type strain. Here again a close relationship is suggested by the similar shape of the two curves.

To further purify the pigments, they were converted to their hydrochloride salts and various analyses were made on this purified material. The chloride, molecular weight, melting points and nitrogen

Figure 14. Spectral curves of acetone extracted pigment of an orange mutant of Serratia marcescens.



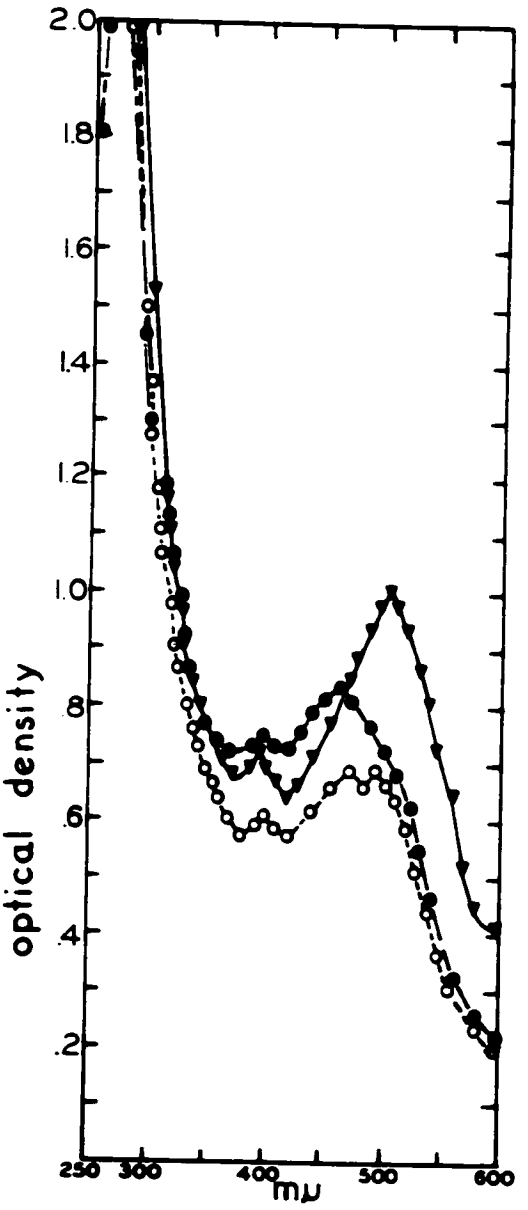

2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
optical density
250
300
400
500
600
mμ

Figure 15. Infrared spectral curves of the whole pigment, the combined red components and the blue component of wild-type Serratia marcescens. Readings were made in chloroform on a Perkin-Elmer double beam recording spectrophotometer, courtesy of Dr. Dan Edwards of the Veterans Hospital (Houston).

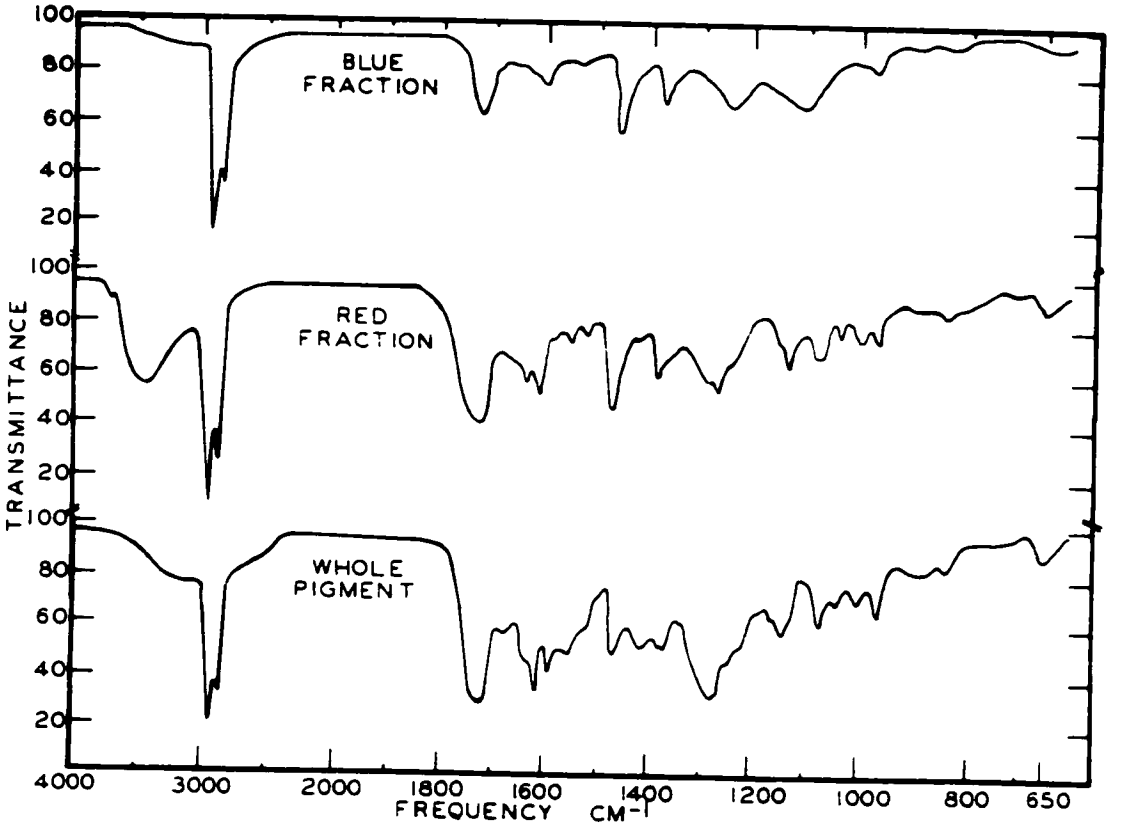
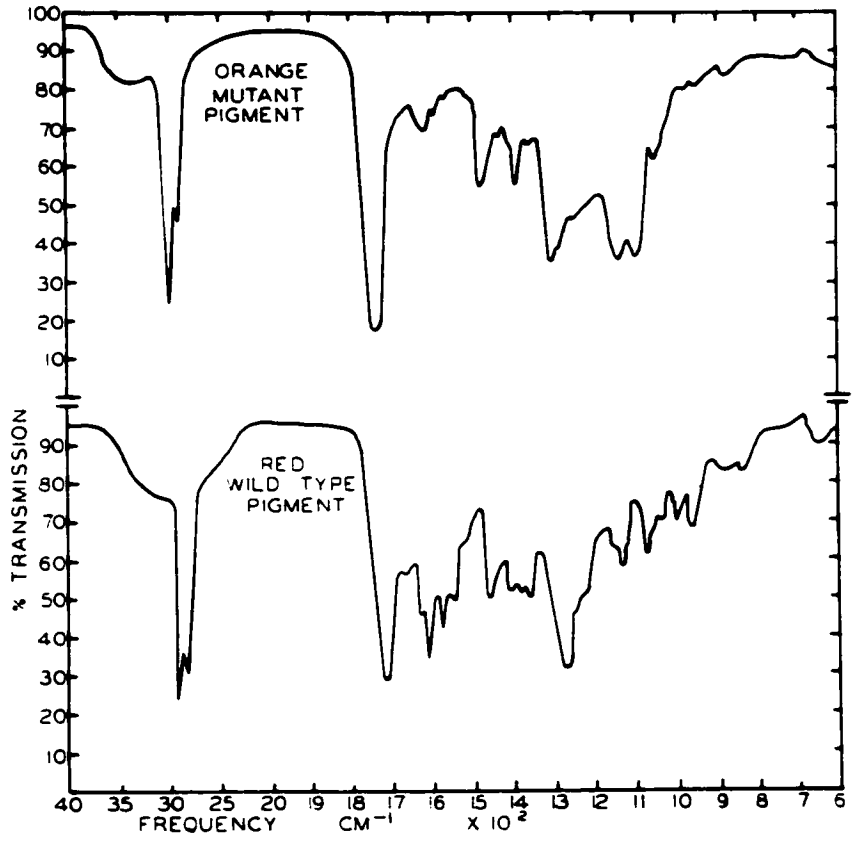

BLUE
FRACTION
RED
FRACTION
WHOLE
PIGMENT
TRANSMITTANCE
100
80
60
40
20
4000
3000
2000
1800
1600
1400
1200
1000
800
650
FREQUENCY CM-1

Figure 16. Infrared spectral curves of the pigment of the orange mutant plotted with the curve of the whole pigment of the wild-type Serratia marcescens.


ORANGE
MUTANT
PIGMENT
RED
WILD TYPE
PIGMENT
% TRANSMISSION
100
90
80
70
60
50
40
30
20
10
40 35 30 25 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6
FREQUENCY CM⁻¹ X 10²

content were determined. These values are listed in Table 5. Samples of the hydrochloride salts of the pigment were sent to Huffman Analytical Laboratories (Wheatridge, Colorado) for an element analysis. The values reported by the Huffman laboratories agreed well, in most instances, with those obtained in this laboratory (Table 5).

The presence of iron and calcium in the pigment samples suggested a possible chemical combination between the pigment and these metals. To test for any chelating properties inherent in the pigments, experiments were made utilizing radioactive calcium (Ca^{45}) and iron (Fe^{59}). The organisms were grown in the presence of each of these isotopes separately, until the amount of blue and red pigments were approximately equal in weight. At this time the pigments were extracted, fractionated and the radioactivity of the intact cells and pigment components determined. The results of these experiments are shown in Table 6. Extraction of the white organisms by identical procedures did not yield any radioactivity. This fact was taken as an indication that the resulting radioactivity in the pigment was not due to simple adsorption. Table 6 shows that the blue fraction had greater chelating properties, based on the specific activity found in these fractions, than the red components. Also, the iron was concentrated in the pigment fraction of the organisms to a much greater extent than in the cellular debris fraction. This, however, was not true of calcium retention in the wild-type organisms. In the orange mutant the pigment incorporated more of both of the isotopes than did the cellular debris. A radioautograph of the wild-type pigment containing either iron (Fe^{59}) or calcium (Ca^{45}) is shown in Figure 17.

Table 5

Analysis of Pigments

	Red fraction %	Blue component %	Orange mutant %
Carbon [1]	64.32	61.18	56.61
Hydrogen [1]	8.00	6.29	7.94
Nitrogen* [2]	8.96	9.73	8.68
Chloride+ [1,2]	7.92	4.70	6.14
Residue [1]	2.25	7.05	6.39
Molecular Weight♦ [1,2]	460	775	360
Melting Point °C [2]	110-115	Decomposes	160-170

* Determined by Kjeldahl analysis (Ma and Zuazaga, 1942)

♦ Determined by Rast's (Shriner, 1948) cryoscopic method.

+ Determined by modification of method by Schales and Schales (1941).

1 Determined by Huffman Analytical Laboratories (Wheatridge, Colorado).

2 Determined in this study.

Figure 17. Radioautogram of the pigment from wild-type Serratia marcescens grown in the presence of either iron (Fe^{59}) or calcium (Ca^{45}) to show the relative distribution of the isotopes in the pigment fractions. Chromatograms were run first in ether:petroleum ether 1:1 (cf. Figure 3) followed by acetone:petroleum ether 1:1 which accounts for the secondary ring of blue pigment.

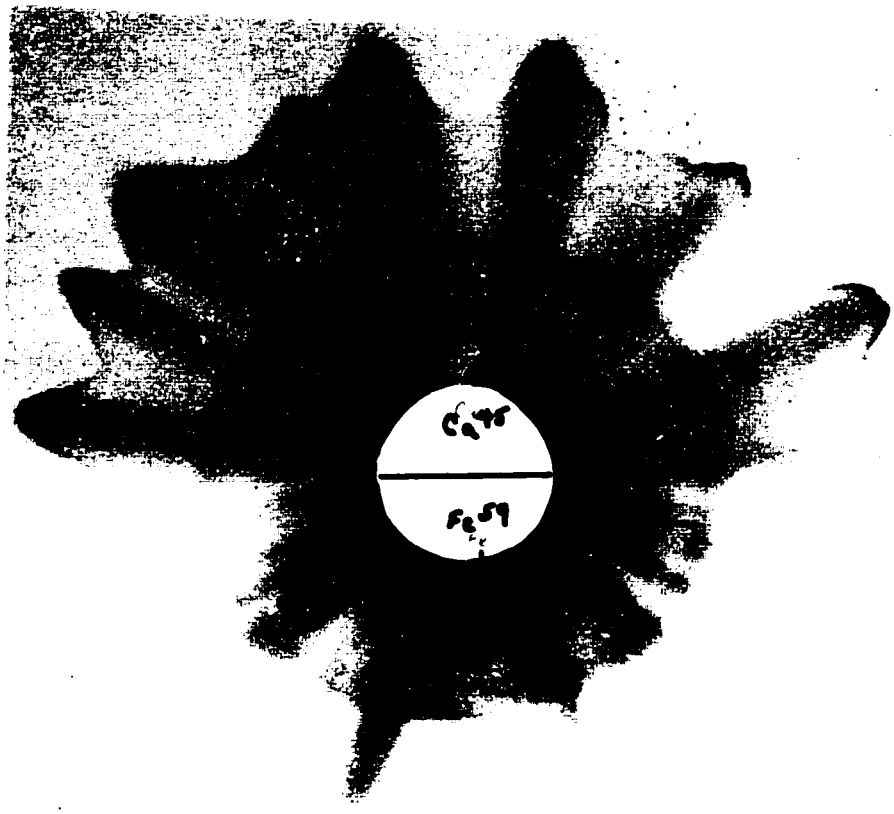


Table 6

Distribution of Ca^{45} and Fe^{59} in Serratia marcescens

Fraction	Weight (mg)	Specific Activity* $\times 10^{-3}$	
		Iron	Calcium
Parent strain cells	750.0	1.59	0.70
Entire red pigment	1.0	1.04	1.17
Blue component	0.51	13.0	1.33
Red component	0.49	2.00	1.00
Orange mutant cells	740.0	1.07	4.50
Orange pigment	0.40	32.0	23.1

* Specific activity is given as cpm, per milligram

Total activity of Fe^{59} was 3.03×10^6 cpm in 70 ml. medium.

Total activity of Ca^{45} was 1.10×10^7 cpm in 70 ml. medium.

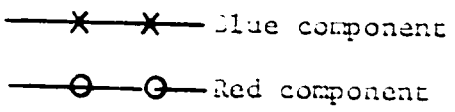
When the wild-type strain was grown for prolonged periods there was a marked increase in color, hence, it was of some interest to determine the nature of the various pigment components which accumulated with time. By means of a spectrophotometric analysis of the isolated pigment components, the variation in pigment concentration with growth of the organisms was studied. The quantity of organisms was determined on the basis of weight. The isolated pigment was chromatographed and the amount of each component was determined spectrophotometrically by comparison to a standard absorption curve (Figure 18). The relation between the whole pigment accumulation and the accumulation of the individual blue and red fractions is shown in Figure 19. The relative absorption of the two components at their maximum wave length (540 mμ) and the absorption of the whole pigment are shown in Figure 20. It may be seen that the light absorption of the red pigment was much greater than that of the blue pigment. This relationship can also be observed by referring to the absorption spectra of equimolar concentrations of the various pigment fractions shown earlier (Figure 13a and b).

The blue pigment appeared later in the growth sequence than did the red pigment, but it soon exceeded the red pigment in quantity, reaching a plateau at about 8 to 10 days and remaining relatively constant for at least 34 days. The viable count and the total weight of organisms for this period of study are presented in Figure 21.

III. METABOLIC STUDIES ON SERRATIA STRAINS.

A. Phosphorus Incorporation and Nucleic Acids.

Figure 16. Standard curves of the red and blue components of prodigiosin in acid alcohol solution.



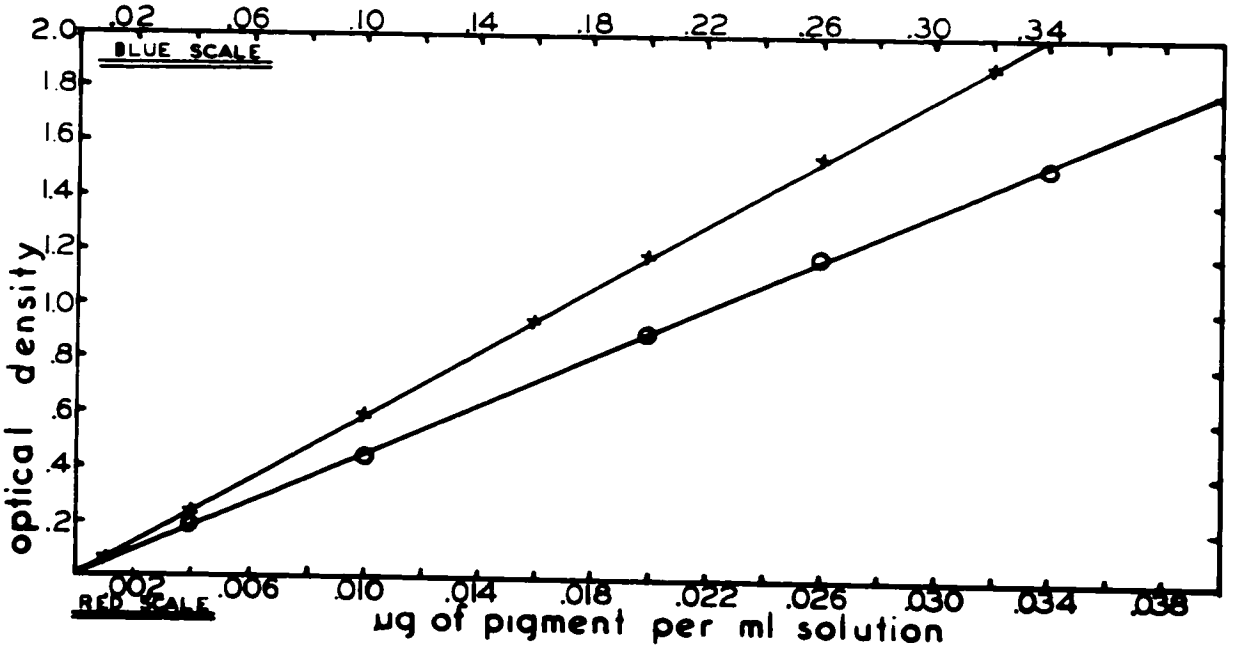

.02
.06
.10
.14
.18
.22
.26
.30
.34
BLUE SCALE
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
optical density
.002
.006
.010
.014
.018
.022
.026
.030
.034
.038
RED SCALE
µg of pigment per ml solution

Figure 19. Weight of whole pigment, the red component and the blue component plotted against time of accumulation in wild-type Serratia marcescens.

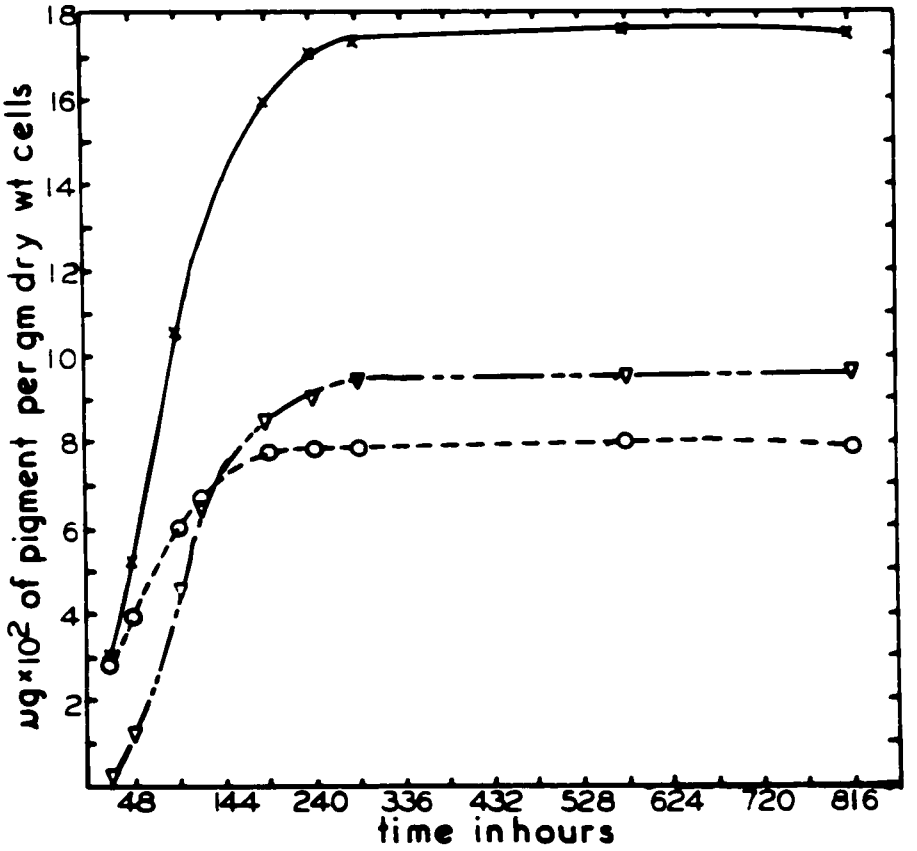
—✕—✕— Whole pigment

--O--O- Red component

—·▽—··▽— Blue component

Figure 20. Relation of light absorbance of the whole pigment, the red component and the blue component plotted against time of accumulation.

Curve designations same as Figure 19.


µg×10² of pigment per gm dry wt cells
18
16
14
12
10
8
6
4
2
48
144
240
336
432
528
624
720
816
time in hours

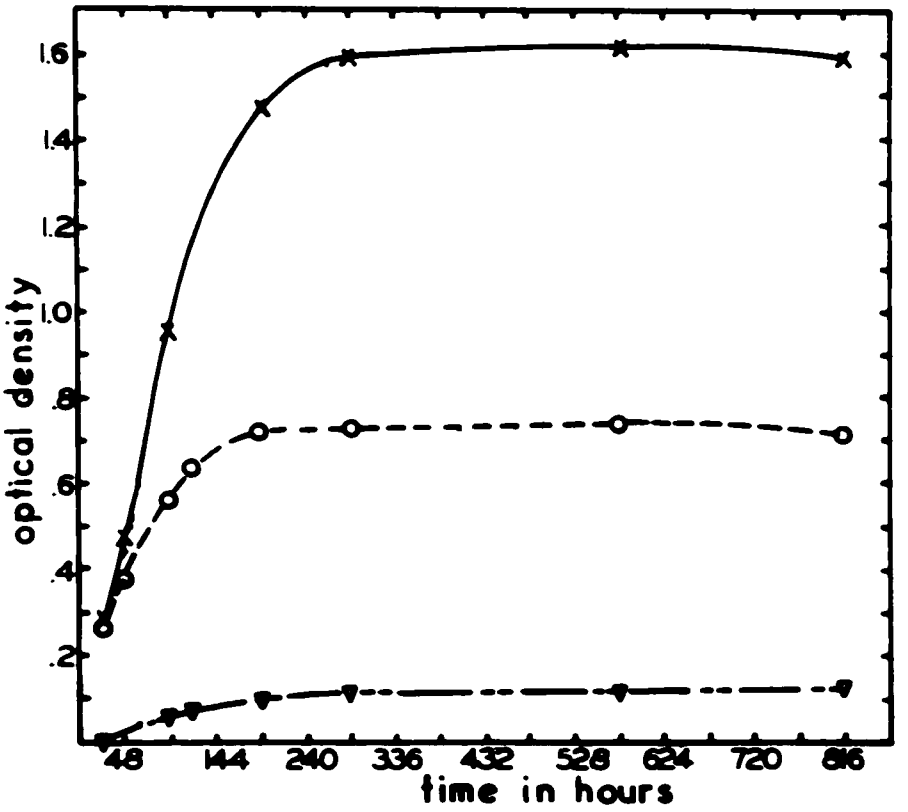
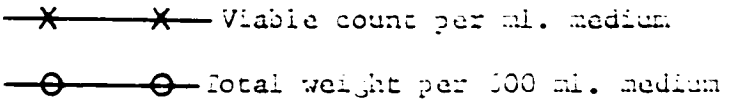
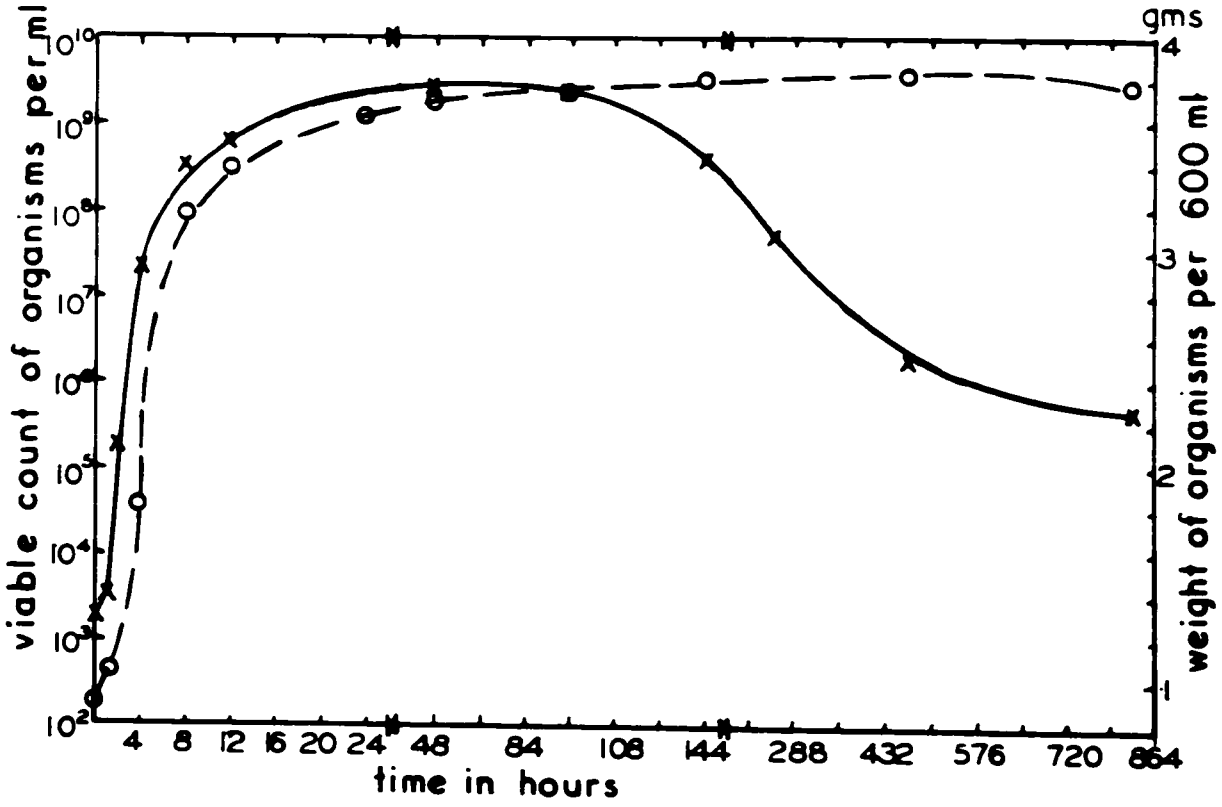

optical density
1.6
1.4
1.2
1.0
.8
.6
.4
.2
48
144
240
336
432
528
624
720
816
time in hours

Figure 21. Plot of viable count of organism in the medium as compared to the total number of organisms over a period of 134 hours.




gms
viable count of organisms per ml
weight of organisms per 600 ml
time in hours
4 8 12 16 20 24 48 84 108 144 288 432 576 720 864

Since facilities did not permit further detailed examination of the molecular configuration of the isolated pigments, the study of the problem of pigmentation in Serratia was switched to an investigation of the metabolism of the organisms, in order to ascertain whether or not there were measurable metabolic differences in the organisms which might account for the difference in pigmentation. This seemed particularly important, since no systematic metabolic study has been reported for Serratia. Such an investigation was necessary in order to describe the nature and scope of metabolic interconversions in this organism.

Initially, phosphorus 32 was used to determine whether or not there were any differences in the phosphorus metabolism of the three strains of organisms. The results of these studies are presented in Table 7a and b. While some small differences will be noted, repeated experiments indicated that these differences were not significant. The DNA fraction of the white organism was observed to be consistantly lower than DNA in the other two strains studied. If the added radioactivity, approximately 100, was accounted for in these experiments.

Due to the difference noted above in the nucleic acid fraction, detailed analyses of the RNA and DNA were made to determine whether there was a difference in the amount of either of these in any of the organisms. The results of these analyses (Table 8) show that no significant quantitative difference existed in the nucleic acids between the various organisms. The high value for the RNA in the early phase of growth paralleled the rapid period of multiplication. The DNA concentration remained rather constant throughout the period of study, while RNA

Table 7a

Total P^{32}-Incorporation by Wild-Type and Mutant Strains of Serratia marcescens

Organisms	cpm. per gram wet weight x 10^5	Per cent uptake
Wild-type (red)	1.34	15.2
Orange	1.14	13.1
White	2.10	17.3

Table 7b

P^{32} Distribution in Cellular Components of Wild-Type and Mutant Strains of Serratia marcescens

	Acid soluble		Lipids		DNA		RNA		Protein	
Organism	cpm/gm x10^4	%	cpm/gm x10^4	%	cpm/gm x10^3	%	cpm/gm x10^4	%	cpm/gm x10^4	%
Wild-type (red)	4.31	25.1	0.94	5.12	10.33	4.33	5.45	29.8	6.49	35.1
Orange	4.70	25.3	1.15	6.20	8.93	3.73	6.10	33.2	5.50	30.0
White	5.14	24.3	1.67	7.96	5.82	2.70	6.69	31.9	6.73	32.1

* Total counts in 70 ml. Tris-buffered medium was 1.2×10^6 cpm.

† Per cent of incorporated P^{32} counts.

Table 3

Variation of Per Cent Concentration* of Nucleic Acids With Time In Strains of S. marcescens

	RNA							
Hours	1	4	8	14	24	40	70	144
Red	2.70 ± 0.20	3.14±0.12	2.94±0.15	2.24±0.38	2.42±0.26	1.65±0.20	1.75±0.15	0.89±0.037
Orange	2.45 ± 0.14	2.50±0.21	2.78±0.20	2.25±0.30	2.35±0.16	1.87±0.12	1.76±0.12	0.84±0.046
White	2.15 ± 0.32	2.60±0.13	2.16±0.25	2.11±0.35	2.13±0.20	1.91±0.09	2.00±0.18	0.83±0.040
	DNA							
Red	0.06 ±0.009	0.09±0.009	0.13±0.015	0.19±0.020	0.19±0.030	0.16±0.028	0.14±0.014	0.16±0.009
Orange	0.08± 0.010	0.10±0.008	0.08±0.012	0.20±0.015	0.20±0.008	0.17±0.022	0.19±0.034	0.18±0.017
White	0.07± 0.009	0.09±0.010	0.10±0.009	0.17±0.015	0.19±0.020	0.14±0.016	0.13±0.012	0.13±0.008

* Based on wet weight

dropped rather suddenly after 24 hours of growth.

b. CO_2 Metabolism

The fact that Serratia marcescens incorporates an appreciable amount of CO_2 (McLean, 1951) prompted investigation on the metabolism of this compound. The amount of $C^{14}O_2$ incorporated into the various fractions was determined initially on 24 hour cell cultures of the different strains. In beginning this work it was desirable to study the effects of the medium and various supplements on the incorporation of the isotope into various fractions of the organisms. McLean (1947-1953) showed that the addition of a variety of nitrogen and/or carbon containing substances significantly decreased the overall incorporation of carbon dioxide ($C^{14}O_2$). The results of the present study are shown in Table 9. It was found that the addition of any amino acids or acetate to the medium as a carbon source decreased the total incorporation of the $C^{14}O_2$ by the organisms.

The incorporation of $C^{14}O_2$ in the wild-type and the two mutant strains was compared. The results of these experiments are given in Table 10. Since considerable overlapping in the values of $CO^{14}O_2$ incorporation was found in four experiments, the differences noted between the wild, white, and orange strains were considered insignificant.

To determine whether these organisms had a Krebs Citric Acid Cycle, the acid fraction was isolated and analysed for di- and tricarboxylic acids. Citric, succinic, fumaric, and malic acids were located and identified by paper chromatography. A radioautograph was then made of the resulting chromatogram. Figure 22 shows the

Figure 22. Citric Acid Cycle Intermediates extracted from Serratia marcescens grown in presence of $C^{14}O_2$.

1. Citric acid
2. Malic acid
3. Succinic acid
4. Fumaric acid


Sec. Butyl Alcohol
Formic Acid

radioautograph from a typical paper chromatogram of these isolated organic acids.

A study of the incorporation of $C^{14}O_2$ into the nucleic acids was also made. The initial concentration of the nucleic acid was determined spectrophotometrically. Chromatograms were then made on the perchlorate hydrolysate of the nucleic acids with respect to total radioactivity and nucleic acid components. The recovery was better than 97% from the chromatograms in all experiments. The isolated components were counted and the specific activity of the individual free bases was determined. The results of these analyses is presented in Table 9. Since there is no color reagent for the location of the nucleic acid bases, a representative diagram and a radioautograph were made from such a chromatogram. This is shown in Figure 23.

A similar study of the incorporation of $C^{14}O_2$ into various cellular fractions was carried out during growth of the organisms, the analysis being made at 1, 4, 8, and 24 hours. Table 10 gives the overall distribution of radioactivity in the various fractions. The activity in the acid soluble fraction, which is recovered with the medium, showed decreased C^{14}-activity with time, as did the alcohol soluble fraction and Bloor's lipid fraction. The RNA fraction did not decrease in specific activity until the 24th hour of growth, while the protein and the DNA fractions continued to increase in specific activity throughout the experimental period. Table 11 gives the specific activity of RNA and DNA on the basis of the weight of these compounds individually.

In Figures 24 and 25 the specific activity of the free bases from

Table 9

Effect of Various Media on $C^{14}O_2$ Incorporation and Growth of Wild-Type S. marcescens in 24 Hour Cultures (100 ml. medium)

Medium	Total % $C^{14}O_2$ incorporated			% Total $C^{14}O_2$ incorporated in organisms		
	Weight of organism mg.	Cells	Acid soluble medium	Alcohol soluble	Lipids	Nucleoprotein
Minimal	382.6	30.00	6.30	4.23	1.39	93.70
Glycine	86.7	19.55	14.61	4.73	3.65	92.10
Acetate	106.0	3.33	2.24	2.55	3.24	94.20
Complete	465.0	29.00	6.33	22.20	1.36	77.90

Figure 23. Tracing of ultraviolet absorbing spots (a) of a chromatogram of nucleic acid derivatives from Serratia marcescens grown in presence of $C^{14}O_2$ and its corresponding radioautograph (b).

1. Guanine
2. Adenine
3. Cytosine
4. Thymidine
5. Thymine
6. Cytidylic acid
7. Uridylic acid

a b

Table 10

Comparison of $C^{14}O_2$ Incorporation of Wild-Type (Red) and Two Mutant Strains of Serratia marcescens Grown 24 Hours in 100 ml. Minimal Medium

	Wt. of organism mg.	Total % Incorporated		% Total $C^{14}O_2$ Incorporated Into Organisms					RNA	DNA
		cells	Acid soluble medium	Alcohol soluble	Lipids	RNA	DNA	Protein	Pu/Py	A+T/G+C
Red	917.00	32.60	7.50	2.75	0.26	13.11	1.80	73.50	1.40	0.70
Orange	914.00	30.65	7.78	2.74	0.31	14.02	2.22	80.60	1.38	0.71
White	893.00	32.20	7.95	1.98	0.22	11.78	2.07	78.90	1.43	0.71

Table 11: * Initial activity of $C^{14}O_2$ was in actual counts: 12.7 x 10^4 in experiment 1 and 1.37 x 10^6 in experiment 2 in 300 ml. urie buffered medium.

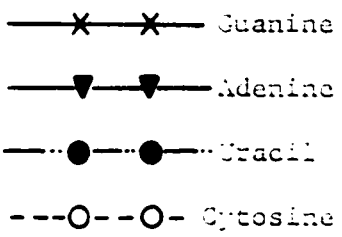
+ Values given are specific activity in cpm per gram wet weight of organisms, adjusted to 10. x 10^4 counts.

Table II

Total $C^{14}O_2$ Incorporated*

Weight of organisms	1.15 gm.	1.42 gm.	2.10 gm.	2.75 gm.
Acid soluble	4.78×10^{4}	3.30×10^{4}	2.48×10^{4}	1.82×10^{4}
Alcohol soluble	7.31×10^{5}	2.06×10^{6}	2.03×10^{6}	2.18×10^{6}
Bloor's lipids	3.65×10^{3}	3.03×10^{3}	2.85×10^{3}	2.35×10^{3}
RNA	4.50×10^{5}	8.78×10^{5}	1.18×10^{6}	1.13×10^{6}
DNA	8.16×10^{4}	2.47×10^{5}	2.75×10^{5}	3.94×10^{5}
Protein residue	1.82×10^{5}	3.19×10^{5}	4.92×10^{5}	5.98×10^{5}
Specific Activity of Nucleic Acids as cpm per mg (dry wt.)				
RNA	1.92×10^{4}	4.06×10^{4}	6.18×10^{4}	5.66×10^{4}
DNA	1.22×10^{4}	2.96×10^{4}	4.78×10^{4}	5.64×10^{4}

Figure 24. Specific activity of the free bases of ribonucleic acid from wild-type Serratia marcescens grown in presence of $C^{14}O_2$ plotted against time of incubation.



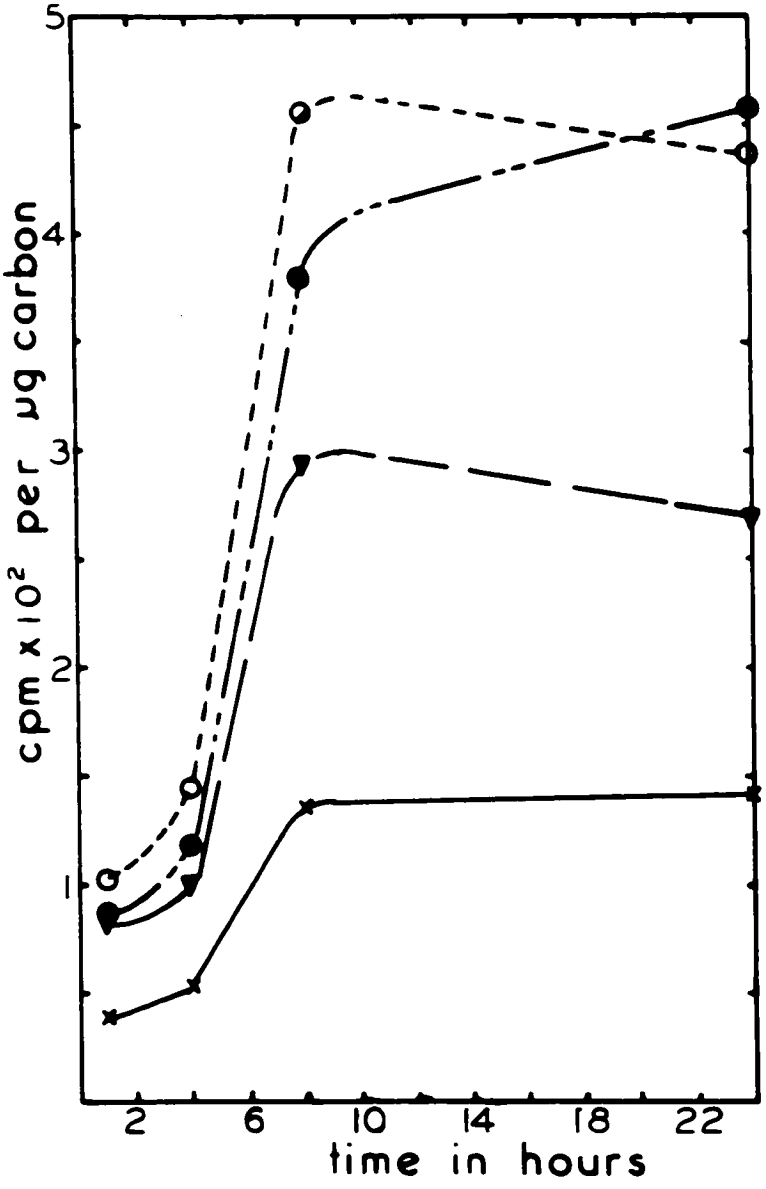

5
4
3
2
1
cpm x 10² per µg carbon
2
6
10
14
18
22
time in hours

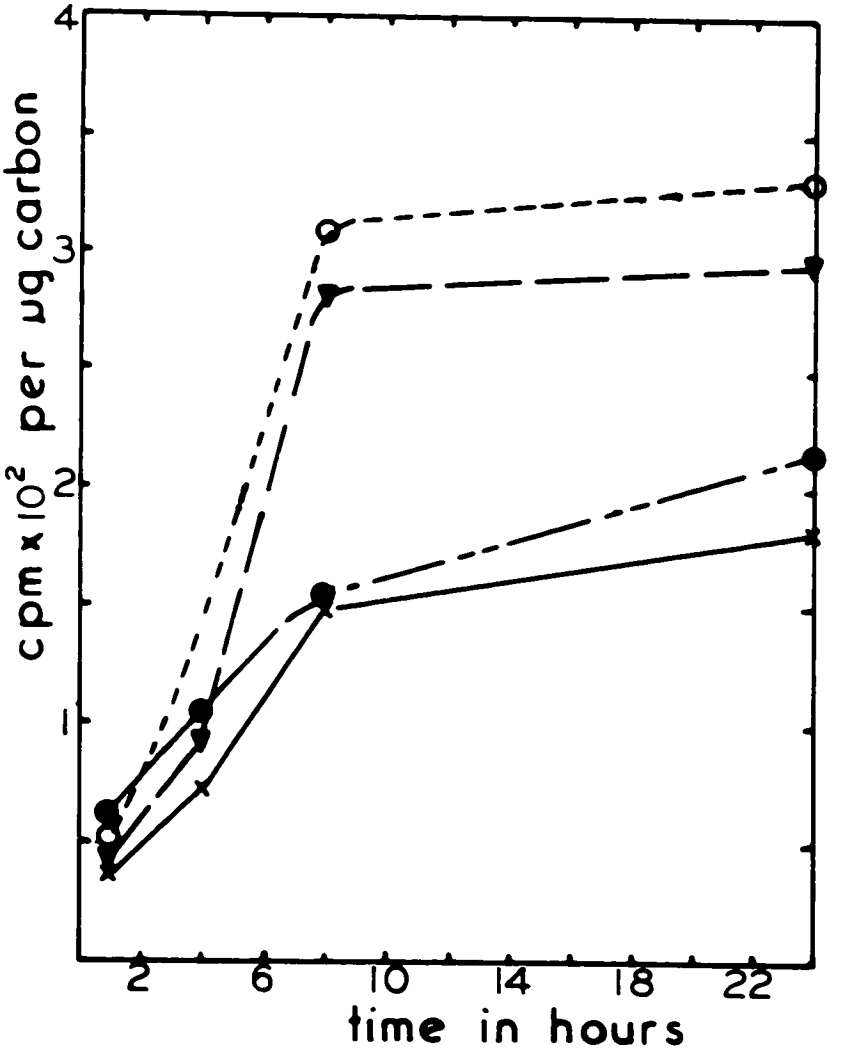
Figure 25. Specific activity of the free bases of deoxyribonucleic acids from wild-type Serratia marcescens grown in presence of $C^{14}O_2$ plotted against time of incubation.

—X—X— Guanine

—▼—▼— Adenine

—··●—··●— Thymine

---O---O— Cytosine


4
3
2
1
cpm x 10² per µg carbon
2
6
10
14
18
22
time in hours

nucleic acids are plotted on the basis of unit carbon content versus time of growth. It will be noted that the pyrimidines, uracil and cytosine contained a much higher specific activity than did the purines, adenine, and guanine; however, the pyrimidine, thymine, had a relatively low specific activity.

There is no free amino acid pool in these organisms. However, the alcohol soluble fraction yielded several peptides which, although not identified as such, were hydrolysed and the individual amino acids identified. These amino acids corresponded to the amino acids which were recovered after hydrolysis of the residual protein from the organism. These are listed in Table 12. A typical paper chromatogram and its corresponding autoradiogram is shown in Figure 26a and b. Figures 27 and 28 show the accumulation of radioactivity in the various amino acids with time. It will be noted that the accumulation of radioactivity into the different amino acids corresponded closely with their degree of metabolic relationship to the carboxylated intermediates of the Citric Acid Cycle. It will be noted that glutamic acid and aspartic acid, which are immediate interconversion compounds between the Citric Acid Cycle and other amino acids, had the highest specific activity at one hour, but the related amino acids quickly surpassed these in specific activity in the proceeding hours of incubation. The relative amounts of the amino acids with respect to the total protein are presented in Table 12. These values can only be considered approximate since the acid hydrolysis would have destroyed any labile amino acids such as tryptophane.

Table 12

Amino Acid Composition of Protein

Amino Acid	Per cent of total protein	Per cent carbon of total protein	Mole-per cent of total protein
Cystine	1.46	0.39	0.67
Citrulline	1.46	1.26	0.91
Argenine	3.13	3.05	2.00
Glutamic	12.10	11.55	9.60
Aspartic	5.96	5.03	4.90
Lysine	4.12	4.76	3.10
Threonine	2.26	2.13	2.10
Isoleucine	3.50	4.70	3.07
Proline	1.37	1.64	1.30
Methionine	2.03	2.35	1.50
Leucine	12.40	15.03	10.40
Valine	3.63	4.35	3.40
Glycine	15.60	11.35	23.30
Serine	4.62	3.67	3.00
Alanine	20.70	19.60	26.60
Tyrosine	2.26	3.15	1.33
Phenylalanine	2.26	3.49	1.50
Histidine	1.11	1.13	0.77

Figure 26a. Paper chromatogram of protein hydrolysate of Serratia marcescens.

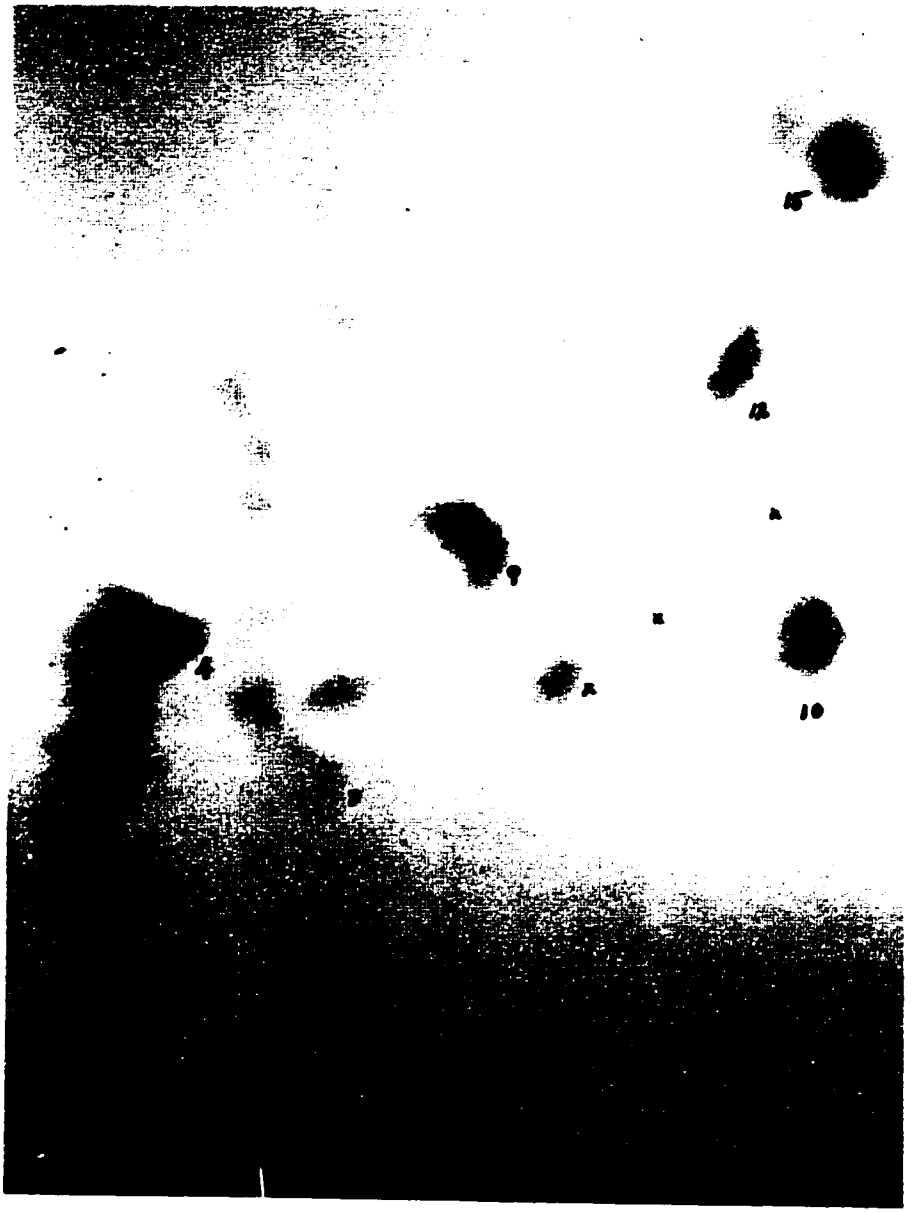
1. Citrulline	7. Serine	13. Tyrosine
2. Arginine	8. Histidine	14. Methionine
3. Aspartic acid	9. Proline	15. Leucine
4. Glutamic acid	10. Threonine	16. Isoleucine
5. Lysine	11. Alanine	17. Phenylalanine
6. Glycine	12. Valine	18. Cystine

Color running from threonine through the methionine-valine spot is background color caused by the diethylamine solvent.


17
11
9
4
3
6
7
10
2
1
5
8
10
Butanol : Methanol : [redacted] 0 10:10:2:5

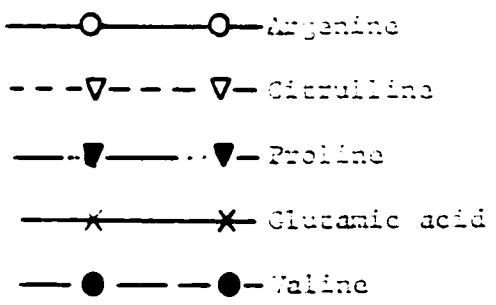
Figure 26b. Radioautogram of protein hydrolysate of Serratia marcescens grown in presence of $C^{14}O_2$.

1. Citrulline
2. Arginine
3. Aspartic acid
4. Glutamic acid
5. Lysine
9. Proline
10. Threonine
12. Valine
15. Isoleucine
K. Unknown degradation products of the hydrolysate.



b

Figure 27. Specific activity of the amino acids of a protein hydrolysate of Serratia marcescens which are related to glutamic acid, (cf. Figure 29) and valine, plotted against time of incubation.



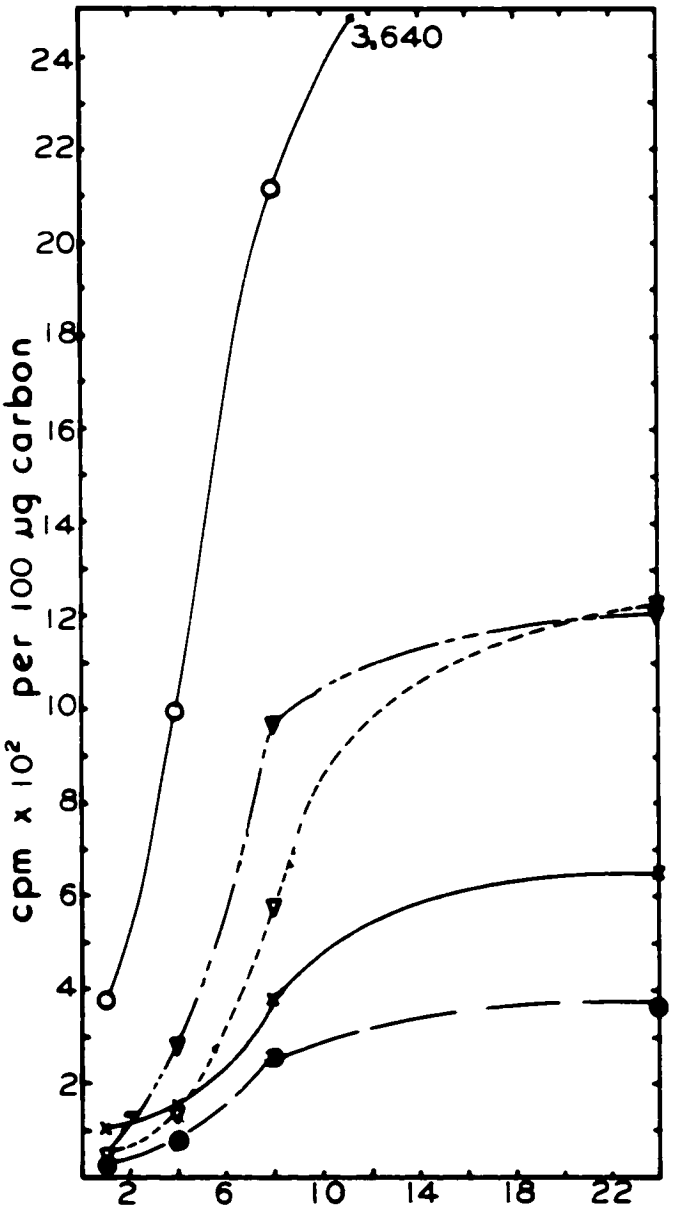
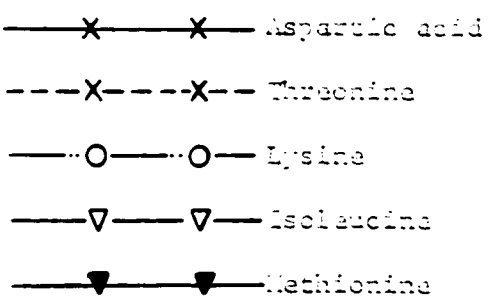
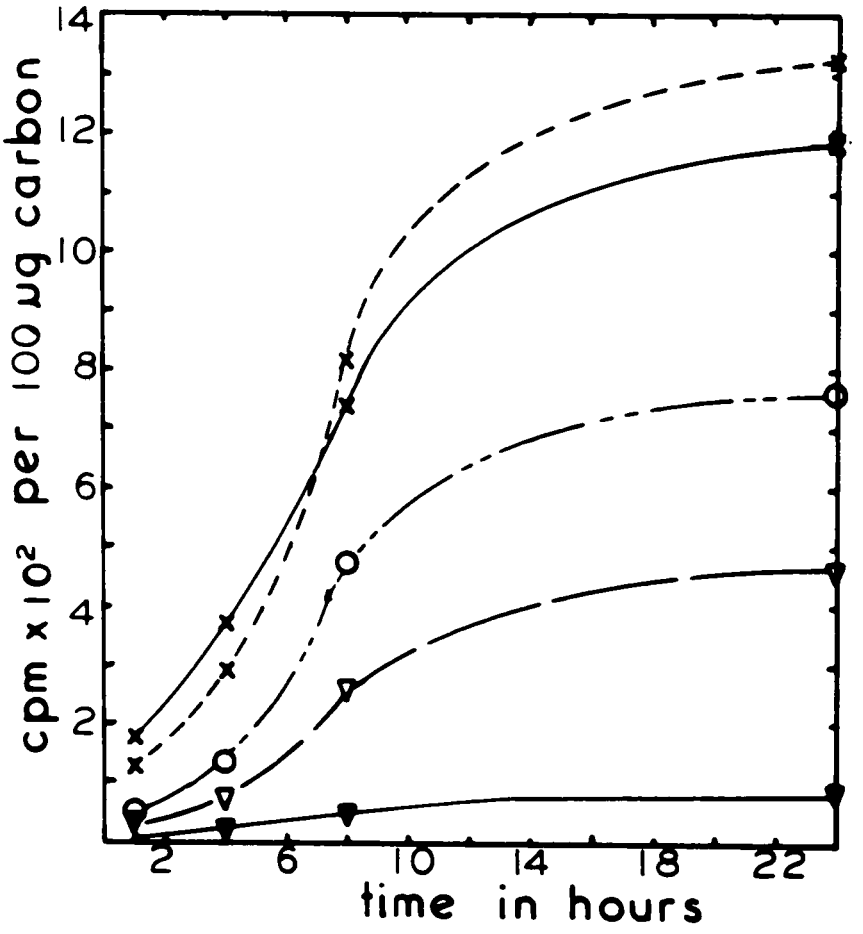

3,640
cpm x 10² per 100 µg carbon
24
22
20
18
16
14
12
10
8
6
4
2
2
6
10
14
18
22

Figure 28. Specific activity of the amino acids of a protein hydrolysate of Serratia marcescens which are related to aspartic acid (cf. Figure 29) (less valine), plotted against time of incubation.




cpm x 10² per 100 µg carbon
14
12
10
8
6
4
2
2
6
10
14
18
22
time in hours

V. DISCUSSION

A. Pigment and Pigmentation.

Many of the results will have to await further basic work before a sound or at least adequate explanation is available, but a few correlations can be drawn from the work which has been done. A report by Merchant (1954) that gamma radiation was useful in sterilizing solutions for tissue culture and the extensive literature on the effect of gamma radiation on various organisms (Salaman, 1947), prompted the present attempt to obtain mutants of Serratia by exposing a culture to radiation from radiosodium source (Na^{22}). Only orange mutants were obtained by this procedure, either from actively multiplying cultures (log phase) or from those at the end of their active growth. Viable counts indicated that gamma radiation did not kill any of these organisms, presumably because the high velocity of the gamma ray permits the rays to go through the organisms without causing ionizations and consequently sterilization. Identity of these mutants was established by various biochemical tests as outlined in Bergey's Manual (1948), and it was established that they were of the genus Serratia.

The need for some biochemical intermediate in a strain of organisms which normally do not require this intermediate constitutes a biochemical mutant.

The phenomenon which has been called syntrophism (Lederberg, 1950), i.e., the supplying of some necessary metabolite to one organism by another when they are cultured near or with each other, has been reported in Neurospora (Houlahan and Mitchell, 1947) and in E. coli (Lampen

et al, 1947). A similar phenomena was found in two mutants in this study, wherein the transmission of some factor by the orange mutant through the agar or across a dialysis membrane, caused certain white mutants to pigment. This was evidence of syntrophic feeding. Some biochemical intermediate is presumably necessary for the white strain to complete the biosynthetic process of pigment formation. A similar phenomenon for Serratia has been subsequently reported by Nizki (1954a and b). All attempts to isolate the agent responsible for the induced pigmentation were unsuccessful. Since this factor is dialyzable, it must be a relatively simple compound of small molecular weight.

The growth of the wild-type red organisms and the stable mutants, both orange and white, indicate that the pigment is not necessary for growth of the organism and is therefore probably a side product of the normal metabolism. This is borne out by growing the organisms either on a glucose medium (Ehrlich, 1934), or extract of beef heart (Goldsworthy, 1936, 1938), or else at a temperature above 35°C (Giolitti, 1949) where pigmentation is inhibited but growth is often accelerated. In further studying the various factors influencing pigment formation, it was found that replacing the phosphate buffer with tris-(hydroxyl, methyl)-aminoethane (tris buffer) enhanced pigmentation. This same effect was noted by Bunting (1949) who reported an enhanced pigmentation when phosphate was omitted from a medium containing 0.5 per cent peptone, which was inhibitory when large amounts of phosphate buffer were present. The cause for this inhibition is not known. Pigmentation in liquid culture was delayed for approximately 24 hours in minimal medium buffered with phosphate (Labrum and Bunting, 1949). In

contrast to this, pigmentation appeared at the end of 4 hours in the "tris" buffered medium in the absence of the inhibitory effect of the phosphate buffer. Addition of various amino acids, vitamins, and co-factors to any of the mutants did not induce red pigment formations in any of the mutants.

Extraction of the pigment from both the mutant and wild-type organisms and subsequent chromatography of the pigment into several fractions, established that prodigiosin is not a single compound, hence it appears that a re-evaluation of the structural configuration of the pigment is necessary. The existence of several components was earlier suggested by Weiss (1949), who chromatographed a butanol extract of the pigment on cellite columns. He demonstrated that the pigment separated into several bands, but overlapping of the different components on the columns and the presence of contaminating lipid substances in the butanol extracts, prevented a complete characterization of these components. The sharp separation and characterization of the different pigment fractions in this study shows quite conclusively that they are individual components of the original red pigment. This is contrary to the report of Nizhi (1954b), who states that the orange fraction isolated from Serratia marcescens is not a component of prodigiosin. The spectral analysis of the pigment shows that the orange component is responsible for a "shoulder" which appears on the spectral curve of the extracted red pigment in acid solution. This shoulder was attributed by Hubbard and Rimington (1950) to an anionic form of the red pigment that was presumed to be present in the acid solution. The absence of this shoulder in the eluted red fraction is

further evidence that all of the orange fractions have been removed and that the orange fraction is a naturally occurring component of the whole pigment. The fact that the red pigments can be isolated from the blue component as a unit, and that they yield three bands on rechromatography identical to those found in the whole pigment, further demonstrates that none of the fractions are decomposition products, but that they are actual components of the original pigment. Additional evidence for this is that a recombined mixture of the isolated pigment fractions behaves chromatographically and spectrophotometrically the same as does the unfractionated pigment.

The spectrophotometric data on the pigment studies agrees quite closely with that of Hubbard and Kimington (1950), demonstrating that the above acetone extracted pigment is similar to the pigment obtained by them. Hubbard and Kimington (1950) compared the spectral properties of a synthetic tripyrrylmethane substance to the extract of the pigment of Serratia marcescens. The similarity of the curves of the pigments and the synthetic tripyrrole indicates that the basic structure of the pigment is a tripyrrylmethene. An elemental analysis of the various pigments also suggests that they are basically tripyrrole derivatives. Further, the molecular weight determinations and the chloride content of the different pigment fractions indicates that the blue component is a polymer, or more specifically, a dimer of some of the red components. The blue component has approximately twice the molecular weight of the red pigment, and it contains only one-half the amount of chloride in the hydrochloride salt as the hydrochloride salt of the red fraction. The same conclusion was suggested from the results of

the time study on the accumulation of the various pigment fractions in the organisms. It was established that the red component appears first, followed by the appearance of a small amount of the blue component. The fact that the amount of the blue pigment then rapidly increases while simultaneously the rate of the red component formation is reduced, suggests that the blue was formed either at the expense of or by a condensation of the red components to form the blue pigment.

The orange component of the whole pigment from the wild-type strain of Serratia marcescens appears very early in the growth phase of the organisms and it is readily isolated from young cultures. This suggests that the orange component may be important in the formation of both the red and blue components, which make up the bulk of the total pigment. It is, however, apparently not identical with the orange pigment of the orange mutant. The orange mutant pigment has a low mobility on paper, whereas the orange component from the red, parent, organism is the most mobile of the pigment fractions; the pigment of the orange mutant remains orange unless exposed to strong acid fumes, while the SO_2 of the air will cause the orange component to become red. The orange fraction is rapidly destroyed by light while the pigment of the orange mutant is relatively stable, since it is destroyed only after prolonged exposure to light. The only similarities between the two pigments is a peak of absorption at 500 mμ in acid solution and that both exhibit fluorescence in the ultraviolet light.

Analysis of the pigments by Huffman Analytical Laboratories (Wheatridge, Colorado) indicated that the pigment contained an appreciable amount of ash. This observation stimulated a study of the metal

binding capacity of the various fractions with isotopic calcium (Ca^{45}) and iron (Fe^{59}). These studies showed that the isotopes were bound to the pigment by chelation similar to the chelates of pyrrole compounds such as hemoglobin, chlorophyll, catalase, and the cytochromes. The significance to the organism of this chelating capacity of the pigments is unknown. Dortels (1927) made a study of the requirements for iron, magnesium, and zinc for growth and pigmentation of several microorganisms. He found that both iron and magnesium were necessary for both growth and pigmentation in Serratia, and stated that in the absence of iron a leucobase was formed which became colored in a very short time upon the addition of ferrous iron salts. Waring and Werkman (1943) verified the results of these earlier workers and showed that both iron and magnesium were essential for pigment production. Bunting and Nemerly (1954), working with the Hy strain of Serratia marcescens, found that the addition of ethylenediamine tetraacetic acid or pyrophosphate markedly increased the occurrance of colorless variants in their cultures. They attributed this phenomenon to the binding of a metal in such a way that it was not available for pigment formation in the colorless colonies.

In order to obtain metal-free pigment, the pigments were routinely treated by washing with ethylenediamine tetraacetic acid (Versene). As in hemoglobin (Lemberg and Legge, p. 475, 1949), the removal of the metals from the pigment did not shift the spectral peaks, indicating that these do not contribute to the chromaphore properties, but do associate with the porphyrin molecule in such a way as to mask its fluorescence. However, removal of metals from porphyrin-like compounds

does change the ratio between the absorption peaks in the ultraviolet region and the peaks in the visible spectrum. The absence of metals in the pigments from Serratia marcescens caused a much stronger absorbance in the ultraviolet region. This increased absorption in the ultraviolet region was probably due to appearance of fluorescence after Versene treatment, since these pigments showed no fluorescence before treatment but did exhibit a marked fluorescence afterwards. This also has been observed with whole blood after addition of acid, thus fluorescence appears due to the liberation of the iron from the hemoglobin molecule. (Lemberg and Legge, p. 47, 1949).

B. Metabolic Studies.

In the study of the metabolic distribution of isotopic phosphorus (P^{32}), it was found there was an insignificant difference between the three strains studied, i.e., red wild-type, the orange mutant, and white mutant. A similar study with P^{32} was made by Roberts and Roberts (1950) and Cowie and Walton (in press) using Escherichia coli. The distribution of the radio-phosphorus between the trichloroacetic acid soluble, lipid soluble, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) fractions, was similar in magnitude to the distribution found in this study of the Serratia strains. (Table 7). Fisher (1954) found that in the trichloroacetic acid (TCA) soluble fraction inorganic phosphate was incorporated into adenosine, mono-, di-, and triphosphates as well as into precursor nucleotides. Approximately 10 per cent of the total incorporated phosphorus (P^{32}) was found in the lipid soluble fraction as phospholipids. The high radioactivity in the ribonucleic and deoxyribonucleic acid fractions was attributed to the

high phosphorus content of these fractions.

Since a small but consistent difference was noted in the radioactivity between the deoxyribonucleic acid fraction of the white organism and that of the wild-type red and mutant orange organisms, a 144 hour growth study was carried out to determine whether there were any significant differences in the amount of nucleic acids in these three strains of Serratia. No significant difference was found to exist. However, in the course of this study, an interesting observation was made; the RNA was at its highest concentration in the cell at the period of most rapid growth, and it decreased in amount as the cells ceased to multiply rapidly. This was not unexpected in the light of the concept that ribonucleic acid is, in part, functional in protein synthesis (Gale, 1956), acting in a way such that the amino acids line up along the RNA molecule, which serves as a template, and are then united into peptide linkeages to form, eventually, proteins. Therefore, the need for RNA is greatest at the time of most rapid protein synthesis. Deoxyribonucleic acid, on the other hand, which is concerned with the reproduction of cells, (Davidson, 1953), remained rather constant throughout the 144 hour period (Table 3). An analysis of the bases in ribonucleic acids gave a ratio of purines to pyrimidines equal to 1.4. The ratio of adenine plus thymine to that of guanine plus cytosine in deoxyribonucleic acid gave values of 0.70. These ratios are in agreement with the reported values of Zamenhof, et al (1952) and Elson and Chargaff (1955) which were 1.28 for RNA and 0.69 for DNA. These values place Serratia in the "GC type" of organisms wherein guanine

and cytosine predominate in contrast to yeast, viruses, and all of the higher organisms which are the "AT type" in which adenine and thymine predominate (Chargaff, 1959 a and b). The significance of this molar difference in thymine is not known at the present time.

The effect of added amino acids, nucleic acid derivitives, and other compounds upon the overall incorporation of $C^{14}O_2$ into Serratia marcescens was studied by McLean et al (1947, 1951 a and b, 1952 and 1955). However, these investigators did not study the distribution of the C^{14} products in the various cellular fractions of this organism. Roberts et al (1955), in a monograph on biosynthesis in Escherichia coli, studied the incorporation and distribution of various isotopically labeled compounds. In this study it was found that when glycine and acetate were used as the sole carbon sources (Table 3), the overall incorporation of $C^{14}O_2$ into the organism was markedly decreased. However, these two compounds had very little effect on the incorporation of carbon dioxide into the nucleoprotein fraction. On the other hand, the use of a glycerol medium, supplemented with yeast and casein hydrolysate, had only a slight depressing effect on the overall incorporation of $C^{14}O_2$, but markedly decreased the incorporation of $C^{14}O_2$ into the nucleoprotein fraction. The reasons for this difference will be discussed later.

On the basis of the above study, a glycerol -"tris" buffered medium was chosen to study $C^{14}O_2$ incorporation into the cellular components of Serratia. Gladstone et al (1935), Rahn (1941) and Lwoff and Monod (1947) have shown that the assimilation of carbon dioxide is essential for some metabolic processes in the organisms concurrent

with growth of the organisms. This need is supplied normally by metabolic CO_2 which is formed by the organisms from the degradation of carbohydrates. It was felt that the phenomenon of CO_2 assimilation may be a means of indicating a metabolic difference in the wild-type organisms as compared to the orange and white mutants. These three strains, grown for a period of 24 hours in a closed system, in the presence of $C^{14}O_2$ (as $NaHC^{14}O_3$), did not exhibit significant differences in the C^{14}-content in any of the cell fractions (Table 10).

It was felt that a time study during growth of the wild-type organism would lead to a better understanding of the biosynthetic processes involved in the assimilation of carbon dioxide in this strain of bacteria. The lag phase of this strain of Serratia marcescens is approximately one hour, followed by a period of rapid multiplication for approximately eight hours. A plateau in the rate of cell multiplication is reached in about 12 hours and continues for at least 24 hours before the difference in viable organisms and total organisms becomes detectable. For this reason, samples of organisms at one, four, eight, and 24 hours after innoculation were analysed, since these intervals represented cellular activity during the different phases of growth.

A complete analysis of the various fractions in Serratia was made by the procedure of Hutchman and Fetty (1955). An ether extraction of the medium and subsequent paper chromatography of the products established the presence of Kreb's cycle di- and tricarboxylic acids in this organism. The acids isolated and identified were aconitic, citric, succinic, fumaric, and malic acids. All of these acids contained

radioactive C^{14}, indicating that the Citric Acid Cycle is present and is one of the modes of CO_2 fixation in this organism.

No free amino acids were found in the acid soluble fraction in this organism. The absence of a free amino acid pool (or the existence of a minute pool) has been reported for other gram negative bacteria by Taylor (1947) and Roberts et al (1955). Analysis of the acid soluble fraction, as well as the alcohol soluble fraction, yielded several peptides, indicating that the amino acids are rapidly converted to peptides. These two fractions were hydrolysed in toto and yielded the same amino acids as were obtained by hydrolysis of the protein fraction. The acid soluble fraction yielded, in addition to the organic acids and peptides, various nucleic acid precursors which were located by their ultraviolet absorption on paper chromatograms. These were not identified or analysed further.

The amino acids obtained by hydrolysis of the protein fraction were chromatographed and analysed quantitatively and the C^{14} concentration was determined. The values found for the amount of amino acids present in the protein can only be considered relative since the more labile amino acids, such as tryptophane were destroyed by the acid hydrolysis (Hawk et al, p. 101, 1951).

It was found that only those amino acids closely associated with the Citric Acid Cycle show appreciable radioactivity. This can be considered as further evidence that the Citric Acid Cycle is in operation in these organisms and that the cycle is responsible in part for CO_2 fixation in these organisms. It is of interest that arginine has the greatest radioactivity of all the amino acids. This is due to the

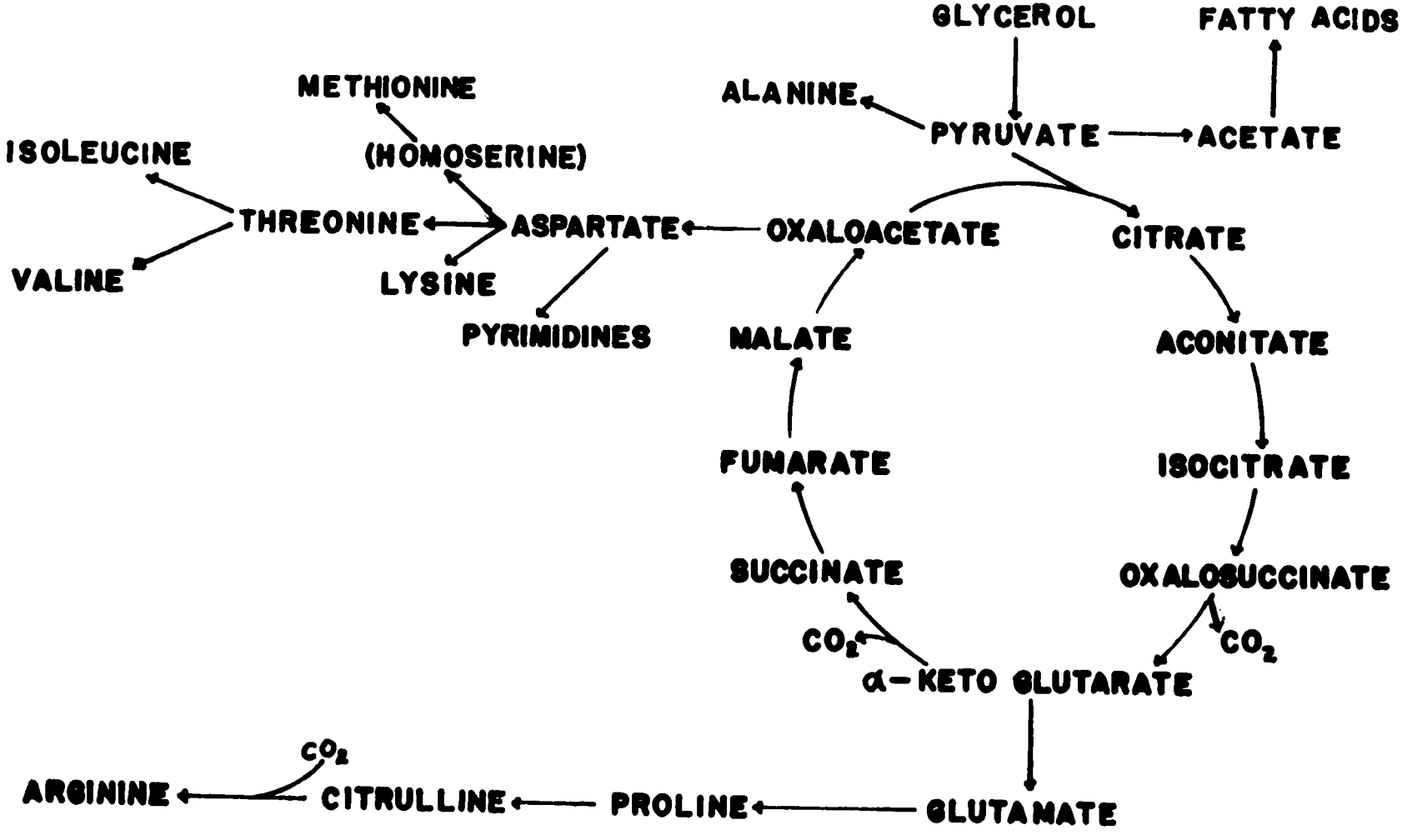
direct carboxylation of the amidino-carbon of the arginine (Roberts et al, 1955). These workers have shown that in E. coli the addition of non-radioactive glutamic acid to the medium results in the labeling of the amidino-carbon only in presence of $C^{14}O_2$, demonstrating that the remainder of the activity in arginine comes from glutamic acid. In this study with wild-strain Serratia, the specific activity of citrulline, the immediate precursor for arginine, has 75 to 80 per cent of the total C^{14} activity (Figure 29), and this value is almost directly comparable to the values reported by Roberts et al (1955) in E. coli.

The relationship of the labeled amino acids to the Citric Acid Cycle is shown in Figure 29. It was found that other amino acids, such as glycine, serine, leucine, tyrosine, phenylalanine, histidine, and cystine contained little or no isotopic carbon.

Determination of the specific activity of the purines and pyrimidines of both ribo- and deoxyribonucleic acids yielded results which are similar to those of Roberts et al (1955) in E. coli. In contrast to the report of Newton et al (1955) on Brucella abortus, however, the specific activity of thymine in these organisms was consistently lower than that of the other pyrimidines, cytosine, and uracil. The low activity of thymine found in this study indicates that possibly the schema for the direct conversion of thymine from uracil may not be the correct sequence for this organism. On the other hand, the high specific activities noted for cytosine, and uracil showed that these pyrimidines incorporated 1.6 times more C^{14} from radioactive carbon dioxide than did the purines, adenine and guanine, in ribonucleic acids at one hour of growth. At four hours this ratio was 1.8, 1.9, and at 24 hours

Figure 23. The interrelationships of the amino acids and the Citric Acid Cycle.

After Roberts et al (1955). Valine relationship after Adelberg (1955).


GLYCEROL
FATTY ACIDS
METHIONINE
ALANINE
ISOLEUCINE
(HOMOSERINE)
PYRUVATE
ACETATE
THREONINE
ASPARTATE
OXALOACETATE
CITRATE
VALINE
LYSINE
PYRIMIDINES
MALATE
ACONITATE
FUMARATE
ISOCITRATE
SUCCINATE
OXALOSUCCINATE
CO_2
CO_2
α-KETO GLUTARATE
CO_2
ARGININE
CITRULLINE
PROLINE
GLUTAMATE

of incubation it was 2.1. The probable reason for the higher radioactivity of the pyrimidines as compared to the purines can be seen in Figures 30 and 31. In the biosynthesis of the pyrimidines, aspartic acid is the immediate precursor for the formation of the pyrimidine ring. In these studies aspartic acid becomes labeled with $C^{14}O_2$, while glycine, which serves as the precursor for the synthesis of the purines, is not labeled. This fact adequately accounts for the difference in the labeling of the two compounds. The high specific activity of both the purines and pyrimidines, as compared to the other cellular fractions, is due to the direct incorporation of $C^{14}O_2$ from the medium. In these studies, as well as those on E. coli (Roberts et al, 1955), the ribose portion of the nucleic acid molecule was not labeled from radioactive carbon dioxide. The interrelationship of the nucleic acids and the amino acids is shown in Figure 32 (Roberts et al, 1955).

The specific activity of both the protein and the nucleic acids show a continuous increase during growth of Serratia marcescens for 24 hours. RNA does show a slight decrease in specific activity at the 24th hour, which may be due to the conversion of some of its constituents to the DNA (Dendich et al, 1950). All other cellular fractions, such as the acid soluble and alcohol soluble, decrease in specific activity due to two simultaneous events. The first of these is the decrease in specific activity of the $C^{14}O_2$ when it is used up in the formation of the intermediates for protein and nucleic acids synthesis, and the second is due to dilution of the $C^{14}O_2$ by the metabolic CO_2 derived from oxidation of the glycerol in the medium. The rate of increase in specific activity is steadily reduced with time, while

Figure 30. The biosynthesis of the pyrimidines showing the interrelationship of $C^{14}O_2$ assimilation and the biosynthesis of the pyrimidines.

'R' Designates ribose

* Designates C^{14}

After Reich (1956)

THE BIOSYNTHESIS OF THE PYRIMIDINES

(ABBREVIATED)

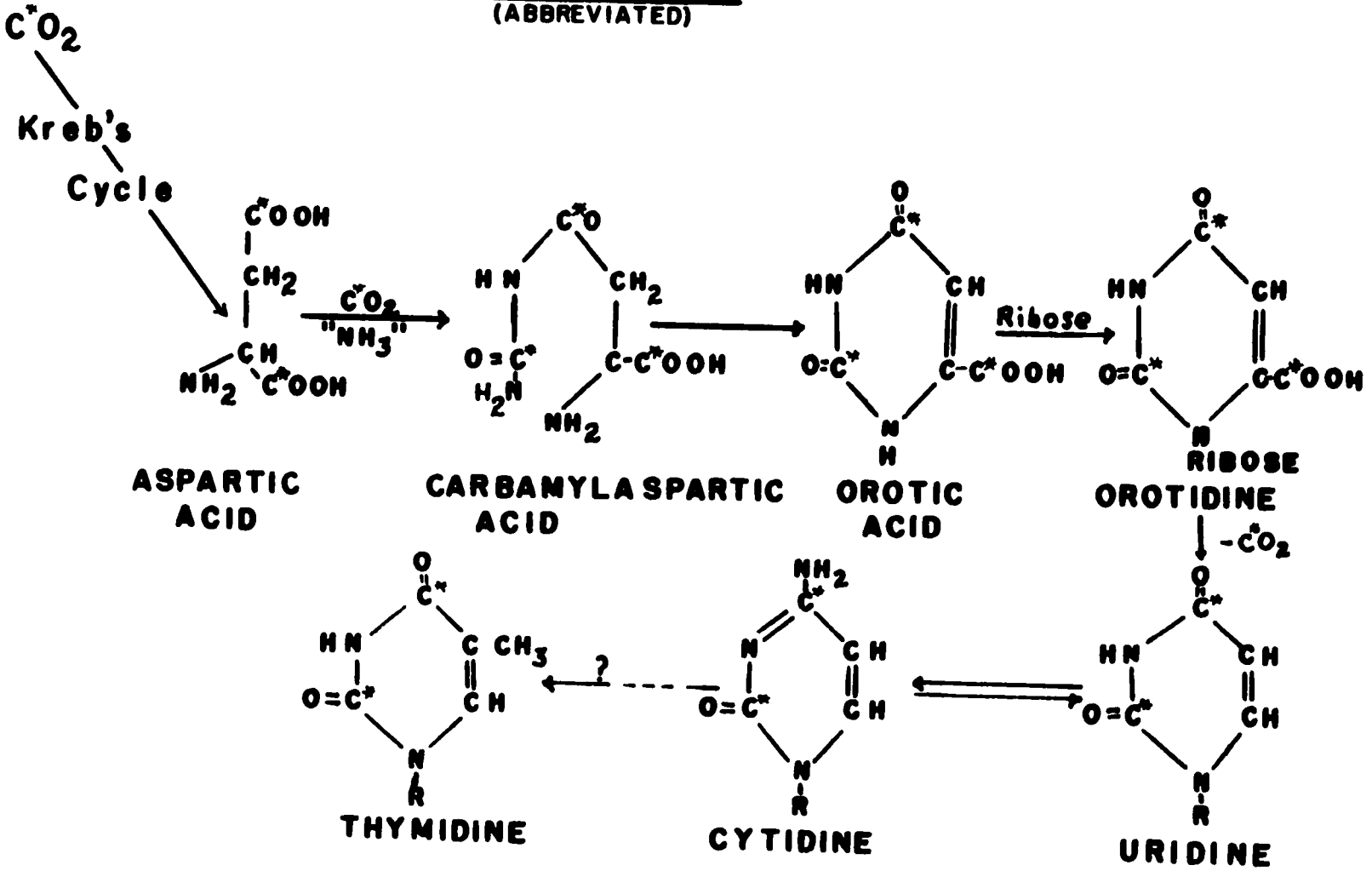


Figure 31. The Biosynthesis of the Purines, showing the point of incorporation of $C^{14}O_2$.

"P" designates phosphate.

* Designates C^{14} label.

After Buchanan (1955)

THE BIOSYNTHESIS OF THE PURINES (ABBREVIATED)

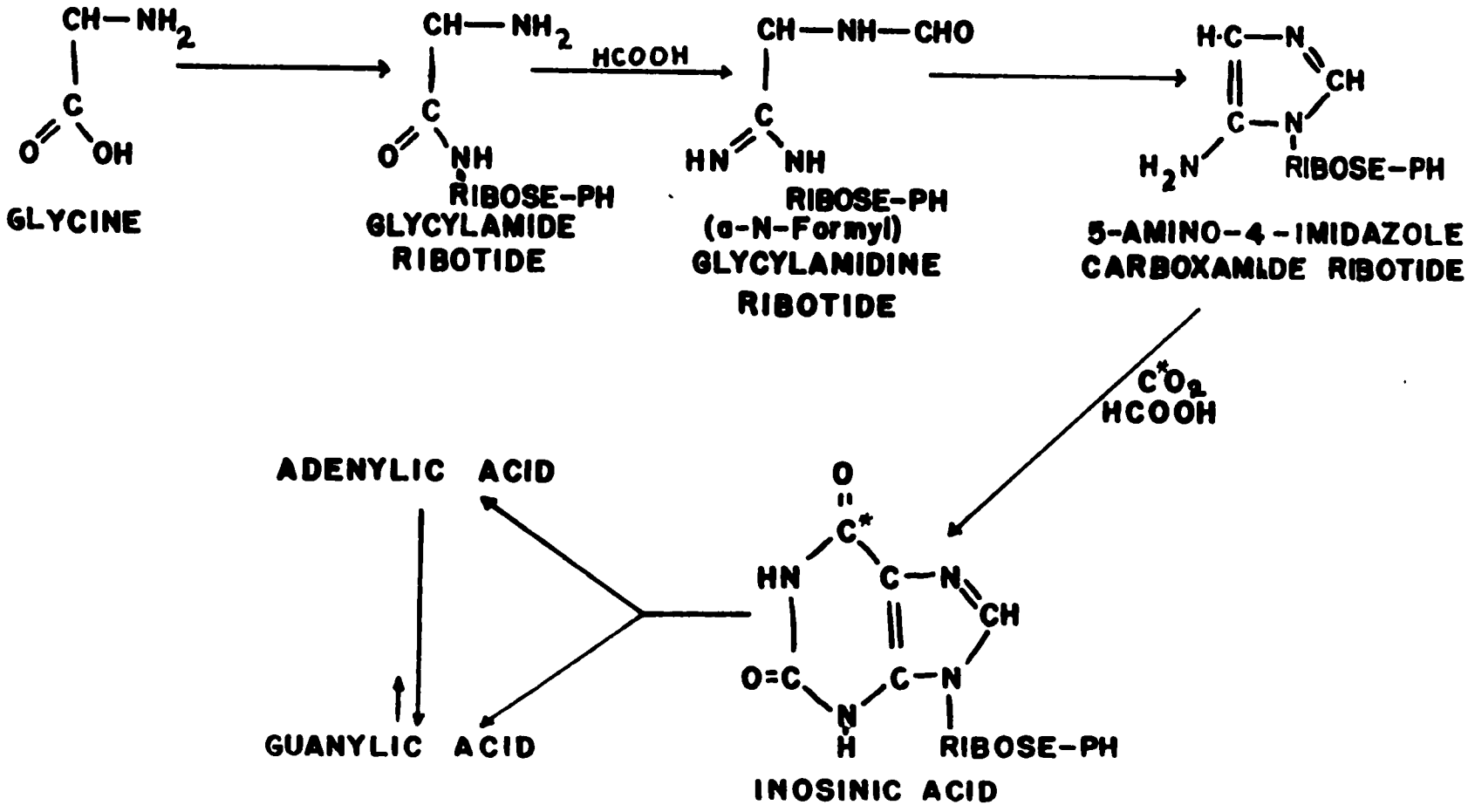


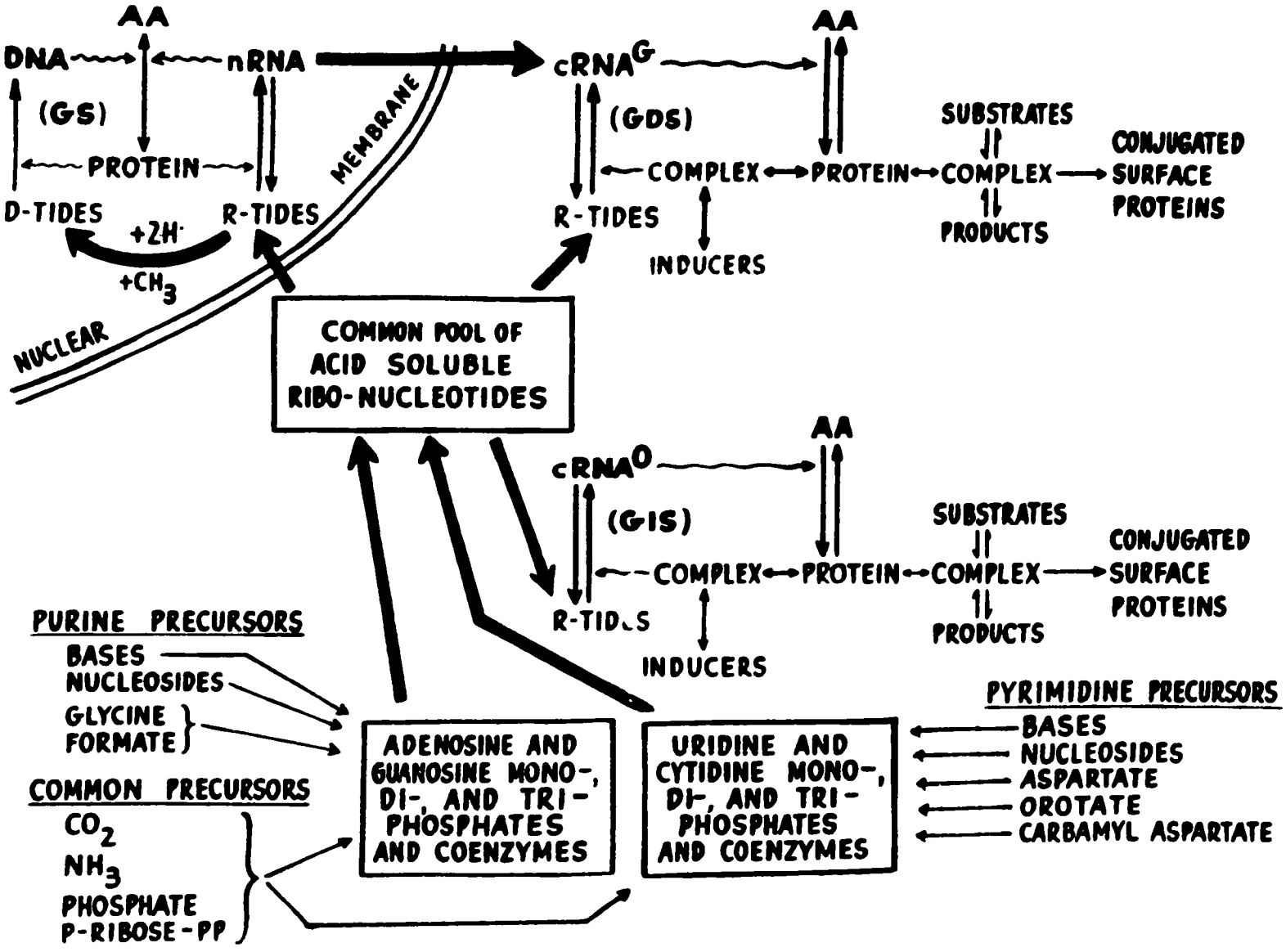
Figure 32. Tentative Relationships Between Genes, Enzyme-Forming Systems, Enzymes, and Cell Surfaces.

This figure is presented to show the relationships between the amino acids, proteins, and the synthesis of these by way of the nucleic acids and intermediates.

Legend

RNA = Ribonucleic Acid
DNA = Deoxyribonucleic Acid
GS = Gene System
AA = Amino Acids
EFS = Enzyme Forming System
GDS = Gene-Dependent EFS
GIS = Gene-Independent EFS

After Potter (1956)


AA
DNA
nRNA
(GS)
PROTEIN
D-TIDES
R-TIDES
+2H
+CH3
MEMBRANE
NUCLEAR
cRNAG
(GDS)
COMPLEX
PROTEIN
R-TIDES
INDUCERS
AA
SUBSTRATES
COMPLEX
CONJUGATED SURFACE PROTEINS
PRODUCTS
COMMON POOL OF ACID SOLUBLE RIBO-NUCLEOTIDES
cRNAO
(GIS)
COMPLEX
PROTEIN
R-TIDES
INDUCERS
AA
SUBSTRATES
COMPLEX
CONJUGATED SURFACE PROTEINS
PRODUCTS
PURINE PRECURSORS
BASES
NUCLEOSIDES
GLYCINE
FORMATE
COMMON PRECURSORS
CO2
NH3
PHOSPHATE
P-RIBOSE-PP
ADENOSINE AND GUANOSINE MONO-, DI-, AND TRI-PHOSPHATES AND COENZYMES
URIDINE AND CYTIDINE MONO-, DI-, AND TRI-PHOSPHATES AND COENZYMES
PYRIMIDINE PRECURSORS
BASES
NUCLEOSIDES
ASPARTATE
OROTATE
CARBAMYL ASPARTATE

simultaneously the rate of cell formation increases. The pigment fraction did not contain any radioactivity. Thus, in view of the work of Wittenberg and Shemin (1950) and Shemin (1955) on porphyrin biosynthesis and that of Hubbard and Rimington (1950) on the biosynthesis of prodigiosin, establishing acetate and glycine as true precursors for these pyrroles, the present results confirm the observation that $C^{14}O_2$ is not converted either to glycine or acetate in these studies with Serratia, and consequently the pigment produced is not labeled.

VI. SUMMARY

A. Pigments.

Several stable mutants of Serratia marcescens were obtained by exposing cultures to gamma ray and ultraviolet irradiation. These mutants were found to be identical to the parent organism with the exception of pigmentation. Mutants were obtained which exhibited rose, yellow, orange and brown pigments in contrast to the deep red pigment of the parent strain.

An interesting relationship was found to exist between some of the pigment mutants in that certain orange mutants caused white mutants to pigment when these were grown near each other in either broth or on an agar plate. The orange mutants did not have the ability to induce pigmentation in white mutants obtained from other strains of Serratia marcescens, thereby demonstrating a strain specificity.

The pigments of the parent red strain, the orange mutant, and the pigment induced in the white organisms were extracted with acetone, purified, and studied. It was found that the pigment from the wild-type organism was chromatographically separable into four components. One component was blue in color and had an Rf. of 0.20. A second component, red in color, was present in small amounts and had an Rf. of 0.43. Another red component exhibited an Rf. of 0.70. The fourth component was orange and possessed an Rf. of 0.89. The orange mutant pigment had a single component which gave an Rf. value of 0.40 in an ether, petroleum ether solvent. The pigment induced in the white organism by growing it with the orange strain had the same four

components that were present in the parent red strain.

All of the pigments were subjected to spectral analysis in the ultraviolet and visible regions of the spectrum. The curve obtained for the whole pigment was almost identical to the curve published by Hubbard and Rimington (1950). These workers considered the pigment to be a single substance. The spectral curve of the whole pigment in acid solution exhibited a prominent shoulder at 500 mμ. Hubbard and Rimington attributed the presence of this shoulder to an anionic form of the pigment. Chromatographic separation and spectral analyses of the various bands of the pigment demonstrated that the orange component had an absorption maximum of 500 mμ, while the red component, when freed of this orange component, exhibited no shoulder at 500 mμ. The fact that recombination of these two components resulted in a spectral curve which exhibited a shoulder at 500 mμ indicated that the orange component is a normal constituent of the whole pigment. The blue pigment had an absorption spectrum which exhibited low, wide absorption maxima. The relative light absorption of the red pigment was ten times greater than that of the blue pigment. The blue fraction caused a reduction in the light absorption of the whole pigment in the ultraviolet region of the spectrum.

A time study showed that the red pigments appear first in the culture followed by an accumulation of the blue pigment which surpasses the red pigment on a weight basis in 5 to 7 days.

Due to the instability of the orange component in light, insufficient quantities were collected for infrared analysis. The pigment was, therefore, separated into the blue and the combined red components by

paper and column chromatography as a unit. The spectral properties of these fractions were run in the infrared region. The similarity of the curves in all regions of the spectrum indicated that they were similar in their basic structure, probably differing only in side groups and configuration of the pyrrole groups.

Elemental analyses for carbon, hydrogen, nitrogen, and chloride as well as the molecular weight of the hydrochloride salts of the different pigments showed definite dissimilarities. The pigments form a monohydrochloride salt. The chloride and molecular weight determinations indicated that the blue pigment is a polymer, or more specifically, a dimer of the red pigments. This might account for the lower mobility of this fraction on paper and column chromatograms. This conclusion is further substantiated on the basis of the similarity of the nitrogen content of the blue and red fractions which would not be altered percentage wise if the condensation of two molecules of similar structure were to occur.

The pigments were found to bind polyvalent metals. A study utilizing the radioisotopes of iron (Fe^{59}) and calcium (Ca^{45}) indicated that iron was preferentially bound in the pigment as compared to the rest of the organism while calcium was equally distributed between the pigment and the cellular debris. It is noteworthy that the blue component binds several times as much iron as does the red component in these organisms. This difference in binding capacity may be attributed to a difference in the number of valences available for the sharing with the metal due to the variation in number of nitrogen atoms.

The pigment from the orange mutant was also analysed chromato-

graphically and spectrophotometrically. The pigment had an extremely high light absorption in the ultraviolet region and exhibited a 500 mμ maximum in acid solution. The similarity of the curves to the natural pigment in the ultraviolet, visible, and infrared regions of the spectrum indicated that the basic structure of this pigment was very similar to the pigments of the wild-type red organism.

The metal binding capacity of the pigment from the orange mutant was tested, using the radioisotopes of calcium (Ca^{45}) and iron (Fe^{59}). In contrast to the pigment from the wild-type organisms, the pigment from the orange mutant bound more of the calcium than it did of the iron. The reason for the binding capacity of all the pigments must await clarification of the structure of the pigments before definite conclusions can be made.

B. Metabolism.

The distribution of phosphorus (P^{32}) in the various fractions of the three strains, parent red, orange, and white mutants, demonstrated no difference in the metabolism of this compound. The relative distribution was found in those fractions which have the greater number of phosphorylated compounds, i.e., the supernatant, which contained both inorganic phosphorus and many phosphorylated intermediates, and the nucleic acid fraction.

Analysis of the molar ratios of the nucleic acid bases gave results comparable to those obtained by other workers for Serratia marcescens (Chargaff, 1951, 1952; Elson and Chargaff, 1955). The organism has a higher concentration of guanine and cytosine than adenine and thymine in contrast to other organisms and is, therefore, termed

a D-C type in accordance with Chargaff's (1951) classification. The amount of deoxyribonucleic acid remains rather constant over a long period of growth while ribonucleic acid reaches a peak at the time of most rapid growth and declines as the cells cease to multiply rapidly.

The incorporation of radioactive carbon dioxide ($C^{14}O_2$) in the organism reaches more than 30 per cent of the added radioactivity in 24 hours when grown in a medium limited in its carbon source. The higher concentration of radioactive carbon is found in the nucleic acid fraction and the amino acid, arginine. It is noteworthy that the di- and tricarboxylic acids associated with the Citric Acid Cycle also are labeled even though their specific activity does not become very high, presumably because of the rapid turnover of these components. The amino acids closely associated with the Citric Acid Cycle, namely glutamic and aspartic acids and the compounds which arise from these, attain a high specific activity while those amino acids more closely related to pyruvate (alanine, glycine, serine) are not labeled with radioactive carbon dioxide ($C^{14}O_2$). In these studies, the ribose was not labeled. The activity of the pigment was found to be comparable to the low activity found in glycine. The amino acid, arginine, has a very high specific activity because of the direct labeling of the amidine group from the medium.

The pyrimidines, uracil and cytosine, have a much greater specific activity than do the purines, adenine and guanine, because of the utilization of aspartic acid as a precursor in the biosynthesis of the pyrimidines.

It is of interest to note that the specific activity of ribonucleic

acid was higher than that of deoxyribonucleic acid in the first hours of growth but the two compounds had the same specific activity at 24 hours. This tends to bear out the conclusion that ribonucleic acid is converted to deoxyribonucleic in the synthesis of the latter (Davidson, 1953). A similar result is seen between the decrease in activity of the acid soluble fraction which contains the peptides and the protein fraction which increases in activity indicating the end product nature of the protein of the organisms.

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