STUDIES ON PIGMENT FRACTIONS PRODUCED BY SERRATIA MARCESCENS BIZIO

by

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INTRODUCTION

Prodigiosin, the pigment of Serratia marcescens Bizio is, perhaps, one of the most studied of the bacterial pigments. A romantic history can be attributed to this pigment from the many accounts of "bloody bread" recorded since the time of Alexander in 332 B.C. Not until the first scientific study of these "bloody spots" in 1819, were they attributed to the pigment of an organism, S. marcescens.

During the past sixty years numerous papers have appeared on prodigiosin in three general areas: (1) the effect of nutrition and environment on the pigment in culture, (2) genetic studies of the differently pigmented cultures, and (3) analysis of the extracted pigment chemically, chromatographically and spectrally. Of recent years, exhibition of in vitro antibiotic activity against protozoa, fungi, and bacteria has opened another area of study.

Chromatographic analyses of this pigment by various workers have shown it to be composed of several fractions. While conducting chromatographic studies of the pigment in this laboratory, it appeared that fractions were undergoing decomposition. It was, therefore, suspected that the fractions obtained by chromatography represented a series of decomposition products. To study this hypothesis, the fractions must first be isolated. Though two general methods of chromatography were used by previous workers, none appeared to be satisfactory. Those methods indicating numerous fractions did not produce complete separation, and the procedure which demonstrated complete separation of fractions only revealed a small

number of fractions possibly indicating a masking of fractions.

The purpose of the thesis, therefore, became twofold. Beside determination of whether the fractions demonstrated by chromatography were a series of decomposition products, a method for complete separation of the fractions by chromatography had to be developed.

A method of chromatography not used by previous workers for this pigment was chosen to obtain the necessary separation. This method, ascending paper chromatography, has the advantage that only small quantities of pigment are required as well as making detection of microquantities possible. The greatest advantage is that ascending chromatography offers a simple method for demonstration of decomposition of fractions. This method is termed "double chromatography."

REVIEW OF THE LITERATURE

Historically, prodigiosin, the pigment of Serratia marcescens Bizio has been of some consequence. Reid (1936) discussed several historical events which may be attributed to the pigmentation of S. marcescens.

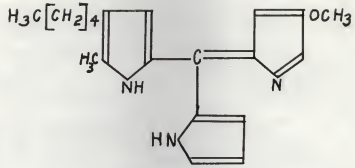
The siege of Troy in 332 B.C. was about to be disbanded because of ominous significance of blood-like drops in the bread. Since the spots were within the bread, an interpretation was offered that this indicated death to the troops within the city. Hence, resulted the triumphant conquest.

An epidemic of red-colored corn meal mush in various localities throughout Italy, in 1819, was notable from the standpoint

of leading to the first scientific explanation. Bizio (1924) conducted the first experiments during the epidemic in an effort to curb the hysteria of the superstitious peasants. He concluded that the red spots were produced by organisms and moreover, stated the necessary conditions for pigmentation as being a damp and warm atmosphere enhanced by putrid exhalations. In addition, he observed camphor, turpentine, and tobacco odors to have no effect on pigmentation, whereas, prolonged sulfur fumes and a temperature of 120° Reáumur (150°C) inhibited pigmentation. This early investigator described the young stage of the organism to be light pink gradually changing to a dark purplish-red which indicated the death of the organism. The examination of the properties of the pigment revealed it to be insoluble in water, soluble in alcohol, and decolorized by light.

Kodama (1929) proposed that prodigiosin was composed of three color elements, red, dark purple, and yellow which differed in solubility and chemical stability against oxidation and reduction. The red and dark purple components were described as large negatively charged particles; the yellow component as small positively charged particles. These components were found to differ in electrolysis and cataphoresis.

The structural formula for prodigiosin was evolved from several studies by Wrede and co-workers by 1934 as a tripyrrylmethene structure. Hubbard and Rimington (1949) presented the formula in its generally accepted form as follows:



The correctness of this structure was questioned by Bunting (1940), Weiss (1949) and Williams et al. (1956) because several different fractions were obtained by these investigators with chromatography.

Bunting (1940) and Weiss (1949) used column chromatography for the development of the fractions, whereas, Williams et al. (1956) and Green et al. (1956) employed circular paper chromatography. The details of the methods and results are found in Experimental Part A, Experiment I.

Prodigiosin in a neutral or acid solution was shown by Hubbard and Rimington (1949) to possess a main sharp spectral absorption band with a maximum at 537 mμ. A slight hump on the decending limb of the curve was observed at about 505 mμ. In an alkaline solution, a much lower and broader band was distinguished at 470 mμ.

Weiss (1949) endeavored to show that various strains of S. marcescens have specific differences as noted by their absorption spectra. He found that several strains could be assigned to three groups on this basis. The three types of absorption spectra occurred

only in strains grown at 23°C; when grown at 30°C, only one type of absorption spectrum was evident.

A change in absorption spectrum with age of a red-purple pigmented strain was next examined by Weiss (1949). The first 24 to 48 hours' growth produced a bright orange pigment with a broad absorption band from 480 mμ to 550 mμ with a small peak at 500 mμ. Increased age gave a visual red pigment, spectral absorption revealing a pronounced peak at 540 mμ. Further aging resulted in a visual purple hue with the absorption spectrum showing a secondary peak at 575 mμ.

Comparison by spectral absorption of the bands separated by chromatography from two strains, led Weiss to conclude that only the yellow bands had spectral absorption similarities, indicating it existed in both strains. A slight variation in the pigment molecule was suggested to explain the slight differences in the absorption spectra of other bands.

Hubbard and Rimington (1950) demonstrated two forms of prodigiosin to exist depending on the hydrogen ion concentration of the medium. The absorption spectrum of the red pigment in an acid solution is characterized by a sharp, main band with a peak from 535 mμ to 540 mμ, and a slight hump on the low wavelength of the curve at 510 mμ. The orange-yellow form, due to an alkaline solution, has a broader band at 470 mμ. These investigators commented on the similarity between the spectral curves of prodigiosin and those of a synthesized tripyrrylmethene, thus, giving support to the structure derived by Wrede and Rothhaas (1934).

Williams et al. (1956) investigated the spectral absorption of extracted prodigiosin in an acid, alkaline, and neutral solution and essentially confirmed the results of Hubbard and Rimington.

Pigment reduction was noted by Hefferan (1904b) when 24- and 48-hour and 10-day growths were exposed to sunlight for 5-, 15-, and 30-minute periods.

The region from 3800Å to 5000Å was specified by Kreitlow (1941) as suppressing pigmentation. Cultures sustained both under blue cellophane or a blue fluorescent lamp lost their pigment.

EXPERIMENTAL PART A. DEVELOPMENT OF AN ASCENDING PAPER CHROMATOGRAPHY METHOD FOR SEPARATION OF THE ORGANIC-SOLUBLE FRACTIONS OF PRODIGIOSIN

General Materials and Methods

Bunting (1940) obtained two fractions of prodigiosin by column chromatography. It was evident that this separation was incomplete as Weiss (1949), Williams et al. (1956), and Green et al. (1956) exceeded this number of fractions.

Weiss using three different strains, each necessitating a different solvent system, obtained 6, 5, and 9 fractions. The chromatogram which resulted in 9 fractions was obtained from the pigment extract of a 21-day-old culture, whereas, the other two chromatograms were obtained from 48-hour cultures.

The method employed by Weiss was inefficient for the proposed study for several reasons. Of concern, was the necessity of processing large quantities of pigment for development with column chromatography. Moreover, if one can assume that the diagrams

represent the actual chromatographic results, then complete separation of all fractions was not realized. The method would also be inefficient for this study on complete isolation of the fractions because it was necessary to cut the column, elute, reconcentrate, and redevelop in order to secure separation in a column of convenient length.

By use of circular paper chromatography, Williams et al. (1956) showed evidence of four components though complete separation was not obtained. In a second study by these workers, Green et al. (1956), in which circular paper chromatography was again employed, but with a different solvent system, complete separation of the four components was realized. This method of chromatography was also inadequate for the proposed study since isolation of fractions would again be difficult. As in the study of Weiss (1949), complete separation required eluting, reconcentrating and redeveloping and, therefore, would also be inefficient. That only four components were obtained by these workers in comparison to the 5, 6, or 9 obtained by Weiss (1949) could have arisen from two factors: (1) the method was inadequate to resolve the pigment into all its fractions, and (2) all the components did not develop under the conditions of cultivation. If due to the first, certainly this method was inadequate for this study. If due to the second, then it was hoped in this study to determine the conditions responsible for the increased number of fractions.

Because of the above inadequacies and because of the advantages of ascending paper chromatography as mentioned in the introduction, development of this method was warranted. The immediate

problem was, therefore, to find the solvent(s) for an ascending paper chromatography method which would completely separate prodigiosin into all of its fractions.

Stock Culture. The bacterium used for this study was S. marcescens, strain Nima, received from Dr. Robert P. Williams, Baylor University, who obtained it from Dr. E. D. Weinberg, University of Indiana. Though it was reported by Kharasch et al. (1936), Goldsworthy and Still (1936), and Weinberg (1951), that certain strains of S. marcescens failed to produce pigmentation on nutrient agar, the stock culture maintained in this laboratory on nutrient agar at 25°C gave excellent pigmentation for a period of over two years.

Cultivation for Pigment Production. Because of the excellent pigmentation of the stock culture on nutrient agar, this medium was used for the cultures from which the pigment would be extracted.

One hundred ml of nutrient agar were distributed into each of ten Blake bottles. Inoculation of the Blake bottles consisted of suspending the growth from a 48-hour nutrient agar slant in 40 ml of sterile, neutral, deionized water. Three ml of the suspension were pipetted into each of the ten Blake bottles which were then gently rotated to distribute the inoculum evenly over the surface of the medium. The inoculated bottles were inverted and incubated in the dark at 25°C.

Harvesting of the Cells. Thirty ml of neutral deionized water were introduced into the first Blake bottle. The growth was scraped with a bent glass rod followed by gentle rocking of the bottle to wash the remaining cells off the medium. This suspension was poured into a second Blake bottle and the process repeated. The process

was continued until all ten Blake bottles were harvested.

The entire harvesting process was repeated using 20 ml of neutral deionized water. The washings from this operation usually removed most of the remaining cells and were pooled with the original washings. Though the process could be repeated as many times as necessary, the object was to obtain as many cells as possible with a minimum of water to facilitate extraction. The pooled suspension was filtered through four layers of cheesecloth to remove any nutrient agar particles that may have been dislodged during harvesting.

Extraction of the Pigment. The method used for extraction was developed by Williams et al. (1956); however, slight modifications were introduced. The modifications were introduced only after demonstrating that no change occurred in the chromatogram as compared with chromatograms of the pigment extracted by using Williams' method.

Four volumes of acetone were added to the cell suspension and the mixture was shaken for one hour. This was centrifuged and the supernatant drawn off. The sedimented cells were resuspended in 50 ml of acetone, shaken for 30 minutes, centrifuged, and the supernatant pooled with the first extract. The second step was repeated until the supernatant showed no evidence of containing any pigment. This usually occurred after the third washing. The pooled supernatant was filtered through a 03 porosity Selas filter.

The pigment in the water-acetone phase was partitioned to Skellysolve B by mixing one volume of the water-acetone solution

with two volumes of Skellysolve B[1] in a separatory funnel. The acetone was removed by adding neutral deionized water to the separatory funnel, shaking, and drawing off the acetone-water phase.

In contrast to the finding of Williams and co-workers in which the acetone-water layer was colorless, pigment was partitioned into the acetone-water layer as evidenced by the reddish-brown color of this layer.

Small volumes of the original acetone-water extract were shaken with Skellysolve B, separated, and the two phases were collected in separate flasks. The flask containing the Skellysolve B was washed several times with neutral deionized water, until the washings were colorless. These washings were pooled into the flask containing the water-acetone fractions. The two pooled flasks were again washed with the opposite solvent, water or Skellysolve B. As these washings were colorless, they were discarded. The result was two flasks, one containing the "water soluble fractions" which will be termed thusly throughout the thesis, and the other containing the Skellysolve B soluble fractions which will be termed the "organic-soluble fractions."

The solutions of both flasks were individually concentrated *in vacuo* at 37°C. Each concentrated solution reduced to 4 to 5 ml was put into stoppered vials and stored in the refrigerator until used for chromatography.

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[1]Although Williams and co-workers used petroleum ether, preliminary studies by this laboratory showed no difference whether petroleum ether or Skellysolve B was used for extraction or chromatography. Therefore, since Skellysolve B was available, it was substituted for petroleum ether where necessary.

Method for Ascending Paper Chromatography. A 4.5 cm by 28 cm ungraduated glass cylinder was selected for the chromatographic chamber. To close the cylinder a number 10 rubber stopper was used into which a screw type brass hook was centered from which to suspend the paper strip.

Paper strips were cut 2.5 x 25 cm from sheets of #1 Whatman filter (chromatographic) paper. With a micropipette, 10 lambda of the concentrated pigment solution were applied to the paper 2 cm from the bottom of the strip and equidistant from both sides. The application of the pigment solution was controlled so that the diameter of the spot was not larger than 5 mm. The concentration of the pigment solution was arbitrarily adjusted by the addition of the appropriate solvent or in vacuo concentration in such a manner that tailing during development was kept to a minimum.

The chamber was saturated for 30 minutes with the desired solvent(s) prior to inserting the paper strip for development. The solvent was brought up to such a level in the bottom of the cylinder that the surface of the liquid was approximately 1 cm from the applied spot.

For ease of comparison of R_f values, the solvent front was usually allowed to ascend the strip to a distance of 20 cm from the applied spot.

Experiment I. Use of Solvents Employed by Previous Investigators

Though previous workers used different chromatographic systems, and their methods were considered unsuitable for the proposed study,

it was desirable to test these solvents in an ascending paper chromatographic system as the beginning of a "trial and error" method for determining the solvents which would separate the fractions.

The results of the chromatography by previous workers are diagrammatically represented on the left of the pages throughout this section. The results obtained employing the same solvents, step for step where possible, as the previous workers, but with ascending paper chromatography are represented by the diagrams on the right.[2]

The R_f values[3] for the results of the paper chromatography are given for only those fractions showing complete separation. R_f values were omitted where evidence existed for two different fractions which were not completely separated because of the difficulty of determining the center of each fraction. Where overlap of these fractions occurred, the probable outline of the fraction is represented by dotted lines based on the contour of the area of the fraction that is separated, the difference in density where the fractions overlap, and the change of color of the overlap representing a mixture of two pure colors. The numbers beside each fraction

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[2]The diagrams representing the work of Weiss (1949) are based on the diagrams appearing in the Journal of Cellular and Comparative Physiology, and the diagrams representing the work of Williams et al. (1956) and Green et al. (1956) are based on photographs appearing in the Journal of Bacteriology. The results of Bunting (1940) are diagrammed from the context of her paper. The diagrams representing the work of this laboratory are drawn to approximately 1/3 scale. The horizontal line above the uppermost spot represents the solvent front.

[3]R_f value = $\frac{\text{distance moved by the spot}}{\text{distance moved by the solvent.}}$ The distance moved by the spot was measured from the center of the spot.

represent the order in which the fractions separated from the applied pigment extract.

Use of Solvents Employed by Bunting. The first chromatographic study was conducted by Bunting (1940) using strain #274. The pigment extract was developed on an aluminum oxide column with petroleum ether (Figure 1).

By use of paper chromatography, four fractions were discernible but were not completely separated (Figure 2).

Use of Solvents Employed by Weiss. The next chromatographic study was conducted by Weiss (1949) with three strains of S. marcescens and three different solvent systems.

With strain JH1, the following 3-step method was employed:

(1) The pigment extract was adsorbed on a MgO column and washed with petroleum ether (Figure 3). To allow the remaining solvents to develop the pigment on the paper chromatograms, the petroleum ether was not allowed to ascend to the top (Figure 4).

(2) The same column was then developed with acetone in petroleum ether. The proportions of acetone were varied from 20 to 80 per cent (Figure 5). For paper chromatography, mixtures of 20, 50, and 80 per cent acetone in petroleum ether were used, each of which was in a different chamber. Each successive concentration was allowed to ascend 5 cm higher than the previous solvent. Figure 6 represents the resulting chromatogram after development with 80 per cent acetone in petroleum ether.

(3) The column was cut, the top brown and pink fractions were eluted and rechromatographed on another column using 1 to 10 per

cent ethanol in petroleum ether (Figure 7). Since no fractions remained at the bottom of the paper strip, development with ethanol in petroleum ether was not necessary. To determine whether ethanol in petroleum ether would effect the separation of fractions, a paper strip with the applied pigment extract was developed successively in three chambers containing 1, 5, and 10 per cent ethanol in Skellysolve B. The result was identical to that shown in Figure 6.

In a second 3-step chromatographic study by Weiss, strain ATCC-60 was used with the following solvent systems:

(1) The pigment extract was adsorbed on a MgO column and washed with petroleum ether (Figure 8). Since all the work reported herein was on strain Nima and since the first two stages of development are the same as above, no diagrams are presented. The result is the same as in Figure 6.

(2) The column was next developed with 20 to 80 per cent acetone in petroleum ether (Figure 9).

(3) The alcohol-soluble portion of the top brown and yellow bands were eluted on another column. It was then developed with acetone and ethanol in petroleum ether (Figure 10) in undescribed ratios. Again since no bands remained at the bottom of the strip in the system of this laboratory, this step could not be completed as such, but another strip with the applied pigment was developed with these solvents in an effort to determine if any fractions could be separated. Here also, the results were similar to Figure 6 except that a long tail was present.

The third 4-step chromatographic study conducted by Weiss was on a 21-day culture of strain JH2A. For standardization this laboratory used 5-day cultures throughout as Williams et al. (1956) and Green et al. (1956) did.

It was found by Weiss that a pigment fraction remained in the cell after extraction with butanol. Using an ethanol-ethyl ether mixture, he was able to extract this fraction and chromatograph it separately (steps 1 and 2 below). Though a small amount of pigment remained in the cells of strain Nima, it could not be extracted with this solvent mixture. Therefore, no comparative chromatograms can be offered.

(1) The pigment extract was adsorbed from petroleum ether on a calcium-celite column (Figure 11).

(2) The column represented by Figure 11 was then developed with 10 per cent ethanol in petroleum ether (Figure 12).

(3) The butanol-soluble fractions were developed with acetone in petroleum ether on another column (Figure 13). The result of this solvent and the following solvent for development of the paper chromatograms is the same as illustrated in Figure 6.

(4) The column represented by Figure 13 was then developed with an ethanol-acetone-petroleum ether mixture (Figure 14).

Use of Solvents Employed by Williams and Co-workers. Williams et al. (1956) obtained the results diagrammed in Figure 15 using ethyl ether and petroleum ether (1:2, v/v). These workers found better separation could be obtained if the paper was rendered basic by treating with ammonia fumes prior to development. Hence, this procedure was used for the comparative studies (Figure 16).

Use of Solvents Employed by Green and Co-workers. Williams and co-workers demonstrated four fractions, though separation of the fractions was not complete. Later these same workers (Green et al. 1956), through use of different solvent systems, were able to realize complete separation. The first solvent mixture consisting of 40 parts heptane, 4 parts tert-butyl alcohol, 3 parts dichloroethane and 94 parts petroleum ether was used to separate the blue fraction from the other three fractions (Figure 17). To separate the combined red fraction into its components, this fraction was eluted, reconcentrated and developed on a second paper with the following solvent mixture: 30 parts heptane, 3 parts tert-butyl alcohol, 2 parts dichoroethane, and 47 parts petroleum ether (Figure 19). It was not necessary to elute the blue fraction from the paper chromatograms as it could be removed by cutting the paper as indicated in Figure 18 and putting the strip with the combined red fraction into another chamber with the solvents listed above. The results are shown in Figure 20.

Since evidence of three fractions was obtained using the solvents employed by Williams et al. (1956) and Green et al. (1956), an attempt was made to resolve the components by varying the proportions of the solvents used by these workers. Also, whether using the same proportion of solvents or the varied proportions of solvents, a duplicate series was run without treating the paper with ammonia prior to development. In no instance were the components further developed.

Discussion. None of the chromatograms obtained by employing the same solvent systems used by previous workers resulted in

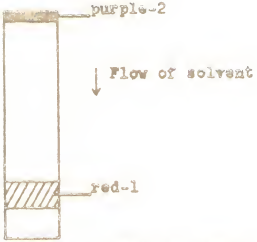


Figure 1. Bunting: Development of aluminum oxide column with petroleum ether.

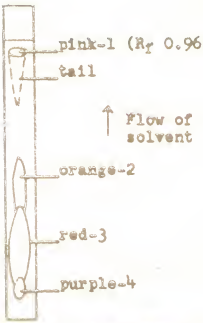


Figure 2. Development of paper strip with petroleum ether.

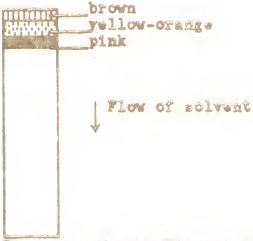


Figure 3. Weiss: Pigment extract of strain JH1 adsorbed on a magnesium oxide column and washed with petroleum ether.

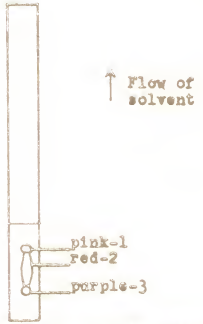


Figure 4. Partial development of paper strip with petroleum ether.

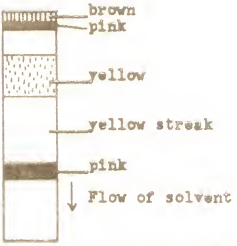


Figure 5. The column represented by Figure 3 developed with 20 to 80 per cent acetone in petroleum ether.

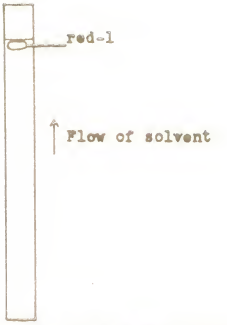


Figure 6. The paper strip represented by Figure 4 after successive developments with 20, 50, and 80 per cent petroleum ether.

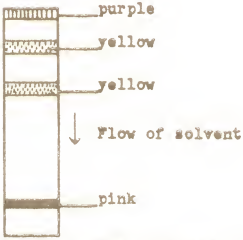


Figure 7. The brown and pink fraction of the above figure eluted and developed on another column with 1 to 10 per cent ethanol in petroleum ether.

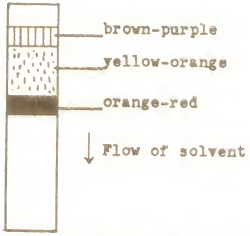


Figure 8. Weiss: Pigment extract of strain ATCC-60 adsorbed on a magnesium oxide column and washed with petroleum ether.

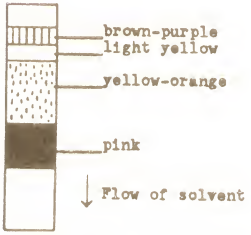


Figure 9. The column represented by Figure 8 developed with 20 to 80 per cent acetone in petroleum ether.

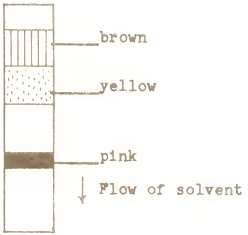


Figure 10. The alcohol-soluble fractions of the top brown and yellow bands in Figure 9 eluted and developed with acetone and ethanol in petroleum ether on different column represented by this diagram.

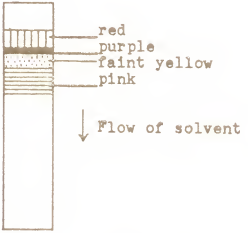


Figure 11. Weiss: Adsorption from petroleum ether on a calcium-celite column of the ethanol-ethyl ether extract from a 21-day culture of strain JH2A.

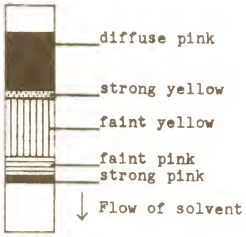


Figure 12. Development of the column represented by Figure 12 with 10 per cent ethanol in petroleum ether.

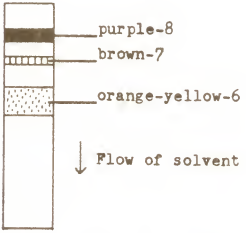


Figure 13. Development with acetone in petroleum ether of the butanol extract from a 21-day culture of strain JH2A.

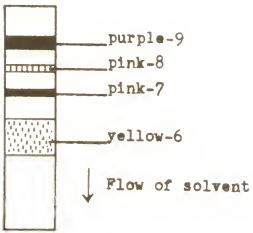


Figure 14. Development of the column represented by Figure 13 with an ethanol-acetone-petroleum ether mixture.

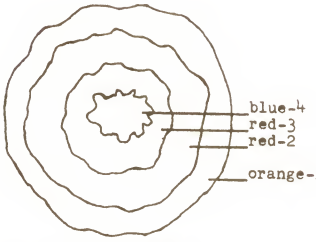


Figure 15. Williams and coworkers: Development of the pigment extract with a 1:2 (v/v) ethyl ether-petroleum ether mixture.

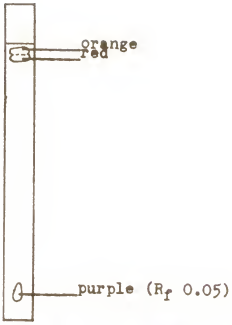


Figure 16. Development of the paper strip with a 1:2 (v/v) ethyl ether-petroleum ether mixture.

[4]This fraction turns red upon exposure to air.

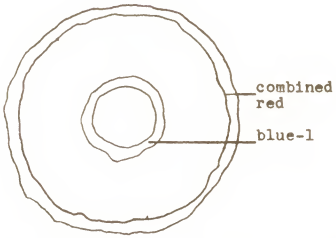


Figure 17. Green and co-workers: Development of the pigment extract with the following solvent mixture: 40 parts heptane, 4 parts tert-butyl alcohol, 3 parts dichloroethane and 94 parts petroleum ether.

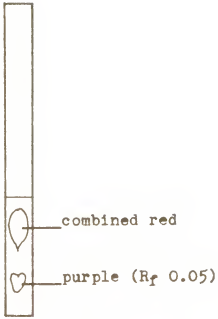


Figure 18. Development of the paper strip with the same solvent mixture used for development of the chromatogram represented by Figure 17.

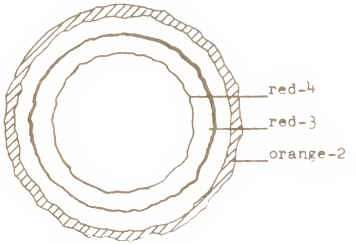


Figure 19. Development of the combined red fraction on the chromatogram represented by Figure 17. This combined fraction was eluted and developed on another paper with the following solvent mixture: 30 parts heptane, 3 parts tert-butyl alcohol, 2 parts dichoroethane and 47 parts petroleum ether.

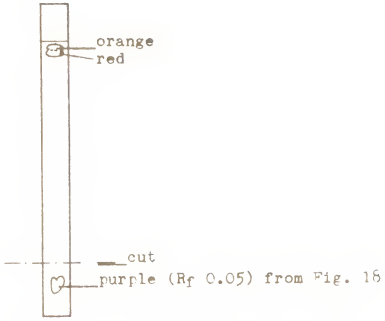


Figure 20. Development of the upper portion of the strip (represented in Figure 18) containing the combined red fractions. The strip was cut above the purple fraction before development with the same solvents used for Figure 19.

complete separation. This is understandable since other details of the systems were different. Varying the proportion of solvents, likewise, did not aid separation.

Using the same solvent as Bunting for development, evidence of four fractions was obtained, whereas, she only obtained two. These four fractions appear to be similar to those obtained by Williams et al. (1956) and Green et al. (1956). Use of the same solvents as these latter workers did not show this similarity, however. The noted difference is that one fraction was called red by these workers, whereas, one fraction was called pink by this laboratory. On the other hand, the orange fraction obtained in their work and in these studies changed to red upon exposure to air.

Experiment II. Studies to Determine the Conditions of Chromatography for Minimizing Tailing

Evidence of four fractions closely akin to those obtained by Williams et al. (1956) and Green et al. (1956) was obtained by use of Skellysolve B as the developing solvent (Figure 2). Since complete separation was not obtained because of the length of tailing, it was desired to find conditions which would minimize tailing.

In the following study of conditions, Skellysolve B was used as the solvent throughout the work. The solvent front of each chromatogram was allowed to rise the same distance (20 cm) from the applied spot. Particulars of materials and methods for each factor will be described in the appropriate section.

Chromatographic Paper. Block et al. (1955) pointed out that choice of paper was important in affecting the amount of tailing.

For this study, Whatman #3 paper, a heavy paper, was compared with Whatman #1 paper. In addition, since speed of solvent flow, which affects adsorption, differs depending on whether the paper is cut with the grain or across the grain, the paper was cut in these two directions and the chromatograms compared.

Comparison of the R_f values obtained on the two thicknesses of paper showed results identical to that of Figure 2 except that the fractions appeared less intense for the thicker paper without reducing tailing. Therefore, throughout the remainder of the study, Whatman #1 paper was used for ease of detection of spots. For equal discernibility of spots using Whatman #3 paper, more pigment would be needed. Use of Whatman #1 paper cut with and across the grain gave results identical to Figure 2. For standardization, the chromatograms for the remainder of the study were cut with the grain.

Chamber Saturation. For satisfactory paper chromatography, many workers feel that chamber saturation is of the utmost importance. The method employed in these studies was to saturate the chamber for 30 minutes prior to placing the paper strip in the chamber. Saturation for a longer period would be superfluous since the chamber has to be opened to insert the paper strip.

To avoid opening the chamber, the solvent was put in the chamber and the paper strip with the applied pigment spot was suspended from a wire hook which penetrated through the rubber stopper. The paper strip was not allowed to touch the solvent. To insure saturation, the junctions between wire and rubber stopper and glass

cylinder and rubber stopper were sealed with plasticine. After saturation for two hours, the paper strip was lowered into the solvent by pushing the wire from which it was suspended down through the rubber stopper.

The results of this type of complete saturation were less rewarding, i.e., less complete separation of spots than the not so fastidious type of saturation. For the remainder of the study the "incomplete type of saturation" was utilized.

Concentration of the Pigment. Heavy concentrations of substances to be chromatographed are known to produce tailing. To determine what concentration of pigment resulted in the least tailing, an aliquot of the original solution was diluted 1:2, 1:4, 1:8, 1:16. Ten lambda of each dilution were applied to the individual paper strips.

Separation was not complete at any concentration used although tailing was somewhat minimized at the lower concentrations. However, at lower concentrations the pink fraction (Figure 2) was barely discernible. Thus, the pigment solution was adjusted to the concentration in which all the fractions were easily discernible at the expense of tailing. In this particular instance, the 1:2 dilution appeared optimum.

Light. It has been generally observed that various light intensities and wave lengths affect the pigmentation of the growing culture. The possibility that light decomposes or alters the color of the fractions during chromatography was therefore considered.

The light intensities to which the chromatograms were exposed were as follows: (1) direct sunlight, (2) diffuse sky light (the

chromatographic chambers were placed at the furthermost distance from the window), (3) strong incandescent light (a 100-watt bulb placed 25 cm from the chromatographic chamber), (4) weak incandescent light (overhead lighting), and (5) total darkness.

The two temperatures available to the laboratory at which the light intensities could be varied were a room at 33°C, and an air-conditioned room at 23°C.

The results for the two temperatures were identical, however, the various light intensities greatly affected at least one fraction (Figure 21).

Least affected was the chromatogram developed in darkness (Figure 21-E). In the absence of light, four distinct fractions were discernible whereas the other light intensities produced various degrees of decomposition. From a comparison of the chromatograms, decomposition is evidenced by the appearance of a tail on a fraction, a long tail replacing a discrete fraction, or disappearance of a fraction. Strong sunlight (Figure 21-A) had the greatest effect as all the fractions were converted to a purple fraction.

Since development in darkness had no noticeable effect on the fractions, this condition was considered to be best for development. Hence, for the remainder of the studies the chromatograms were developed in the dark.

Temperature. It was established in the study on light that the fractions were decomposed least when developed in the dark. It was also shown that the chromatograms developed in the dark at 23°C or 33°C were identical. Apparently, this temperature range

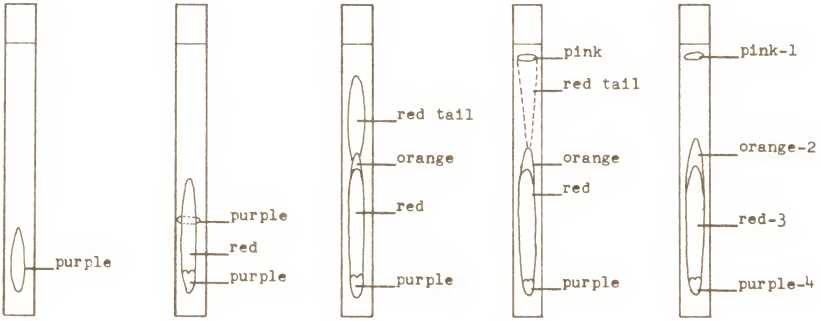


Figure 21. The effect of various light intensities on prodigiosin fractions during development.

was not critical for the development. It was the purpose of this study to determine whether fractions 2, 3, and 4 (Figure 21) could be further separated by development at temperatures more extreme than the 23 to 33°C temperature range.

Chromatograms were developed at constant temperatures of 3°C, 23°C, 33°C, and 37°C. Four strips of the same sample were developed simultaneously and in the dark.

Both chromatograms developed at 3°C and 37°C were inferior to those developed at either 23°C or 33°C. This was evidenced by increased tailing at 3°C and 37°C.

The remaining studies were developed at approximately 23°C since this temperature could be most easily maintained in the laboratory. The range of variation during the course of study was 23 to 27°C.

Treatment of Paper with Ammonium Hydroxide. Skellysolve B appeared promising as a solvent for development of the pigment even though the development had failed to yield complete separation with this solvent. Williams et al. (1956) pointed out that treatment of the paper with ammonia vapor enhanced separation. Such pre-treatment was therefore considered as an adjunct to development with Skellysolve B.

Pigment was applied to the paper strip which was then suspended in a large enclosed chamber having the bottom covered with ammonium hydroxide. The strip was not allowed to be immersed in the ammonium hydroxide. After a period of saturation for one hour in the dark, the strip was developed in Skellysolve B.

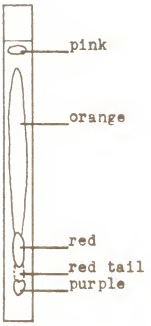
As an alternative method of treatment with ammonium hydroxide, 5 ml of ammonium hydroxide were placed in the bottom of the chromatographic chamber. A glass receptacle with a diameter, smaller than the chamber but wide enough to accomodate the paper strip was placed on the bottom. The height of the receptacle was such that the ammonium hydroxide did not spill into it. Five ml of Skellysolve B were then placed in the small receptacle. The paper strip was suspended above the Skellysolve B and saturation was allowed to occur for 30 minutes after which the paper strip with applied pigment spot was lowered into the Skellysolve B and developed.

Figure 22 presents the chromatograms after the two types of treatment with ammonium hydroxide. As a control (untreated with ammonium hydroxide) see Figure 21-E.

It is immediately evident from the diagrams that both treatments with ammonium hydroxide produced a change in the chromatogram as compared with the control. Also, the two treatments with ammonium hydroxide produced results differing from each other.

Discussion. Skellysolve B showed promise as a solvent for development of the organic-soluble fractions of prodigiosin. Four fractions were easily discernible; however, they were not completely separated because of the length of tailing. Several conditions which affect the development of the chromatograms were therefore studied in an effort to minimize tailing with this solvent.

The paper was first considered from the standpoint of thickness and grain. Whether Whatman #3, a heavy paper, or Whatman #1



A.
Paper treated with NH_4OH prior to development.

B.
Chamber saturated with NH_4OH during development

Figure 22. Treatment of the chromatogram with ammonium hydroxide.

was used or the paper was cut with or against the grain resulted in no difference in the amount of tailing. Perhaps if the chromatograms were developed to a greater height, these factors may have been important.

The chromatograms approached better separation with the "incomplete type of saturation". During the course of the "complete type of saturation" in which the paper strip with applied pigment spot was suspended but not immersed in the solvent, the pigment spot, because of the volatility of Skellysolve B, diffused three to four times the size of its original application. Possibly this factor resulted in the poorer chromatogram. During the "incomplete type of saturation" this spreading phenomenon did not occur.

The concentration of the pigment extract was next considered. The sample size was adjusted to that concentration which minimized tailing and yet did not extinguish the smaller fractions to the point of being difficult to observe. Hence, with every batch of pigment, this arbritary adjustment must be made.

From the study on light it was immediately evident that various degrees of decomposition occurred depending on the type of light. Total darkness (Figure 21-E) appeared to be the optimum condition for development of this pigment as the fractions appeared to be unaffected.

Subjection of the chromatogram to overhead incandescent lighting, resulted in a photochemical change of the pink fraction (Figure 21-D). As the pink fraction ascended, it was gradually converted to a red tail. Since this red tail did not move as the

Skellysolve B progressed up the column, it had an R_f similar to the purple fraction.

Development of the chromatogram in diffuse sky light (Figure 21-C) appeared to affect the chromatogram to a slightly greater degree. By the time the pink fraction reached the same height as when exposed to weak incandescent light, it was entirely converted to the red tail.

Strong incandescent light apparently produced a still greater photochemical change. As soon as the pink fraction was separated, it was rapidly converted to a red fraction which did not move further. Soon after, it changed to a purple fraction which also did not move. On the basis of color and R_f (nonmovement with Skellysolve B) this purple appeared to be identical to the purple, fraction 4 (Figure 21-E).

The most drastic change occurred when the developing chromatogram was exposed to strong sunlight (Figure 21-A). As the pigment began to ascend the chromatogram, it was entirely converted to the purple fraction. The change was so rapid that the sequence could not be noted.

Based on the above observations, it appeared that the sequence of decomposition occurred as follows. The fractions are numbered according to Figure 21-E.

$$\text{Pink-1} \longrightarrow \underset{\text{(Intermediate)}}{\text{Red tail}} \longrightarrow \text{Purple-4}$$

The red intermediate fraction does not appear to be the same as the red-3 fraction since once formed, it does not move up the

strip like the red-3 fraction when using Skellysolve B as the developing solvent. It may be a fraction of fleeting stability, as it does not appear when the chromatogram is developed under stable conditions (i.e. darkness). Furthermore, this fraction disappeared upon storage of those chromatograms revealing this fraction. It was believed that this red intermediate was converted to the purple fraction as in Figure 21-B, but was not detectable by the human eye. It may well be that the molecules comprising the red tail were so diffuse that upon conversion to the purple molecules, they were not detectable on a white background. In a later study in which the various solvents employed dispersed the concentrated purple fraction over a relatively short distance, the purple became difficult to see.

Further consideration is given this problem in a later section.

The photochemical effect was eliminated in the study of temperature since all chromatograms were developed in the dark. Thus, it was believed that the increased tailing at 3°C and 37°C was probably due to the chamber saturation since Skellysolve B is composed of several different hydrocarbons with differing boiling points. Evidently, the range of 23 to 33°C produced the optimum saturation with each hydrocarbon fraction of the Skellysolve B.

Treatment of paper and applied pigment spot by two methods with ammonium hydroxide did not produce complete separation. Each method, nevertheless, presented a particular advantage (compare Figure 22-A and B with Figure 21-E). Treatment of the paper strip

with ammonium hydroxide prior to development (Figure 22-A) appeared to separate the orange fraction better than simultaneous use of ammonium hydroxide and the solvent in the chamber. Also, almost complete separation of the purple fraction was realized. The chromatogram which was treated with ammonium hydroxide during development (Figure 22-B) yielded complete separation of the purple fraction at the expense of no separation of the other three fractions. Because of the advantage with each type of saturation, it was deemed feasible in the search for a developing solvent to employ both types of saturation in addition to saturation with the pure solvent vapor.

As a result of this study, the following conditions were found to be optimum or utilizable for purposes of standardization (1) Whatman #1 paper cut with the grain: (2) "incomplete" type of saturation; (3) pigment extract arbitrarily adjusted to minimize tailing without extinguishing the smaller fractions; (4) development in the dark at approximately 23°C; and (5) development in triplicate with three types of saturation (a) pure solvent, (b) paper strip pretreated with ammonium hydroxide, and (c) saturation with ammonium hydroxide in addition to the developing solvent. These conditions were used throughout the remainder of the study unless otherwise stated.

Experiment III. Use of a Variety of Single Solvents for Development

Not being able to resolve the organic-soluble fractions completely under various conditions of chromatography when Skellysolve B was used as the developing solvent, it was necessary to resort to other solvents.

Materials and Methods. The conditions used for chromatography were those considered to be optimum or for purposes of standardization as listed in the discussion of the previous experiment.

The solvents tested[5] consisted of alcohols, hydrocarbons, cyclic compounds, and a variety of others. These were as follows:

(1) Acetic acid, glacial*
(2) Acetone
(3) iso-Amyl alcohol
(4) tert-Amyl alcohol
(5) Benzene
(6) n-Butanol
(7) Carbon tetrachloride
(8) Chloroform
(9) m-Cresol
(10) Decane*
(11) Ethanol
(12) Ethylene chloride
(13) Ethyl ether
(14) Heptane*
(15) n-Hexane*
(16) 2-4, Lutidine
(17) Methanol
(18) Pentane*
(19) n-Propanol
(20) 2-Propanol
(21) Pyridine
(22) Skellysolve B
(23) Toluene
(24) Water
(25) Xylene (commercial)

Results. The results of this study are listed in Tables 1 to 3. Table 1 contains the results of saturating the chamber with the solvent only; Table 2 the results of saturating the chamber with the solvent and ammonium hydroxide simultaneously; and Table 3 the results of treating the paper strip and applied pigment spot

[5]The solvents with an asterisk were not run in triplicate. The straight-chain hydrocarbons studied gave results, similar, if not identical to Skellysolve B. Since Skellysolve B was most available to this laboratory, it was the only straight-chain hydrocarbon solvent further studied.

with ammonium hydroxide for one hour prior to development. Only those solvents which produced a distinct separation of at least one fraction are recorded in the tables. Those solvents not recorded either did not move the applied spot, or moved the entire sample spot with no or only partial separation.

The criteria for purity of fraction was a discrete entity, without excessive tailing and of a homogeneous color. The homogeneity of color must be emphasized. Invariably, when a spot consisted of more than one fraction, a difference in density of various areas or difference of color at the fringes was noted. Such spots were listed as combined followed by the most predominating colors. Where evidence existed for the spot being composed of several fractions because of partial separation as Figure 21-E, fractions 2, 3, and 4 or Figure 22-B (combined yellow and red), the fractions were also listed as combined but followed by the colors of the fractions partially separated. The R_f value was measured from the center of the entire combined spot although it may have been spread for some distance along the paper strip.

Since the color of the saturated spot in the chamber often differed from that when exposed to air, both colors were recorded.

Discussion. It has been demonstrated in a previous section that four fractions were in evidence with Skellysolve B as a developing solvent. Only one fraction, pink, was clearly separated by this solvent. Twenty-four other solvents of varying chemical structure were chosen in an effort to obtain a complete separation of the organic-soluble fractions. Although the chromatograms were

Table 1. Fractions obtained by development in the chamber saturated with a single solvent only.

Solvent	Color of spot in chamber	Color of spot exposed to air	R_f value
Benzene	combined (red) purple	combined (red) purple	0.95 0.05
Carbon tetrachloride	combined {pink / orange} purple	combined {pink / red} purple	0.82 0.05
Chloroform	combined (red) purple	combined (red) purple	0.95 0.08
Decane	pink combined {orange / red / purple}	pink combined {red / red / purple}	0.79 0.19
Ethyl ether	combined (red) purple	combined (red) purple	0.97 0.05
Heptane	pink combined {orange / red / purple}	pink combined {red / red / purple}	0.93 0.08
n-Hexane	pink combined {orange / red / purple}	pink combined {red / red / purple}	0.90 0.30

Table 1. Concluded.

Solvent	Color of spot in chamber	Color of spot exposed to air	R_f value
Pentane	pink	pink	0.08
	combined {orange, red, purple}	combined {red, red, purple}	0.28
Skellysolve B	pink	pink	0.94
	combined {orange, red, purple}	combined {red, red, purple}	0.28
Toluene	combined {pink, orange}	combined {pink, red}	0.72
	purple	purple	0.08
Xylene	combined {pink, orange}	combined {pink, red}	0.68
	purple	purple	0.05

Table 2. Fractions obtained by development in the chamber saturated with a single solvent and ammonium hydroxide.

Solvent	Color of spot in chamber	Color of spot exposed to air	R_f value
Benzene	combined (orange) purple	combined (red) purple	0.97 0.08
Carbon tetrachloride	combined (orange) purple	combined (red) purple	0.97 0.09
Chloroform	combined (orange) purple	combined (red) purple	0.97 0.09
Ethylene chloride	combined (orange) purple	combined (red) purple	0.97 0.05
Ethyl ether	combined (orange) purple	combined (red) purple	0.97 0.06
Skellysolve B	combined {orange, red} purple	combined {red, red} purple	0.91 0.05
Toluene	combined (orange) purple	combined (red) purple	0.90 0.05
Xylene	combined (orange) purple	combined (red) purple	0.91 0.06

Table 3. Fractions obtained by development of paper pretreated with ammonium hydroxide.

Solvent	Color of spot in chamber	Color of spot exposed in air	R_f value
Benzene	combined (orange) purple	combined (red) purple	0.97 0.08
Carbon tetrachloride	combined {pink orange} purple	combined {pink red} purple	0.91 0.07
Chloroform	combined (orange) purple	combined (red) purple	0.97 0.14
Ethylene chloride	combined (orange) purple	combined (red) purple	0.97 0.05
Ethyl ether	combined (orange) purple	combined (red) purple	0.97 0.06
Skellysolve B	pink combined {orange red purple}	pink combined {red red purple}	0.98 0.40
Toluene	combined {pink orange} purple	combined {pink red} purple	0.86 0.05
Xylene	combined {pink orange} purple	combined {pink red} purple	0.83 0.05

developed in triplicate with two types of ammonium hydroxide treatment, no one solvent was successful for securing a complete separation. However, with several solvents, a different fraction, purple, could be clearly separated.

The straight-chain hydrocarbons, pentane, hexane, heptane, and decane with no other treatment of the paper or chamber gave chromatograms similar if not identical to Skellysolve B (Figure 21-E) under identical conditions. Thus, a pure pink fraction was obtained at the top of the strip by all these solvents. On the lower portion of the strip in descending order were orange, red, and purple spots which were not completely separated.

It is now possible to separate completely two fractions by using two solvents in succession. The combinations are numerous. Care must be used in selecting a combination from the tables that the proper sequence in regard to treatment with ammonium hydroxide is used. For example, if the purple is separated first by using any of the solvents listed in Table 2 in which the chamber is saturated with ammonium hydroxide, then the pink fraction cannot be separated after the extreme basic treatment of the paper. The pink and purple may be separated in any sequence using combinations of solvents in Tables 1 and/or 3. It was found preferable to develop first with Skellysolve B as the completed chromatogram displayed smaller fractions.

Typical of the many possible combinations is the result shown in Figure 23. Skellysolve B (no treatment with ammonium hydroxide involved) was first used to separate the pink fraction. After the

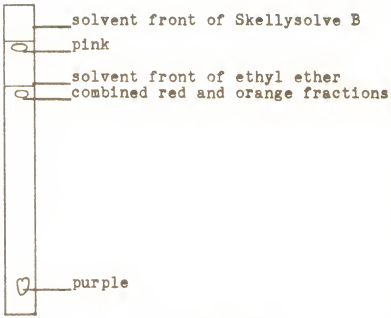


Figure 23. Separation of two fractions by development with two solvents in succession.

pink fraction reached the uppermost portion, the strip was removed, dried in the dark, and redeveloped with the second solvent, ethyl ether (no treatment with ammonium hydroxide involved).

It would be possible with the method just described to use square chambers with square paper. The first phase could be developed with two solvents to line the fractions on one side of the paper. The paper could be turned in a second chamber so that the side with the fractions would be on the bottom. Various other solvents could then be tested to separate the red from the orange fraction.

Rather than explore this possible method of separation, it was decided to test solvent combinations in an effort to array the fraction on one strip. Use of solvent combinations was deemed feasible as the added partitioning effect may enhance separation. This will be studied in the next experiment.

Examination of Tables 1 to 3, showed that the following solvents did not separate a fraction under any condition: acetic acid, acetone, iso-amyl alcohol, tert-amyl alcohol, n-butanol, m-cresol, ethanol, 2-4 lutidine, methanol, n-propanol, 2-propanol, pyridine, and water. It is noteworthy that the "organic-soluble fractions" of prodigiosin are not developed by polar solvents. Of the solvents just listed, water was the only solvent which did not move the applied spot.

Experiment IV. Use of a Mixture of Solvents for Development

As demonstrated in the previous experiment, two pure fractions could be obtained by use of numerous combinations of single

solvents. The red fraction 3 and orange fraction 2 (Figure 21-E) were not separated by any one single solvent.

It was the purpose of this experiment to separate in one operation all four fractions by using a mixture of solvents. If this could not be accomplished it was at least desired to separate the red fraction from the orange, thereby, in conjunction with the previous separation, obtain four well-defined fractions.

Materials and Methods. It is evident that the number of possible solvent mixtures is astronomical. Therefore, since Skellysolve B showed four fractions very near separation, it was chosen as the basic solvent to which other solvents would be added to improve the partitioning effect, thereby, allowing for the possible completed separation. The solvents added to Skellysolve B were:

(1) Acetone
(2) iso-Amyl alcohol
(3) tert-Amyl alcohol
(4) Benzene
(5) n-Butanol
(6) Carbon tetrachloride
(7) Chloroform
(8) m-Cresol
(9) Ethanol
(10) Ethylene chloride
(11) Ethyl ether
(12) 2-4 Lutidine
(13) Methanol
(14) n-Propanol
(15) 2-Propanol
(16) Pyridine
(17) Toluene
(18) Xylene (commercial)

A 1:1 mixture was chosen as the test mixture. If a particular mixture showed promise of separation, then it could be varied over a range of percentages to find the optimum combination of the two component solvents.

As before, the chromatograms were developed in triplicate under the following conditions: (1) chamber saturated with solvent mixture only, (2) chamber saturated with solvent mixture and ammonium hydroxide, and (3) chamber saturated with solvent mixture with the paper and applied pigment spot pretreated for one hour with ammonium hydroxide.

Results. The three treatments are tabulated in Tables 1 to 3 respectively in the same manner as the previous experiment.

Discussion. Though complete separation into four distinct fractions was not realized with any of the solvent mixtures, it is readily seen from Tables 4 to 6 that four solvent combinations each produced three separate spots. The solvent mixtures involved were: (1) benzene and Skellysolve B, (2) carbon tetrachloride and Skellysolve B, (3) toluene and Skellysolve B, and (4) xylene and Skellysolve B. The separation into three spots occurred whether the paper was untreated or treated with ammonium hydroxide but not when the chamber was saturated with ammonium hydroxide.

The spots were arrayed in descending order: (1) pink, (2) combined, and (3) purple. The spot designated combined represented the orange and red fractions (2 and 3) shown in Figure 21-E. The appearance of this combined fraction varied depending on: (1) the particular solvent combination, (2) whether the paper was treated with ammonium hydroxide, and (3) whether it was exposed to air.

Thus, the results obtained by use of solvent combinations were identical to the results obtained using development with two separate solvents (i.e. the alignment of the same three spots). However,

Table 4. Prodigiosin fractions obtained by development in the chamber saturated with a solvent mixture only.

Solvent mixture in 1:1 ratio	Color of spot in chamber	Color of spot exposed in air	R_f value
Skellysolve B plus Benzene	pink	pink	.97
	combined (orange)	combined (red)	.92
	purple	purple	.03
Skellysolve B plus Carbon tetrachloride	pink	pink	.95
	combined (orange)	combined (red)	.70
	purple	purple	.05
Skellysolve B plus Ethylene chloride	combined {orange / red}	combined {red / red}	.97
	purple	purple	.06
Skellysolve B plus Ethyl	combined (orange)	combined (red)	.95
	purple	purple	.05
Skellysolve B plus Toluene	pink	pink	.97
	combined {orange / red}	combined {red / red}	.38
	purple	purple	.03
Skellysolve B plus Xylene	pink	pink	.98
	combined {orange / red}	combined {red / red}	.66
	purple	purple	.02

Table 5. Prodigiosin fractions obtained by development in the chamber saturated with a solvent mixture and ammonium hydroxide.

Solvent mixture in 1:1 ratio	Color of spot in chamber	Color of spot exposed in air	R_f value
Skellysolve B plus Benzene	combined (orange) purple	combined (red) purple	.98 .05
Skellysolve B plus Carbon tetrachloride	combined (orange) purple	combined (red) purple	.97 .06
Skellysolve B plus Chloroform	combined (orange) purple	combined (red) purple	.95 .09
Skellysolve B plus Ethylene chloride	combined (orange) purple	combined (red) purple	.97 .06
Skellysolve B plus Ethyl ether	combined (orange) purple	combined (red) purple	.95 .05
Skellysolve B plus Toluene	combined (orange) purple	combined (red) purple	.97 .05
Skellysolve B plus Xylene	combined (orange) purple	combined (red) purple	.92 .05

Table 6. Prodigiosin fractions obtained by development of paper pretreated with ammonium hydroxide.

Solvent mixture in 1:1 ratio	Color of spot in chamber	Color of spot exposed in air	R_f value
Skellysolve B plus Benzene	pink	pink	.97
	combined (orange)	combined (red)	.80
	purple	purple	.03
Skellysolve B plus Carbon tetrachloride	pink	pink	.97
	combined (orange)	combined (red)	.64
	purple	purple	.03
Skellysolve B plus Chloroform	combined (red)	combined (red)	.97
	purple	purple	.07
Skellysolve B plus Ethylene chloride	combined (orange)	combined (red)	.97
	purple	purple	.06
Skellysolve B plus Ethyl ether	combined (orange)	combined (red)	.97
	purple	purple	.02
Skellysolve B plus Toluene	pink	pink	.97
	combined (orange)	combined (red)	.58
	purple	purple	.03
Skellysolve B plus Xylene	pink	pink	.96
	combined (orange)	combined (red)	.62
	purple	purple	.02

the method using a solvent mixture was more efficient in that development could be accomplished in one operation, whereas, the previous required two operations.

Experiment V. Complete Separation of the Organic-Soluble Fractions.

In Experiment IV, it was found that four solvent mixtures produced three spots. Two of these spots appeared to be pure fractions (pink and purple), whereas, the third appeared to be an unseparated red and orange fraction. In every instance, early in the development the red and orange fractions were clearly distinguishable but unseparated. As development was completed, the individuality of these fractions composing the center spot disappeared. By varying the percentage (v/v) of the solvent mixed with Skellysolve B, it was anticipated in this study that the red and orange fraction would separate, meanwhile maintaining the isolation of the pink and purple fraction.

Materials and Methods. Of the four solvent mixtures giving three spots, toluene and Skellysolve B (1:1, v/v) was chosen for further study on the basis that the middle spot was more nearly developed at an intermediate distance from the pink and purple fractions. Should the results from this mixture be unrewarding then the study would be continued with the other three solvent mixtures.

Ten ml each of a 10, 25, 50, 75, and 90 per cent (v/v) toluene in Skellysolve B mixtures were distributed in five chambers. A duplicate series was set up for which the paper and pigment spot

were pretreated with ammonium hydroxide for one hour. Chambers saturated with ammonium hydroxide as in the usual procedure were omitted since complete separation did not appear promising. The chromatograms were developed employing those conditions considered optimum (Experiment III).

Results. The two series of chromatograms (paper untreated and paper treated with ammonium hydroxide) are diagrammed in Figures 24 and 25, respectively.

The chromatograms presented in Figure 24 gave the first indication that the orange-2 and red-3 fractions shown in Figure 22-E, were the same component of prodigiosin. The color change from red to orange was caused by subjection of the red fraction to a different H ion concentration. As the slow-moving red fraction ascended the paper strip, the H ion concentration of the paper being different from the extract changed the leading edge of the red fraction to a faster moving orange fraction.

The chromatogram represented by Figure 24-E evolved through the same sequence of changes as the chromatograms from left to right. That is, as the red was converted to orange, the red fraction diminished and the orange fraction increased. Each chromatogram in the series went through the same sequential steps, left to right, as those preceeding.

For the other series diagrammed in Figure 25 in which the paper was treated with NH_3, the same can be said. Here, however, the applied spot and paper strip were pretreated with ammonium hydroxide causing some of the red fraction to be converted to orange before development of the chromatogram. This can be seen

by comparison of Figure 24-A with 25-A which also represents the picture presented early in the development of Figure 24-E and Figure 25-E.

Further verification that the red and orange are the same component is that intense treatment with ammonium hydroxide converts the red-3 to an orange fraction which then ascends the paper strip at a rate comparable to the orange-2 fraction.

Without realization, complete separation of the organic-soluble fractions had been secured many times over in Experiment III by use of two single solvents in succession and in Experiment IV by use of four different solvent combinations. In all cases, a pink fraction was arrayed at the top, an intermediately located orange and/or red fraction and a purple fraction at the bottom.

"Complete separation", as used above, should perhaps be qualified since it was noted that a small weak red fraction periodically occurred masking a small area of the purple fraction. Should the same solvent system be used on consecutive days, this fraction was not always found.

By use of single solvents for development of the chromatograms on which the weak red fraction appeared, it was found that iso-amyl alcohol (paper untreated or pretreated with ammonium hydroxide) separated the remaining fractions from it.

As a result of these studies, the following methods have been developed for the complete separation of the organic-soluble fractions:

(1) Develop the applied pigment spot with iso-amyl alcohol (paper untreated with ammonium

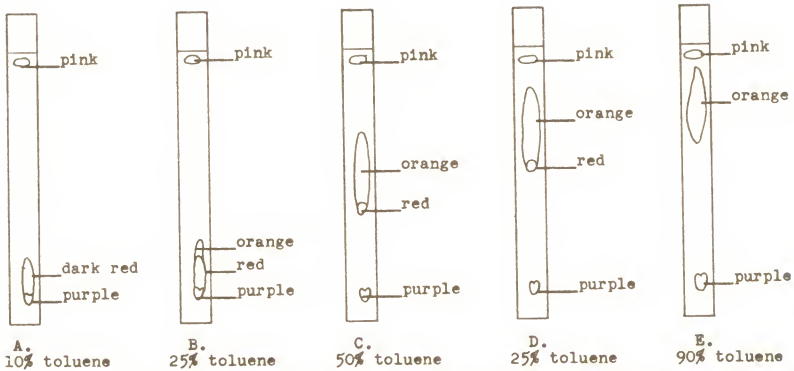


Figure 24. Chromatograms of organic-soluble components of prodigiosin on untreated paper developed with various toluene-Skellysolve B solvent mixtures.

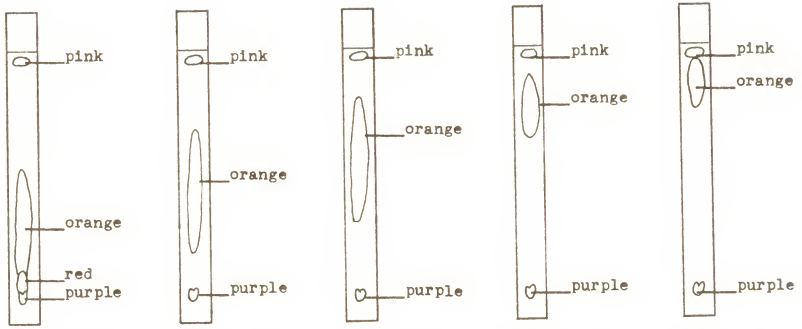


Figure 25. Chromatograms of organic-soluble components of prodigiosin on paper pretreated with ammonium hydroxide developed with various toluene-Skellysolve B solvent mixtures.

hydroxide) to separate the weak red fraction, if present, from the remaining combined fractions. This development is shown in Figure 26.

The combined fractions should be permitted to ascend only to that height at which separation is complete. This is necessary to allow for a sufficient length of the paper strip to complete the separation with other solvents.

(2) Dry the iso-amyl alcohol-developed strip in the dark.

(3) Develop the chromatogram represented by Figure 26 with the two single solvents in succession listed in Experiment III or with the combined solvents listed in Experiment IV.

When using the two single solvents in succession, it may be necessary to cut the strip as indicated by the broken line in Figure 26. The first solvent Skellysolve B does not move the weak red fraction, however, the second single solvent may move it up into the other fractions. Only when the second single solvent moves the red fraction, will it be necessary to cut the strip to prevent the weak red fraction from interfering with the completion of the separation. This problem does not occur when the solvent mixtures are used.

Examples of chromatograms obtained employing both methods are shown in Figures 27 and 28, respectively. The resulting chromatograms demonstrate the complete separation of the organic-soluble fractions.

The development by use of two single solvents successively proceeds as follows:

(a) Develop the upper portion of the chromatogram represented by Figure 26 with Skellysolve B (Figure 27-A).

(b) Dry the strip in the dark.

(c) Redevelop with ethyl ether (Figure 27-B). The solvent front employing ethyl ether is allowed to ascend just above the top of

the strip is shown in Figure 27-B to show the complete array of fractions.

The procedure for development with the solvent mixtures involved adjustment of the concentration of the pigment extract and/or adjustment of the concentration of the solvent mixed with Skellysolve B in order to obtain the orange-red (pH variable) fraction intermediate between the purple and pink as discussed in Experiment IV. The results in Figure 28 were obtained by development of the chromatogram represented in Figure 26 with 50 per cent toluene in Skellysolve B after adjustment of the concentration of the pigment extract.

Discussion. Hefferan (1904a) noted that cultures of S. marcescens were violet-red on an acid medium and orange-red on an alkaline medium. Bunting (1940) found the red band obtained by column chromatography to be bright red in acid and yellow in alkali. By treatment of the pigment extract composed of all fractions with buffered solutions, Weiss (1949) found prodigiosin to be rose-red to orange-red on the acid side of the pH scale and orange-yellow to yellow on the alkaline side.

The orange and/or red, pH-dependent fraction-3 observed in this study was believed to be responsible for the above color changes noted by Hefferan, Bunting and Weiss. None of the other fractions obtained showed a change of color when treated with N/1 hydrochloric acid or N/1 sodium hydroxide. When treated with N/1 sodium hydroxide, however, the orange (pH variable fraction) changed to yellow. Bunting demonstrated that the red band obtained by column chromatography produced this color change, also in alkaline solutions. However, this red band was probably composed of other fractions since she only obtained a purple and a red fraction

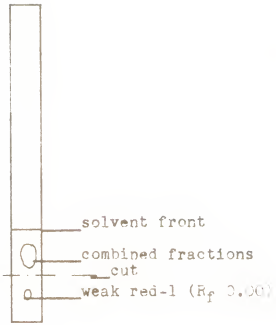
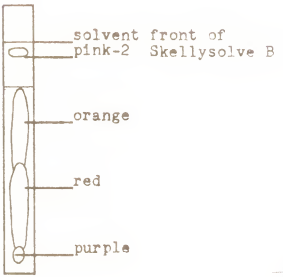
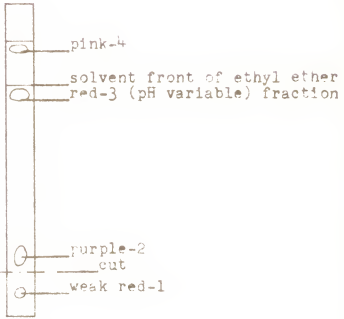


Figure 26. Separation of the weak red fraction by development with iso-amyl alcohol.



A.
Development of top portion of strip (Figure 26) with Skellysolve B.



B.
Development of Figure 27-A with ethyl ether. The bottom portion of strip that was removed is diagrammed to demonstrate the complete array of fractions.

Figure 27. Complete separation of the organic-soluble fractions by employing single solvents in succession.

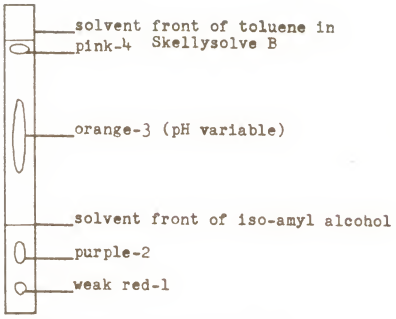


Figure 28. Complete separation of the organic-soluble fractions by separation of the weak red fraction with iso-amyl alcohol followed by development with 50 per cent toluene in Skellysolve B.

in comparison to the several obtained by Weiss (1949), Williams et al. (1956), Green et al. (1956), and this laboratory.

One may assume that the initial pigment extract used for this study was in an acid condition since it was deep red. According to Williams et al. (1956), Whatman #1 chromatography paper is slightly acid. Hence, as the acid red-3 fraction moves up the nearly neutral paper strip, it will acquire the pH of the paper. At pH 6.5, the pigment extract prepared by Weiss showed a orange-red color and at 7.0, it was orange. This, therefore, accounts for the more acid red fraction turning orange as it ascends the slightly acid chromatography paper.

The orange fraction when exposed to air turned red similar to the acid condition. When treated with ammonia fumes it returned to the orange state. Upon exposure to air again, it reverted to the red form. This phenomenon of change of color was recognized by Williams et al. (1956). No mention was made by these workers that the orange fraction was a different form of the red-3 fraction. It does not seem likely that upon exposure to air, that the CO_2 could produce acid conditions sufficient for this color change as the dissociation constant of carbonic acid is 3.5×10^{-7} at $18^{\circ}C$. One of the difficulties in the appraisal of the phenomenon is that the pH of the paper and the pigment cannot be accurately obtained.

Two methods were used to obtain the complete separation of the fractions. Each required the use of iso-amyl alcohol for separation of the first, weak red fraction. They differed in that one required two more developments (use of single solvents)

whereas, the other method required only one more development (use of a solvent mixture) to complete the separation. The method which employed the use of the solvent mixture appeared to be more efficient since a total of only two developments were required, whereas, the method which employed single solvents required a total of three developments. However, the method which employed the solvent combination required that the pigment concentration and/or the percentage of solvent mixture be adjusted to develop the pH variable red-orange-3 fraction intermediate in position between purple-2 fraction and pink-4 fraction. Within limits, the adjustment of the concentration of the pigment extract for development with single solvents is not necessary. Another advantage of the single solvent method is that fraction 3 (pH variable) occupies a much smaller area when several of the single solvents are employed.

To obtain the chromatograms for the diagrams of Figure 24, the concentration of the pigment extract was adjusted in such a manner that the orange-red pH variable fraction could be arrayed intermediate between the pink and purple fractions when using 50 per cent toluene in Skellysolve B.

Both of the described methods resulted in the fractions being arrayed in line on a paper strip. Such paper strips can be used in the Spinco Analytrol, permitting quantitative studies of the fractions.

The above methods can also easily be adapted to two-phase chromatography by development with a different solvent in each direction, resulting in an even greater dispersion of the spots.

EXPERIMENTAL PART B. DEVELOPMENT OF AN ASCENDING PAPER CHROMATOGRAPHY METHOD FOR SEPARATION OF THE WATER-SOLUBLE FRACTIONS OF PRODIGIOSIN

It was found in this study that upon extraction of the water-acetone layer with petroleum ether a reddish-brown pigment remained in the water-acetone phase. Since the pigment remained soluble in the water after the evaporation of the acetone, this pigment could not be partitioned into Skellysolve B like the other organic-soluble fractions. It was the purpose of this study to characterize these fractions by ascending paper chromatography.

Materials and Methods

The solubility of the water-soluble fractions was tested by evaporation of aliquots of the extract in test tubes. Solvents were added and shaken to determine whether the dried pigment would go into solution. The solvents tested were: methanol, ethanol, n-propanol, n-butanol, and acetone.

The method for chromatography of the concentrated extract was identical to that employed for the organic-soluble fractions. The solvents tested to develop these fractions were:

(1) Acetone
(2) Methanol
(3) Ethanol
(4) Skellysolve B
(5) Water, neutral deionized

Results

The dried pigment in contrast to the organic-soluble fractions which gave a red amorphous film appeared upon examination with a

hand lens to consist of purple crystals and yellow amorphous material. The overall appearance was brown. The dried pigment was found to be soluble in methanol, ethanol, n-propanol, and n-butanol, as well as water, but not acetone. It was noted on prolonged drying that the purple fraction formed a purple precipitate which was not soluble in any of the above solvents or Skellysolve B.

Complete separation was obtained by development of the applied brown spot with neutral deionized water. This resulted in a purple and yellow spot as diagrammed in Figure 29.

Acetone and Skellysolve B failed to develop the fractions. The applied brown spot remained unchanged. Both methanol and ethanol produced long, brown streaks.

Neither the yellow nor the purple spot underwent a color change when treated with N/1 NaOH or N/1 HCl.

Discussion

By use of the same criteria for purity, used in Experimental Part A, Experiment III, the water-soluble fractions appeared to be composed of two fractions, purple and yellow.

Bunting (Figure 1, this thesis), Williams et al. (Figure 15, this thesis), Green et al. (Figures 17 and 19, this thesis) failed to demonstrate these water-soluble fractions.

Since Williams and co-workers and Green and co-workers used a modification of Bunting's medium, the possibility exists that the nutrients necessary for production of the water-soluble fractions were not present. The media used by these workers were elaborate, however. This laboratory was able to obtain the water

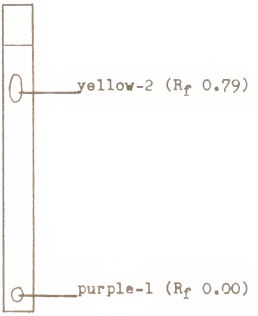


Figure 29. Complete separation of the water-soluble fractions using neutral, deionized water as the solvent.

soluble fractions on a simple salt medium designed by Dewey and Poe (1943).

Since the method of extraction employed by Williams et al. (1956) and Green et al. (1956) was used in this study, this factor does not appear to be the reason for the failure of these previous investigators to obtain these fractions.

Moreover, the pigment was extracted from five-day-old cultures by Williams et al. and Green et al., as well as this laboratory. The time factor for production of these fractions, therefore, does not account for the absence of these fractions by the previous workers.

Weiss (1949) apparently obtained these fractions as demonstrated by the separation of the brown fraction into a purple and yellow fraction (Figures 3 and 7, 13 and 14). With his method of extraction and development, he was not able to recognize that the total fractions of prodigiosin could be separated into water and organic-soluble fractions. Duplication of his extraction in this laboratory demonstrated that all the fractions including the water-soluble were extracted into the n-butanol even in the presence of water. The next step considered by Weiss was extraction into petroleum ether from n-butanol. Since n-butanol is fully miscible in petroleum ether, this was not a fractionation but a mixture. Thus, a n-butanol-petroleum ether mixture was applied to the column and not merely a petroleum ether extract as he stated. In the light of this, it appears that the brown band which was developed into a purple and yellow band are the same

as the brown spot which was developed into a purple and yellow spot in this study.

If the water-soluble fractions were not unknowingly discarded by Williams et al. and Green et al., then a study is warranted to determine what conditions are necessary for the production of these fractions. Also, a further characterization of these fractions and a determination of their relationship to the organic-soluble fractions is warranted.

EXPERIMENTAL PART C. THE STABILITY OF THE ORGANIC-SOLUBLE AND WATER-SOLUBLE FRACTIONS OF PRODIGIOSIN

It was noted in Experimental Part A, Experiment II, that exposure to various light intensities decomposed certain fractions of prodigiosin. This study was designed to further elucidate this photochemical decomposition. Since light may catalyze oxidation, it was therefore, imperative to study whether oxidation of the fractions also occurred in the dark.

Decomposition of fractions during chromatography can easily be determined from a method applied by Schwarz and Bitancourt (1957) which they termed "double chromatography". Essentially, it is two-phase chromatography except that the solvent used for development of the fractions in the first dimension is also used to develop the fractions in the second dimension.

The details of the procedure are as follows: The applied spot is developed, thereby, arraying the fractions along one side of the chromatogram. The chromatogram is then dried and the side having the arrayed fractions is immersed into the same solvent and

developed as before, taking care that the solvent front reaches the same height as in the first development. The chromatogram is then removed from the chamber. If each spot has the same R_f value as before, then decomposition did not occur. Each spot will, therefore, be located on the diagonal of the square. A small allowance should be made if the spot is not centered on the diagonal since the solvent front is slightly curved with the crest in the center of the paper. If decomposition has occurred, the spot will be found other than on the diagonal. This would be expected because a change in chemical structure should result in a different R_f value when the same solvent is employed.

Materials and Methods

Chromatographic Chamber for the Organic-Soluble and Water-Soluble Fractions. The chromatographic chamber used for the study was a Fischer Scientific museum jar type chromatographic chamber.

Chromatographic Paper for the Organic-Soluble and Water-Soluble Fractions. Number 1 Whatman filter (chromatographic) paper was cut into 23.5 cm squares. A border 1.0 cm from the edge on all sides was ruled, one border serving as the level to which the paper would be immersed in the solvent and the opposite border, the level to which the solvent front would be permitted to ascend. When the paper was turned on side for the second development, the borders served the same purpose. The pigment was spotted in one corner, 3 cm from either edge.

Lighting Conditions for Manipulation of Chromatograms. A small fluorescent lamp in the far corner of the room was the only

light used during manipulation of the chromatograms. In addition, the chromatograms were shielded as much as possible from this small amount of light. During development, total darkness was maintained.

Methods for the Organic-Soluble Fractions. A preliminary double-chromatogram was developed in the dark to determine whether decomposition would occur during the time required for the development in both dimensions. As shown by Figure 31, no decomposition occurred although the total time from the beginning of the first development to completion of the second development was approximately five hours.

This chromatogram serves as the control for both the studies of the effect of various light intensities and oxidation in the dark.

Since no decomposition occurred in the dark, the fractions arrayed during the development must be stable in the dark at least for the five-hour period of time necessary for development. Therefore, by subjecting the arrayed fractions from the first development to conditions which may induce decomposition, then the second development should demonstrate the fractions which decomposed from exposure to the particular condition. The decomposition of the fraction is observed from the change in R_f value and also from a change in color. By use of this method, it was thereby anticipated that the sequence of decomposition could also be determined.

The light intensities to which the chromatograms were exposed during the interval from the first development to the second development are as in Experimental Part A, Experiment II: (1) direct

sunlight, (2) diffuse sky light, (3) strong incandescent light, and (4) weak incandescent light.

The time interval for exposure to light was one hour except for direct sunlight which was 10 minutes. The reason for this shorter time was that one hour was too drastic, changing each of the arrayed fractions through the entire sequence of decomposition to the purple state. Hence, with over 10 minutes' exposure to sunlight no sequence of decomposition could be determined by the second development.

To determine whether the organic-soluble fractions were oxidized upon storage in the dark, the chromatogram after the first development was stored for five days in the dark at $32^{\circ}C \pm 3$ before completion of the second development. As already demonstrated, the control chromatogram did not undergo decomposition during the five hours of development in the dark.

The solvent system chosen for development of the chromatograms was the method which employed a solvent mixture (Experimental Part A, Experiment V.) To simplify the method, the first step was omitted in which the weak red-1 fraction was separated from the purple-2 fraction with iso-amyl alcohol. Should the weak red-1 fraction be an intermediate in decomposition, it would appear again and be further decomposed to the next sequential fraction. By employment of any one of the solvent mixtures recommended, the weak red-1 fraction did not move from the isolated purple and, therefore, would not interfere with the interpretation of this study.

The solvent mixture used for development of the fractions was toluene in Skellysolve B (25:75, v/v). This mixture moved the

orange-3 fraction to an intermediate position between the pink-4 and purple-2 fraction in the larger museum jars.

Orange fraction number 3 presented a problem. As shown in Figure 28, when developed with toluene in Skellysolve B, it extended over a rather long distance. This distance is longer if the concentration of the pigment is heavier. Development in the second direction, if no decomposition occurred, would result in the spot occupying a square with sides equal to the length of the first development. Because of this diffuseness, the spot would be difficult to see. This large diffuse spot could be avoided by use of a second solvent mixture, ethyl ether and Skellysolve B (1:2, v/v), which would not move the purple-2 or weak red-1 spot and yet concentrate the orange-3 fraction into a smaller area much the size of the original spot. If no decomposition occurred, development of this orange fraction in the second dimension with toluene in Skellysolve B would result in a spot with shape and R_f value similar to that produced in the first development with this solvent mixture. Likewise, development in the second dimension with ethyl ether and Skellysolve B would again produce a small spot similar in R_f value and shape to that produced by the first development with this solvent. Use of this second solvent would serve as an additional test for the decomposition of the orange-3 fraction. This, however, is merely accessory to the main purpose of producing an easily readable double-chromatogram.

The solvent front for the ethyl ether-Skellysolve B mixture was allowed to run only to a height immediately above the orange-3

fraction since this solvent would also move the pink-1 fraction. The solvent front for development in the second dimension with the ethyl ether-Skellysolve B mixture was measured to be the same as in the first direction.

Before the development in each dimension with the first solvent mixture (toluene in Skellysolve B) the paper was treated with ammonium hydroxide. Again a change was necessary. Instead of the usual treatment for one hour with ammonium hydroxide, five minutes was found to be optimum. Previously, the paper was treated by suspending the strip in long cylinders. Because of the width of the present chromatograms, it was necessary to treat the paper in the museum jars in which more intimate contact with the ammonium hydroxide was obtained. It was found that excessive treatment with ammonium hydroxide of the paper after applying the sample or the developed chromatograms produced results similar to the chromatograms developed with the chamber saturated with ammonium hydroxide; the orange fused with the pink and remained unseparated. The purpose of this treatment was to ascertain that the pH variable fraction (Figure 28, fraction 3) was converted entirely to the orange form during development. Decomposition of this fraction may occur in only one form or the other (red or orange). This would insure that both forms were subjected to the condition responsible for decomposition because the orange turns red in air.

To reduce the length of time from the beginning of development to completion, three chromatographic chambers were maintained in the dark. Two were for each solvent mixture and one for the ammonium hydroxide.

The major steps in the procedure for development of the organic-soluble fractions by double-chromatography may be summarized as follows:

(1) Prior to the development of the chromatogram for test purposes, adjust the concentration of the pigment, the concentration of the toluene-Skellysolve B mixture, and the time of treatment of the paper with ammonium hydroxide so as to obtain well-separated fractions upon the first development.

(2) Treat the paper and applied pigment spot with ammonium hydroxide vapors for the optimum time.

(3) Develop in the first direction with the appropriate toluene and Skellysolve B mixture.

(4) Develop with ethyl ether and Skellysolve B (1:2, v/v) mixture to concentrate the long orange spot to a small area.

(5) Subject the chromatogram to the condition (light, aging, etc.) to be tested.

(6) Treat the chromatogram with ammonium hydroxide vapors for the same time as the first treatment.

(7) Turn the chromatogram so that the arrayed spots are on the bottom and immerse to the same depth as for the first development.

(8) Develop in the second dimension with the toluene and Skellysolve B mixture permitting the solvent front to rise to the exact height as the first development with this solvent.

(9) Develop in the second dimension with the ethyl ether and Skellysolve B mixture permitting the solvent front to reach the same height as the first development with this solvent.

(10) Interpret the results.

It was pointed out (Experimental Part A, Experiment V) that treatment of the orange fraction (Figure 28) with N/1 NaOH converted it to a yellow color. It, therefore, was suspected that

the yellow fraction obtained by development of the water-soluble fractions may have been the soluble basic salt of the orange fraction. That this could be a likely occurrence is substantiated by the fact that the medium became alkaline with the aging of the culture.

To test this hypothesis, an applied pigment spot of the organic-soluble pigment was treated with N/1 sodium hydroxide and developed with neutral, deionized water. Should the orange fraction which was now yellow be the same, it would ascend the strip to the same R_f value as the yellow water-soluble fraction.

Methods for the Water-Soluble Fractions. The control chromatogram was developed in both dimensions in the dark (Figure 30). Since no decomposition occurred, this chromatogram was suitable for a control as explained for the organic-soluble fractions.

To determine decomposition by exposure to light, the chromatogram after development of the first dimension in the dark was exposed to direct sunlight for one hour. The chromatogram was then developed in the second dimension in the dark. Since no decomposition was noted from treatment with direct sunlight (the most drastic light treatment) subjection to the lesser light intensities was omitted.

Determination of oxidation during storage of the pigment in the dark paralleled the study conducted with the organic-soluble fractions. After development of the first dimension, the chromatogram was stored for five days in the dark. Development in the second dimension was then completed.

The only solvent necessary for development of the double-

chromatogram was neutral, deionized water, as demonstrated in Experimental Part B.

Results

Stability of the Organic-Soluble Fractions. The results of the chromatograms are diagrammed in Figures 31 to 33 which are drawn to 1/4 scale.

It is immediately evident that decomposition of the organic-soluble fractions occurred by the finding of spots above and below the diagonal. This was found to occur whether the chromatograms of the first dimension were exposed to various light intensities or to storage for five days in the dark.

A further observation may be included at this time. When the completed chromatograms were exposed to direct sunlight for five to six hours (this time will vary greatly depending on the concentration of the pigment used) all of the fractions disappeared.

Development with water of the orange fraction which was converted to yellow with N/1 sodium hydroxide did not move this fraction from where it was applied.

Stability of the Water-Soluble Fractions. As noted by the alignment of the fractions on the diagonal (Figure 37), the water-soluble fractions in contrast to the organic-soluble fractions were found to be stable even though subjected to most drastic treatment with sunlight. By comparison, the organic-soluble fractions were decomposed by exposure to direct sunlight for 10 minutes, whereas, the water-soluble fractions were found to be stable when exposed for one hour.

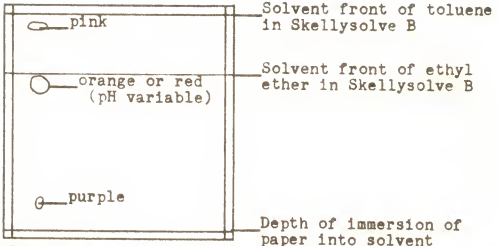


Figure 30.[6] Development of the first dimension. This diagram represents the first dimension for the following chromatograms of the organic-soluble fractions represented by Figures 31 to 36.

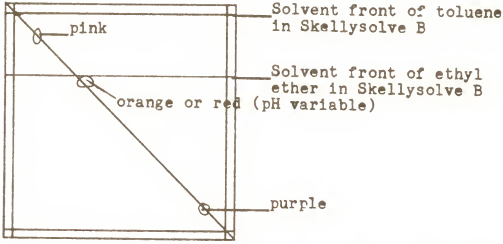


Figure 31. Control chromatograph. Development of the second dimension of the chromatogram represented by Figure 30. Both dimensions were developed in the dark with no intermediate treatment between the two developments.

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[6]The chromatograms represented by Figures 31 to 36 were obtained by rotating the chromatograms represented by Figure 30, after the indicated treatment, counterclockwise so that the arrayed fractions were on the bottom. When placed in this position, they were then developed. All of the diagrams are drawn as they appear in position in the chamber after completion of development.

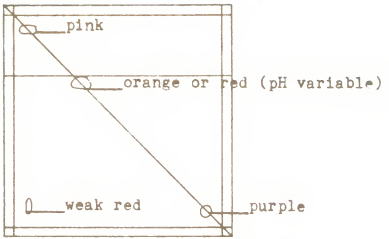

Figure 32. Development of the second dimension after the chromatograms represented by Figure 30 were exposed to weak incandescent light and another to strong incandescent light for one hour.

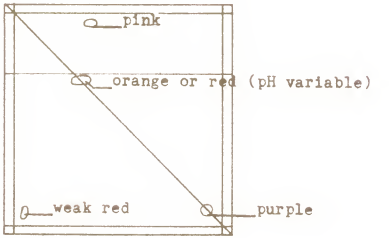

Figure 33. Development of the second dimension after the chromatogram represented by Figure 30 was exposed to diffuse sky light for one hour.

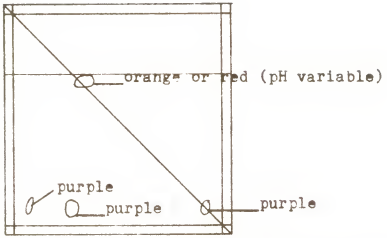

Figure 34. Development of the second dimension after the chromatogram represented by Figure 30 was exposed to direct sunlight for ten minutes.

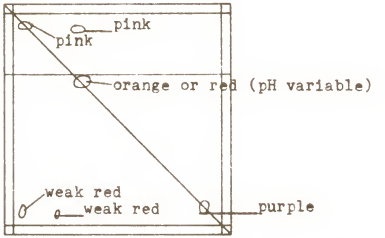

Figure 35. Development of the second dimension after the chromatogram represented by Figure 30 was stored for 5 days in the dark.

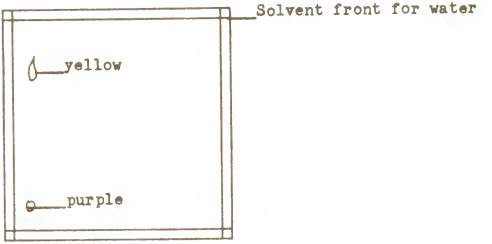

Figure 36[7]. Development of the first dimension for (1) the control (no intermediate treatment), (2) for exposure to direct sunlight for 1 hour, and (3) for storage for five days prior to development of the second dimension.

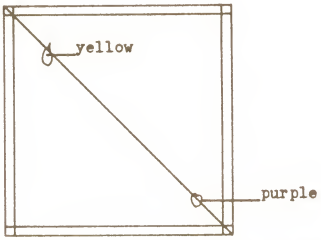

Figure 37. The second dimension of three chromatographs with identical results: (1) development of a chromatogram represented by Figure 36 with no intermediate treatment, the control chromatogram, (2) development of a chromatogram represented by Figure 36 after exposure to direct sunlight for one hour, and (3) development of a chromatogram represented by Figure 36 after storage in the dark for five days.

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[7]The same in regards to position applies to these diagrams as stated in footnote 6.

Discussion

Although, various light intensities were used, it was not the purpose of finding a quantitative relationship between decomposition and light intensity. It was hoped that by use of various light intensities, that at some one particular light intensity for a particular fraction that the decomposition would be stepwise and not so drastic as to break the fractions down completely to the end product (purple-4) as was done in Figure 21-A.

From Figure 33, one finds that the orange or red (pH variable) fraction decomposes to the pink as noted both by R_f and color.

The pink fraction in Figure 33 did not move up to the diagonal as it should if no decomposition occurred but was decomposed to the weak red fraction. That this was the same weak red as appeared in Figure 28 was established, by other than color, from development with iso-amyl alcohol which failed to move it.

In Figure 34, the pink not only was changed to the weak red but was further decomposed to purple. That there are no other intermediate steps between weak red and purple can reasonably be assumed since no other fractions have appeared during any of the chromatograms produced throughout the entire study. That the purple does not decompose to the weak red can be deducted from the fact that the purple from the first dimension was never demonstrated to decompose in any chromatogram.

From the above, the following sequence of decomposition can be proposed:

$$\text{(H ion conc.)} \downarrow\uparrow \begin{matrix}\text{red (acid)}\\ \text{orange}\end{matrix} \longrightarrow \text{pink} \longrightarrow \text{weak red} \longrightarrow \text{purple}$$

It is generally observed that as cultures of S. marcescens age in the dark over a rather long period of time, a change from orange through shades of red to purple occurs. This agrees with the sequence established in this study and the fact that oxidation of chromatograms in the dark is very slow.

To complete the sequence, two other observations which were pointed out must be added: (1) the change of the orange fraction to yellow by treatment with sodium hydroxide, (2) the change of the fractions to colorless upon prolonged exposure to sunlight. It was observed that as the well-separated fractions (pink, orange or red-pH variable fraction, and weak red) were subjected to direct sunlight for an extensive time that these fractions before becoming colorless appeared to pass through the above sequence, left to right. The purple fraction was the last to occur before the colorless product resulted.

Hence, the complete sequence of possible changes that can occur during chromatography is:

$$\begin{matrix}\text{red (acid)}\\ \downarrow\uparrow\\ \text{orange} \longrightarrow \text{pink} \longrightarrow \text{weak red} \longrightarrow \text{purple} \longrightarrow \text{colorless}\\ \downarrow\\ \text{yellow (base)}\end{matrix}$$

The above sequence may explain the failure of young cultures when exposed to direct sunlight to produce pigment.

The possibility must be considered that as the pigment is produced it is decomposed rapidly to the colorless.

The question whether a temperature of 37°C also produced decomposition of pigment to the colorless state may be answered by the technique employed in this study. Hefferan (1904), Ramchandani (1929), Goldsworthy and Still (1936), and Novelli (1953) all found pigment production unsatisfactory at 37°C. Perhaps the interpretation given in Experimental Part A, Experiment II, that the tailing of the chromatogram developed at 37°C was the result of a change in saturation of the chamber was incorrect.

Since light produces a photochemical change, it may be challenged whether the spectral curves of fractions conducted by Weiss (1949) Green et al. (1956), and Monk (1957) are not curves of mixed decomposed fractions. Besides the light from the spectrophotometer, the light from the room during the preparation of the sample could have produced a change. Monk (1957) observed during spectral analysis that a change in a peak from 470mμ to 535mμ occurred over a period of 30 minutes. This change of the orange fraction to the red supports the findings of this laboratory in which the orange fraction was decomposed to pink.

The results indicated that the water-soluble fractions were unrelated to the organic-soluble fractions and, therefore, imply two separate pigment systems. Factors which did not appear to play a role in the formation of the water-soluble fractions were discussed in Experimental Part B.

Upon the completion of a suitable method of chromatography, it was possible to verify the hypothesis that the fractions of

prodigiosin are the result of a sequence of decomposition which occurs in the dark and is catalyzed by light.

Quantitative studies could also be applied to this work in the form of control of light intensity and time and measurement of the relative amount of decomposition by use of a Spinco Analytrol.

The condition responsible for the intermittant appearance of the weak red fraction needs further study.

SUMMARY

Prodigiosin obtained from Serratia marcescens strain Nima was separated by extraction into two distinct types of fractions: water-soluble and organic-soluble.

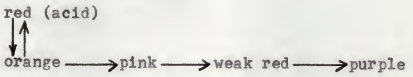
The reddish-brown, water-soluble extract when dried appeared brown but consisted of a yellow amorphous fraction, and a crystalline purple fraction. The red, organic-soluble extract when dried formed a red amorphous film.

A method of ascending paper chromatography was developed for each of the two groups of pigments in which well-separated fractions of each group were produced. Development of the water-soluble fractions resulted in a fast-moving yellow and a stationary purple fraction. The organic-soluble fractions were arrayed from top to bottom: pink, orange or red (depending on the H ion concentration), purple, and a weak red that appeared intermittantly.

By utilization of a method termed "double chromatography" the stability of the two groups of fractions was studied. The water-

soluble fractions were found to be stable to the conditions to which the organic-soluble fractions were subjected. However, the organic-soluble fractions were very unstable in the presence of light and only partly stable when stored in the dark for long periods.

By the double-chromatography method, the following sequence of decomposition was determined for the organic-soluble fraction of prodigiosin:



It was also demonstrated that the orange fraction was converted to yellow in the presence of N/1 sodium hydroxide and the purple was converted to colorless after several hours exposure to direct sunlight.

ACKNOWLEDGMENTS

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Sincere thanks are due my wife for typing the manuscript.

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STUDIES ON PIGMENT FRACTIONS PRODUCED BY SERRATIA MARCESCENS BIZIO

by

JOSEPH POERIO

B. S., University of Illinois, Urbana, Illinois, 1951

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

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Department of Bacteriology

KANSAS STATE UNIVERSITY
OF AGRICULTURE AND APPLIED SCIENCE

1959

During preliminary chromatographic studies of prodigiosin, the pigment of Serratia marcescens strain Nima, it appeared that decomposition of the fractions was occurring. It was therefore hypothesized that the fractions arrayed by various chromatographic procedures were a sequence of decomposition products.

To test this hypothesis, a method termed "double chromatography" was employed which is a modification of two-dimensional ascending paper chromatography. Instead of development with two different solvent systems, the same solvent system is used for both dimensions. The fractions are arrayed in the first dimension. If development in the second dimension arrays the fractions such that the same R_f value is obtained then no decomposition occurred. However, if the R_f value of a particular fraction changed, then decomposition occurred.

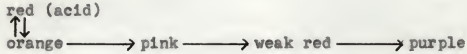
As no method of ascending paper chromatography was previously developed for this pigment, the immediate problem was to develop a method in which the fractions could be clearly separated.

Extraction of the pigment produced two distinct types of fractions, water-soluble not heretofore studied as such, and organic-soluble. The reddish-brown, water-soluble extract when dried appeared brown. Examination with a hand lens showed it to be composed of a yellow amorphous component and a purple crystalline component. The red organic-soluble extract when dried formed a red amorphous film in an evaporating dish.

A method of ascending paper chromatography was developed for each of the two groups of fractions. Of the solvents tested,

neutral, deionized water developed the water-soluble fractions into a fast-moving yellow fraction and a stationary purple fraction. Development of the organic-soluble fractions could be accomplished by use of several single solvents in succession or a single solvent and one of four solvent mixtures in succession. The developed chromatograms resulted in the following fractions arrayed from top to bottom: pink, orange or red (pH variable), purple and weak red.

During the course of development of the ascending paper chromatography method, it was noted that various intensities of light decomposed the fractions, whereas the fractions were stable in the dark for the period of time necessary for development of the chromatogram. By use of double chromatography, the first dimension chromatograms developed in darkness were subjected to various light intensities. From development in the second dimension (in darkness), it was possible to determine the sequence of decomposition. This was found for the organic-soluble fractions to be:



In addition, treatment of the orange fraction with N/1 sodium hydroxide changed it to yellow and exposure of the purple fraction to direct sunlight for several hours decomposed it to colorless.

There was no indication that decomposition occurred in the dark during the five-hour period for development. Storage of the

first dimensional chromatogram of the organic-soluble fractions in the dark for five days did, however, produce decomposition.

The water-soluble fractions were demonstrated to be stable to the conditions which produced decomposition of the organic-soluble fractions.

The proposed hypothesis that the fractions arrayed by chromatography were stages in the sequence of decomposition was verified for the organic-soluble fractions but not for the water-soluble fractions.