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A Study of the Separation of Pigments of Euglena 9 by Chromatographic Adsorption Techniques and a Determination of Their Absorption Spectra

By Ruth W. Helmick

The flagellate protozoans, Euglena have been observed to exhibit different pigments but few comparative studies have been made concerning them. If there is a difference in the pigments of the various species of Euglena, a method of separation of these pigments or identification would be of interest and value to those working in the fields of physiology and taxonomy.

The purpose of the present study is to determine the absorptive spectra of various pigments, isolated by chromatographic adsorption techniques from bacteria-free cultures of *Euglena* 9. The original culture was obtained from Dr. Luigi Provasoli of Haskins Laboratories, New York, New York. The following methods were used, (1) extraction of the pigments from the *Euglena* 9 cultures, (2) separation of the pigment mixture into individual pigments by chromatographic adsorption, and (3) determination of the absorptive spectra of the individual pigments.

Materials and Methods

Preparation of the Glassware. All of the glassware used in the experiments were washed with a detergent (*Joy*) and rinsed with tap water. The usual potassium dichromate and sulfuric acid solution was used followed by seven tap water rinses and three separate washes of distilled water. The glassware was air dried.

Culturing the Organisms. The Provasoli medium was used for culturing the organisms. It consisted of 0.1 per cent Thiopeptone, (Wilson and Co., Chicago), 0.1 per cent Sodium Acetate, (Coleman and Bell, c.p.), and 0.2 per cent Trypticase, (Baltimore Biological Co.). This solution was adjusted to pH 6.0-6.1. The 125 cc of media were placed into 250 cc flasks and sterilized by autoclaving at fifteen pounds pressure for twenty minutes. A loopful of the green growth of *E.* 9 from a sterile agar stock slant was transferred to a flask of the media, using bacteriologically sterile techniques. These new stock cultures were examined for sterility before inoculations were made. The flasks of growth media were inoculated in a sterile room with 1 cc of the new stock cultures throughout the experiments. The cultures were placed near a north win-

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dow and allowed to grow at room temperature for two weeks. The cultures were examined for sterility under high power of a compound microscope (400x).

Extraction of the Pigments from Euglena 9 *Cultures.* The liquid medium from one culture flask was placed in centrifuge tubes and centrifuged for five minutes or more to separate for media from the swimming organisms. The supernatant liquid was discarded. A 1 : 1 mixture of acetone and ethanol was added and the mixture was shaken vigorously. This solution was centrifuged five or more minutes and the grass-green supernatant liquid was saved. The suitability of the solutions used for extraction was determined by comparison of pigment remaining in an organism after extraction by microscopic examination. The acetone-alcoholic extract was mixed with a small amount of the 9 : 1 petroleum ether-benzene mixture and shaken well in a separatory flask. The colorless acetone-alcoholic solution was discarded and the grass-green petroleum ether-benzene solution was washed three times with the slow addition of distilled water.

Separation of the Pigment Extract into Individual Components by Chromatographic Adsorption Techniques. A 9 : 1 mixture of petroleum ether, Skellysolve B, (Skelly Oil Co., Lyman, Okla.), (b.p. 60°.0-65°.0) and benzene (b.p. 80°.0-92°.0 C) was chosen as the solvent. The *Euglena* 9 pigment extract was adsorbed on the following absorbents: confectioner's sugar (containing 3 per cent cornstarch) ; Hyflo-supercel; calcium carbonate; activated alumina; powdered sucrose (c.p.) ; powdered sucrose and calcium carbonate; powdered sucrose, calcium carbonate, and activated alumina. The last arrangement of a mixed adsorption column was used to determine all data for separation of the Euglena pigments. The chromatographic adsorption equipment described by Winterstein and Stein (Zechmeister and Cholnoky, 1948, p. 59) was used. In addition, a separatory funnel was used to regulate the amount of pigment mixture and solvent that flowed down the column. A 1 x 20 cm glass tube was connected by cellulose tape to make an air tight seal between this tube and the adapter which directed the solvent into the suction flask. The adapter and suction flask were connected by a one-hole rubber stopper. A water faucet aspirator provided the suction. The crystalline sucrose (c.p.) was ground until the crystals were reduced to a fine powder. The activated aluminum oxide (Alcoa Aluminum Ore Co.) (F-20 mesh) was activated by heating at 400° F for four hours in a hot air oven. The calcium carbonate (c.p.) (Fisher Scientific Co.) was used as it was obtained

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in a powdered form. Before the column was packed, a piece of cotton was placed in the bottom of the glass tube and the tube was gently tapped with a wooden rod slightly smaller than the internal diameter of the tube. About 1 cm of cooled activated alumina was placed on top of the cotton and gently tapped down. Tapping was continued until a fine powder was raised, care being taken to pack the layer evenly on all sides. The 20 cm glass tube was packed with the chemicals in the following proportion: top one-half, sucrose; middle one-fourth, calcium carbonate; and the bottom one-fourth, activated alumina. The 9:1 mixture of petroleum ether and benzene was poured onto the side of the glass tubing until the entire column was wet and about 1 cm of liquid covered the top. Suction was applied during the packing and wetting processes. The *Euglena* 9 pigment extract in a 9:1 petroleum ether-benzene solution was allowed to flow slowly over the column maintaining 1 cm of liquid over the absorbent. When this level was reached the pigment extract was washed downward by the addition of the 9:1 petroleum ether-benzene solvent. The column was developed into well defined colored bands of the individual pigments after about one to two hours of development under suction. The glass tube with the column was removed and examined in daylight. It was also examined in a darkened room under ultraviolet light to determine the presence of bands which were invisible in daylight. The locations of the bands were recorded as observed and used as a guide in the separation of the column into the individual pigment bands. The bands were removed by placing a pice of cotton between a wooden rod and the sucrose and pushing while the column was wet. As the adsorbent was pushed out slowly, the various pigment bands in it were cut apart using a scapel and saved with the adsorbent on both sides of it. The rose and blue bands (ultraviolet light observation) were removed as one color band. The following pigment bands were extracted from the adsorbant with absolute alcohol: top grass-green pigment on the sucrose, yellow pigment on sucrose, pink pigment on sucrose, gray-green pigment on sucrose, and orange pigment at calcium carbonate and alumina junction. The rose and blue pigments on alumina were extracted with chloroform. These solutions were poured from the absorbent, placed in corked test tubes which were wrapped with heavy paper, and left in a dark cabinet until absorptive spectrophotometric determinations could be made.

Determination of the Absorptive Spectra of the Individual Pigments. The absorptive spectra for each of the six pigments were

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determined by the use of the Beckman (Model DU) spectrophotometer. Matched absorption cells were used with the solvent in one light beam and the pigment solution in the other. Spectrophotometric readings were taken from 220 millimicrons to 1500 millimicrons.

Results and Interpretation of Data

The colors of the six pigments separated from the extract of *Euglena* 9 as examined in the daylight and the fluorescent color observed in ultra-violet light are summarized in Table I. The zones indicate the order in which the pigments were adsorbed on the chromatographic adsorption column beginning at the top.

Table 1

Observations of Pigments Under Daylight and Ultraviolet Light.

Zone Number	Adsorbent	Color in Daylight	Fluorescent Color in Ultraviolet Light
I	sucrose	grass-green	purple
II	sucrose	yellow	blue-purple
III	sucrose	pink	pink-purple
IV	sucrose	gray-green	rose
V	calcium carbonate and alumina junction	orange	orange
VI	alumina	rose	one blue band one rose band

Twenty-five extractions of *Euglena* 9 cultures were made. The last fourteen were made with a 1:1 acetone-alcohol solution and then the pigments were transferred to a 9:1 petroleum ether-benzene solution. The acetone and alcohol mixture was removed from the petroleum ether-benzene solution by several washings of distilled water. Two other methods of extraction were studied. Four extractions were made using the 9:1 petroleum ether-benzene solution before addition of ethanol, and seven extractions were made using only ethanol. These methods were observed to be slower than the 1:1 acetone-alcohol method and incompletely extracted the pigments from the organisms.

Several adsorbents were studied. It was found that only the green bands were adsorbed to the powdered sucrose (c.p.). The remaining pigments passed through the column, producing a yellow filtrate. Some pigments were observed to pass through the column of powdered sucrose and calcium carbonate. Indefinite bands were produced with columns of confectioner's sugar (containing 3 per

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cent cornstarch) and Hyflo-supercel. The green bands were observed to change to lighter colors on columns of calcium carbonate and alumina. The mixed adsorption column of powdered sucrose, calcium carbonate and alumina were found to adsorb all pigments and produced definite bands. A 4:1 and a 19:1 solvent mixture of petroleum ether and benzene were found to be too slow or too rapid for proper adsorption of the pigments. The 9:1 mixture was found to produce good pigment adsorption. The 1:1 acetone-alcohol pigment extract was transferred to the 9:1 petroleum ether-benzene solution before columning. An emulsion was observed to form when the mixture of these two solutions was shaken while washing with distilled water. No emulsion was formed when the water was added slowly down the side of the separatory funnel with little shaking. The pigmented zones formed on the column were separated with adsorbents, which were all colorless bands, as observed in the daylight, with the exception of the pinkish-gray alumina. Under ultraviolet light in the presence of the solvent used, sucrose was light purple, calcium carbonate was purple, and activated alumina was yellowish tan. The distance between the colored zones varied with the length of time allowed for development of the column and the velocity of flow of the solvent. If allowed to develop for greater periods of time, the lower bands in the sucrose were observed to be carried into the next adsorbent layer and the other pigments were absorbed successively lower on the column. Zone VI on activated alumina in daylight was observed to be rose in color, but under ultraviolet observation was found to be composed of two bands. A top blue band (under ultraviolet light) extended slightly above the rose area (daylight color) and into one-half the distance of the rose area. Below this in the remaining one-half of the rose (daylight color) was a rose band (ultraviolet color.) These two ultraviolet bands were too close to be cut apart with accuracy. Zone VI was found to be more easily dissolved in chloroform than ethanol.

Graphs of the absorption spectra of each of the six pigments separated by chromatographic adsorption techniques are given on Plates I and II. The data obtained by spectophotometric determinations are presented to show per cent transmittance of light. Spectrophotometric readings were determined at intervals of 10 millimicrons. Near regions of maxima and minima, readings were taken from 5 to 1 millimicrons to determine these points more accurately. The spectra of all pigments were determined from 220 to 1500 millimicrons but only principal curve areas were graphed to show maxi-

mum and minimum absorption. Table II summarizes the absorption maxima and minima as determined spectrophotometrically for the six pigments separated from *Euglena* 9. The readings given are the average of three separate determinations of a sample using unknown concentrations of the pigment. The data used for these determinations, which were graphed on Plates I and II, were selected from eighteen of the sixty pigments separated from the twenty-five extractions of *E.* 9 in this study upon the basis of extraction of pigments from the organism, chromatographic adsorption separation, accurate cutting apart of the column, and length and method of storage of the dissolved pigment. It was believed that all these factors might influence the adsorption spectra of the pigments.

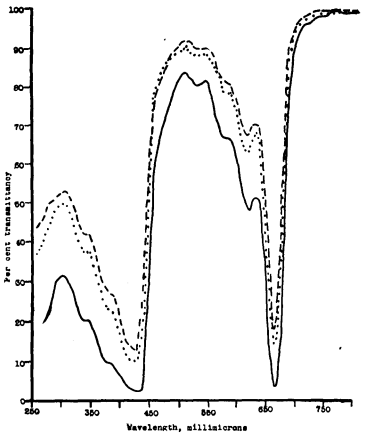

FIGURE 1. ABSORPTION SPECTRA OF THREE SAMPLES OF GRASS GREEN PIGMENT ADSORBED ON SUCROSE.

Solvent = ethanol; concentration unknown.

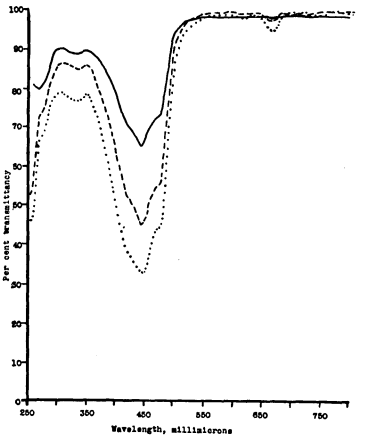

FIGURE 2. ABSORPTION SPECTRA OF THREE SAMPLES OF YELLOW PIGMENT ADSORBED ON SUCROSE.

Solvent = ethanol; concentration unknown.

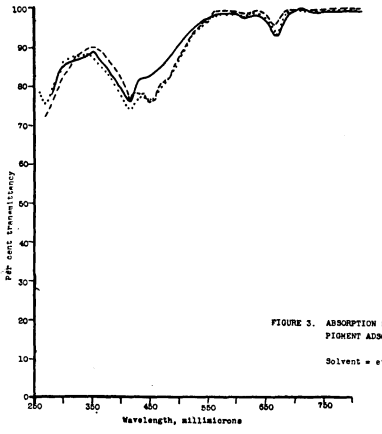

FIGURE 3. ABSORPTION SPECTRA OF THREE SAMPLES OF PINK PIGMENT ADSORBED ON SUCROSE.

Solvent = ethanol; concentration unknown.

Plate I. Absorption Spectra of Pigments Separated from *Euglena* 9.

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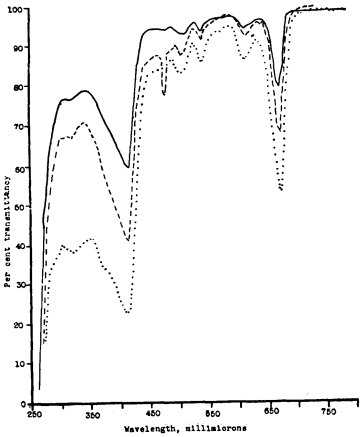

FIGURE 4. ABSORPTION SPECTRA OF THREE SAMPLES OF GREY GREEN PIGMENT ADSORBED ON SUCROSE.

Solvent = ethanol; concentration unknown.

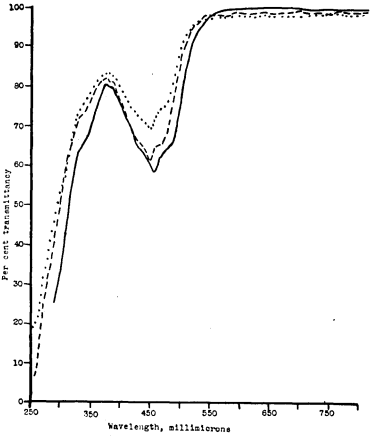

FIGURE 5. ABSORPTION SPECTRA OF THREE SAMPLES OF ORANGE PIGMENT ADSORBED AT JUNCTION OF CALCIUM CARBONATE AND ALUMINA.

Solvent = ethanol; concentration unknown.

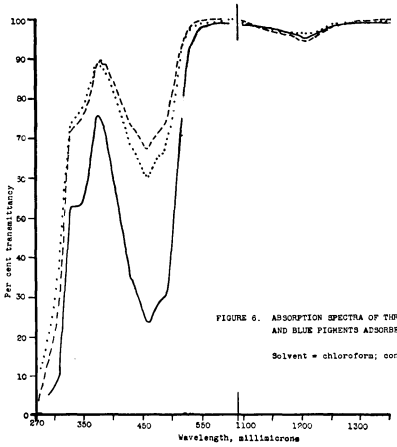

FIGURE 6. ABSORPTION SPECTRA OF THREE SAMPLES OF ROSE AND BLUE PIGMENTS ADSORBED ON ALUMINA.

Solvent = chloroform; concentration unknown.

Plate 2. Absorption Spectra of Pigments Separated from *Euglena* 9.

Discussion

Because of the risk of change or destruction of many plant and animal pigments great care had to be exercised in the procedures involving the pigments. When ethanol alone was added to the organisms for extraction, it was observed that some green coloring remained in the organisms showing incomplete extraction of pigments. Incomplete extraction was again observed using the 9:1 petroleum ether-benzene solution. The pigments were removed completely and rapidly (within a few minutes) with the 1:1 acetone-alcohol extraction. This method is suggested as a means of extraction of pigments from the *Euglena* 9 organisms.

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Table 2

Prinicpal Absorption Maxima and Minima for Pigments Separated from *Euglena* 9.

Zone	Pigment	Maxima	Minima
I	grass-green	428	305
		534	512
		585	549
		620	590
		665	635
			770
II	yellow	340	310
		448	350
		580	570
		665	630
			690
III	pink	270	347
		415	435
		450	580
		610	630
		670	700
IV	gray-green	320	310
		412	342
		475	461
		500	490
		539	528
		610	580
		670	630
			710
V	orange	450	378
			590
VI	rose and blue	460	378
		1210	560
			1300

The 9:1 ratio of petroleum ether and benzene was chosen as the solvent for adsorption columning as it flowed down the column at a proper rate for adsorption of definite bands of pigments upon the adsorbents. It was observed upon shaking vigorously of the mixture of the 9:1 petroleum ether-benzene and the 1:1 acetone-alcohol solution when distilled water was added, that a green-yellow pigmented emulsion was formed. It is recommended that the distilled water be added slowly down the sides of the separatory funnel, when quantitative determinations are needed so as to completely recover all pigments from the 1:1 acetone-alcohol extract.

Periods of over development of the column caused the pigments to be washed into the next adsorbent layer. This might cause decomposition of the pigments such as reported by Winterstein and Stein (Zechmeister and Cholnoky, 1948, p. 89) that chlorophyll undergoes changes on calcium carbonate and alumina. It was observed that with short periods of time for the development of the

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adsorption column into colored bands, that the pigments were not separated enough to be easily cut apart and obtained in pure samples. This would influence the accuracy of the spectrophotometric determinations which might follow separation of pigments by this method.

Long storage of pigment solutions in strong light was observed to alter the spectrophotometric determinations. This was also reported by Zscheile and Comar in 1941. Poor separation of the pigment solutions from the adsorbent caused turbid solutions unless they were allowed to settle upon standing. It was observed that these turbid solutions produced transmittancy curves with poorly defined maxima and minima. However, the characteristic shapes of the curves were the same.

In the study of the effect of solution concentration upon transmittancy curves, Webb and Ferguson (1941) show that there is an increase in transmittancy with a decrease in the concentration of the solution used. Using intensity of color observed as the basis for concentration determination, this same phenomena was exhibited in the extracts of *Euglena* 9.

The characteristic spectral curve shape and band maxima and minima of Zones I and IV occurred approximately in the region of the curves of chlorophyll A and chlorophyll B of Zscheile (Mellon, 1950, p. 423). The pigment position on the adsorbent of Zone I and IV corresponds to those reported by Winterstein and Stein (Strain 1945, p. 121). of chlorophyll B and chlorophyll A. From this evidence it is concluded that Zone I was composed of chlorophyll B and Zone IV of chlorophyll A. Winterstein reports (Zechmeister and Cholnoky, 1948, p. 92) that carotenes from leaf extracts are adsorbed on alumina. The Zone VI from the *Euglena* 9 extract was adsorbed on the alumina of the column. In solution this pigment was yellow in color and may be composed of carotenes. Zone VI was thought to be a mixture of two pigments from ultraviolet observations. Therefore, it is suggested that seven pigments may be isolated from *E.* 9 if Zone VI could be separated. An analysis of the individual pigment bands as to the physical and chemical properties would aid in the further identification of these pigments.

The absorption maxima found for the six pigments separated from *Euglena* 9 are in satisfactory agreement with the absorption maxima determined by Baas-Becking and Ross (1926) for whole *Euglena sp.* They report these maxima at: 468, 476, 528, 543, 586, 613, 622, 670 millimicrons. From this it is suggested that if

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a chromatographic adsorption analysis was made for an organism and the absorption maxima for the individual pigments were previously known, the extract of the entire organism would produce absorption curves characteristic of the superimposed individual pigments. This would facilitate identification of the organism.

The indefinite bands of pigment on columns of confectioner's sugar and on Hyflo-supercel may be due to decomposition of the pigments upon these two substances. It was necessary to choose a column which would adsorb and not decompose the pigments in the extract. No references were found concerning the use of the same mixed chromatographic adsorption column used in these experiments for separating pigments of any Euglena organism. Several investigators report the use of chromatographic adsorption and spectral absorption methods with protozoans. Kylin in 1927 examined the alcoholic extracts of *Euglena sp.* and found three modified red haematochrome pigments not found in green leaves. Gunther (1928) examined the spectra of chlorophyll of the pyrenoids of *Euglena terricola, E. geniculata, E. proxima, E. sanguinea,* and *E. lucens* and found that they differed from that of the higher plants. Seybold and Hulsbruck (1943) investigated algal groups and found *Euglena gracilis* contain chlorophyll A and chlorophyll B. Commoner (1950) employed ultraviolet microspectophotometric methods to determine spectra of the cytoplasmic area and the nuclear area of living cells of *Euglena sp.* Strain, Manning, and Hardin (1943) found *Euglena gracilis* contained chlorophyll B but no chlorofucine (chlorophyll C) by chromatographic adsorption and spectral absorption methods. Tischer (1944) used calcium carbonate and powdered sugar in purification of the carotenoids of *Haematococcus pluvialis.* In isolating the pigment, euglenarhoden from *Euglena heliorubescens,* Tischer (1936) used alumina. It is suggested that the arrangement of the pigment bands upon an adsorption column of sucrose, calcium carbonate, and alumina with further determination of pigment spectra might be an aid to the identification of the Euglenoids.

Summary

1. The separation of six pigments of *Euglena* 9 by the use of a mixed adsorption column of powdered sucrose, calcium carbonate, and activated alumina developed with a 9:1 petroleum ether-benzene solution is described.

2. A 1:1 acetone-alcohol solution was found to extract the *Euglena* 9 organism.

3. The fluorescent colors of the six pigments separated and a

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possible seventh pigment are listed as observed under ultraviolet light.

4. The absorption maxima and minima for the six individual pigments of the organism are given as determined spectophotometrically.

5. Two of the pigments separated from the *Euglena* 9 organism are believed to be chlorophyll A and chlorophyll B. Other pigments separated are unidentified.

6. It is suggested that chromatographic adsorption and absorption spectroscopy might be aids to the speciation of the Euglenoid organisms.

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