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THE ABSORPTION SPECTRA OF SUSPENSIONS OF LIVING MICRO-ORGANISMS

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INTRODUCTION

Absorption spectra of suspensions of micro-organisms are of considerable interest in a wide variety of biological experiments. Although many measurements are reported, they generally suffer from serious broadening of the absorption bands, lack of definition in the fine structure and raising of the apparent absorption curve over the whole range of wavelength, even where there is no absorption band. The difficulty lies in the great light scattering by the cell suspension.

In determining the absorbance (optical density) of suspensions, the transmitted light is usually measured without consideration as to whether it represents all or only a part of the light emerging from the suspension. Let us consider two extreme cases: 1) in which all transmitted light passing through the suspension is detected by the measuring procedure, and 2) in which only that part of the transmitted light that proceeds in the same direction as the incident light is measured. We shall designate these two kinds of transmitted light by "total transmitted light" and "specularly transmitted light," and the corresponding absorbance by "total absorbance" (E_t) and "specular absorbance" (E_s), respectively.

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In the instrument commonly employed for these measurements, a part of the total transmitted light escapes the detector. Consequently, the measured absorbance is intermediate between E_s and E_t, and depends on the optical arrangement of the instrument used. Naturally, E_t and E_s have different physical implications. Almost all of the specularly transmitted light is composed of the light which does not hit the cells in suspension since that light which does is scattered out of the direct beam. Therefore, specular absorbance will not depend markedly on wavelength. Total absorbance will depend markedly on wavelength according to the absorption spectrum of each cell in the suspension because total transmitted light contains all light (refracted, reflected and scattered) other than the light absorbed in the cells.

E_s can be measured by using the device recently reported by Rose,[1] in which only the fraction of transmitted light that goes through a long collimator with small slits was caught by the detector. To measure E_t, we considered using a light detector with a sufficiently large window associated with a relatively short path length to catch all scattered light. However, such a detector is not available incorporated in a spectrophotometer. The purpose of the present paper is to describe a new technique which enables us to measure E_t very simply and to demonstrate that the spectrum of the suspension of micro-organisms measured by E_t gives well-defined absorption bands.

METHOD

In analyzing growth curves of algae, Tamiya, Shibata, et al.[2] obtained improved average absorbance measurements in the visible region using opal glass

(1) H. F. Rose, Nature, 169 (1952) 287.

(2) H. Tamiya, E. Hase, K. Shibata, A. Mituya, T. Iwamura, T. Nihei and T. Sasa, "Algal Culture, from Laboratory to Pilot Plant," Carnegie Institution of Washington Publication 600 (1953) 219-220.

to diffuse uniformly both the specularly transmitted and scattered emergent light regardless of incident direction. A limited absorption spectrum of Chlorella was reported. This application is now extended to the measurement of complete absorption spectra of suspensions and translucent substances in a physically definitive manner.

The principle of the method is illustrated in Figure 1. The sample and blank cell compartments are both provided - on the sides of light transmission - with identical opalescent plates (A in Figure 1) which diffuses uniformly all the light as it leaves the cuvette. If the scattered light emerging from the opalescent plate used with the cell containing solvent alone has the same angular distribution functions as the light from the opalescent plate used with the suspension (irrespective of whether or not light is scattered in the cuvettes), the quantities of light from the two opalescent plates in each case must be proportional to the light intensities emerging from the cuvettes at corresponding solid angles. We can, therefore, measure E_t, provided that the diameters of the windows of the detectors for solvent light beam and suspension light beam and also the distances between the cell and the detector are kept identical.**

The required properties of the opalescent plate are uniform, moderate and constant opalescency over a wide range of wavelengths. Too much opalescency makes the wavelength range of observation narrower because of the low light intensity beyond the opalescent plate and causes a broadening of abosrption

(**) More precisely, the intensity of total transmitted light through the suspension with the opalescent plate is different from the intensity of the light without the opalescent plate. A part of light reflected from the opalescent plate will go back and forth between suspension and opalescent plate, and this effect will cause the increase of total transmitted light, which gives the decrease of E_t; however, this effect was found to be very small compared with the height of the absorption band. The detail of this effect will be published in another report.

bands owing to the wide slit requirement of the monochromator. An opalescent material having these properties was found to be an oil-impregnated filter paper made by the following procedure: A thick piece of filter paper (Whatman 3 MM) was dipped into pure paraffin oil and was allowed to drain overnight, after which the excess of paraffin oil was removed by blotting. The oil-impregnated filter paper was sandwiched between two quartz plates for use. The purpose of dipping the filter paper into paraffin oil was to decrease the high opalescency of the paper to a suitable value. We may consider using thinner and less opalescent filter paper without treatment, but, usually, thin filter paper has holes in it.

By using this oiled paper, we can measure the absorption spectra of a suspension of micro-organisms from 220 mμ. to 1300 mμ., which is the limit due to the strong absorption band of water. For observation in the visible region, the commercial opal glass, one side of which has an opalescent coating, gave a result slightly better than that obtained with oiled paper.

For the ultraviolet and visible regions, the measurements were made with a Cary Recording Spectrophotometer (Model 11), and for the near infrared region, with a Beckman Recording Spectrophotometer (Model DK). The positions of 1 cm. quartz cells for solvent and suspension were carefully adjusted to make the distances between cell and detector the same.

RESULTS AND DISCUSSION

A unicellular algae, Chlorella pyrenoidosa was used as the sample testing the technique. The data of the test are shown in Figure 2, in which curve A is the absorption spectrum with no opalescent plate and curve B is the spectrum with oiled paper, using the same suspension of Chlorella for both experiments. For comparison, an aliquot of the same suspension of Chlorella was centrifuged

and extracted with a volume of hot ethanol equal to twice the original volume of suspension, and the absorption spectrum of the extract was observed in the usual way (C in Figure 2). The concentration of cell extract is expressed in terms of the original algal suspension by considering the concentration of the extract to be equal to that of the cell suspension, when the same total volume of packed cells was used in each case, and when the volume of ethanol used in making the extract was equal to the total volume of the cell suspension.

It will be clear from these data that curve B shows every detail of the absorption spectrum of the extract, while curve A shows only the existence of the main peak of chlorophyll. We cannot estimate the exact position of even the main peak from data obtained without the oiled papers, because, in curve A, the absorption curve is superimposed on the high scattering curve and is greatly deformed. On the other hand, it is possible to consider the differences between the position of the absorption maxima in vivo and in vitro by using the data obtained with oiled paper. The shift of the chlorophyll band in the red region was estimated to be 13 mμ. (665 mμ. in vitro ⟶ 678 mμ in vivo) in Chlorella. Five values have been given for this peak in vivo: 680, 668, 672, 675 and again 680 mμ.,[3] the average value of which is 675 mμ. A similar shift to longer wave lengths is observed in the other bands of chlorophyll. The possible significance of this shift has been discussed in the light of a similar shift observed with pure chlorophyll microcrystals.[4] The band at

(3) E. Katz and E. C. Wassink, Enzymologia, 7 (1939) 108; W. Noddack and H. J. Eichhoff, Z. Physik. Chem., A185 (1939) 241; R. Emerson and C. M. Lewis, Gibson Island A.A.A.S. Symposium on Phytosynthesis (unpublished) (1941); E. C. Wassink and J. A. H. Kersten, Enzymologia, 12 (1946) 3; A. Seybold and A. Weissweiler, Botan. Arch., 43 (1942) 252.

(4) E. E. Jacobs, A. S. Holt, J. Chem. Phys., 20 (1952) 1326; E. I. Rabinowitch, E. E. Jacobs, A. S. Holt and R. Krumhout, Zeit. f. Phys., 133 (1952) 261.

475 mμ. has been attributed to the absorption of luteol[5] and is also shifted towards the red in vivo.

Recently, an attempt to get clear absorption spectra of the suspensions of micro-organisms was made by Barer, et al.[6] by adding protein to the suspensions in order to adjust the refractive index of the medium to the same value as the refractive index of the cells in suspension. Following this idea, the absorption spectrum of the same sample of Chlorella was measured by adding egg albumin to the suspension up to a concentration of 50%. The spectrum obtained was found to be improved compared with curve A, but was less sharp and higher than the spectrum with opalescent oiled paper (curve B).

The spectra of various micro-organisms

Since all previously published spectra of living micro-organisms suffered from scattering effects, it seemed worth while to scrutinize the absorption spectra of the suspensions of several important micro-organisms by this new method. The suspensions of 11 kinds of colored and colorless micro-organisms were chosen for this purpose. These living cells were separated from culture medium by centrifugation (15 min. at 3000 rpm), resuspended in 0.9% NaCl aq. solution at a definite concentration and used for measurement. Some of them were extracted by hot ethanol and the absorption spectrum of each extract was compared with the spectrum of the corresponding suspension, with particular attention to the difference of the positions of the absorption maxima between in vitro and in vivo. The data obtained are shown in Figures 3-10. The positions of the peaks are listed in Table I where parentheses indicate values for less well-defined bands.

(5) E. I. Rabinowitch, "Photosynthesis," II, 1 (1951) 706.

(6) R. Barer, K. F. A. Ross and S. Tkaczyk, Nature, 171 (1953) 720.

As will be seen from Figure 3, the absorption spectrum of Scenedesmus is quite similar in shape and position of the peaks to that of Chlorella. Euglena gracilis (Figure 4) has a little different absorption spectrum from these two. The distinct difference is around 480 mμ., which may indicate that Euglena has a different kind or combination of carotenoids from that of Chlorophyceae. Further difference is observed in the spectra in vitro in the ultraviolet region; namely, Euglena has three bands from 260 mμ. to 280 mμ., while Chlorella and Scenedesmus have only one weak band.

One sample for which it is difficult to observe the absorption spectrum is the suspension of the streptomycin-bleached Euglena (Figure 5) because it has β-carotene in the tiny eyespot in the cell, and much scattering by other parts of the cell. Two different concentrations of the suspension were measured to see the peaks in the ultraviolet and visible regions, which are quite different in their height. Figure 5 shows the typical absorption bands of carotene between 420 mμ. and 480 mμ. in a streptomycin-bleached Euglena. The absorption spectrum in the ultraviolet region bears a remarkable resemblance to that reported for eleostearic acid.[7]

There are several opinions regarding the variations in the positions of absorption peaks for different species of purple bacteria in the near infrared region. Our measurements gave a difference of 2 mμ. between the positions for Rhodospirillum rubrum and Rhodopseudomonas capsulatus (Figures 6 and 7). More differences are observed for the peaks of carotenoids of these bacteria. The in vivo shift of the chlorophyll peak in the near infrared region was found to be about 100-102 mμ. toward the red. The extract of Rhodospirillum by hot ethanol has a sharp peak at 424 mμ., which may be due to a decomposition product

(7) L. J. N. van der Hulst, Rec. Trav. Chim. Pays-Bas, 54 (1935) 639, 644.

of the original pigments. It is, however, interesting to note the close correspondence of this peak with the Soret band reported for cytochrome f.[8]

Three bands of Porphyridium cruentum at 568, 552 and 502 mμ. and the bands of Synechococcus cedrorum at 624 mμ. are due to the absorption of phycoerythrin and phycocyanine, respectively, which do not appear in the ethanol extract (Figures 8 and 9).

To test the applicability of this technique to the ultraviolet region, measurements were made with colorless micro-organisms; namely E. coli B, purified bacterial Virus $T_{2r}+$ and commercial Fleishman's yeast (Figure 10). A strong absorption band was observed at about 260 mμ in these samples. The fact that both bacterial Virus and a desoxypentose nucleic acid solution (Figure 10) has this band, indicates that this band arises primarily from the absorption of nucleic acid in the cell. The broadening or the absorption of E. coli and yeast on the long wave side is to be attributed to the aromatic amino acids present in the protein. The greater part of the absorption around 260 mμ. by colored samples described previously might also be due to nucleic acid.

The same difficulty in obtaining clear absorption spectra occurs with erythrocyte suspensions. Heparinized rat blood was diluted 265 times with 0.9% NaCl (aq.) solution. Without the opalescent plates the spectrum of the resulting suspension was too high to see the detailed structure as is apparent in Figure 11. With the opalescent plates, we can measure exactly the difference between the absorption peaks of CO-treated and normal blood suspensions. In Table II the positions of those peaks are listed. The same erythrocytes were washed with a saline solution and the hemoglobin was extracted with distilled water. The positions of the absorption peaks of the resulting extract of hemoglobin coincide quite well with that of the original suspension.

(8) H. E. Davenport and R. Hill, Proc. Roy. Soc. B, 139 (1952) 327.

The spectra of leaves and other translucent substances.

So far, we have shown the application of this technique to suspensions of micro-organisms or blood. It should be noted that this technique can be used with other kinds of suspensions and also with other forms of translucent materials such as a piece of leaf or petal. The spectra of a green leaf or Glycine soja (soybean) and its ethanol extract and of a red leaf of Prunus cerasifesa var. pissardii and its hot water extract were observed in the visible region (Figure 12). The data for Glycine soja without oiled paper show how difficult it is to observe the spectrum without a device to overcome the effects of scattering. The amount of the in vivo red shift in these leaves is similar to that of green algae. The shoulder around 540 mμ. in the spectrum of Prunus shows the presence of anthocyanine, which is clearly observed in the hot water extract. Observations were also made of the absorption bands of pigments in flower petals. Figure 13 shows the clear absorption bands of three kinds of petals: Epiphyllum, Saintpaulla and Eschscholtzea californica.

Another type of organism studied was a filamentus alga. The spectrum of a brown alga, Ectocarpus siliculsus, pressed between two sheets of quartz plate, was observed using oiled papers. The difference between the spectra of the filaments and that of their ethanol extract around 500 mμ. suggests the presence of protein-bound pigments in the whole alga (Figure 14).

The technique with opalescent plates can be used not only with biological materials but also in physico-chemical investigation. A suspension of crystals of zinc tetraphenyl porphine was selected as a sample and was prepared in the following way: The crystals were suspended in water and subjected to 9 Kc. supersonic vibration for 45 min. in order to reduce the particle size. After

~15 min. the remaining fine suspension was decanted for use. The observed data (Figure 15) indicate not only the shift of absorption peak between suspension and solution but also the great change of the relative intensities of those peaks. It is thus possible to obtain sharply defined absorption spectra of powdered solids, making possible a much broader scope for solid state studies. Another curve, shown in Figure 12, was obtained to see the phenomenon of metachromacy. A piece of filter paper (Whatman No. 4) was dipped into the benzene solution of zinc tetraphenyl porphine, dried in the air and the absorption spectrum was observed using an untreated white paper as the control. It was found that we can measure precisely the positions of the absorption maxima in this case also and, therefore, the estimated value is accurate enough to permit discussion of the small color shift induced by adsorption of the dye.

SUMMARY

A new method is described for obtaining sharp absorption spectra from suspensions of micro-organisms. The principle of this technique is to measure the total absorbance of the suspensions by attaching opalescent plates to cuvettes for both solvent and suspension. It must be emphasized that this technique does not involve any treatment of the suspension or any change of the optical system of the spectrophotometer for measurement. We can obtain a clear absorption spectrum of living micro-organisms with the commonly-used spectrophotometer just by attaching opalescent plates to the cuvettes. The applications to the other translucent materials such as suspensions of blood and crystals and a piece of leaf or petal have been shown.

ACKNOWLEDGMENT

The authors wish to express their thanks to Dr. R. C. Fuller and Dr. R. E. Norris of this laboratory for isolating and culturing many of the organisms used in this work, and also to Dr. I. Watanabe, Virus Laboratory, University of California, for giving us samples of E. Coli, virus and DNA.

Table I

Organism	λ_{max} in mμ. in vivo	in vitro (in ethanol solution)
Scenedesmus and Chlorella	678 ---------- **	665
	(626)* ----------	618
	(592) ----------	(586)
		(540)
	(475) ----------	468
	438 ----------	435
	(418) ----------	(418)
	(382) ----------	(378)
	340 ----------	336
	262 - - - - - **	264
		228
Euglena	676 ----------	666
	(626) ----------	620
	(590) ----------	(586)
		(538)
	484 ----------	475
	435 ----------	435
	(416) ----------	(416)
	(384) ----------	(378)
	(338) ----------	338
		(295)
		280
		269
	260 - - - - - -	260
Astasia		280
		269
	253	258
Streptomycin-bleached Euglena	490 ----------	478
	460 ----------	448
	432 ----------	422
		(295)
	(282) ----------	281
	270 - - - - - -	269
		(250)

Table I-b

Rhodospirillum	882	———	780
	(800)	———	(700)
	588		602
	550	———	528
	514	———	496
	486	———	468
			424
	376	———	366
			338
	323	———	318
	(312)	———	(306)
	272	- - - - - -	272
Rhodopseudomonas	880	———	780
	(800)	———	(700)
			(602)
	588	———	(586)
	506	———	486
	476	———	458
	(448)	———	(428)
	374	———	365
	262	- - - - - -	268
Porphyridium	680	———	666
	630	———	618
	568		588
	552		
	502	———	482
	(466)		
	438	———	434
	(420)	———	(418)
	(384)	———	(380)
	340	———	336
	(310)		
	270	- - - - - -	266
Synechococcus	676	———	666
	624		620
	496		588
			476
	438	———	434
	(420)	———	(418)
	(384)	———	(380)
	340	———	336
	262	- - - - - -	266

Table I-c

Yeast	261		
E. Coli B	261		
Bacterial virus T_{2r}^+	260		
Deoxy pentose nucleic acid			258***
Glycine soja	676	----------	665
	(626)	----------	618
	(592)	----------	(584)
			(540)
	(470)	----------	468
	436	----------	435
Prunus cerasifera var. *Pissardii*	676		
	624		
	(544)	----------	536****
	490		
	438		

(*) Parentheses show the values of the rather obscure bands.

(**) The solid lines (----) indicate the corresponding absorption maxima. The dotted lines (- - -) show rather doubtful correspondency.

(***) In phosphate buffer solution.

(****) The data for water extract.

Table II

Original blood	CO-treated blood
578	568
544	540
414	420
342	342
271	273

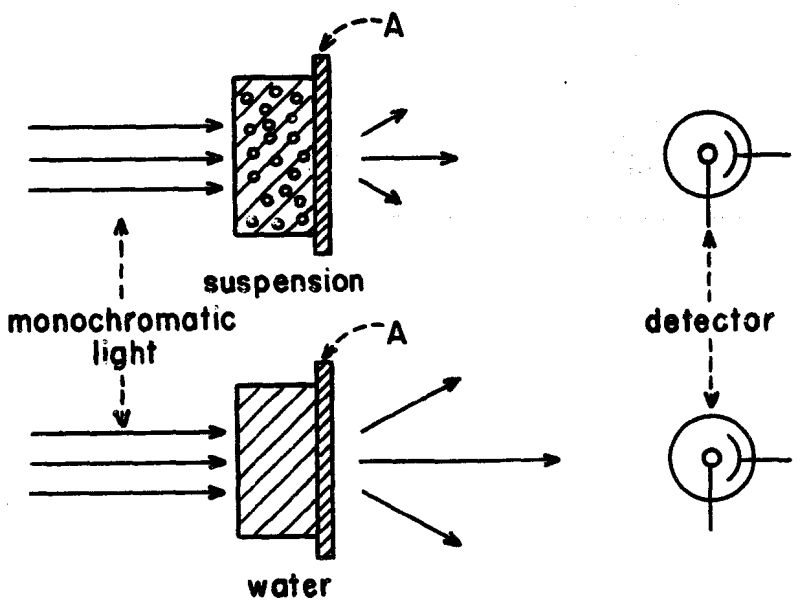

Fig. 1 Diagram showing relation of opalescent plates (A) to optical system in spectrophotometer.

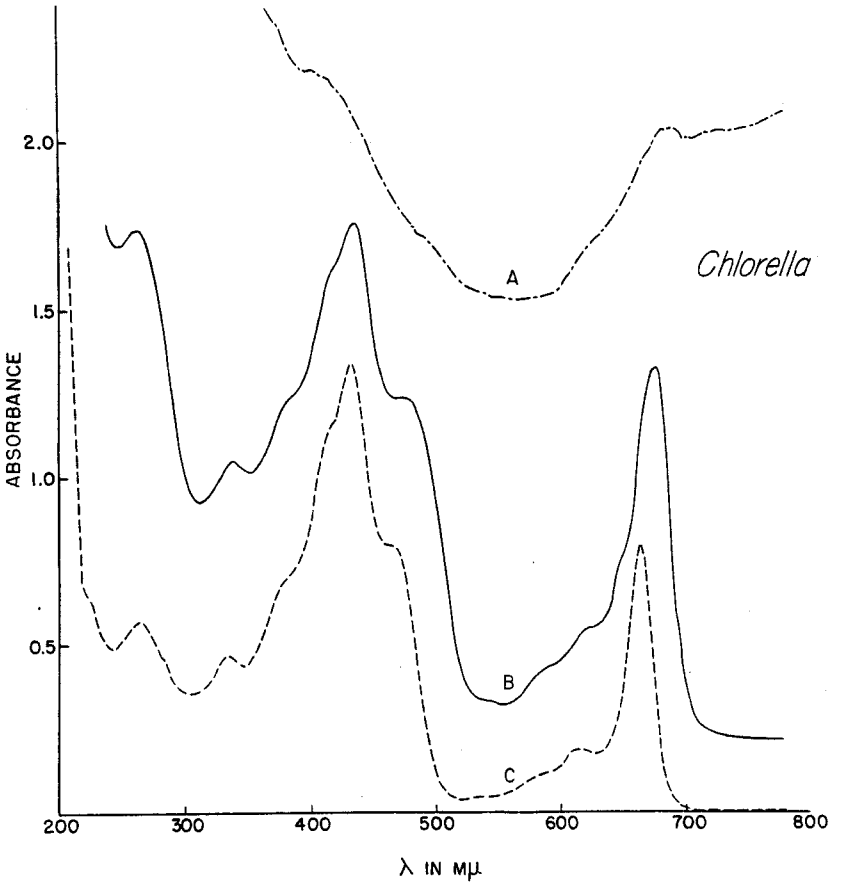

Fig. 2 Absorption spectra of Chlorella suspension and extract
A, cell suspension (0.51%), without opalescent plate.
B, cell suspension (0.51%), with opalescent plate.
C, alcohol extract.

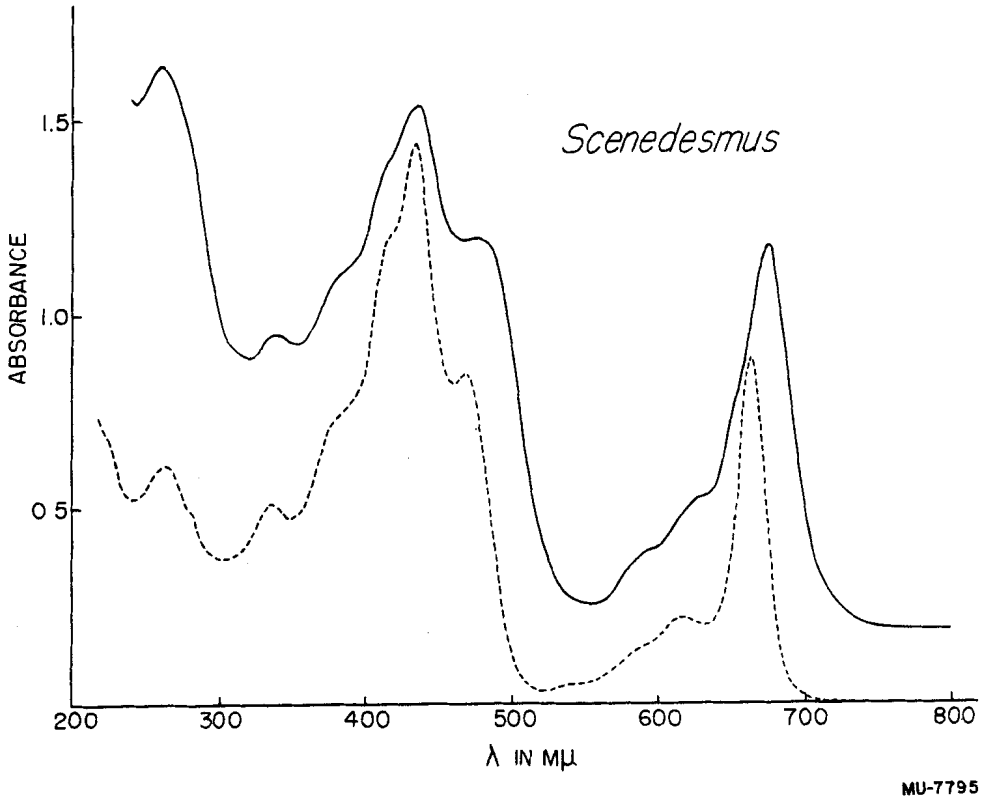

Fig. 3 Absorption spectra of Scenedesmus suspension and extract _____, cell suspension (0.25%) - - - -, alcohol extract (0.125%).

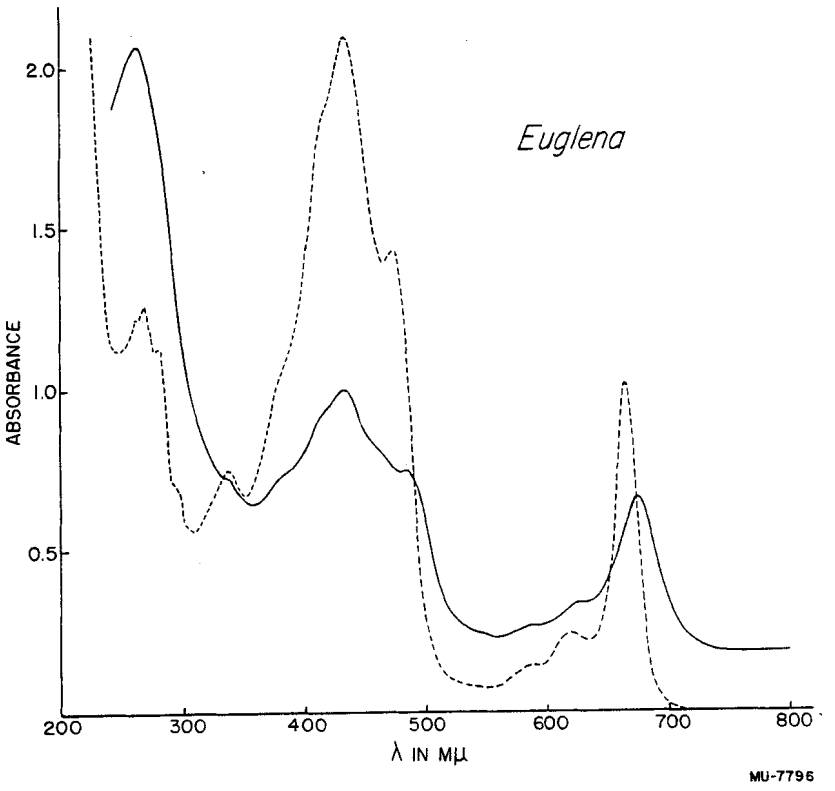

Fig. 4 Absorption spectra of Euglena suspension and extract. _____, cell suspension. - - - -, alcohol extract.

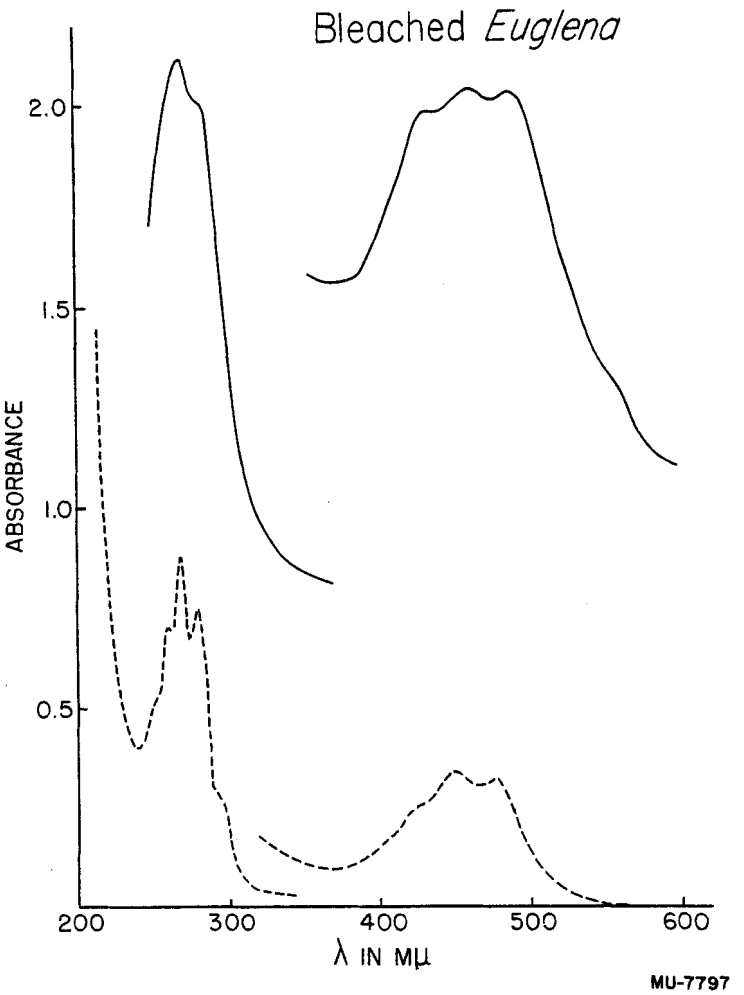

Fig. 5 Absorption spectra of streptomycin-bleached Euglena. _____, cell suspension. - - - -, alcohol extract. The cell concentration was changed at 350 mμ to increase the relative height of the visible absorption bands.

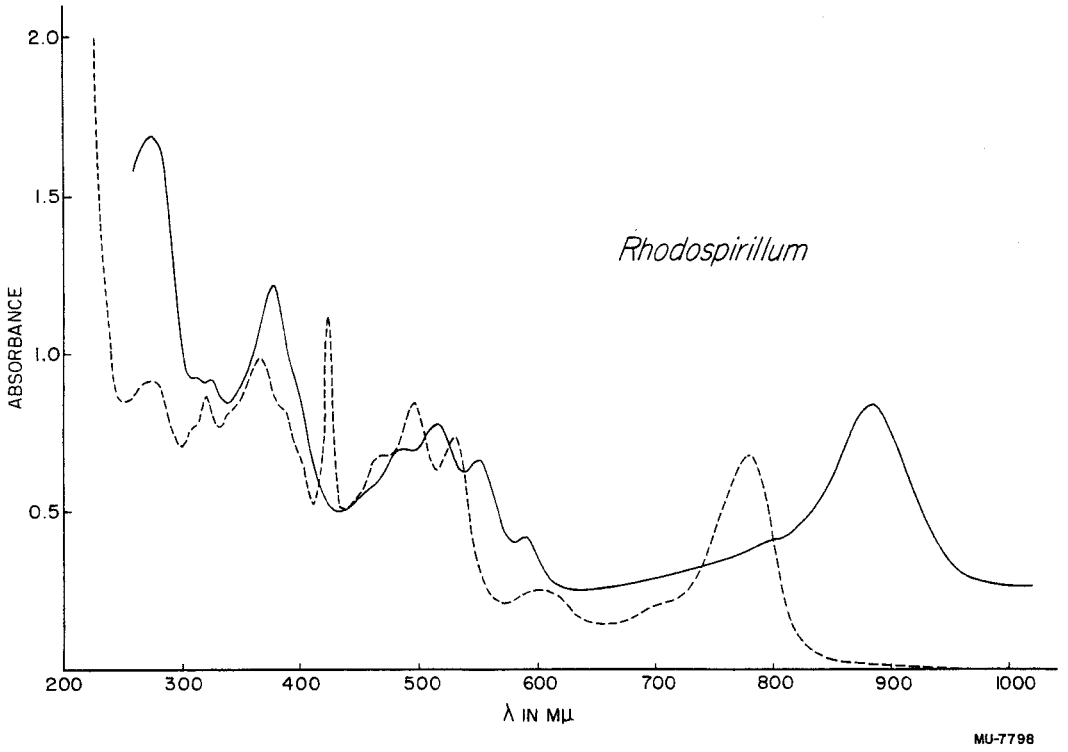

Fig. 6 Absorption spectra of Rhodospirillum suspension and extract. _____, cell suspension (0.5%). - - - -, alcohol extract (0.5%).

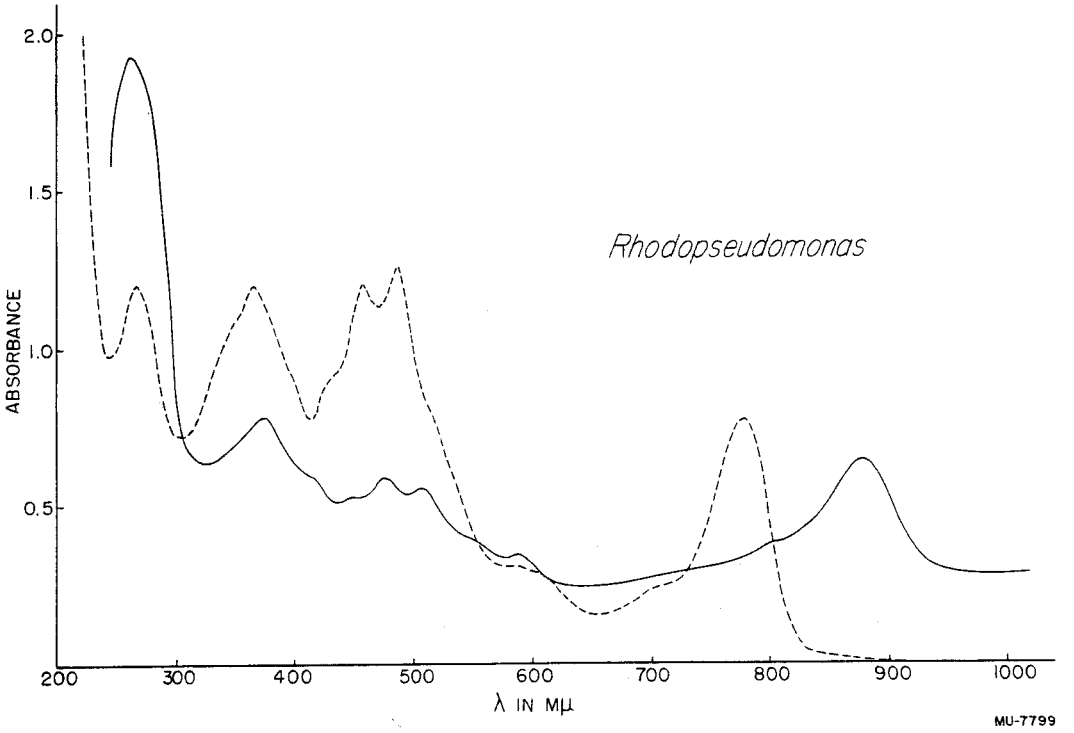

Fig. 7 Absorption spectra of Rhodopseudomonas suspension and extract. _____, cell suspension (1.0%). - - - -, alcohol extract (4.0%).

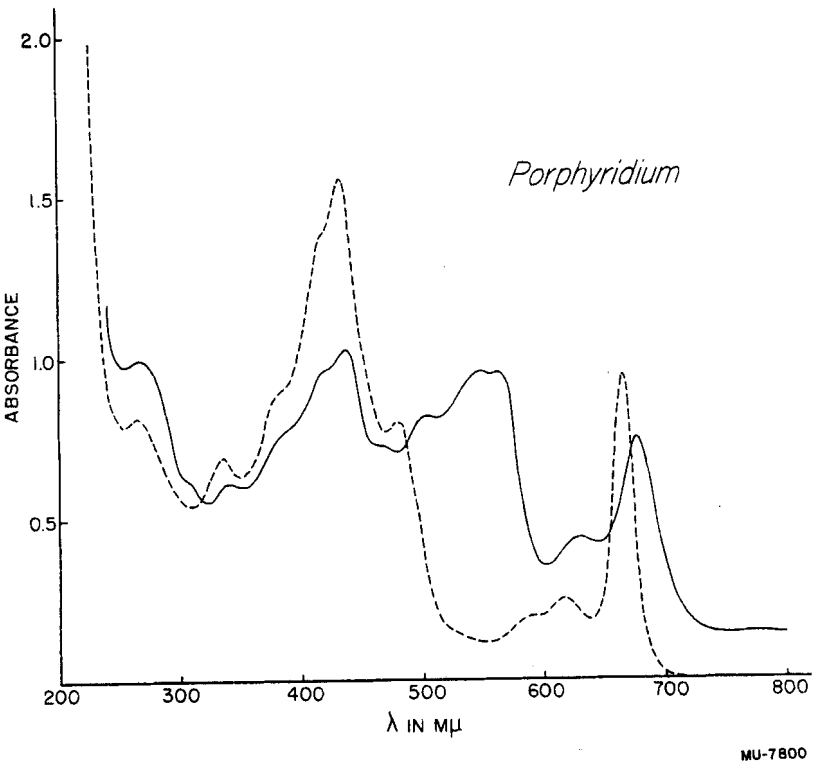

Fig. 8 Absorption spectra of Porphyridium suspension and extract. _____, cell suspension (8%). - - - -, alcohol extract (8%).

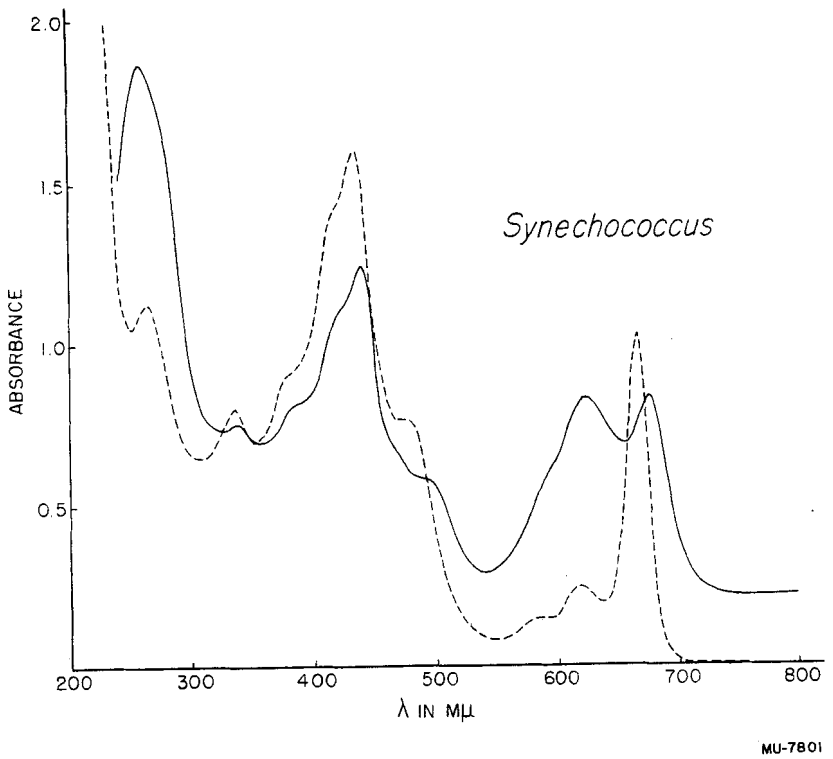

Fig. 9 Absorption ~~spectra~~ of Synechococcus suspension and extract. _____, cell suspension (4%). - - - -, alcohol extract (4%).

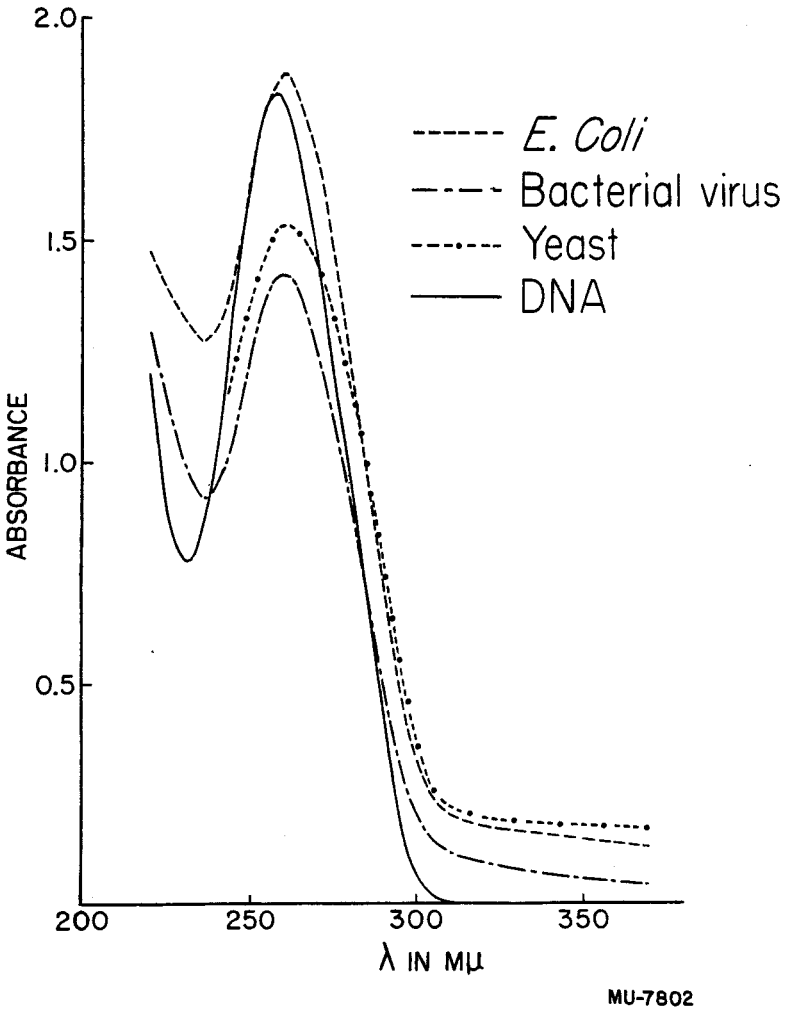

Fig. 10 Absorption spectra of E. Coli, bacterial virus and yeast suspension, and DNA solution.

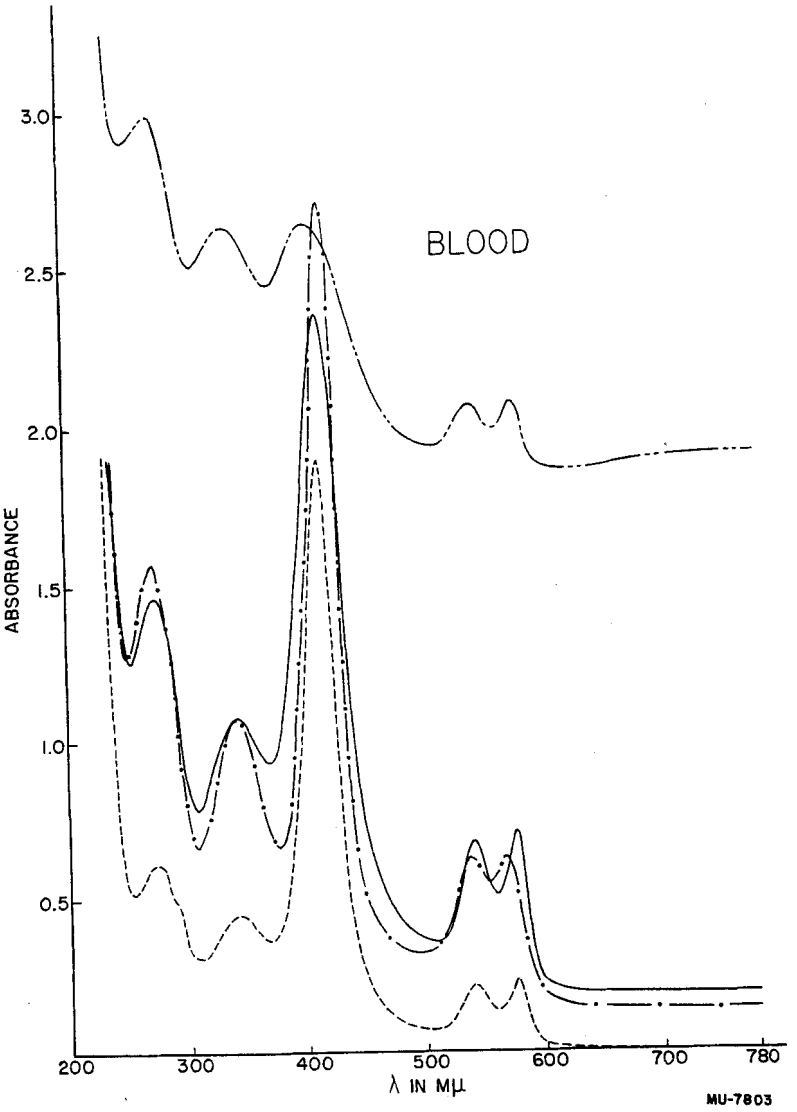

Fig. 11 Absorption spectra of rat blood suspension. _____, blood suspension, HbO_2 (dil. 1:256). - - - -, water extract (HbO_2). ___ .. ___ .. ___, blood suspension, HbO_2 (dil. 1:256) without oiled paper. ___ . ___ . ___, blood suspension saturated with CO, HbCO (dil. 1:256).

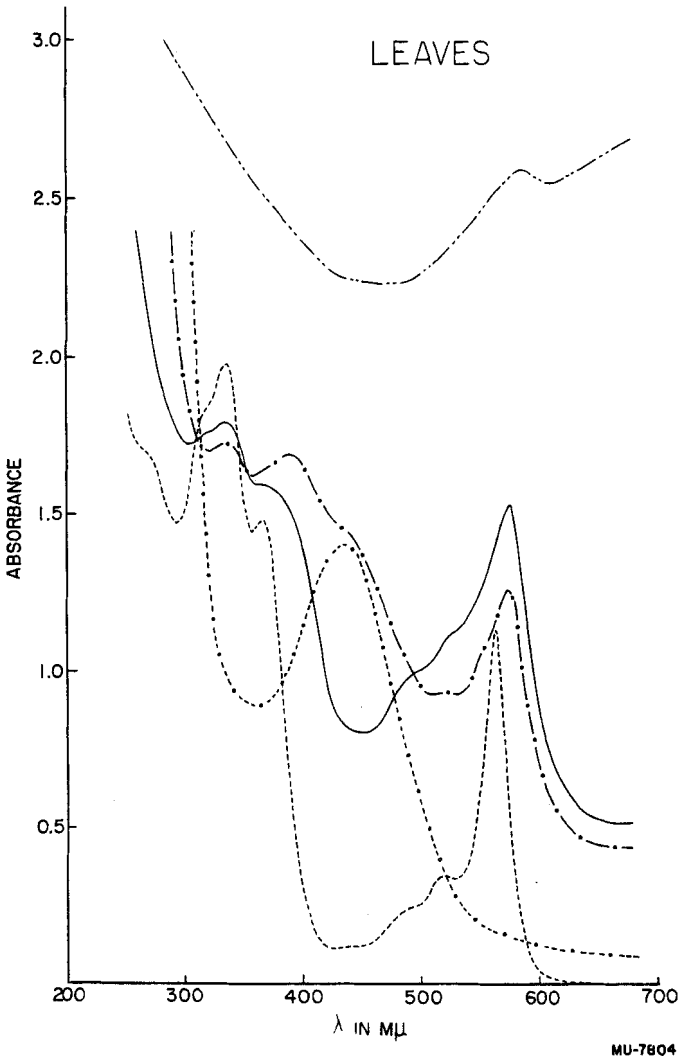

Fig. 12 Absorption spectra of leaves with opalescent plate ___ . . ___ . . ___ , Glycine soja leaf without oiled paper. _____ , Glycine soja leaf. - - - -, alcohol extract from Glycine soja. ___ . ___ . ___ , Prunus cerasifesa var. Pissardii leaf. - - -. ---. - - -. , water extract from Prunus cerasifesa.

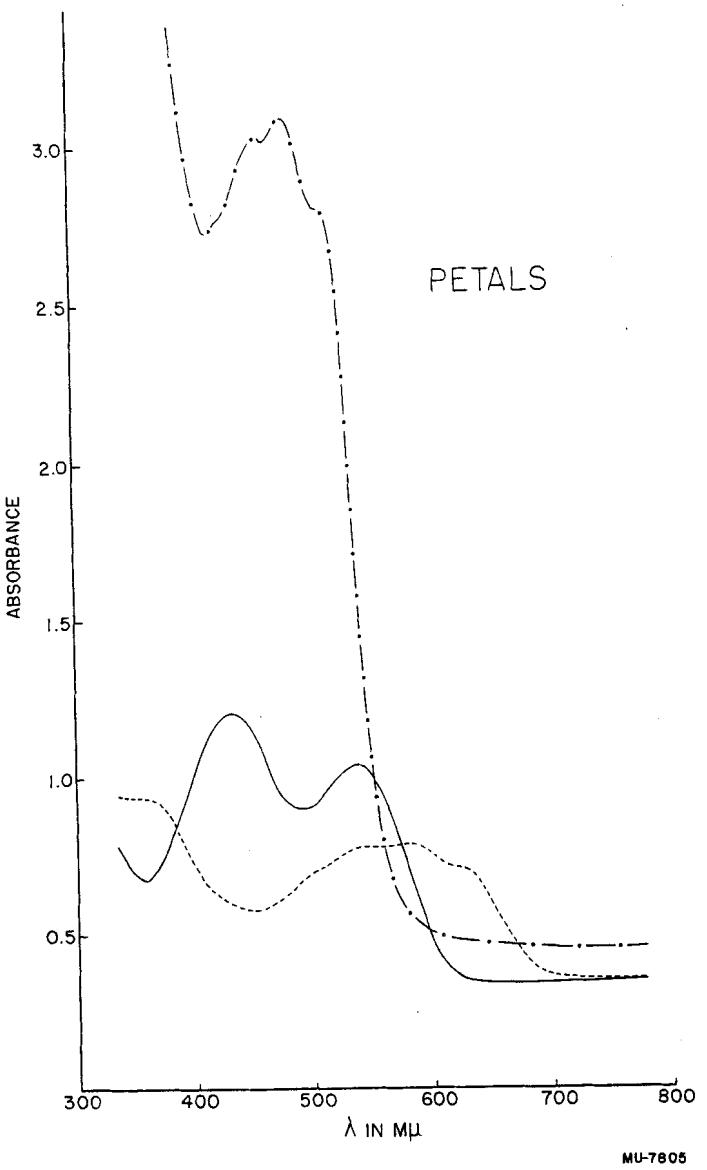

Fig. 13 Absorption spectra of petals with opalescent plate. _____, Epiphyllum petal. ----, Saintpaulla petal (African violet). ___. ___. ___, Eschscholtzea californica (California poppy) petal.

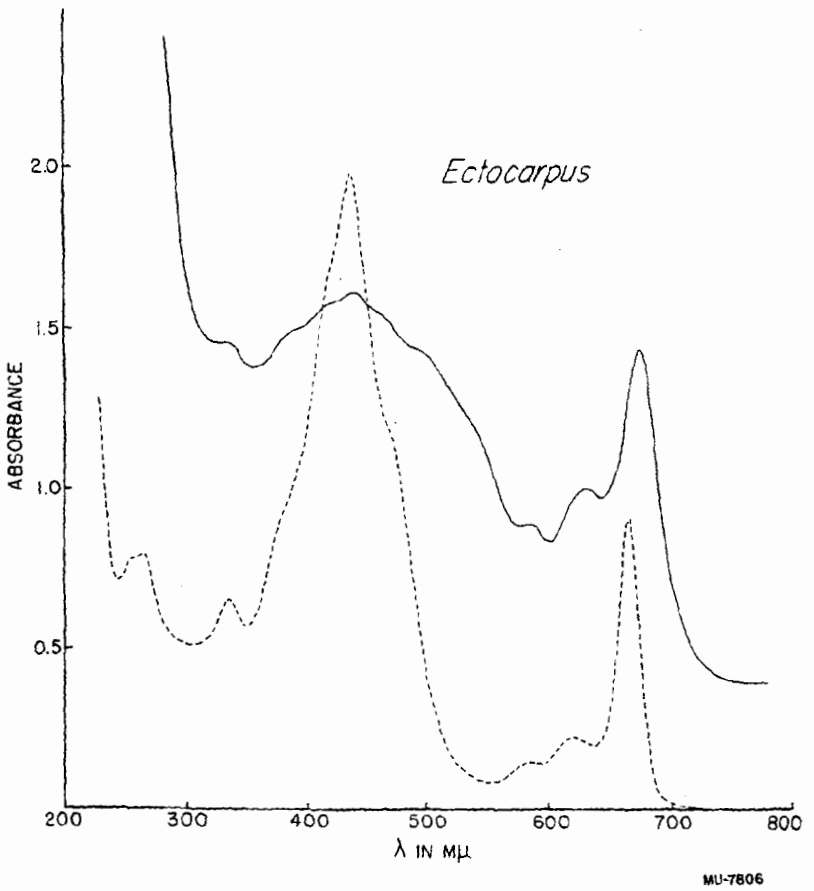

Fig. 14 Absorption spectra of Ectocarpus siliculosus _____, filaments. - - - -, alcohol extract.

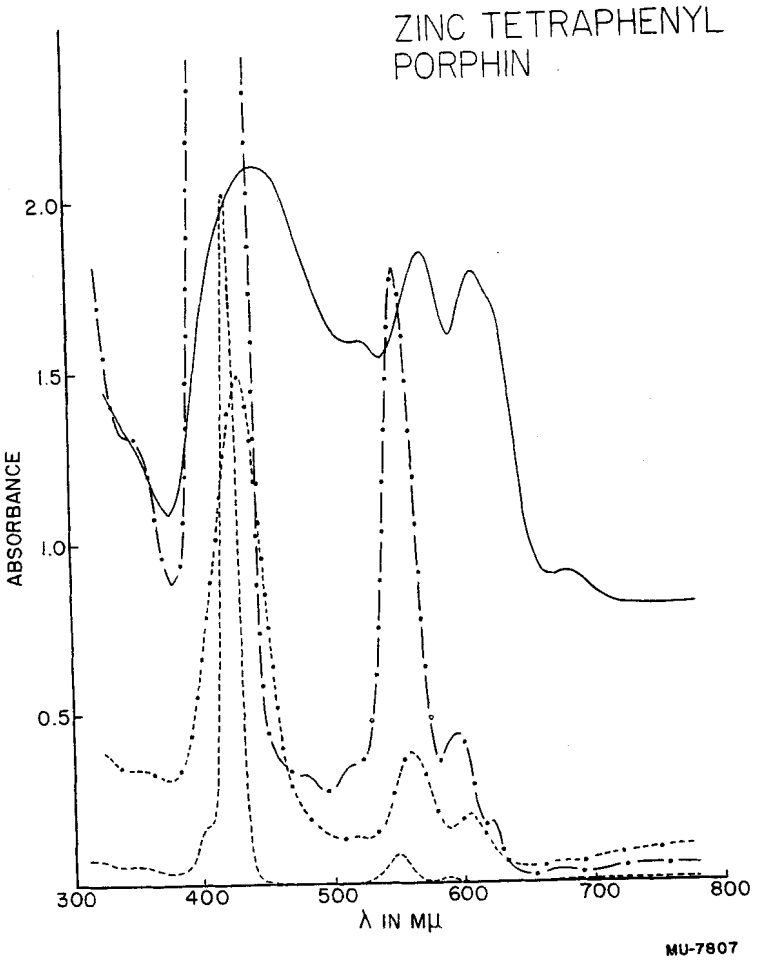

Fig. 15 Absorption spectra of zinc tetraphenyl porphin. _____, suspension in water. - - - -, solution in benzene (0.000368%). ___ . ___ . ___, solution in benzene (0.0092%). - - -. - - -. - - -, zinc tetraphenyl porphin adsorbed on filter paper.