STUDIES ON THE PIGMENTS OF SOME PLASMODIAL SLIME MOULDS

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by

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ABSTRACT

Gel permeation chromatography on Sephadex LH-20 and G-10 in aqueous alcoholic solvents has provided a non-destructive method of isolating a consistent pigment preparation from Physarum flavicomum (pigment A-2) and Physarum polycephalum (pigment 3). Both pigments have 'indicator properties' and correspond to substances implicated in light-induced sporulation of several slime moulds. Pigment A-2 (P. flavicomum) has been shown to be homogeneous from electrophoresis studies. The unusual, reversible bathochromic shift of the visible absorption spectrum on protonation is described. Analysis suggests an empirical formula $C_{12}H_{13}NO_5$ for the protonated pigment. The pigment is not a Schiff's base, contains no amino acids, has a modified polyene chromophore and a molecular weight corresponding to at least twice the empirical formula. Catalytic hydrogenation gives a colourless compound with a pH dependent ultraviolet absorption spectrum which is probably due to the presence of a substituted 2,4-pyronone chromophore. A partial structure based on spectroscopic and analytical data and chemical degradation is proposed.

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INSTRUMENTATION

Ultraviolet/visible absorption spectra were measured using a Perkin-Elmer PE202 or Unicam SP800 spectrophotometer.

Infrared absorptions were recorded in solution in either chloroform or carbon tetrachloride on a Perkin-Elmer 237B spectrophotometer.

Mass spectra were recorded on a Hitachi Perkin-Elmer RMU-6E mass spectrometer.

Nuclear magnetic resonance spectra were determined on a Varian HA-100 spectrometer.

Melting points are determined on a Kofler block heater and are uncorrected.

INTRODUCTION I

A study of cellular differentiation in simple unicellular systems should provide valuable information towards a more detailed understanding of the same processes in more complex systems. Such knowledge of the biochemical behaviour of cells and tissues grown in axenic culture may be applied towards a better understanding of abnormal cellular behaviour, and may help explain why normal cells become cancerous, and the factors that govern this change. As material for biochemical studies on normal growth and differentiation in simple systems, the plasmodia of some Myxomycetes, of which there are about 450 known species (1), are ideally suited. In these organisms, some of which have been grown in axenic culture (2,3,4) synchronous nuclear divisions occur at well defined intervals, and hence the biochemical changes accompanying these mitoses can be followed experimentally in the suitably equipped laboratory. This therefore offers a major advantage over most tissue cultures which usually contain cells that are in all stages of the mitotic cycle. The Myxomycetes or acellular slime moulds are fungus-like organisms whose life cycle can be conveniently divided into two phases, an assimilative phase and a propagative or reproductive phase. The assimilative phase exists in the form

of a free living multinucleate acellular mobile mass of protoplasm called the plasmodium, and this may be found in a variety of colours (1). In the propagative phase, the organism has undergone a morphological change, and consists of a mass of spores borne in a simple or complex membraneous cellular spore case (1). Several pigmented and non-pigmented Myxomycetes have been used in analytical studies of the process of differentiation, since under appropriate conditions the plasmodia undergoes SPORULATION, (which may also be synchronous), a simple form of differentiation (5).

Available evidence to date seems to indicate that in most pigmented Myxomycetes and some non-pigmented ones light is required to initiate this fruiting process (5), although other factors such as age of plasmodia, nutrition, and temperature may be important. Gray (1938) (6) first demonstrated that light is required before many pigmented plasmodia will sporulate, and he also showed that of the non-pigmented plasmodia he studied, none required light exposure for differentiation. Reinhard (cf.29) (1952), Straub (7) (1954) and Lieth (8) (1954) demonstrated that the reddish brown, sometimes cream coloured plasmodium of Didymium nigripes also required light for sporulation. Similar light requirement for sporulation was observed for Physarella oblonga (cf.29), Physarum gyrosum (9), and Physarum nicaraguense (cf.29), and Gray's earlier finding that the yellow pigmented plasmodium of Physarum polycephalum has an absolute light requirement for sporulation was confirmed by other

workers (10). It is generally agreed that light in the 350-500 nm region is required to induce fruiting (5), and this process is usually accompanied by the gradual disappearance of the plasmodial pigments, and the appearance of reproductive spores which are themselves coloured, ranging from yellow to black. The plasmodial pigments have therefore been implicated as possible photocatalysts for this transformation from a dynamic steady state to a new but more static steady state, and the hope is that clarification of this process might also throw some light on the mechanism of carcinogenesis.

The discovery that P. polycephalum (2), P. flavicomum (3), and P. rigidum (4), can be grown in axenic culture in the laboratory on a semi-defined medium has accelerated the study of the biology and chemistry of these organisms in the last decade. A further impetus for the study of these slime moulds has been the possibility that they produce toxins and antibiotic substances. For example, Seifriz (cf.11) studying P. polycephalum observed that when two plasmodia approach each other under favourable conditions, and when the approach is gradual, they halt a short distance between each other establishing a zone of definite width between them, and never fuse. This is interpreted to mean that exotoxins are secreted by the plasmodia. Locquin and Prevot (cf.11), Locquin (cf.11) and Sobels (cf.11) have reported the production of antibiotic substances from various species of Myxomycetes. Some are antibacterial, and some are effective against yeasts and

filamentous fungi. Texera (cf.11) on the other hand found no antibiotic activity when he tested aqueous and other extracts from plasmodia of P. polycephalum and Fuligo septica against bacteria, while Buchberger (cf.11) reported a Fuligo plasmodium to be rich in vitamin B_1. Our laboratory has been concerned with the isolation, purification and structure determination of the pigments and other metabolites elaborated by various Myxomycetes, but particularly by P. flavicomum and P. polycephalum.

INTRODUCTION - II

The Chemical Nature of the Pigments.

As early as 1889, Zopf (12) extracted the pigments from certain species of Myxomycetes and finding they were fat soluble, called them lipochromes. In 1935 Seifriz and Zetzmann (13) showed that the pigments of P. polycephalum possessed indicator properties and suggested that they may belong to a group of respiratory ferments known as flavones, lyochromes or flavins. A few years later Gray (6) demonstrated that blue light (blue line of a mercury arc at 436 nm) was an important factor in the fruiting of P. polycephalum and other Myxomycetes. He further showed that the pH range of 3.0 and 4.0 was most successful in inducing fruiting of P. polycephalum during studies over a pH range 3.0 to 8.0. This seemed to suggest that a pigment component was involved in inducing this metamorphosis and which functioned better in an acidic medium. In 1959 Wolf (14) using the methanol extraction method of Allman (15) was able to separate two pigment components from P. polycephalum by column chromatography on alumina. His observations are summarised in Table 1.

Table 1. Electronic absorption spectra of P. polycephalum pigments

Pigment	Absorption spectra in methanol (nm)			Infrared absorption (cm^{-1})
	Neutral	Acidic	Basic	
Component 1.	380	420	380	3400, 1600
	245	260-80	265	1440, 1120, 880
Component 2.	340-45	330-340	330-350	3300, 2900, 2300
	260	260	265	1750, 1560, 1420, 1050
				920, 830

On the basis of the electronic data of the isolated components, Wolf concluded that both pigments are pteridines. Biological verification of component 1 as a pteridine was obtained using the flagellate Crithidia fasciculata as a test organism, since it is known that this organism requires pteridines as a growth factor. Wolf did not identify either component 1 or 2 with any known compound of the pteridine group, and provided no further chemical evidence or data to support his conclusions. Wolf, however, suggested that of the two pigments, component 1 best fitted the properties of a photoreceptor since its absorption maximum in acidic methanol (420 nm) was close to the wavelength of light (436 nm) used by Gray to induce fruiting in P. polycephalum (6). Further work on the chemical nature of the pigments of P. polycephalum came to light with the publication of C.F. Dresden's doctoral thesis in 1959 from Prof. F.M. Strong's laboratory at Wisconsin (16). Dresden grew this organism on oatmeal, and attempted to

purify the pigments of P. polycephalum by a series of solvent fractionations, and by large scale paper chromatography. He found at least four closely related pigments in a 100-fold concentrate of the original aqueous acetone extract of the plasmodia which had been grown for about five days. His purest component (A-3) behaved like a fairly strong organic acid, being insoluble in aqueous acid solution, but soluble in a 5% aqueous sodium bicarbonate solution. He obtained a pK_a of 4.7 for this pigment preparation, using a spectrophotometric method described by Bendich (17), and this value was very similar to the acid dissociation constant of organic acids in general. This orange pigment exhibited a single absorption maximum at 390 nm with no absorption at longer wavelengths up to 800 nm. The peak at 390 nm was shifted to 410 nm by acidifying the methanolic solution; its spectral behaviour is thus similar to component 1 isolated earlier by Wolf. There was, however, no increase in the extinction coefficient on acidification. The infrared spectrum of the pigment did not show any fine absorption maxima, and specific functional groups could not be fully characterised.

Chemical analysis of the best preparation of the pigment showed carbon 55.59%, hydrogen 7.18%, nitrogen 3.12% and iron 1.10% while phosphorus, sulphur and halogens were absent. The iron content of the pigment corresponded to a minimum molecular weight of 5600. Five amino acids were

detected after acid hydrolysis of this pigment preparation with 6N HCl. They were characterised as glycine, glutamic acid, leucine, valine and proline in the solvent systems 2-butanone / propionic acid / water (75:25:30) and butanol / acetic acid / water (4:1:1). However, no chemical structure was advanced for this pigment.

Paper electrophoresis of Dresden's preparation A-2 using a pH 8.5 tris (hydroxymethyl amino methane) buffer (0.05M) separated it into 3 bands; a faster moving but rather diffuse band that migrated towards the anode, and one band which remained at the origin, but no explanation was evident for the third band.

Kuraishi (18) also at Wisconsin reported in 1961 the mass culture of the plasmodia of P. polycephalum in 15-litre and 150-litre fermentation tanks using the semi-defined medium developed by Daniel and Rusch (2). He was able to obtain three pigments having absorption maxima at 418, 379 and 360 nm which he designated A, B, C respectively and which were extracted by 80% acetone. After removal of the acetone, the aqueous solution was brought to pH 5 by concentrated hydrochloric acid, and the resulting red orange precipitate was washed first with water and then skellysolve C, and finally dissolved in absolute acetone. The acetone soluble pigments were then chromatographed on various adsorbents (acid activated Florisil, heat activated Florisil, DEAE

cellulose and CM cellulose). In concentrated solution, pigment A was converted into pigment B, and B into pigment C. Pigment A, which had a peak at 418 nm, showed no fluorescence, no absorption at 280-240 nm and yielded no amino acids after hydrolysis. Kuraishi suggested that the pigment is neither a pteridine as proposed by Wolf, nor a peptide type pigment as suggested by Dresden. E.N. Brewer (19), continuing the work on these pigments at Wisconsin, was able to obtain "three highly purified polyene pigments" A, B, C from aqueous acetone extracts of the plasmodia of the slime mould P. polycephalum. Brewer also attempted to purify the pigments by a series of solvent fractionations. A summary of the spectroscopic properties of pigments A, B, C as obtained by Brewer is given in Table 2. Pigment A exhibited an absorption spectrum characteristic of an all trans conjugated hexaene, with absorption maxima at 342, 356 and 374 nm with an $E^{1\%}_{1cm}$ of 1100, and this is in good agreement with the published data for known conjugated hexaene antibiotics exemplified by mediocidin, isolated from the mycelium of several strains of Streptomyces mediocidus (20). Reviews of the chemistry of polyene antifungal antibiotics can be obtained in two reports published by Vining in 1960 (21), and by Oroshnik and Mebane in 1963 (22). Pigment B is also a polyene compound containing nitrogen (ca. 4%), and is quite different from pigment A. A noticeable feature of pigment B is the pH dependence of its absorption spectrum,

i.e. the pigment is behaving like an indicator. In neutral solution the absorption maximum is 384 nm, ($E^{1\%}_{1cm}$ 1010) and this is shifted to 390 nm ($E^{1\%}_{1cm}$ 1190) on acidification, i.e. the pigment shows a bathochromic shift of + 6 nm, and an extinction increase of approximately 17%. Pigment B is therefore very similar to component 1 isolated by Wolf and to pigment A-3 described by Dresden. Brewer's pigment B is water insoluble, but soluble in aqueous alkalis, and Brewer contends that the pigment is amphoteric and is isolated as the zwitterion since the water washed product exhibited an ultraviolet spectrum intermediate between those determined in acidic and alkaline solution in both form and extinction, although the virtual insolubility of the pigment in acid makes this conclusion doubtful. Infrared analysis of pigment B indicatesthe presence of olefinic, amine or hydroxyl groups, but aldehyde-ketone and aromatic bands are absent. Brewer also isolated a third pigment, pigment C, which he considered an isolation artefact but which seemed to have similar spectral properties to the pigment isolated by Seifritz and Zetzmann in 1935 and by Wolf in 1955. However, the virtual identity of the infrared spectra of pigments B and C indicates that they are closely related, An additional double bond in the chromophore, perhaps arising by oxidation, may be responsible for the difference between the two pigments. None of these pigments contains peptide or carbohydrate components. Pigment C on catalytic

Table 2. Summary of ultraviolet-visible spectra of pigments of P. polycephalum according to Brewer (19).

λ_{max}(nm)* $E^{1\%}_{1\ cm}$

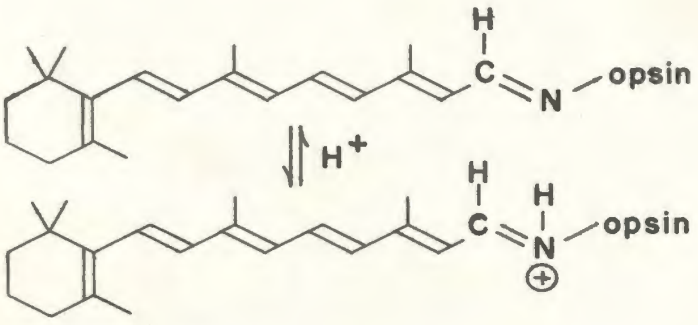
Pigment	Neutral solution	Acidic solution	Alkaline solution
A	374 (850)	no change	no change
	356 (1100)	"	"
	342 (980)	"	"
	264 (235)	"	"
B	384 (1010)	390 (1190)	355 (780)
	260 (235)	257 (190)	264 (340)
	220 (290)	221 (255)	- - - - -
C	403 (1230)	418 (1320)	385 (860)
	256 (255)	289 (190)	254 (180)
		308 (200)	
Reduced C	284 (350)	284 (350)	284 (350)
	242 (290)	227 (240)	245 (345)
	230 (385)		
Esterified C	- - - -	374	- - - - -
		347	
		333	

* $E^{1\%}_{1\ cm}$ = Absorption ($\log_{10} I_o/I$) of a 1 per cent solution in a cell with a 1 cm. path length; I_o and I are the intensities of the incident and transmitted light respectively.

hydrogenation yielded a colourless derivative (m.p. 97-99°) which crystallised from methanol/water and is the first crystalline product reported for this pigment group but Brewer was, however, unable to characterize this compound.

In a review published in 1966, Daniel (23) reported the isolation of a pigment component from P. polycephalum which absorbs maximally at 380-390 nm in alkaline solution, and at 415 nm in acid solution. This component reacts with hydroxylamine, 2,4-dinitro-phenylhydrazine, and more slowly with semicarbazide, with subsequent loss of light absorption in the 390-415 nm region. Reductionswith sodium borohydride, sulphite, dithionite and Fe^{++} - mercaptoethanol also lead to the loss of the 415 nm absorption. Daniel has therefore suggested that a carbonyl group is an essential part of the chromophore, and that destruction of the carbonyl group results in the loss of the characteristic absorption of the pigment. He also pointed out that the indicator yellow derivative (now called N-retinylidene-opsin) obtained by the action of light on the visual pigment rhodopsin, and related Schiff base derivatives show a number of similar spectral and chemical properties although molar absorptions and solubility properties of indicator yellow and his pigment differ. N-retinylidene-opsin is a Schiff base formed by the reaction of the aldehyde group of retinaldehyde with an amino group of the opsin complex, and was originally called 'indicator yellow' because of its behaviour on

changing pH. In alkaline solution it exists as an uncharged Schiff's base (I) with an absorption maximum near 365 nm, so appearing colourless; in acid solution it is protonated to the yellow conjugate acid (II) absorbing near 440 nm (24), a change of ca. 75 nm.



(I)

(II)

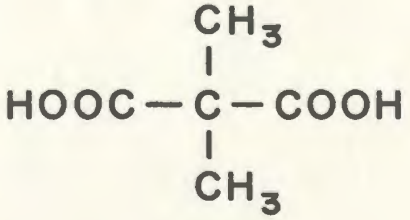
Daniel expressed the opinion that the strongly basic nitrogen function observed by Brewer could be explained on the basis of a quaternary form of a Schiff's base. Daniel further obtained strong colour reactions on treating his pigment with concentrated sulphuric acid indicative of a conjugated polyene system and this is in agreement with similar observations made by Brewer. Daniel also reported a small but definite bathochromic shift from 416 nm to 430 nm when one purified component was reacted with $SbCl_3$/MeOH, a colour test normally associated with the detection of vitamin A, a conjugated polyenic alcohol.

Watson (25) continued to work on samples of pigment A isolated earlier by Brewer at Wisconsin, and was able to separate it further into 3 components by column chromatography on silicic acid. Component 1 appeared to be a conjugated tetraene (λ_{max} 302 nm), component 2 (λ_{max} 327 nm) is a conjugated pentaene, while component 3 which he

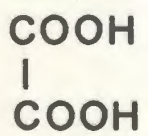
renamed A-1 was classified as a conjugated hexaene. Pigment A-1 had a maximum absorbance at 356 nm, and its spectrum was identical with that of Brewer's pigment A, and similar to that shown by several conjugated hexaene antibiotics (21). The ninhydrin test for amino acids and peptides was negative as was the ferric chloride test for phenols; although the Molisch test for carbohydrates was inconclusive because of the H_2SO_4 - polyene colour, and the anthrone test inconclusive because of the HCl-polyene colour, Watson suggested that no carbohydrate moiety was present in the pigment.

Infrared analysis of this pigment suggested the presence of -NH, olefinic -CH, and a carboxyl group. The highest mass ion in the mass spectrum of the acetate of pigment A-1 was at 314, (315 at lower electron voltage), but this probably was not the molecular ion.

Watson isolated a mixture of organic diacids by oxidation of the pigment with potassium permanganate/manganese, and then with silver oxide for conversion of the aldehydes to the corresponding carboxylic acids. Vapour phase chromatography of the diesters prepared gave 2 components whose retention times were identical to similar esters of dimethyl malonic acid (III) and oxalic acid (IV).

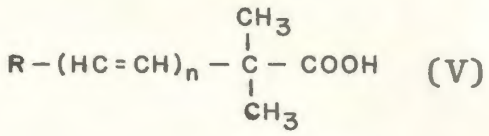


(III)



(IV)

He suggested that part of pigment A-1 must have the structure



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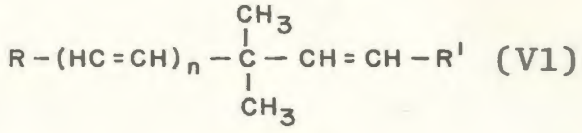


(V1)

which by exhaustive oxidation would give the diacids (III) and IV). Watson's work on P. polycephalum was terminated when his original supply of pigment A, left him by Brewer, was exhausted, and he was unable to repeat the isolation of the same pigment from a new culture of the slime mould (supplied by Dr. H.P. Rusch, University of Wisconsin). This situation is not unusual in work on mould metabolites, and is an extra difficulty which must be surmounted.

Work on the chemistry of the pigments from Myxomycetes other than P. polycephalum is even less documented. Nair and Zabka (26) (1966) extracted the pigments with either boiling methanol or 95% ethanol from the plasmodia of the white slime moulds Physarum gyrosum and Didymium squamulosum, the brown slime mould D. iridis, and 2 yellow species Physarella oblonga and Physarum polycephalum. The pigments were purified by paper partition chromatography using the following solvents systems:-

1. the organic phase of n-butanol/acetic acid/water (4:1:5)
2. n-butanol/butyric acid/water (2:2:1)

3. 3N. ammonium hydroxide.

In system 1, 3 components were obtained for Physarella oblonga, 5 components for Physarum polycephalum and 5 components for D. iridis. The absorption spectra of the crude extracts of the slime mould pigments were recorded in methanol and are given in Table 3.

Table 3. Absorption spectra of pigments from some Myxomycetes (26)

Mould	Plasmodium colour	Wavelengths of absorption (nm, methanol)	
		major peaks	minor peaks*
P. gyrosum	white	335	235-240
D. squamulosum	white	-	250-255*
Physarella oblonga	yellow	335	405
Physarum polycephalum	yellow	380	250-55, 400-405 335
D. squamulosum	yellow	370	290
D. iridis	brown	335	220, 240, 280, 290, 530
D. squamulosum	brown	335,345*	280, 290, 525-530, 220* 245*

*inflexion in curve

These workers concluded that one pigment in P. gyrosum is a flavone and also suggested that one pigment in D. iridis and one in P. polycephalum are phenolic in nature. Sobels (27) found that the pigment(s) of the orange yellow plasmodia of her strain II of Badhamia utricularis to be readily soluble in 96% alcohol, and results from paper chromatography and

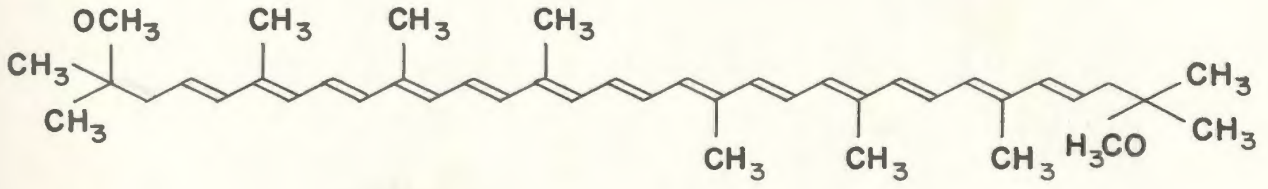
absorption spectra (335, 375-380, minor peaks at 247, 265, 275 nm) indicated that the plasmodia contain two pigments of a flavone nature.

Lieth (1954) (8) was able to separate four pigments by electrophoresis from an extract of the red-brown plasmodium of D. nigripes.

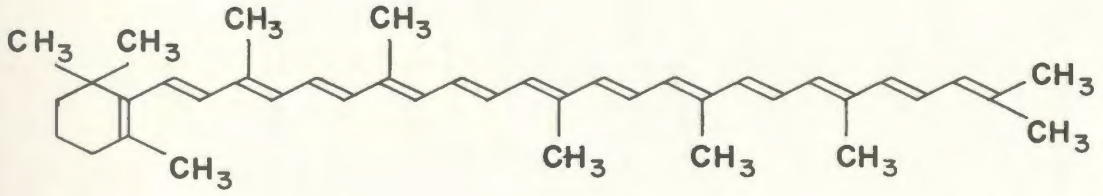
Rakoczy (28) suggested that the pigments of P. nudum are similar to those of P. polycephalum reported by Wolf after studying the effect of light on fruiting, and stated that it is component 2, (λ_{max} 340-45 nm) and not component 1 (λ_{max} 380 nm) which may be involved in the absorption of radiant energy.

LeStourgeon (1970) (29) homogenised the plasmodia of 50 twelve-day old cultures of P. flavicomum with absolute methanol; the clear yellow supernatant obtained after centrifuging was reduced in volume and chromatographed on alumina. Two broad yellow bands were eluted with chloroform-methanol (90:10) and their absorption spectra in methanol were found to be maximum at 420 nm, with shoulders at 405 and 430 nm. Plasmodia collected after an eight-hour illumination period with white light appeared paler in colour, and only one broad pigment band was isolated using the same chromatographic technique, suggesting that the pigment composition was modified, and some chemical changes had taken place. This pigment had an absorption maximum at 420 nm but possessed

definite shoulders at 430, 455 and 480 nm. Two carotenoid pigments, spirilloxanthin (rhodoviolascin) (VII) and torulene (VIII) have been reported to be found in the coral-red plasmodia of another Myxomycete, Lycogala epidendrum (30).



(VII)



(VIII)

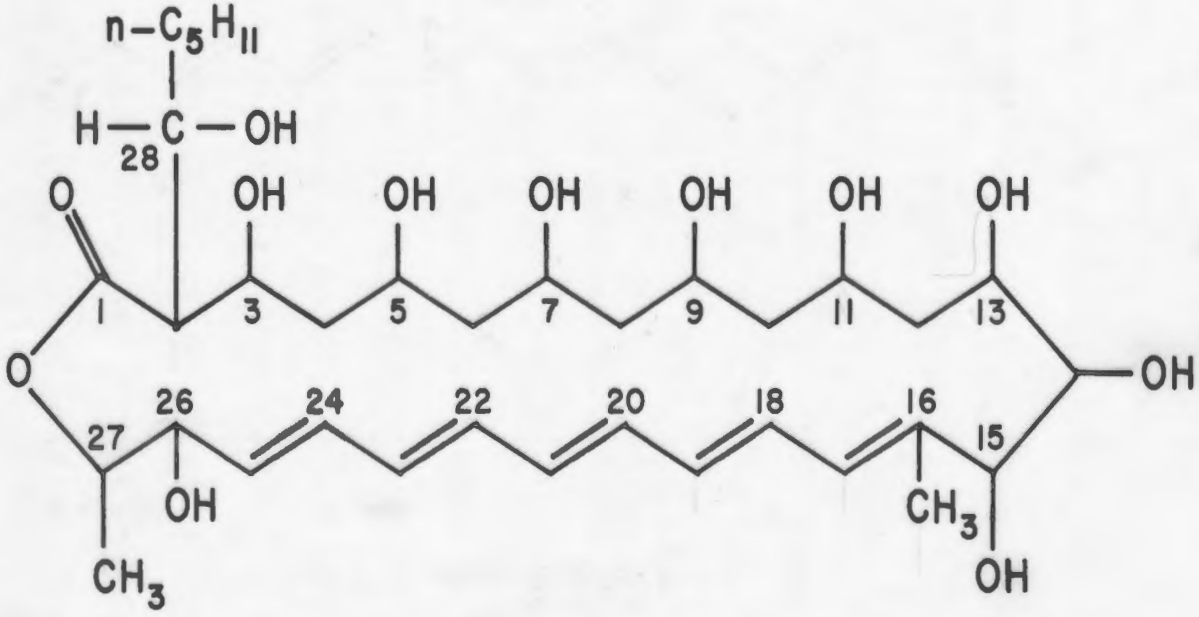
It is interesting to note that spirilloxanthin has also been isolated from Rhodospirillium rubrum, other purple bacteria, mutants of Neurospora crassa and Chromatium sp, while torulene has been found in Rhodotorula rubra, and is a characteristic pigment of red yeasts (31).

The available evidence to date indicates that P. polycephalum contains a mixture of at least three conjugated polyenes (19), Brewer's pigment A being classified as a conjugated hexaene, while pigment B is a nitrogen containing heptaene, perhaps in conjugation with an azomethine link.

In recent years a large number of macrocyclic antibiotics of a highly unsaturated nature, produced mainly by Streptomyces sp., have been isolated and some of them have had their structures elucidated. These conjugated polyenes possess characteristic ultra violet and visible spectra which can be used diagnostically to determine the number of double bonds present in the metabolites. In addition, these macrolides are highly oxygenated, usually containing a large number of hydroxyl groups. Table 4 (32) lists the absorption spectra of some representative polyenes and (1X) shows the structure of fungichromin, a conjugated pentaene macrolide elucidated by Cope and coworkers (33).

Table 4. Classification of polyene antibiotics (32).

Antibiotic	Polyene moiety	Absorption maxima (nm)			Solvent
Nystatin	Tetraenes	292	304.5	318	EtOH
Rimocidin		291	304	318	80% MeOH
Antimycoin		291	304-5	318	EtOH
Chromin		292.5	305	320	EtOH
Eurocidin	Pentaene	318	333	351	-
Flavacid	Hexaenes	341	358	379	EtOH
Mediocidin		337-8	356	377	-
Candicidin		358-9	379.5	401.5	EtOH
Candidin		363	383	406	EtOH
Ascosin	Heptaenes	358	377	399	EtOH
Trichomycin		364	384	406	EtOH
Candimycin		362	382	406	MeOH



FUNGICHROMIN (IX)

DISCUSSION

It has already been suggested that the pigments from P. polycephalum were conjugated polyenes, and an attempt was made by Brewer (19) to further categorise them. Of these pigments, the structure of the one thought most likely to be intimately involved in the sporulation process was Brewer's pigment B, postulated by Daniel (23) to be a heptaene in conjugation with a modified carbonyl function, possibly an azomethine group, to account for the significant bathochromic shift on protonation. A study of the pigments from some Myxomycetes (mainly members of the Physarales P. polycephalum and P. flavicomum) was commenced in this laboratory by Baker and Bullock, using the polyene "Schiff's base" postulate as a reasonable working hypothesis (34). A pigment preparation was isolated by chromatography on silicic acid from P. polycephalum which showed a small bathochromic shift on protonation and which seemed to correspond to pigment B isolated by Brewer. When the plasmodial pigments from P. flavicomum were separated on silicic acid, a pigment preparation 'indicator' pigment (chart 2) was obtained and its absorption spectrum was similar to that of a pigment isolated by the same method from P. polycephalum. Several degradation experiments were initially carried out on this pigment preparation (alkaline hydrolysis with dilute sodium hydroxide, potassium hydroxide, and barium hydroxide, and acid hydrolysis with hydrochloric acid) but the variability of the products obtained precluded any meaningful conclusions. It was on this basis that we felt that a milder purification scheme might provide a consistent isolate on which we could concentrate.

Isolation and purification of the plasmodial pigments of Physarum flavicomum and Physarum polycephalum.

The chemical study of the metabolites from the plasmodia of Myxomycetes (of which there are about 450 known species (1)), lagged behind the study of secondary metabolites from the so called true fungi. This impetus was probably provided by Sir Alexander Fleming's first observation of the antagonism between a strain of Pencillium and a Staphylococcus and the eventual isolation of penicillin by Florey and Chain (35). The isolation of streptomycin, a broad spectrum antibiotic of current medical application from a culture of Streptomyces griseus by Waksman and coworkers in 1944 was the result of an extensive screening program at Rutgers State University, and since then the search for metabolites with useful biological action has escalated (36).

Allman (15) reported that the pigments from the plasmodia of the yellow P. polycephalum could be extracted with methanol and this method of isolation, followed by paper chromatography and alumina column chromatography, was subsequently used by Wolf (14) to isolate two plasmodial pigments from P. polycephalum. The spectral properties of these pigments have been summarised in Table 1. The wide R_f range of these two components, obtained from paper chromatography, 0.44 - 0.62 for component 1, and 0.67 - 0.77 for component 2, suggests that these pigments may not be homogeneous. On an alumina column component 1 was

strongly adsorbed and remained within 2 to 3 cm from the top of the column; it could not be eluted with organic solvents, but was eluted with 0.1N HCl. Acidic treatment of the pigment should be avoided in view of the fact that very little was known about the nature of the pigment. The absorption maximum of component 1, λ_{max} 380 nm in neutral methanol (pigment obtained by the paper chromatographic method) was shifted to 420 nm in acidic methanol, and this bathochromic shift of 40 nm was accompanied by a small hyperchromic increase. Component 2, λ_{max} 260 and 340-45 nm in neutral methanol, was only weakly adsorbed on alumina and was readily eluted with methanol.

Methods of extraction and purification of plasmodial pigments employed by Dresden.

Since the details of the various methods employed by Dresden are available only in thesis form (16), it was considered desirable to discuss some of these methods in some detail. Dresden (16) recognised the need for a more comprehensive study of these plasmodial pigments from P. polycephalum, but suffered from an additional difficulty because the organism had to be grown on surface culture, and this limited the amount of plasmodium and hence pigment he had to work with. The subsequent development of a liquid semi-defined growth medium by Daniel and Rusch (2) for P. polycephalum was therefore a significant breakthrough in the study of this organism, and

simplified the situation for later workers studying the chemistry and biochemistry of this and other Myxomycetes.

1. Acetone extraction of fresh stationary cultures (16)

The mycelium was homogenised with acetone in a Waring blender for 5 min, this crude extract designated A-1 had an $E^{1\%}_{1\ cm}$ of 10.6 where E is defined as the absorbance at 400 nm of a solution of this preparation at a concentration of one gram (dry weight) per 100 cm^3 in acetone.

In neutral solution the preparation showed peaks at 255 and 390 nm in methanol, shifting to 410 nm on acidification, a bathochromic shift of 20 nm.

Dresden attempted to purify this extract on acid washed alumina eluted with 0.5% HCl in ethanol but found that there was strong pigment adsorption on the column, a potentially significant loss if only a small amount of pigment is available. In a further attempt to purify the pigment Dresden dissolved extract A-1 in water, adjusted the pH to 8.0 with sodium hydroxide and then this solution was acidified to pH 1.0 resulting in the formation of a bright red flocculent precipitate now designated preparation A-2. Although this preparation had an $E^{1\%}_{1\ cm}$ of ca. 1000, the possibility that the treatment with alkali and then acid could substantially alter the structure of the pigment cannot be overlooked. Sublimation of this preparation (45 min, 120°, 0.2 nm Hg pressure) was not pursued further because of the large loss

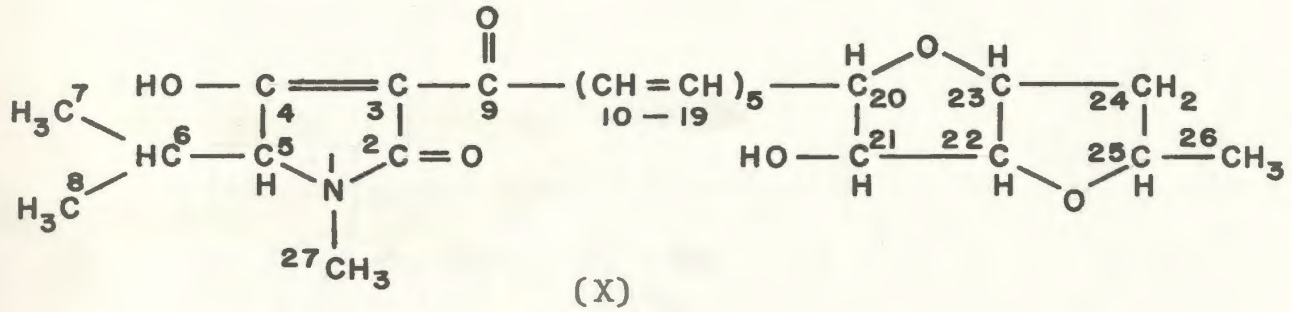
due to thermal decomposition. The most successful purification of the pigment achieved by Dresden was by paper chromatography of A-2 in the solvent system methanol/water/benzene/butanol (20 : 11.1 : 10 : 10), which resolved the pigment into four pigmented bands. A dark orange red band R_f 0.70 contained most of the pigment and was designated A-3 ($E^{1\%}_{1\ cm}$ 1500).

The absorption spectrum of A-3, 390 nm in neutral methanol, also exhibited a bathochromic shift of 20 nm on protonation, but this was accompanied by a small hypochromic shift. This hypochromic shift of Dresden's pigment A-3 on acidification is to be contrasted with the usual hyperchromic effect (ca. 40%) observed in this study of the related pigments from P. flavicomum and P. polycephalum in our laboratory. The paper chromatographic study was reproducible, giving a non-crystalline substance which was homogeneous and chromatographed as a single well defined spot in three different solvent systems. Dresden reported a 60% recovery of his pigment A-3 after purification by paper chromatography.

Acid acetone extraction of fresh stationary cultures.

Dresden extracted the plasmodia with acetone/sulphuric acid and obtained an orange red precipitate with an $E^{1\%}_{1\ cm}$ 516, and this preparation was also subjected to paper chromatography. The precipitation of the pigment by acid indicated that pigments with acidic properties could be obtained,

although it is possible that some degradation may have occurred. However, it must be pointed out that a mixture of 5% HCl in benzene was successfully used by Howard and Raistrick (37) to extract erythroskyrine (X), a polyene pigment produced by the mycelia of Penicillium islandicum, the acidity of the pigment being due to the enolisation of the β diketone system.



(X)

Several other extraction and purification methods were tried by Dresden and these included

(a) extraction of the dried mycelia at 95° with dilute acetic acid. Both the acid extraction and the elevated temperature were undesirable as again the possibility of pigment decomposition would be increased.

(b) extraction of the fresh mycelia with potassium arsenate buffer pH 7 (0.007 M), and then precipitation of the pigment by acidifying the clear yellow filtrate to pH 2 with concentrated sulphuric acid.

(c) extraction of the plasmodia with dilute sodium hydroxide solution to give a clear yellow brown filtrate. This filtrate

was adjusted to pH 7 with 85% phosphoric acid and then the pigment precipitated with 25%, 50% and 75% ammonium sulphate. The red precipitate obtained from these fractionations had an $E^{1\%}_{1\ cm}$ of ca. 400, which was certainly no improvement over the paper chromatographic method used to obtain pigment A-3 ($E^{1\%}_{1\ cm}$ 1500) from the same organism.

(d) Dresden also dried the plasmodia obtained from P. polycephalum in a drying oven at 30 mm Hg pressure for 4 h, while the temperature for the first two hours was maintained at 70°. Although he did obtain a yellow orange product after dryness, this method suffered from the obvious disadvantage of unknown thermal stability of the pigment. This dried orange product was then saponified with aqueous methanolic potassium hydroxide for one hour, and after extraction with ether (which was discarded), the aqueous alkaline solution was acidified to produce a bright red precipitate. Although saponification is one of the most effective methods of removing unwanted lipids and chlorophylls, the former often abundant in marine invertebrates and the latter invariably in photosynthetic organisms, use of this procedure in the purification of these plasmodial pigments could result in structural changes and must therefore be considered highly undesirable.

Dresden (16) however, recommended acetone as the most suitable solvent for extraction of these plasmodial pigments, a choice which was found very satisfactory in our study.

Extraction and purification method of Brewer (19)

Perhaps the most significant advance in the study of the Myxomycetes was the development of a semi-defined medium by Daniel and Rusch (2) for growing P. polycephalum. It made possible the growth of the organism on a much larger scale and virtually eliminated the problem of getting rid of other metabolites contributed by the oatmeal medium, a problem which Dresden had to contend with. The larger amounts of plasmodia meant the availability of increased quantities of pigments if only a satisfactory method could be developed for the purification of these pigments. Brewer (19) was able to grow one hundred litre cultures of P. polycephalum, and he extracted the pigments by homogenising the plasmodia in a Waring blender with four volumes of acetone. Filtration of this aqueous acetone extract and removal of the acetone produced an orange red acid soluble material. Brewer redissolved this pigmented extract with n-butanol, and found that on further dilution with ether a pigment component λ_{max} 356 nm, which he called pigment A could be easily extracted into 0.1N HCl. Pigment A could be extracted back into butanol, or into mixtures of butanol with either ether or ethyl acetate; water was also a good solvent although N HCl, 5% NaOH or Na_2CO_3 gave poor yields of the pigment. Brewer precipitated pigment A out of solution by adding ether to the butanol solution, and obtained a dark brown powder by lyophilising

this precipitate. Pigment A, which was not obtained earlier by Dresden possessed an $E^{1\%}_{1\ cm}$ 1100, and was assumed to be homogeneous. Elemental analysis of his best preparation showed C 59.85, H 7.31, O 14.37, N 7.34, and Cl 6.57, with phosphorus and ash negligible. The interpretation of chloride in the pigment should be treated with reserve since hydrochloric acid was used as an extraction solvent during the purification. The assumption must also be made here (perhaps incorrectly) that there is little or no structural rearrangement taking place in the presence of dilute acid. Watson's later demonstration (25) that Brewer's pigment A could be separated into three components cast some doubt on the homogeneity of the preparation and consequently of the isolation method.

A second compound, pigment B, was found to be readily extractable from the butanol/ether solution with aqueous base e.g. 5% $NaHCO_3$, or an alkaline buffer but Brewer (19) chose to use a pH 7.2 buffer solution for his continued purification of the pigment. This pigmented buffer extract was then acidified, and the pigment easily transferred into ethyl acetate which after evaporation produced an orange amorphous material. The absorption spectrum of pigment B has been reported in Table 2, with maxima at 384 nm ($E^{1\%}_{1\ cm}$ 1010) which changes to 390 nm ($E^{1\%}_{1\ cm}$ 1190) on acidification.

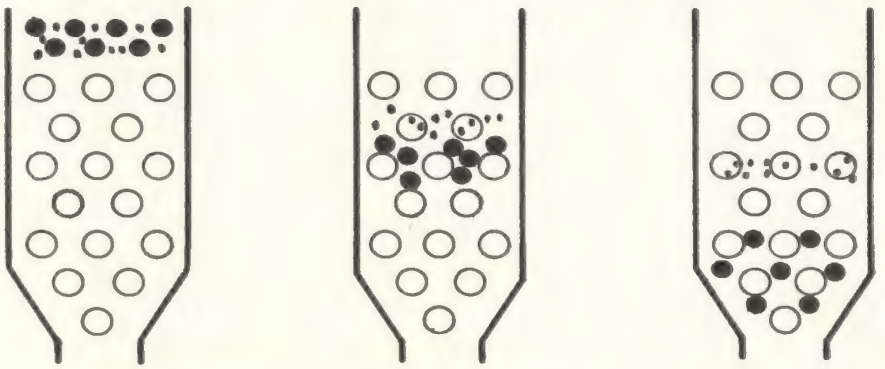
A pigment C, λ_{max} 403 nm (neutral methanol) shifting to 418 nm on acidification, was also isolated by Brewer, although this preparation was considered an isolation artefact resulting from pigment B.

Daniel (23) suggested that the pigments isolated from P. polycephalum which show a bathochromic shift on protonation are polyene Schiffsbases. If this suggestion is correct, then treatment of the pigment with dilute acid is certainly not desirable, in view of the fact that Schiff bases are readily hydrolysed to the corresponding carbonyl and the amine components. For example, N-retinylidene opsin, an intermediate in the bleaching of the visual pigment rhodopsin and thought to be a polyene Schiffsbase, is readily hydrolysed to retinaldehyde and opsin (24). The available evidence to date then indicated that although consistent products were obtained in some cases, there was some variation in the preparation among workers who had employed paper chromatography, thin layer and column chromatography or solvent extraction for the purification of these pigments.

Isolation of the pigments from P. flavicomum by the sodium bicarbonate hydrochloric acid method, and purification on silicic acid.

Since no work had been reported on the separation of the plasmodial pigments from the closely related P. flavicomum, it was decided to investigate this organism more fully to see if the pigments could be purified more easily, since varying success seemed to have been achieved by other workers (16,19,23,34) investigating P. polycephalum. In our study the plasmodia of P. flavicomum were homogenised with acetone and after filtration the aqueous acetone was reduced in volume. This pigment mixture was redissolved in 10% $NaHCO_3$ and then acidified with hydrochloric acid to give a brown precipitate (Chart 2). This mixture was chromatographed on a column prepared from silicic acid and eluted with ethyl acetate, followed by mixtures of ethyl acetate and methanol. A pigment component absorbing maximally at 390-410, which shows a small bathochromic shift to 414 nm as well as a small hyperchromic increase on acidification was isolated and several degradation experiments were carried out on this component. The non-reproducibility of these reactions necessitated a decision to attempt to develop an improved separation method if consistent results were to be obtained. Thus it was decided to investigate other purification methods with the hope that a more homogeneous and consistent pigment preparation could eventually

be produced. Gel filtration (fractionation according to molecular size) which was introduced as a chromatographic technique in 1959 was being recognised as a simple and efficient procedure in both analytical and preparative work. The method is very mild and is therefore suitable for the separation of labile biological substances, a classification which we felt encompassed these slime mould pigments. The commercially available Sephadex is a dextran polymer where the dextran macromolecules are cross linked to give a three dimensional network of polysaccharide chains. A gel filtration experiment can be summarised in the following way and schematically shown in Fig 1.



Large dots represent the large molecules.
Small dots represent the small molecules.
Open circles represent the Sephadex particles.

Fig. 1. Schematic representation of separation on a Sephadex gel bed.

Molecules which are larger then the largest pores of the swollen Sephadex, i.e. above the so-called exclusion limit, are unable to penetrate the gel particles and they

therefore pass through the bed in the liquid phase outside the particles and are eluted first. Smaller molecules, however, penetrate the gel particles to a varying extent depending on their shape and size. Thus, molecules are eluted from the Sephadex bed in order of decreasing molecular size, although other factors such as aromaticity and adsorption of substances containing carboxyl and hydroxyl groups, usually lead to higher elution volumes than can be expected from the molecular size only.

Gel filtration of plasmodial pigments of P. flavicomum on Sephadex G-25 F.

The pigment preparation obtained by acetone extraction of the plasmodia of P. flavicomum was chromatographed on a Sephadex G-25 F column (Chart 3). Three coloured bands were eluted off the column by a pH 7.5 phosphate buffer, and their absorption spectra are reported in Table 5 below.

Table 5 . Absorption spectra of pigments of P. flavicomum in order of elution from Sephadex G-25 by phosphate buffer.

Pigment	Absorption maxima (nm) in phosphate buffer		
	Buffer (pH 7.5)	Buffer (pH<3)	Buffer (pH>12)
Band I	351	351-3	353
Band II	359-62	406 (365)	359-62
Band III	390, 407	407 (388), (430)	407, (388), (430)

( ) = shoulder

Band I.

Since it was decided to direct more attention to the pigment preparation that showed a bathochromic shift on protonation, only preliminary results are reported here for this first pigment band off the Sephadex G-25 column. The absorption spectrum of this strongly fluorescent band, λ_{max} 351 nm in pH 7.5 buffer, is very similar to that of pigment A isolated by Brewer from P. polycephalum (λ_{max} 80% acetone) 356 nm, although absence of the polyenic fine structure may be a consequence of the spectrum being measured in aqueous buffer as opposed to acetone. The pH independence of the absorption spectrum is in agreement with Brewer's pigment A. This band* is eluted first off the column and this would indicate a much larger molecular weight than for the other pigments, assuming there are no other interactions between the pigment and the gel. A positive ninhydrin reaction was recorded for this preparation but this could be due to protein impurities in the fractions since no attempt was made to purify the combined extract further.

Band II.

This distinctly orange band was obtained over several fractions with an elution volume of 450-600 cm^3 of buffer, and in contrast to the previous band does not give a ninhydrin

*Elution volume, 230 ml.

reaction. The absorption maximum λ_{max} 359-362 nm in aqueous buffer was shifted to λ_{max} 406 nm, with a small shoulder at 365 nm on acidification, a bathochromic shift of ca. 45 nm. Similar bathochromic shifts on protonation (20-40 nm) have been recorded previously for pigments isolated for P. polycephalum (14,16).If this change on protonation was due to the presence of an azomethine group as Daniel suggested (23), then this pigment band corresponded to pigment B isolated from P. polycephalum by Brewer (19). The pigment could not be extracted from aqueous solution into non-polar solvents (e.g. ether or benzene), and it was not considered desirable to precipitate the pigment by acidification as was done previously. Evaporation of the aqueous buffer on a rotary evaporator was slow at 37°, and consequently the fractions were lyophilised. This was a useful technique because it avoided the use of heat; however, it extended the time period necessary for purification considerably. Furthermore, it was necessary to remove the phosphate salt by desalting, and this too added to the length of the purification scheme. The use of Sephadex G-25 however indicated that a satisfactory purification scheme was possible and recovery was good, because there was little adsorption of the pigment on the Sephadex column especially when compared with silicic acid. The separation of the pigments was attempted on Sephadex G-15, and also on Sephadex G-25F using other buffers, but these

offered no significant improvement in the aim of achieving a single homogeneous pigment.

Purification of pigments on Sephadex LH-20.

The development of Sephadex LH-20 (obtained by alkylation of most of the hydroxyl groups of Sephadex G-25) extended the use of this dextran gel to organic solvents in addition to water and aqueous buffers, since Sephadex LH-20 swells in water and most organic solvents. If this gel could be used with aqueous alcohols to separate these plasmodial pigments, then the fractions eluted off this LH-20 column could be combined and evaporated to dryness under reduced pressure in a short time, thereby decreasing the possibility of pigment decomposition. At the same time, desalting of the plasmodial extract could be achieved since Sephadex LH-20 was only a modified G-25 gel. The use of Sephadex gels for the separation of these plasmodial pigments was first developed in this study and later used by other workers in this laboratory for the separation of related Myxomycete pigments (38). The plasmodial extract (P. flavicomum) after lyophilization was separated by gel filtration on a column prepared from Sephadex LH-20 (Chart 4). Four pigments were eluted from this column by 80% aqueous methanol, and these are listed in Table 6 . Pigment 2 designated "Schiff's base" pigment, which exhibited the characteristic bathochromic shift on protonation, was selected for further purification and study.

Table 6. Absorption spectra of the pigments of P. flavicomum in order of elution from Sephadex LH-20 by aqueous methanol.

Pigment	Absorption maxima (nm) in methanol		
	Neutral	Acidic	Basic
1	(340)		(340)
	356-57	no change	356-57
	(377-78)		(377-78)
	277-78		
2	374	412	372
3	-	(366)	-
	388	386	384
	-	(404)	-
4	410-414	no change	no change

( ) denotes shoulder

Similarly when the lyophilised plasmodial extract from P. polycephalum was chromatographed on Sephadex LH-20 eluted with 80% methanol, four pigments could also be consistently isolated. Table 7 lists the absorption spectra of these pigments, while Figs. 3,4,5 are representative spectra of pigments 1,2,3 from P. flavicomum respectively.

Table 7 . Absorption spectra of the pigments of P. polycephalum in order of elution from Sephadex LH-20 by aqueous methanol.

Pigment	Absorption maxima (nm) in methanol		
	Neutral	Acidic	Basic
1	278, 283	278, 283	242, 294
	358-59	358-59	358-59
	(339-40)	(339-40)	(339-40)
	(378-79)	(378-79)	(378-79)
2	388-390	388-390	no change
	408	408	
3	390-94	418-420	388-390
4	406-414	424	386

( ) = shoulder

Pigment 2, P. flavicomum, and pigment 3, P. polycephalum seem to be related since their absorption spectra both show bathochromic shift of 30-40 nm, and a hyperchromic increase on protonation. Consequently, these pigments have been designated "Schiff's base" pigments. These pigments are also related to pigment 1 isolated by Dawson (38) from another Myxomycete Badhamia utricularis, since this too shows a similar bathochromic shift on protonation.

Choice of pigments for further study.

Pigment 2, P. flavicomum and pigment 3, P. polycephalum ("Schiff's base" pigments) purified by Sephadex LH-20 were chosen for further study for several reasons. It was also decided to concentrate on pigment A-2* from P. flavicomum, and to relate its properties to pigment 3 from P. polycephalum.

1. The bathochromic shift displayed by these pigments on protonation was similar to that shown by polyene Schiff's bases e.g. retinylidene-opsin and various synthetic retinylidene derivatives (39).

2. Their absorption maxima in acid solution were close to the wavelength of light found by Gray (6) and Daniel and Rusch (10) to induce sporulation in P. polycephalum. In this context, however, it is interesting to note that Le Stourgeon (29) has reported that in P. flavicomum, red light (610 to 700 nm) was more effective than blue light in inducing sporulation in twelve-day old cultures of photosensitised plasmodia. (LeStourgeon has suggested that the pigments may function as a screening system for light of a certain wavelength.)

3. The demonstration that a pigment component showing the same spectral shift of pigments 2, P. flavicomum and pigment 3, P. polycephalum on protonation was being produced by plasmodia grown for 24 or 48 h by the surface culture method of Mohberg and Rusch (40).

4. The fact that after sporulation there is complete dis-

*see page 41 for definition

appearance of the yellow plasmodial pigments would suggest that these pigments may be intimately involved in this remarkable biochemical transformation from an actively growing cell mass to the dormant reproductive spore-bearing phase.

Purification of pigment 2, P. flavicomum on Sephadex G-10 eluted with 50% ethanol.

It was felt that additional chromatography on a Sephadex gel with a lower exclusion limit might further fractionate pigment 2. Sephadex G-10 with an exclusion limit of 700 (c.f. Sephadex LH-20 with an exclusion limit of 5000) can also be used with lower aqueous alcohols, and so an attempt was made to further purify pigment 2 on this gel. When pigment 2 was rechromatographed on Sephadex G-10 eluted with 50% ethanol/water, two pigmented bands were obtained, designated

(i) pigment A-1,

(ii) pigment A-2.

Pigment A-2

This orange pigment was easily separated from the faster moving less polar brown pigment, designated A-1. Pigment A-2 was adsorbed as an orange band on the gel and not eluted with the void volume, suggesting that its molecular weight should be less than 700 assuming that other factors like hydrogen bonding and aromaticity are negligible. (These factors tend to increase the elution volume of the substance and hence give an incorrect estimate of the molecular weight.) The pigment was

soluble in water, indicating the presence of very polar or ionic groups, but was also soluble in mixtures of lower alcohols and water. The absorption spectrum of pigment A-2 (Fig. 7) was very similar to that of pigment 2 and also to that of other related pigments reported in P. polycephalum (16) and Badhamia utricularis (38). Pigment A-2 shows an absorption maximum (in 50% ethanol/water, pH ca. 12) in the near ultraviolet at 372 nm, ($E^{1\%}_{1\ cm}$ 2120) which is shifted to the visible at 412 nm, ($E^{1\%}_{1\ cm}$ 2560) on acidification. Another absorption maximum at 250 nm ($E^{1\%}_{1\ cm}$ 520) in the same solvent also showed a bathochromic shift to 286 nm ($E^{1\%}_{1\ cm}$ 320) on acidification. This is in contrast to the usual hypsochromic shift shown by phenolic compounds when the phenoxide ion is neutralised with acid (phenol in alkaline solution has λ_{max} 235 and 287 nm while, in neutral solution, absorbs at 210.5 and 270 nm).(41 The absorption spectrum of the pigment was found to be influenced by the polarity of the solvent; for example the pigment shows a broad absorption band at 380 nm in water which shifts to 416-418 nm on acidification with only a 16% increase in extinction. Similar observations have been recorded by Dawson (38) for a related pigment from Badhamia utricularis. The $E^{1\%}_{1\ cm}$ value of 2080 found for pigment A-2 from P. flavicomum in this study was much higher than the values reported for similar pigments from P. polycephalum ($E^{1\%}_{1\ cm}$ 1500 (16), and 1010 (19)) and this is interpreted to represent a significant improvement in the purity of this preparation.

Some advantages of using Sephadex.

1. Since the method is mild, recovery of the pigments after chromatography on both Sephadex LH-20 and G-10 is almost quantitative as there is no adsorption loss on LH-20, and very little on G-10. Furthermore, there seems to be little or no decomposition of these pigments on either gel.
2. The same columns can be reused provided they have been properly washed with the eluting solvent after chromatography of one sample. Consequently the use of Sephadex is economical both in time and money.
3. In this work, the various pigmented bands are easily visible on the white background of the gel making collection of 'bands' relatively simple. Aluminium foil was used at all times to screen the pigments from light.

Additional advantages include (i) protein comes off fast, thereby getting rid of a troublesome contaminant for any natural product. (ii) Salts are eluted very slowly, thereby getting rid of a contaminant common in any isolation method for natural products. However, the use of Sephadex does have at least one disadvantage - the low loading permitted for reasonable separation compared with silica, alumina, etc. While it would be too long to detail various specific applications of Sephadex LH-20 or G-10, it is interesting to note that Sephadex LH-20 has been used in the separation of the carotenoid pigments from Sarcinia lutea (42), in the separation of chlorophyll (43) and also the antibiotic actinomycin (44).

Similarly the separation of campesterol and β-sitosterol has been reported on an alkylated Sephadex LH-20 by Elliot and Hyde (45). Vining (46) has recently reported the separation of gibberellin A, and dihydrogibberellin A, by argentation partition chromatography on a Sephadex G-25 column, and suggests that the method may be equally valuable for the separation of other unsaturated substances, especially polar compounds which are acid sensitive and require solvent mixtures containing acetic acid.

Cellulose acetate electrophoresis of pigment A-2 P. flavicomum.

As a further test of the purification of the plasmodial pigments on the combination Sephadex gels, pigment A-2 was subjected to cellulose acetate electrophoresis in buffers ranging from pH 8.9 to 1.1 (Fig.8). A single pigmented band possessing overall anodic mobility (with no residue at the origin) was obtained in buffers from pH 5.1 and above, although the electrophoretic mobility decreased as more acidic buffers were used. Between pH 4.0 and 2.0 only a single orange band was obtained but there was a significant increase of pigment residue at the origin, while at pH 1.1, there was no movement of the now orange pigment although there was slight spreading of the band because of diffusion. It is also important to note there was no cathodic migration of the pigment in this acidic buffer, indicating that the pigment was now behaving

as a neutral species. Since only one band was obtained after cellulose acetate electrophoresis of pigment A-2, this study suggests that this pigment obtained after chromatography on Sephadex G-10 was homogeneous. The electrophoresis of the pigment was pH dependent, but at all pH's the compound appeared homogeneous. The pH dependence study (discussed in detail later) casts doubt on Daniel's "Schiff's base" hypothesis (23).

Cellulose acetate electrophoresis of pigment 3, P. polycephalum.

This related pigment from P. polycephalum was also subjected to cellulose acetate electrophoresis and Fig. 21 illustrates the separation of this preparation. In buffers of pH 8.9, 8.0 and 7.0, the major pigment obtained was a single yellow band along with a narrow yellow band both migrating to the anode. At pH 6.0 and 5.1, three pigments were obtained (one orange and two yellow), all possessing a net negative charge while at pH 4.0, there was no movement of the now orange pigment. Similarly at pH 3.0, 2.0 and 1.1 there was no net movement to either cathode or anode suggesting that the pigments were now neutral. It is interesting to observe, however, that even at pH 4.0 there was no net movement of the pigment in contrast to the behaviour of pigment A-2 from P. flavicomum which still migrated to the anode at this pH. This can be interpreted to mean that pigment A-2 from P. flavicomum is a

slightly stronger acid than the related pigment 3 isolated from P. polycephalum. It was already pointed out that pigment 3 from P. polycephalum could not be separated into distinct bands on Sephadex G-10 in this study because of adsorption effects and diffusion of the pigment on this column. It is therefore not surprising that pigment 3 may be a mixture of possibly related polyene pigments and this is partially supported by the separation of this pigment by thin layer chromatography into three components (Table 32). It may also be extremely fortuitous that pigment 3 from P. polycephalum was also resolved into three components by cellulose acetate electrophoresis in buffers of pH 5.1 and 6.0. Baker (34) had earlier isolated a pigment preparation from P. polycephalum by silicic acid column chromatography, possessing similar spectral properties to the 'indicator' pigment from P. flavicomum (Chart 2).

'Indicator pigment' refers to pigment which shows a bathochromic shift on protonation and which is obtained after silicic acid chromatography of the crude plasmodial extract (P. flavicomum and P. polycephalum).

Pigment A-3 (P. flavicomum)

This pigment λ_{max} 414 nm (80% methanol) was obtained after chromatography of pigment A-2 on Dowex 50W-X8 cation exchange resin (p.164).

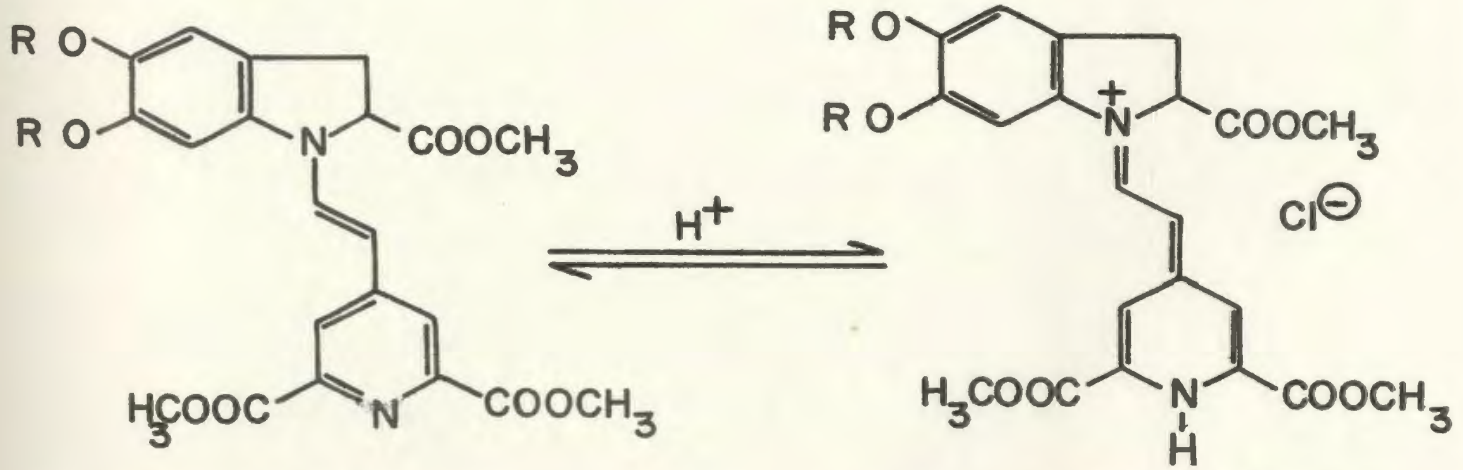
'Schiff's base' pigments from P. flavicomum and P. polycephalum.

It has already been suggested that a pigment preparation isolated by Daniel (23), which was related to Brewer's pigment B, was a polyene in conjugation with an azomethine group, and this reasonable postulate formed the working hypothesis of our investigations of the plasmodial pigments. The bathochromic shift, (25-40 nm), and the accompanying hyperchromic increase on protonation, are similar to shifts observed with authentic polyene Schiff's bases e.g. synthetic N-retinylidene-amino acids and other amine compounds so thoroughly investigated by Morton and coworkers (39,47). The nitrogen content (3-5%) reported by Dresden (16) and Brewer (19) for their P. polycephalum pigment augments the Schiff's base postulate, while a recent analysis of pigment A-3 (P. flavicomum) which reported a 5.7% nitrogen content, seemed to support this hypothesis (48). Pigment A-2 (P. flavicomum) λ_{max} (MeOH/NaOH) 372 nm exhibits a bathochromic displacement of $\sim$ 40 nm on protonation and in this highly purified pigment, is accompanied by a hyperchromic increase of ca. 40%. Similarly, pigment 3, (P. polycephalum), λ_{max} (MeOH/NaOH) 388-90 nm shows a bathochromic shift of approximately 30 nm on protonation.
Table 8 lists (i) the absorption spectra of related Schiff's base pigments from several Myxomycetes and their approximate change on protonation, (ii) their absorption maximum in alkaline solution and (iii) the approximate change in the absorption maximum in basic and acidic methanol.

Table 8 . Long wavelength absorption bands in the electronic spectrum of some related Myxomycetes pigments.

Myxomycete	Pigment	Absorption maximum (nm) MeOH/$OH^{\ominus}$	MeOH/H^{+}	$\Delta\lambda$(nm)	Reference
P. flavicomum	A-2	372	412	40	
P. polycephalum	3	388	420	32	
P. polycephalum	1	380	420	40	(14)
P. polycephalum	A-3	390	410	20	(16)
P. polycephalum	B	355	390	35	(19)
P. polycephalum	C	385	418	33	(19)
P. polycephalum	1	380-390	415	25-35	(23)
Badhamia utricularis	1	375	420	35	(38)

Several polyene Schiff's bases were synthesised from retinaldehyde and α-furylacrylaldehyde (49) with various amines and amino acids, and the change in their absorption spectra on protonation recorded (Tables 28 and 29). Schiff's bases derived from retinaldehyde generally showed a bathochromic shift of ∿ 75-98 nm, observations which are similar to those reported by Ball et al (39), while those derived from α-furylacrylaldehyde are displaced to the red by 50-60 nm, and this is always accompanied by a hyperchromic effect. Dawson (38) has also reported a shift of 57-62 nm for Schiff's bases derived from 2,4,6-octatrienal with various aliphatic amines and amino acids. Similarly, a bathochromic shift of about 94-110 nm, accompanied by a hyperchromic increase of 40% has been reported to occur when (XI) and related compounds are protonated to give the quaternary ammonium salt (50). These compounds have been characterised as part of a study on the red-violet water-soluble pigments from families of the Centrospermae and named betacyanins.



(XI), R=H, $\lambda_{max}^{(MeOH)}$ 410nm

(XIa), R=H, $\lambda_{max}^{(MeOH/H^+)}$ 520nm

(XII), R = OCH_3, $\lambda_{max}^{(MeOH)}$ 403nm

(XIIa), R = OCH_3, $\lambda_{max}^{(MeOH/H^+)}$ 513nm

Origin of the bathochromic shift on protonation.

Pitt et al (47) have suggested that the bathochromic displacement of Schiff's bases on protonation is a consequence of proton addition to the nitrogen atom to form a quaternary ammonium salt. The positive charge can be formally localised at different positions on the polyene and therefore several contributing forms can be written for any protonated polyene Schiff's base, compared to one main form for the non-protonated compound. The case of the betanidin derivatives studied (50) is rather different since protonation brings into conjugation two aromatic chromophores, thereby shifting the long wavelength absorption maximum to the red.

In a study of the u.v. spectra of some azomethines and related compounds, Brocklehurst (51) noted that when a solution of N-benzylidene-aniline (XIII) is protonated, there is a shift in absorption from an inflexion at λ_{max} (EtOH) 310 nm (ε8200) to λ_{max} (EtOH) 335 nm (ε19,500) (note the intensity increase). A similar intensity increase was observed for N-phenyl-benzylidene-nitrone (λ_{max}EtOH) 316 nm (ε=20,000). To account for the bathochromic shift it was proposed that nonprotonated N-benzylidene-aniline is not planar since the nitrogen lone pair occupies an orbital at right

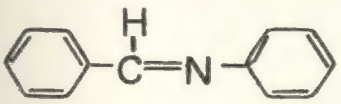
angles to the main π electron system. The protonated form of the azomethine and the nitrone resemble trans-stilbene implying that the azomethine assumes a planar configuration on protonation and a normal stilbene type spectrum results. Since coplanarity is required for the most effective overlap of the π-orbitals and increased ease of the $\pi \rightarrow \pi^*$ transition, then the protonated azomethine which shows an increased extinction at 335 nm should exist predominantly in the planar form. Brocklehurst (51) also compared the u.v. spectra of

(XIV) 2-phenyl-benzimidazole

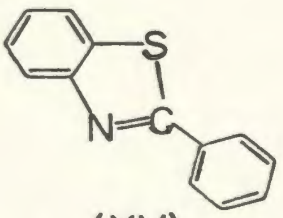
(XV) 2-phenyl-benzothiazole

(XVI) 2-phenyl-benzoxazole

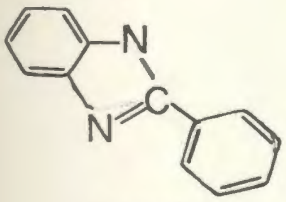
with those of N-benzylidene-aniline and trans-stilbene.



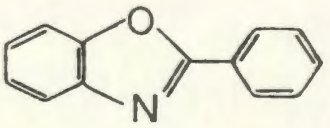
(XIII)



(XV)



(XIV)



(XVI)

In compounds, (XIV), (XV), and (XVI) the configurations about the -N=C- linkage are fixed due to the five-membered ring and they are all completely planar.

Table 9 . Absorption data for compounds of the azomethine type in ethanol (nm).

Compound	λ_{max} (nm)	ε
2-phenyl-benzimidazole	301	23,300
2-phenyl-benzothiazole	295	20,700
2-phenyl-benzoxazole	299	22,400
trans-stilbene	298	27,500
N-benzylidene-aniline	310	8,200

Thus when the N-benzylidene-aniline molecule is made to assume a planar configuration, e.g. by protonation, the intensity of the band in the 300 nm region increases appreciably. The spectra of 2-phenyl-benzimidazole, 2-phenyl-benzothiazole, and 2-phenyl-benzoxazole, on the other hand, are very similar in this region to trans-stilbene, since they are already planar. [These observations support the idea that the N-phenyl group of benzylidene-aniline is out of the plane of the main conjugated system.] If pigment A-2 (P. flavicomum) and pigment 3 (P. polycephalum) do contain an azomethine group then protonation of the nitrogen lone pair would bring about maximum overlap of the π electrons of the conjugated polyene with perhaps another chromophore, thereby causing both a bathochromic shift and a hyperchromic increase (∿ 40%).

The normal bathochromic shift on protonation of the related polyene Myxomycete pigments (20-40 nm) is low for normal conjugated Schiff's bases although some workers (52) prepared Schiff's bases derived from crotonaldehyde, sorbaldehyde, and 5-methyl-2,4-hexadienal with n-butylamine, and reported a bathochromic shift of 27 to 56 nm on protonation, in acetonitrile. Since the bathochromic shift of the Myxomycete pigments is small compared to the change in authentic polyene Schiff's bases, it seems reasonable to assume that the environment of a polyene Schiff's base chromophore within the pigment itself might modify the shift. Such effects are known, although the shift to longer wavelengths is usually much greater than in these Myxomycete pigments; for example, in the imine prepared from retinaldehyde and phosphatidylethanolamine (PE) it has been suggested that internal protonation of the nitrogen atom by the phosphoric acid group of PE (53) might be partly responsible for the anomalous absorption (500 nm) compared with simple polyene Schiff's bases of the same size (ca. 440 nm in acid solution). It was also shown recently that similar bathochromic effects to those observed on protonation occur when retinylidene-n-butylamine was adsorbed on to silica gel or when a methanol solution was cooled to -78° (49). On the other hand, Hirtenstein and Akhtar (54) reported that Schiff's bases obtained from all-trans retinaldehyde and either ethanolamine or lysine which normally absorb at λ_{max} 437 nm in aqueous solution can absorb up to λ_{max} 490 nm in aqueous solution containing

0.2M trichloroacetic acid (TCA) frozen to -150°, and suggested as a possible explanation the specific interaction of TCA with the retinylideneiminium chromophore. With hydrochloric acid, acetic acid, and monochloroacetic acid, no bathochromic shift in the spectrum to λ_{max} 490 nm was observed from -150° and 10°C. No similar anion specificity is observed with the plasmodial pigments. The spectral data and the changes associated with protonation of these pigments and known conjugated polyene Schiff's bases indicate that the Myxomycete pigments might contain an azomethine group, although the quantitative comparison (of both bathochromic and hyperchromic effects) is not good. It was consequently decided to investigate other properties of pigment A-2 (P. flavicomum), and pigment 3 (P. polycephalum) so that further comparisons could be made with similar properties of simple polyene Schiff's bases, and with other related Myxomycete pigments. Possible methods of reaching a decision on the presence or absence of a conjugated azomethine in the pigments include

(a) pK_a measurements

(b) typical azomethine degradations

(c) Infrared and Raman spectra

(d) N.m.r. studies of azomethine link (proton is found in very low field similar to aldehydic proton).

pK_a of the pigments and some Schiff's base conjugate acids.

The pK_a of pigment A-2 (P. flavicomum) was determined in aqueous buffer by a spectroscopic method (17) at the following wavelengths (i) loss of 380 nm maximum with decreasing pH, and found to be ca. 3.4 and (ii) appearance of the maximum at 416 nm with increasing acidity and found to be ca. 2. Similarly, values of about 4.8 (by following the loss of 390 nm maximum with decreasing pH) and 3.6 (by following the appearance of the maximum at 420 nm) for the related pigment 3 from P. polycephalum, although later electrophoretic analysis which resolved the pigment into at least 3 bands has cast some doubt on the homogeneity of this preparation. Dresden (16) had earlier reported a pK_a of 4.7 for his related P. polycephalum pigment, and on this evidence suggested the presence of a carboxyl group, although he was unable to prepare a methyl or ethyl ester. Dawson (38) has found a pK_a of 4.0 for his related pigment 1 from Badhamia utricularis in aqueous solution. Kosower and Sorensen (55) determined the pK_a of the conjugate acids from 2,4-hexadienylidene-n-butylamine and crotonylidine-n-butylamine and found values of 8.5 and 8.6 respectively. Similarly the pK_a of octatrienylidene-t-butylamine and retinylidene-t-butylamine were found to be 8.6 and 7.4 respectively in 90% methanol (38). It seems unlikely that the pK_a of the pigment as reported by Dresden relates to the dissociation of a carboxyl group.

The spectral behaviour of the pigment suggests that the dissociation of the relevant group is intimately associated with the large bathochromic shift on protonation and a reversible hypsochromic shift in alkaline solution, whereas the dissociation of carboxyl groups in conjugation with an extended polyene chromophore has little effect on the absorption spectrum. The pK_a of pigment A-2 is suggestive of a stronger acid than a simple carboxylic acid and is not in agreement with the value expected for a simple protonated polyene Schiff base, 2,4-hexadienylidene-n-butylamine and crotonylidene-n-butylamine being far more basic. Nigrifactin, a polyene Schiff's base produced by Streptomyces (strain No. FFD-101), has been found by Terashima *et al* (56) to have a pK_a of 8.8 in 50% aqueous ethanol. However the possibility that the acidity of a protonated nitrogen can be increased by the presence of electron withdrawing groups cannot be entirely eliminated. Table 10 lists the pK_a's of conjugate acids of a variety of compounds, and though not meant to be exhaustive it gives some indication of potential acidic chromophores.

In the curves used to determine the pK_a of pigment A-2 (*P. flavicomum*) (Fig.13) it should be noted that two fail to pass through an isosbestic point. Both of these curves relate to very acidic solutions, which strongly suggests that a second reaction (presumably following protonation and catalysed by

acid) occurs to give a modified product. The full hyperchromic effect is only observed with relatively strongly acidic solutions (i.e., pH < 1.1). It follows therefore that the "pK_a" ($\sim$ 2) measured from appearance of the 420 nm absorption is a composite number which is probably lower than the true pK_a value for the acidic system present. Alternatively it could represent a second more acidic functional group in the molecule. The curves in Fig.13 which relate to solutions of pH > 2.0 pass through an isosbestic point and it is suggested that these curves relate to a simple protonation reaction. On this assumption, the pK_a of the native pigment in P. flavicomum is about 3.4.

A similar observation was made for pigment 3 (P. polycephalum) and Fig.22 clearly shows the effect. In this pigment a "true" pK_a of 4.8 is obtained, with the 'acid modified' value being 3.6, the former value agreeing with Dresden's spectrophotometric measurement (16).

TABLE 10. pK_a of conjugate acids of some model compounds

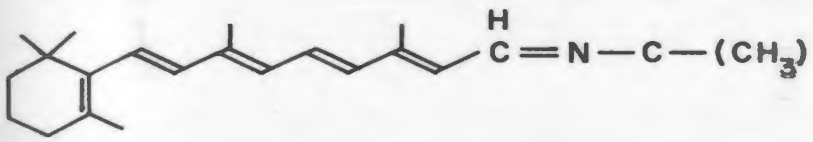
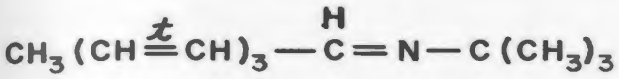
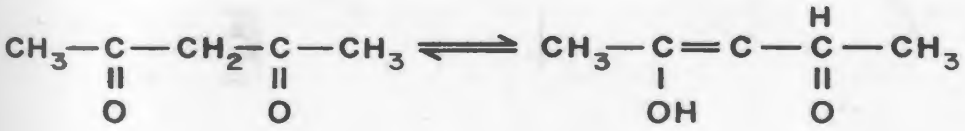
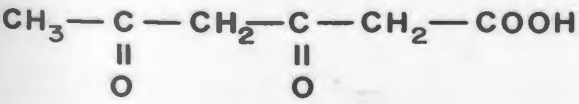
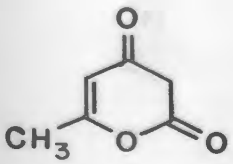
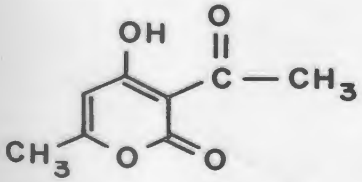
Compound	No.	pK_a	Ref.
	(XVII)	8.6	38
	(XVIII)	7.4	38
	(XIX)	8.8	56
	(XX)	5.3	57
	(XXI)	8.94	58
	(XXII)	10.49	58
	(XXIII)	3.3	59
	(XXIV)	5.0	60
	(XXV)	5.3	60

Table 10. pK_a of conjugate acids of some model compounds (continued

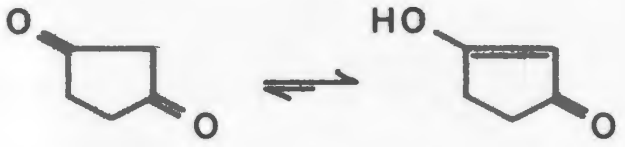
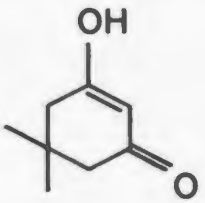
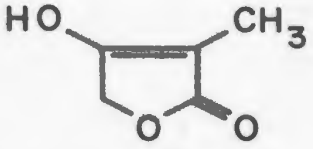
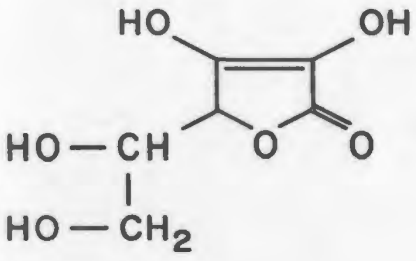
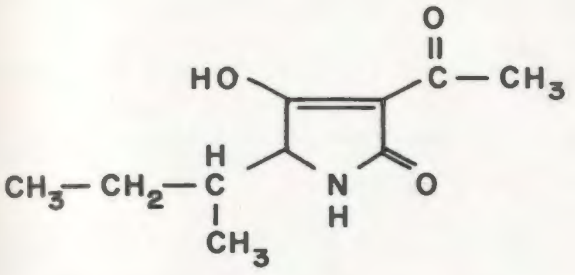
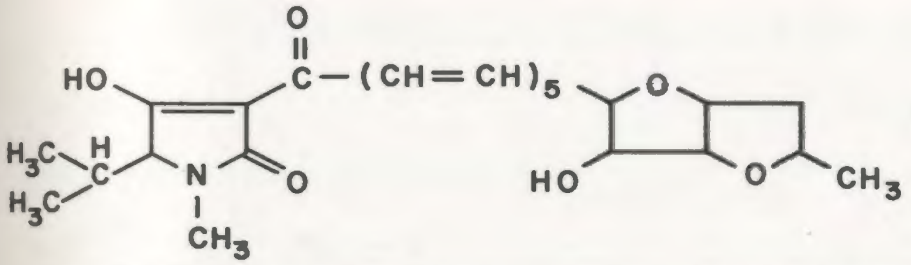
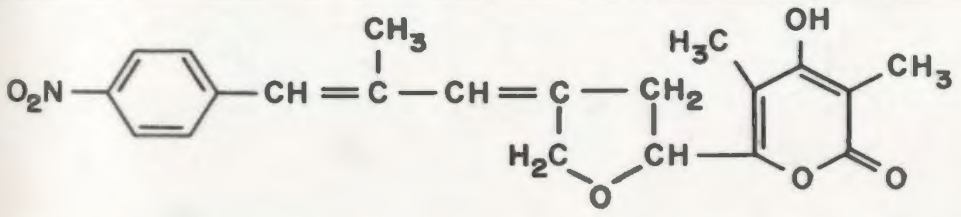
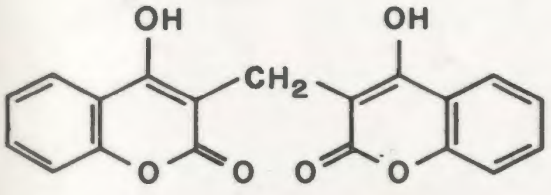
Compound	No.	pK_a	Ref.
	(XXVI)	4.5	61
	(XXVII)	6.21	62
	(XXVII)	6.2	62
	(XXIX)	3.76	63
	(XXX)	3.6	64
	(XXXI)	4.85	65

Table 10. pK_a of conjugate acids of some model compounds (continued

Compound	No.	pK_a	Ref.
	(XXXII)	3.35	66
	(XXXIII)	-	67
	(XXXIV)	4.6	68
	(XXXV)	7.2	69

Reaction with sodium borohydride.

In the early stages of this work, sodium borohydride was found to reduce pigment A-2 P. flavicomum rapidly (and also pigment 3 from P. polycephalum). In both these cases the reduced product shows a polyene type of spectrum (Table 11), accompanied by a decrease in the absorbance. Daniel (23) had earlier reported the same reaction of a pigment isolated from P. polycephalum, causing the loss of the 415 nm maximum.

Table 11. Spectral changes on reduction of pigment A-2 P. Flavicomum and pigment 3 P. polycephalum by sodium borohydride in ethanol.

Myxomycete (pigment)	Absorption maxima in basic ethanol (nm)	Absorption maxima in ethanol after reduction (nm)
P. flavicomum (A-2)	250, 372	272, 282, 304, 319
P. flavicomum (A-3)	385, (380) (430)	244 (broad), 304, 320, 334, 376
P. polycephalum (Pigment 3)	390	270, 283, 301, 317, 331

( ) = shoulder

In the later stages of this work, isolates of pigment A-2 could not be reduced with borohydride under the same experimental conditions. However, when pigment A-3 (obtained by protonation of pigment A-2 on a cation exchange resin column) was treated with the reducing agent the solution was decolourised after 5 min, again producing a polyene type spectrum which

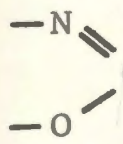
exhibited strong yellow fluorescence under u.v. light. The apparently related pigment 1 from B. utricularis was unaffected by treatment with sodium borohydride (38), although it was observed that after t.l.c. on silica gel, the pigment was rapidly decolorised giving a solution with an absorption maximum at 235 nm which shifted to 266 nm in acid, together with very weak absorption bands at 300, 311, 325 (infl.) and 343 nm. It is known that sodium borohydride reduces polyene Schiff's bases (70), the site of action, even in the presence of large excess of reducing agent, being only the imine bond (71), and this has been confirmed in the various Schiff's base models studied here. The inability of pigment A-2 to react with borohydride in the later isolates could perhaps be due to some modification in the nature of the chromophore, the pigment isolated earlier being slightly different than the later isolate. However, protonation of the pigment seems to modify the chromophore rendering it now susceptible to attack by borohydride producing a product with a conjugated polyene type spectrum. It is interesting to note that Bownds and Wald (71) have found that rhodopsin (λ_{max} 578 nm) is not attacked by borohydride; however, reduction of the Schiff's base linkage occurs only after the visual pigment has been irradiated to form metarhodopsin II (λ_{max} 380 nm), the reduction product absorbing at 330 nm (cf. retinol λ_{max} 328 nm). A similar reactivity to borohydride has been observed for the visual purple pigment isolated from the membrane of

Halobacterium halobium (72). More importantly synthetic Schiff's bases made from retinaldehyde with various amino acids, brought to acid pH so that their λ_{max} is ca. 440 nm, characteristic of the protonated species (Table 28), react very rapidly with sodium borohydride at room temperature(71). The unreactivity of the visual pigments towards borohydride has been rationalised on the grounds that steric shielding of the imine bond by the protein opsin renders it immune to attack by borohydride, since there is evidence that configurational changes in opsin accompany the bleaching of cattle rhodopsin (73). It has already been suggested from the study of the pK_a of the conjugate acid of pigment A-2 P. flavicomum, that perhaps a rearrangement catalysed by strong acid is taking place. It is quite conceivable therefore that pigment A-3 which could perhaps be a rearrangement product is now susceptible to attack by the borohydride ion. Alternatively, after protonation in strong acid, another functional group(s) in pigment A-3 which when attacked by borohydride, isolates the polyene chromophore from another chromophore with which it was previously in conjugation. This would tend to indicate that the earlier pigment isolates were already in the rearranged form or that the mould is producing a pigment with similar spectral characteristics but different chemical reactivity to borohydride before protonation.

Resonance Raman spectroscopy of pigment A-2 (P. flavicomum)

So far no definite conclusions as to the presence in pigment A-2 of an azomethine group in conjugation with the polyene chromophore could be reached, and an attempt was made to clarify this situation by use of resonance Raman spectroscopy. The resonant enhancement of certain Raman active vibrations occurs when molecules are excited by light of a wavelength lying near an electronic transition and the effect provides a structural probe at unusually low concentration. In this study the region 1,500-1700 cm^{-1} which contains C=C and C=N stretching vibrations was emphasised in pigment A-2 from P. flavicomum, although the region below 1,500 cm^{-1} which contains C-C stretching and C-H stretching modes was also examined. Spectra were measured for pigment A-2 in methanol and water, and the effect of protonation on the resonance Raman bands in both solvents was determined. The spectral information is summarised in Table 26. Rimai and coworkers have made a detailed Raman spectroscopic study of various retinals (74) and their Schiff's bases (75). These workers found that those vibrational modes most strongly enhanced are contributed by C=C and C-C stretching in the conjugated chain, and these occur near 1,570 and 1,200 cm^{-1} respectively. In addition, they assigned C=O and C=N stretching vibrations in retinaldehyde and retinylidine-imines at 1673 and 1627 cm^{-1} respectively. On protonation of the Schiff's base, the C=N bond stretching frequency at 1627 cm^{-1} is replaced by a broader band at 1654

cm^{-1} as expected for a protonated $C=\overset{+}{N}-H$ bond. Mendelsohn has recently examined the photoreceptor-like pigment of the halophytic bacterium Halobacterium halobium, since the primary structure of the chromophore is retinylllysine, being much the same as rhodopsin (76). A band at 1623 cm^{-1} assigned to the C=N stretch of the Schiff's base appears at the same frequency as in unprotonated retinylhexylamine (76). The strongest peak in the "purple membrane" Raman spectrum at 1531 cm^{-1} has no counterpart in the spectra of the model Schiff's bases, all of which have intense C=C stretching vibrations above 1550 cm^{-1}. Examination of resonance Raman spectrum of pigment A-2 in methanol shows a band at 1618 cm^{-1} of medium intensity which is assigned to a C=N stretching frequency, while a sharp strong band at 1584 cm^{-1} is assigned to C=C stretching vibrations of a conjugated polyene, further confirming the presence of such a system in pigment A-2. Other bands were also observed at 1145, 1160, 1260 and 1565 cm^{-1}. The resonance enhanced Raman spectrum of the protonated pigment (MeOH/HCl) revealed that the band assigned to the C=N stretch did not shift and was observed at the same value as the unprotonated sample (1618 cm^{-1}). This would suggest that although a C=N group is present and in conjugation with the polyene, it is not a simple Schiff's base link, because it has already been demonstrated by Rimai and coworkers that in protonated Schiff's bases, the $C=\overset{+}{N}-H$ groups 'resonate' at ~1654 cm^{-1} (75). It was found in this study that 2-methyl benzoxazole does not show resonance Raman, but shows Raman bands at 1578 (C=C) and 1618 (C=N) cm^{-1}, and these bands did not shift when acid was

added. Since the band at 1618 cm^{-1} is found in both pigment A-2 and 2-methyl benzoxazole, it is tempting to suggest that the  group may be present in pigment A-2.

Oxidation of pigments

(a) Reaction of 'indicator' pigment P. flavicomum and P. polycephalum with silver oxide and methyl iodide.

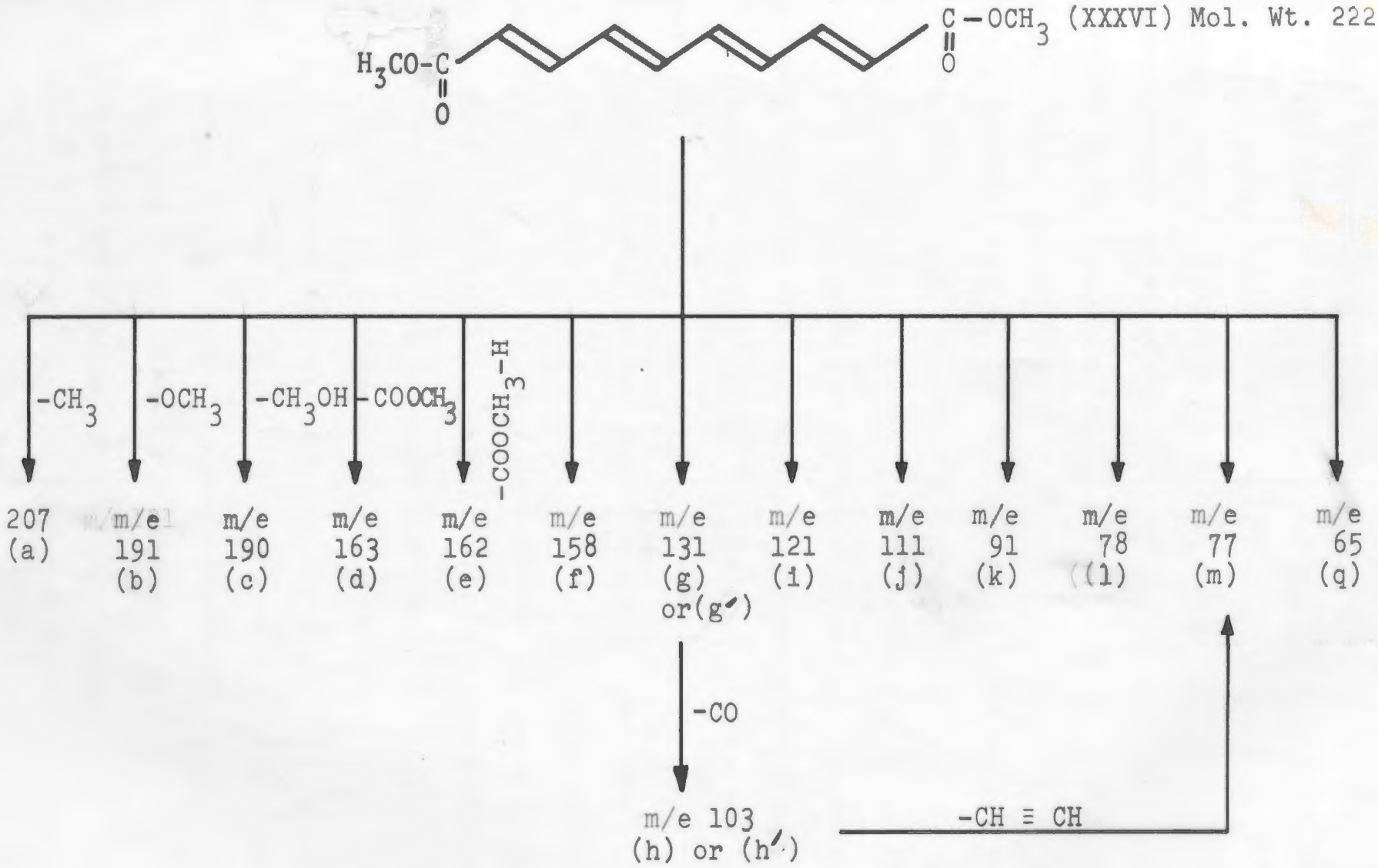
During the early part of this study the 'indicator' pigment obtained from P. flavicomum after silicic acid chromatography (Chart 2) was reacted with freshly prepared silver oxide and excess methyl iodide. All-trans dimethyl 2,4,6,8-decatetraene-1, 10-dioate (XXXVI) was isolated and characterised by comparison of its u.v., i.r., and mass spectra with those of an authentic sample prepared from the diacid provided by Dr. D.S. Tarbell. Earlier, Baker and Bullock (34) in our laboratory had obtained a mixture of polyene diesters from a pigment preparation isolated in the same way from the related mould P. polycephalum. This mixture was characterised as containing all-trans dimethyl 2,4,6,8-decatetraene-1, 10-dioate (XXXVI), (mol. wt. 222) and all-trans dimethyl 2,4,6,8,10-dodecapentaene-1, 12-dioate (XXXVII) (mol. wt. 248), on the basis of mass spectral data and the characteristic u.v. spectra of these compounds.

(b) Reaction of pigment A-2 (P. flavicomum) with silver oxide/methyl iodide.

When pigment A-2 (P. flavicomum) was treated with silver oxide and excess iodide under similar experimental conditions, all-trans dimethyl 2,4,6,8-decatetraene-1, 10-dioate was isolated as one of the degradation products. Occasionally

another polyene diester, characterised by mass spectrometry as all-trans dimethyl 2,4,6,8,10-dodecapentaene-1, 12-dioate could be isolated in trace amounts along with diester (XXXVI) as the major product. Assignment of structure of diester (XXXVI) is also supported by its (a) U.v. spectra $\lambda_{max}(CHCl_3)$ 292, 305, 321 and 337 nm, characteristic of an all-trans conjugated tetraene containing a carbonyl function (77). (b) I.r. spectra ($CHCl_3$) 1706 (carbonyl stretching frequency of an α,β-unsaturated ester), 1620 (C=C stretching frequency), 1265 (C-O stretch), and 1005 cm^{-1} assigned to the trans-C-H out of plane vibration in conjugated systems. (c) N.m.r. τ 3.0 - 4.2 (8H, olefinic) and τ 6.2 (6H, assigned to CH_3 of $COOCH_3$). The mass spectrum of this compound shows principal fragmentation ions at m/e 222, 221, 191, 190, 163, 158, 131 (base peak when mass spectrum scanned at 160°), 121, 103, 91, 78, 77 and 65, although when the spectrum is scanned at 190°, the base peak is represented by an ion at m/e 121. Fig. 2 (p.69) summarises the important fragmentation ions obtained. Peaks appearing at m/e 207 ($M-CH_3$) ion (a), 191 ($M-OCH_3$) ion (b), and 163 ($M-COOCH_3$) ion (d), are probably due to cleavage of appropriate C-C and C-O single bonds, while the ion at m/e 190 ($M-OCH_3-H$) ion (c), may be due to loss of methanol as a neutral species. An attempt will be made here to explain the fragmentation pattern of diesters (XXXVI) and (XXXVII), since their interpretation has much relevance to the mass spectrum of pigment A-2 (P. flavicomum) discussed later.

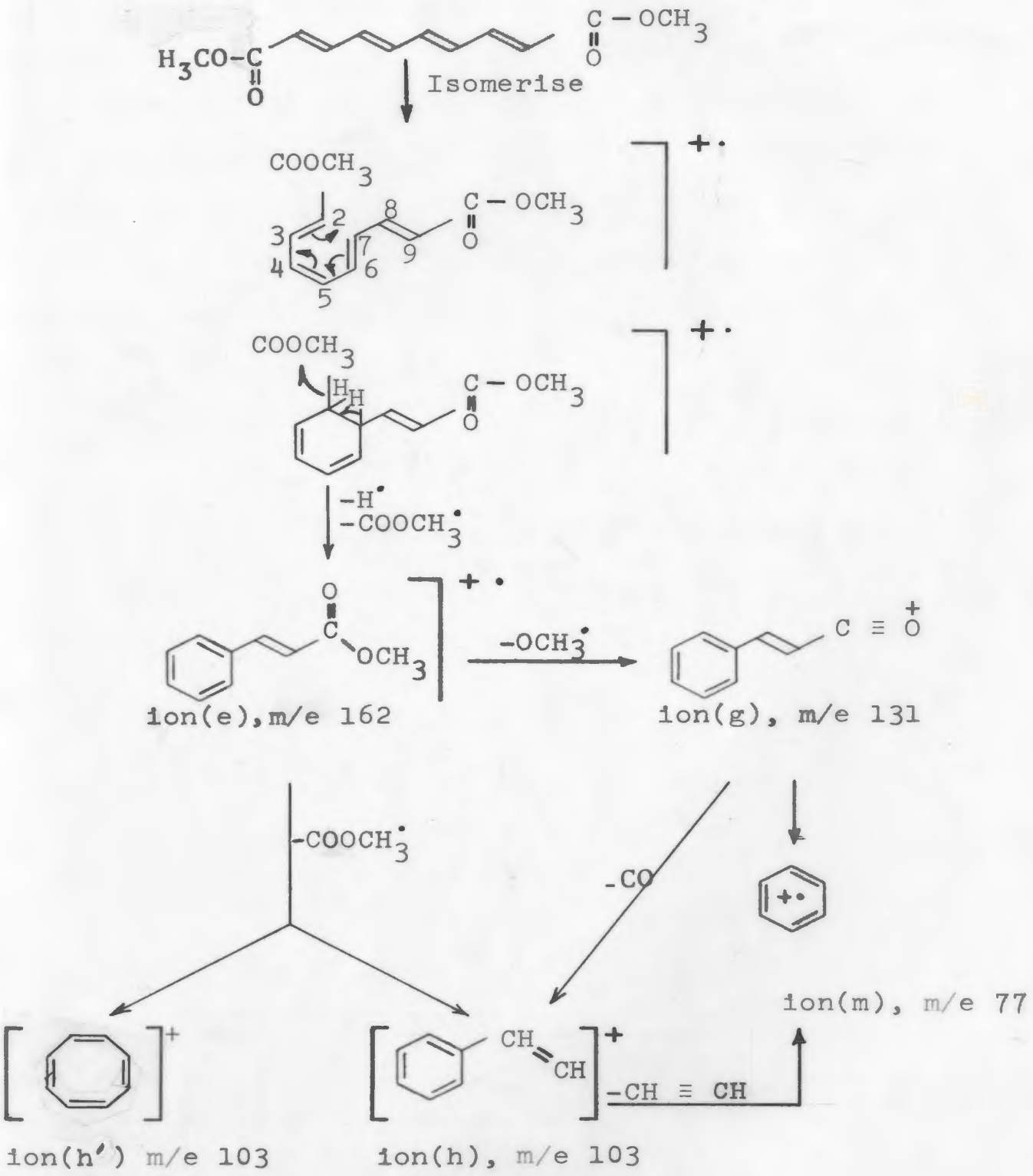
Fig. 2. **Mass spectral fragmentation of all-trans dimethyl 2,4,6,8-decatetraene-1,10-dioate.**



Ions at m/e 162, 131, 103 and 77.

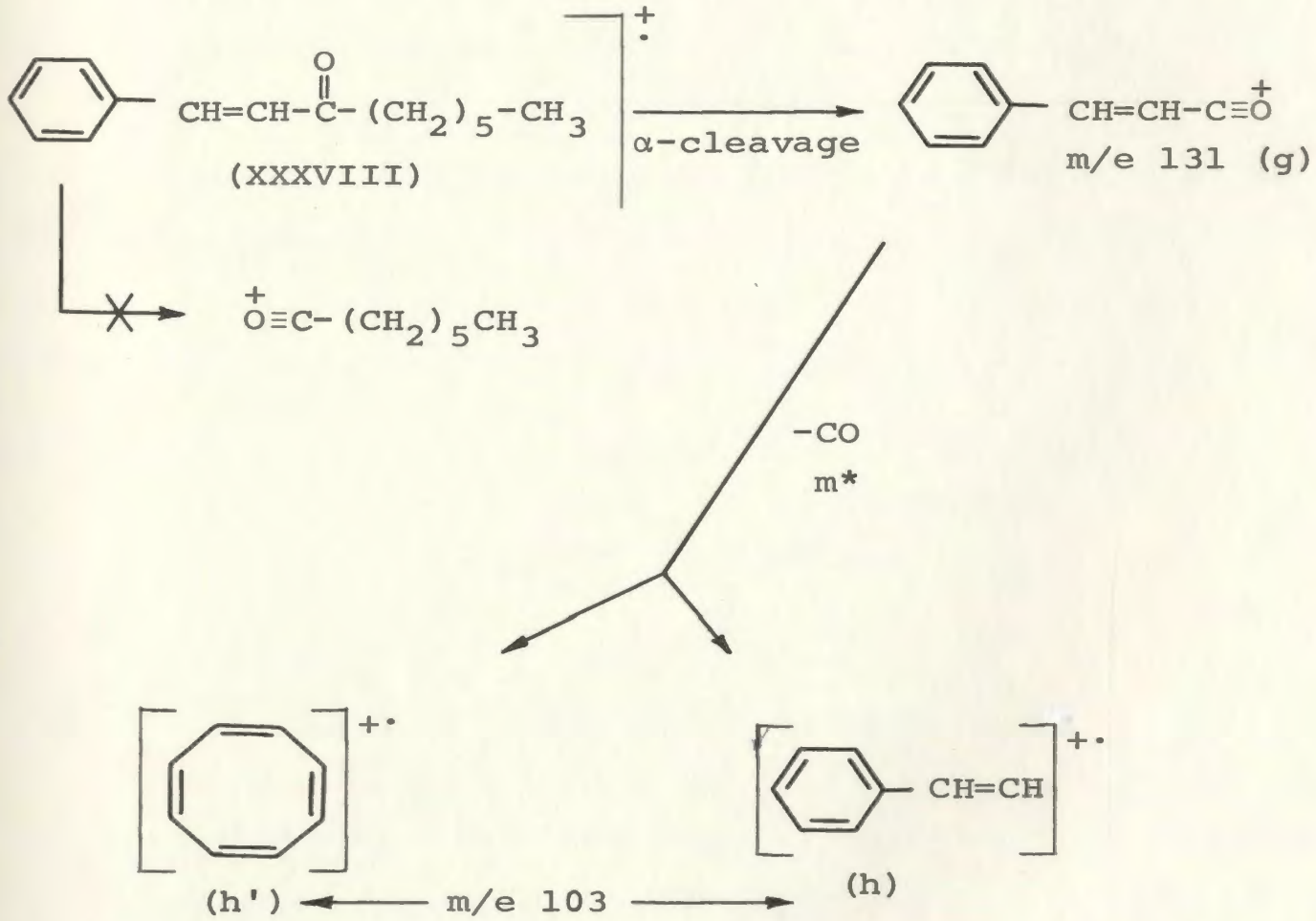
The base peak in most samples of diester (XXXVI) examined occurs at m/e 131, while ion (e), m/e 162 accounts for ∿ 20% of the base peak. A mechanism which attempts to explain the formation of ion (g) m/e 131 suggests that this ion arises from ion (e) m/e 162. Such a scheme requires isomerisation of the all-trans polyene system in (XXXVI) to a trans, cis, cis, trans-configuration, an arrangement which would facilitate cyclisation to produce an intermediate dihydrobenzene derivative (Scheme 1). The driving force for the formation of ion (e) m/e 162, as a styryl intermediate is probably provided by (i) loss of carbomethoxy and hydrogen radicals expelling acetic acid* as a neutral species and (ii) stabilisation of the positive charge by formation of an aromatic intermediate (e). Loss of a methoxy radical from ion (e) would then account for the formation of ion (g) or (g') at m/e 131. Scheme 1 summarises a possible mechanism for formation of ions (e), and ions (g) m/e 162, (h) or (h') m/e 103, and (m) m/e 77, derived from (e). Ion (e) at m/e 162 ($C_{10}H_{10}O_2$) is significant (∿ 20%) in the fragmentation of the diester (XXXVI), while in the high resolution mass spectrum of pigment A-2, an ion at m/e 162 accounts for only 2-3% of the base peak (Appendix I). Ion (e), at m/e 162 then corresponds to the molecular ion of methyl cinnamate $[C_6H_5CH{=}CHC(=O)OCH_3]^{\ddagger}$. The base peak at m/e 131, ion (g), is due to loss of a methoxy radical from (e), and such an ion appears in the spectra of several cinnamates which have been investigated

*or methyl formate



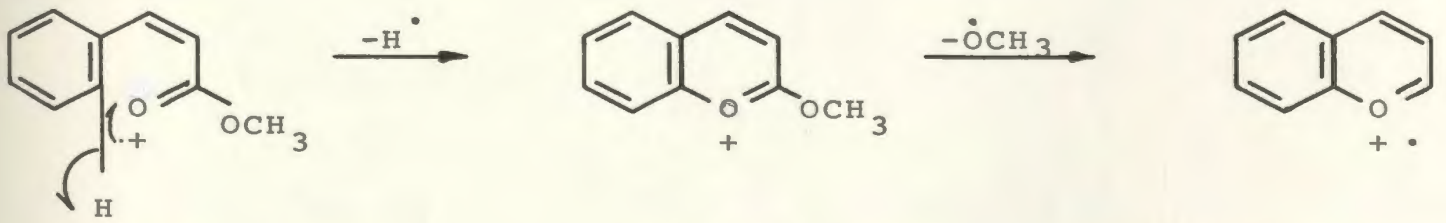
Scheme 1. Formation of ions at m/e 162, 131, 103 and 77.

by Emery (78). More recently the mass spectrometry of a series of nuclear substituted styryl ketones e.g., (XXXVIII) has been investigated by Smith and coworkers (79), and it was shown that in compounds of this type, the base peak corresponds to ion (g) m/e 131, which then loses CO to form ion (h) or (h') m/e 103 (Scheme 2). This sequence has been confirmed by the presence of the appropriate metastable ion formed during the fragmentation of (XXXVIII); however, a similar metastable ion (at m/e 80.8) was not observed in the spectrum of the diester (XXXVI).



Scheme 2. Formation of ions at m/e 131 and m/e 103.

An assumption is made here that ion (g) m/e 131, and ion (h) or (h') m/e 103, obtained in the spectrum of the ketone (XXXVIII) correspond to similar ions recorded in the spectrum of the diester (XXXVI) and also in the spectrum of pigment A-2 (P. flavicomum). However, accurate mass analysis obtained for pigment A-2 tends to support this assumption (see p.294). A similar assumption is made for ions occurring at m/e 78 and m/e 77. An alternative explanation for the ion at m/e 131 can be rationalised by the formation of a benzopyrylium ion (g') obtained from ion (e) by cyclisation, following loss of the methoxy group (Scheme 3). There is some evidence for such a scheme occurring in the mass spectral fragmentation of methyl cinnamate (80a) with formation of an intermediate pyrylium ion at m/e 161 through loss of an aromatic hydrogen atom; such an ion at m/e 161 (16%) is found in the mass spectrum of diester (XXXVI) Fig.14.

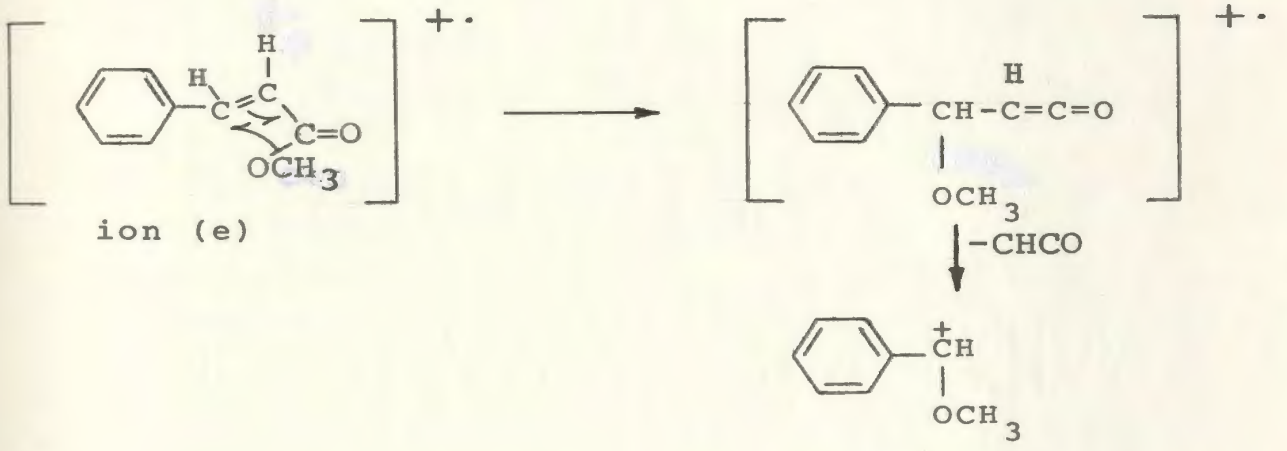


m/e 162, ion (e) m/e 161 m/e 131, ion(g') (C_9H_7O)

Scheme 3. Possible mechanism for formation of ion (g') m/e 131.

Genesis of m/e 121: Assuming that ion (e) m/e 162 is formed by cyclisation of the polyene diester (Scheme 1) then ion (i), m/e 121, can be rationalised by loss of CHCO preceded

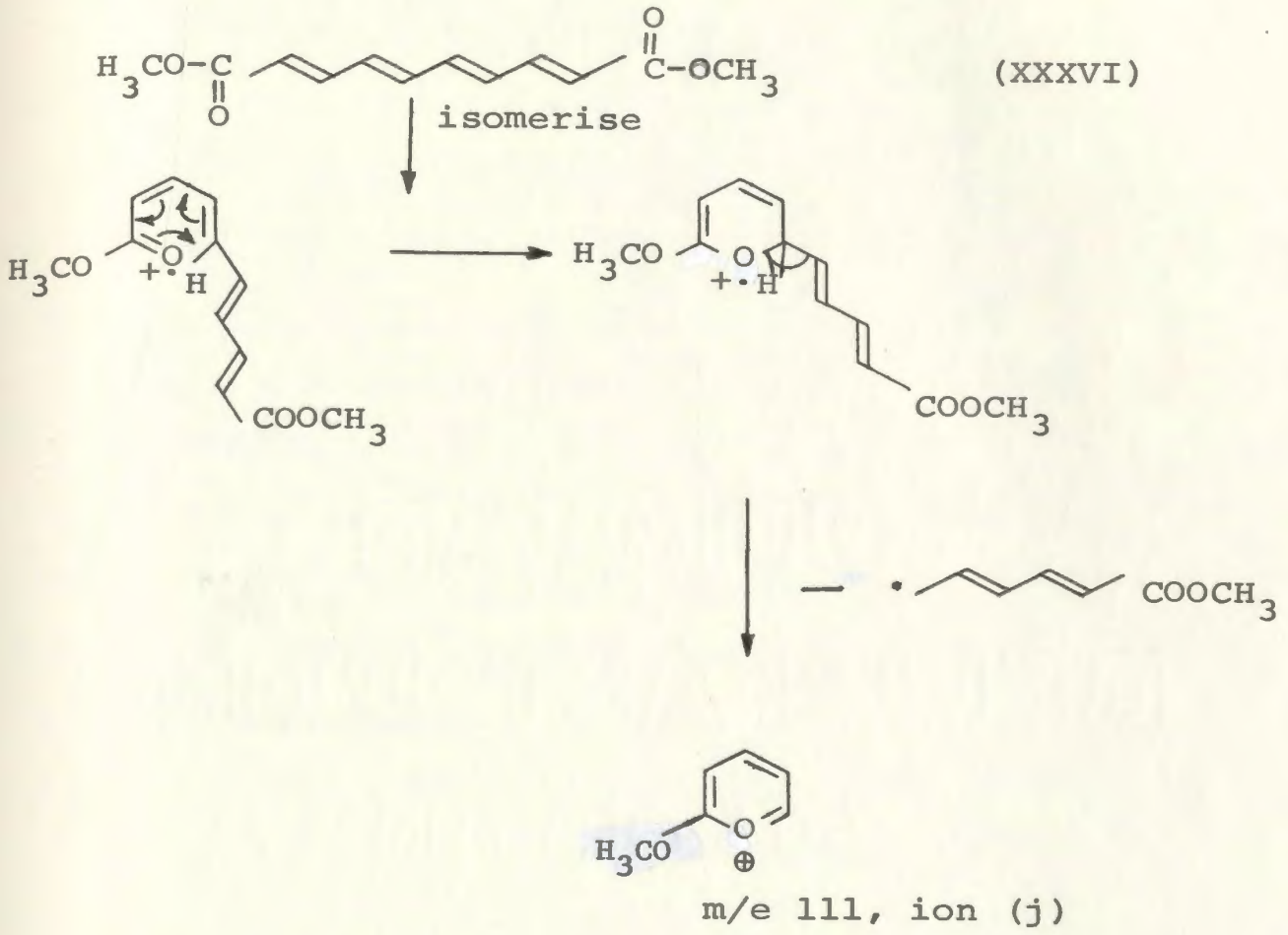
by a methoxyl group migration in ion (e) (Scheme 4). The formation of the ion at m/e 121 in methyl cinnamate arises by a 1,3-methoxyl shift and subsequent bond cleavage, ion (e) to (i), Scheme 4, and was established by deuterium labelling and exact mass measurements (80a). Ion (i) has been found to be a significant fragmentation product (35%) in the mass spectrum of the diester (XXXVI) scanned at 160°, while at higher temperatures (190°), it has been found to be base peak, suggesting that its formation may be thermally promoted.



Scheme 4. Formation of ion (i) m/e 121.

Ion at m/e 111: The formation of ion (j), m/e 111 by isomerisation and cyclisation of the all-trans diester (XXXVI) is summarised in Scheme 5. A mechanism for formation of ion (j) might involve initial isomerisation of one of the trans double bonds to a cis-configuration and such an arrangement would facilitate cyclisation (Scheme 5). Cleavage of the 5,6-bond in the cyclised intermediate would then produce

the stable pyrylium ion (j), m/e 111.

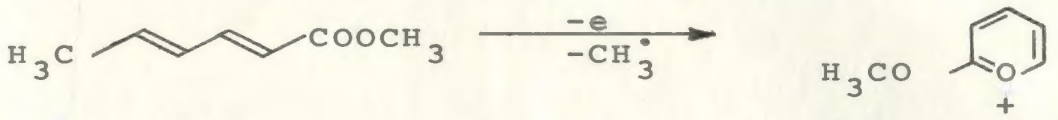


m/e 111, ion (j)

Scheme 5. Formation of ion (j) m/e 111.

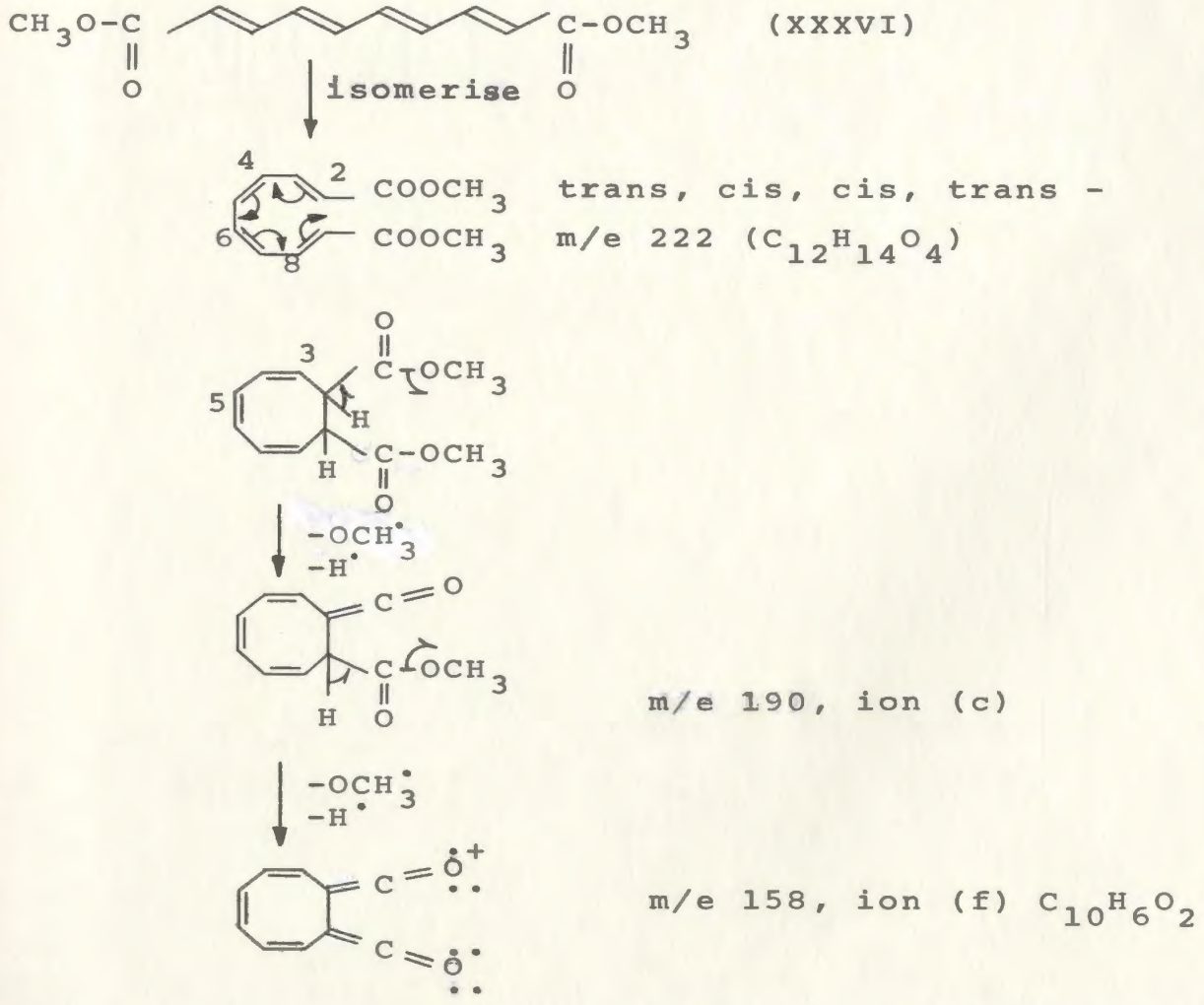
Such a scheme is rationalised on the basis that in the spectrum of methyl sorbate (XXXIX), loss of a methyl radical (established by deuterium labelling) is very pronounced since cyclisation of the resulting ion can now afford a completely aromatic pyrylium ion (j) (80b). On this basis, it is suggested that ion (j), m/e 111, occurring in the fragmentation of the diester (XXXVI) is similar if not identical to the pyrylium

ion obtained in the fragmentation of methyl sorbate.



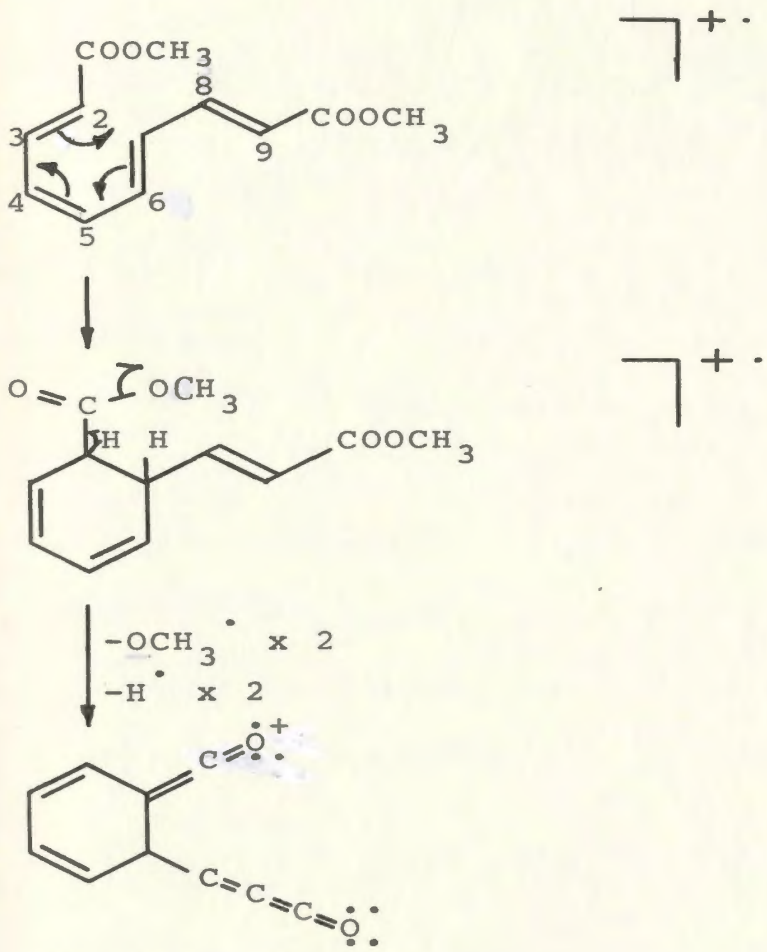
(XXXIX) ion (j) m/e 111

Genesis of ion at m/e 158: the origin of this ion arising from the fragmentation of the polyene diester (XXXVI) is more difficult to rationalise, but it seems to be formed by successive losses of (OCH_3-H). Although there is no absolute evidence or mass spectral data from model compounds, a possible scheme involving initial isomerisation of the all-trans system to a trans, cis, cis, trans-configuration followed by cyclisation to produce a "cyclooctatriene" system can be postulated (Scheme 6).



Scheme 6. Formation of ion (c), m/e 190 and ion (f), m/e 158.

Another possible fragmentation scheme for the formation of ion (f) m/e 158, is outlined in Scheme 7 below. For cyclisation to occur, it is necessary that the geometry of the polyene is right and this is achieved by isomerisation of the all-trans polyene system to a trans, cis,trans, trans-configuration (Scheme 7).



m/e 158, ion (f)

$C_{10}H_6O_2$

Scheme 7. Alternative mechanism for formation of ion at m/e 158.

In addition to these main fragments, an ion (m) corresponding to m/e 78 is present in the spectra of the diesters (XXXVI) and (XXXVII) and 2,4,6,8-decatetraene 1, 10-dioic acid (XL). A possible explanation for the formation of this ion (m) in the diester and also in the mass spectrum of pigment A-2 (P. flavicomum), where it is 84% of the base peak, will be discussed later. Ion (m) m/e 77 corresponding to a phenyl group probably arises from ion (h) m/e 103 by loss of the neutral acetylene (Scheme 1). It is also pertinent to note that in the mass spectrum of the tetraene dicarboxylic acid (XL), mol. wt. 194, prominent ions occur at m/e 158 (17%), m/e 131 (79%), m/e 103 (95%) and m/e 77 (73%), data which support a common fragmentation pathway for these ions in diester (XXXVI) and its corresponding diacid.

(c) Reaction of pigment A-2 with silver oxide/ethyl iodide.

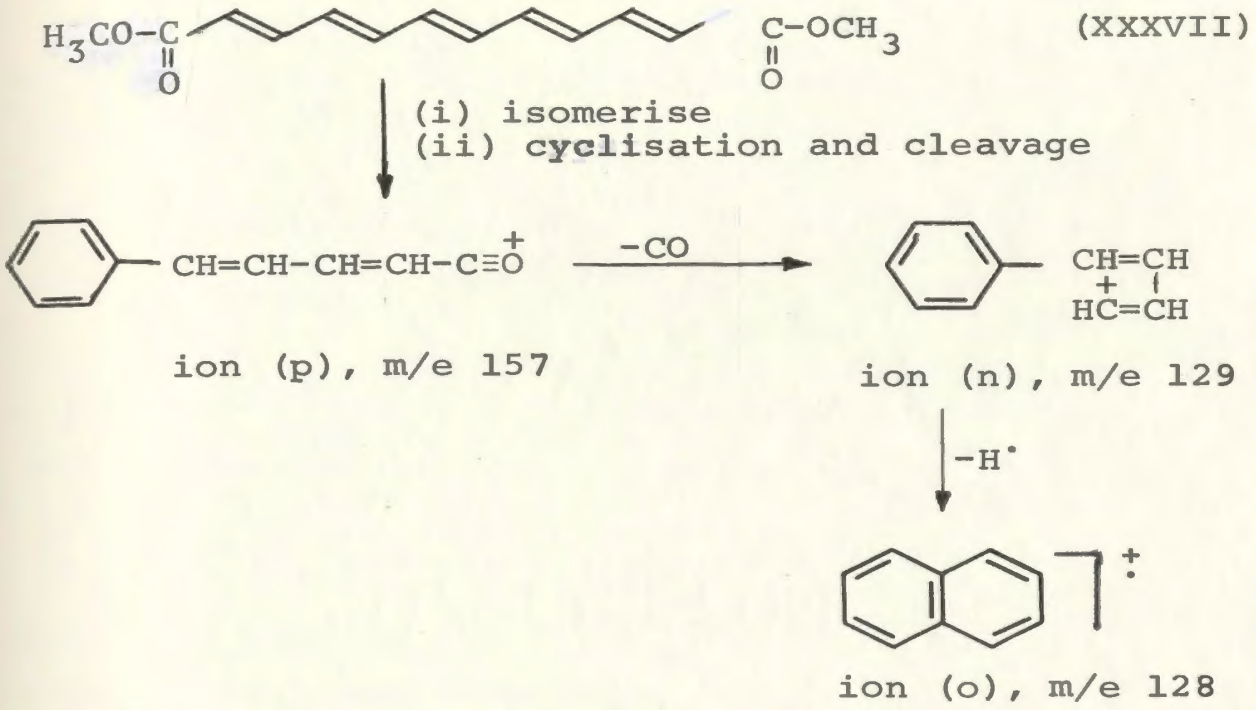
The polyene diethyl esters (XLI) and (XLII) (trace), were isolated when pigment A-2 (P. flavicomum) was treated with silver oxide and ethyl iodide. Diester (XLII) was present in very small amounts, and was detected by mass spectrometry by the ion at 276 which corresponded to its molecular weight. The major component of this mixture was the diethyl ester (XLI), and the mass spectral fragmentation pattern of the mixture is analogous to that of the dimethyl ester (adjusting for the difference in molecular weight) and is summarised on p.226. Interestingly, the base peak for these diethyl esters corresponds to the ion at m/e 131, which is also the most intense

peak for the dimethyl ester (XXXVI). An ion at m/e 131 (14 %) analysing for C_9H_7O is also present in the high resolution mass spectrum of pigment A-2 (Appendix II).

(d) Reaction of pigment 3 (P. polycephalum) with silver oxide/ methyl iodide.

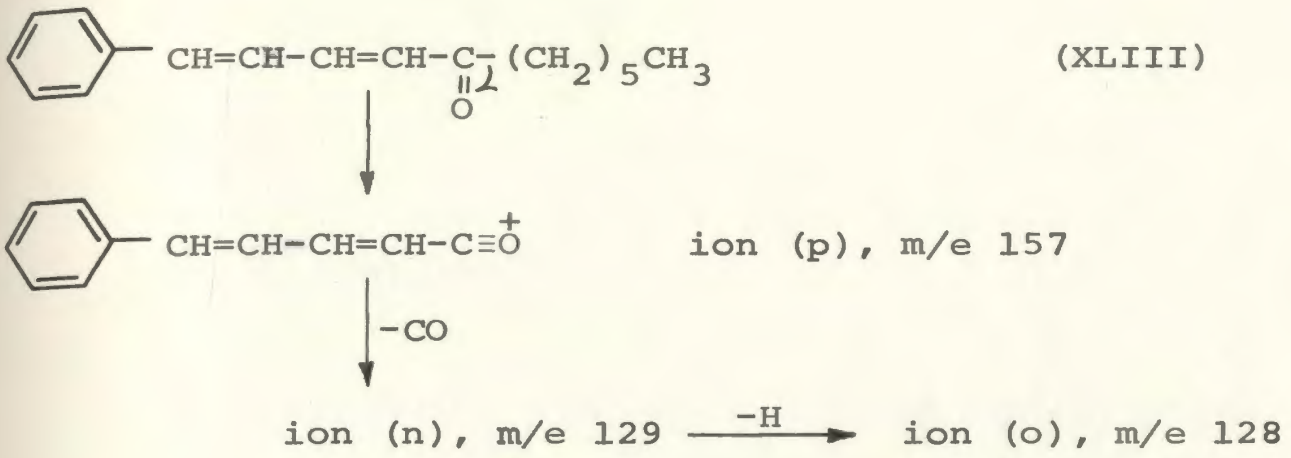
The mass spectrum of the mixture of polyene diesters $(CH_3O\text{-}\overset{\overset{O}{\|}}{C}\text{-}(CH{=}CH)_{\overline{n}}\overset{\overset{O}{\|}}{C}\text{-}OCH_3$, (XXXVI), n=4 and (XXXVII), n=5 obtained when pigment 3 (P. polycephalum) and 'indicator' pigment from the same organism were reacted with silver oxide/ methyl iodide shows ions corresponding to m/e 248 (molecular ion for (XXXVII), m/e 222 molecular ion for (XXXVI)), 185, 157, 158, 131, 129, 128, 121, 111, 103, 91, and 77 in addition to ions due to loss of methoxy, and carbomethoxy radicals resulting from cleavage of the appropriate C-C and C-O bonds. The approximate ratio for these diesters was estimated by the ratio of the relative abundance of the molecular ions and found to be 3:2. Ions at m/e 158 (f), 131 (g or g'), 121 (i), 111 (j), 103 (h or h') probably arise from the same fragmentation mechanism postulated earlier for the diester (XXXVI) isolated from P. flavicomum. In addition to ions listed above, intense ions corresponding to m/e 129 ion (n), 128 ion (o) are found in the mass spectrum of the polyene diester mixture obtained from P. polycephalum, which are relatively insignificant in the diester (XXXVI) isolated from P. flavicomum. If the postulate that cyclisation of the polyene diesters occurs in the mass spectrometer giving rise to aromatic species, then ions at m/e 129 and m/e 128 represented by ions (n) and (o) are formed from

fragment (p) m/e 157, which arises from the cyclisation of the n=5 polyene diester (XXXVII) (Scheme 8).



Scheme 8

Ion (o) represented by a naphthalene structure is formed from ion (n) by loss of H·. Such a scheme has been suggested following the mass spectral study of 1-phenyl-1, 3-undecadien-5-one (XLIII) by Smith et al (79) where α-cleavage of the C_5-C_6 bond takes precedence over cleavage of the C_4-C_5 bond, and the positive charge is retained as in ion (p) (Scheme 9).

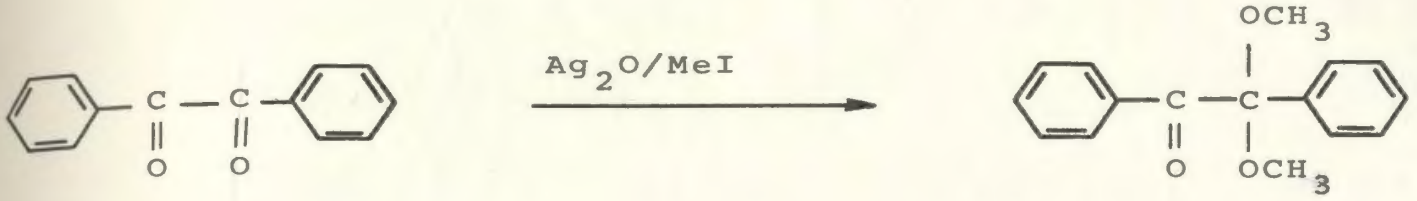


Scheme 9

The transition from (p) to (n) by loss of CO in the study by Smith *et al* (79) has been confirmed by the presence of the appropriate metastable ion during the fragmentation of the conjugated ketone (Scheme 9).

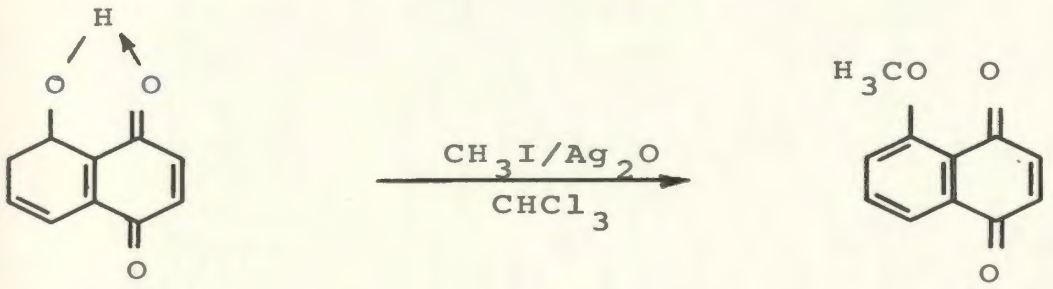
Some reactions of silver oxide and evidence for the chromophore.

Silver oxide is usually used as a mild oxidising agent; for example, (i) hydroquinones are oxidised to the corresponding quinones, and (ii) aldehydes to the corresponding carboxylic acids. A detailed survey of other reactions is given by Fieser and Fieser in their classical volumes 'Reagents for Organic Synthesis' (81a). The combination reagent, silver oxide/methyl iodide was introduced for exhaustive methylation of carbohydrates (81a) but has been useful in other contexts. For example, 1,2-diketones are converted to monoketals, e.g., benzil (XLIV) is converted to (XLV) (81a).



(XLIV) (XLV)

The strongly chelated peri hydroxyl group of juglone (XLVI) can be methylated with methyl iodide and silver oxide in chloroform but not in DMSO, to (XLVII).

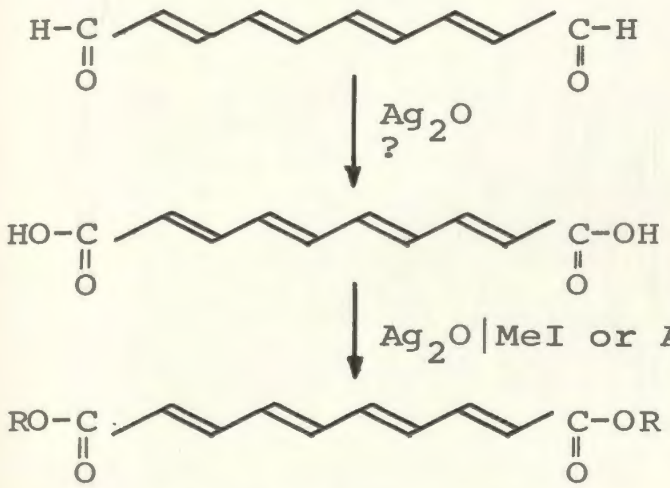


(XLVI) (XLVII)

Conclusions from Ag_2O reaction

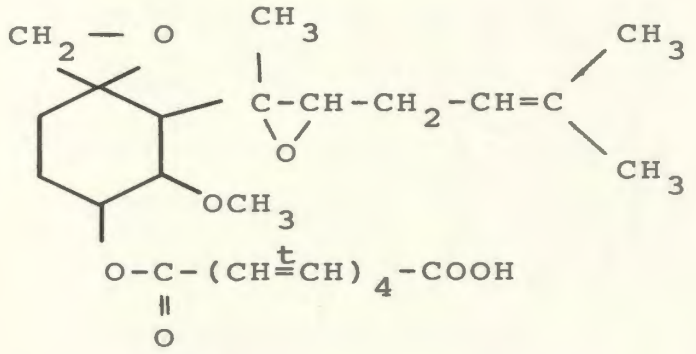
Because the polyene diester (XXXVI) was obtained after Ag_2O/MeI treatment of pigment A-2 (P. flavicomum), intermediate formation of a dialdehyde (XLVII) is a distinct possibility since oxidation and esterification would give rise to the corresponding ester. When pigment A-2 (P. flavicomum) is treated with Ag_2O/EtI under similar reaction conditions, the polyene diethyl esters(XLI) and (XLII) are obtained. The isolation of the dimethyl and diethyl polyene esters when methyl and ethyl iodide are respectively used, shows that alkylation of the polyene intermediate is occurring at both ends of the chain, therefore

suggesting that the intermediate must either be a dialdehyde or diacid, a half aldehyde acid, but not a half ester aldehyde or acid.



The change in colour of the reaction mixture to a silvery grey during the Ag_2O/MeI reaction suggests a change of oxidation state of silver oxide (to Ag°), but does not necessarily imply that it is the dialdehyde (XLVIII) which is being oxidised. It is possible that other oxidation reactions on the pigment involving silver oxide are taking place. The presence of the polyene chromophore as a separate entity and not in conjugation with another chromophore can be ruled out because the absorption spectrum of pigment A-2 (P. flavicomum) shows a broad maximum at 380 nm; in other words the polyene must be in conjugation with another chromophore. This contrasts with fumagillin (XLIX)

λ_{max}MeOH 336 and 351 nm where the polyene chromophore is the only u.v. active group, and this metabolite exhibits a spectrum typical of a conjugated pentaene (77).



(XLIX)

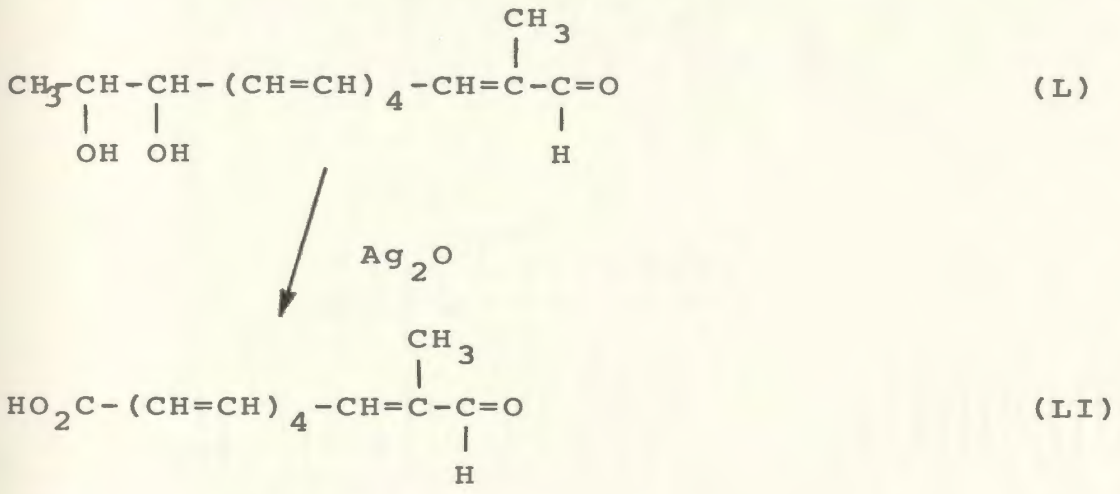
Hence in pigment A-2 (P. flavicomum) at least one bond cleavage (but possibly two) occurs under these conditions to give the corresponding carbonyl (or carboxyl) functional group, even assuming that the other end of the polyene chromophore is a carboxylic acid. It must be stated that although other data indicate that pigment A-2 from P. flavicomum is acidic (solubility in sodium bicarbonate, sodium hydroxide, pK_a measurements, and electrophoresis studies) there is nothing to suggest that a carboxyl group terminates one end of the polyene system. (The possibility that an aldehyde terminates the polyene system will be ruled out in the following section which discusses the oxidation of pigment A-2 (P. flavicomum) with sodium periodate. If then there is bond cleavage with Ag_2O/MeI, the degradation product immediately after cleavage could conceivably be a

(a) polyene dialdehyde

(b) polyene aldehyde acid

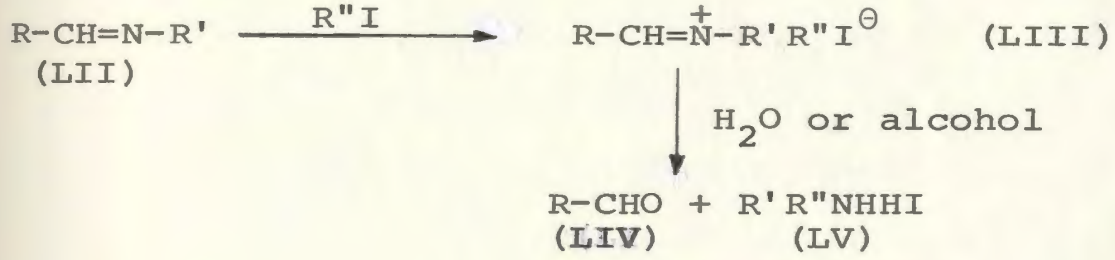
(c) polyene dicarboxylic acid.

A rather unusual C-C bond cleavage involving the use of silver oxide has been reported by Dhar, Thaller and Whiting (82) in their structure determination of lagosin, a polyene macrolide antibiotic produced by a Streptomyces species. These workers found that when the C_{15} diol (L) was treated with silver oxide prepared from silver nitrate and sodium hydroxide, the polyene aldehyde acid (LI) was isolated.



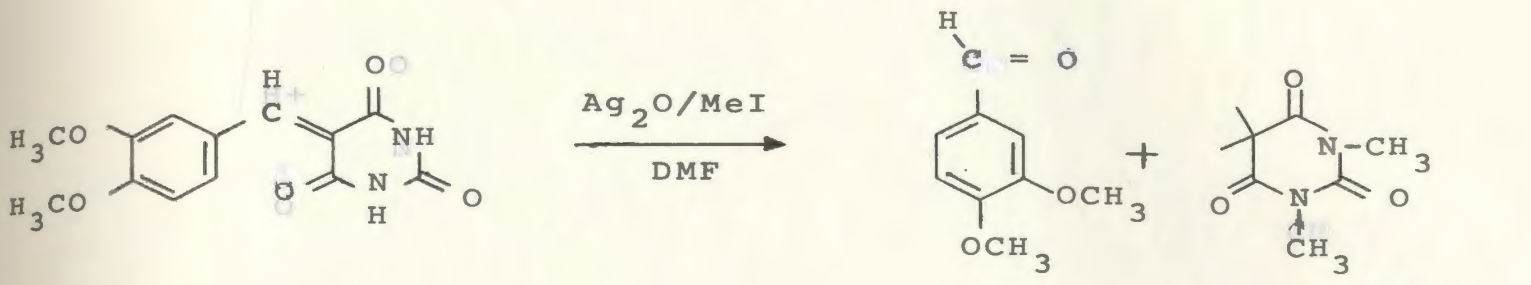
Cleavage of the C-C bond of the diol occurs in this instance, the aldehyde presumably formed is oxidised to the acid, while at the other end of the molecule, the aldehyde group is not oxidised further. No mention was made of the fate of the 2-carbon unit (lost either as acetaldehyde or acetic acid). These workers (82) reported no precedent for the C-C bond cleavage of the α-glycol nor was an explanation offered for the failure of silver oxide to oxidise the aldehyde function at C-1 to the corresponding acid, a reaction which was routinely performed by Cope et al. in their studies leading to the structure of

fungichromin (IX)(33). It is therefore unlikely that pigment A-2 contains a 1,2-diol function. If the assumption is made however that pigment A-2 P. flavicomum and pigment 3 (P.polycephalum) are Schiff's bases (LII) then treatment of either of these pigments with an alkyl iodide should form a quaternary ammonium iodide salt (LIII) which after hydrolysis with water or alcohol would generate the aldehyde (LIV) and the corresponding amine hydriodide (LV)(83). The aldehyde could then be oxidised to the corresponding acid by silver oxide and then alkylated to give the dialkyl ester.



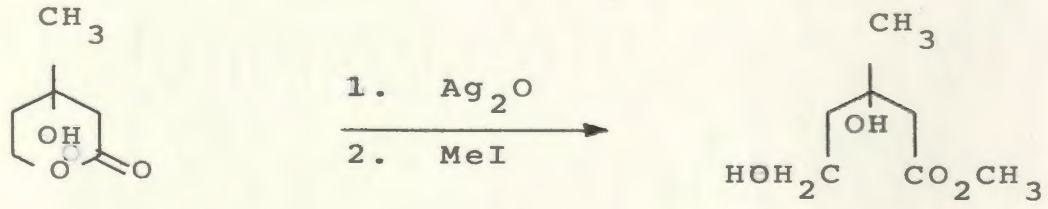
This would be consistent with the presence of a polyene azomethine chromophore in the pigment. It has been shown that when 9-anthranylidene-p-chloroaniline is treated with Ag_2O/MeI for 48 h, an ion at m/e 236, corresponding to the molecular ion of the methyl ester of 9-anthranilic acid could be detected by mass spectrometry, suggesting that Ag_2O/MeI can cleave an azomethine bond. Further evidence which suggests the non-specificity of the Ag_2O/CH_3I reaction

is the finding by Ethier and Neville (84) that benzylidene derivatives of barbituric acids (LVI) are cleaved by the reagent in refluxing DMF (90 min.).



(LVI) (LVII), 80% (LVIII), 45%

What is surprising is the fact that it is the aldehyde (LVII) and not the corresponding acid (or ester) that is isolated in good yield. It has also been reported (85) that when the dry silver salt of mevalonolactone was treated with excess methyl iodide, mevalonic methyl ester was obtained (LIX)⟶(LX), a reaction which may be significant for the plasmodial pigments.



(LIX) (LX)

It is also known that allylic alcohols in the carotenoid series are oxidised by silver oxide to the corresponding α,β-unsaturated ketone (86). Thus it seems that while dialkyl polyene esters can be obtained from Ag_2O/CH_3I or

Ag_2O/EtI degradation of 'Schiff's base' pigments the nature of the reaction remains unclear, and the results do not rigorously define the nature of the functional groups attached to the polyene system. However, the silver oxide/methyl iodide and silver oxide/ethyl iodide oxidative alkylation of these 'Schiff's base' pigments from P. flavicomum and P. polycephalum demonstrates clearly that (i) these pigments possess an all-trans conjugated polyene system as an integral part of their structure, (ii) the functional groups attached at either end of the polyene system are either aldehydic or carboxyl groups, or are potentially capable of giving rise to such groups after cleavage and oxidation.

Oxidation of pigments with sodium periodate in aqueous methanol

The presence of a conjugated polyene in pigment A-2 (P. flavicomum) was confirmed by the isolation of all-trans dimethyl 2,4,6,8-decatetraene-1, 10-dioate (XXXVI) following reaction with Ag_2O/MeI, but the non-specificity of this reaction prevented any definite conclusions about the nature of the groups to which this polyene system was attached. Reports that sodium periodate was used with good success to establish the nature of the chromophore in two polyene macrolides lagosin (82) and fungichromin (33) prompted us to investigate the action of this reagent on pigment A-2, although there was no other evidence to suggest it was a polyene macrolide.

(a) Characterisation of degradation products after oxidation of pigment A-2 for 88h.

Table 26 summarises the effect of excess sodium periodate on both the absorption spectra and the extinction increase (after protonation) of pigment A-2 with time. Reaction with periodate for 88h followed by ether extraction before and after acidification gave (a) neutral ether extract, and (b) acidic ether extract respectively.

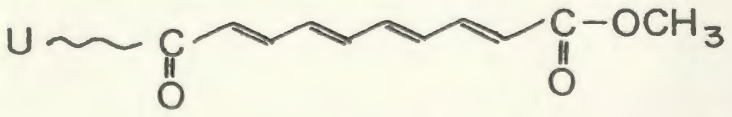
Neutral ether extract.

Diazomethane methylation of the neutral ether extract followed by t.l.c. on silica gel produced four bands.

Band I: this compound possessed an ion at m/e 222 (molecular ion), with fragment ions at m/e 191, 190, 163, 162, 158, 131, 121, 103, 78 and 77. U.v. peaks (MeOH) at 303, 317, 332 and 350 nm suggest the presence of a conjugated polyene system (77).

This degradation product was found to be identical to all-trans-dimethyl 2,4,6,8-decatetraene-1,10-dioate (XXXVI). Spectroscopic evidence for the structure of this diester has already been discussed.

Band II: this degradation product possesses a u.v. spectrum similar to diester (XXXVI), with peaks (MeOH) at 303, 317, 333 and 350 nm and suggests that a polyene dicarbonyl chromophore is common to both these degradation products. Its mass spectrum (Fig.20) shows an ion at m/e 328 (possibly the molecular ion); ions at 297 and 269 due to loss of methoxy and carbomethoxy radicals from the molecular ion indicate a methyl ester, while an ion at m/e 296 is probably due to loss of methanol as a neutral species. Significantly, the ion at m/e 131 is the base peak in this degradation product, and is also the most abundant ion in the mass spectrum of all-trans dimethyl 2,4,6,8-decatetraene-1,10-dioate (XXXVI). Other important ions are found at m/e 162, 158, 121, 105, 103, 91, 79, 78, 77, 71, 69, 59, 55 and 43 and many of these are common to the mass spectrum of diester (XXXVI). This mass spectral similarity along with similar u.v. properties at once suggests that the second degradation product (LXI) must contain a conjugated polyene dicarbonyl system.



(LXI)

U = 137 a.m.u.

The unknown fragment U therefore corresponds to 137 mass units. The degradation product (LXI) possesses an abundant ion at m/e 253 (95%) produced by loss of 75 mass units from the molecular ion. Such a loss is not found in the mass spectrum of diester (XXXVI) and by inference must be contained in portion (U) of degradation product (LXI). It is unfortunate that neither i.r. nor n.m.r. spectra could be obtained for (LXI), a fact which makes absolute deductions difficult. An attempt was made to calculate the carbon content of (LXI) by using the relative abundance of the parent ion (P), and ions P+1, and P+2, (328, 329 and 330) in the general equation devised for calculating the carbon content using the ^{13}C isotope ratio of carbon (87). A maximum value of 27 carbon atoms was obtained for (LXI), but this produces a molecular weight in excess of 328. Similarly, when ions at m/e 253, 254 and 255 were used as parent ion P, and ions P+1 and P+2 respectively, an estimate of 20 carbon atoms was obtained. The fragment corresponding to 75 mass units could possibly be represented by molecular formulae C_3H_9ON, $C_3H_7O_2$, C_5HN or C_6H_3. A high resolution mass spectrum for (LXI) could not be obtained.

Band III. The amount of this degradation product (LXII) isolated by t.l.c. allowed determination of its u.v. spectrum at 207, 240, 251 and 265 nm and mass spectrum. An ion at 314 (77%) is probably the molecular ion, although this is not certain, while the ion at m/e 299 (100%) could be due to loss of a methyl radical from m/e 314.

What is significant is an ion at m/e 239 (61%) which corresponds to the loss of 75 a.m.u. from m/e 314. It is therefore possible that the group corresponding to 75 mass units is common to (LXI) and (LXII), and is expelled as a neutral species in both cases. It is, however, not possible to assign a structure to degradation product (LXII).

Band IV (LXIII)

The u.v. spectrum λ_{max}(MeOH) 208, 230 and 259 nm and mass spectrum of this product were recorded. The highest peak in the mass spectrum was found at m/e 345. Very little structural information could however be deduced.

Acidic ether extract.

T.l.c. separation of the methylated extract revealed four compounds, which were identical to bands I-IV obtained in the purification of the neutral ether extract.

(b) 48 h sodium periodate oxidation of pigment A-2.

Oxidation for 48 h, acidification, ether extraction followed by t.l.c. of this methylated extract produced five bands (a-e) which included esters (XXXVI) and (XXXVII) but not (LXI).

Potassium carbonate/sodium periodate oxidation of pigment 2.

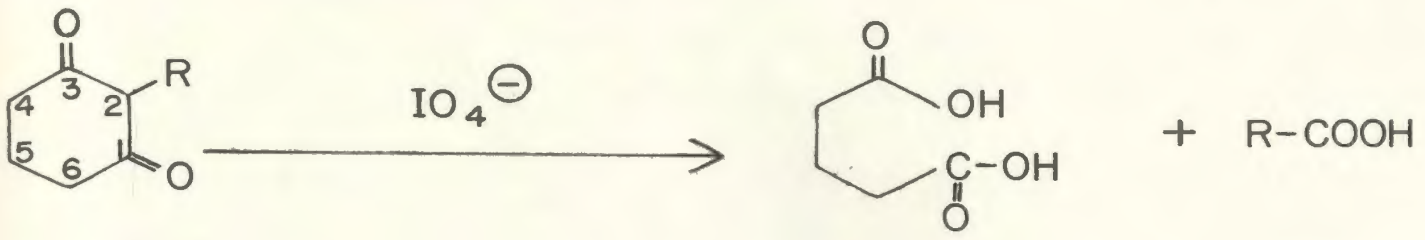
The absorption spectrum of pigment 2 λ_{max} 370 nm was unchanged after treatment with K_2CO_3 and still showed bathochromic and hyperchromic shifts on protonation, suggesting that the functional group responsible for this change was still intact, and not affected by K_2CO_3. Subsequent reaction with

periodate for 72 h exposes a polyene with u.v. peaks characteristic of a conjugated pentaene (77). Although several degradation products were isolated, and some of them characterised, conclusions on the nature of the pigment would be open to doubt because of the heterogeneous nature of the preparation.

Functional groups in pigment A-2.

Since all-*trans* dimethyl 2,4,6,8-decatetraene 1,10-dioate (XXXVI) and polyene ester (LXI) were obtained following periodate oxidation and CH_2N_2 methylation, the oxidation state prior to methylation must be at the carboxylate level. This is a logical conclusion because sodium periodate as an oxidising agent does not oxidise conjugated polyene aldehydes to the corresponding carboxylic acids (82, 88). Therefore, potential groups leading to a carboxylic acid can be considered in the light of this reaction. A 1,2-diol group at either end of the conjugated polyene is ruled out since a conjugated mono- or di- aldehyde has not been isolated in any of the sodium periodate degradations as would be expected if pigment A-2 contained a 1,2-diol group. Since it is known that 1,2-diketone groups are converted to the corresponding carboxylic acid (89) by periodate, this function was next considered. The hydroxylamine/nickel acetate test (107c) for aliphatic 1,2-diketones was negative, and other 1,2-ketones were excluded on the basis of the negative reaction to o-phenylenediamine (120). An α-hydroxy ketone function was next

considered, as oxidation by periodate gives a mixture of the corresponding aldehyde and carboxylic acid (89) e.g., benzoin is converted to benzaldehyde and benzoic acid. The negative reaction of the pigment with blue tetrazolium in alkali, however, excludes the α-hydroxy ketone function (81c). So far then, several potential carbonyl functions have been eliminated. The presence of a β-diketone in pigment A-2 seems an attractive possibility because of the potential of introducing an extra double bond by enolisation, a situation which might explain the shift to longer wavelengths of these "Schiff's base" pigments on protonation. A positive ferric chloride test, and an emerald green precipitate with saturated copper acetate are consistent with the presence of an enolic system in pigment A-2. Earlier work (90) has shown that periodates do not react with esters of malonic and acetoacetic acids, while more recently (91) three acyclic 1,3-diketones, 2,4-pentanedione, 1-phenyl-1, 3-butanedione and 1,3-diphenyl-1, 3-propanedione were shown to reduce periodate very slowly. While it seems that sodium periodate does not react with acylic 1,3-diketones, the reagent oxidises cyclic five-or six-membered 1,3-diketones (91), the oxidation product being a carboxylic acid and carbon dioxide if C-2 is unsubstituted, and a mixture of dicarboxylic and monocarboxylic acids if C-2 is substituted with an alkyl group, e.g., (diagram on next page)



(LXIV) (LXV) (LXVI)

The isolation of the polyene dicarboxylic acid ester (XXXVI) from periodate oxidation/CH_2N_2 methylation implies that the oxidation product prior to methylation must be the corresponding diacid (XL). The presence of a cyclic β-diketone system would be consistent with these results. However, a cyclic β-diketone system other than a cyclic 1,3-dione must be postulated in order to accommodate the other available evidence, since the possibility that hydrogenated pigment A-2 (P. flavicomum) contains a 6-membered cyclic 1,3-dione, e.g. cyclohexane-1, 3-dione, has already been eliminated on the grounds of spectral changes at different pH, pK_a and absence of a sharp isosbestic point. It is clear, however, that the functional group(s) responsible for the bathochromic shift on protonation is being oxidised by periodate, i.e. the "hyperchromic increase" (on protonation) of pigment A-2 falls off with time (Table 26). Secondly, the shift of the absorption maxima from 380 nm (of pigment A-2) to 337 nm (of the polyene) implies that the polyene system must be in conjugation with another chromophore, which is isolated from the polyene after periodate oxidation. This chromophore can then be oxidised further.

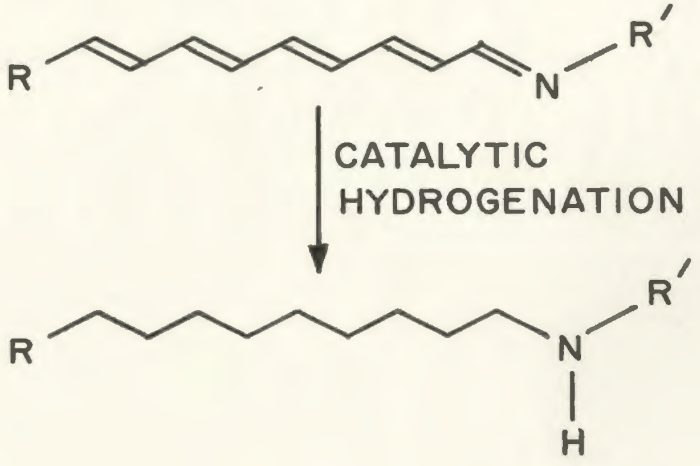
Catalytic hydrogenation of pigment A-2 (P. flavicomum) and pigment 3 P. polycephalum.

When a yellow methanolic solution of pigment A-2 was hydrogenated at room temperature with either platinum oxide or 5% palladium on charcoal, the solution was completely decolourised. Removal of the catalyst by filtration gave a colourless solution that was strongly fluorescent (yellowish green). Examination of the u.v. absorption revealed a new chromophore exhibiting two maxima at 245 and 280 nm in neutral solution. Since conjugated or isolated double bonds (and an azomethine group) would now be saturated, it seems likely that the new chromophore may be aromatic and not reduced by hydrogenation with either 5% Pd/C or Adams catalyst (platinum oxide) suggesting that most (if not all) the double bonds are in conjugation with the new reduced chromophore. Its absorption spectrum, like that of the parent pigment was also pH dependent, and in acid solution only a single absorption maximum at 277 nm was observed, with a corresponding loss of the 245 nm peak. In alkaline solution, however, both maxima (at 245 and 278 nm) were present and these could be reconverted to the single maximum at 277 nm on protonation, indicating that this pH dependence was reversible* (Fig. 9). The u.v. spectrum of hydrogenated pigment 3 (P. polycephalum), shows similar spectral behaviour to pigment A-2 (P. flavicomum) in neutral, basic and acidic solutions (Fig. 25) and Table 12. The spectral

*The spectral changes could be reversed after both acidification and basification.

behaviour of hydrogenated pigment A-2 was re-examined in aqueous buffer solutions over a considerable pH range and a series of curves obtained. Fig. 10 is a composite representation of these changes. Between pH 3.3 to 10, both maxima were present, while at pH 2.2, 1.1 and <1, only the single maximum at 277 nm was obtained. The pK_a of hydrogenated pigment A-2 was determined at the following wavelengths (i) loss of the 245 nm maximum with decreasing pH and found to be $\sim$ 2.3, (ii) appearance of the 280 nm maximum with increasing acidity and found to be about 2.4. The pK_a of the native pigment A-2 suggested that it was a strong acid and elemental analyses of pigment A-2 and the protonated form (48) from P. flavicomum indicated a very high oxygen content (24 - 29%). An oxygen content from 25 - 33.5% was reported by other workers (16,19), for related pigments from P. polycephalum. The behaviour of hydrogenated pigment A-2 at different pH's was very similar to that of triacetic acid lactone (XXIV) (3,4-dihydro-6-methyl-pyran2,4-dione) previously investigated by Bu'Lock and Smith (92) with a view to confirming the structure of yangonin (LXXVI), a 4-methoxy α-pyrone. These workers examined the u.v. spectra of triacetic acid lactone over a pH range 1.1 to 10, with the hope of detecting its tautomeric equilibria. In aqueous solutions of pH 1-4 the lactone (XXIV) shows a single absorption maximum closely similar to that of the α-pyrone (XXIVa), with no sign of the presence of the tautomeric 2-hydroxy-4-pyrone (XXIVb). Above pH6, when the lactone can only exist as

a single species (the derived anion), absorption maxima at 276 and 233 nm were observed, therefore suggesting that in the anion, both the electronic transitions observed individually in the α- and γ-pyrones are simultaneously possible. If such a 2,4-pyrone system is present in the hydrogenated pigment, then it would be reasonable to suggest that it is more acidic than triacetic acid lactone and this is partially supported by the pK_a (∿ 3.4) value of the native pigment, and ∿ 2.4 for the hydrogenated pigment. It is also interesting to note that triacetic acid lactone in alkaline methanolic solution also exhibited the same yellowish green fluorescence under long range u.v. light although not as intense as the hydrogenated pigment. This may suggest that perhaps an additional chromophore (aromatic) may be present in the pigment. Assuming then that A-2 (P. flavicomum)and pigment 3 (P. polycephalum) were conjugated Schiff's bases, catalytic hydrogenation would saturate the olefinic double bonds, and convert the azomethine link to a secondary amine, e.g.



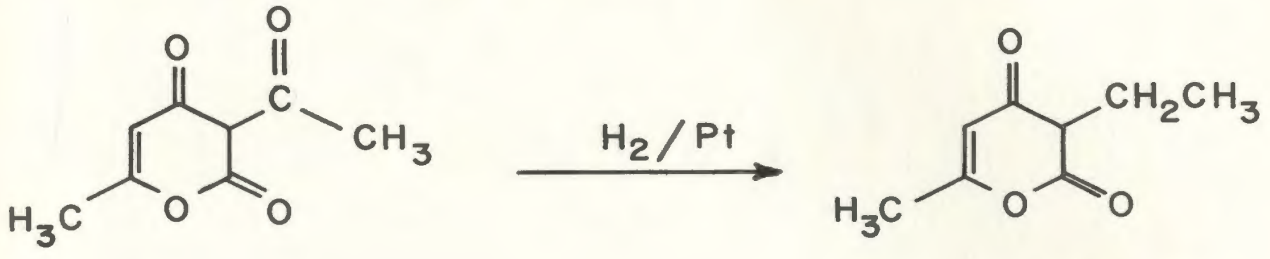
The basicity of the hydrogenated pigment should be increased although no pK_a change was observed by u.v. examination of this product in aqueous buffers at different pH's unless one was looking at some chromophore attached to nitrogen.

The acidity of pigment A-2, the recovery of a pH dependent chromophore after hydrogenation, and the high oxygen content prompted us to examine and compare spectral properties of several oxygen containing compounds, e.g., tetronic acids, which are often metabolites of fungi. Although the pK_a values of tetronic acids (Table 10), are comparable with our pigment, the pH dependence of the chromophore of our reduced product clearly does not correspond to a tetronic acid. The possibility that the reduced chromophore might be a cyclic β-diketone was considered but neither the pK_a (cf. pK_a for cyclohexane-1, 3-dione at 6.21) nor the pH dependence is in agreement with similar data for the reduced chromophore in pigment A-2 (P. flavicomum).

Reduction of carbonyl group by catalytic hydrogenation.

If then pigment A-2 (P. flavicomum) and the related pigment 3 from P. polycephalum do contain the 2,4-pyrone nucleus after reduction, the possibility that the parent nucleus is substituted by another carbonyl function should not be ignored. For example, it has been reported by Malachowski and Wanczura (93) that hydrogenation of dehydroacetic acid (LXIX) in methanol

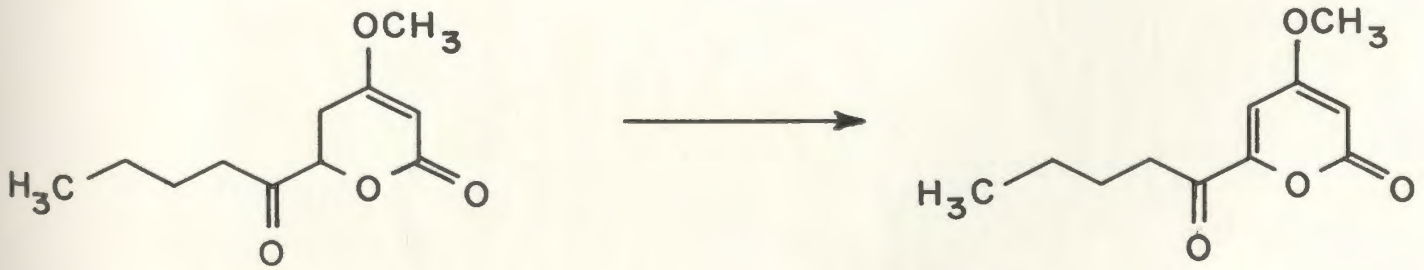
for 4 h with platinum oxide gave a 43% yield of the ethyl compound deoxodehydroacetic acid (LXX) an observation which was later confirmed by Berson (60).



(LXIX) (LXX)

It has also been shown in our laboratory that when retinaldehyde is hydrogenated in methanol containing platinum oxide, the allylic aldehyde is hydrogenolysed to the methyl derivative, in addition to the side chain double bonds being reduced. Thus, of the models so far presented, only triacetic acid lactone (XXIV) shows a pH dependent chromophore closely resembling the hydrogenated pigment.

The isolation from an unidentified fungus of a new 5,6-dihydro-2-pyrone derivative (LXXI) has recently been described. This compound was dehydrogenated to the 4-methoxy-2-pyrone derivative (LXXII) (94).



(LXXI) (LXXII)

The absorption maximum of (LXXII) λ_{max}(EtOH) 223, 309 nm is at longer wavelength than perhydro pigment A-2 (λ_{max}280 nm), although it is possible that a C-6 substituted carbonyl group on the pyrone ring would be catalytically reduced (93) during hydrogenation. Other pyrones which might be considered include 5-acetyl-6-methyl pyronone, investigated by Butt and Elvidge (95).

Table 12. Summary of ultraviolet spectra of hydrogenated pigment from some Myxomycetes and of a possible chromophore (nm) (methanol)

Pigment	Neutral solution	Acidic solution	Alkaline solution	Reference
A-2 (P. flavicomum)	245, (0.77) 280, (0.74)	- 277 (0.80)	245 (0.87) 278 (0.93)	
3 (P. polycephalum)	245-50 (0.96) 270-76 (0.96)	- 272 (1.01)	244 (1.13) 275 (1.04)	
C (P. polycephalum)	230 242 284	227 - 284	- 245 284	(19)
Triacetic acid lactone	233 283	225 283	234 280	(92)

( ) = absorbance

Table 13. Ultraviolet absorption spectra of the lactone (XXIV), and hydrogenated pigment A-2 in aqueous buffer (nm).

Buffer solution (96)	NaOAc - HCl				KH_2PO_4 - NaOH		$Na_2B_4O_7$ - NaOH-KCl		
pH	1.1	3.3	4.2	5.2	6.1	7.4	8.0	9.0	10.0
Lactone (XXIV)	284 -	283 -	282 -	277, 230	276, 233	276, 233	276, 233	276, 233	276, 233
Hydrogenated pigment	277 -	280, 245	280, 245	280, 245	280, 245	280, 245	281, 244	280, 245	281, 245

Methylation of pigment A-2 and A-3(P. flavicomum)

Dresden (16) obtained a pK_a of 4.7 for the related P. polycephalum pigment, a value which was similar to the pK_a of carboxylic acids, and therefore he rationalised that this pigment should form an ester. However, he was unable to prepare a methyl ester by treating an ethanolic solution of the pigment with diazomethane at 0°, although there was a hypsochromic shift of the absorption maximum from 410 nm to 337 nm. Dresden was also unable to prepare an ethyl ester by refluxing the pigment in ethanol containing dry hydrogen chloride.

Attempted methylation of pigment A-2 with diazomethane.

When a cold methanolic ether solution of pigment A-2 was treated with CH_2N_2 at ~ 4°, and the absorption spectrum determined after 24 h, the usual bathochromic shift to 412 nm was still obtained after protonation, accompanied by a hyperchromic increase. The 'methylation product' was insoluble in chloroform or carbon tetrachloride but soluble in water, 50% ethanol/water and methanol, indicating that the pigment was still very polar or ionic which therefore suggested that almost all of pigment A-2 was recovered unchanged.

Cation exchange chromatography of recovered product and remethylation with diazomethane.

When the sample recovered above was chromatographed on a cation exchange column, the absorption maximum shifted from 380 nm to 414 nm as expected; the pigment was now soluble

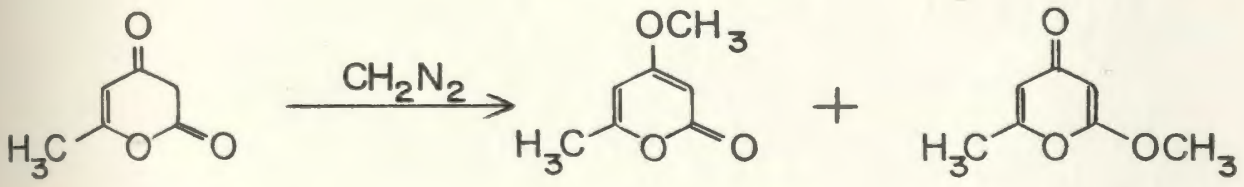
in chloroform, ethanol and methanol but insoluble in water, suggesting the pigment anion was protonated to give a preparation akin to A-3. The i.r. spectrum ($CHCl_3$) Fig. 17 showed strong bands at 1710, 1670, 1620, 1590 (infl.) and 1580 cm^{-1}, indicating the presence of carbonyl, aromatic and olefinic stretching frequencies. When this protonated product was remethylated with CH_2N_2, the absorption spectrum λ_{max} (MeOH) 372 nm was unchanged on acidification, although there was a small hyperchromic increase. It follows therefore that the functional group responsible for the typical bathochromic shift may have now been converted to the methyl derivative. The i.r. spectrum showed a broad band at 3500 - 3150 cm^{-1} probably due to an N-H stretching frequency. Strong bands at 1712 and 1672 cm^{-1} (of equal intensities) could be assigned to carbonyl stretching frequencies, while an intense band at 1010 cm^{-1} is usually assigned to the trans C-H out of plane stretching frequency in conjugated polyenes.

Methylation of pigment A-3

When a methanolic ether solution of pigment A-3 (protonated pigment A-2 obtained directly after chromatography on a cation exchange resin column) was treated with an ethereal solution of CH_2N_2 for 24 h, the absorption maximum after work-up was recorded at 364 nm, (shoulder at 390 nm), the shorter wavelength peak being now pH independent. It has previously been shown that the dissociation of the relevant group in pigment A-3 alters its spectral properties causing a large hypsochromic

shift (∿ 40 nm) in alkaline conditions whereas the dissociation of carboxyl groups in conjugation with a polyene chromophore generally causes a small hypsochromic change (3-5 nm) in the absorption spectrum. Although any carboxyl group present in the pigment would be converted to its methyl ester, this change would not be expected to result in a large hypsochromic shift after methylation; on the contrary, methyl esters of conjugated polyene acids generally possess absorption maxima about 4-8 nm higher than the corresponding free acids;(e.g. 2,4,6-octatrienoic acid has λ_{max} 294 nm, while its methyl ester has λ_{max} 302 nm. It is also important to note that the methylated product has approximately the same absorption maxima in both neutral and alkaline conditions. Thus it seems that an acidic functional group(s), which may be more acidic than a carboxyl function, has been methylated and this is consistent with the pK_a value of 3.4 obtained for the native pigment. (The significance of two apparent pK_a values will be discussed later). It has already been suggested on the basis of the spectral behaviour of the hydrogenation product of pigment A-2 in neutral, acidic and alkaline conditions that the pigment might contain a pyrone moeity as an integral part of the chromophore, since its ultra-violet spectra showed changes reminiscent of triacetic acid lactone (XXIV). Since an understanding of the nature of these methylation reactions is considered relevant to this discussion, a fairly detailed survey is included here of changes in the

absorption and infrared spectra on the methylation of 2,4-pyrononones (used synonymously with 2,4-pyrones). The course of the diazomethane methylation of some 2,4-pyrononones has been extensively investigated by workers in several laboratories, since these pyrone systems are being discovered in secondary metabolites that are produced by both higher plants and fungi. A group of Polish workers (97) examined the methylation of triacetic acid lactone (XXIV) with diazomethane and concluded that 6-methyl-4-methoxy-α-pyrone (LXXIII) (72%, λ_{max} (EtOH) 280 nm as well as 6-methyl-2-methoxy-γ-pyrone)(LXXIV) (20%, λ_{max} 240 nm) are produced in the methylation reaction.



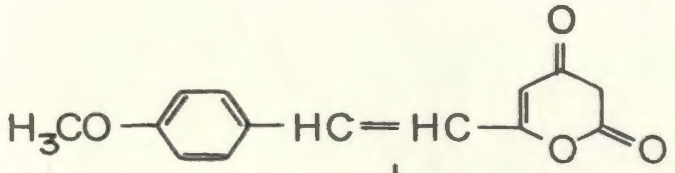
(XXIV) (LXXIII) (LXXIV)

The separation of the two isomers (LXXIII) and (LXXIV) was accomplished by taking advantage of the fact that γ-pyrones form pyroxonium salts (98), the hydrochloride of the 2-methoxy-γ-pyrones being insoluble in ether. When Wiley and Jarboe (99) reported that they were only able to obtain the 4-methoxy-α-pyrone (LXXIII), another group (100) reinvestigated the diazomethane methylation of triacetic acid lactone and obtained a 64% yield 6-methyl-4-methoxy-α-pyrone (LXXIII) λ_{max} (EtOH) 280 nm, and a 19% yield of 6-methyl-2-methoxy-γ-pyrone (LXXIV) λ_{max} 240 nm,

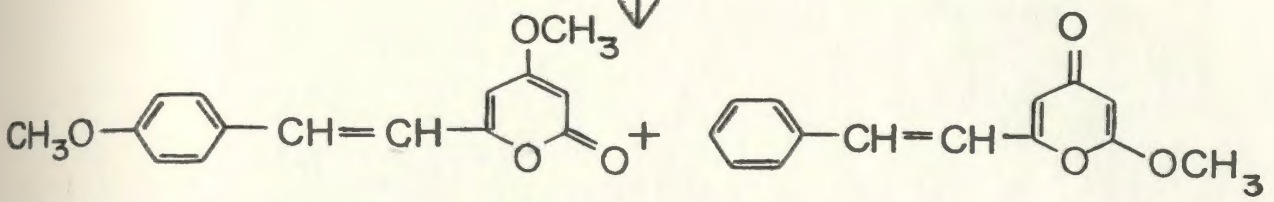
and their physical and chemical constants were in excellent agreement with the values reported by the Polish workers. This work was extended to the methylation of 6-phenyl-2, 4-pyronone and again the major product isolated was 6-phenyl-4-methoxy-α-pyrone, λ_{max} (EtOH) 314 nm, and a small amount of 6-phenyl-2-methoxy-γ-pyrone, λ_{max} (EtOH) 276 nm. In both cases the α- and the γ-pyrones were isolated and there is a difference of 38 to 40 nm between the longer wavelength maximum represented by the α-pyrone derivative and the shorter wavelength maximum represented by the γ-pyrone derivative.

Application of these findings to the methylation of pigment A-3 would suggest that the methylated product (λ_{max} 364, 390 nm) might be a mixture of the enol methyl ethers of the α- and γ-pyrone whereas the protonated pigment (λ_{max} (MeOH) 412 nm, would seem to exist predominantly in the 4-hydroxy-α-pyrone form in tautomeric equilibrium with the 2-hydroxy-γ-pyrone, in conjugation with a polyenic system. In addition to the differences in their absorption spectra, α- and γ-pyrone structures have been assigned on the basis of their infrared spectra in the carbonyl region. The i.r. spectrum ($CHCl_3$) of pigment A-3 (Fig.16) shows bands at 1710 and 1670 cm^{-1} which can be assigned to carbonyl stretching frequencies. The presence of all <u>trans</u>- conjugated double bonds can be inferred from the i.r. active bands at 1625, 1600 and 1010 cm^{-1}.

As part of their investigations on the chemistry of rosewood (Aniba species) Herbst et al (100) reported the isolation of anibine, 4-methoxy paracotoin and 5,6-dehydrokawain. Assignment of structure in some of these pyrone derivatives was based in part on the i.r. spectra. These workers showed that γ-pyrone derivatives exhibit their first carbonyl band near 6.0μ (1667 cm^{-1}) while α-pyrones absorb near 5.80μ (1724 cm^{-1}) (somewhat variable depending on the solvent; see Table 14), but typical of unsaturated six-membered lactones. (These workers, however, cautioned that their assignment refers only to the first absorption band in the carbonyl region since all 4- methoxylated α-pyrones they studied, also show an intense band near 6.05μ (1653 cm^{-1}) which has been assigned to the enol ether grouping (100). In an attempt to establish unequivocally the structure of yangonin, Chmielewska et al (101) treated the 4-hydroxy-compound yangonolactone with diazomethane and obtained two substances, only one of which (LXXVI) was identical with the natural yangonin.



(LXXV)

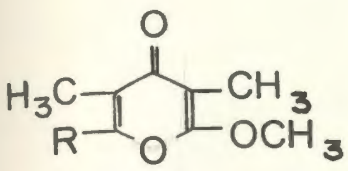


(LXXVI)

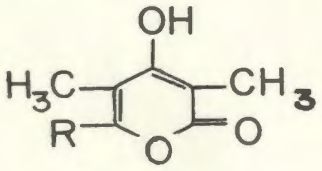
(LXXVII)

Compound (LXXVI) showed a peak in the i.r. at 1724 cm^{-1} and was formulated as the α-pyrone, while the γ-pyrone structure was assigned to the isomeric second product (LXXVII) (C=O stretch at 1667 cm^{-1}). Bu'Lock and Smith (92) also investigated the diazomethane methylation of triacetic acid lactone and their findings were fully consistent with those of Herbst et al (100). Bu'Lock and Smith cite the i.r. absorption data of several α- and γ- pyrones and Table 14 lists some of them.

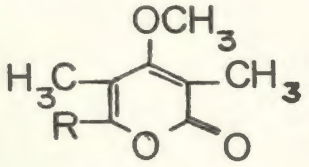
Hirata et al (68) reported that when aureothin (LXXVIII) is warmed in ethanolic hydrochloric acid, a demethylation occurs giving desmethylisoaurethin (XXXIV).



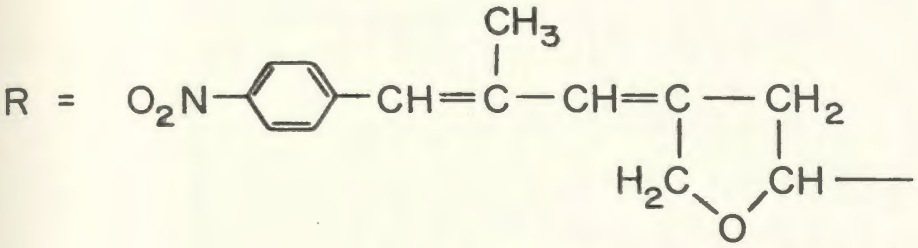
aureothin
(LXXVIII)



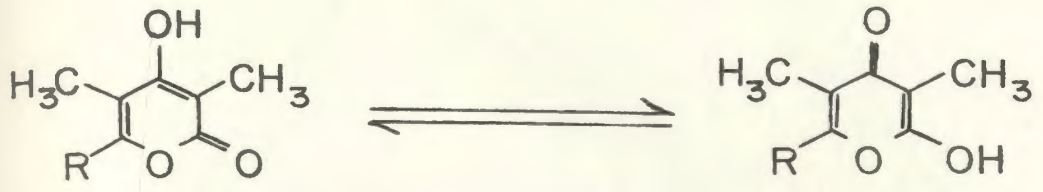
desmethyl isoaureothin
(XXXIV)



isoaureothin
(LXXIX)



The short wavelength maximum λ_{max} 248 nm (log ε 4.26) [also 346 nm (log ε 4.30)] of (LXXIX) is more consistent with a γ-pyrone structure. This assignment is valid since the pyrone system is isolated from the conjugated chromophore in all the aureothin derivatives. Later work has shown that when (XXXIV) is reacted quickly with CH_2N_2 in ether, the α-pyrone isoaureothin (LXXIX) is produced in 100% yield. However, if the CH_2N_2 solution is added over 18 h, a 98% yield of isoaureothin (LXXIX) is obtained, but more significantly a 2% yield of the γ-pyrone aureothin (LXXVIII) can be isolated. These results suggest that desmethylisoaureothin (XXXIV) exists in solution as a tautomeric mixture of (XXXIV) and (XXXIVa).



(XXXIV) (XXXIVa)

It was also shown that methylation of the sodium salt of (XXXIV) with dimethyl sulphate in dry acetone gave nearly equal amounts of aureothin (LXXVIII) and isoaureothin (LXXIX), although methylation of the sodium salt of triacetic acid lactone under the same conditions gave only the stable isomer 6-methyl-4-methoxy-α-pyrone (68). It seems possible, therefore, that production of α- or γ-enol ethers from 2,4-pyronone systems is markedly dependent on other substituents on the ring. Table 14 shows some examples of i.r. data related to α- and γ-pyrones, while Table 15 lists the u.v. spectra of some pyrone isomers. On the basis of the foregoing argument, methylated pigment A-3 could conceivably be a mixture of enol ethers of the α- and γ-pyrones, the peak at 1710 cm^{-1} being assigned to the C=O group of the α-pyrone, while the peak at 1670 cm^{-1} could be assigned to the carbonyl group of the γ-isomer, although u.v. - vis. absorption spectra suggest that the predominant isomer may be the enol methyl ether of the γ-pyrone. Alternatively, the absorption at 1670 cm^{-1} in methylated

pigment A-3 could be assigned to the C=N stretching frequency (1690 - 1640 cm^{-1}) (102) although the absence of a bathochromic shift on protonation (if the methylated product still contained an azomethine link) suggests that the 1670 cm^{-1} peak is not due to C=N. The purification of methylated pigment A-2 was attempted on silica gel, but separation was poor, although three bands were obtained (see experimental). Decomposition of the methyl esters may have occurred on the adsorbent, and this should be borne in mind in further attempts to isolate the α- and γ-pyrones.

Silylation of pigment A-2

Silylation of pigment A-2 resulted in a product whose absorption maximum (362 nm, shoulder at 410-418 nm) was now pH independent. The i.r. spectrum showed carbonyl bands at 1710 and 1690 cm^{-1} which could be interpreted as representing the carbonyl stretch of a mixture of α- and γ-trimethylsilyl enol ethers.

Acetylation

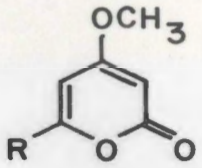
The absorption maximum of pigment A-2 after acetylation λ_{max} (MeOH) 370 nm, weak shoulders at 414 and 444 nm, was also unchanged on protonation. The i.r. band at 1755 cm^{-1} could be due to the C=O stretch frequency of an enol acetate, and this is in agreement with values for carbonyl stretching frequencies of enol acetates of 2,4-pyrone systems present in other natural products (103).

Table 14. Infrared absorption spectra (carbonyl bands in cm^{-1}) of some α- and γ- pyrones.

α-Pyrones	Number	C=O	Reference
4-Methoxy-6-methyl-	(LXXIII)	1722, 1736	(92)
anibine	(LXXX)	1733	(100)
dehydrokawain	(LXXXI)	1712	(100)
yangonin	(LXXVI)	1709	(100)
hispidin O-trimethyl ether	(LXXXII)	1701	(103)
citreoviridin	(LXXXIII)	1702, 1689	(104a, b)
luteoreticulin	(LXXXIV)	1688	(105)
isoaureothin	(LXXIX)	1700	(68)
desmethylisoaureothin	(XXXIV)	1682	(68)
γ-pyrones			
γ-pyrone	(LXXXV)	1670	(106)
2,6-Dimethyl-	(LXXXVI)	1672	(92)
2-Methoxy-6-methyl-	(LXXIV)	1677, 1692	(92)
3-Hydroxy-2-methyl-(maltol)	(LXXXVII)	1656	(92)
Pseudoyangonin	(LXXVII)	1667	(92)
Aureothin	(LXXVIII)	1668	(68)

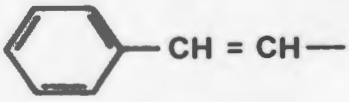
Table 15. Absorption maxima of some α- and γ-pyrones.

Compound	λ_{max}EtOH(nm)	Reference
4-Methoxy-6-methyl-2-pyrone	280	(92)
2-Methoxy-6-methyl-4-pyrone	240	(92)
4-Methoxy-6-phenyl-2-pyrone	314	(100)
2-Methoxy-6-phenyl-4-pyrone	276	(100)
4-Methoxy-6-(p-methoxystyryl)-2-pyrone	360	(101)
2-Methoxy-6-(p-methoxystyryl)-4-pyrone	345	(101)

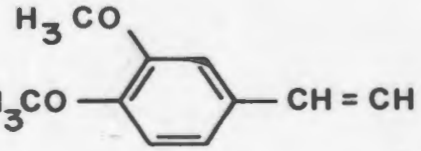


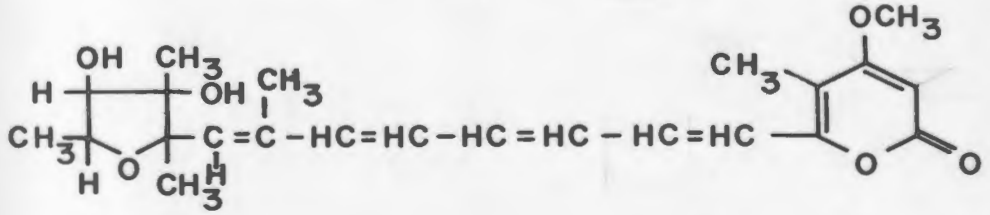
R = CH_3 (LXXIII)

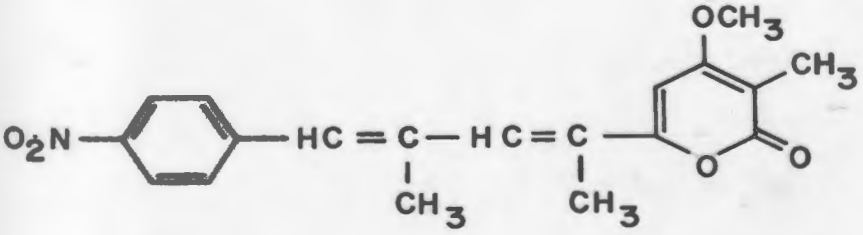
R = 3 — pyridyl (LXXX)

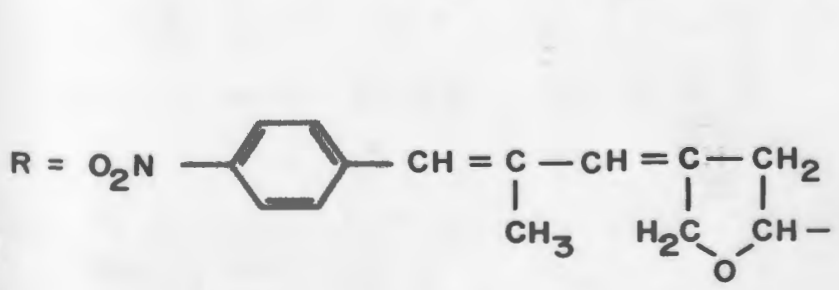
R =  (LXXXI)

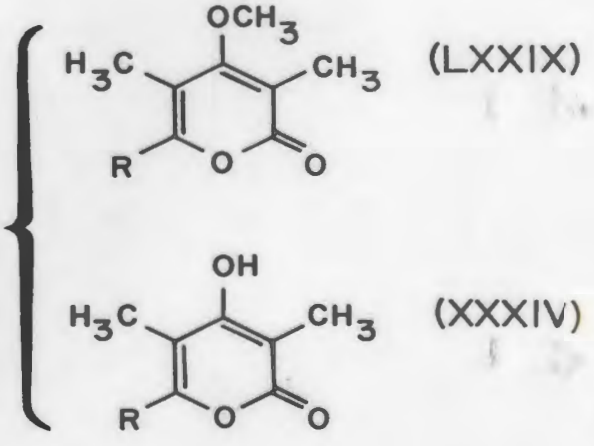
R =  (LXXVI)

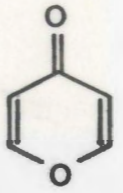
R =  (LXXXII)

 (LXXXIII)

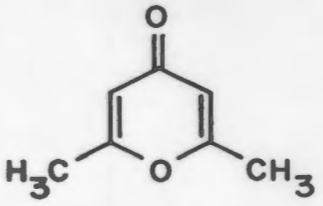
 (LXXXIV)



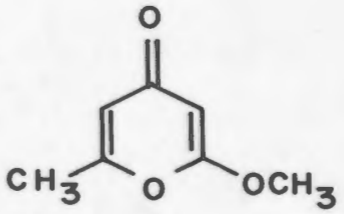




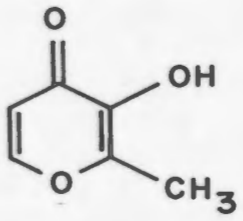
(LXXXV)



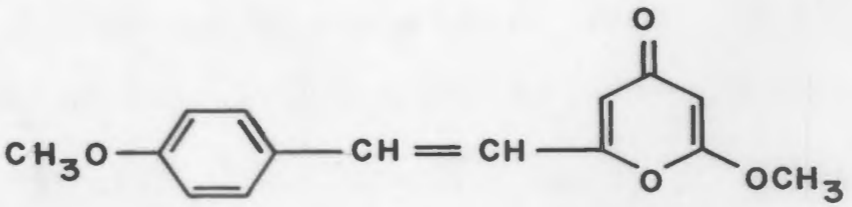
(LXXXVI)



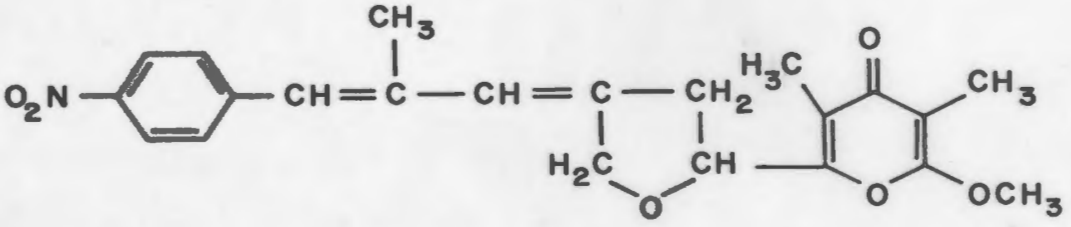
(LXXIV)



(LXXXVII)



(LXXVII)



(LXXVIII)

Reaction of pigment A-2 with dilute hydrochloric acid.

Pigment A-2 was heated with dilute acid for 17 h under N_2. The ether extract (extract I) exhibits a spectrum characteristic of a conjugated polyene with peaks at 315, 329, and 344 nm, similar to that of 2,4,6,8-decatetraene-1, 10-dioic acid (XL). Extract I did not react with sodium borohydride, suggesting that the conjugated polyene was not extended by either aldehyde or ketone functions. The highest peak in the mass spectrum was recorded at m/e 279, but it is doubtful whether this is the molecular ion. Hydrogenated extract I showed weak i.r. bands at 3670 and 3570 cm^{-1} (OH stretch), while the band at 1730 cm^{-1} could be due to a carboxyl group. The mass spectrum of the reduced product possessed ions at m/e 314 to m/e 43. It was not possible to characterise this degradation product. The u.v. spectrum of the aqueous solution (extract II) λ_{max} (MeOH) 258 nm, was shifted to 265-272 nm in alkaline solution, perhaps suggesting the presence of a phenol. Paper chromatographic examination resolved extract II into at least 3 components, all exhibiting strong white fluoresence. A positive reaction to 2,4- D.N.P. was obtained with extract II (carbonyl containing compound present). Extract II gave a reddish brown precipitate with 2,3,5-triphenyltetrazolium chloride indicating the presence of a reducing sugar (107a). The test for ketohexoses (107b) was negative. The u.v. spectra of several compounds (Table 27) were examined with the hope of identifying extract II (at least tentatively).

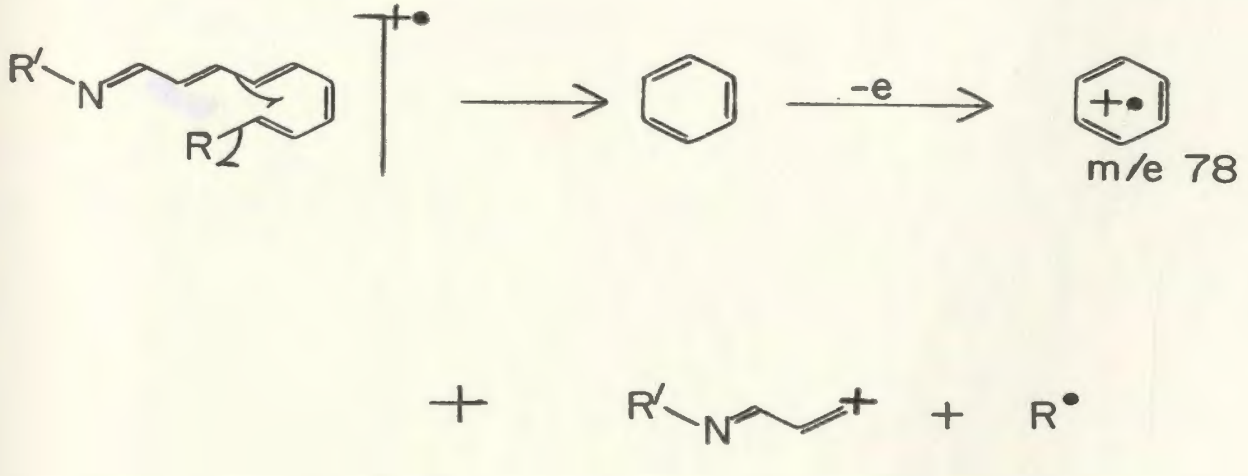
Reaction of pigment 2 with (a) dilute hydrochloric acid (b) dilute sodium hydroxide.

In an experiment early in this study, pigment 2 was heated with hydrochloric acid (2N) on a steam bath for 2 h, and after cooling, the mixture was made alkaline with sodium hydroxide (2N) and refluxed for 45 min. Acidification followed by extraction with CH_2Cl_2 produced a yellow oil with λ_{max} (MeOH) 315, 332 and 350 nm (polyene type spectrum). Catalytic hydrogenation of this oil gave a crystalline solid m.p. 61-63° with no u.v. spectrum. The i.r. spectrum indicated free and hydrogen bonded OH (3490 and 3300-3000, 2750-2400 cm^{-1}) and a carboxyl function (1740 and 1705 cm^{-1}). Two components were detected by g.l.c. and these were characterised as stearic acid (M^+ 284, major) and arachidic acid (M^+ 312, trace). CH_2N_2 methylation of these acids gave the corresponding methyl esters (M^+ 314, and M^+ 326).

High resolution mass spectrum of pigment A-2 (P. flavicomum).

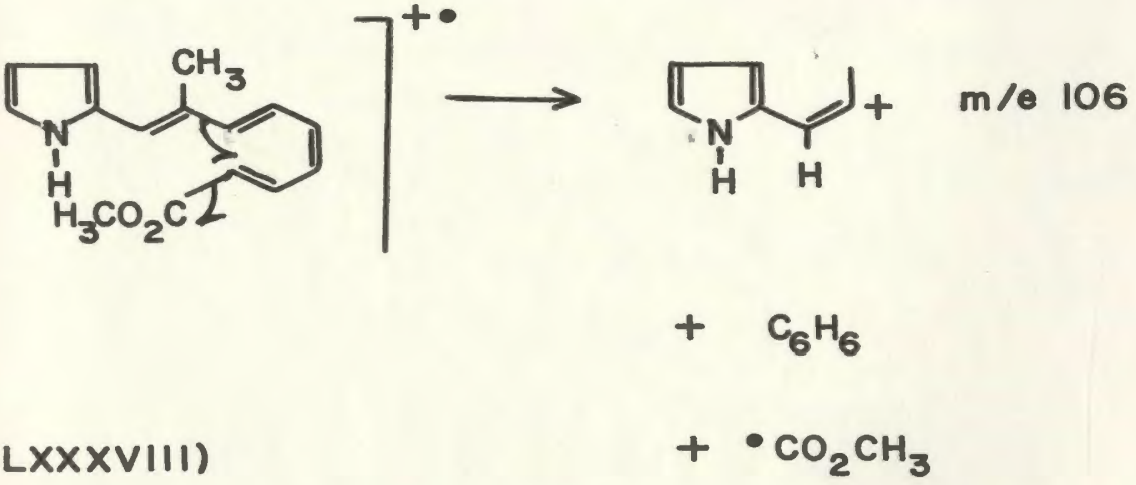
A high resolution mass spectrum (HRMS) of pigment A-2 was obtained at 315° (Appendix I) and the highest peak was recorded at m/e 409, although it is doubtful whether this corresponds to the molecular ion. The base peak at m/e 91 analyses for a $C_7H_7^{\oplus}$ fragment (for accurate masses see Appendix II) and this probably corresponds to a tropylium ion, implying the presence of an aromatic system in the pigment

capable of forming such an ion. Also present is a strong peak at m/e 107, (86%) analysing for C_7H_7O and differing from the ion at m/e 91 by sixteen mass units. It is, therefore, tempting to suggest that ions at m/e 91 and 107 are due respectively to benzyl- and benzyloxy- groups in pigment A-2, although confirmatory chemical evidence is lacking. A strong peak at m/e 78 (84%) analysing for C_6H_6 is probably due to the formation of benzene (as a positively charged species); several plausible mechanistic schemes can be advanced and these are summarised in Schemes 10a to 10d. It has been shown that pigment A-2 contains an all-trans tetraene system, possibly in conjugation with a modified C=N- group. This is utilised in Scheme 10a, but requires isomerisation of the polyene to a trans, trans, cis, trans, arrangement prior to cyclisation and cleavage.



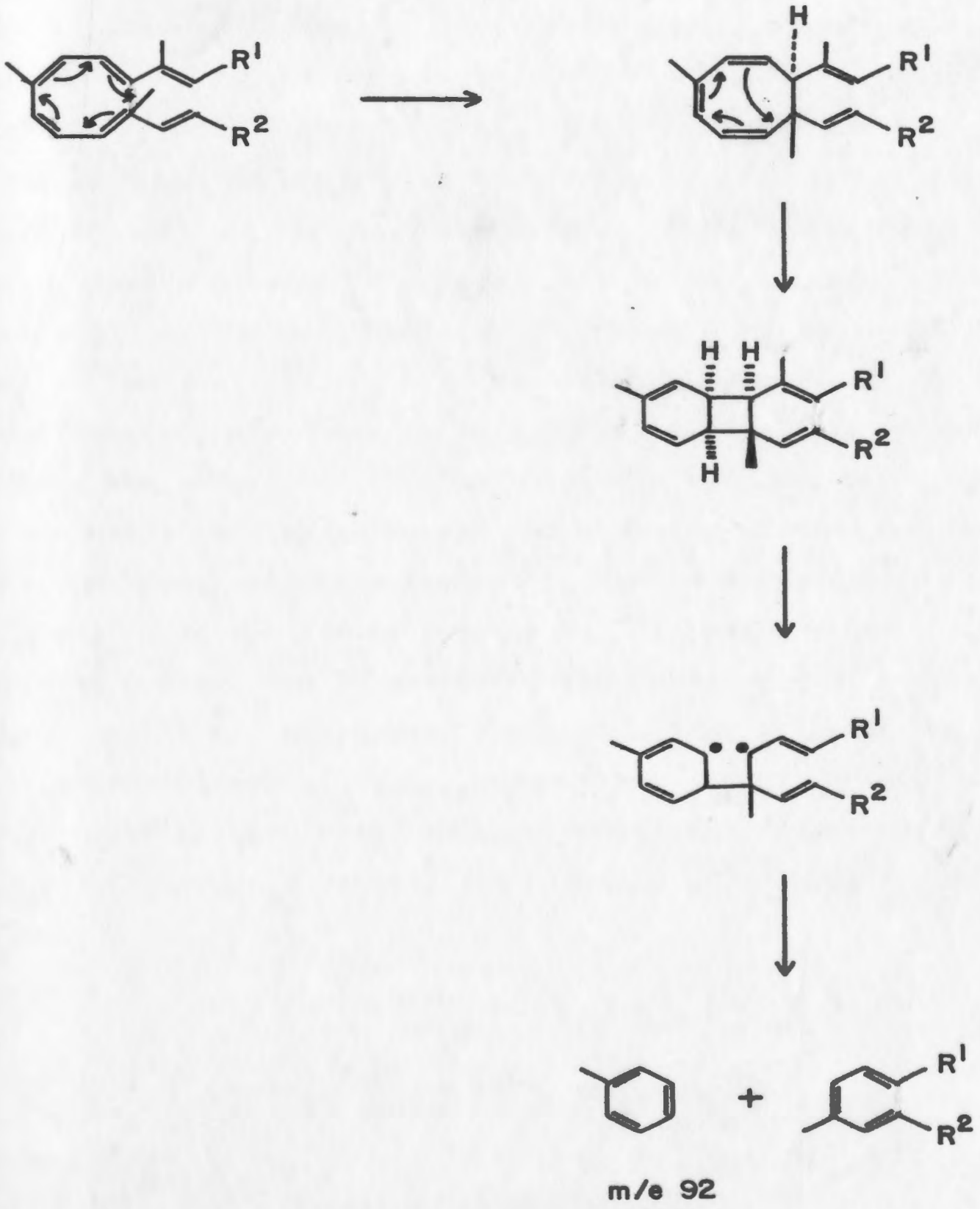
Scheme 10a

The mechanistic approach for formation of the ion at m/e 78 is patterned after a scheme suggested by Badar et al., (108) as a reasonable explanation for loss of benzene in the conjugated tetraenoate (LXXXVIII). In this case however, (Scheme 10b) the positive charge is retained on the pyrrole portion of the molecule while benzene is expelled as a neutral molecule, and this differs from the fragmentation of pigment A-2 where the ion at m/e 78 is expelled as a positively charged species. This difference in the charge retention ability requires that benzene offers preferential charge stabilisation over the rest of the molecule in pigment A-2, although the reason for this is not clear.



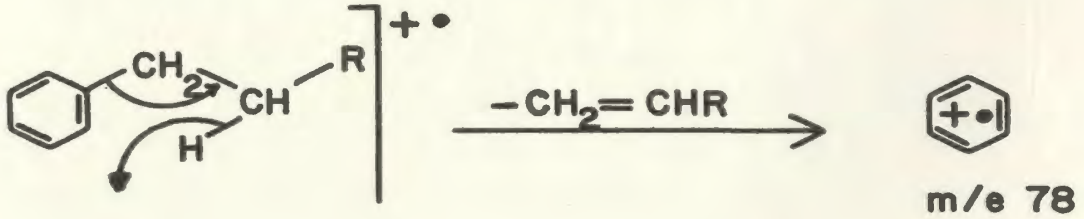
(LXXXVIII)

Scheme 10 b



Scheme 10 c

Thermal expulsion of aromatic entities from conjugated polyene systems is found in the carotenoid field where in some cases ions at (M-92) and (M-106) arising from loss of toluene and m-xylene from the molecular ion are abundant (109, 110). Scheme 10c rationalises the thermal formation of toluene from an arbitrarily folded and cis- and trans- isomerised polyene chain in a carotenoid; thermal formation of benzene from an unbranched polyene is then not difficult to visualise. A number of cis-trans- isomerisations known to occur in polyenes under thermal conditions has to precede the operation of this mechanism (111). Since the exact configuration and conformation of the chain undergoing the cyclisation is not known, the arrangement of the chain drawn in Scheme 10c is arbitrary. Another approach attempts to explain the formation of the ion at m/e 78, not from cyclisation of the polyene chain, but from an aromatic system already present in the pigment. One possibility involves an internal hydrogen exchange reaction as depicted in Scheme 10d (112) would require the presence of structure (LXXXIX) in pigment A-2.



(LXXXIX)

Scheme 10 d

Intermediate (LXXXIX) would also satisfy the requirements for the formation of the ion at m/e 91 which is the base peak of pigment A-2.

Ion at m/e 131

It has been suggested that the ion at m/e 131 ion (g), which is the base peak in the polyene esters (XXXVI) and (XXXVII), is formed from a tetraene carbonyl intermediate (Scheme 1). In the HRMS of pigment A-2, an ion is found at m/e 131 (14%) analysing for C_9H_7O, and its formation is probably similar to that shown for ion (g) postulated as a cinnamoyl ion. This would be consistent with the presence of at least one carbonyl group in conjugation with the tetraene in the pigment.

Ion at m/e 158

It has been suggested that the ion at m/e 158 ($C_{10}H_6O_2$) found in diesters (XXXVI) and (XXXVII) could be due to a cyclic 8-membered intermediate (Scheme 6). This intermediate requires carbonyl groups extending each end of the tetraene chain. The HRMS of pigment A-2 possesses an ion at m/e 158 (7%) analysing for a $C_{10}H_8NO$ fragment. Assuming then that an ion at m/e 131 (C_9H_7O) is derived from the ion at m/e 158, then the 27 a.m.u. difference could be due to loss of HCN implying the presence of a C=N group in the pigment. Ions at m/e 161 ($C_{10}H_{11}NO$) (34.5%), m/e 160 ($C_{10}H_{10}NO$) (56%), and m/e 159 ($C_{10}H_9NO$) (25%) all seem to lose a single hydrogen

radical successively to give the ion at m/e 158 ($C_{10}H_8NO$), and it is noticeable that these ions all possess oxygen and nitrogen. Other significant ions in the HRMS of pigment A-2 are recorded at m/e 147 (55.8%) which analyses for C_9H_9NO and at m/e 133 (25%) analysing for C_8H_7NO, a difference of one CH_2 group. Further, an ion at m/e 117 (26%) which corresponds to C_8H_7N is probably derived from loss of oxygen from the ion at m/e 133, indicating a structural relationship of these ions, although their full significance remains unclear at this stage. An ion at m/e 103 (17%) analysing for C_8H_7, in the HRMS of pigment A-2 is probably due to formation of ion (h) or (h') as in the mass spectrum of diester (XXXVI) (Scheme 1). Loss of acetylene from this fragment would give a phenyl radical m/e 77 ion(m), and indeed such an ion (85% of base peak) which analyses for C_6H_5 is present in the HRMS of pigment A-2.

CONCLUSIONS: PARTIAL CHEMICAL STRUCTURES

Analysis and molecular weight

Gel permeation chromatography on Sephadex LH-20 and G-10 in aqueous alcoholic solvents has provided a non-destructive method of isolating a consistent pigment preparation from P. flavicomum (pigment A-2) and P. polycephalum (pigment 3). Both pigments have 'indicator' properties and correspond to substances implicated in light-induced sporulation of several slime moulds (5). Pigment A-2 (P. flavicomum) appears to be essentially homogeneous (from electrophoresis studies), and of mol. wt. less than 750 (from its elution properties on Sephadex G10). Pigment 3 (P. polycephalum) was shown by electrophoresis to be a mixture of at least three components and will not be considered further here. Analyses and other data suggest that native pigment A-2 (P. flavicomum) is an alkali metal salt which gives a free acid (pigment A-3) on protonation. Most recent analyses (48) (on samples prepared by Mrs. J. Hillier) confirm that pigment A-2:

(i) is highly unsaturated

(ii) is highly oxygenated

(iii) contains nitrogen

(iv) has Na:N ratio of 1:1.

Amino-acids (-ve ninhydrin reaction of acid hydrolysate), and pyrrole and indole derivatives (negative reaction to Erhlich's reagent) are absent, so the nitrogen must be present in some other function. The protonated pigment (A-3, P. flavicomum)

has empirical formula $C_{12}H_{13}O_5N$, emp. form. wt. 251, whilst the sodium salt analyses moderately well for $C_{12}H_{16}O_7N$ Na(48). Degradation studies clearly support a higher molecular weight for these pigments than is suggested by these formulae. An estimate of the molecular weight of pigment A-3 which assumes that two nitrogen atoms are present per molecule would be 502. An ion at m/e 409 (1.16%) was recorded in the high resolution mass spectrum of pigment A-2, but it is doubtful whether this corresponds to the molecular ion.

Unsaturation.

The empirical formula of A-3 (P. flavicomum) corresponds to 7 units of unsaturation. Various degradations produce substances with typical polyene spectra and all the standard tests for alkenes are positive. Treatment of pigment A-2 with Ag_2O/CH_3I or $NaIO_4$ followed by CH_2N_2 produces mainly all-trans dimethyl 2,4,6,8-decatetraene-1,10-dioate (XXXVI) with small amounts of all-trans dimethyl 2,4,6,8,10-dodecapentaene-1, 12-dioate (XXXVII) confirming the presence of a tetraene with either carbonyl or potential carbonyl functions at either end. Neither terminal function is an ester, since reaction of pigment A-2 with Ag_2O/CH_3CH_2I produces a diethyl ester of the all-trans acid (XL). The absorption spectrum of pigment A-2 (P. flavicomum)(Table 8) suggests that the tetraene system is conjugated to another chromophore, at least at one end.

The azomethine system

Resonance Raman spectroscopy of pigment A-2 in neutral and acidic methanol shows the presence of a non-basic, C=N-group

in conjugation with the polyene chromophore. Thus the simple Schiff's base postulate of Daniel (23) is incorrect. The carbon and/or nitrogen atoms of the C=N- system must be connected to groups which render the nitrogen neutral. Raman spectroscopy suggests —O\C=N— as a serious possibility.

Measurement of the pK_a of the pigment by the spectroscopic method (17) and the lack of electrophoretic mobility of pigment A-2 at pH 1.1 confirm that protonation of this pigment does not occur through a Schiff's base system.

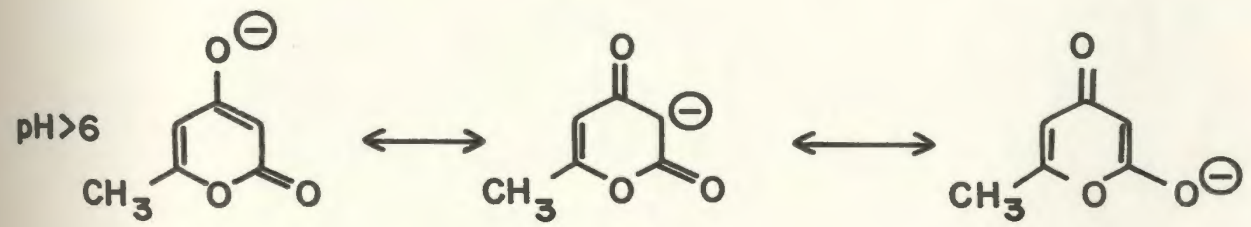
Nature of the acidic function(s).

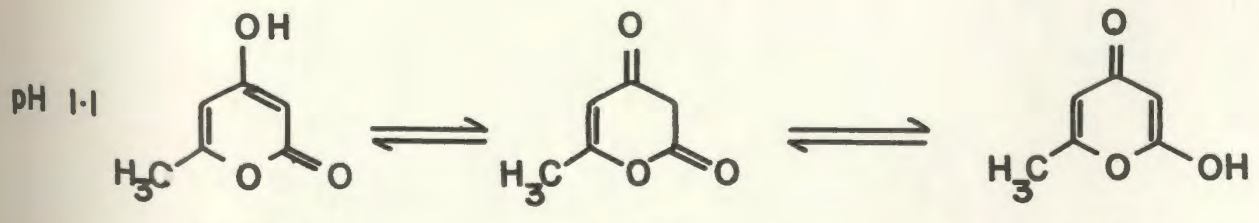
For mol. wt. 502, pigment A-3 (P. flavicomum) must have two acidic systems (since there would be two sodium atoms in pigment A-2). The pK_a determinations of pigment A-2 are ambiguous, and the lack of a true isosbestic point (Fig.13) at low pH clearly implies that protonation is followed by another reaction. This has been confirmed recently by Mrs. J. Hillier (48) who has shown that treatment of pigment A-2 (P. flavicomum) with dilute acid in a stopped-flow apparatus (observing the peak at 414 nm), produces a first order reaction in which the 414 nm absorption declines after an initial "instantaneous" rise. The function whose acidity is observable spectroscopically cannot be a -COOH group because ionisation of polyene carboxylic acids does not produce electronic spectral changes analogous to those of the pigment (however, the second acidic function could be a carboxylic acid). The bathochromic, hyperchromic shift of the 380 nm peak is very unusual since in general, salts absorb at longer wavelengths than undissociated acids. Thus, one of the

acidic functions is a rather stronger acid than a carboxylic acid, and also part of a chromophore with anomalous pH dependence.

Possible additional chromophores.

The Schiff's base postulate was made to accommodate the ultraviolet/visible spectral changes of the pigment on protonation and is incorrect. Hydrogenation of pigment A-2 (P. flavicomum) produces a colourless product whose u.v. spectrum is also pH dependent. Perhydro A-2 shows maxima at 245 and 280 nm at pH 7.4; below pH 1.1 only a single peak at 277 nm is observed. This shift does not appear to be merely a simple protonation, but more likely also involves the establishment of an equilibrium. Few systems with similar characteristics have been reported; one example, triacetic acid lactone (XXIV) shows exactly analogous changes (92), (pH 1.1 λ_{max} 284 nm; pH > 6 λ_{max} 233, 276 nm). A possible interpretation of the pH dependence of triacetic acid lactone chromophore is given below.





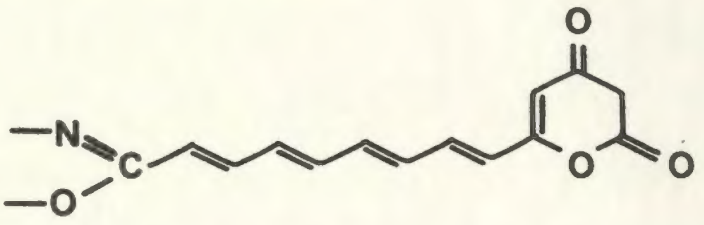
(XXIVa) (XXIV) (XXIVb)

major form very minor very minor

The high resolution mass spectrum of pigment A-2 (P. flavicomum) has a base peak at m/e 91 corresponding to the tropylium ion $C_7H_7^{\oplus}$, and it is therefore possible that the pigment contains an aromatic system. This remains unproven, and in view of the ready cyclisations of conjugated polyenes to aromatic species in mass spectrometers (vide supra), must be treated with caution.

Partial structures.

It is proposed that most of the data recorded here are consistent with the polyene-2,4-pyronone partial formula (XC) for pigment A-2, form. wt. 257.



(XC)

The biogenesis of the skeleton can be readily rationalised as being derived from acetate, though the C=N- function is unusual. A polyene -2,4-pyronone chromophore has been found in the neurotoxin citreoviridin (LXXXIII) and tracer studies have established that the carbon skeleton is acetate derived (113). Other possible functions which must be considered in a full structure for pigment A-2 include

(i) a carboxyl group

(ii) a sugar

(iii) another non-basic nitrogen containing function.

Further studies.

The molecular weight of the pigment must be precisely established. Mass spectral examination of silylated pigment A-3 might be one approach, and this method might also give an estimate of the number of hydroxyl groups in the pigment. In view of the high oxygen content of the pigment, substances which are highly oxygenated e.g., sugars should be looked for carefully. The nitrogen containing fragment should be isolated and characterised, and this may possibly be achieved by careful examination of the products obtained by acid or alkaline hydrolyses of the pigment. Sodium periodate oxidation of the pigment (on a larger scale) should be pursued; the structure of degradation product (LXI) (mol. wt. 328) should be established. The presence or absence of other carbonyl containing degradation products in the aqueous layer after periodate oxidation should also be demonstrated.

Finally, pigment A-2 should be tested for biological activity in view of the similarity of its chromophore to citreoviridin, a polyenic 4-methoxy 2-pyrone with demonstrated neurotoxic activity (104).

EXPERIMENTAL I

GROWTH OF P. FLAVICOMUM AND P. POLYCEPHALUM

The original cultures of the yellow slime mould Physarum polycephalum Schw., and the orange slime mould Physarum flavicomum Berk., were kindly provided by Dr. C.J. Alexopoulos, Department of Botany, University of Texas at Austin. As received, these slime moulds were growing on oat flakes suspended in solid agar, and were both successfully induced to grow on the semi-defined liquid medium developed by Daniel and Rusch for P. polycephalum (2). The cultures were maintained in our laboratory as:

(a) stationary cultures
(b) 50-cm^3 liquid shake flask cultures
(c) 500-cm^3 liquid shake flask cultures
(d) twelve-litre fermentor cultures.

(a) Stationary cultures.

(i) Oatmeal cultures.

Oatflakes (3-4 g, Ogilvie) moistened with water (2 cm^3) were placed in Erlenmeyer flasks (500 cm^3) fitted with cotton wool plugs. After autoclaving at 125° for 15 min., followed by cooling to room temperature, the flasks were innoculated aseptically by transferring small pieces of plasmodia (approximately 1 cm square) from a previous actively growing culture. This transfer was effected by means of a

flamed nichrome wire loop. After 4-5 days growth, the plasmodia had migrated to cover the entire oatmeal surface, at times even attempting to grow against the walls of the flasks and upwards through the cotton wool plugs. Maximum growth usually coincided with maximum pigmentation in each organism. Oatflake cultures of both organisms were maintained during the entire study as a source of the same culture, in case of loss due to contamination in the other culture methods that are described below.

(ii) Agar culture

Both P. polycephalum and P. flavicomum were grown on sterilised agar plates in petri dishes (10, 15 cm diameter) prepared essentially after the method of Ross and Sunshine (3). The organisms were grown for 4-5 days in the dark at 25°, after which time transfers of actively growing plasmodia were made to freshly sterilised agar plates. Plasmodia from these agar plates were used for growing the organisms in liquid shake flask culture, and it was therefore essential that the growing plasmodia be contaminant free. Consequently cultures of both organisms were purified by the standard MIGRATION TECHNIQUE (114) used in microbiological laboratories, and when shown to be contaminant free, used as inoculum for the liquid shake flask cultures.

(b) 50-cm^3 liquid shake flask cultures

Plasmodia taken from cultures which had been purified on agar plates were used to inoculate Erlenmeyer flasks

(250 cm^3) containing sterilised liquid growth medium (50 cm^3) fitted with cotton wool plugs. These flasks were placed on a 120 revolutions/min. rotary shaker (Model G-10, New Brunswick Scientific Co. Inc.) in a 25° incubation room and were grown for 3 days.

(c) 500-cm^3 liquid shake flask cultures

Microplasmodia obtained after allowing the 50-cm^3 three day old cultures to settle were then used to innoculate Fernbach flasks (2800 cm^3 capacity) each containing 500 cm^3 of sterilised liquid growth medium. Each flask was innoculated with a plasmodial suspension (10 cm^3) which had been allowed to settle for 5 min. After 4-5 days growth the plasmodia were harvested and extracted as described on p.140.

The composition of the liquid medium is given in Table 16, and is similar to the medium developed by Daniel and Rusch (2) for growing P. polycephalum in a semi-defined medium. Initial attempts to grow P. flavicomum in liquid medium met with very little success; however addition of a 10^{-2}M solution of quinic acid to the growth medium as reported by Ross and Sunshine (3) seems to sustain growth of this organism. These authors also observed that addition of quinic acid increased the yield of pigment, and prolonged the time necessary for sporulation. Quinic acid was without any noticeable effect on the growth rate of P. polycephalum, and this is in agreement with the findings of Ross and Sunshine.

Table 16 gives the composition of the liquid medium used for growing P. flavicomum and P. polycephalum.

Table 16. Composition of liquid culture medium for the Myxomycetes P. flavicomum and P. polycephalum.

Component	Amount
Casein hydrolysate[a]	10 g
Glucose (anhydrous, Baker)	10 g
Yeast extract (Difco)	1.5 g
KH_2PO_4	2.0 g
Quinic acid (10^{-2}M)[b]	1.92 g
Haematin solution (0.04% in 0.15N NaOH)[c]	10.0 cm^3
Supernatant fluid of salt suspension[d]	30.0 cm^3
Citric acid (0.5M)/1.0M NaOH buffer (pH 4.6)	27.0 cm^3
Antifoam GE-60[e]	0.1 cm^3
Penicillin (Wycillin G)[f]	0.1 cm^3
Distilled water to	1000.0 cm^3

(a) Bactotryptone (Difco Laboratories, Detroit, Michigan).

(b) Only for P. flavicomum (3).

(c) Autoclaved separately and added aseptically to each flask. Usually haematin solution (5 cm^3) was added to each flask containing growth medium (500 cm^3). Recrystallised haemin (bovine or equine) was obtained from Sigma Chemicals.

(d) Supernatant fluid of salt suspension was prepared as follows. All components were reagent grade.

Salt solution A

$CaCl_2\ 2H_2O$ (10 g)	all dissolved in
$MgSO_4\ 7H_2O$	distilled water
Citric acid monohydrate (20 g)	(300 cm^3) and
Hydrochloric acid (conc. 2 cm^3)	called salt solution A

Salt solution B

$FeCl_2\ 4H_2O$ (2 g)	all dissolved in
$MnCl_2\ 4H_2O$ (1.4 g)	distilled water
$ZnSO_4\ 7H_2O$ (0.56 g)	(100 cm^3)

The two solutions A and B were combined and diluted to 500 cm^3 with distilled water. After settling, the supernatant fluid was poured off and used as "the supernatant fluid of salt suspension" in Table 16.

(e) General Electric Co. antifoam.

(f) Added after culture medium was cooled to 25°.

The slime moulds were routinely grown in Fernbach flasks (2800 cm^3) containing culture medium (500 cm^3) on a gyro-rotatory shaker (Model G-10, New Brunswick Scientific Co. Inc.) at 120 revolutions/min. After 4-5 days growth, the plasmodia were processed by the method described on p.140. Table 17 records the approximate yield of wet plasmodia, and the dry weight after lyophilisation.

Table 17. Yield of wet plasmodia and dry weight after lyophilisation (P. flavicomum)

Volume of media	Growth period (h)	Wet plasmodia (g)	Plasmodia after lyophilisation (g)	Dry plasmodia/ 100 g wet weight
4x500 cm^3 (2L)	96	77.5	1.58	2.11
4x500 cm^3 (2L)	124	170.0	5.40	3.17
4x500 cm^3 (2L)	96	90.0	2.52	2.80
4x500 cm^3 (2L)	120	200.0	5.90	2.95

The average yield of lyophilised plasmodial extract was 2.1 - 3.2 g. per 100 g wet plasmodia

(d) Twelve-litre fermentor cultures

Both P. flavicomum and P. polycephalum were grown in a Micro Ferm Laboratory fermentor (12L, New Brunswick Scientific Co. Inc., New Brunswick, N.J.). The liquid medium was sterilised for 20 min. at 125° and 15 p.s.i. in an autoclave (Model AE-30-10, New Brunswick Scientific Co. Inc.) and cooled to room temperature before use. Each 12L fermentor was innoculated with microplasmodia obtained from moulds that were growing for four days in Fernbach flasks (500 cm^3), Wycillin G (300,000 IU/cm^3, 1 cm^3) was routinely added to each 12L growth medium to reduce the risk of contamination by other organisms. The air flow was maintained at 10-15 cm^3/min., the rate of mechanical agitation was 80-100 revolutions/min. for P. flavicomum and 180 revolutions/min. for P. polycephalum and the temperature was thermostatically controlled at 25°.

The organisms were grown for four days, and the extraction of the plasmodia is as described for the liquid shake flask cultures (500 cm^3) (p.140). The yield of wet plasmodia from a 12L fermentor after centrifuging varied from 700-1000 g.

Growth of large plasmodia of P. Flavicomum and P. polycephalum on millipore filters.

Large plasmodia (12-15 cm. diameter) were grown in our laboratory by Dr. Eric Bullock essentially after the method of Mohberg and Rusch (40) for growing large synchronous plasmodia of P. polycephalum. The plasmodia were grown in metal trays on a millipore filter supported by filter paper on a stainless steel screen. Plasmodia were started from a ring of innoculum to allow inward and outward migration and were incubated on a rocker so that nutrient medium could flow back and forth, wetting the undersurface of the plasmodia. The plasmodia were grown for periods of 24, 48 and 72 h, and were immediately frozen in liquid nitrogen and then lyophilized. The absorption spectra of an aqueous extract of these preparations were determined, and even after 24 h growth, a pigment component that showed the typical bathochromic and hyperchromic shift (reminiscent of pigment A-2, p.163) could be detected in P. flavicomum.

Extraction of pigments from P. Flavicomum and P. Polycephalum.

The microplasmodia obtained after growing the slime moulds for 4-5 days were allowed to settle for 15 min. Fusion of the microplasmodia resulted in one large plasmodial mass which was separated from the liquid culture medium by decantation. It was found that if the growing period was extended beyond five days, there was excessive production of "slime", and this made fusion and subsequent separation of the microplasmodia from the growth medium very difficult, and consequently very undesirable. The plasmodial mass was then centrifuged for 1 min. at 10,000 r.p.m. using an "International" centrifuge, and the resultant plasmodial residue blended with equal volumes of acetone using a Waring blender. Other extraction solvents, e.g. methanol or ethanol worked equally well, but it was decided to adopt acetone as the solvent in all subsequent extractions. Unpublished studies in our laboratory (115) have used distilled water as the extraction solvent with good results. The acetone extract was then filtered using a large Büchner funnel to give an orange-red aqueous acetone solution and a pale yellow solid residue which was discarded. The acetone was then removed at 37° under reduced pressure on a rotary evaporator to give an aqueous suspension of the plasmodial pigments. This suspension was then exhaustively extracted with petroleum ether (30-60°) (8x1 volume) at room temperature under

reduced light. Very little pigment was extracted from the aqueous layer by the organic solvent. Traces of petroleum ether were removed from the aqueous residue by evaporation under reduced pressure.

Isolation of pigments.

1. Sodium bicarbonate/hydrochloric acid method.

The aqueous suspension of the pigments was dissolved in aqueous sodium bicarbonate (10%, w/v) to give a homogeneous red solution which was cooled to 0° and acidified with hydrochloric acid (2N). A brown solid precipitated and this was removed from the orange red aqueous solution by suction filtration. The aqueous filtrate was extracted with ethyl acetate (4 x 100 cm^3) and this pigment extract was combined with the solution of pigment obtained by dissolving the brown solid previously obtained. The combined ethyl acetate solution was reduced in volume, and applied on a chromatographic column packed with silicic acid.

A summary of the isolation and purification is given in Charts 1 and 2.

Column chromatographic separation of pigments of Physarum flavicomum on silicic acid.

Method.

Silicic acid (150 g, MCB) was slurried with ethyl acetate (400 cm^3) and the suspension was made up into a long glass chromatographic column (70 x 2.5 cm) equipped with a teflon stopper. The size of the packing after several washings with the solvent was (42 x 2.5 cm). A pigment mixture (620 mg) which had been obtained by the sodium bicarbonate/hydrochloric acid/ethyl acetate isolation procedure was suspended in ethyl acetate (35 cm^3) and carefully applied to the silicic acid column which was then eluted successively with ethyl acetate, 10% methanol/ethyl acetate, 25% methanol/ethyl acetate, and finally with absolute methanol. The elution rate was maintained at ca. 50 cm^3/hr, and several (10) fractions were collected. The absorption spectra (in methanol) of the various fractions are recorded in Table 18.

At this stage, fractions eluted from the silicic acid column that showed a bathochromic shift on acidification were combined, and used as a purified component 1. Several degradation experiments were carried out on this purified pigment component, with inconsistent results. It was therefore felt that the purification method outlined above was not rigorous enough, and this would make any conclusions on the nature of the pigment subject to serious doubt. Secondly

the basic and acidic conditions employed during the purification could substantially alter or degrade the chromophore, and it became desirable to introduce a separation procedure that was mild enough to reduce the risk of decomposition, and at the same time to attempt to isolate the pigment components in as native a form as possible. The introduction of gel filtration, i.e. fractionation according to molecular size, and its use in the purification of labile biological substances such as proteins, enzymes and hormones provided an alternative method of purification of the pigments produced by these slime moulds.

Table 18. Absorption spectra of fractions eluted from silicic acid column ('indicator pigment', P. flavicomum)

Fr.	Volume	Colour	Weight (mg)	Absorption maxima (mm) methanol	methanol/HCl
1	100	colour-less	-	-	-
2	100	yellow	64	365	365
3	100	orange	117	390-410	410-414
4	100	orange	66	390-410	412-414
5	70	orange	18	390-410	404-416
6	70	orange	36	384	384 (408)
7	100	orange	11	390-410	412
8	100	orange	18	408	412
9	100	orange	50	390	412
10	100	yellow	110	375	375

Fraction	Eluting solvent
1-5	ethyl acetate
6,7	10% methanol/ethyl acetate
8,9	25% methanol/ethyl acetate
10	absolute methanol

CHART 1. EXTRACTION OF PIGMENTS FROM PLASMODIA OF THE MYXOMYCETES PHYSARUM FLAVICOMUM AND PHYSARUM POLYCEPHALUM WITH AQUEOUS ACETONE.

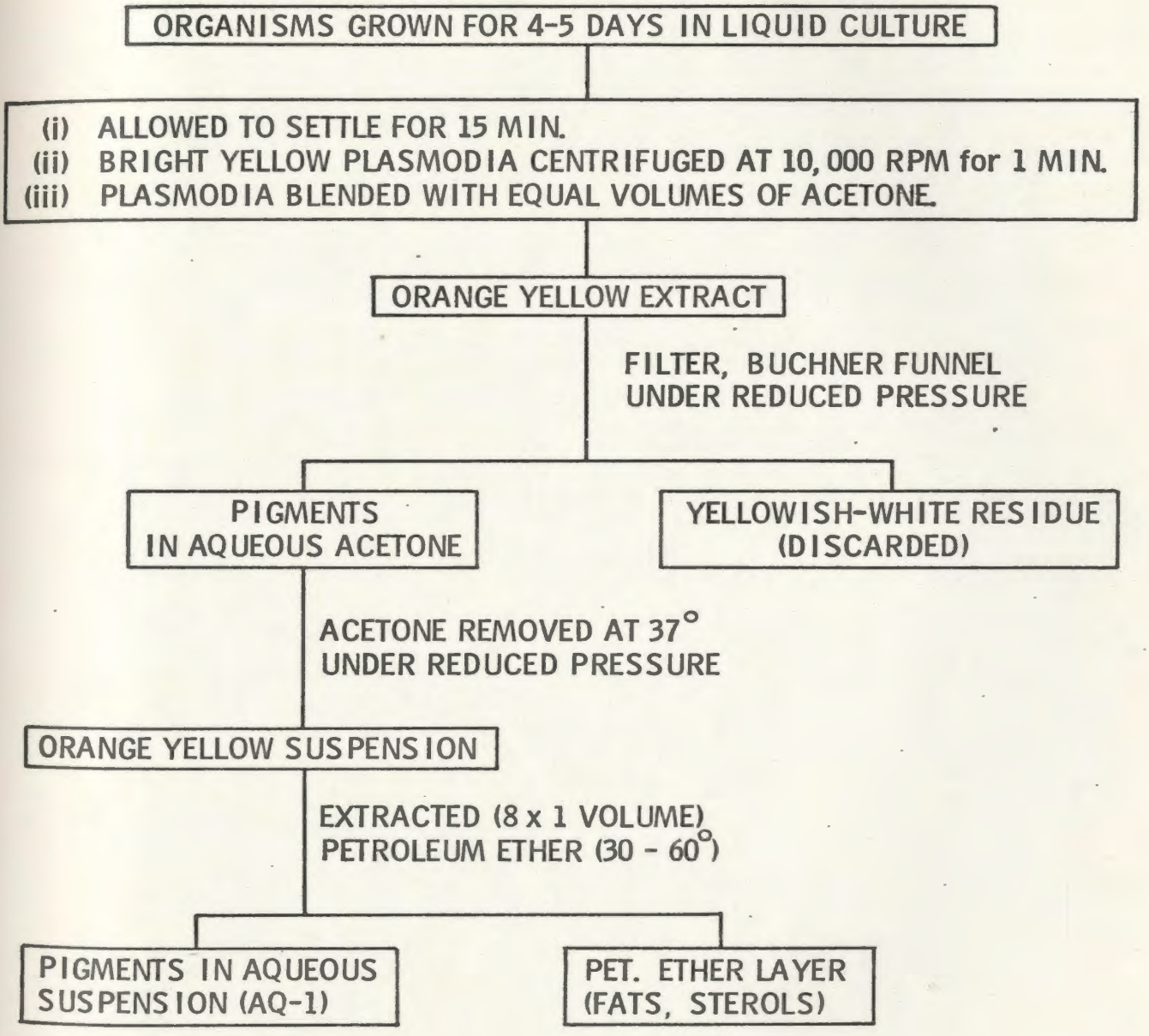
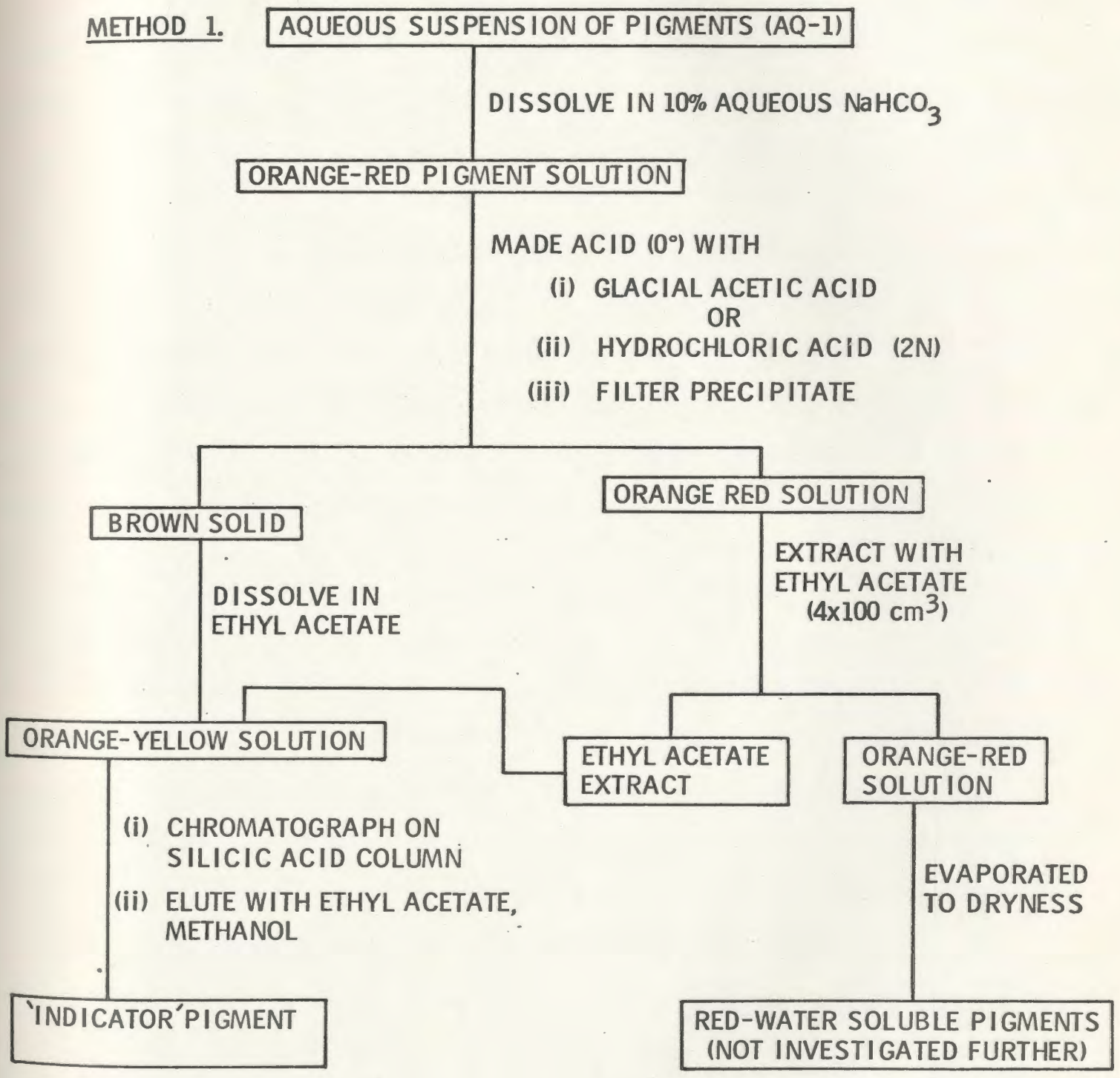


CHART 2. ISOLATION AND PURIFICATION OF PLASMODIAL PIGMENTS FROM P. FLAVICOMUM AND P. POLYCEPHALUM.



Separation of pigments of P. flavicomum by column chromatography using various Sephadex gels.

The aqueous pigment extract obtained after ether* extraction (AQ-1) was first diluted with ethanol and evaporated to dryness. It was necessary to add ethanol as attempted evaporation of the aqueous suspension without ethanol resulted in considerable frothing. The orange residue was then redissolved in methanol, filtered to remove inorganic salts, and the filtrate evaporated to dryness to give an orange-red solid. This pigmented residue was then dissolved in a minimum volume of the appropriate phosphate buffer and filtered, and this filtrate was used for further purification of the pigments. This method is summarised in Chart 3.

(a) Gel filtration of pigments using Sephadex G-25F, eluted with phosphate buffer (pH 7.5).

Sephadex G-25F, (44 g) [particle diameter 20-80 μ, Pharmacia (Canada) Ltd., Montreal] was suspended in the phosphate buffer (300 cm^3) and allowed to stand for 24 h to permit the gel to expand fully. The swollen Sephadex was freed from excess aqueous buffer, and the suspension was made up into a chromatographic column (Sephadex K26/40, 40x2.6 cm) and allowed to settle for 1-2 h. The buffer was slowly passed through until the gel bed had completely settled and then for a further 4 h. Just prior to sample application, most of the eluant above the gel was removed by suction using a Pasteur pipette, the column

*petroleum ether

outlet was opened, and the remaining eluant drained away. After closing the outlet, the pigment mixture (110 mg) was carefully applied to the top of the gel bed using a sample applicator and the outlet opened. After the sample had drained into the bed, the gel surface (and the column wall in contact with the sample) was washed with a small amount of phosphate buffer (1-2 cm^3). The column was then filled with buffer and connected to an eluant reservoir, and the flow rate was maintained at 100 cm^3/h. This method of sample application was used in all subsequent chromatography on Sephadex gels. Two pigmented bands, a light yellow and a bright orange band could be clearly seen after 90 min, but further elution produced a third more polar light yellow band. These three pigmented bands were collected over fifteen fractions (30-40 cm^3 each). Table 19 summarises the absorption spectra of these fractions (nm, phosphate buffer) and their spectral behaviour in aqueous solution at pH 3, and pH = 12.

Table 19. Absorption spectra of fractions eluted from Sephadex G-25 by aqueous phosphate buffer (P. flavicomum).

Fraction	Elution volume (cm^3)	Absorption spectra (nm, phosphate buffer) pH 7.5	pH < 3	pH ≈ 12
1-4	230	351	351, (425)	351
5	260	355-61	364, (406-7), (425)	355-361
6	290	359-62	406-7 (365)	359-62
7	320	364, 264-7	406-7 (424)	364, 264-7
8-12	320-479	Mixture of pigments		
13	513	390, 407	407 (388) (430)	407, (388), (430)

( ) = shoulder

When the pigment mixture was chromatographed on a column prepared with Sephadex G-25F (50 g) but with the eluting solvent phosphate buffer pH 7.1, a similar separation was obtained.

(b) Gel filtration of pigments using Sephadex G-15.

Sephadex G-15 (particle diameter 40-120 μ) has a molecular weight exclusion limit of 1500, compared to an exclusion limit range of 1000-5000 for Sephadex G-25F. However, these exclusion limit values are usually quoted for dextrans, peptides and globular proteins.

Purification of the pigments was also attempted on a chromatographic column prepared with Sephadex G-15 (75 g) and eluted with phosphate buffer pH 7.0. There was considerable adsorption of the pigments on this gel although 40 fractions (25 cm^3 each) of pH 7.0 buffer were collected and a further 14 fractions when the buffer was changed to pH 8. This purification method was not pursued further because of this adsorption effect.

(c) Separation of pigments on Sephadex G-15 eluted with sodium acetate (0.1M).

A column was prepared from Sephadex G-15 (75 g) and sodium acetate (0.1M) as described for the Sephadex G-25 F. Pigment extract (170 mg) was carefully applied to the surface of the gel bed, and the column eluted with sodium acetate (0.1M) with a flow rate of 100 cm^3/h. Two pigmented bands were collected with a total elution volume of 1700 cm^3 of solvent and distributed over 29 fractions. The separation of the pigments was similar to that summarised in Table 19.

Purification of the pigmented bands after chromatography.

The virtual insolubility of these pigments in non-polar solvents like hexane, benzene or ether prevented their re-extraction from the aqueous buffers into the organic phase. Acidification of the aqueous pigment solution followed by extraction into ethyl acetate would have been satisfactory but it was decided to avoid the use of acid in any purification step. Removal of the water (at 37°) from the aqueous buffer solution on a rotary evaporator was usually slow at this

temperature, and it was felt that this might increase the possibility of pigment decomposition. It was therefore decided to lyophilise the pigment fractions after chromatography but this too was time consuming.

Furthermore, the use of an aqueous phosphate buffer in the purification of the pigment necessitated the removal of the various salts before any meaningful studies could be carried out on the pigment. This desalting process can be achieved using Sephadex gels, e.g. G-25, and water as the eluting solvent, but this would obviously lengthen the purification process, and one had to contend with a further loss of pigment due to adsorption. However, the introduction of Sephadex LH-20, an alkylated dextran polymer which could be used with organic solvents as well as water, prompted us to modify our purification scheme. The use of Sephadex LH-20 and the choice of a suitable organic solvent (or an aqueous solvent mixture) would eliminate the need for desalting of the pigments, and the dried purified pigment can be obtained simply by evaporation of the organic eluting solvent. A purification scheme, (Chart 4, p.168) involving lyophilization* of the crude pigment extract followed by column chromatography on Sephadex LH-20 and then on Sephadex G-10 using various aqueous alcohols as eluting solvent, was worked out. The method was very reproducible, and was consequently adopted as the method of choice in the purification of these plasmodial pigments.

*The aqueous pigment solution (AQ-1) was freeze-dried in a lyophilizing apparatus specially constructed by Mr. Douglas Seymour, Scientific Glassblower, Memorial University. His help is gratefully acknowledged.

Gel filtration on Sephadex LH-20 (P. flavicomum pigments)

Sephadex LH-20 (170 g) was suspended in methanol/water (80/20 v/v) (800 cm^3) and allowed to stand for 24 h at room temperature to allow maximum swelling of the gel. The suspension was made up into a chromatographic column (35 x 5 cm) and the solvent was slowly passed through until the bed had completely settled, and then for a further 4 h. The pigment mixture was dissolved in the minimum amount of solvent, filtered and the orange-red filtrate applied to the column, care being taken not to disturb the surface of the gel bed. The column was eluted at a flow rate of 100 cm^3/h and several distinct pigment bands were eluted and separated (Table 20). Chromatographic columns of varying length and width were also used, with similar results.

Pigment 1.

This was the first pigment to be eluted as a diffuse yellowish brown band. It exhibited strong yellowish white fluorescence under ultraviolet light, and gave a positive ninhydrin test, but this could be due to protein impurities present in the fractions corresponding to pigment 1.

Pigment 2.

This pigment usually eluted as an orange band. Its absorption spectrum shows both a bathochromic shift and hyperchromic increase on acidification, and this pigment was selected for further purification since it was thought that a single passage through the Sephadex LH-20 gel would be inadequate for complete purification. Pigment 2 was

designated "Schiff base pigment" because of its reversible change of absorption spectra in acidic and basic solutions.

Pigment 3.

This pigment component is usually present as a very minor component of the pigment mixture, and is eluted as a narrow yellow band immediately following the orange band containing mainly pigment 2. The absorption spectrum of pigment 3 is pH independent, although in acid solution, additional shoulders at 366 nm and 404 nm appear, but with the main absorption maximum at 386 nm. It is interesting to note that the absorption maxima of amphotericin B, (in methanol) a known conjugated heptaene whose structure was established in 1957 by Walters, Dutcher and Wintersteiner (116) occurs at 383 nm, with shoulders at 364 and 408 nm. Only a preliminary investigation was carried out on pigment 3.

Pigment 4.

This was eluted as a yellow band and was the last to be eluted off the Sephadex column. Its absorption maximum, 412 nm in methanol, was also pH independent (cf. pigment 3), and was similar to the absorption maximum of pigment 2 in acid solution, perhaps indicating a structural relationship.

Table 20. Properties of pigments eluted from Sephadex LH-20 by aqueous methanol (80%)

P. flavicomum

Pigment (in order of elution)	Colour	Fluores.	Ninhydrin	Absorption max. (nm) (methanol) Neutral	Acidic	Basic
				(340)		(340)
1.	yellow	yellow	+ve	356-57	no change	356-57
		white		(377-78)		(377-78)
				277-78		292
2. "Schiff's base pigment"	orange	orange	-ve	374	412	372
3	orange	orange	-ve	-	(366)	-
	yellow			388	386	384
				-	(404)	-
4	yellow		-ve	410-414	no change	no change

( ) denotes shoulder

The yield of pigment 2 obtained usually represented 1-3% of the total plasmodial extract applied on the Sephadex LH-20 column.

Figs. 3, 4 and 5 represent the absorption spectra of pigments 1, 2 and 3 P. flavicomum respectively.

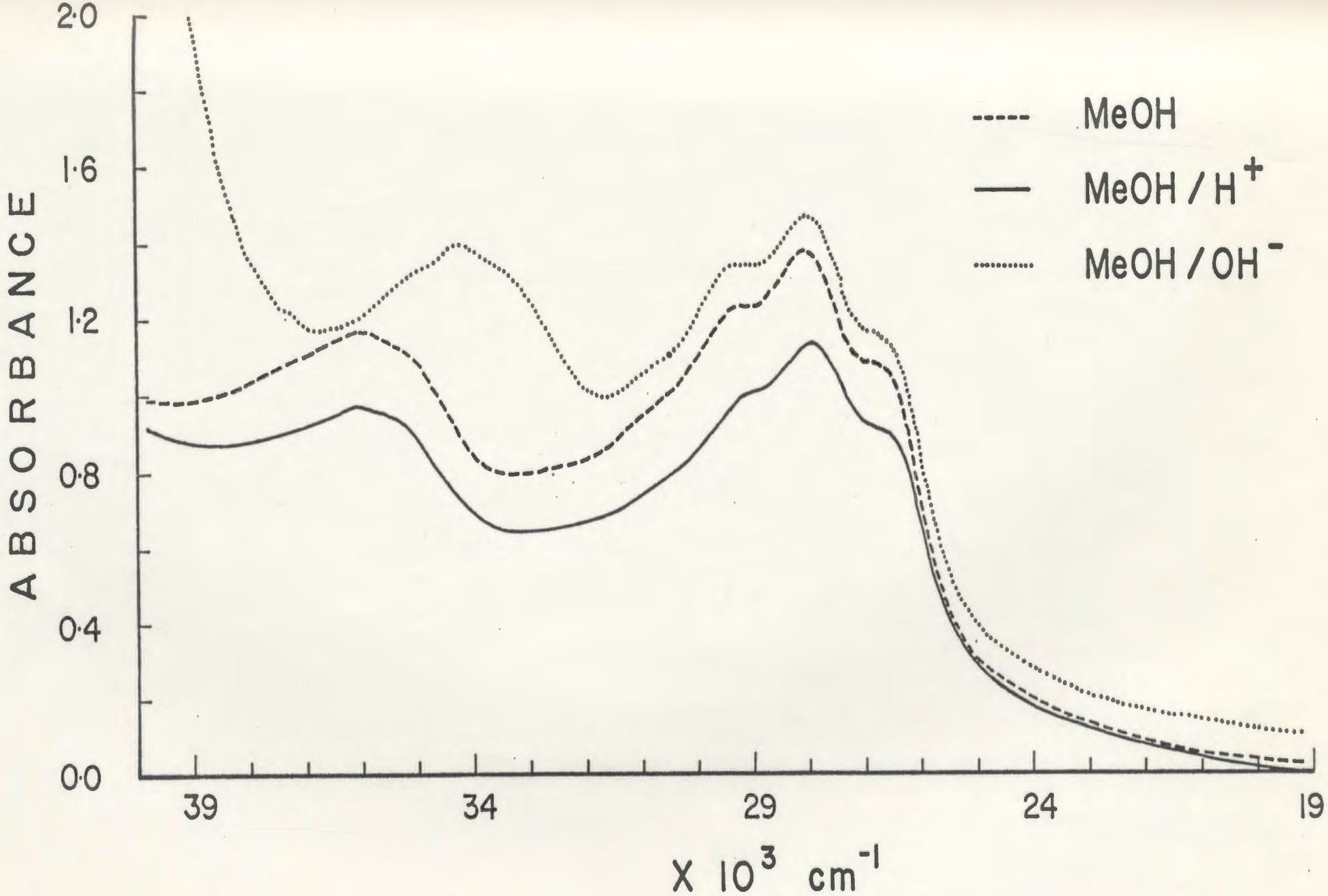


Figure 3. Absorption spectrum of pigment I (P. flavicomum) (cm^{-1})(90% MeOH)

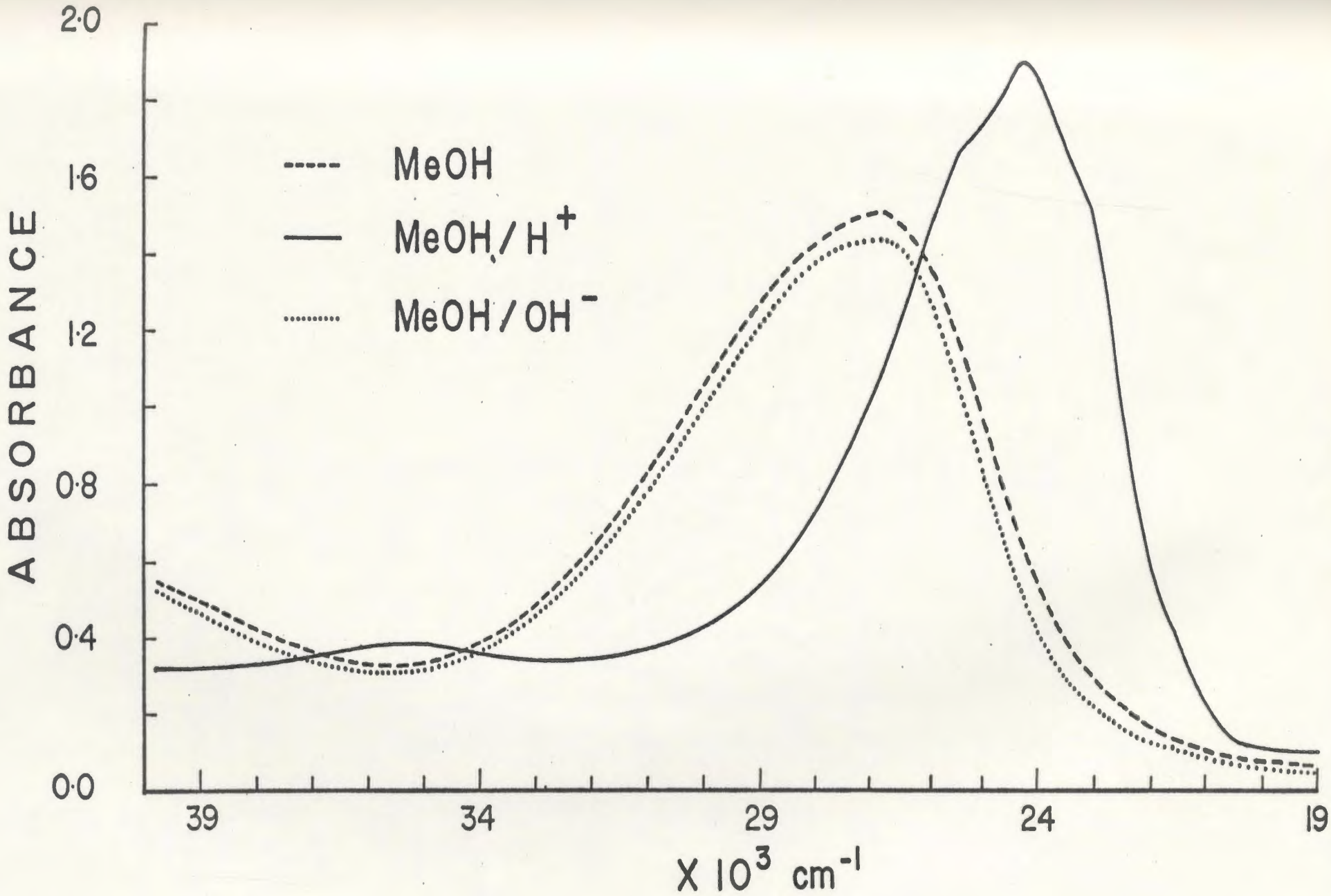


Figure 4. Absorption spectrum of pigment 2 (P. flavicomum) (cm^{-1}) (MeOH)

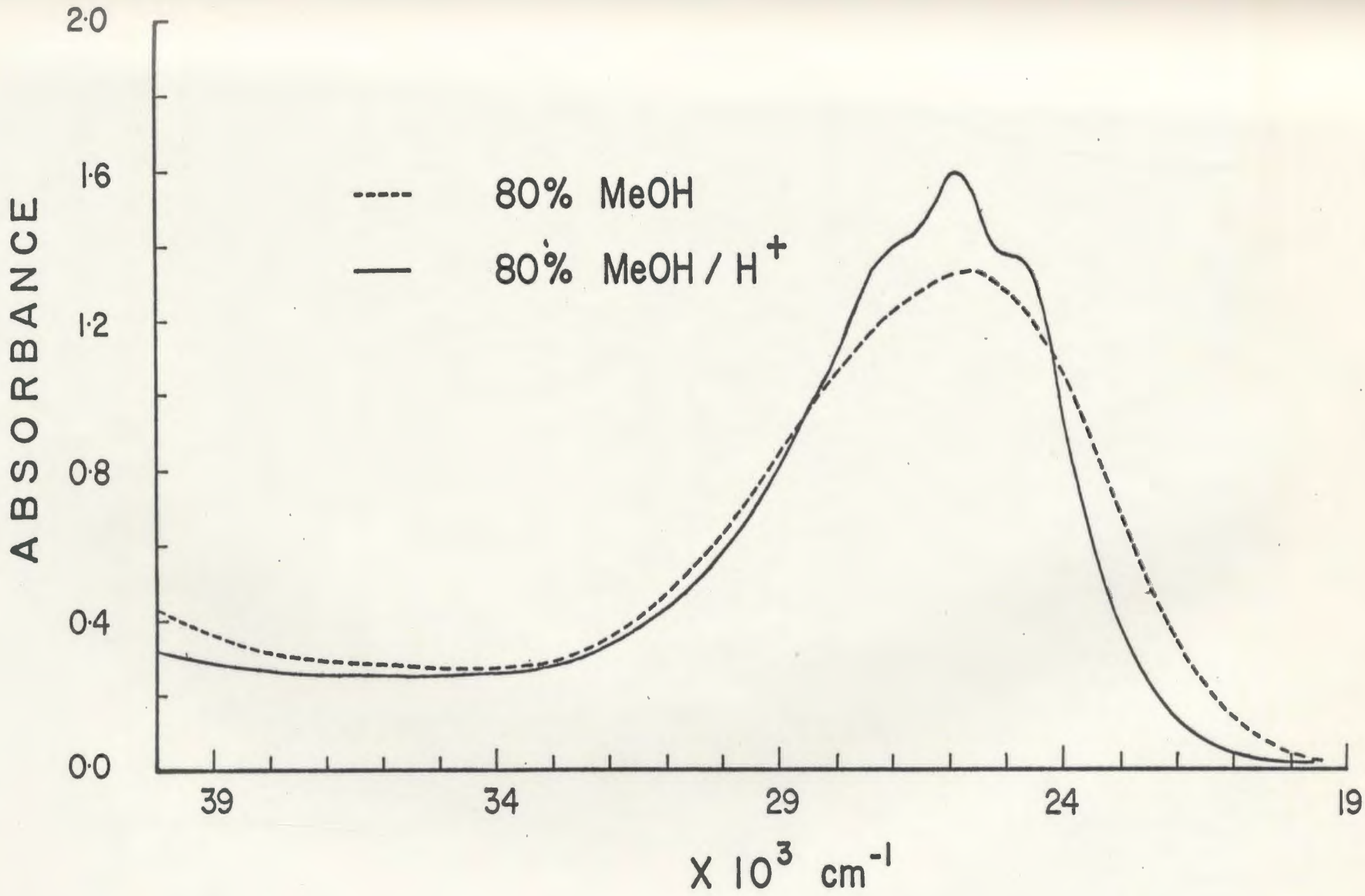


Figure 5. Absorption spectrum of pigment 3 (P. flavicomum)(cm^{-1}) (80% MeOH)

Further purification of pigment 2 on Sephadex G-10 eluted by ethanol/water (50%, v/v).

Sephadex G-10 (40 g) was suspended in ethanol/water (1:1, v/v) and allowed to stand for 24 h at room temperature to allow maximum swelling of the gel. The suspension was made up into a chromatographic column (30 x 2.5 cm) and prepared as described for the Sephadex LH-20 column.

Fractions of pigment 2 which had been obtained from the Sephadex LH-20 column and which gave a negative ninhydrin and the typical acid/base shift (increase in intensity and shift to longer wavelength on protonation) were combined and evaporated to dryness under reduced pressure at 37°. The methanol/water azeotrope was removed initially, and the remaining water was distilled with 95% ethanol. During this time the flask was covered with aluminium foil to exclude light. The dried orange pigmented extract was dissolved in the minimum amount of solvent and applied to the column. It was sometimes necessary to warm the mixture in a water bath at 37° in order to obtain a completely homogeneous solution before column application. The elution rate was maintained at 60 cm^3/h, and two distinct pigment bands were eluted and separated;

(i) A faster moving yellowish brown band designated pigment A-1,

(ii) A slower moving bright orange band designated pigment A-2.

The total volume of solvent required for complete elution of the pigments from this column was approximately 250 cm^3. If the pigments had not decomposed, there was little or no adsorption on the Sephadex column, and therefore the column could be re-used after it had been washed with 500 cm^3 of solvent. It was the usual practice in our laboratory to wash the column until there was no residue left after evaporation of the washings. By this time the gel was completely white, and this was taken as a good indication of its cleanliness.

Other separations using Sephadex G-15 and various mixtures of ethanol/water and methanol/water were tried, but the G-10/50% ethanol method was chosen as the one giving reproducible and consistent results.

Table 21. A typical separation of pigment 2 on the Sephadex G-10 column eluted with 50% aqueous ethanol.

Fr.No.	Volume (cm^3)	Colour	Weight (mg)	Absorption spectrum (nm) (50% ethanol)		
				Neutral	Acidic	Nomenclature
1	50	colourless				
2	12	light brown		284-287	no change	
3	11	light brown	20	363-365		A-1
4	7	brown				
5	30	brown				
6	14	orange-yellow				
7	20	orange				
8	18	orange				
9	13	orange	20	380	414	A-2
10	33	orange				
11	12	orange				
12	20	yellow	2			

The change in absorption maxima of pigment A-2 on protonation was 36-38 nm in methanol and this was accompanied by an intensity increase (hyperchromic shift) of 35-42% in the purest preparation. This intensity increase on protonation was used as a guide as to the purity of pigment A-2, i.e. the larger the increase, the purer the sample. The absorption spectrum of pigment A-2 is shown in Fig. 7.

On some occasions during the purification of pigment 2 on Sephadex G-10, fractions corresponding to pigment A-2 were obtained with absorption maxima at 255-260 and 380 nm, the ratio of these maxima being 1:1.6. The decrease in the extinction of the lower wavelength absorption peak was used as a further criterion of purity of pigment A-2, although the chromophore due to this maximum may be an integral part of the pigment itself.

Pigment A-1.

Fractions corresponding to pigment A-1 were collected but were not investigated further. The absorption spectra of pigment A-1 is shown in Fig.6.

Pigment A-2.

Ultraviolet-visible spectra

Absorption maxima (nm)	($E^{1\%}_{1\ cm}$)	Solvent
253-254	(440)	50% ethanol/water
380-381	(2080)	" " "
286	(320)	50% ethanol/water/H^+
412	(2560)	" " " "
250	(520)	50% ethanol/water/$OH^\ominus$
372	(2120)	" " " "

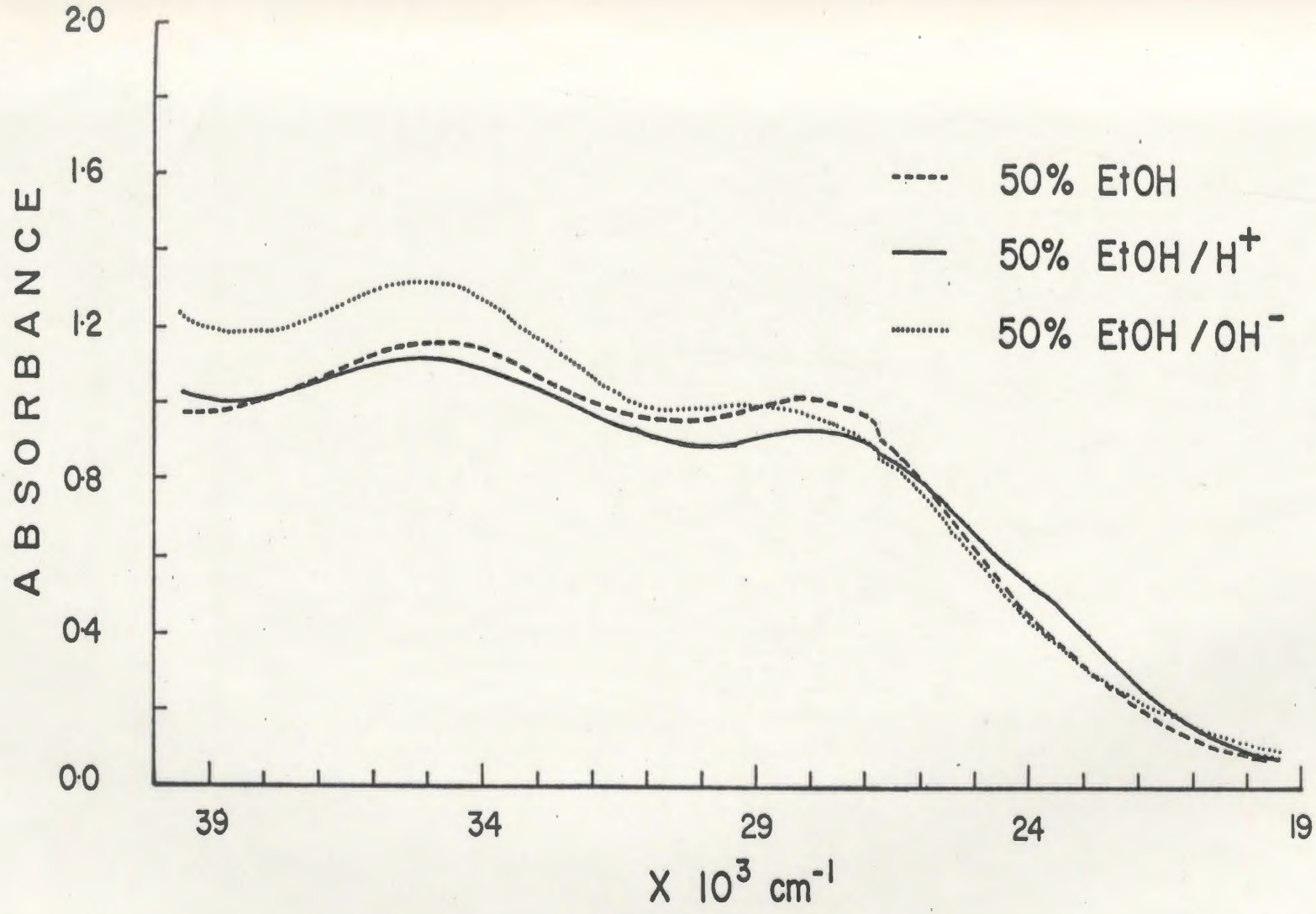


Figure 6. Absorption spectrum of pigment A-1 (P. flavicomum) (cm^{-1}) (50% EtOH)

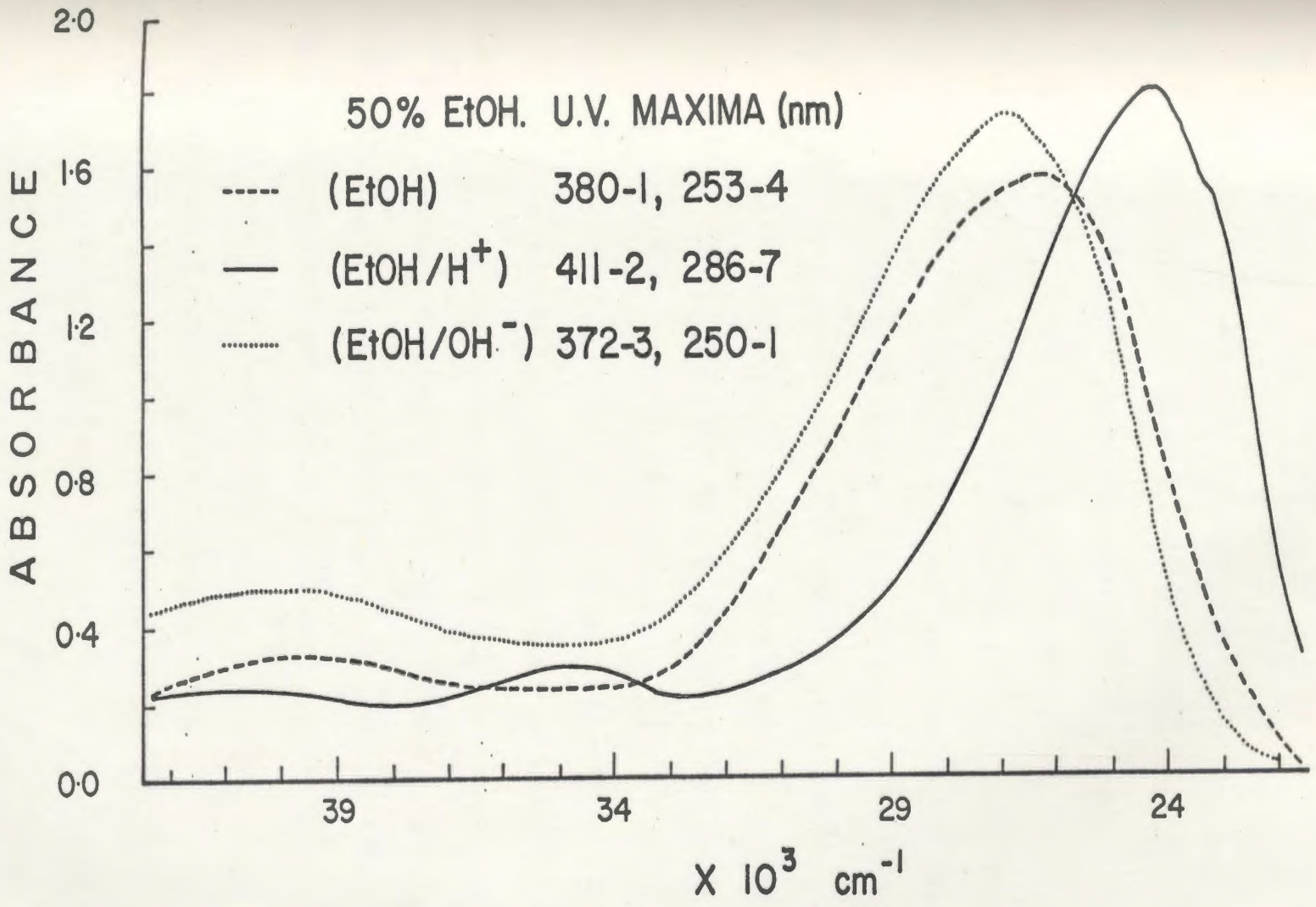


Figure 7. Absorption spectrum for pigment A-2 (P. flavicomum) (cm^{-1}) (50% EtOH)

Chromatography of pigment A-2 P. flavicomum on Dowex 50W-X8 cation exchange resin.

Dowex 50W-X8 cation exchange resin (25 g) (20 - 50 mesh, Baker) was washed several times with distilled water and then prepared into a chromatographic column (20 x 1 cm). The resin was washed successively with distilled water, sulphuric acid (2N), distilled water and finally with 80% methanol/water until the washings were neutral to universal indicator paper (pH 1-12). An aliquot of pigment A-2 dissolved in a minimum amount of 80% methanol was applied, and the column slowly eluted with the same solvent. The single absorption maximum of the pigment after chromatography was 414 nm (80% methanol), indicating that pigment A-2 had been protonated. This absorption spectrum was similar to that of the protonated form of the pigment obtained when a drop of HCl (conc.) is added to a methanolic solution of A-2. The protonated pigment resulting after passage through the Dowex column is designated pigment A-3. The methanolic solution was evaporated to dryness and an orange brown residue was obtained. Pigment A-3 was soluble in methanol, ethanol, and aqueous alcohols, partially soluble in chloroform and dichloromethane, and insoluble in ether.

U.v. λ_{max} (95% EtOH) 414, shoulders at 390 nm and 430 nm; u.v. λ_{max} (50% EtOH) 414 nm; u.v. λ_{max} (50% EtOH/NaOH) $\sim$ 385 nm.

I.r. ($CHCl_3$) 3495, 3400-3100 (H-bonded OH) 3080 (weak), 2915, 2850, 2700-2400 (H-bonded OH), 1735 (shoulder) 1712 (C=O) 1620, 1580, 1545 (weak), 1455, 1420, 1290-1200 (broad), 1125, and 1075 cm^{-1}.

I.r. (CH_2Cl_2) a similar spectrum was obtained in this solvent.

Mass spectrum (170°): a very weak spectrum was obtained, with no significant fragmentation at this temperature, the highest recorded a.m.u. being 226.

Mass spectrum (265°): again a weak spectrum was obtained, the most intense peak recorded at m/e 64.

Mass spectrum (320°): peaks at m/e 139 (28%); 121 (8%); 113 (18%); 111 (14%); 98 (26%); 91(22%); 79 (12%); 78 (32%); 77 (18%); 76 (18%); 73 (14%); 71 (12%); 70 (14%); 69 (28%); 66 (24%); and 64 (100%).

Dr. Gregory recorded that most of the sample still seemed to be present on withdrawal, but somewhat browner.

A sample of pigment A-3 was treated with diazomethane, (detailed on p.205) and a mass spectrum attempted.

Mass spectrum (160-240°, scan 235°): m/e 223 (6.9%); 185 (10.3%); 175 (20.7%); 171 (13.8%); 167 (17.2%); 166 (17.2%); 153 (13.8%); 143 (17.2%); 139 (41.4%); 135 (22.4%); 133 (27.6%); 131 (17.2%); 121 (38%); 111 (38%); 103 (20.7%); 91 (24.1%); 87 (17.2%); 83 (34.5%); 81 (24.1%); 79 (20.7%); 78 (13.5%); 77 (34.5%); 75 (20.7%); 74 (31.0%); 71 (27.6%);

69 (72.4%); 68 (24.1%); 67 (24.1%); 59 (6.2%); 55 (51.7%); 53 (34.5%); 45 (2.9%); 44 (51.7%); 43 (65.5%); 42 (58.6%) and 41 (48.3%).

Another scan of the sample at 290° showed little difference over the scan at 235°.

CHART 3. ISOLATION AND PURIFICATION OF PIGMENTS ON SEPHADEX GELS P. FLAVICOMUM.

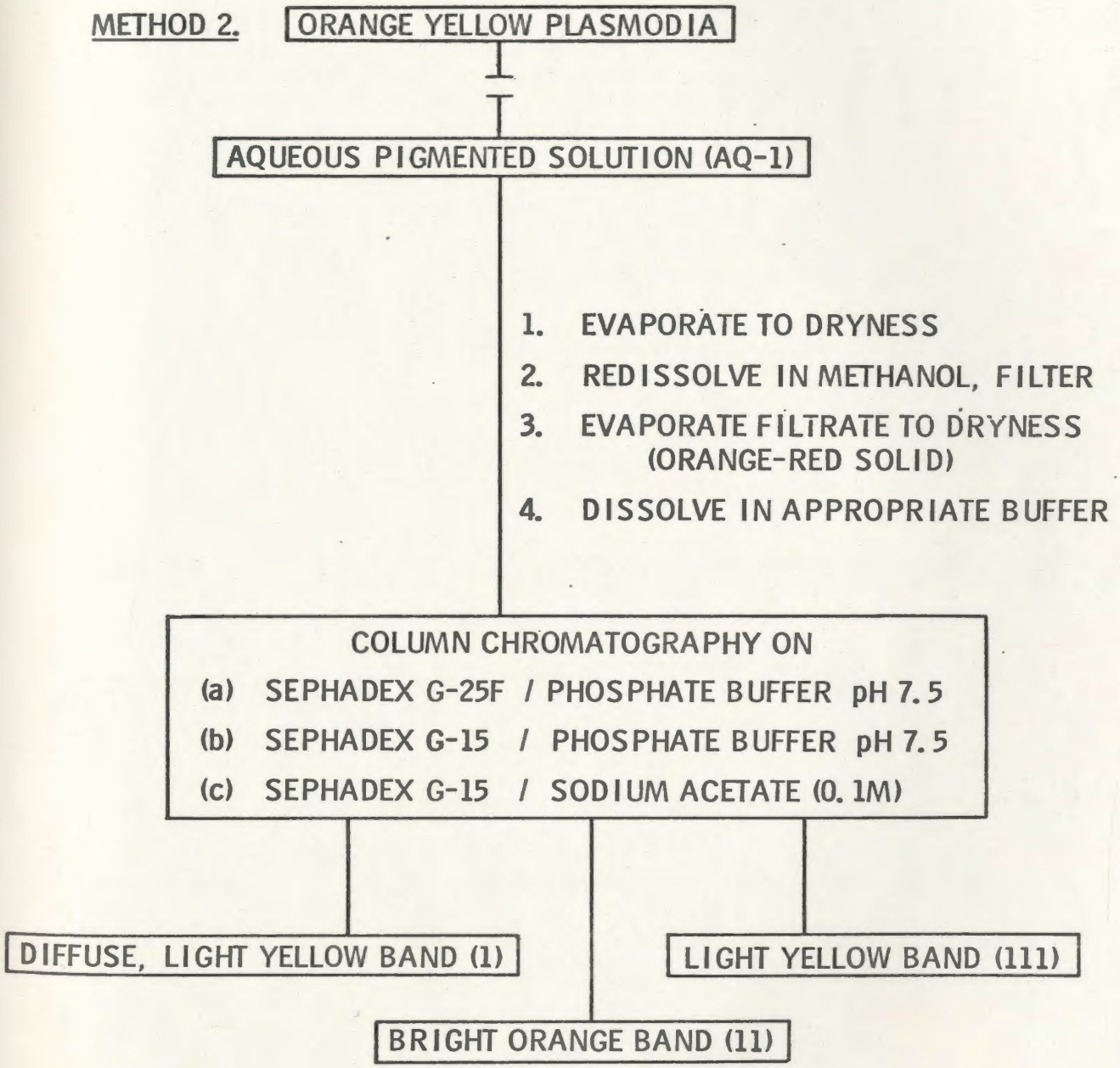
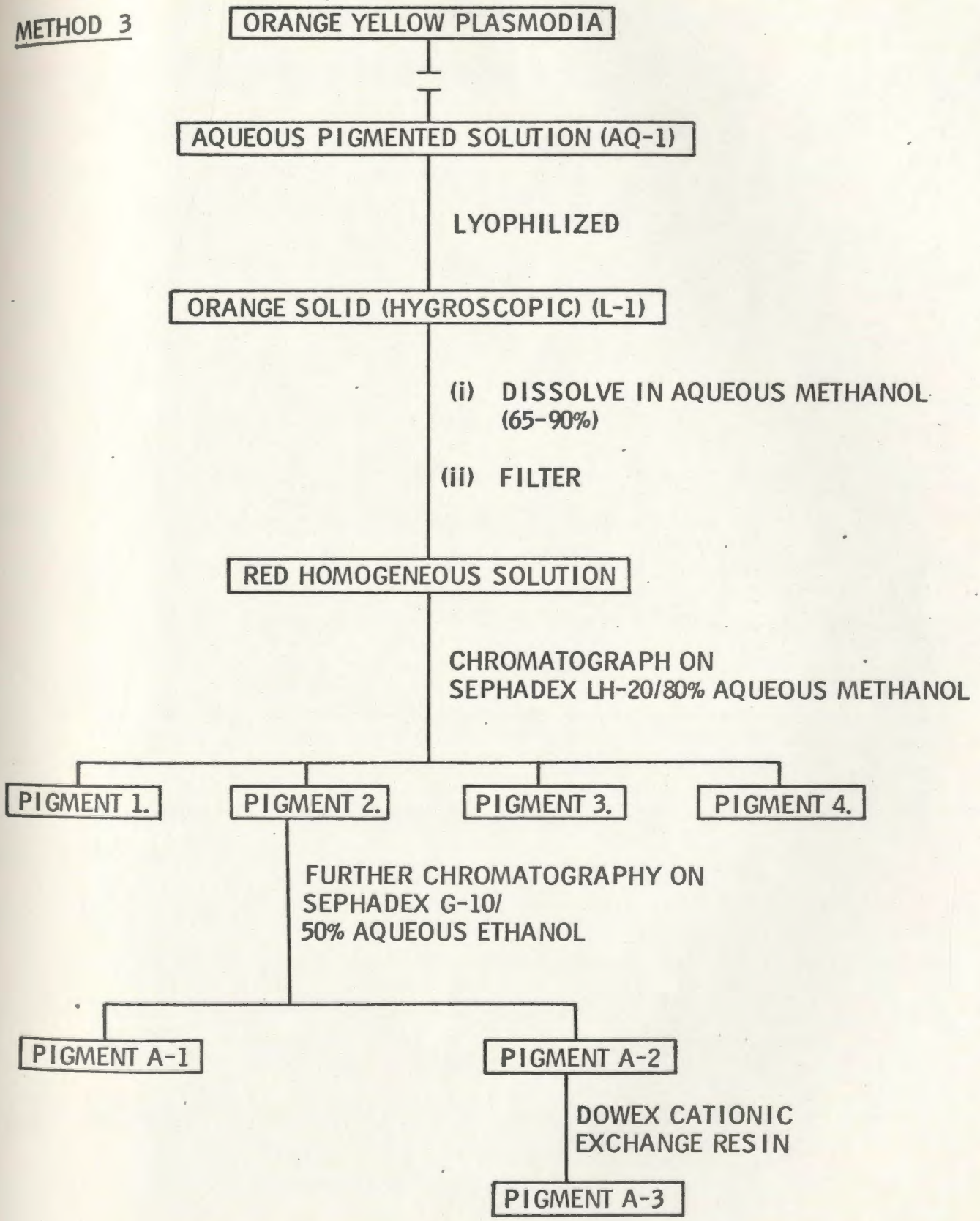


CHART 4. ISOLATION AND PURIFICATION OF PIGMENTS ON SEPHADEX G-10 AND SEPHADEX LH-20 P. FLAVICOMUM.

METHOD 3



Column chromatography of ethyl acetate extract of crude pigments on Sephadex LH-20.

Freeze dried plasmodial extract (L-1, 5 g) was exhaustively extracted with ethyl acetate (300 cm^3), and this was evaporated to dryness at 37° to give an orange red solid which was then redissolved in 80% aqueous methanol (30 cm^3) and filtered. The orange red filtrate was then chromatographed on Sephadex LH-20, and eight fractions were collected and examined by spectroscopy.

Fractions 1, 2.

The absorption spectra of these fractions corresponded to pigment 1 (p.154), and gave a positive ninhydrin test.

Fraction 3.

Its absorption spectra $\lambda_{max}^{methanol}$ 370 nm, with shoulders at 392 and 351 nm, seem to indicate the presence of a conjugated heptaene (32). The absorption maxima of this pigment are shifted by 14 nm to the red compared to the absorption maxima of the routinely obtained pigment 1. This is in effect a new conjugated polyene pigment, and the extra double bond probably arises by oxidation. This pigment was encountered on only one occasion.

Fractions 4 and 5.

Both fractions showed absorption maxima at 410 nm, with a shoulder at 392 nm in 80% methanol. On acidification the absorption maxima at 410 nm was unchanged, (shoulders at 392, 432 nm), although there was a small hyperchromic shift, suggest-

ing a structural similarity to pigments 2 and A-2.

Fraction 6.

This fraction showed $\lambda_{max}^{methanol}$ 388 nm changing to λ_{max} 386 nm, but with new shoulders appearing in the spectrum at 366 and 403 nm in acidified methanol. This spectrum is similar to that shown by some conjugated heptaenes (116).

Separation of pigments from plasmodia grown by the millipore filter surface culture method. P. flavicomum.

Freeze dried plasmodia (2 g) obtained from ca. 10 g of wet plasmodia that were grown for 24 h were finely ground with clean sand (5 g) in a mortar and pestle. The plasmodial powder was then stirred magnetically with acetone (25 cm^3) and water (15 cm^3) for 30 min. The aqueous acetone suspension was filtered, and evaporated to dryness (467 mg) and this dried pigment extract was then redissolved in 80% methanol/water and filtered. The filtrate was extracted three times with petroleum ether (30-60°) and removal of the residual petroleum ether from the aqueous methanolic solution was achieved by bubbling N_2 through. The pigment solution was applied on a chromatographic column (28 x 5 cm) packed with Sephadex LH-20 and eluted with 80% aqueous methanol. Initial separation revealed the presence of two main bands, a light yellow diffuse band, and a slower-moving orange yellow band, but as separation proceeded, five bands were clearly discernible. Eleven fractions were collected.

Fractions 1 and 2.

These were colourless, corresponding to an elution volume of 100, and 175 cm^3 of solvent respectively.

Fractions 3 and 4. (80 mg)

These were eluted as a light yellow band (band I) whose absorption spectrum corresponding to that of pigment 1.

Fraction 5. (117 mg) (band II)

The solution was light yellow and possessed a broad absorption maximum at 264-278 nm. It was intensely fluorescent

(yellowish green) and had a pungent 'amine' smell characteristic of plasmodia of P. flavicomum.

Fraction 6. (18 mg)

This solution was also intensely fluorescent, and possessed a broad absorption maximum at 266-72 nm (band III).

Fraction 7. (5 mg) (band III).

Exhibited yellowish green fluorescence and possessed a single absorption maximum at 262 nm.

Fractions 8-10. (5.0 mg)

These fractions were collected from an orange band (band IV) over a total elution volume of 80 cm^3. The absorption spectrum of band IV was similar to that of pigment 2, the isolation of which is described on p168. The absorption maximum changes from 376 nm in neutral methanol to 414 nm on acidification, with shoulders at 398 and 430 nm, and with a 34% increase in intensity.

Fraction 11. (3.5 mg) (band V).

This was eluted last from the column as a yellow band, and exhibited maxima at 384 and 404 nm, and these were unchanged on adding acid or alkali. Fractions 3-7 gave a positive ninhydrin test, while fractions 8-11 were negative.

This preliminary study on the 24 h plasmodia was initiated to determine at what stage some of these pigments were produced. The results seem to suggest that the same pigments produced by plasmodia grown in liquid culture for 4-5 days are also produced even after 24 h, but more important-

ly the 'Schiff base pigment' (pigment 2) is also produced after this short growth period. The physiological significance of this observation is not known at this time.
The yield of pigment from fractions 8-10 represented 0.25% of the lyophilised plasmodia.

Cellulose Acetate Electrophoresis of Pigment A-2 at different pH.

The instrument used was an electrophoresis apparatus (Model No.51170-1, Gelman Instrument Company, Michigan). Cellulose acetate strips (12x1 cm, Sepraphore III, Gelman) were presoaked in buffer at the pH of run, and then blotted to remove excess buffer before the sample of pigment was applied. The operating voltage was maintained at 300V for all runs except at pH 1.1, when it was reduced to 250V, with a corresponding increase in the current.

The total current passed through the solution increased from 4 mA to 30 mA from pH 8.9 to pH 1.1, and the approximate running time for each sample was 20 min. Buffers used were standard buffer solutions (117). Fig. 8 illustrates the separation of pigment A-2 in buffers pH 8.9 to 1.1, and Table 22 gives the distance travelled from the origin. Pigment A-2 migrated as a single yellow band towards the anode between pH 8.9 to 5.1 indicating an overall negative charge on the pigment. The anodic mobility of the pigment decreased progressively as the more acidic buffers were used, and at the end of the run at pH 4.0 there was some pigment residue at the origin. At pH 1.1 there was little movement of the pigment to either the anode or the cathode, most of the pigment remaining at the origin.

Table 22. Electrophoretic separation of pigment A-2 on cellulose acetate in buffers of different pH.

Buffer pH	Pigment component	Distance travelled to anode from origin (cm)
8.9	(a) yellow	6.2
8.0	(a) yellow	4.7
7.0	(a) yellow	4.7
6.0	(a) yellow	4.7
5.1	(a) yellow	3.8
4.0	(a) orange yellow	3.2
	(b) orange pigment residue	0.2 (diffuse)
3.0	(a) orange band	2.0
	(b) orange residue	0.1
2.0	(a) orange band	1.4
	(b) orange residue major	0.0
1.1	(a) orange residue	0.0

Fig. 8

Cellulose Acetate Electrophoresis
Pigment A-2 P. FLAVICOMUM at diff. pH's

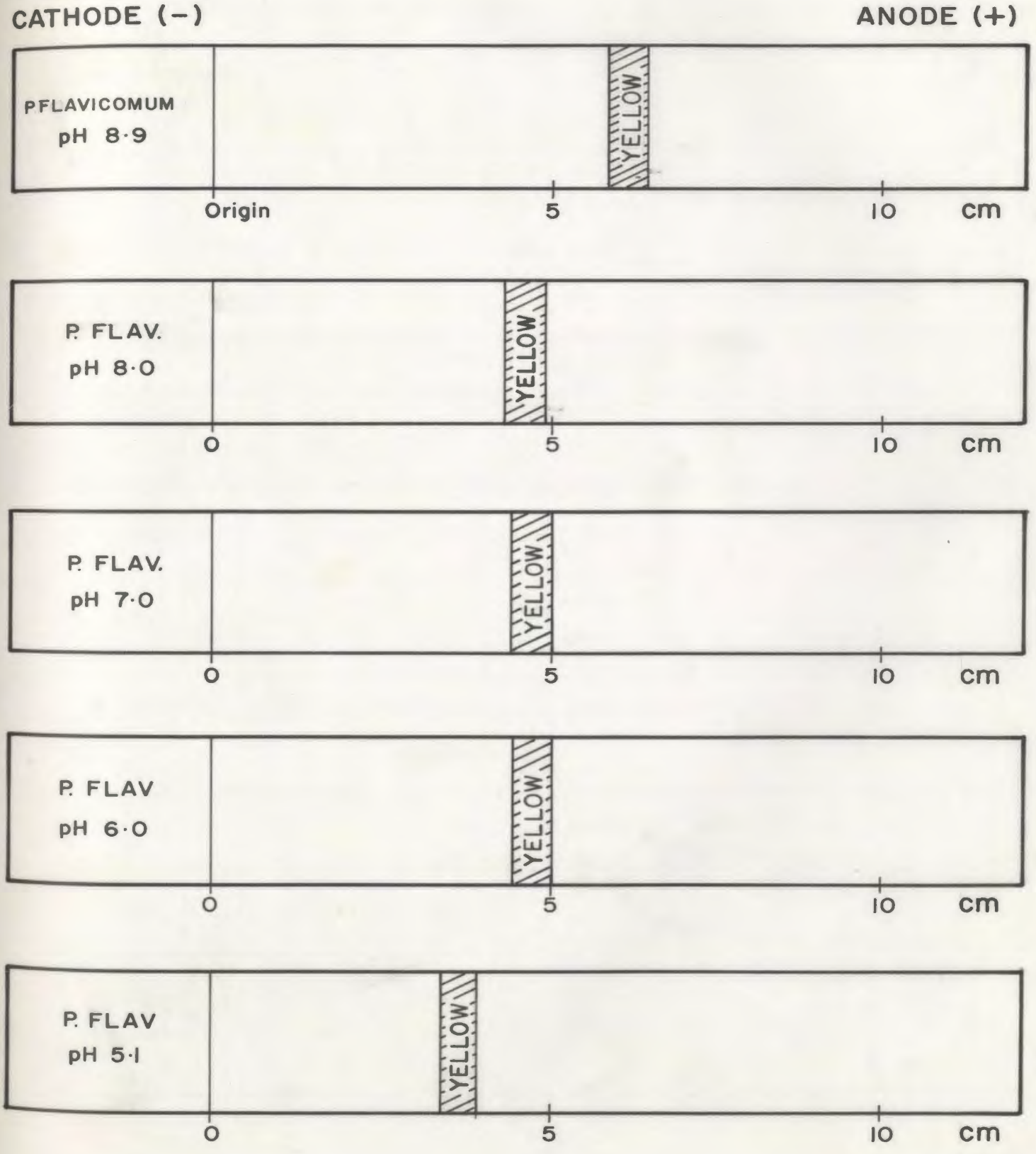
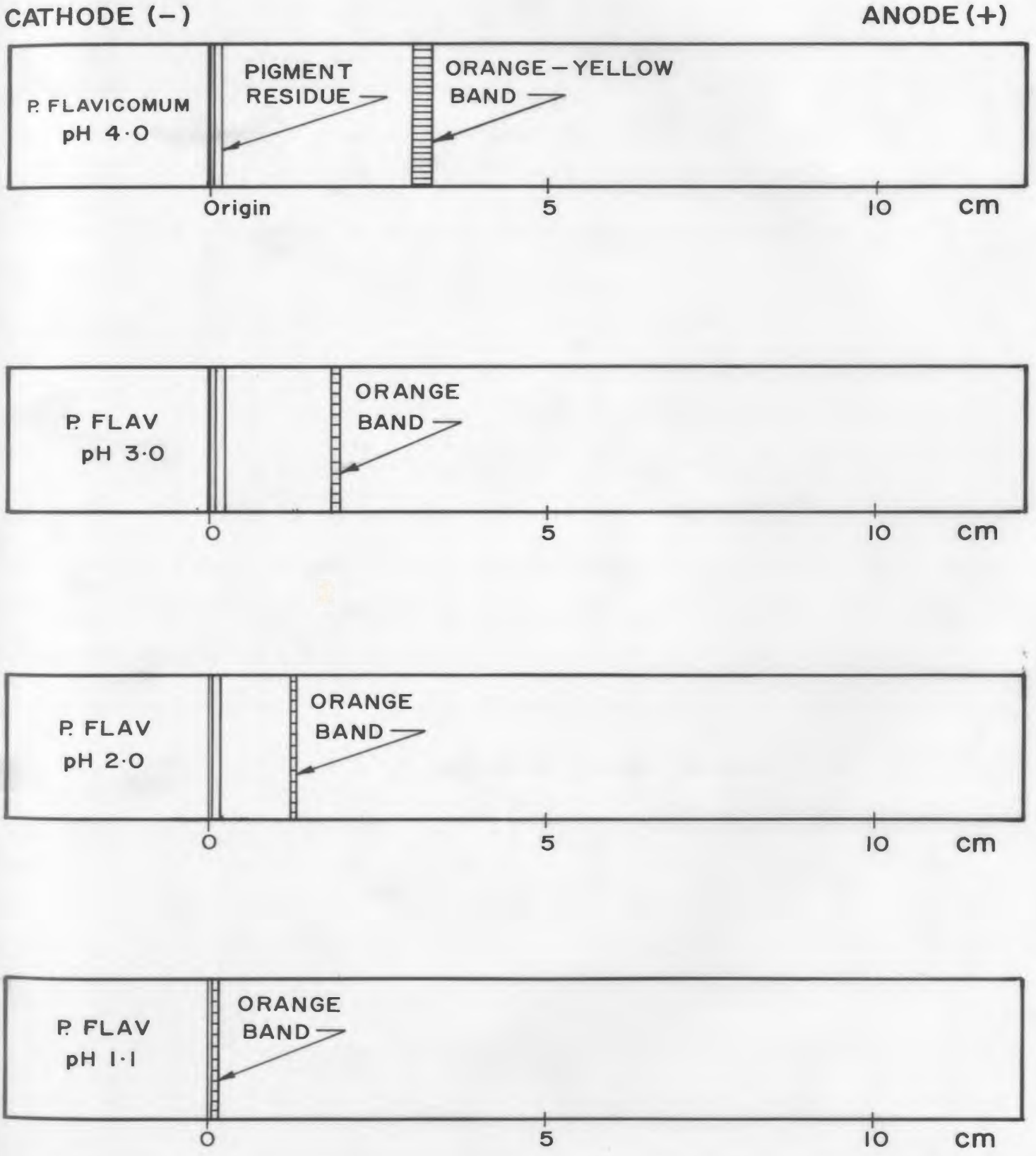


Figure 8 (continued)

Cellulose Acetate Electrophoresis

Pigment A-2 P. FLAVICOMUM at diff. pH's



Absorption spectroscopy after Cellulose acetate electrophoresis at pH 8.9 and pH 2.5.

(i) Buffer (pH 8.9)

The single pigment band which had migrated to the anode was eluted from cellulose acetate strips with 80% methanol-water after the strips were blotted dry between sheets of filter paper. This pigment possessed an absorption maximum at 370 nm (80% aqueous methanol) which was shifted to 414 nm on acidification, a change characteristic of the native pigment.

(ii) Buffer(pH 2.5)

At this pH there was a slight anodic migration of the pigment preparation applied resulting in a diffuse band at the origin. This pigmented band was eluted with 80% methanol as before. It possessed a single absorption maximum at 414 nm, which remained unchanged after acidification with hydrochloric acid (2N); there was, however, a small hyperchromic shift (14%) on acidification indicating that the pigment isolated after cellulose acetate electrophoresis was not completely protonated at pH 2.5.

Physical and chemical properties of pigment A-2.

Melting point.

This orange amorphous solid begins to darken at 125-130°, becoming darker to 143°, and finally black and oily at 147-148°.

Solubility.

This pigment was soluble in water, aqueous alcohols, lower alcohols, acetone, pyridine, dimethyl formamide, tetrahydrofuran and insoluble in hexane, petroleum ether, ether, chloroform, carbon tetrachloride and benzene.

Elemental analysis of pigment A-2.

(i) Samples of pigment A-2 were submitted for elemental analysis to Alfred Bernhardt Microanalytisches Laboratorium, West Germany.

Analysis No.	%C	%H	%O	% Residue	Total %
1	49.38	2.04	-	21.98	73.40
2	40.19	6.33	-	36.01	82.53
3	50.22	4.54	23.80	18.20	96.76

There was no substance left for nitrogen determinations, as the amount of sample submitted was very small. A significant ash content was recorded in all three samples. In analysis no.3, the elemental composition adds up to only 96.76%, and it may be possible that nitrogen is present in this pigment preparation. (Nitrogen was later found to be present in an

analysis of the protonated pigment (see below).

(ii) Elemental analysis of pigment A-3.

The pigment preparation sent for analysis was prepared by Mrs. Judith Hillier (48). Pigment A-2 obtained as described previously (p.168) was protonated to give pigment A-3 (λ_{max} 412 nm in methanol) by chromatography on a Sephadex G-10 column which had been prewashed with 50% ethanol/water acidified to approximately 0.05M in HCl. Pigment A-2 (λ_{max} 380 nm in ethanol/water) was then applied and eluted with 50% ethanol/water, and pigment A-3 was obtained as an orange band. Fractions corresponding to this protonated pigment were combined and reduced in volume under reduced pressure, and this usually resulted in the precipitation of a bright orange solid which was centrifuged off. This bright orange solid is designated pigment A-3. Obviously an assumption is made here that the pigment preparation obtained by protonation of pigment A-2 on a Dowex cationic exchange resin (p.164) is the same as the pigment obtained by chromatography of A-2 on an acidified Sephadex G-10 column.

Elemental analyses of protonated pigment

	%	
Carbon	54.88	
Hydrogen	4.96	
Oxygen	29.04	
Nitrogen	5.37	(The weighing of the nitrogen analyses was not dried, and this was accounted for in calculation C and D)
Residue	4.16	(grey)
Total	98.41	
% drying loss	0.92	

A. Uncorrected analyses of protonated pigment

C 54.88 H 4.96 O 29.04 N 5.37

Empirical formula = $C_{12}H_{13}O_{4.8}N$, Empirical molec. weight = 247.8

If N = 3, formula is $C_{36}H_{39}O_{14.4}N_3$, then M.W. = 743.4

If N = 4, formula is $C_{48}H_{52}O_{19.2}N_4$, then M.W. = 991.2

Calculated for $C_{12}H_{13}O_{4.8}N$	C	H	O	N
	54.88	4.96	29.04	5.37
Found:	58.16	5.25	30.99	5.65

B. Corrected for C, H, O, N analyses only, by assuming no residue was present.

Corrected analyses C 58.23 H 5.26 O 31.80 N 5.70

Empirical formula $C_{12}H_{13}O_{4.9}N$, molec. weight = 249.4

If N = 3, formula is $C_{36}H_{39}O_{14.7}N_3$ M.W. = 748.2

N = 4, formula is $C_{48}H_{52}O_{19.6}N_4$ M.W. = 997.6

	C	H	O	N
Calcd. for $C_{12}H_{13}O_{4.9}N$	50	5.22	31.44	5.61
Found (after correction)	58.23	5.26	31.80	5.70

C. Calculation after correcting for nitrogen analyses on undried sample.

Since % loss on drying = 0.92, then corrected nitrogen composition = $\frac{5.37 \times 100}{99.08} = 5.42$

Analyses is now C 54.88 H 4.96 O 29.04 N 5.42

Empirical formula $C_{12}H_{13}O_{4.7}N$, atomic weight = 246.2

If N = 3, formula is $C_{36}H_{39}O_{14.1}N_3$ M.W. = 738.6

N = 4, formula is $C_{48}H_{52}O_{18.8}N_4$ M.W. = 984.8

	C	H	O	N
Calcd. for $C_{12}H_{13}O_{4.7}N$	58.54	5.28	30.54	5.69
Found	54.88	4.96	29.04	5.42

D. Corrected analyses for nitrogen, and assuming no residue was present.

Corrected analyses C 58.20 H 5.26 O 30.80 N 5.75

Empirical formula is $C_{12}H_{13}O_{4.7}N$, atomic weight = 246.2

If N = 3, formula is $C_{36}H_{39}O_{14.1}N_3$ M.W. = 738.6

If N = 4, formula is $C_{48}H_{52}O_{18.8}N_4$ M.W. = 984.8

	C	H	O	N
Calcd. for $C_{12}H_{13}O_{4.7}N$	58.54	5.28	30.54	5.69
Found:	58.20	5.26	30.80	5.75

E. Calculation of double bond equivalents (D.B.E.) for formula $C_{36}H_{39}O_{14.1}N_3$

For $C_aH_b(O_c)N_d$: D.B.E. = $\frac{(2a+2) - (b-d)}{2}$

For $C_{36}H_{39}O_{14}N_3$ = 19

Reactions of pigment A-2 and pigment A-3(Physarum flavicomum).

Functional group analysis.

The following chemical tests were performed on small aliquots of pigment A-2 dissolved in either aqueous methanol or ethanol.

(i) Test for unsaturation

(a) Bromine water.

The yellow colour of the bromine water (0.1 cm^3) was completely discharged on adding 2 drops of a solution of the pigment.

(b) Baeyer's solution (0.5% $KMnO_4$)

The deep purple colour of the reagent (1 drop) changed immediately to dark brown on adding one drop of the pigment solution.

(c) Concentrated sulphuric acid.

The pigment solution first changed to orange, then red, and progressively through brown until finally a black solution was obtained.

(d) Concentrated hydrochloric acid.

A bright orange pigment was obtained which retained its colour after 5 min. When this test was performed on filter paper, the orange protonated pigment could not be eluted with cold water, indicating that the protonated pigment was water insoluble.

(ii) Test for carbohydrates

(a) Molisch's test.

The reagent was prepared as described in (118), but the test was scaled down appropriately. No violet ring was detected at the interphase of the two liquid layers, but the top layer became orange after two min. When the mixture was poured into water an orange colour was produced, suggesting that no carbohydrate was present.

(b) Test with 2, 3, 5-triphenyl tetrazolium chloride.

Test for reducing sugars (107a).

Pigment solution (2 drops) was mixed in a micro test tube with 0.5% aqueous 2, 3, 5-triphenyl tetrazolium chloride (2 drops) and sodium hydroxide (0.5 N, 1 drop), and the mixture was heated on a water bath for 2 min. The yellow colour of the mixture slowly turned to brown, with finely divided brown particles in suspension. A similar test using fructose solution (2 drops) gave a deep pink colour in the cold, becoming darker red after heating, and eventually forming a red precipitate.

(c) Anthrone test for ketoses.

The reagent was prepared from anthrone, glacial acetic acid, ethanol phosphoric acid and water as described by Stahl (119).

Pigment solution (1 drop) was applied to a silica gel plate (GF254), anthrone reagent (2 drops) was added producing a bright orange pigmented spot with a yellow diffuse

ring around the spot. The plate was heated at 110° for 5 min. and a yellow-orange spot was produced. It was, however, difficult to draw any conclusions because of the interaction of the polyene chromophore with the acid in the reagent. Reaction with fructose, an α-ketose produced a yellow spot.

(d) Test for ketohexoses with stannous chloride sulphuric acid and urea.

The reagent was prepared by dissolving urea (4 g) and stannous chloride (0.2 g) in 40% sulphuric acid (10 cm^3). Pigment solution (2 drops) was treated in a micro crucible with the reagent (10 drops) and a bright orange colour was immediately produced. The mixture was then heated for 2 min, and on cooling the orange colour persisted. A similar test was performed using fructose, and a pale blue colour was obtained. According to the author (107b), the mechanism of this colour test is still unknown.

(iii) Tests for carbonyl group.

(a) 2,4-Dinitrophenylhydrazine solution.

An approximately 0.1M solution of the reagent in ethanol and 85% phosphoric acid was prepared according to the method described by Fieser and Fieser (81b). Two drops of the reagent was added to the pigment contained in a depression in a heated porcelain tile. An immediate red precipitate was produced.

(b) p-Nitrophenylhydrazine solution

The reagent was prepared as described (81b) except that p-nitrophenylhydrazine was used. An immediate red precipitate was obtained.

(c) Test for aliphatic 1,2-dioxo compounds (107c)

Pigment solution (2 drops) was treated in a micro test tube with the reagent (2 drops) prepared from hydroxylamine hydrochloride (1 g), sodium acetate (1 g) and water (2 cm^3). The mixture was warmed for 1 min. on a water bath and a drop of the still yellow solution was placed on filter paper, and nickel acetate solution (5%, 1 drop) added. The pigment remained yellow, although a very faint pink ring could be observed around the main pigment spot when the filter paper was placed over ammonia fumes. A similar test using 2,3-butanedione (diacetyl) produced a red colour immediately on adding the nickel acetate solution.

(d) Reaction with o-phenylene-diamine

Following the method of Morrison (120), an aqueous solution of pigment A-2 (6.0 mg) was added to a solution of o-phenylene diamine (50 mg) in 10% acetic acid (4 cm^3). A brown precipitate was immediately formed, and the solution was allowed to stand for 24 h at room temperature in the dark. The brown precipitate was filtered and its absorption spectrum in methanol determined, and this showed no change. It seemed that the unreacted pigment was precipitated out in acid

solution and no quinoxaline derivative was formed.

In another experiment the brown solid corresponding to the unreacted pigment was dissolved in ethanol (5 cm^3) and glacial acetic acid (0.1 cm^3) and o-phenylene diamine (50 mg) added, and the mixture heated for 30 min. and allowed to stand overnight. After filtering the absorption spectrum determined in methanol showed no change indicating that no reaction had taken place. This was taken as evidence for the absence of an α-diketone group in the pigment.

Reaction of o-phenylene diamine with benzil, an α-diketone, produced the expected quinoxaline (m.p. 125°).

(e) Reaction with blue tetrazolium. (81c)

Blue tetrazolium is a pale yellow water-soluble substance which in the presence of a trace of alkali, oxidises aldoses, ketoses and other α-ketols, and is thereby reduced to the water-insoluble, intense blue diformazan. A few drops of a 0.5% aqueous solution of the reagent and 1 drop of NaOH (2N) were added to a aqueous solution of pigment A-2. The mixture was warmed on a steam bath, but the colour of the solution remained yellow after 5 min.

Reaction of benzoin, (an α-hydroxy ketone) with blue tetrazolium under the same conditions produced a blue colour immediately, while reaction with fructose also produced a blue colour after 2 min. Since this test is specific for α-hydroxy ketones, a negative reaction is interpreted to mean that this group is not present in the pigment.

(iv) Miscellaneous

Test for enols

(a) Methanolic ferric chloride

Pigment solution (1 drop) was applied on filter paper, and neutral methanolic ferric chloride solution (1 drop) allowed to diffuse toward the pigment. A dark brown ring was formed at the junction of pigment and reagent and this was interpreted as a positive reaction for an acidic group, e.g. enol. Similar brown colourations were produced with acetyl acetone and cyclohexane 1,3-dione. When this test was repeated with the pigment in a culture tube, a reddish brown precipitate was obtained.

(b) Aqueous copper acetate

Pigment solution (5 drops) was vigorously shaken with cold saturated aqueous copper acetate (1 cm^3). An emerald green solution with a finely divided precipitate was obtained, suggesting the presence of an enol. The solution or precipitate was not fluorescent under UV light. A similar test using acetyl-acetone produced a pale blue precipitate almost instantaneously while ethylbenzoylacetate gave a green precipitate.

Test for phosphate

The reagents were prepared according to the method described in (107d). One drop of the aqueous pigment solution was mixed with calcium oxide (5 mg) and heated in a small platinum crucible. The crucible was gently heated at first and then at red heat for 5 min. After cooling two drops of nitric acid (2N)

were added to dissolve the residue and this solution placed on quantitative filter paper containing ammonium molybdate solution. After 2 min. a drop of benzidene solution was added, and the paper held over ammonia vapour. A yellow colour was formed, indicating that phosphate was absent.

Test for amino acids.

(a) Ninhydrin

Pigment solution (1 drop) was applied to a clean filter paper, allowed to dry, and then sprayed with ninhydrin reagent (0.1%, Merck), and again allowed to dry. The paper was then heated in an oven at 110° for 5 min, but no deep purple colour was formed.

(b) Acid hydrolysis with hydrochloric acid (6M)

Pigment A-2, (2 mg) λ_{max} (H_2O/HCl) 414 nm was heated at 100°C for 26 h in a sealed tube with 6M HCl (2 cm^3). The now black mixture was diluted with water and then evaporated to dryness and this procedure was repeated until the residue was completely free of HCl. A ninhydrin test on an aqueous solution of the residue was negative, indicating that the pigment did not contain any protein as part of its structure.

Test with p-dimethylaminobenzaldehyde for pyrrole or indole derivatives (Erhlich's test).

Pigment solution (2 drops) in 50% EtOH was mixed with p-dimethylaminobenzaldehyde (2 drops, 5% solution in conc. HCl). Two drops of water were added and a pink colour was produced which was very similar to the colour produced when the pigment was reacted with conc. HCl alone. Similar tests with the reagent with indole produced a wine red colour immediately which slowly turned brown, while 2-acetyl pyrrole produced a pink colouration.

Reaction of pigment A-2 with excess methyl iodide

Working on the assumption that the pigment A-2 was a conjugated polyene azomethine, an attempt was made to react this pigment with excess methyl iodide, to produce the corresponding quaternary ammonium salt. Pigment A-2 (1 mg) dissolved in a minimum amount of methanol was treated with excess methyl iodide (5 cm^3) and the mixture allowed to stand at room temperature for 24 h, and then taken to dryness. The absorption spectrum in methanol revealed a broad maximum (357-409 nm) making any conclusions on the nature of the reaction (if any) very difficult.

Exchange reaction with t-butylamine

If pigment A-2 was indeed a conjugated polyene azomethine, it was argued that it should undergo an exchange reaction with excess t-butylamine, a primary amine. It was hoped that at least the molecular weight of the new Schiff base could be obtained by mass spectrometry. Consequently pigment A-2 (0.5 mg) was dissolved in ethanol (2 cm^3), excess t-butylamine was added and the mixture allowed to stand over 4A molecular sieves for 24 h. The solution was then decanted, and the excess t-butylamine removed under reduced pressure. The absorption spectra were recorded in methanol.

Pigment after	Absorption spectra (nm) methanol	
	methanol	methanol/HCl
22 1/2 h	356	412, (394), (433)
72 h	360	412, (394), (433) (305) (320)

( ) = shoulder

In a similar experiment the mixture was refluxed for 2 h, and examined as previously described. A broad absorption maximum at 357-370 nm was shifted to 412 nm on acidification, with shoulders at 394 and 433 nm.

Sodium borohydride reduction of pigment A-2.

(i) A solution of pigment A-2 in methanol (6 cm^3, absorbance 1.6- 1.8 in 1 cm cell) was treated with sodium borohydride (5 mg). The colour of the solution disappeared immediately, but the solution was left to stand a further 15 min. The absorption spectrum showed the presence of a reduced chromophore, absorbing at 272, 282, 304 and 319 nm in methanol which upon correlation with other known polyene compounds indicated a conjugated tetraene (77). There was a corresponding decrease in the total absorbance.

(ii) When the sodium borohydride reduction was repeated later on another sample of the pigment, there was no change in the absorption spectrum nor in the total absorbance, indicating that no reaction had taken place. This seemed to suggest that some modification of the pigment structure had occurred.

(iii) Sodium borohydride reduction of pigment after protonation with Dowex cation exchange resin (protonated pigment now called A-3).

A solution of pigment A-2 (λ max 380 nm in 50% EtOH) was passed through a small column of Dowex 50W-X8 cation exchange resin (Baker) to effect complete protonation of

the pigment. The absorption maximum of this new pigment preparation was shifted to 414 nm (now called pigment A-3) (p.164). To a solution of pigment A-3 in 95% ethanol (6 cm^3, absorbance 1.3 in 1 cm cell) sodium borohydride (5 mg) was added. The colour of the solution discharged immediately.

The absorption spectrum revealed the presence of a reduced chromophore absorbing at 304, 320, 334 nm in ethanol, and a corresponding loss of the single absorption maximum of 414 nm. The reduced chromophore suggests the presence of a conjugated pentaene in pigment A-3. There were also broad maxima at 244 and 376 nm.

Catalytic hydrogenation of pigment A-2 P. flavicomum.

Pigment A-2 (ca. 4 mg) was dissolved in methanol (10 cm^3) to give a yellow solution to which was added 5% Pd/C (10 mg, MCB). This solution was then deaerated under reduced pressure and then connected to a hydrogenator at room temperature and pressure. After about 1 h, the colour of the solution was completely discharged, but the reaction was continued for a further 51 h. The total hydrogen uptake was 20 cm^3 (after subtraction of hydrogen required to saturate the catalyst only). The solution was filtered and the filtrate which showed strong yellowish green fluorescence under UV light was evaporated under reduced pressure to give a colourless oil. Attempted crystallisation from methanol water gave an oil.

Table 23. Absorption spectra of hydrogenated pigment A-2, (nm) in methanol.

Neutral	Basic	Acidic
245 (0.77)	245 (0.87)	-
280 (0.74)	278 (0.93)	277 (0.80)

( ) = absorbance in 1 cm cell

The spectral change reported in Table 23 was reversible, i.e. the absorption maxima 245, 280 nm and 245, 278 nm in neutral and basic methanol respectively, were converted to a single absorption maximum at 277 nm in acidic methanol, with a corresponding loss of the peak at 245 nm. Other hydrogenation experiments using platinum oxide as catalyst were carried out with essentially the same results.

Changes in the absorption spectra of hydrogenated pigment A-2 at different pH.

A standard solution of the hydrogenated pigment A-2 in methanol, which when acidified had an absorbance of ca. 0.9 in a 1 cm cell at its absorption maximum, was prepared. An aliquot of this solution (5 cm^3) was evaporated completely to dryness under reduced pressure at 35° and the residue was redissolved in the appropriate buffer solution (5 cm^3). The electronic absorption spectrum was recorded, and a series of curves was obtained over a range of pH values. The buffers were made up as described by Vogel (96).

Table 24. Absorption spectra of hydrogenated pigment A-2 at different pH.

pH of buffer	Buffer solution	Absorption maxima (nm) in different buffers (Ref. 96)			
1.1				277	(0.84)
2.0		245	(0.60)	278	(0.73)
3.3	NaOAc-HCl	245	(0.71)	280	(0.65)
4.2		245	(0.74)	280	(0.65)
5.2		245	(0.74)	280	(0.64)
6.1		245	(0.75)	280	(0.66)
7.4	KH_2PO_4-NaOH	243	(0.75)	280	(0.62)
8.0		244	(0.76)	281	(0.67)
9.0	$Na_2B_4O_7$-NaOH-KCl	245	(0.76)	280	(0.69)
10.0		245	(0.76)	281	(0.73)

( ) = absorbance

Fig. 9. Ultraviolet absorption spectrum of hydrogenated pigment A-2 (P. flavicomum).

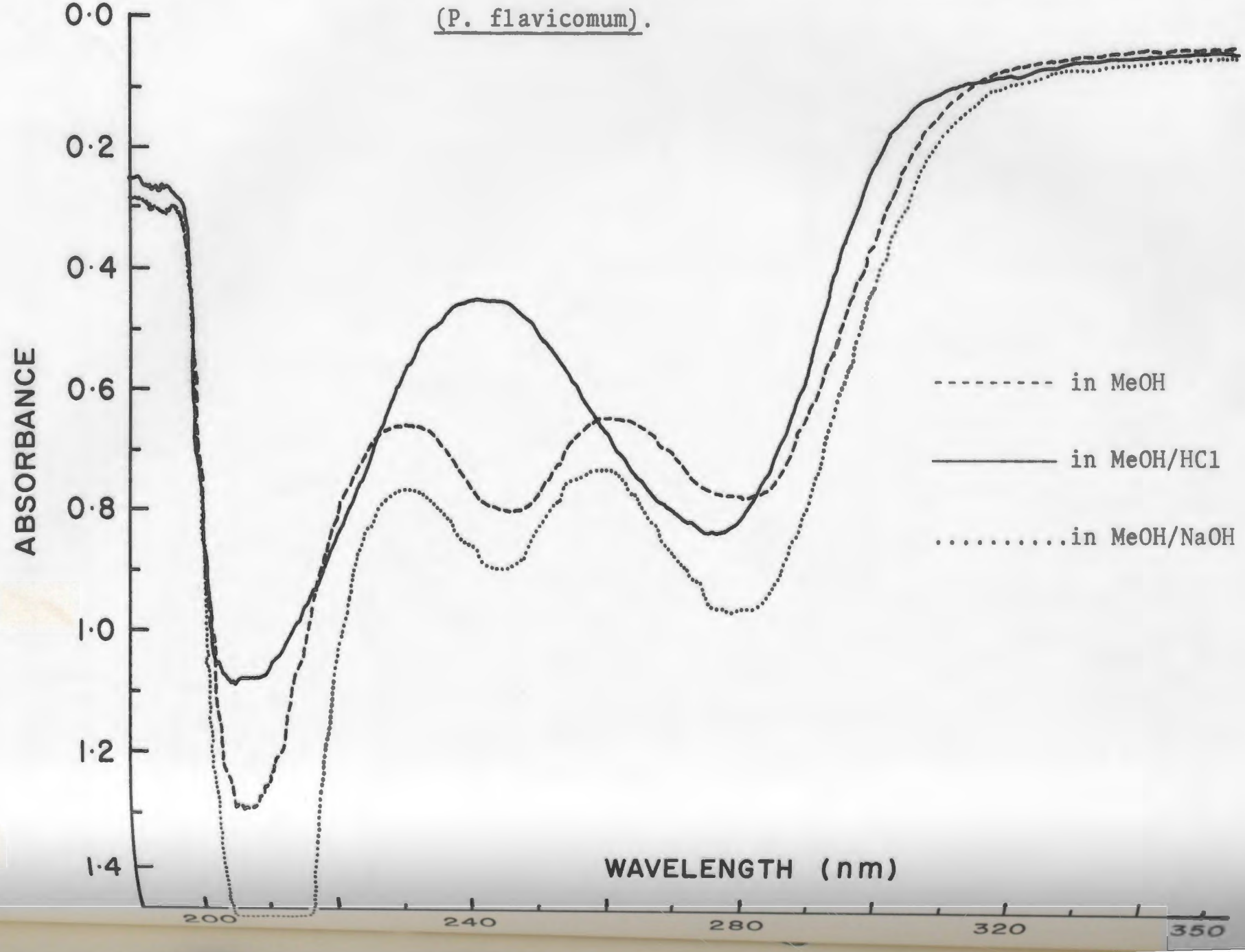
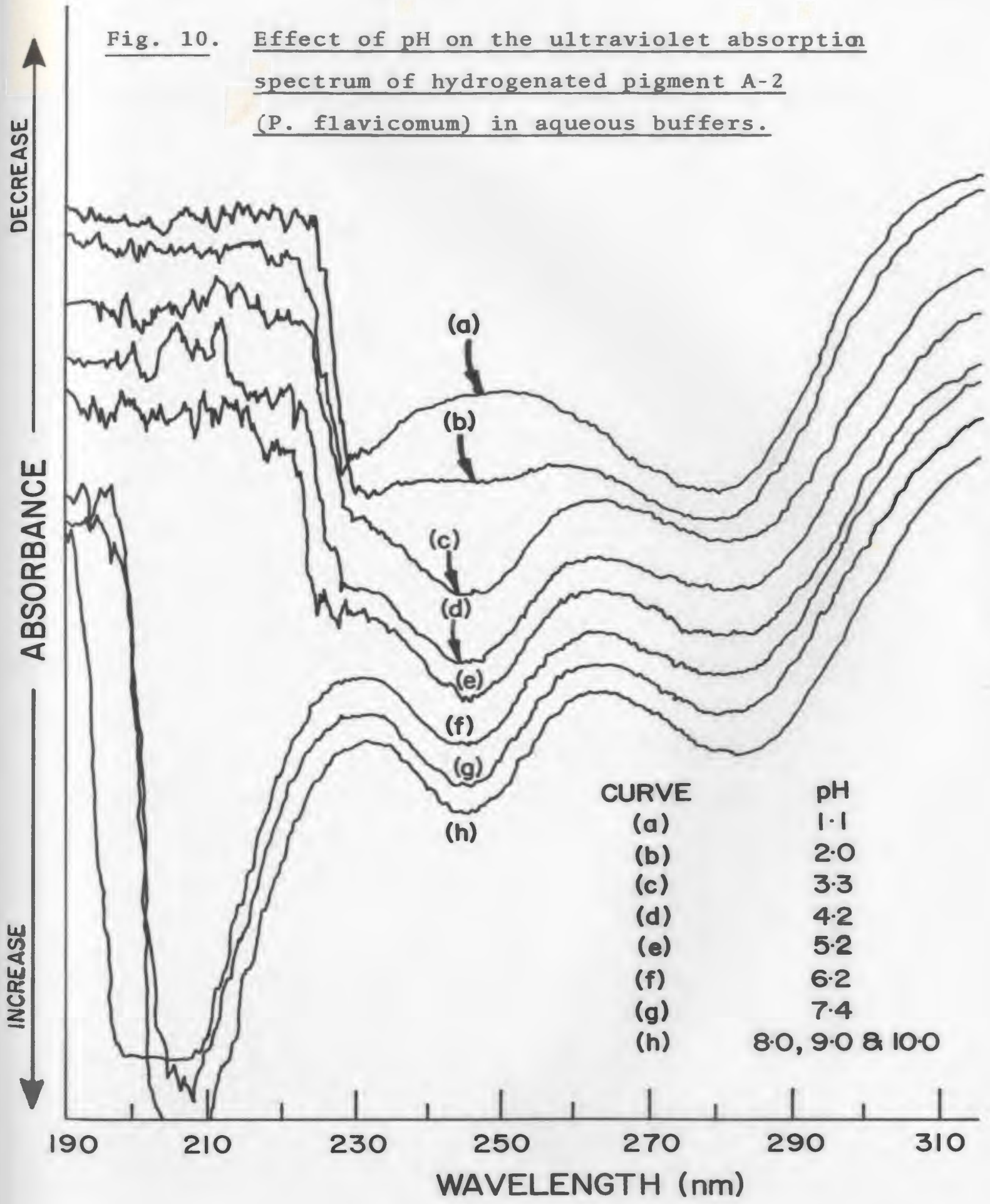


Fig. 10. Effect of pH on the ultraviolet absorption spectrum of hydrogenated pigment A-2 (P. flavicomum) in aqueous buffers.



Spectrophotometric determination of acid dissociation constant of hydrogenated pigment A-2.

A standard solution of hydrogenated A-2 in methanol, with an absorbance of ca. 1.2 in a 1 cm cell was prepared. A fixed volume of this solution was evaporated to dryness, and redissolved in the appropriate buffer (5 cm^3). The electronic absorption spectrum was recorded and a series of curves were obtained over a range of pH values. The absorbance of each solution at 245 and 380 nm was plotted against the pH of the solution, and the inflexion point taken as the approximate pK_a of the hydrogenated pigment.

pK_a of hydrogenated pigment

The pK_a of the hydrogenated pigment was determined by following (i) the loss of the 245 nm maximum with decreasing pH and found to be ca. 2.3 and (ii) the appearance of the maximum at 280 nm with increasing acidity and found to be approximately 2.4. Curves showing the change in the absorption spectrum with pH and the change in absorbance at 245 and 280 nm are given in Figs. 10, 11 and 12.

Fig. 11. Absorbance of hydrogenated pigment A-2 (P. flavicomum) at 245 nm in aqueous buffers.

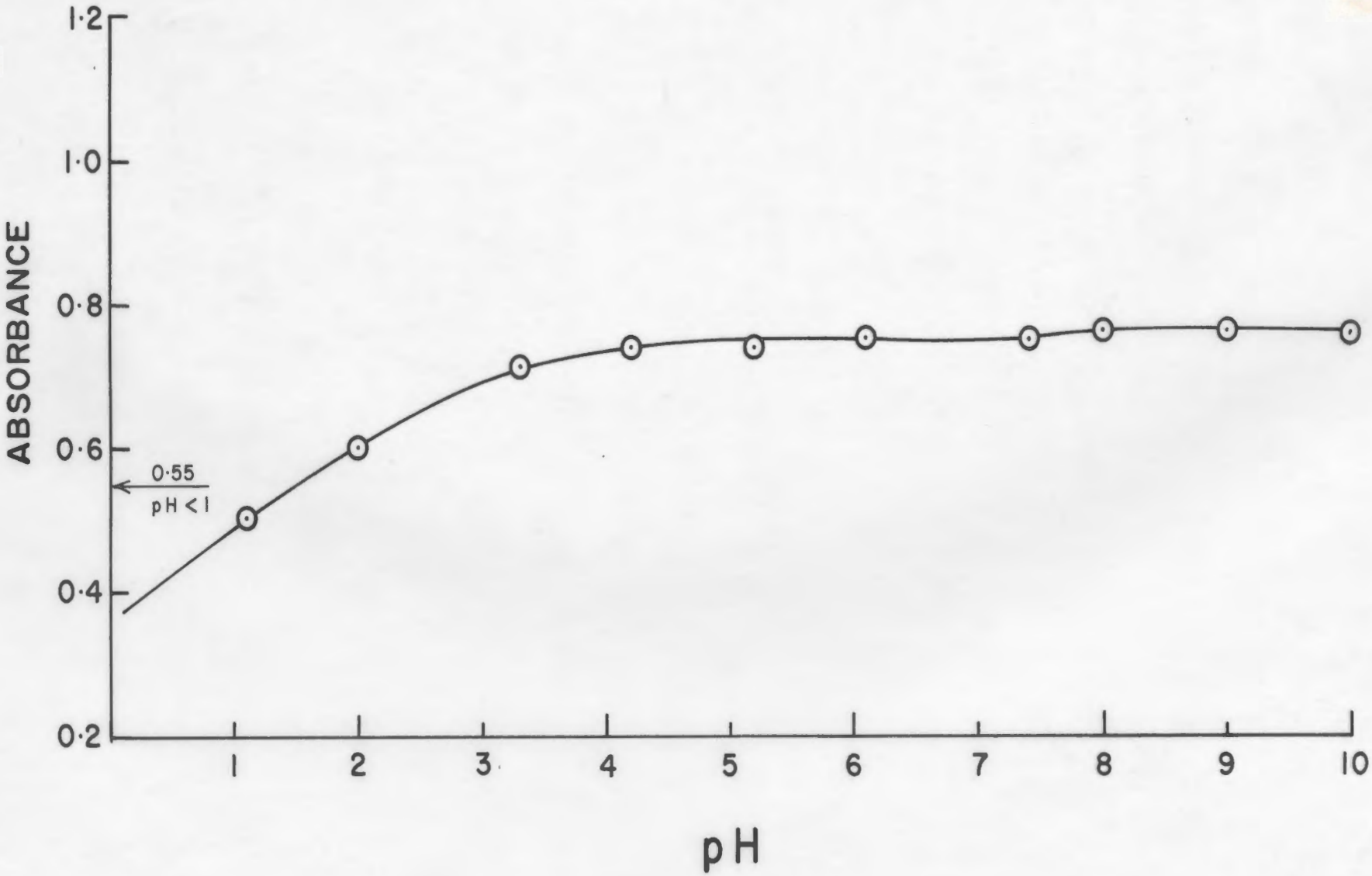
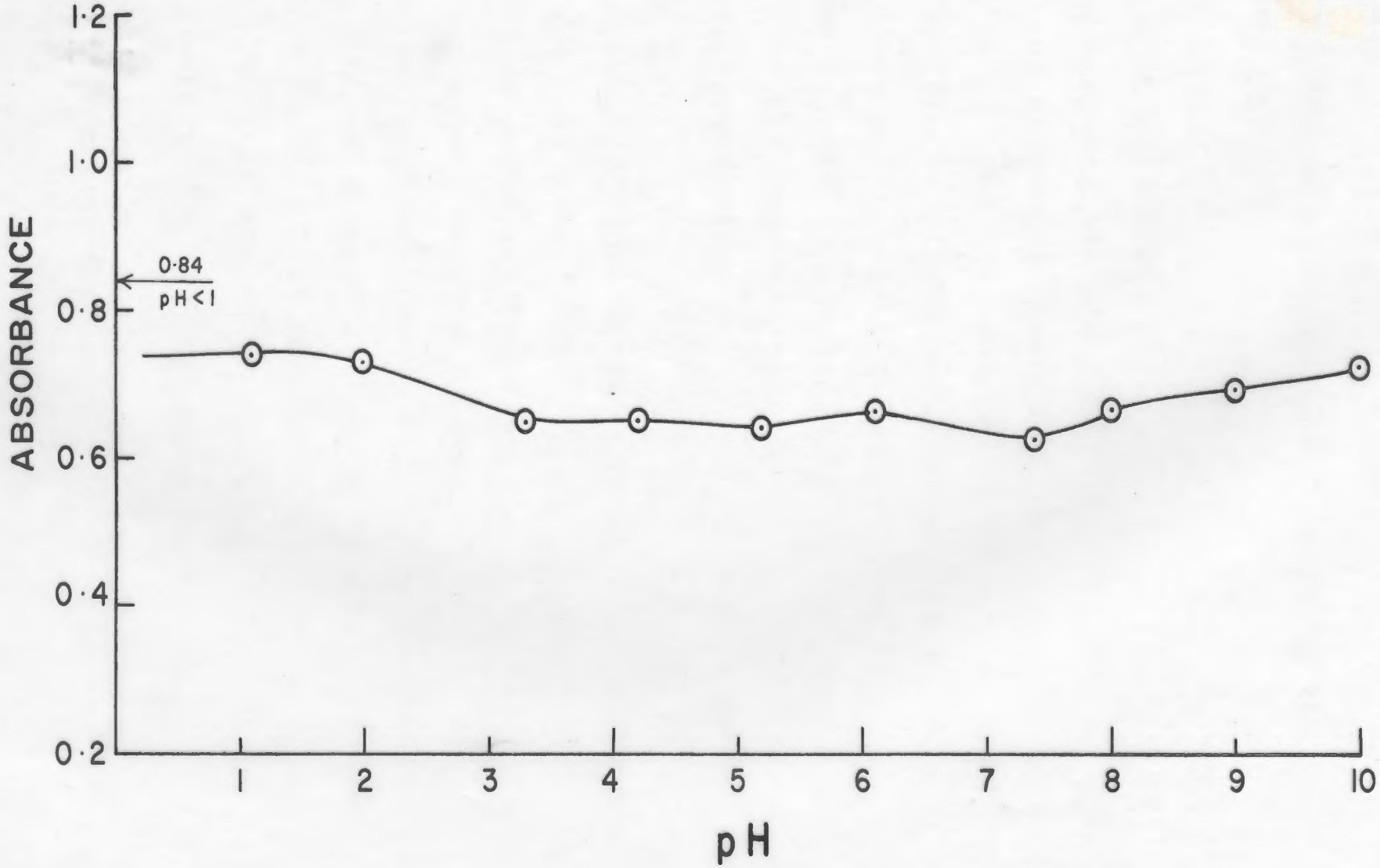


Fig. 12. Absorbance of hydrogenated pigment A-2 (P. flavicomum) at 280 nm in aqueous buffers.



Spectrophotometric determination of acidic dissociation constant of pigment A-2.

A standard solution of pigment A-2 in methanol, which when acidified had an absorbance of ca. 1.4 in a 1 cm cell at its absorption maximum, was prepared. An aliquot of this solution (5 cm^3) was evaporated completely to dryness under reduced pressure at 35° and the residue was redissolved in the appropriate buffer (96). The electronic absorption spectrum was recorded and a series of curves were obtained over a range of pH values. The absorbance of each solution at 380 nm and 416 nm was plotted against the pH of the solution, the inflexion point being taken as the pK_a of the pigment.

pK_a of pigment A-2.

The pK_a of pigment A-2 was determined by this spectroscopic method by following (i) loss of the 380 nm maximum with decreasing pH and found to be approximately 3.4 and (ii) appearance of the maximum at 416 nm with increasing acidity and found to be about 2. Curves showing the change in the absorption spectrum with pH and the change in absorbance at 380 nm and 416 nm are given in Figs. 13, 14 and 15.

Fig. 13. Effect of pH on the visible absorption spectrum of pigment A-2 (P. flavicomum) in aqueous buffers.

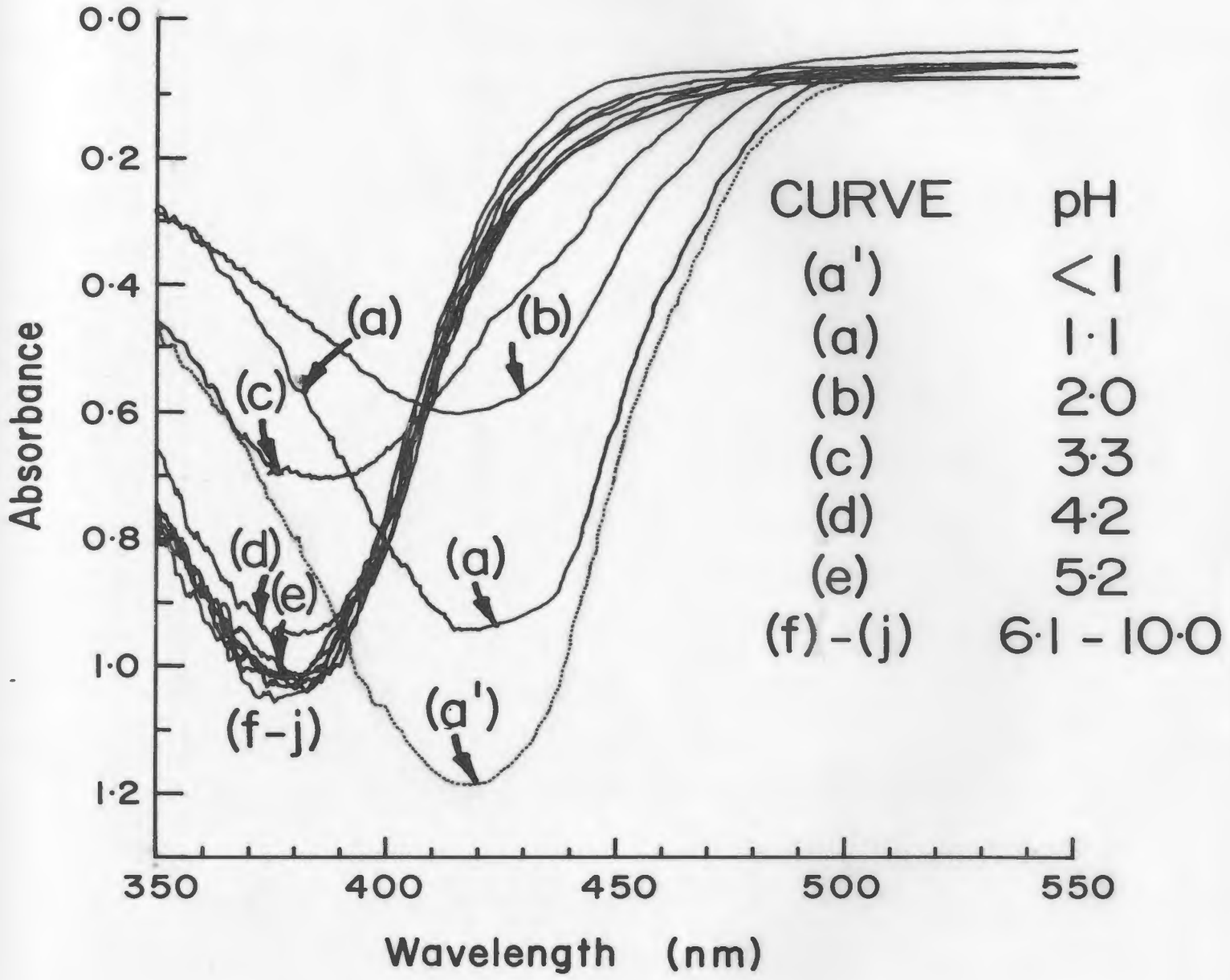


Fig. 14. Absorbance of pigment A-2 (P. flavicomum) at 380 nm in aqueous buffer solutions.

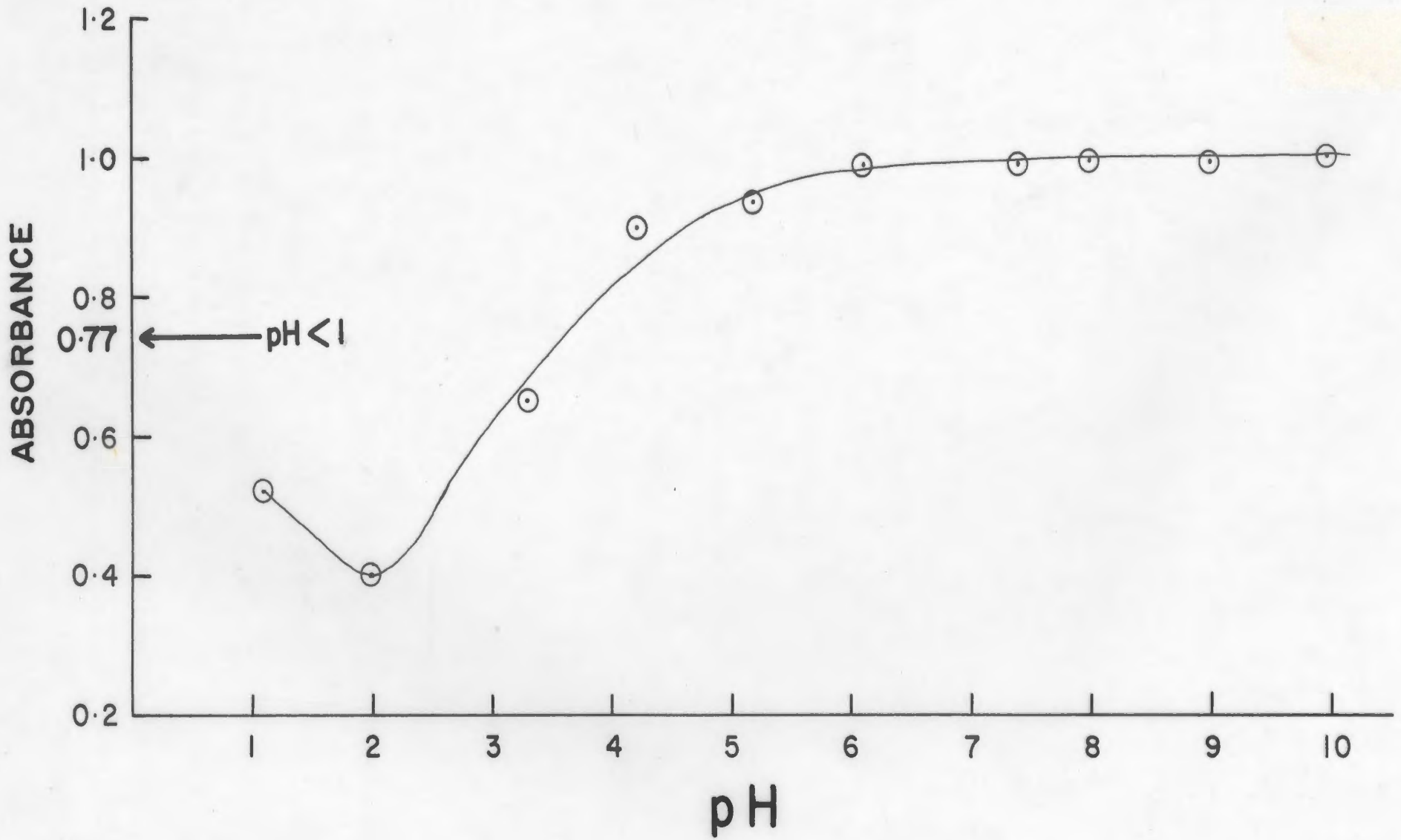
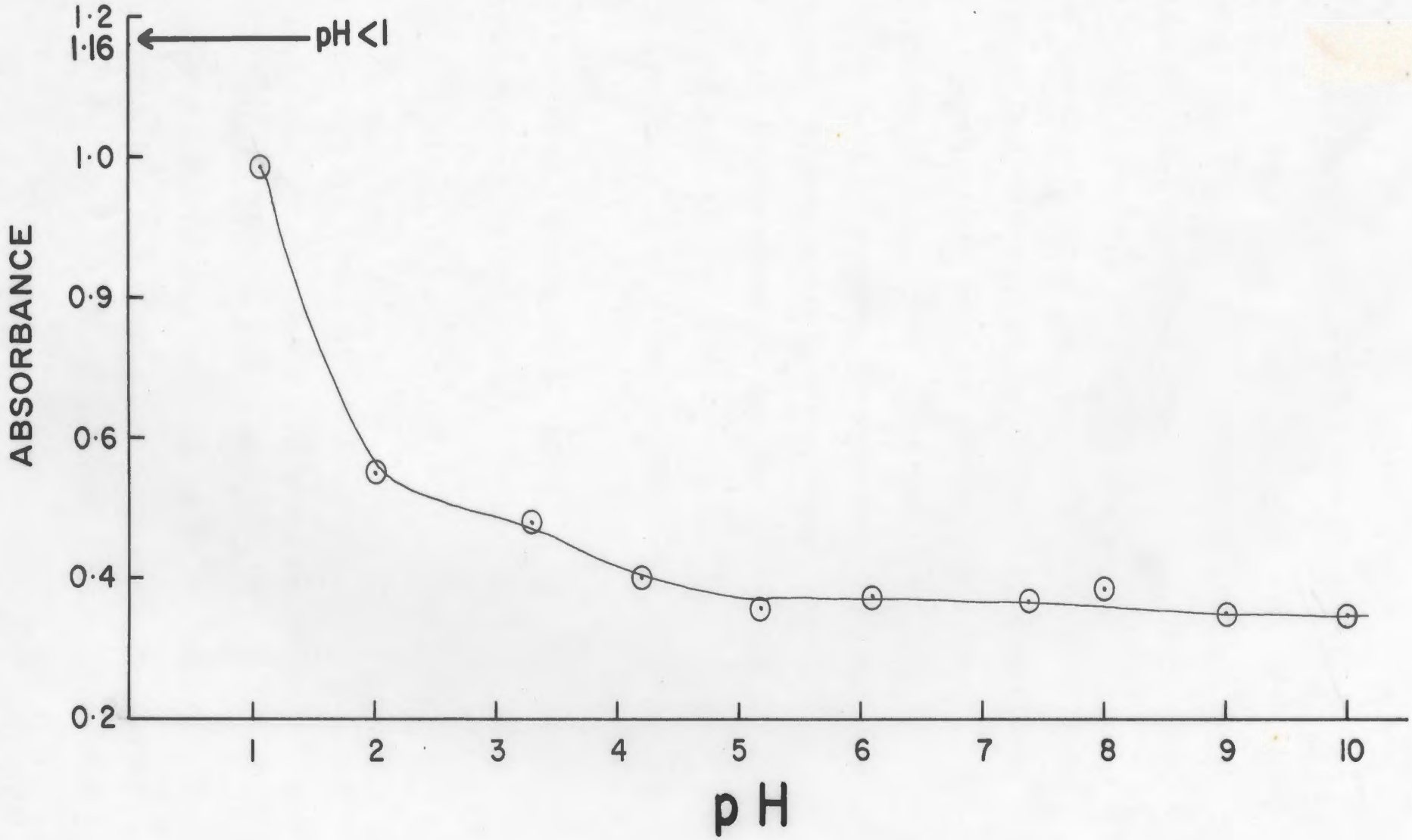


Fig. 15. **Absorbance of pigment A-2 (P. flavicomum) at 416 nm in aqueous buffer solutions.**



(i) Methylation of pigment A-3 with diazomethane.

Pigment A-3 (∿ 3 mg) λ_{max} (MeOH) 414 nm, was dissolved in methanol/ether (5:1) (1.5 cm^3) and the yellow solution cooled to 4° (p.206). Small volumes of twice distilled diazomethane in ether were added to the pigment solution, but this time there was a vigorous evolution of nitrogen. After evolution of gases had ceased, the solution was left overnight at ~ 4°. The solution was evaporated to dryness, and its absorption spectrum λ_{max} (MeOH) 364 nm, shoulder at 390 nm, was not shifted on acidification, indicating that perhaps the functional group(s) responsible for the reversible shift in acid and alkali had been converted to the methyl derivative. The product was soluble in ethanol and methanol, but was now soluble also in chloroform so that an infrared spectrum was obtained (Fig. 16).

I.r. ($CHCl_3$) (3110-3440, broad, NH?), 2975, 2910, 2810, 1710 (C=O, α,β-unsatd. ester), 1670 (C=O), 1660 (infl.), 1635, 1625, 1600 (C=C), 1440, 1365, 1340, 1310, 1270, 1175, 1135, 1110 and 1010 (C=C$\swarrow^{H}$ out of plane stretch), cm^{-1}.

Attempted purification of the product on silica gel GF254 plates (20x20 cm, 400μ) developed with benzene/ethyl acetate/acetone (6 : 4 : 1) produced the following:

(a) A yellow pigmented diffuse band, remaining at the origin, (R_f 0.0), and containing a significant amount of the sample applied. U.v. λ_{max} (MeOH) 215 and 260-290 nm (broad), but

no other distinct peaks were visible. It seemed that extensive degradation of the pigment had occurred on the adsorbant, indicating that perhaps silica gel t.l.c. was not a wise choice for attempted separation.

(b) A strongly fluorescent white band, (R_f 0.30).

U.v. λ_{max} (MeOH) 208 (solvent?) and 264-65 nm.

(c) A yellow diffuse pigmented band, (R_f 0.45).

U.v. λ_{max} (MeOH) 321, 337 and 352 nm, (very weak shoulders) perhaps indicating a conjugated polyene chromophore; however, the lack of sharpness of these peaks was taken as further evidence that the methylated product was being degraded on the silica gel.

(ii) Methylation of pigment A-2 with diazomethane.

Pigment A-2 (~ 5 mg) was dissolved in methanol/ether (5:1) (2 cm^3) and the yellow solution cooled to ~ 4° in an ice bath. Small volumes of twice distilled diazomethane in ether prepared from Diazald, (Aldrich) were added to the pigment solution to give a slight excess of diazomethane, after which the flask was fitted with a small plug of cotton wool and left overnight at ~ 4°. The solution was then evaporated to dryness, and its absorption spectrum λ_{max} (MeOH) 380 nm was shifted to 412 nm on acidification with hydrochloric acid (2N, 2 drops). This 'methylation product' was soluble in water, and in aqueous alcohols, but was insoluble in carbon tetrachloride or chloroform, solubility properties characteristic of the unreacted pigment. An infrared spectrum could not be

obtained in either carbon tetrachloride or chloroform.

Chromatography of "methylated product A-2" on a Dowex cation exchange resin column.

The product obtained from the previous methylation attempt was chromatographed on a column prepared from Dowex cation exchange resin (p.164). The dried protonated pigment (~ 4.0 mg), λ_{max} (MeOH) 390 and 412 nm obtained, was now soluble in methanol, ethanol, and chloroform but insoluble in water. The infrared spectrum (2000-800 cm^{-1}) is shown in Fig.17. I.r. ($CHCl_3$) (2000-800 cm^{-1} region): 1710 (c=O of α,β unsatd. ester), 1670 (C=O), 1620 (C=C), 1590, 1580 (C=C), 1550, 1305, 1275, and 1130 cm^{-1}.

Remethylation of protonated pigment with diazomethane.

The protonated pigment (ca. 4 mg) (recovered above) was redissolved in methanol/ether (5:1) and treated with small portions of diazomethane in ether. Bubbles were evident as each portion of diazomethane solution was added, and the reaction mixture was left for 24 h. The absorption spectrum λ_{max} (MeOH) 372 nm was now pH independent.
I.r. ($CHCl_3$) 3500-3150 (broad, NH), 2975 (shoulder) 2930, 2812, 1715, 1672, 1635 (infl.) 1625), 1465, 1440, 1270 and 1010 cm^{-1}. T.l.c. examination of this product on silica gel GF254 (20x20 cm, 400μ) developed with benzene/ethyl acetate/acetone (6 : 4 : 1) produced:

(a) A yellow diffuse pigmented band (R_f ~ 0.0), remaining at the origin containing a large proportion of the sample applied. The spectral characteristics were similar to that reported for (a) p.205.

(b) A strongly fluorescent white band (R_f 0.30), recovered as an oil.

U.v. λ_{max} (MeOH) 210 and 264 nm.

(c) A yellow diffuse band (R_f 0.56) recovered as a yellow oil.

U.v. λ_{max} (MeOH) 310, 315 and 350 nm.

It can be emphasised here that silica gel seems to degrade the pigment, and it is suggested that further separation of the methylated product be attempted on a less active adsorbent. [In the preparation of these silica gel coated thin layer plates, the standard practice in our laboratory was to activate the plates for 60 min. at 110°C.]

Fig. 16. Infrared spectra of methylated pigment A-3 (P. flavicomum) (cm^{-1}) ($CHCl_3$).

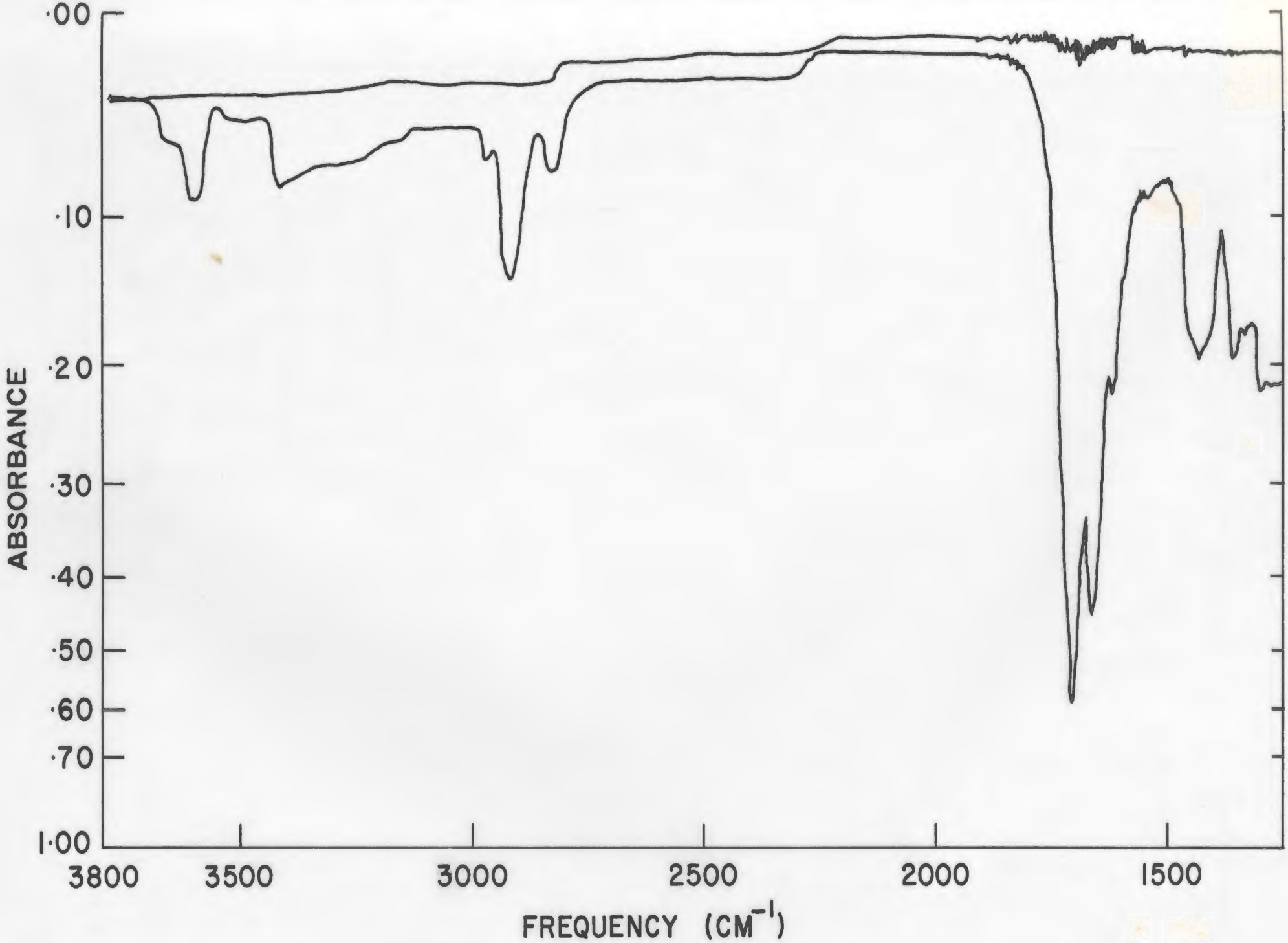


Fig. 16 (cont'd). Infrared spectra of methylated pigment A-3 (P. flavicomum) (Cm^{-1}) ($CHCl_3$)

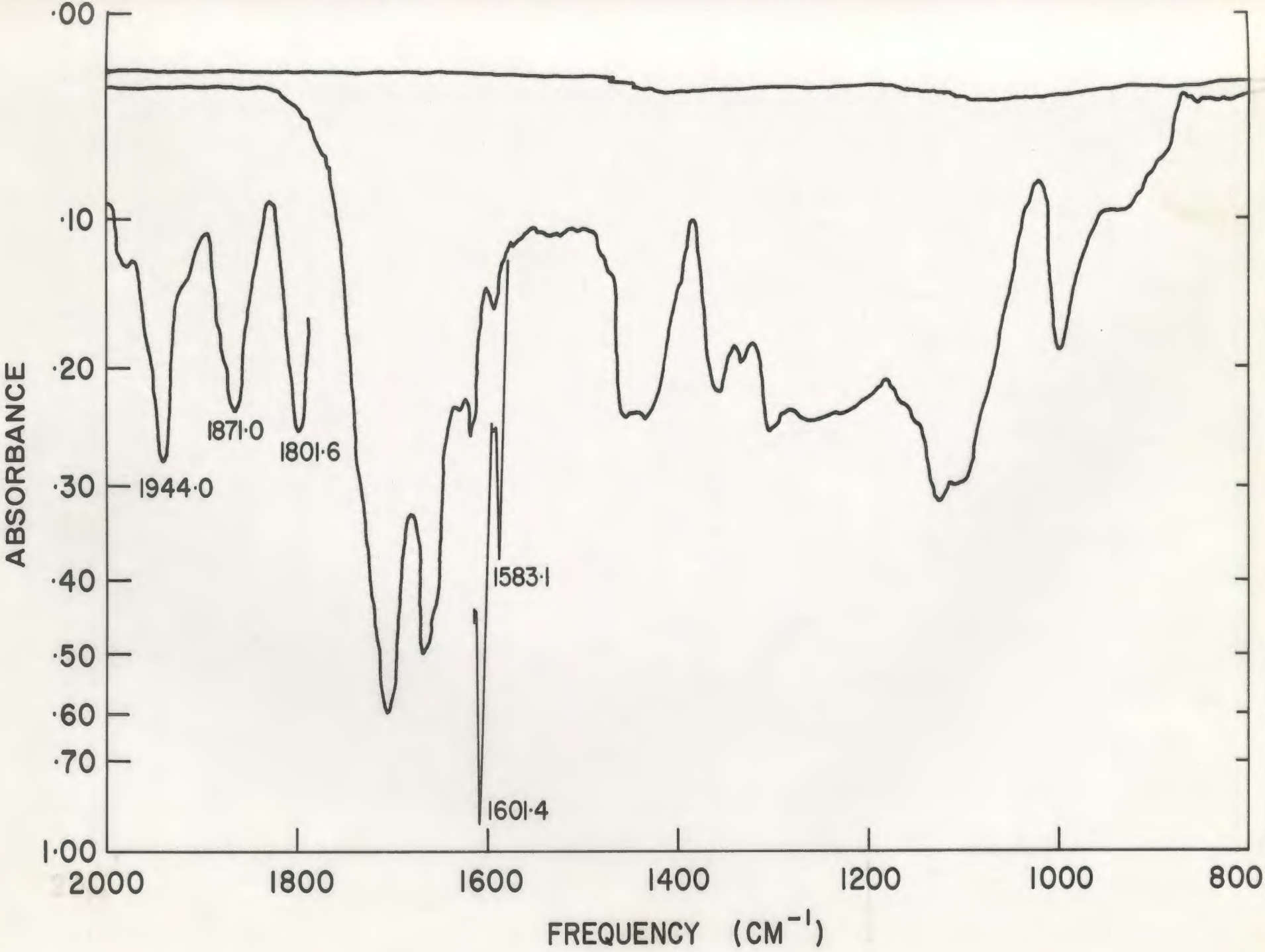
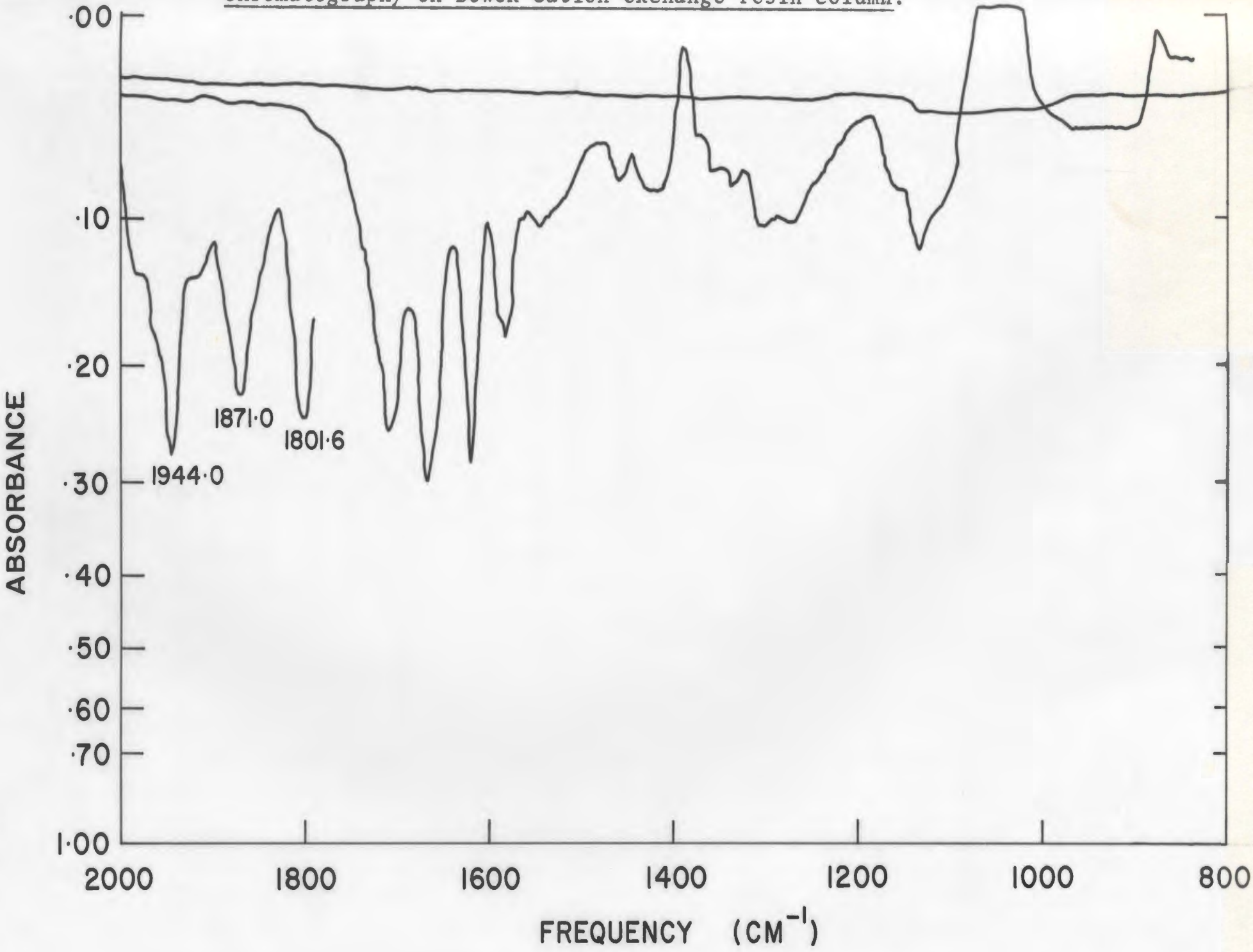
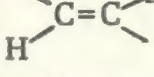


Fig. 17. Infrared spectrum (2000-800 cm^{-1}) of "methylated product A-2" after chromatography on Dowex cation exchange resin column.



Silylation of pigment A-2

Pigment A-2 (2 mg) was treated with *Tri-Sil (0.5 cm^3, Pierce Chemical Company) in a small glass vial and the mixture was vigorously shaken for 30 sec, and then allowed to stand at room temperature for a further 5 min. in a desiccator containing anhydrous silica gel. The reaction mixture was then evaporated to dryness under nitrogen and dried in vacuo. U.v. λ_{max} (MeOH) 362 and 410-418 nm (shoulder), which did not change on protonation. This suggests that the functional group(s) responsible for the bathochromic shift on protonation of pigment A-2 has been derivatised.

I.r. ($CHCl_3$) 3470 (weak), 3240-3000 (broad), 2970 (weak), 2940 (weak), 2910, 2840, 2800-2300 (broad, H bonded OH?), 1710, 1690 (C=O), 1620 (strong, C=C), 1590 (C=C), 1550, 1490, 1415, 1375, 1315, 1255, 1110, 1065, 1015 (trans = C–H out of plane stretch in ), and 845-870 (broad) cm^{-1}.

Mass spectrum (200°): m/e 369 (1.4%); 341 (5%); 314 (1.4%); 3.3 (4.6%); 299 (1.7%); 296 (1.5%); 295 (4.4%); 282 (2.7%); 281 (5.9%); 239 (2.1%); 235 (2.1%); 223 (2.1%); 221 (5.9%); 208 (4.8%); 207 (20.5%); 155 (5.6%); 150 (6.5%); 149 (54.9%); 148 (91.5%); 147 (100%); 145 (6.2%); 141 (7.3%); 133 (9.6%); 132 (13.5%); 131 (34.2%); 129 (30.4%); 127 (11.1%); 125 (4.8%); 123 (2.5%); 120 (8.0%); 117 (21.0%); 111 (7.5%); 103 (7.5%);

*Tri-Sil is a commercial silylating agent containing trimethylsilylchloride (Me_3SiCl) and hexamethyldisilazane ($(CH_3)_3$ Si–NH-Si$(CH_3)_3$) in pyridine.

99 (15.2%); 97 (13.5%); 93 (12.3%); 89 (8.5%); 85 (32.6%); 83 (12.0%); 82 (13.7%); 81 (6.3%); 80 (13.8%); 79 (40.0%); 78 (17.7%); 76 (6.1%); 75 (12.0%); 74 (11.1%); 73 (54.1%); 72 (5.2%); 71 (13.7%); 70 (8.7%); 69 (14.4%); 67 (8.5%); 66 (6.3%); 60 (5.9%); 59 (8.2%); 57 (5.6%); 56 (9.0%); 55 (15.6%); 53 (11.0%); 52 (26.6%); 51 (28.7%); 50 (14.5%); 47 (5.4%); 46 (8.5%); 45 (21.5%); 44 (11.8%); 43 (30.0%); and 41 (12.7%). The highest discernible peak in the spectrum was at m/e 410.

Acetylation of pigment A-2

Pigment A-2 (5 mg) dried overnight under vacuum over P_2O_5 was dissolved in dry pyridine (0.25 cm^3) and acetic anhydride (0.5 cm^3). The pyridine was twice distilled over CaH_2 and acetic anhydride was distilled over anhydrous sodium acetate. This mixture was left for 48 h in the dark at room temperature during which time the colour of the solution changed from a light orange to a darker orange brown. Ethanol (2 cm^3) was added, and the acetylation mixture reduced in volume under N_2. This residue was diluted with water (2 cm^3) and extracted with methylene chloride (3 x 25 cm^3); most of the pigment was transferred to the organic phase which was now orange. The methylene chloride extract was washed to neutrality and dried overnight over anhyd. $MgSO_4$. Some pigment was adsorbed on the drying agent but could be eluted with methanol/methylene chloride (9:1).
U.v. λ_{max} (MeOH) 370 nm (unchanged on acidification).
I.r. ($CHCl_3$) 3030, 2970, 2840, 1755 (C=O of enol acetate?) 1725 (C=O), 1670 (C=O), 1615, 1580, 1260-1180 (broad) and 1005 cm^{-1}.

Raman spectra of pigment A-2 (P. flavicomum).

Raman spectra were recorded on a Coderg PHO spectrophotometer after sample excitation with one of the 461.9, 472.7, 488.0 and 514.5 nm lines of a Coherent Radiation model-52 krypton-argon mixed gas laser. Samples were contained in a 1 cm^3 cuvette. A 4.0 cm^{-1} slit width was used.

Table 25. Resonance Raman shift (cm^{-1}), pigment A-2 (P. flavicomum).

A-2 methanol	A-2 (methanol/HCl)
1145 medium	1145
1160 medium	1160
∿ 1260 weak	∿1260
∿ 1565 shoulder	∿1565 shoulder
1584 sharp, strong	1584
1618 medium	1618

Resonance Raman (RR) spectra for pigment A-2 and pigment A-2/H^+ were virtually identical with the exception that the resonance effect was greater in the acidified sample due to the shift of the absorption band to the visible. The samples in the methanol did not decompose. Table 25 summarises the relevant data.

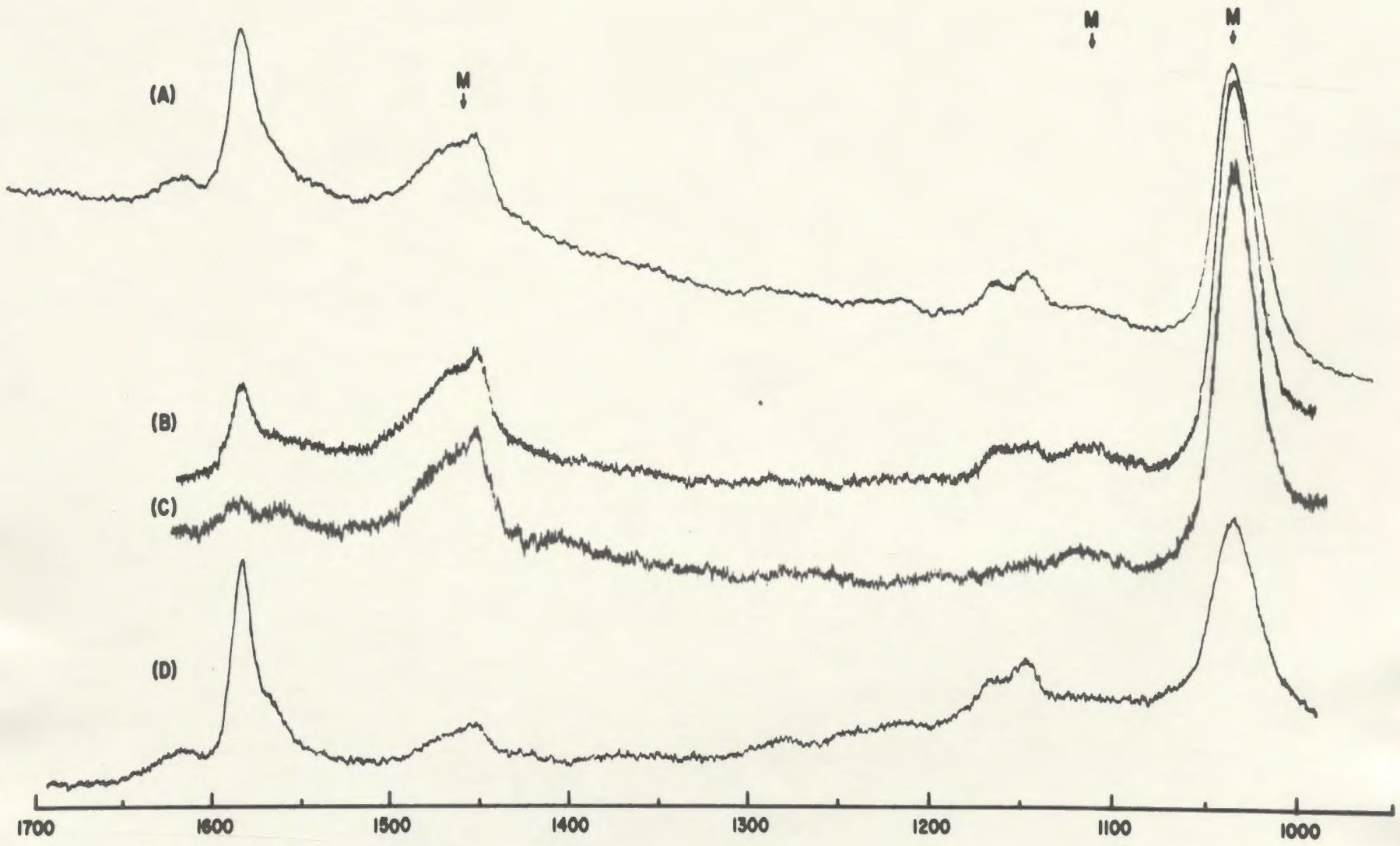
Spectra were also recorded for samples in H_2O and H_3O^+. Results identical to those in methanol were obtained if the spectra were scanned immediately but once in the laser light

these samples bleached and the resonance Raman effect fell off sharply. The intense yellow colour of the protonated sample reverted to the pale yellow colour of the neutral sample after about 15 min. in the laser beam. 2-Methyl benzoxazole did not show resonance Raman but shows Raman bands at 1578 (C=C) and 1618 (C=N) cm^{-1}, and these bands did not shift when acid was added. Fig. 18 is a composition diagram of the Resonance Raman spectra of pigment A-2 in methanol, and in methanol/HCl.

Fig. 18. Resonance Raman Spectra of A-2 in Methanol(cm^{-1}).

A. 461.9 nm excitation
B. 488.0 nm excitation } A-2 unprotonated
C. 514.5 nm in excitation
D. 488.0 nm excitation A-2 protonated with HCl

M. denotes Methanol band (can be used as internal intensity standard).


(A)
(B)
(C)
(D)
M
M
M
1700
1600
1500
1400
1300
1200
1100
1000

Oxidation.

Oxidation/methylation with freshly prepared silver oxide and excess methyl iodide.

Preparation of silver oxide.

Sodium hydroxide (pellets, 1.5 g) was dissolved in water (20 cm^3) and this solution was added to silver nitrate (5.9 g, Baker) dissolved in water (20 cm^3). The brown precipitate which was immediately formed was allowed to stand at room temperature for 10 min, and filtered on a Büchner funnel under reduced pressure. This brown solid was repeatedly washed with water to neutrality (indicator paper pH 1-12) and then successively with methanol and finally diethyl ether. The silver oxide (~ 4 g) was air dried at room temperature and was used in this state without further purification. The preparation of silver oxide and its use as a reagent in this pigment study and subsequently was developed by Dr. P.M. Baker in our laboratory (34).

Reaction with silver oxide and excess methyl iodide.

(a) On ethyl acetate extract of pigment (indicator pigment).

During the early stages of this work, the pigment obtained after silicic acid chromatography of the ethyl acetate extract was used without further purification. Pigment (25 mg) in a round bottomed flask (250 cm^3) was mixed with freshly prepared silver oxide (4 g) and suspended in methyl iodide (2 cm^3, Baker).

An appropriate glass stopper was firmly secured with masking tape, and the flask was left at room temperature in the dark for 5-7 days. The flask was shaken at intervals and more methyl iodide (2-5 cm^3) added during the course of the reaction; during this time the colour of the reaction mixture usually changed from a dark brown to a silvery grey. The reaction mixture was then exhaustively extracted with chloroform (5x50 cm^3) and the combined extract reduced in volume to give a dark brown oil. This was then chromatographed on silicic acid in a glass column (40 x 2.5 cm) and eluted successively with benzene and increasing proportions of ethyl acetate in benzene. Thirty seven fractions were collected. Fractions 6 and 7 on evaporation gave a pale yellow crystalline solid, m.p. 212-213°.

U.v. λ_{max} ($CHCl_3$) 305, 320, 335 and 352 nm, peaks which seem characteristic of a conjugated tetraene system containing a carbonyl group at each end (77).

Mass spectrum (160°): m/e 223 (M+1), 9%; 222 (M), 61%; 207 (M-CH_3) 5%; 191 (M-OCH_3), 32%; 190 (M-OCH_3-H), 18%; 163 (M-$COOCH_3$), 39%; 162 (M-$COOCH_3$-H), 23%; 158 (M-64), 43%; 131 (M-91), 100%; 121 (M-101) 35%; 103 (M-118), 22%; 91 (M-131) 25%; 77 (M-145) 37%. (Fig. 19).

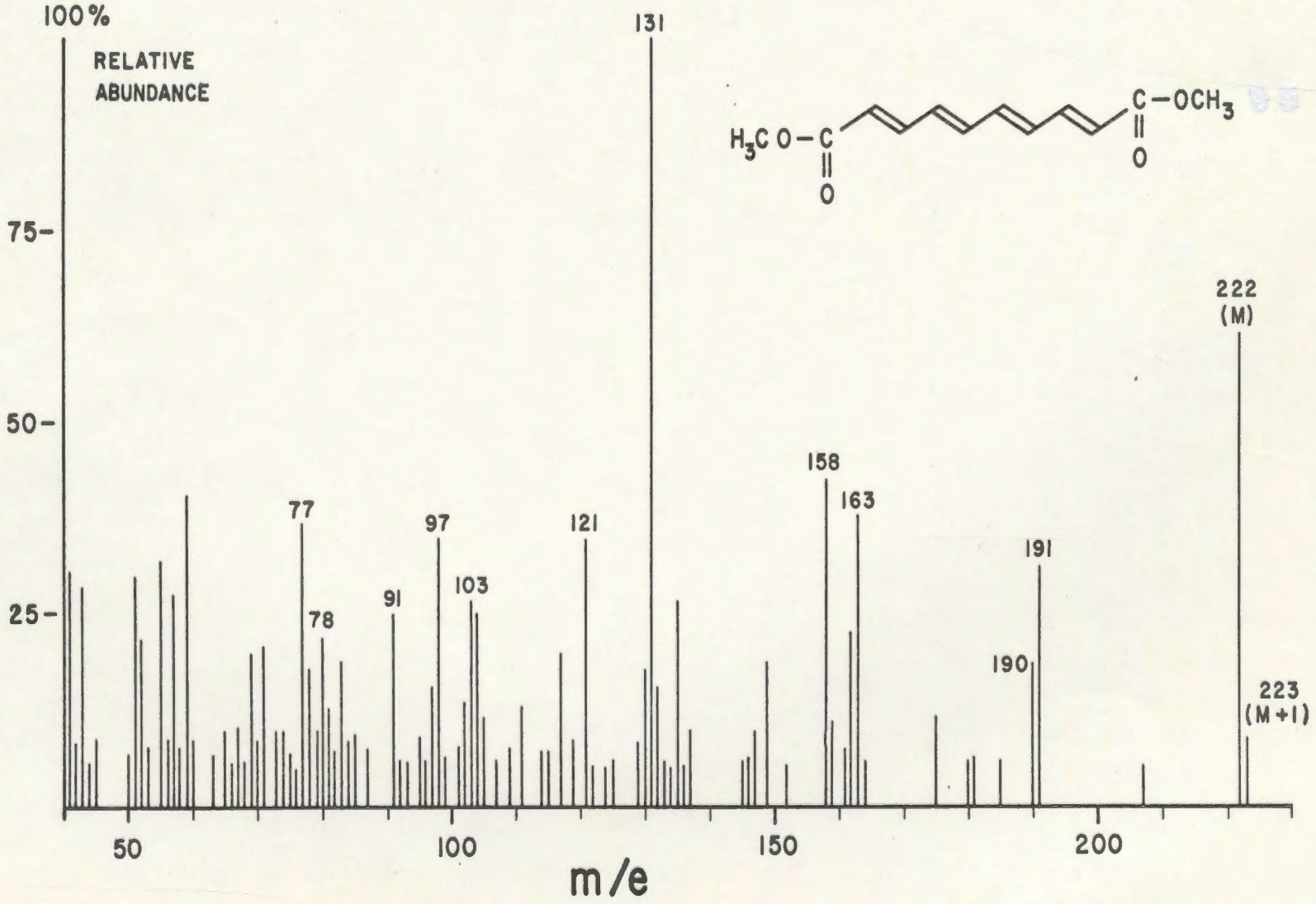
This compound was characterised as all-<u>trans</u> dimethyl 2,4,6,8-decatetraene-1, 10-dioate by comparing its u.v., i.r., n.m.r., mass spectrum, and t.l.c. behaviour and mixture melting point with an authentic sample, m.p. 214°, (121)

the diacid of which was kindly supplied by Dr. D.S. Tarbell. The diester was also further characterised by catalytic hydrogenation to the known dimethyl sebacate. Further useful information from the remainder of the fractions was not forthcoming.

Mass spectrum of 2,4,6,8- decatetraene 1,10-dioic acid (at 160°).

M/e 194 (M), 100%; 176 (M-H_2O), 65%; 158 (M-36), 17%; 131 (M-63), 79%; 130 (M-64), 17%; 104 (M-90), 34%; 103 (M-91), 95%; 91 (M-103), 31%; 79 (M-115), 38%; 78 (M-116), 36%; and 77 (M-117), 73%.

Fig. 19. Mass spectrum of all-trans dimethyl 2,4,6,8-decatetraene-1,10-dioate (XXXVI).



(b) Oxidation/methylation of pigment A-2 with silver oxide/ methyl iodide.

Pigment A-2 (10 mg) was mixed with freshly prepared silver oxide (4 g), excess methyl iodide added (5 cm^3) and the mixture left for 4-5 days at room temperature in the dark. The flask was periodically shaken and during this time the black silver oxide slowly changed colour to a metallic grey. Exhaustive extraction with chloroform (5x25 cm^3) produced a strongly fluorescent yellow solution which was evaporated to dryness and chromatographed on silica gel GF254 plates, (20x20 cm, 400μ) developed with benzene/ethyl acetate (8:2). Three fractions were collected:

(a) several narrow yellow pigmented non-fluorescent bands (R_f 0.0 - 0.70) were detected and eluted together which, after evaporation, produced a yellow oil.

U.v. ($CHCl_3$) 318, 354 and 370 nm.

Mass spectrum (250°): peaks corresponding to m/e 101, 121, 131, 139, 156, 193 and 249 were recorded in the mass spectrum scanned at 250°. The spectrum was very weak, and no useful information could be deduced.

(b) a fluorescent yellow band, ($R_f \sim 0.75$), crystallised from chloroform to give yellow solid m.p. 212°.

U.v. λ_{max} ($CHCl_3$) 292, 305, 321, 337 and 355 nm.

I.r. ($CHCl_3$) 2940, 2835, 1706 (C=O of ester), 1620, 1430, 1310, 1265, 1160, 1125 and 1005 cm^{-1}.

N.m.r. ($CDCl_3$; 100 MHz, accumulated 60 sweeps) τ 3.0 - 4.2 (8H, multiplet, not resolved), τ 6.2 (6H, singlet, $COOCH_3$).

Mass spectrum (190°): m/e 222 (M), 39%; 221 (M-1), 56%; 207 ($M-CH_3$), 4.7%; 191 ($M-OCH_3$), 15%; 190 ($M-OCH_3-H$), 14%; 163 ($M-COOCH_3$), 45%; 162 ($M-COOCH_3-H$), 16%; 158 (M-64), 27% 131 (M-91), 61%; 121 (M-101), 100%; 111 (M-111), 14%; 103 (M-119) 43%; 91 (M-131), 46%; 78 (M-144), 17%; 77 (M-145), 31%.

This compound was characterised as all-<u>trans</u> dimethyl 2,4,6,8-decatetraene-1, 10-dioate.

(c) yellow fluorescent band (R_f 0.87).

U.v. λ_{max} ($CHCl_3$) 275, and 283 nm, weak bands at 321, 336, and 355 nm.

Mass spectrum (195°): m/e 264 (2.9%); 263 (14.2%); 223 (6.8%); 207 (6%); 206 (5.8%); 205 (8.3%); 150 (11.4%); 149 (100%); 133 (8.8%); 132 (4%); 131 (1%); 121 (3.4%); 104 (5.5%); 91 (7.7%); 87 (7.8%); 77 (10.8%); 74 (12.6%); 57 (12.5%); 56 (8.5%); and 43 (5.4%).

In some cases, ions of less than 5% relative abundance will be recorded when interpretation of data becomes ambiguous. This mass spectrum is almost identical to that of fraction 2, p.223 obtained in an oxidation/methylation experiment conducted for 48 h in the presence of sodium hydroxide (2N, 3 drops).

Oxidation/methylation with silver oxide/methyl iodide in the presence of NaOH (2N).

"Schiff's base" pigment (pigment 2, ∿ 20 mg) λ_{max} (MeOH/HCl) 388, 409 nm, (and used as isolated from the Sephadex LH-20 column, and not purified on Sephadex G-10) was mixed with freshly prepared silver oxide, excess methyl iodide and sodium hydroxide (2N, 3 drops). The mixture was allowed to stand at room temperature in the dark for 48 h, and then extracted exhaustively with chloroform to give a yellow product (6.2 mg). Column chromatography on alumina (Act III, 6% H_2O) eluted with ethyl acetate separated the methylation product into 2 fractions:

(i) a faster moving yellow band (∿ 1.5 mg); u.v. λ_{max} (EtAc) 301, 318, 332, 349, 380 and 406-407 nm.

(ii) pale yellow (∿ 4.0 mg); u.v. λ_{max} (EtAc) 301-2, 318, 332, 350, 380-81, and 406 nm; u.v. λ_{max} (cyclohexane) 300-1, 316-17, 331-32, 350, 381 and 412 nm.

I.r. (CCl_4) 3010, 2948, 2920, 2850, 1725 (C=O of ester), 1687 (C=O), 1625 (C=C), 1455, 1440, 1265 (C-O), 1200, 1140, 1125, 1078, 1040 and 1010 cm^{-1}.

Mass spectrum (170°): m/e 264 (1%); 263 (10.9%); 223 (2.7%); 222 (3.1%); 207 (4.9%); 206 (1.4%); 205 (3.7%); 163 (4.5%); 158 (4.3%); 149 (100%); 133 (10.7%); 131 (10.1%); 121 (5.7%); 103 (6.4%); 93 (5.8%); 91 (4.7%); 77 (12.9%); 57 (14.6%); and 43 (6%).

Oxidation/ethylation of pigment A-2 with silver oxide and excess ethyl iodide.

Pigment A-2 (11 mg) was mixed with freshly prepared silver oxide (4 g) and excess ethyl iodide (5 cm^3, Baker) added. The mixture was allowed to react at room temperature in the dark. After ca. 15 h, the pigment suspension was strongly fluorescent yellow, and after 4 days the mixture was exhaustively extracted with chloroform (5 x 25 cm^3), and the combined extract (also strongly fluorescent) was reduced in volume to give a yellow oil. Chromatography of this extract on silica gel GF254, (20x20 cm, 400μ) developed with benzene/ethyl acetate (6:4) revealed five yellow pigmented bands.

1. yellow ($R_f \sim 0.06$).

U.v. λ_{max} (MeOH) 230 and 290 nm (weak shoulders).

Mass spectrum: a very weak mass spectrum was obtained for this fraction, (175-280°) and the highest mass number was recorded at m/e 129. Consequently this fraction was not investigated further.

2. yellow, ($R_f \sim 0.63$).

U.v. λ_{max} (MeOH) 360 - 380 nm.

Mass spectrum (172-178°): 280 (5.7%); 242 (5%); 228 (6.5%); 227 (26%); 226 (13%); 210 (15.4%); 199 (8.9%); 198 (23.6%); 184 (8.9%); 183 (31.7%); 182 (100%); 170 (16.3%); 168 (16.3%); 155 (10%); 154 (38.2%); 127 (15.4%); 126 (17.9%); 99 (13%);

98 (10.6%); 97 (30.9%); 85 (13%); 83 (16.3%); 72 (13.0%); 71 (20.3%); 70 (10.6%); 69 (20.3%); 57 (28.5%); 56 (30%); 55 (21.1%).

3. yellow ($R_f \sim 0.69$)

U.v. λ_{max} (MeOH) 360-380 nm (broad).

Mass spectrum (175°: a very weak spectrum (70 eV) was obtained for this 'degradation product', the highest atomic mass unit being recorded at m/e 242, with additional peaks at 226, 199, 184 (base peak), 102, 98, 97, 83, 69 and 55. It should be pointed out that a malfunction was detected in the instrument, which was not corrected in time for the sample to be re-run.

4. yellow (R_f 0.78).

U.v. λ_{max} (MeOH) 350-370 nm (broad) no change on acidification.

Mass spectrum: again a very weak mass spectrum was obtained. for this fraction (220°) and the highest atomic mass unit was recorded at m/e 299. This fraction was not further investigated.

5. yellow (R_f 0.86).

U.v. λ_{max} (MeOH) 316, 335, 350, and 384 nm (shoulder).

A methanolic solution exhibited strong yellowish-green fluorescence under u.v. light.

I.r. ($CHCl_3$) 3015, 2970, 2905, 2820, 1715, 1625, 1305, 1125 and 1005 cm^{-1}.

N.m.r. ($CDCl_3$; 100 MHz, accumulated 36 sweeps) τ 3.0-4.2 (8H, multiplet, not resolved).

Mass spectrum (175-182°): 276 (M_1), 7.2%; 250 (M_2) 41.6%; 205 (M_2-OC_2H_5), 28%; 204 (M_2-OC_2H_5-H), 16%; 177 (M_2-$COOC_2H_5$), 26.4%; 176 (M_2-$COOC_2H_5$-H), 25.6%; 158 (M_2-92), 17.6%; 132 (M_2-118), 50.4%; 131 (M_2-119), 100%; 121 (M_2-130), 10.4%; 103 (M_2-147), 38.4%; 91 (M_2-159), 38.4%; 77 (M_2-173), 29.6%.

This fraction was characterised as all trans-diethyl 2,4,6,8-decatetraene-1,12 dioate (M_2=250), and perhaps containing a trace amount of all trans-diethyl 2,4,6,8,10-dodecapentaene-1,12-dioate (M_1=276).

(i) Reaction of pigment A-2 with neutral sodium periodate in aqueous methanol for 88 h.

Pigment A-2 (22 mg) λ_{max} (50% MeOH/H_2O) 380 nm, was dissolved in methanol/water (1:1) v/v (10 cm^3). Sodium metaperiodate (214 mg, ca. 0.001 M) dissolved in water (1.5 cm^3) was added all at once to the pigment solution and the mixture was allowed to stand (with occasional shaking) at room temperature in the dark for 88 h. The course of the reaction was followed by absorption spectroscopy on small aliquots removed from the reaction mixture. There was a slow deposition of a white crystalline solid (presumably sodium iodate) while the solution changed from an orange yellow to a pale yellowish brown colour.

Table 26. Changes in the absorption spectra with time during oxidation of pigment A-2 in aqueous methanol.

Time (min)	Absorption spectra (nm) (∿50% MeOH)		Hyperchromic increase (%)
	50% MeOH/H_2O	50% MeOH/H_2O/H^+	
0	380 (0.79)	416-418 (0.92)	16.6
20	376 (0.83)	416 (0.86)	3.5
180	318, 337, 353, 370	318, 337, 353, 390*, 414*, 434*	-
1500	318, 332, 347	318, 332, 347	-

( ) = absorbance

* = weak shoulders

The reaction mixture (which exhibited intense yellowish green fluorescence after 24.5 h and onwards) was reduced in volume on a rotary evaporator at room temperature (∿ 25°C), diluted with water (15 cm^3) and extracted with ether (4x30 cm^3) to give a pale yellow solution, strongly fluorescent green under long wavelength u.v. light. The combined ether extract was washed to neutrality with small portions of water, (added to aqueous solution) and dried overnight with $MgSO_4$ (∿ 2 g). This was designated (a) neutral ether extract. The aqueous solution remaining after the neutral ether extraction which was still pigmented (yellowish brown) was acidified with H_2SO_4 (2N, 4 drops) and this acidified solution was exhaustively extracted with ether (5x30 cm^3). The combined ether extract was now washed to neutrality with water (5x2 cm^3) and dried over $MgSO_4$. This extract was designated (b) acidic ether extract. The aqueous residue remaining after these ether extractions was designated (c) aqueous solution.

(a) Neutral ether extract.

The dried ether solution was filtered, evaporated to dryness to give a yellow solid (∿ 5 mg) which was dried overnight in a desiccator under reduced pressure. The dried sample was dissolved in a small volume of ether/methanol (4:1, v/v) cooled to ∿ 4°, and treated with a freshly prepared solution of diazomethane in ether, prepared from 'Diazald' (Aldrich). The diazomethane solution in ether was doubly

distilled to remove any basic impurities. The methylation mixture was left overnight in the dark at ∿ 4°, evaporated to dryness, and redissolved in a small volume of 95% ethanol. This yellow ethanolic solution was chromatographed on thin layer plates (20x20 cm) uniformly coated (500μ) with silica gel GF254 (Brinkmann Instruments (Canada) Ltd.), developed with benzene/ethyl acetate (9:1); the distance travelled by the solvent front from the origin was 15 cm, while the approximate running time was 50 min. The plates were dried using the cold air from a portable hair dryer, and four fluorescent bands were visible under short wavelength u.v. light.

		R_f
Band I	bright red fluorescence	0.48
Band II	narrow, pale red fluorescence	0.54
Band III	narrow, pale purple fluorescence	0.86
Band IV	broad, deep purple fluorescence	0.91

Each band was eluted from the plate with dichloromethane/methanol (9:1) filtered to remove the silica gel, evaporated to dryness under reduced pressure and then under vacuum overnight.

The spectral data for these bands are recorded below.

Band I (R_f 0.48).

U.v. λ_{max} (MeOH) 303, 317, 332 and 350 nm.

Mass spectrum (175°): m/e 223 (M+1), 18%; 222 (M), 100%; 207 (M-CH_3), 9%; 191 (M-OCH_3), 53%; 190 (M-OCH_3-H), 42%; 163 (M-$COOCH_3$), 70%; 162 (M-$COOCH_3$-H), 64%; 158 (M-64), 50%; 131 (M-91), 88%; 121 (M-101), 59%; 103 (M-119), 84%; 78 (M-144), 82%; 77 (M-143), 82%; 69 (M-153), 53%. This compound has the same R_f as the known compound all-trans dimethyl 2,4,6,8-deca-tetraene-1, 10-dioate R_f (0.47) on silica gel GF254; its mass spectrum was almost identical to that of an authentic sample.

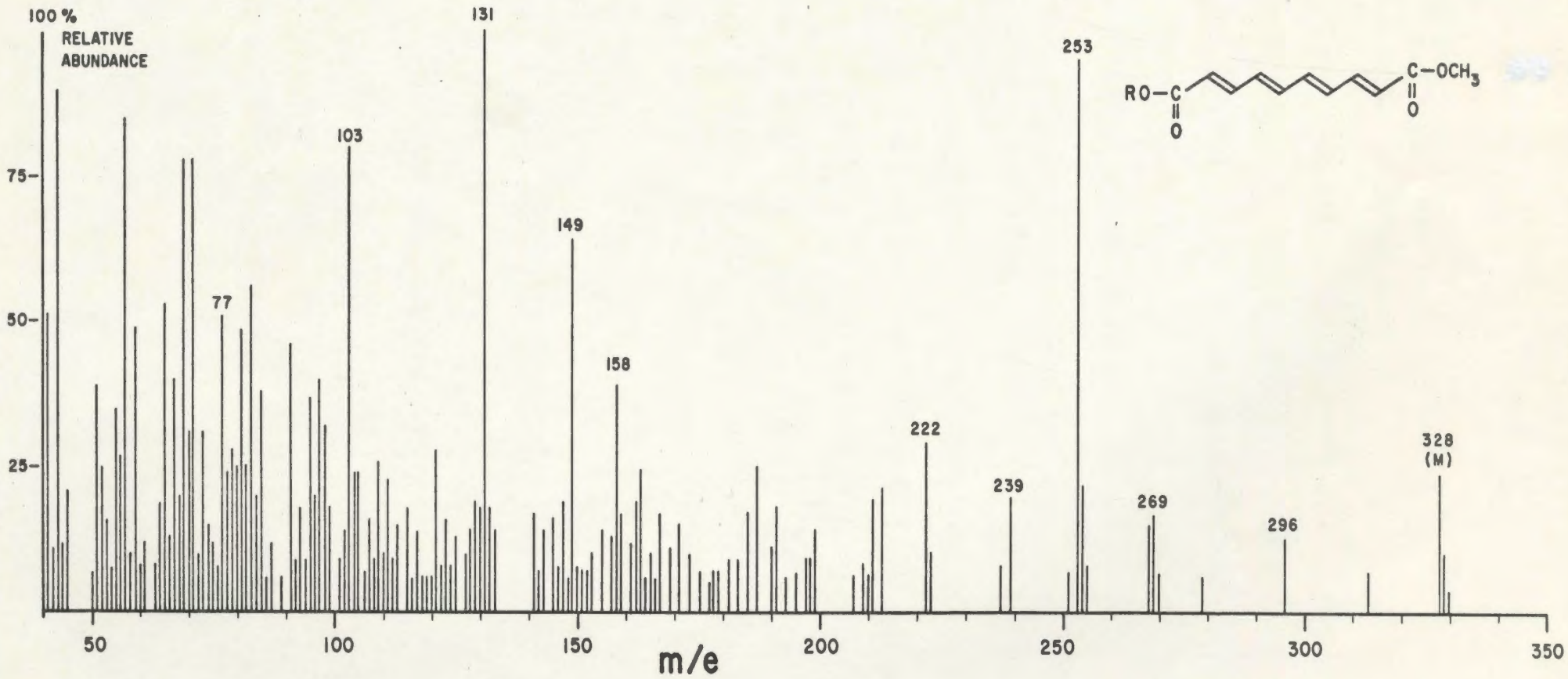
Band II (R_f 0.54).

Band II was less polar than band I.

U.v. λ_{max} (MeOH) 303, 317, 333 and 350 nm, shoulder at 245 nm; the fine structure of this spectrum was remarkably similar to that of band I (R_f 0.48).

Mass spectrum (125°) Fig. 20 : m/e 330 (M+2), 3.8%; 329 (M+1), 10%; 328 (M), 34%; 313 (M-CH_3), 7%; 296 (M-CH_3OH), 13%; 269 (M-$COOCH_3$), 17%; 253 (M-75), 95%; 239 (M-89), 20%; 223 (M-105), 10%; 222 (M-106), 29%; 213 (M-115), 21%; 191 (M-137), 18%; 131 (M-197), 100%; 121 (M-207), 28%; 105 (M-223), 24%; 104 (M-224), 23%; 103 (M-225), 80%; 91 (M-237), 46%; 77 (M-251), 51%; 75 (M-253), 12%; 74 (M-254), 15%; 73 (M-255), 31%; 72 (M-256), 10%; 71 (M-257), 78%; 70 (M-258), 31%; 69 (M-259), 77%; 59 (M-269), 49%; 57 (M-271), 85%; 56 (M-272), 27%; 55 (M-273), 35%; 51 (M-277), 39%; and 43 (M-285), 85%.

Fig. 20. Mass spectrum of sodium periodate degradation product (LXI) from pigment A-2 (*P. flavicomum*).



Band III (R_f 0.86).

The amount of sample isolated from this band was very small and only enough for the determination of its absorption and mass spectra.

U.v. λ_{max} (MeOH) 207, 240, 251 and 265 nm (all weak shoulders).

Mass spectrum (100°) Fig. : m/e 318 (M+4), 12%; 317 (M+3), 10%; 315 (M+1), 316 (M+2), 21%; 314 (M), 77%; 313 (M-1), 7%; 312 (M-2), 14%, 299 (M-15), 100%; 241 (M-73), 24%; 240 (M-74), 71%; 239 (M-75), 61%.

Band IV (R_f 0.91).

Again, the amount of sample isolated here was very small and only its absorption and mass spectra were determined.

U.v. λ_{max} (MeOH) 208, 230 and 259 nm (weak shoulders).

Mass spectrum (120°) m/e 345 (3%); 336 (5%); 330 (5%); 323 (5%); 320 (6%); 316 (5%); 310 (5%); 309 (6%); 308 (7%); 307 (5%); 306 (6%); 305 (5%); 304 (5%); 302 (5%); 296 (5%); 295 (7%); 294 (7%); 293 (5%); 292 (7%); 291 (6%); 290 (5%); 289 (5%); 287 (5%); 282 (5%); 281 (8%); 280 (8%); 279 (6%); 278 (7%); 277 (6%); 276 (5%); 273 (5%); 267 (9%); 266 (8%); 265 (6%); 264 (7%); 263 (7%); 262 (5%); 261 (6%); 259 (6%); 254 (7%); 253 (12%); 252 (9%); 251 (7%); 250 (7%); 249 (8%); 248 (5%); 247 (7%); 246 (5%); 245 (6%); 239 (14%); 238 (8%); 237 (8%); 236 (7%); 235 (9%); 234 (6%); 233 (8%); 232 (5%); 231 (7%); 229 (5%); 227 (5%); 226 (6%); 225 (14%); 224 (10%); 223 (10%); 222 (8%); 221 (11%); 220 (7%); 219 (11%); 218 (6%); 217 (9%); 215 (6%); 213 (5%); 211 (15%); 210 (10%); 209 (13%); 208 (9%); 207 (13%); 206 (8%); 205 (12%); 204 (5%); 203 (9%);

202 (5%); 201 (5%); 199 (6%); 198 (5%); 197 (20%); 196 (12%); 195 (15%); 194 (10%); 193 (17%); 192 (8%); 191 (15%); 190 (6%); 189 (10%); 187 (7%); 185 (7%); 184 (6%); 183 (23%); 182 (14%); 181 (18%); 180 (11%); 179 (20%); 178 (10%); 177 (14%); 176 (5%); 175 (8%); 173 (9%); 171 (11%); 170 (8%); 169 (28%); 168 (16%); 167 (23%); 166 (12%); 165 (24%); 163 (17%); 162 (5%); 161 (10%); 160 (5%); 159 (13%); 158 (5%); 157 (10%); 156 (8%); 155 (33%); 154 (17%); 153 (27%); 152 (15%); 151 (28%); 150 (10%); 149 (27%); 148 (7%); 147 (12%); 146 (5%); 145 (16%); 144 (6%); 143 (12%); 142 (8%); 141 (40%); 140 (20%); 139 (39%); 138 (19%); 137 (36%); 136 (12%); 135 (22%); 134 (10%); 133 (20%); 132 (6%); 131 (15%); 130 (6%); 129 (10%); 128 (9%); 127 (47%); 126 (30%); 125 (60%); 124 (27%); 123 (50%); 122 (12%); 121 (20%); 120 (14%); 119 (27%); 118 (6%); 117 (9%); 115 (7%); 114 (6%); 113 (67%); 112 (24%); 111 (67%); 110 (38%); 109 (70%); 108 (13%); 107 (19%); 106 (12%); 105 (25%); 100 (7%); 99 (21%); 98 (40%); 97 (43%); 96 (58%); 95 (29%); 94 (16%); 93 (23%); 92 (7%); 91 (22%); 86 (14%); 85 (52%); 84 (56%); 83 (42%); 82 (14%); 81 (100%); 79 (26%); 77 (11%); 72 (18%); 71 (23%); 70 (23%); 69 (53%); 68 (30%); 67 (21%); 66 (5%); 61 (20%); 58 (21%); 57 (40%); 56 (24%); 55 (65%); 54 (16%); 53 (14%); 43 (21%); and 42 (30%).

(b) Acidic ether extract.

After removal of the $MgSO_4$ by filtration, the ether extract was evaporated to dryness and was redissolved in a small volume of ether/methanol (4:1) as was done for the neutral

ether extract. Treatment of this solution with excess diazomethane in ether at ∿ 4° and subsequent t.l.c. on silica gel revealed four fluorescent bands, and their spectral data are recorded below.

Band I (R_f 0.45)

U.v. λ_{max} (MeOH) 303, 318, 333 and 350 nm.

Mass spectrum (168°): m/e 222 (M), 100%; 207 (M-CH_3), 9%; 191 (M-OCH_3), 54%; 190 (M-OCH_3-H), 51%; 163 (M-$COOCH_3$), 77%; 162 (M-$COOCH_3$-H), 68%; 158 (M-64), 48%; 131 (M-91), 90%; 121 (M-121), 54%; 103 (M-119), 76%; 91 (M-131), 76%; 79 (M-143), 29%; 78 (M-144), 57%; 77 (M-145), 97%; and 69 (M-153), 41%.

Band II (R_f ∿ 0.54)

U.v. λ_{max} (MeOH) 305, 318, 334 and 350 nm.

Mass spectrum (120-135°): m/e 330 (M+2), 4%; 329 (M+1), 12%; 328 (M), 43%; 313 (M-CH_3), 7%; 297 (M-OCH_3), 5%; 296 (M-CH_3OH), 13%; 269 (M-$COOCH_3$), 17%; 268 (M-$COOCH_3$-H), 14%; 253 (M-75), 100%; 131 (M-197), 51%; 131 (M-197), 24.3%; 103 (M-225), 39%; 91 (M-237), 39%; 77 (M-251), 51%.

Band III (R_f ∿ 0.86).

Because of the small amount of material in this degradation product only the absorption and mass spectra could be determined, and these were similar to band III (neutral ether extract) already described.

Band IV (R_f ∿ 0.90)

A very small amount of this degradation product was isolated, and only absorption and mass spectra were determined, and

these were similar to band IV (neutral ether extract) described.

(c) Aqueous solution.

U.v. λ_{max} 222 nm. The u.v. spectrum (after 2 weeks) of this solution which had been kept in the dark at room temperature showed a maximum at 450 nm, but this fraction was not investigated further.

(ii) Reaction of pigment A-2 with neutral sodium periodate in aqueous methanol for 48 h.

Pigment A-2 (25 mg) was dissolved in 50% aqueous methanol (5 cm^3) and sodium periodate (107 mg) added, and the mixture was kept at room temperature in the dark. After 30 min, there was deposition of a white crystalline precipitate. A further 400 mg of sodium periodate was added 30 min. later, and then allowed to react (with occasional shaking) for 48 h. The reaction mixture was reduced in volume, diluted with water (4 cm^3) and acidified with H_2SO_4 (2N). Exhaustive extraction with ether (5x25 cm^3) produced a pale yellow strongly fluorescent solution which was washed to neutrality, and dried overnight. After evaporation, the extract was redissolved in ether/methanol (4:1) cooled in an ice bath and treated with an ethereal solution of diazomethane. The methylated product was chromatographed on silica gel GF254, developed with benzene/ethyl acetate (7:3). Five bands were eluted with dichloromethane/methanol, 9:1 (v/v).

(a) ($R_f \sim 0.27$), yellow oil.

U.v. λ_{max} (MeOH) 303 nm (broad).

I.r. ($CHCl_3$) 1735, 1708, 1620, and 1005 cm^{-1}.

Mass spectrum (152°): m/e 223 (6%); 153 (13%); 135 (29%); 127 (39%); 115 (15%); 95 (100%); 91 (25%); 59 (47%); 57 (26%) and 55 (39%).

(b) ($R_f \sim 0.38$), yellow oil.

U.v. λ_{max} (MeOH) 303 nm (broad).

I.r. ($CHCl_3$) 1735, 1708, 1620 and 1005 cm^{-1}.

Mass spectrum (140°): the highest a.m.u. was recorded at m/e 181.

(c) ($R_f \sim 0.74$), yellow crystalline solid m.p. 205-207°.

U.v. λ_{max} (MeOH) 302, 318, 333, and 350 nm.

I.r. ($CHCl_3$) 1700 and 1623 cm^{-1}.

Mass spectrum (122°): m/e 223 (M+1), 13%; 222 (M), 50%; 207 (M-CH_3), 12%; 191 (M-OCH_3), 33%; 190 (M-OCH_3-H), 21%; 163 (M-$COOCH_3$), 33%; 158 (M-64), 46%; 131 (M-91), 100%; 121 (M-101), 33%; 111 (M-111), 83%; 103 (M-119), 73%; 79 (M-143), 39%; 78 (M-144), 23%; and 77 (M-145), 48%, characterised as all <u>trans</u>-dimethyl 2,4,6,8-decatetraene-1, 10-dioate.

(d) ($R_f \sim 0.81$), yellow crystalline needles m.p. 211-214°.

U.v. λ_{max} (MeOH) 302, 318, 333 and 350 nm.

I.r. ($CHCl_3$) 1700, 1622, 1305, 1125 and 1005 cm^{-1}.

Mass spectrum (128°): m/e 248 (M_1) 1.8%; 222 (M_2) 51%;

131 (M_2 - 91), 100%. The mass spectrum of this fraction was very similar to fraction (c), and was characterised as containing diester (XXXVI) as the major product, along with a trace of diester (XXXVII).

(e) ($R_f \sim 0.83$ - 1.0) yellow solid, m.p. 194 - 208°.
U.v. λ_{max} (MeOH) 300, 316, 333, and 350 nm.
I.r. ($CHCl_3$) 2910, 2840, 1720, 1625, 1125 and 1010 cm^{-1}.
Mass spectrum (120°): m/e 298 (3.9%); 294 (8.1%); 270 (8%); 264 (3.0%); 255 (2.7%); 241 (3.0%); 239 (4.8%); 227 (6.3%); 222 (7.7%); 199 (5.7%); 185 (5.7%); 171 (4.8%); 157 (4.5%); 143 (16.3%); 129 (8.9%); 115 (6.6%); 101 (9.8%); 97 (37.4%); 96 (30.9%); 95 (36.8%); 91 (30.4%); 87 (62.6%); 83 (40.8%); 82 (33.3%); 81 (37.4%); 80 (14.0%); 75 (22.4%); 74 (100%); 71 (41.5%); 69 (53.0%); 57 (59.2%) and 55 (68.0%).

Reactions with sodium periodate (continued).

(iii) Potassium carbonate/sodium periodate oxidation of pigment 2 P. flavicomum in aqueous methanol.

The method employed here is essentially that used by Cope and Johnson (88) investigating the structure of fungichromin (IX) a polyene macrolide isolated from the genus Streptomyces. (Fungichromin was shown to contain a mcarocyclic lactone, which after hydrolysis exposes a 1,2-diol system which is oxidised by periodate to the corresponding aldehyde.)
Pigment 2 (129 mg) λ_{max} (MeOH) 370 nm was dissolved in methanol (20 cm^3) and potassium carbonate (200 mg, Baker) dissolved in water (5 cm^3), was added. The solution was refluxed on the

steam bath for 30 min, and after cooling the absorption spectrum of a small aliquot was found to have a single maximum ∿ 372 nm. (A portion of this solution in alkali was left for 35 days at room temperature, but the spectrum was unchanged after this time suggesting that the original chromophore was still intact). Sodium periodate (600 mg) in water (20 cm^3) was slowly added to the bulk of the pigment solution, and the resulting mixture was allowed to stand in the dark at room temperature for 72 h. During this time the orange solution slowly changed to brown; there was also a white crystalline precipitate (presumably sodium iodate) formed during the reaction. The pale yellow reaction mixture was extracted with ether (4x20 cm^3) while basic and then acidified with sulphuric acid (2N) at 0°C. This acidified solution was then exhaustively extracted with ether (5x20 cm^3) and the combined ether extracts washed to neutrality with water and dried over $MgSO_4$. The respective fractions are now designated

(a) basic ether extract

(b) acidic ether extract

(c) aqueous residue (not investigated further).

(a) Basic ether extract

U.v. λ_{max} (MeOH) 224-232 (broad) and 270-272 nm.
I.r. ($CHCl_3$) 3500 (OH), 3400-3000 (H--O-H), 2915, 2845, 2750-2400 (broad), 1712, 1470, 1450 and 1380 cm^{-1}.
N.m.r. ($CDCl_3$, 60 MHz) τ8.73.

Mass spectrum (115°): 284 (4.8%); 270 (3.0%); 264 (5.8%); 257 (6.7%); 256 (33.0%); 241 (3.0%); 227 (7.3%); 213 (18.5%); 199 (10.6%); 185 (20.9%); 171 (17.3%); 157 (16.7%); 143 (15.5%); 129 (43.6%); 122 (24.2%); 115 (18.6%); 111 (24.8%); 101 (18.8%); 99 (16.7%); 98 (26.7%); 97 (43.6); 96 (18.2%); 95 (15.5%); 87 (43.9%); 85 (37.6%); 84 (29.7%); 83 (55.5%); 82 (17.3 %); 81 (20%); 74 (52.1%); 71 (61.8%); 70 (25.8%); 69 (64.5%); 60 (80.0%); 57 (100%); and 55 (94.5%).

(b) Acidic ether extract

This yellow ether extract divided into two parts, and the following investigation carried out on one part.

U.v. λ_{max} (MeOH) 302, 316, 331 and 347 nm, the spectrum showing a small hypsochromic shift on adding NaOH (2N, 2 drops) to 298, 312, 326 and 342 nm.

Reaction with sodium borohydride.

A solution of the extract in methanol (5 cm^3, absorbance 1.9 in 1 cm cell) was treated with sodium borohydride (5 mg). The u.v. spectrum exhibited peaks at 298, 312, 326 and 342 nm, which were shifted to 302, 316, 331 and 347 nm on acidification (as for acidic ether extract above) suggesting that a reducible carbonyl function, if present, was not in conjugation with the chromophore. A similar spectral behaviour was recorded for 2,4,6,8- decatetraene 1,8-dicarboxylic acid, a degradation product of fumagillin.

Examination of the acidic ether extract.

The acidic extract (oil) was dissolved in aqueous sodium bicarbonate solution (10%) which was now re-extracted with ether (2x20 cm^3). Acidification of the bicarbonate solution with HCl (2N) followed by exhaustive ether extraction (5x20 cm^3), washing to neutrality, drying over $MgSO_4$ and finally evaporation produced a yellow oil.

U.v. λ_{max} (MeOH) 302, 316, 331 and 347 nm.

Extraction into bicarbonate and re-extraction (after acidification of the aqueous solution) into ether suggested that this degradation product contained at least one carboxyl group.

Treatment of the yellow oil with diazomethane.

The yellow oil was redissolved in ether/methanol (4:1), cooled to $\sim 4°$ in an ice bath and treated with a solution of diazomethane in ether. Evaporation of this methylation product was followed by column chromatography on alumina (Act III, 10x1 cm) eluted with 5% methanol in benzene, and three fractions were collected.

Fraction 1 (2 mg)

U.v. λ_{max} (MeOH) 302, 317, 331 and 347 nm.

I.r. ($CHCl_3$) 2955, 2925, 2855, 1712, 1625, 1600, and 1010 cm^{-1}.

Mass spectrum (150°): m/e 315 (M ?) 5%; 297 (9%); 222 (17%); 203 (8%); 191 (8%); 191 (8%); 190 (6%); 186 (12%); 185 (100%); 131 (31%); 121 (8%); 113 (26%); 112 (42%); 111 (14%); 103 (19%); 98 (21%); 97 (20%); 85 (16%); 84 (15%); 83 (28%); 71 (53%); 70 (36%); 69 (24%); 57 (69%); 55 (28%) and 43 (19%). This

fraction probably contains some of fraction 2, as several of the peaks are common to both spectra.

Fraction 2 (2 mg)

U.v. λ_{max} (MeOH) 303, 318, 333, and 350 nm, unchanged after treatment with sodium borohydride.

I.r. ($CHCl_3$); 2975, 2945, 2910, 2825, 1710, 1628, 1575, 1460, 1435, 1355, 1312, 1270, 1165, 1130 and 1012 cm^{-1}.

Mass spectrum (150-155°): m/e 222 (M), 57%; 207 (M-CH_3), 2.7%; 191 (M-OCH_3), 24%; 190 (M-OCH_3-H), 17%; 163 (M-$COOCH_3$), 27%; 162 (M-$COOCH_3$-H), 18%; 158 (M-64), 55%; 131 (M-91), 100%;

This degradation product was characterised as all-trans dimethyl 2,4,6,8-decatetraene-1,10-dioate.

Fraction 3.

U.v. λ_{max} (MeOH) 305-309 (broad), 350 (shoulder), and 210-222 nm (broad).

I.r. ($CHCl_3$) 3650, 3575, 3520, 3375, 2975, 2945, 2925, 2855, 2850, 1712, 1620, 1600, 1460, 1435, 1400, and 1005 cm^{-1}.

Mass spectrum (152-160°): m/e 315 (1%); 297 (2%); 205 (5%); 185 (27%); 153 (5%); 149 (8%); 143 (5%); 139 (5%); 133 (5%); 131 (34%); 121 (27%); 111 (35%); 107 (30%); 99 (18%); 98 (23%); 97 (48%); 96 (24%); 95 (68%); 93 (33%); 85 (39%); 84 (25%); 83 (76%); 82 (79%); 81 (52%); 79 (36%); 78 (16%); 77 (29%); 75 (51%); 71 (70%); 69 (67%); 57 (100%); and 43 (88%).

Oxidation of pigment A-2 with potassium permanganate in acetone.

The method used here was essentially according to Nagel, Steyn and Ferreira (113) for the oxidation of citreoviridin, a polyene neurotoxin isolated from Penicillium pulvillorum. Pigment A-2 (∿ 4 mg) dissolved in a mixture of acetone (2 cm^3) and water (1 cm^3) was treated with powdered $KMnO_4$ (ca. 17 mg) over a period of 3 h at 5-15° until the solution remained a dark red. Water (2 cm^3) was added and SO_2 gas passed through the solution until it became clear. The solution was acidified with hydrochloric acid (6M) and extracted with ether (3x5.0 cm^3). The aqueous residue exhibited strong white fluorescence. The combined ether extract was taken up into 10% $NaHCO_3$, and this was then acidified with hydrochloric acid (12 M) and extracted again with ether. The ether layer was washed to neutrality, dried and evaporated to dryness.

U.v. λ_{max} (Ether) 275 nm; when the u.v. spectrum was remeasured 24 h later, the maximum at 275 nm had almost disappeared, being replaced by a weak shoulder at 275-280 nm. This decomposition was probably due to the presence of a trace of acid in the solution, although this is pure speculation. However, further work was not possible.

Aqueous residue:-

This solution was very strongly fluorescent yellow-green under u.v. light.

U.v. λ_{max} (MeOH) 210 and 258 nm.

An aliquot of the aqueous extract was resolved into three components by paper chromatography (3MM, Whatman), developed with the upper layer of the solvent mixture n-butanol acetic acid/water (4 : 1 : 5).

(i) R_f 0.23 (exhibiting moderate white fluorescence)

(ii) R_f 0.43 (pale white fluorescence)

(iii) R_f 0.51 (exhibiting strong white fluorescence).

Test with p-dimethylaminobenzaldehyde (Erhlich's test) (107e)

Aqueous extract (2 drops) was reacted with p-dimethylaminobenzaldehyde in conc. HCl (5 drops). A brown colour was obtained, showing that pyrroles and indoles were absent.

Acid hydrolysis of pigment A-2 with 2N hydrochloric acid.

Pigment A-2 (14 mg) was dissolved in water (16 cm^3) and hydrochloric acid (2N, 1 cm^4) added, producing a bright orange solution. The reaction mixture was refluxed under nitrogen for 17 h, and the nitrogen effluent was passed into a solution of 2,4- dinitrophenylhydrazine in acidified ethanol. After cooling, the reaction mixture was extracted with ether (3x25 cm^3) to give a bright yellow solution which was strongly fluorescent under u.v. light. This solution was washed to neutrality, and dried over 4A molecular sieves (instead of $MgSO_4$) in an effort to expedite the drying process but some product loss was encountered although most of it could be extracted from the drying agent by methanol. This organic extract was called extract I. The aqueous suspension was filtered to give a yellowish brown solution that was evaporated to dryness (extract II) and a reddish brown precipitate (extract III) λ_{max} (MeOH) 414 nm (unreacted protonated pigment) that was not further investigated. The absence of a precipitate in the 2,4, DNP solution was interpreted to mean that no volatile carbonyl compound was produced during the hydrolysis.

Extract I.

This yellow solution was evaporated to dryness to give a yellow oil ($\sim$ 2 mg) and absorption spectra in both ether and methanol were determined.

U.v. λ_{max} (ether) 300, 315, 328 and 345 nm; λ_{max} (MeOH) 315, 329 and 344 nm, and this typical polyene system was not modified

after reaction with sodium borohydride in methanol, the solution then possessing λ_{max} (MeOH/$NaBH_4$) 312, 328 and 342-45 nm reverting to λ_{max} (MeOH) 314, 331 and 347 nm on acidification.

Mass spectrum (150°): the highest mass unit was recorded at m/e 279, (12.3%) of base peak 149 (100%), and 167 (27.4%) which suggested that it was mainly a spectrum of contaminants in the sample. This extract was, however, catalytically reduced with 5% Pd/C in methanol for 24 h at room temperature.

I.r. ($CHCl_3$) (weak spectrum) 3670, 3570 (OH), 2950, 2920, 2850, 1730 (weak), 1605 (shoulder) and 1600 cm^{-1}.

Mass spectrum (128-130°): m/e 314 (M?), 2.5%; 299 ($M-CH_3$) 4.2%; 239, 12%; 199, 32.8%; 196, 16.4%; 166, 29.1%; 165, 18.6%; 157, 32.3%; 139, 16.9%; 138, 25.3%; 125, 80%; 107, 29.5%; 101, 15.1%; 98, 51.3%; 97, 54.7%; 87, 53.9%; 85, 19.7%; 84, 41.3%; 83, 14.8%; 74, 100%; 71, 22.5%; 69, 41.7%; 57, 37.8%; 55, 70.3% and 43, 62.3%.

Extract II.

This yellow aqueous solution obtained after ether extraction was evaporated to dryness and could be redissolved in either methanol or water and both solutions were intensely fluorescent (yellow green). A brown colour was obtained when a small aliquot of this extract was reacted with ninhydrin, but this could not be taken as positive proof for the presence of amino acids. U.v. λ_{max} (MeOH) 258 nm, changing to 265-272 nm in sodium methoxide, suggested the presence of a phenolic OH.

The u.v. spectra of several possible models for this chromophore were investigated with the hope of identifying this product (if only at least tentatively). Table 27 lists the absorption spectra of these possible models.

Mass spectrum (150°): a very weak spectrum was obtained, with peaks at 211, 149, 141 and 71, thus making any meaningful interpretation almost impossible.

Table 27. Ultraviolet spectra of water soluble acid hydrolysis product and some possible chromophores.

Compound	Ultraviolet spectra (nm) methanol or water	
	Acidic	Basic
extract II	208, 257	208 (intense) 266-72 (broad)
p-hydroxy benzoic acid	208, 255	208, 274
3,4-dihydroxy toluene	264	265-269
O-amino-phenol	210-16, 272,	243, 298
nicotinic acid	256, 263, 270	256, 263, 270
sorbic acid	257	253
cytidine +	213, 284	233, 275
uridine +	207, 262	207, 222, 262
guanosine +	255, 278	263
adenine	214, 262	221, 270 (shoulder)

+ in water

(a) Test with 2,3,5-triphenyltetrazolium chloride.

When extract II (2 drops) was heated with the reagent (as described previously), a pink solution with a reddish brown precipitate was obtained, while a blank remained colourless, suggesting a reducing sugar may be present. Feigl (107a) states that aldehydes, hydrazine, hydroxylamine, sulphites, tartaric and citric acid are completely inactive towards this reagent.

(b) In a test for ketohexoses (107b) no reaction was observed when extract II (2 drops) was reacted with stannous chloride, sulphuric acid and urea reagent (5 drops); however, a blue colour was produced with fructose, while a blank test solution remained colourless.

(c) Extract II (2 drops in water) was treated with 2,4-DNP solution. The solution darkened with some evidence of a precipitate, suggesting the presence of a carbonyl containing compound.

Paper chromatography.

An aliquot of the aqueous extract was resolved into three components on paper chromatography (3MM, Whatman) developed with the upper layer of n-butanol/acetic acid/water (4 : 1 : 5)

(i) R_f 0.23 (strong white fluorescence)

(ii) R_f 0.37 (strong white fluorescence)

(iii) R_f 0.46-0.93 (broad streak, exhibiting strong white fluorescence).

Test with p-dimethylaminobenzaldehyde (107e)

Extract II (3 drops) was treated with p-dimethyl-aminobenzaldehyde 5% in conc. HCl (5 drops) in a porcelain plate. A pale yellow solution resulted, contrasting with a bright violet-purple colouration obtained when indole was used as a test sample. The test was therefore negative. In other acid hydrolyses experiments, a degradation product which was characterised as p-hydroxy benzoic acid mainly by u.v., t.l.c. and mass spectrometry of the methyl ester and ether, but it is uncertain whether this product is an authentic component of the native pigment.

Hydrolysis of pigment 2 with (a) hydrochloric acid (2N) and (b) sodium hydroxide (2N).

Pigment 2, (∿ 100 mg) isolated by Sephadex LH-20 column chromatography, was refluxed on a steam bath for 2 h with hydrochloric acid (2N, 20 cm^3); the solution was cooled to room temperature, neutralised with sodium hydroxide (2N) and a further 10 cm^3 of alkali added. This basic solution was now refluxed for 45 min, cooled to room temperature and exhaustively extracted with dichloromethane (5x60 cm^3) after acidification to give (a) an organic extract and (b) an aqueous residue u.v. λ_{max} (MeOH) 315, 330, 350 and 389-400 nm.

(a) organic (CH_2Cl_2) extract.

This extract was washed to neutrality, dried over $MgSO_4$ for 24 h and evaporated to dryness to give a yellow oil (-15 mg). U.v. λ_{max} (MeOH) 315, 332, and 350 nm (polyene type spectrum). Catalytic hydrogenation of a methanolic solution of this extract with platinum oxide (30 mg) for 24 h gave a white crystalline solid (∿ 20 mg) m.p. 61-63° with no u.v. spectrum indicating that the polyene had been completely saturated. I.r. ($CHCl_3$) 3490 (free OH), 3300-3000 (broad, H-bonded OH), 2910, 2845, 2750-2400 (broad, H-bonded OH), 1740, 1705 (C=O) and 1465 cm^{-1}.

G.l.c. (Pye Chromatograph, column 1% OV-17, 4.5m x 6.6mm) separated the hydrogenated product into a major component with a trace amount of another compound.

Mass spectrum (100°) m/e 312 (M'), 1.2%; 285 (M+1), 6.2%; 284 (M), 25.9%; 256 (M-28), 21.2%; 241 (12.1%); 227 (M-57), 10%; 213 (M-71), 14%; 199 (M-85), 13%; 185 (M-99), 26%; to 57 (M-227), 100%, fragmentation pattern typical of a mixture of a long chain fatty acid (M=284, major) and (M'=312, trace) (122), but perhaps containing a primary alcohol which could be obtained from the catalytic reduction of a conjugated polyene aldehyde. (The current working hypothesis was that the chromophore of pigment 2 consisted of a conjugated polyene Schiff's base.) The product recovered above was methylated with CH_2N_2 in ether at ∿ 4°, to give a white solid after evaporation. This compound was then refluxed with p-phenylazobenzoyl chloride in pyridine for 90 min (123), diluted with water, cooled, and extracted with methylene chloride (3x10 cm^3), to give a lower organic layer and an (upper) aqueous yellow extract λ_{max} (EtOH) 326 and 441-45 nm.

The methylene chloride extract was washed to neutrality, and then evaporated to dryness giving a white solid, m.p. 39.1°. I.r. ($CHCl_3$) 2910, 2840, 1725 (C=O of satd. ester) cm^{-1}. Mass spectrum (63°): m/e 326 (M'), 1.2%; 299 (M+1), 4.7%; 298 (M), 19.8%; 270 (M-28), 6.7%; 255 (M-43), 7.7%; 241 (M-57), 3.8%; 227 (M-71), 4.5%; 213 (M-85), 4.5%; 199 (M-99), 9.9%; 185 (M-113), 5.7%; 171 (M-127), 3.8%; 157 (M-141), 4.5%; 143 (M-155), 18.2%; 129 (M-169), 6.7%; 87 (M-211), 61.9%; 75 (M-223), 29.4%; 74 (M-224), 100%; 69 (M-229), 10%; 57 (M-241), 13.2%; 55 (M-243), 14%; and 43 (M-255), 18.8%.

This product was characterised as a mixture of methyl stearate (M, 298, major), and methyl arachidate (M, 326, trace).

Acidification of the yellow aqueous extract precipitated a cream coloured solid (m.p. 245-246°) characterised as p-phenyl-azo benzoic acid (m.p. 248.5-249.5° (57b)).

Preparation of retinylidene-t-butylamine.

The method used was similar to that reported by Irving and Leermakers (49). All trans-retinaldehyde (10 mg, Sigma) was dissolved in methanol (2 cm^3), excess t-butylamine added (5 cm^3) and the solution was allowed to stand over 4A molecular sieves for 24 h. The solution was then decanted, and the excess amine and solvent removed under reduced pressure, giving solid retinylidene-t-butylamine. Mass spectrum, m/e 339 (M), 16 %; 324 (M-CH_3), 5%; 282 (M-butyl-H), 5%; 268 (M-butyl-H-N), 7%, 103 (M-236)100%. Other Schiff bases derived from retinal and various amines were also prepared, and their absorption spectra, measured before and after protonation, are given in Table 28. Schiff bases formed from glycine, lycine and serine were prepared by the method of Ball et al (39), in which the mixture is made alkaline with aqueous NaOH (2N), allowed to stand for 30 min, and their absorption spectra then measured.

Sodium borohydride reduction of retinylidene-t-butylamine.

Retinylidene-t-butylamine (5 mg) was dissolved in 95% ethanol (5 cm^3) and sodium borohydride (10 mg) was added. The yellow colour of the solution was discharged immediately, but the solution was allowed to react for a further 10 min. The absorption spectrum showed the presence of a reduced chromophore absorbing at 328 nm (cf. retinol λ_{max} (EtOH) 328 nm).

Preparation of α-furylacrylylidene-t-butylamine.

The method used was identical to that described for the preparation of retinylidene-t-butylamine but using furylacrylaldehyde (25 mg) and t-butylamine (5 cm^3) in methanol (5 cm^3). The absorption spectra of the various other Schiff bases prepared are given in Table 29 .

Treatment of 9-anthranylidene-p-chloroaniline with silver oxide/methyl iodide.

9-Anthranylidene-p-chloroaniline (250 mg) (kindly provided by Dr. S.J. Griffiths, Dept. of Chemistry) was treatment with silver oxide (4g) and excess methyl iodide for 48 h. The mixture was extracted with chloroform (5x20 cm^3), which was washed with 0.1N HCl, and then H_2O to neutrality and dried over $MgSO_4$. Examination of the evaporated extract by mass spectrometry showed an ion at m/e 236, (possibly the molecular ion of 9-anthranilic acid methyl ester).

Table 28. Table of Schiff bases derived from retinaldehyde in neutral and acidic methanol.

Amino compound	Absorption max. methanol (nm)	Absorption max. methanol/HCl* (nm)	Δλ
methylamine	360	436	76
n-butylamine	370	440	70
t-butylamine	366	442	76
cyclohexylamine	368	440	72
benzylamine	368	446	78
aniline	398	496	98
glycine	370	450	80
lysine	370	446	76
serine	370	450	80

*solution colour changed to orange-yellow on adding HCl.

Table 29. Table of Schiff bases derived from α furylacryl-aldehyde in neutral and acidic methanol.

Amino compound	Absorption max. in methanol (nm)	Absorption max. in methanol/HCl (nm)	Δλ
methylamine	307	358	51
n-butylamine	307	359	52
t-butylamine	305	359	54
cyclohexylamine	309	360	51
benzylamine	310	365	55
ethylene diamine	310	370	60
aniline	394	396*	2*
glycine	314	267, 366	52
lysine	313	265, 364	51

*incomplete reaction?

Catalytic hydrogenation of retinaldehyde with Adams catalyst.

Retinaldehyde (100 mg, Sigma) was dissolved in methanol (20 cm^3) to give a yellow solution and platinum oxide (60 mg, MCB), suspended in methanol was carefully added. The mixture was then set up on a hydrogenator at room temperature and pressure, as described on p.193. After 48 h, the catalyst was removed by filtration and the now colourless solution was evaporated to dryness and then purified by t.l.c. on silica gel GF254 plates (20x20 cm, 400μ) eluted with benzene to give a colourless solid (30 mg).

I.r. (CCl_4) 3010, 2950, 2855, 1455, 1370, 1355, and 1110 cm^{-1}. Notably absent is a carbonyl stretching frequency of retinaldehyde at 1664 cm^{-1}.

Mass spectrum (150°) 278 (M) 26%; 263 (M-CH_3) 29%, 69(M-209)100%.

The hydrogenation product has therefore been assigned as 3,7-dimethyl-1-(2',6',6'-trimethyl-1'-cyclohexen-1-yl)-nonane. An ion corresponding to m/e 280 (8.9%) has been assigned as 3,7-dimethyl-1-(2',6',6'-trimethyl-1'-cyclohexyl)-nonane.

EXPERIMENTAL II

Culture and isolation of pigments from plasmodia of Physarum polycephalum.

The Myxomycete Physarum polycephalum was grown in shake flask liquid culture (500 cm^3) as described for P. flavicomum. The extraction of the plasmodial pigments has already been summarised in Chart 1, while Table 30 summarises the yield of lyophilised plasmodial extract per 100 g wet plasmodia.

Purification of plasmodial pigments from P. polycephalum.

The plasmodial pigments from P. polycephalum were previously purified in our laboratory (34) by column chromatography on silicic acid in a glass column eluted with ethyl acetate etc. (Chart 2). Concurrent studies on the separation of the pigments of P. flavicomum on various types of Sephadex gels allowed us to make a preliminary study of the purification of the pigments from the related P. polycephalum. The lyophilised plasmodial extract was chromatographed on Sephadex LH-20, and at least four pigments could be consistently obtained. Pigment 3 was selected for further study but only preliminary results are reported. Chart 5 summarises the isolation and purification of these plasmodial pigments from this organism.

Table 30. Yield of wet plasmodia and dry weight after lyophilisation (P. polycephalum).

Volume of media	Growth period (h)	Wet plasmodia (g)	Plasmodia after lyophilisation (g)	Dry plasmodia/ 100 g wet wgt.
4 x 500 cm^3 (2L)	96	155	2.90	1.87
4 x 500 cm^3 (2L)	96	139	2.50	1.80
4 x 500 cm^3 (2L)	68	82	2.25	2.70
4 x 500 cm^3 (2L)	94	214	4.74	2.21

The average yield of lyophilised plasmodial extract per 100 g wet plasmodia was 1.8 - 2.7 g.

Column chromatography of plasmodial pigments of P. polycephalum on Sephadex LH-20 eluted with 80% aqueous methanol.

Sephadex LH-20 (175 g) was suspended in 80% aqueous methanol (800 cm^3), and the gel was allowed to swell at room temperature for 24 h. This swollen gel was now used to prepare a glass chromatographic column (dimensions 35 x 5 cm) after complete settling of the gel. Lyophilised plasmodial extract (4.7 g) was extracted with 80% methanol (30 cm^3) and the pigmented solution filtered. This filtrate was then chromatographed as described for P. flavicomum with an approximate flow rate of 100 cm^3/h. Four distinct pigment bands were evident.

Band I - a fast moving yellowish brown band, but darker brown in some samples.

Band II - a narrow yellow band.

Band III - a bright orange pigmented band.

Band IV - a narrow yellow band.

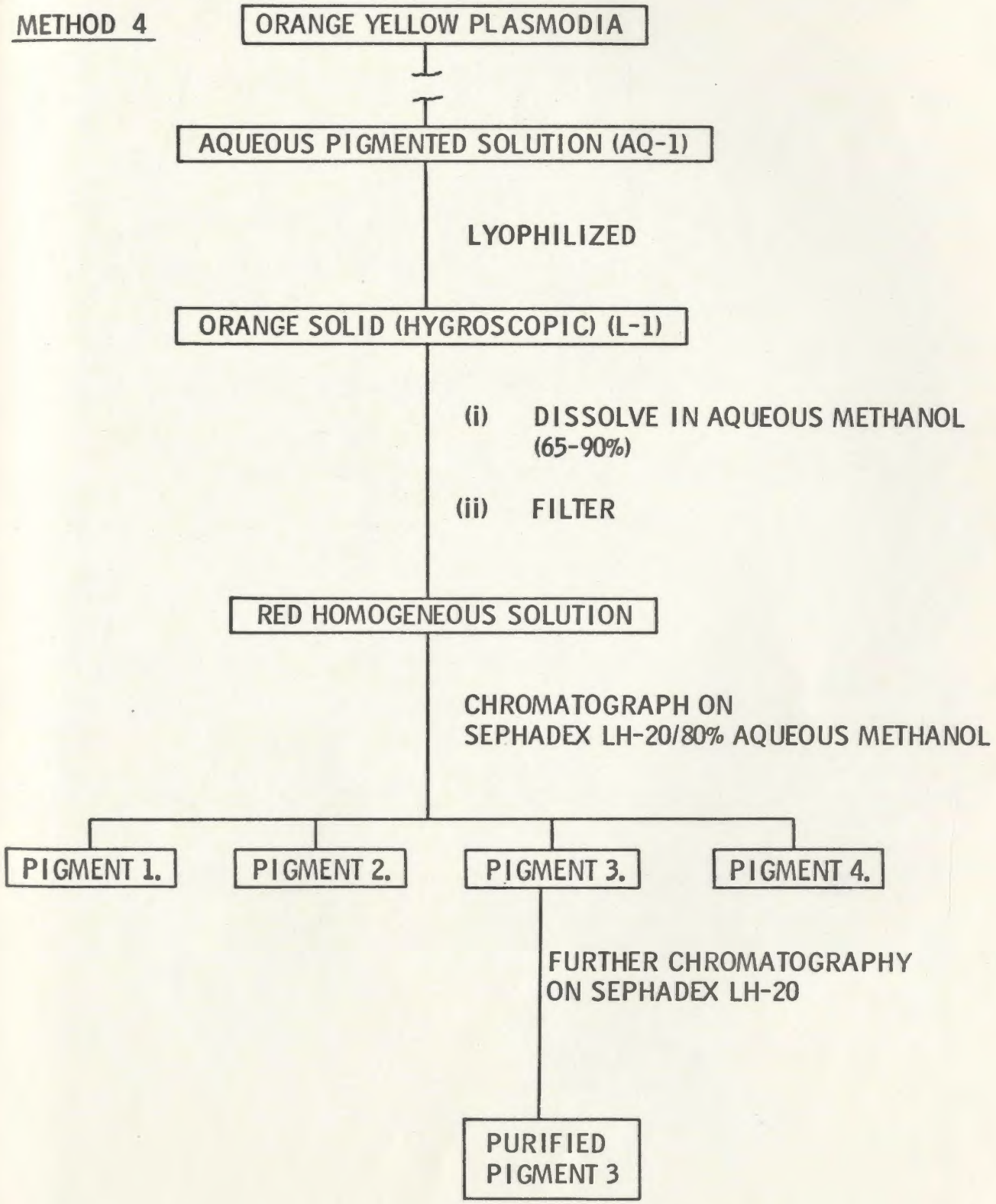
Eight fractions were collected, and they were classified into 4 main pigments mainly on the basis of u.v. spectroscopy. Table 31 lists some properties of these pigments.

Table 31. Separation of plasmodial pigments of P. polycephalum on Sephadex LH-20.

Fraction	Elution volume (Cm^3)	Pigment band and colour	Pigment No.	Ninhydrin	Fluorescence	Absorption spectra (nm) 80% methanol Neutral	Acidic	Basic
1	210	colourless		-	-	-	-	-
2	211-450	yellowish brown diffuse band band I	1	+ve	strong yellow-green	278, 283 358-59 (339-40) (378-79)	278, 283 358-59 (339-40) (378-79)	242, 294 358-59 (339-40) (378-79)
3	451-550	interband pale yellow	-	+ve	strong yellow green	-	-	-
4	551-625	yellow band band I	2	-ve	yellow	388-90 408	388-90 408	no change
5	626-660	yellow band band II	2	-ve	orange	388-90 410	388-90 410	no change
6	660-715	main orange band band III	3	-ve	weak orange	390-94	418-20	388-90
7	716-735	orange band part of band III	3	-ve	weak orange	410-424	424-450	not measured
8	736-770	orange yellow band (mixture of band III and band IV	3,4	-ve	weak orange	406-414	424	386

( ) = shoulder

CHART 5. ISOLATION AND PURIFICATION OF PIGMENTS ON SEPHADEX G-10 AND SEPHADEX LH-20 P. POLYCEPHALUM.



Thin layer chromatography of pigments 2,3 and 4 from P. polycephalum after elution from Sephadex LH-20.

Pigments 2,3, and 4 obtained after chromatography on Sephadex LH-20 were subjected to t.l.c. on silica gel GF254, (20 x 20 cm, 400 μ) developed with methanol/chloroform/acetic acid (50 : 13 : 3). The distance travelled by the solvent was approximately 15 cm, in ∿ 45 min. The pigmented bands were eluted with dichloromethane/methanol (9:1) after the plates were dried. Table 32 summarises the relevant data for the various pigments after separation.

Table 32. Thin layer chromatography of plasmodial pigments obtained after chromatography on Sephadex LH-20 (P. polycephalum).

Pigment	Solvent system	Colour, band	R_f	Absorption maxima (nm) methanol	
				Basic	Acidic
2	Methanol/chloroform/acetic acid (50:15:3)	(i) main yellow	0.40	384 (297-98), (328) (341-42)	377-78 (290), (312), (328), (345), (406-7)
	Several other bands were seen under u.v. light, but only the pigmented bands were eluted here.	(ii) narrow yellow	0.55	-	-
		(iii) faint yellow	0.86	-	-
3	"	(i) yellow	0.23	377-78	420
		(ii) main yellow brown	0.50	381-82	420, (198), (442).
		iii) narrow yellow	0.93	330, (315), (346), (373)	330, (318), (351) (382)
4	"	(i) minor yellow	0.20	-	-
		(ii) major yellow brown	0.42	404-5, (379), (422)	425-26, (445)
		iii) minor yellow	0.86	-	-

( ) = shoulder

Further column chromatography of pigment 3 P. polycephalum on Sephadex G-10.

Sephadex G-10 (34 g), swollen in 95% ethanol, was used to prepare a chromatographic column (12 x 2.5 cm). Pigment 3 (∿ 10 mg) was dissolved in the solvent, and this orange red pigmented solution was carefully applied to the gel surface. Further elution with the solvent (flow rate ∿ 60 cm^3/h) resulted in no distinct band separation, but produced a rather diffuse mixture extending the length of the column, although a more concentrated orange "band" appeared to be strongly adsorbed at the top of the column. This extensive diffusion effect along the length of the column was also observed when 50% ethanol/water was used as the eluting solvent. This is to be contrasted with the separation of pigment 2 (P. flavicomum) on Sephadex G-10, where there is distinct band separation and the gel becomes clean and white after separation.

When the eluting solvent was changed to 1% HCl in ethanol, the orange pigment band could be obtained in the protonated form, although there was little further improvement in separation. Table 33 lists the absorption spectral properties of pigment 3 chromatographed on Sephadex G-10 and eluted with 95% ethanol.

Table 33. Absorption spectra of fractions eluted from Sephadex G-10 column pigment 3 (P. polycephalum).

Fr.	Volume (Cm^3)	Colour	Absorption spectra (nm) 95% ethanol Neutral	Acidic
1	35	Colourless	-	-
2	25	yellow	400-410	418-420
3	25	yellow	414-418	420 (394), (446)
4	25	yellow	410-418	418 (394), (446)
5	25	yellow	416-420	
6	25	yellow	400-416	
7*		orange	-	416

( ) = shoulder * eluted with 1% HCl/95% EtOH.

Subsequently, pigment 3 from P. polycephalum was rechromatographed on a small Sephadex LH-20 column (15 x 2.5 cm) and the pigment used in these preliminary experiments was obtained from the middle third of the orange band. This pigment solution λ_{max} (MeOH) ∿ 392-394 nm, shows the typical bathochromic increase to 418-20 nm on protonation, accompanied by a hyperchromic increase (29%). A corresponding blue shift to 391 nm (MeOH) along with a hypochromic shift was observed when the solution was made alkaline, indicating a reversible

change. Pigment 3 was soluble in methanol, ethanol and aqueous, alcohols, pyridine, acetone, dimethyl formamide, but insoluble in hexane, petroleum ether (30-60°) carbon tetrachloride and benzene. M.P. 143-160° (sample goes darker red) and progressively blackens to 200° with no sharp melting point.

Cellulose acetate electrophoresis of pigment 3, (P. polycephalum).

Electrophoresis of purified pigment 3 obtained from Sephadex LH-20 column chromatography was carried out as described for pigment A-2 P. flavicomum. The same buffers were used and the approximate running time was 20 min. The separation of the pigments is illustrated in Fig. 21 . At pH 8.9 and 8.0, pigment 3 could be separated into a major yellow pigment component, and a minor yellow component both pigments migrating towards the anode. In a pH 7 buffer, there were two distinct bands again migrating to the anode, while in buffers pH 6.0 and 5.1, three pigment components were separated, all migrating to the anode. At pH 4, there was a small anodic migration (0.3 cm from origin) while the bulk of the sample remaining at the origin was bright orange, suggesting that the pigment had been protonated at this pH. From pH 3.0 to 1.1, the orange pigment stayed at the origin as a diffuse band. There was, however, no cathodic migration of the pigment in these buffers.

Table 34. Electrophoretic separation of pigment 3 (P. polycephalum) on cellulose acetate in buffers of different pH.

Buffer pH	Pigment component	Distance travelled to anode from origin (cm)
8.9	(a) yellow (trace)	1.3
	(b) yellow (major)	6.2
8.0	(a) yellow (trace)	1.3
	(b) yellow (major)	4.7
7.0	(a) bright yellow (major)	4.7
	(b) yellow (minor)	4.9
6.0	(a) yellow (trace)	1.3
	(b) bright orange	3.3
	(c) yellow	4.9
5.1	(a) orange (major)	1.0
	(b) yellow	2.5
	(c) yellow	3.8
4.0	(a) orange	0.3
3.0	(a) orange	0.0
2.0	(a) orange	0.0
1.1	(a) orange	0.0

Cellulose Acetate Electrophoresis
"Schiff Base" Pigment 3 P. Polycephalum at diff. pH's.

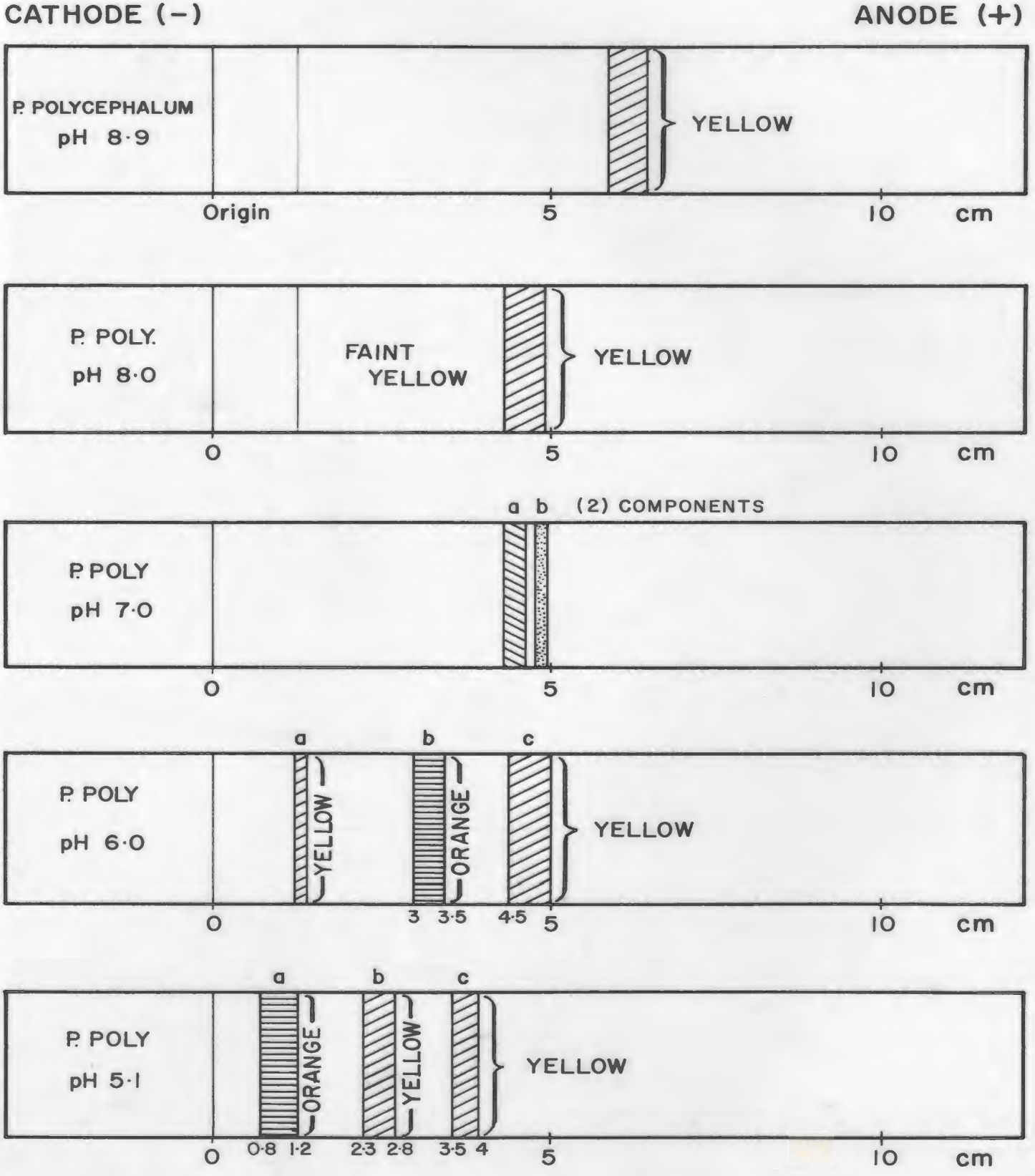
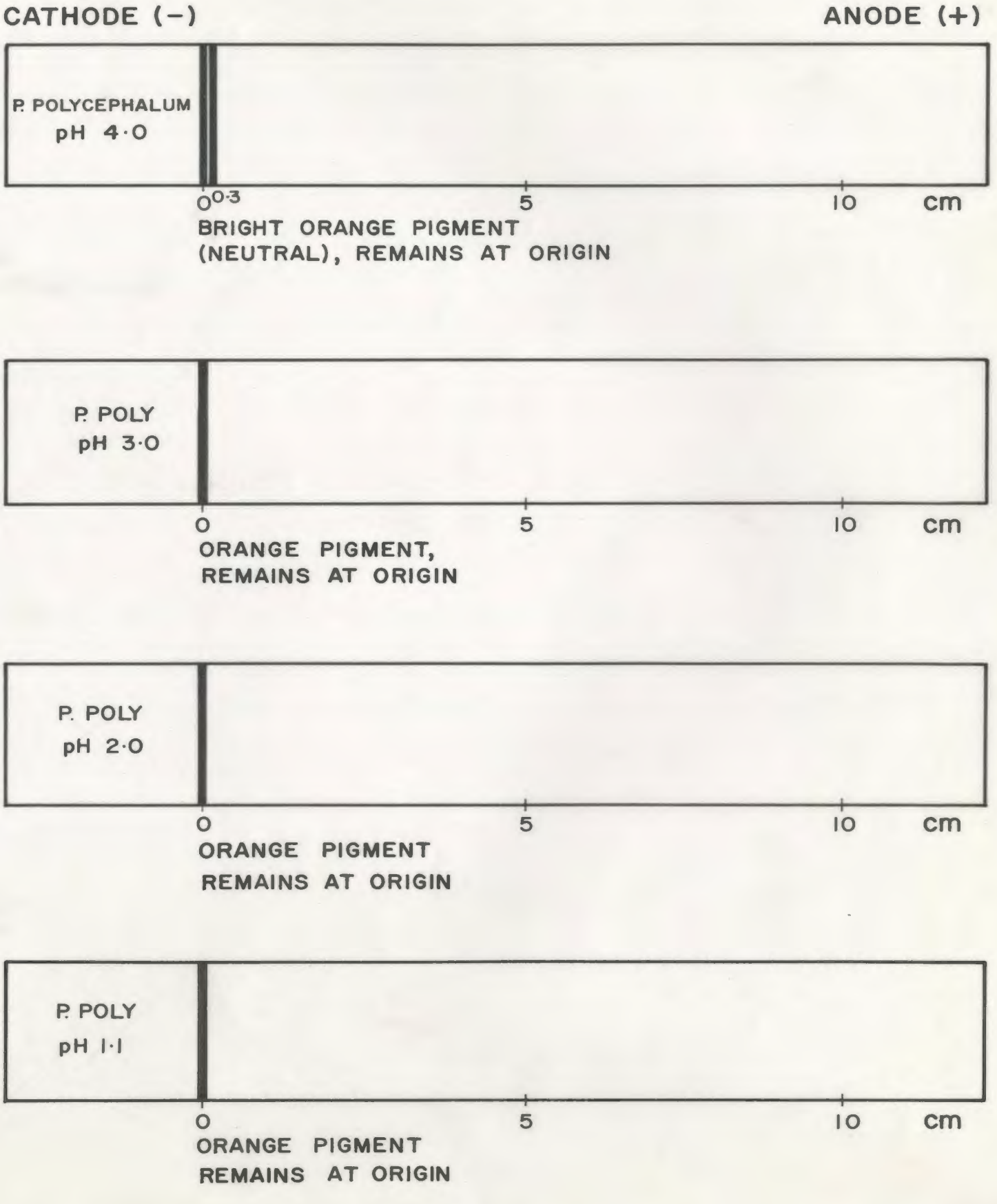


Figure 21 (continued)

Cellulose Acetate Electrophoresis
"Schiff Base" Pigment 3 P. Polycephalum at diff. pH's.



Spectrophotometric determination of acid dissociation constant of pigment 3.

A standard solution of pigment 3 in methanol, which when acidified had an absorbance of Ca 1.4 in a 1 cm cell at its absorption maximum was prepared. An aliquot of this solution (5 cm^3) was evaporated completely to dryness under reduced pressure and the residue redissolved in the appropriate buffers as described for pigment A-2, P. flavicomum. The absorbance of each solution at 420 and 390 nm were plotted against the pH of the solution, the inflexion point being taken as the pK_a of the pigment.

pK_a of pigment 3.

The pK_a's of the pigment were determined at

(i) loss of the 390 nm maximum with decreasing pH and found to be ca. 4.8.

(ii) appearance of 416 nm maximum with increasing acidity and found to be ca. 3.6. Curves showing the change in the absorption spectrum with pH, and the change in absorbance at 390 and 416 nm are given in Fig. 22, Fig. 23, and Fig. 24.

Fig. 22. Effect of pH on the visible absorption spectrum of pigment 3 (P. polycephalum) in aqueous buffers.

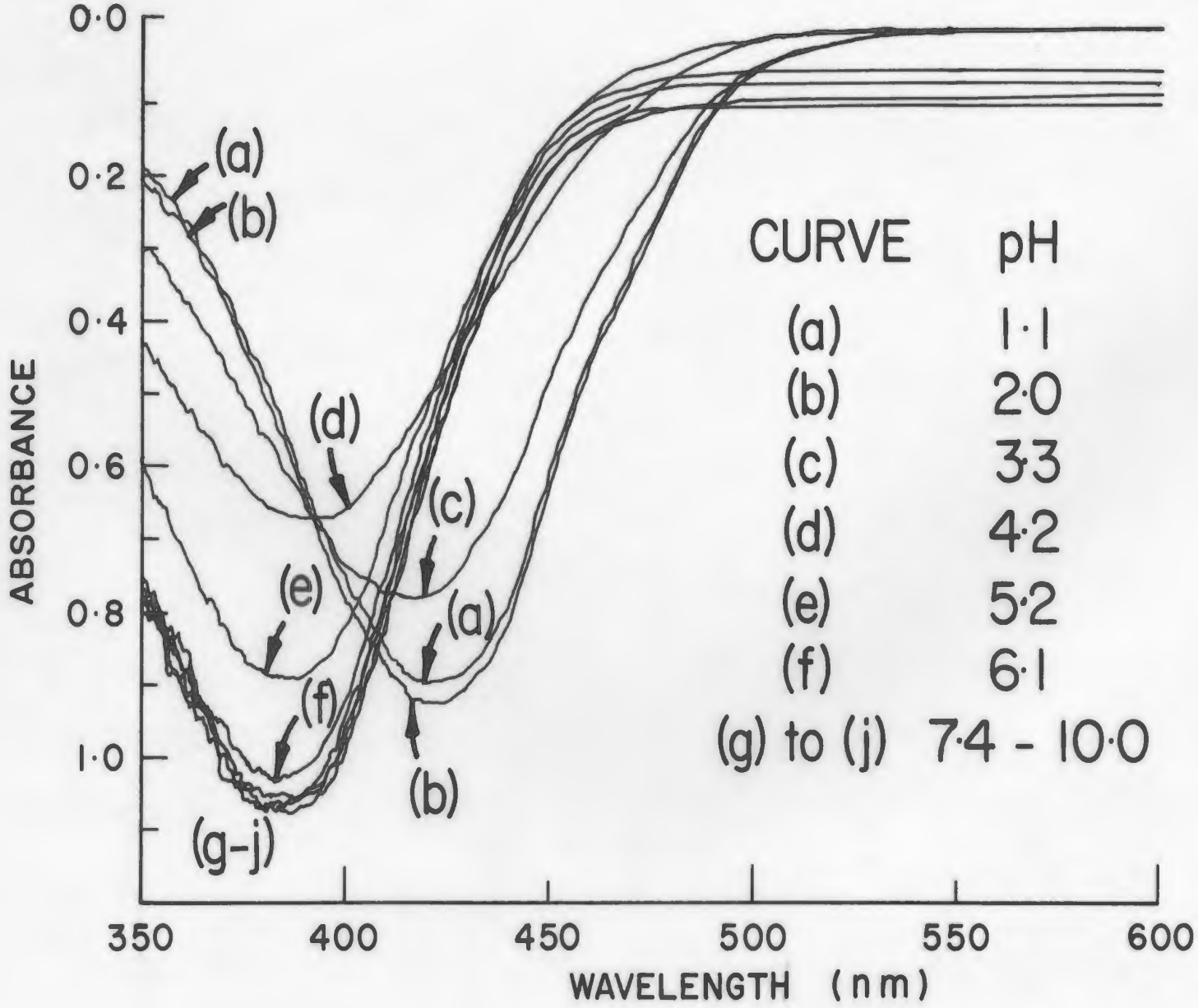


Fig. 23. Absorbance of pigment 3 (P. polycephalum) at 390 nm in aqueous buffer solutions.

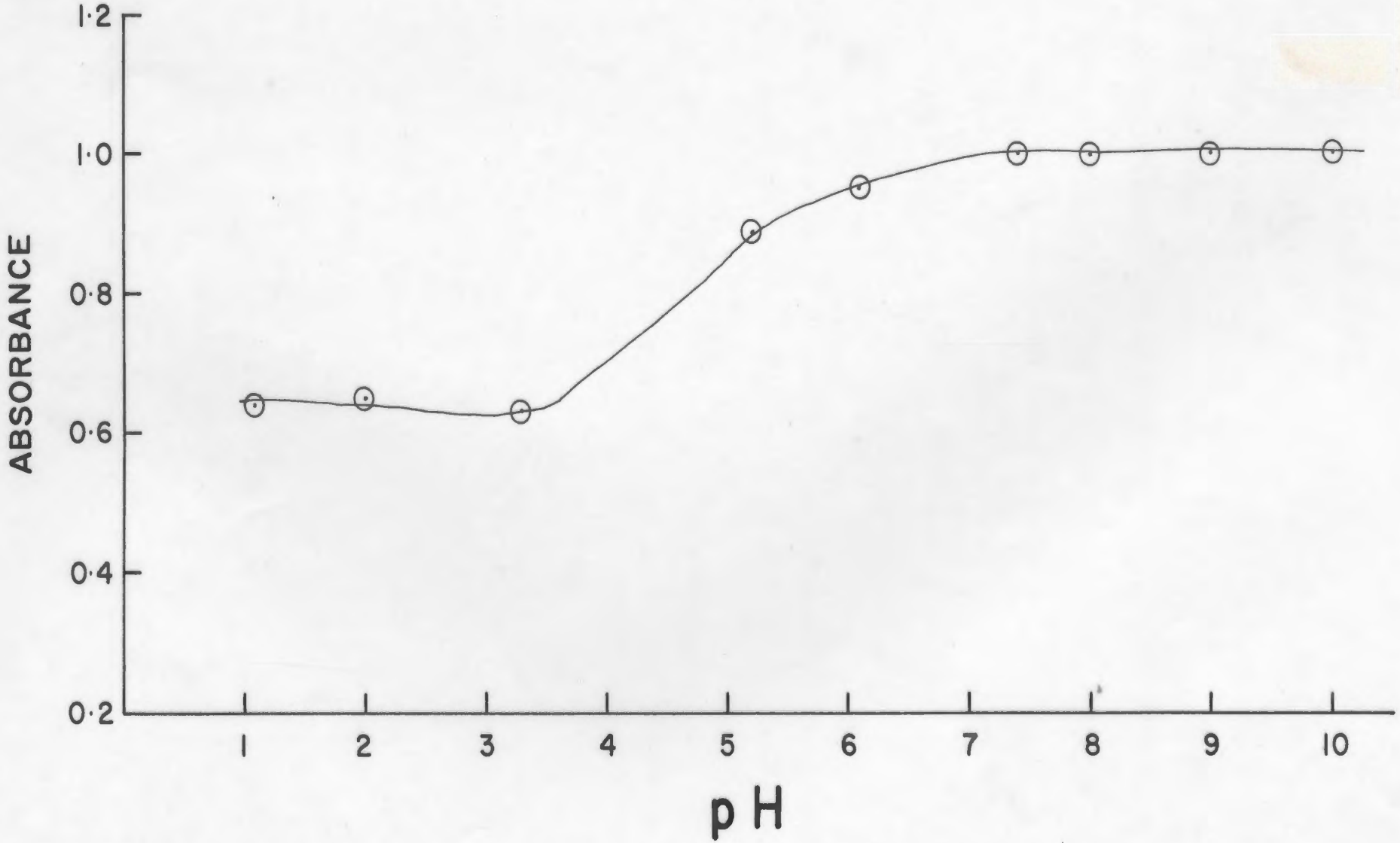
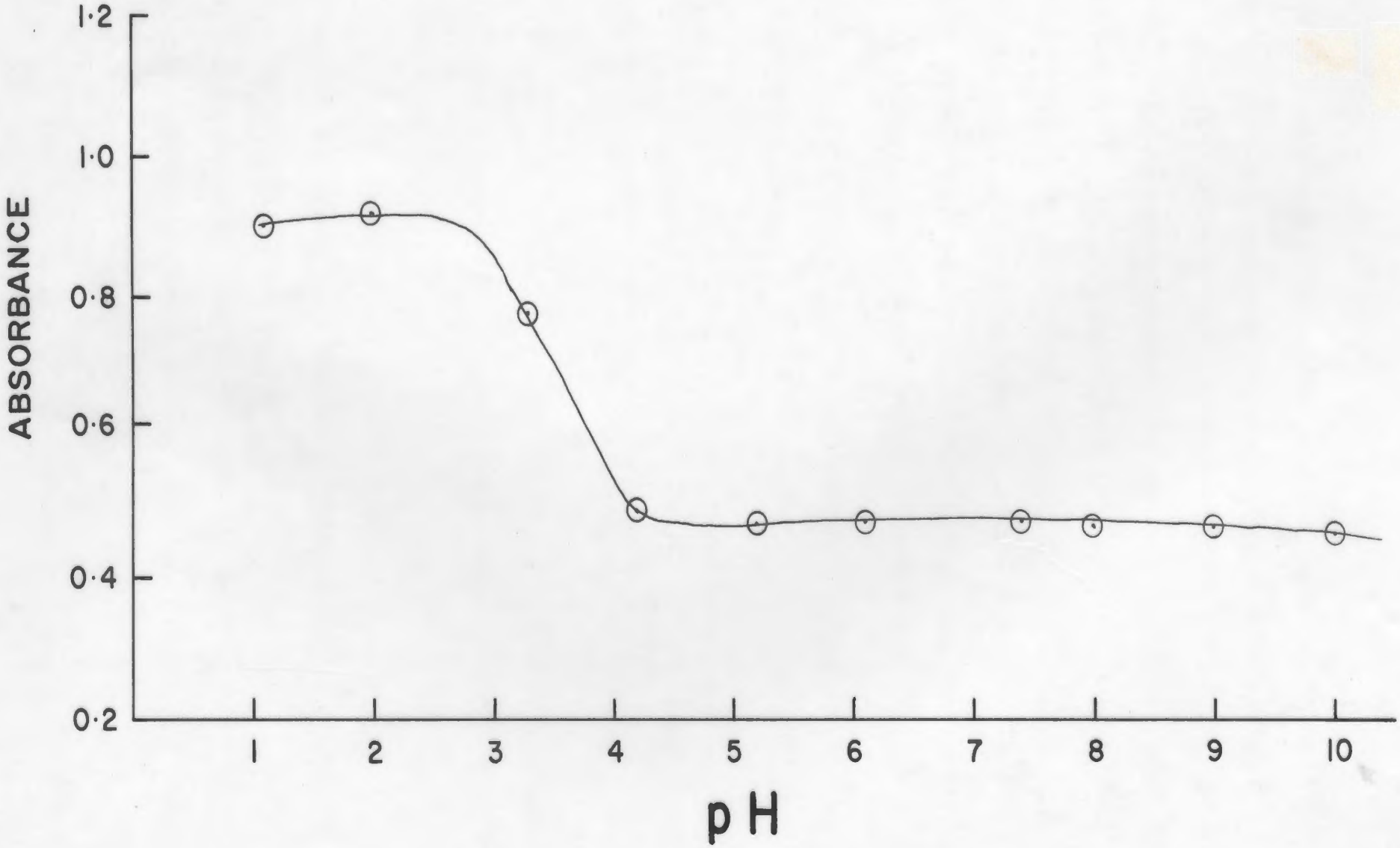


Fig. 24. Absorbance of pigment 3 (P. polycephalum) at 420 nm in aqueous buffer solutions.



Catalytic hydrogenation of pigment 3.

Pigment 3 (10 mg) was dissolved in methanol (10 cm^3) to give an orange solution to which palladium on charcoal (5% palladium, 10 mg) was added. The flask was then connected to a hydrogenator at room temperature, evacuated as described for the hydrogenation of P. flavicomum and allowed to react for 24 h. The resulting colourless solution obtained after filtration exhibited very strong yellowish white fluorescence. The ultraviolet spectrum measured in methanol, in methanolic containing hydrochloric acid and sodium hydroxide.

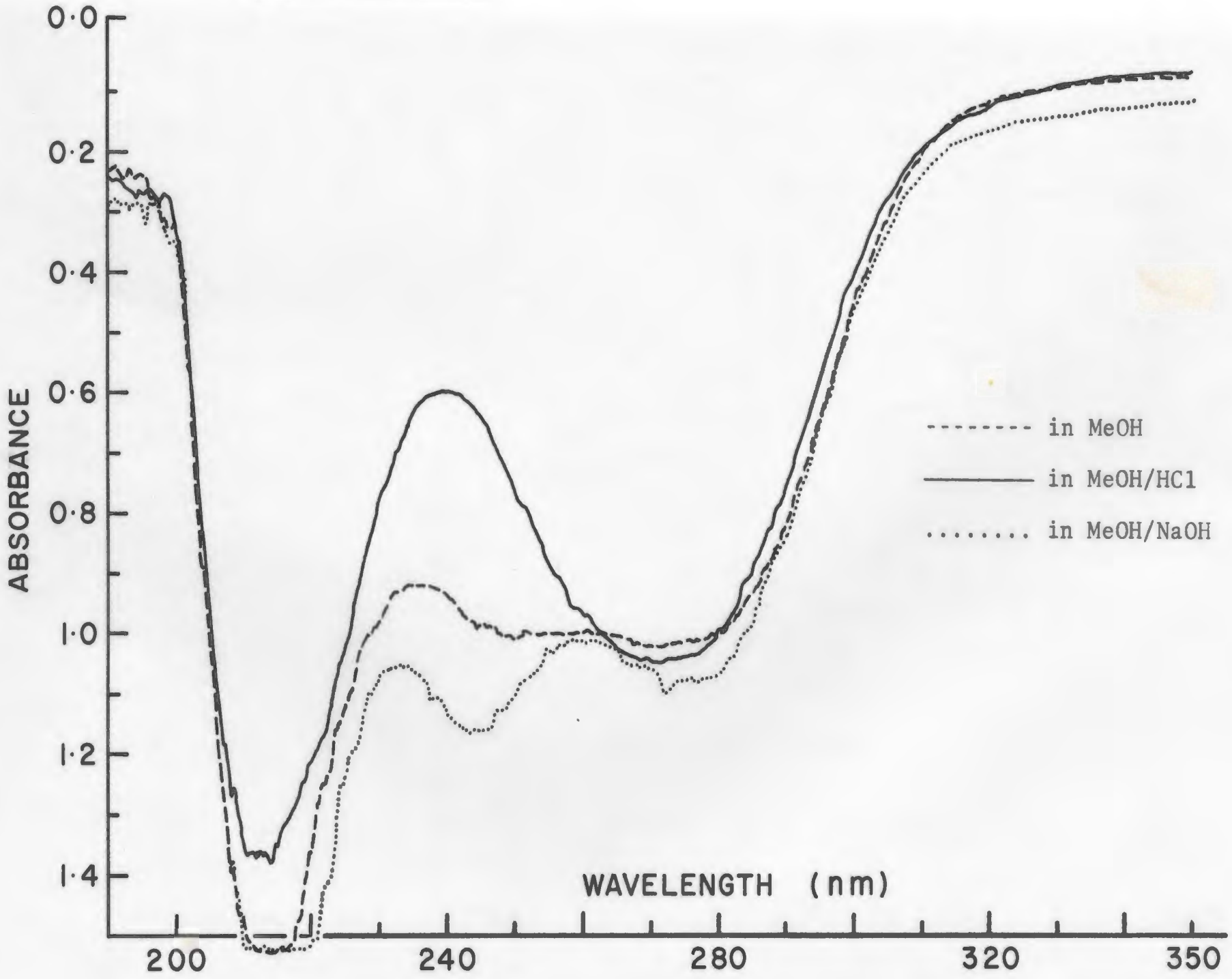
Table 35. Ultraviolet spectra of hydrogenated pigment 3 in methanol (nm).

Pigment	Neutral	Acidic	Alkaline
Hydrogenated pigment 3	245-50 (0.96) 270-76 (0.96)	- 272 (1.01)	244 (1.13) 275 (1.04)

( ) = absorbance

When the hydrogenation was carried out using platinum oxide instead of palladium on charcoal on pigment 3 isolated from another batch of plasmodia, u.v. peaks at 245 and 283 nm were observed in neutral solution.

Fig. 25. Ultraviolet absorption spectra of hydrogenated pigment 3 (P. polycephalum).



Methylation of hydrogenated pigment with diazomethane.

Hydrogenated pigment 3 (∿5 mg) dissolved in methanol was treated at 4° with an ethereal solution of diazomethane and allowed to stand for 24 h, and then evaporated to dryness. U.v. λ_{max} (MeOH) 250-265 nm, unchanged in acid or alkali.

I.r. ($CHCl_3$) 3650, 3550-3100 (broad), 2920, 2825, 1725, 1655, 1600, 1450, and 1365 cm^{-1}.

Sodium borohydride reduction of pigment 3.

(i) Solution of pigment 3 in methanol (5 cm^3, absorbance ∿ 1.5 in 1 cm cell) was treated with sodium borohydride (5 mg). The yellow colour of the solution was rapidly discharged and the solution was left to stand for a further 15 min. The absorption spectrum showed the presence of a reduced chromophore absorbing at 270, 283, 301, 317 and 331 nm in methanol which seemed to suggest the presence of a conjugated pentaene. This reaction could be easily repeated during the early course of this work.

(ii) However, when the sodium borohydride reduction was repeated later on another sample of the pigment, there was no significant change in the absorption spectrum, indicating that no reaction had taken place, and suggesting that some modification of the pigment structure had occurred.

Oxidation/methylation of pigment 3 (P. polycephalum) with silver.oxide and excess methyl iodide.

Pigment 3 (20 mg) was treated with freshly prepared silver oxide (4 g) and methyl iodide (5 cm^3) added. This reaction mixture was worked up as described for pigment A-2 (P. flavicomum). The chloroform extract was separated by t.l.c. on silica gel G developed with benzene/ethyl acetate (3:1) into 2 bands. The main yellow band showed:
U.v. λ_{max} ($CHCl_3$) 304, 321, 337, 355, and 387 nm.
Mass spectrum (160°): m/e 248 (M_1), 18.6%, 222 (M_2), 27.1%; 191 (M_1-OCH_3), 23.6%; 190 (M_2-CH_3OH), 10.7%; 189 (M_1-$COOCH_3$), 16.4%; 185 (93.9%); 163 (M_2-$COOCH_3$), 26.4%; 158 (M_2-64), 30%; 157 (M_1-91), 64.3%; 131 (M_2-91), 100%; 129 (M_1-119), 71.4%; 128 (M_1-120), 40%; 121 (M_2-101), 58.6%; 112 (54.3%); 111 (M_2-111), 45.7%; 103 (M_2-119), 92.9%; 91(M_2-131), 53.6%; 79 (M_2-143), 31.4%; 78 (M_2-144), 44.3%; and 77 (M_2-145), 71.4%.
This degradation product consists of a mixture of all-trans dimethyl 2,4,6,8,10 decatetraene-1, 10 dioate (M_2, mol. wt. 222) and all-trans dimethyl 2,4,6,8,10 dodecapentaene-1, 12-dioate (M_1, mol. wt. 248).

APPENDIX I Run 1, 30°C

Mass spectral data for pigment A-2 (P. flavicomum) at different temperatures.

BASE PEAK				LAST PEAK			
NO.	MASS	INT	SAMPLES	NO.	MASS	INT	SAMPLES
153	149.02464	60	27	246	49.99681	62	30

SUM OF INTENSITIES - 571

NO.	MASS	INT	SAMPLES
25	408.94679	6.67	13
133	177.05368	5.00	9
141	164.92873	3.33	8
142	163.04008	16.67	16
144	162.93360	20.00	18
151	150.02699	6.67	9
153	149.02464	100.00	27
159	137.13124	5.00	10
166	127.14993	5.00	9
167	127.01703	5.00	9
168	126.01116	5.00	9
169	125.13397	3.33	7
170	124.12338	6.67	12
172	123.11781	8.33	14
173	121.10135	6.67	7
174	121.02836	6.67	11
179	117.07213	3.33	7
184	111.11489	5.00	10
185	110.10944	5.00	8
186	109.10066	15.00	15
187	105.03249	3.33	9
189	100.93384	3.33	7
192	97.10119	26.67	16
194	96.09342	11.67	11
195	96.08819	5.00	9
196	95.08548	26.67	24
197	95.01112	65.00	25
199	93.07013	6.67	10
201	91.05438	28.33	15
203	85.10214	15.00	15
205	83.08648	31.67	19
207	82.07846	16.67	15
209	81.07025	18.33	17
211	79.05416	10.00	13
212	77.03903	20.00	

Run 2, ~ 150°

BASE PEAK				LAST PEAK			
NO.	MASS	INT	SAMPLES	NO.	MASS	INT	SAMPLES
219	58.04300	1308	61	238	49.99681	41	32

SUM OF INTENSITIES - 2162

NO.	MASS	INT	SAMPLES
118	181.01086	0.23	9
122	177.05670	0.38	9
123	177.04972	0.46	12
128	164.92934	0.46	12
129	163.03960	1.99	25
131	162.93066	0.54	11
135	150.02873	0.38	9
137	149.02352	3.98	24
143	137.13120	0.15	7
153	121.10597	0.23	8
161	111.11736	0.15	8
162	110.03616	0.15	8
163	109.10407	0.23	9
164	109.09975	0.23	9
165	107.08600	0.31	9
166	105.06953	0.38	9
169	98.07423	1.38	21
170	97.09975	0.46	7
172	96.09524	0.23	9
173	95.08642	0.76	17
174	95.08172	0.15	9
175	95.01150	2.68	24
177	91.05365	0.69	16
179	85.09985	0.23	8
181	84.09415	0.15	7
182	84.05244	0.23	11
183	83.08686	0.69	13
184	83.04927	3.59	24
185	83.01138	0.92	19
186	82.07808	0.76	17

Run 3, 280°

NO.	MASS	INT	SAMPLES	NO.	MASS	INT	SAMPLES
343	78.04766	329	38	445	49.99681	27	29

SUM OF INTENSITIES - 5381

NO.	MASS	INT	SAMPLES
81	222.09088	2.13	14
82	221.08356	3.95	22
89	210.11120	0.61	7
90	209.08557	5.78	19
94	205.08857	1.52	9
99	196.07599	1.52	14
100	195.06891	3.65	15
102	194.05714	1.52	10
106	192.18945	0.61	7
108	186.09423	0.61	8
110	184.07447	2.13	16
111	183.06666	1.52	9
113	182.07225	0.91	7
115	181.08737	1.22	7
116	181.07419	0.61	8
117	181.01103	0.61	7
119	180.07910	1.82	12
120	178.07707	1.52	11
121	178.06364	0.91	7
122	177.05680	0.91	8
124	174.08914	1.22	10
125	174.05190	0.61	7
127	173.08559	3.65	14
128	173.07535	0.91	7
129	172.07472	2.74	16
130	171.13591	0.61	9
133	168.08010	0.61	7
134	168.06764	11.55	29
136	167.07227	3.34	12
138	166.06742	0.61	7
139	165.06845	2.74	18
140	164.93135	1.52	14
141	164.06101	0.61	7
142	163.05291	0.61	7
143	163.04022	9.12	21
145	162.93261	2.43	18

Run 3 continued -

NO.	MASS	INT	SAMPLES
147	161.08334	3.34	18
148	161.04795	2.43	14
149	160.07556	7.90	33
150	160.05376	1.52	10
151	160.03714	0.61	8
152	159.06844	24.01	27
153	158.07144	1.52	14
154	158.06092	4.56	17
155	157.06441	0.91	8
156	155.07234	1.22	11
157	155.04955	0.91	10
159	154.07550	1.22	10
160	154.06831	1.22	8
161	153.07038	3.95	20
162	152.06175	3.04	14
163	152.04630	0.61	7
166	149.04736	1.52	14
167	149.02349	15.50	30
168	148.09023	0.91	7
169	148.07205	0.91	9
170	148.05108	0.91	9
171	147.06784	12.16	28
172	147.04665	0.61	7
173	147.03415	2.43	16
175	146.06074	13.07	42
176	145.06680	3.34	22
177	145.05287	30.09	38
179	144.08142	1.82	11

Run 4, 300°

NO.	MASS	INT	SAMPLES	NO.	MASS	INT	SAMPLES
283	147.06915	177	32	452	92.99521	87	24

SUM OF INTENSITIES - 5355

NO.	MASS	INT	SAMPLES
16	408.94559	2.26	12
77	261.10617	1.13	7
81	251.13194	1.69	7
82	251.09547	1.13	7
86	248.11610	1.69	8
87	248.10555	3.39	15
93	237.12319	1.69	9
95	236.11031	2.26	11
96	236.10049	2.26	9
98	235.09961	3.95	13
99	234.09267	2.82	10
100	234.08434	1.69	8
101	233.08602	3.95	14
107	228.08175	1.13	7
108	227.09586	1.69	8
109	225.07945	2.26	7
111	224.10585	3.95	10
113	223.10013	14.12	22
114	222.09066	14.69	26
115	221.08324	9.04	20
116	220.07791	2.82	10
121	216.08251	2.26	11
122	215.12729	1.13	7
123	215.08376	2.26	12
124	215.06834	1.13	7
125	213.07666	2.26	8
128	211.06172	3.95	9
129	210.09860	2.82	12
130	210.08879	5.65	14
132	209.08269	19.21	25
133	208.08190	2.82	11
134	208.07084	4.52	12
135	207.06820	1.69	9

Run 4 continued -

NO.	MASS	INT	SAMPLES
136	206.10735	1.13	8
138	204.09238	1.13	7
139	204.08074	4.52	13
140	203.09599	1.69	7
141	203.08306	2.26	10
142	202.07728	3.39	10
143	202.06701	3.39	13
144	201.11121	2.82	9
145	201.07639	2.82	9

Run 5, ~ 315°

BASE PEAK				LAST PEAK			
NO.	MASS	INT	SAMPLES	NO.	MASS	INT	SAMPLES
380	91.05496	258	29	445	69.99857	85	26

SUM OF INTENSITIES - 5505

NO.	MASS	INT	SAMPLES
19	408.94482	1.16	10
57	299.13188	1.16	7
78	261.11308	1.55	11
79	259.09681	1.16	8
85	247.09600	1.55	11
86	246.09211	1.94	9
90	242.09792	0.78	7
92	238.09315	0.78	8
93	237.11020	3.49	11
95	236.10725	1.94	12
97	235.09783	1.94	14
98	233.08443	1.55	8
105	223.09914	8.91	21
106	222.09256	8.91	21
107	221.08251	6.20	22
108	220.07863	1.16	7
113	216.08308	0.78	7
114	215.08746	1.94	12
115	213.06895	1.16	7
118	211.09590	2.71	13
119	210.08939	2.71	10
120	209.09436	0.78	7
121	209.08371	8.91	20
122	208.07878	3.10	15
125	204.08465	1.55	8
126	204.07832	1.16	8
127	203.07792	1.16	9
128	202.07842	3.49	17
129	201.11920	0.78	7
130	201.05752	1.55	8
133	197.08749	1.16	9
134	197.08126	1.16	7
135	196.07680	6.20	20
136	195.07917	2.71	13
137	195.06898	3.49	14
139	194.06133	1.55	13
141	193.08845	1.94	11
143	192.09262	1.94	10
144	192.08139	1.55	10
145	191.08337	3.10	12

Run 5 continued -

NO.	MASS	INT	SAMPLES
146	191.07447	0.78	8
147	190.06630	1.94	10
148	189.11604	1.55	10
149	189.07889	1.16	7
150	187.10150	2.71	9
151	187.09610	1.16	8
154	185.08238	2.33	11
155	184.07415	3.10	14
156	183.08005	1.16	8
157	183.06529	2.71	14
159	182.07380	3.10	13
160	182.06193	1.94	13
162	181.08621	4.26	22
163	181.06508	7.75	19
165	180.09093	1.16	7
166	180.08124	8.14	18
167	179.08336	1.16	7
169	178.07168	2.71	15
171	176.06032	1.94	11
172	175.09808	7.75	24
175	174.09206	9.30	25
177	173.08412	17.05	24
178	172.09142	1.16	9
179	172.07624	13.57	26
180	171.06914	1.94	10
181	170.10779	0.78	7
182	170.09536	1.16	7
183	170.07613	1.55	11
184	170.06072	1.94	12
186	169.06562	1.55	7
188	168.07919	1.94	11
189	168.06941	4.65	17
190	167.08735	3.88	16
191	167.07472	6.20	25
193	166.07841	1.94	10
194	166.06829	4.65	17
195	165.07106	11.24	19
196	164.93309	1.94	11
197	164.04892	0.78	7
198	163.05388	2.33	12
199	163.03860	10.85	18
201	162.93329	2.33	11
202	162.08704	2.71	9
204	161.08288	34.50	49
205	161.04532	1.55	10
206	160.07596	55.81	41
207	160.03856	1.16	10

Run 5 continued -

NO.	MASS	INT	SAMPLES
208	159.08132	1.94	9
209	159.06784	25.19	28
210	158.07312	1.55	10
211	158.06094	7.36	22
212	157.08928	1.55	9
213	156.09235	2.71	14
214	156.08419	3.10	15
215	156.07803	1.16	8
216	155.08966	1.94	9
217	155.08358	1.55	11
218	155.07264	0.78	8
220	154.07555	1.94	7
221	154.06525	5.04	18
222	153.06980	7.36	19
223	153.05715	2.33	12
224	152.06247	8.53	20
225	151.05524	5.43	18
228	149.04442	1.94	11
229	149.02237	7.36	13
230	148.07359	5.04	16
231	148.01499	1.16	8
233	147.06795	55.81	28
235	146.07876	1.16	8
236	146.06174	48.45	46
237	145.08920	2.33	15
238	145.06485	7.75	22
239	145.05240	21.71	22
241	144.09368	2.71	14
242	144.08109	5.04	13
243	144.05680	4.65	22
244	144.04654	1.55	7
246	143.08451	3.49	17
247	143.07349	5.81	22
249	142.07775	8.91	21
250	142.06842	3.10	14
251	141.06982	11.63	23
252	141.05707	5.43	22
253	140.06155	1.94	11
254	140.05422	1.94	9
255	140.04804	1.94	10
256	139.05470	7.36	21
258	136.08878	4.26	20
259	135.08025	1.16	7
261	134.07210	3.10	17
262	134.05796	3.49	20
263	134.01210	1.55	10
264	133.06360	3.10	8
265	133.05284	25.19	28

Run 5 continued -

NO.	MASS	INT	SAMPLES
267	132.08384	3.10	15
268	132.07648	1.55	10
269	132.05830	7.75	16
270	132.04422	11.24	20
272	131.08562	5.04	23
273	131.07322	14.73	22
274	131.04952	14.34	25
277	130.07799	4.65	18
278	130.06566	21.71	26
279	129.07041	16.28	26
280	129.06313	1.55	9
281	129.05715	2.71	14
282	128.06176	21.32	25
283	128.05098	4.65	12
284	127.05495	7.75	25
285	126.04791	2.33	9
286	126.04300	1.55	8
288	123.07463	0.78	8
289	123.06687	2.71	10
290	122.07255	22.09	29
291	122.05929	2.33	9
292	122.03320	1.16	8
293	121.08953	3.10	13
294	121.06544	20.16	22
295	121.05897	1.55	9
296	120.09302	5.81	23
297	120.05722	9.30	27
299	119.08847	2.71	10
300	119.07574	0.78	7
301	119.07147	1.16	9
302	119.04950	7.75	19
303	119.03643	2.33	8
306	118.08050	1.16	7
307	118.07438	1.16	8
308	118.06570	14.34	29
309	118.04149	1.55	8
310	117.07062	12.02	24
311	117.05846	25.97	27
313	116.06266	9.30	27
314	116.05010	11.63	34
315	115.05523	33.33	26
316	115.04271	3.10	10
317	114.04886	3.88	11
318	114.04195	1.94	13
319	114.03348	1.16	8
321	113.03902	5.81	18
324	111.06863	1.94	8
325	110.07257	2.71	12

Run 5 continued -

NO.	MASS	INT	SAMPLES
326	110.03697	4.26	20
327	109.10174	1.16	7
328	109.96536	1.55	14
329	109.06005	2.33	10
330	109.05111	4.65	11
331	108.08019	1.55	11
332	108.05689	48.06	39
333	108.04410	1.55	10
334	107.08505	1.16	9
335	107.07431	1.94	9
336	107.04912	86.43	44
337	106.07748	11.63	24
338	106.06450	6.59	21
339	106.04098	17.44	24
340	105.06997	20.54	24
341	105.05747	9.30	23
342	105.03405	27.91	28
344	104.06089	15.12	30
345	104.04924	19.77	30
346	104.02647	3.88	15
347	103.05449	17.44	22
348	103.04131	6.59	26
349	102.04714	9.69	28
350	102.03640	0.78	7
351	101.04043	3.88	14
354	99.11680	1.16	9
355	99.03400	0.78	8
356	99.03067	2.33	9
357	99.02295	1.55	9
358	98.07286	0.78	7
359	98.03628	3.10	17
360	97.10053	1.94	12
361	97.05316	3.10	17
362	97.02776	1.16	8
364	96.09522	0.78	7
365	96.05818	4.26	17
366	96.04693	1.55	10
367	95.08553	5.04	15
368	95.04773	8.14	26
369	95.01145	16.67	27
370	94.53579	0.78	7
371	94.06478	5.43	18
372	94.04203	37.21	24
373	93.06962	5.04	17
374	93.05813	5.04	20
375	93.03452	1.94	10

Run 5 continued -

NO.	MASS	INT	SAMPLES
377	92.06225	33.72	34
378	92.05105	1.94	12
379	92.02563	5.43	22
380	91.05496	100.00	29
381	91.04236	3.49	18
382	90.04616	20.16	36
383	89.53508	1.16	7
384	89.03914	28.68	22
385	88.03070	3.49	15
386	87.04395	3.88	13
388	86.03638	12.02	23
389	86.01754	0.78	7
390	86.01476	1.94	9
391	85.10135	2.33	12
392	85.05243	4.65	19
393	85.02948	4.26	18
395	84.09407	1.94	11
396	84.05808	1.16	7
397	84.04657	1.16	11
398	84.01867	1.16	8
399	83.53734	1.94	7
400	83.53475	1.55	7
401	83.08578	7.75	21
402	83.04890	3.88	20
403	83.03883	1.16	8
404	83.03561	3.10	13
405	83.01091	5.04	15
406	82.07823	6.59	25
407	82.04260	3.49	17
408	82.03752	3.49	13
409	82.02922	1.55	9
411	81.52476	0.78	7
412	81.06980	10.08	21
413	81.03404	5.81	16
415	80.06295	9.30	23
416	80.05847	1.55	8
417	80.05060	6.98	14
418	79.05372	46.51	41
419	79.03836	0.78	7
420	78.04678	84.11	44
421	78.03523	3.88	15
422	78.03198	1.55	9
423	77.03874	84.88	43
424	77.02082	1.94	10
425	76.53522	1.16	8
426	76.03120	26.36	27
427	76.01770	0.78	8
428	75.94367	5.04	17
429	75.02278	11.63	23
430			

Run 5 continued -

430	75.01061	2.71	11
432	74.03706	1.55	10
433	74.01610	12.02	23
435	73.53491	1.16	8
436	73.02928	11.24	23
437	72.02068	18.22	27
438	71.08640	3.88	17
439	71.04954	2.71	15
440	71.03753	5.43	16
441	71.01503	1.55	8
442	70.07828	6.59	20
443	70.04180	3.49	17
445	69.99857	85.00	26

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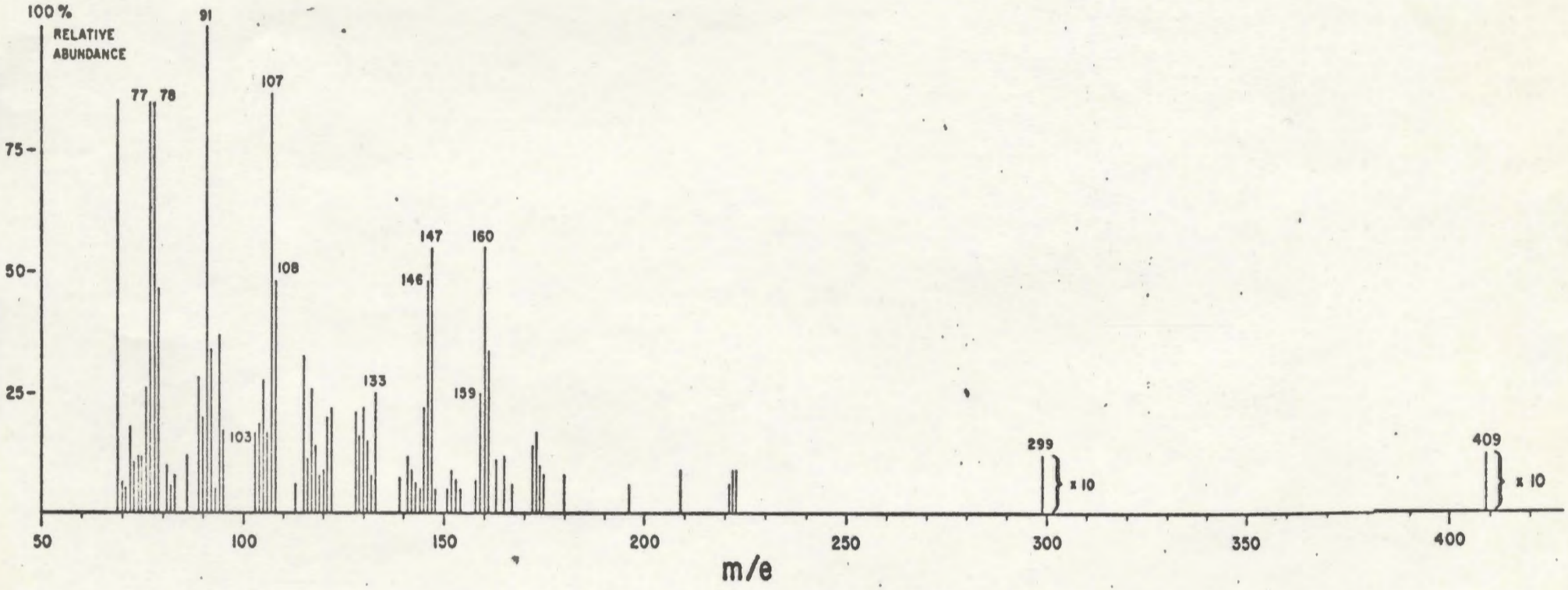
Fig. 26. High resolution mass spectrum of pigment A-2 (P. flavicomum).



APPENDIX II

MOLECULAR FORMULAE FOR ACCURATE MASSES IN PIGMENT A-2 (*P. FLAVICOMUM*) (124).

NO.	ACCURATE MASS	INTENSITY	MOLECULAR FORMULA	ACCURATE MASS
93	237.11020	3.49	$C_{14}H_{13}N_4$	0.11402
			$C_{11}H_{15}N_3O_3$	0.11132
			$C_{13}H_{17}O_4$	0.11268
			$C_{15}H_{13}N_2O$	0.10278
105	223.09914	8.91	$C_{15}H_{13}NO$	0.0997
			$C_{13}H_{11}N_4$	0.0983
106	222.09256	8.91	$C_{15}H_{12}NO$	0.0919
			$C_{13}H_{10}N_4$	0.0905
107	221.08251	6.20	$C_{15}H_{11}NO$	0.0841
			$C_{13}H_9N_4$	0.0827
121	209.08371	8.91	$C_{12}H_9N_4$	0.08272
			$C_{14}H_{11}NO$	0.08406
122	208.07878	3.1	$C_{12}H_8N_4$	0.07489
			$C_{14}H_{10}NO$	0.07623
128	202.07842	3.49	$C_{11}H_{10}N_2O_2$	0.07422
			$C_{16}H_{10}$	0.07825
135	196.07680	6.20	$C_{13}H_{10}NO$	0.07623
			$C_{11}H_8N_4$	0.07489
136	195.06898	3.49	$C_{10}H_{11}O_4$	0.06573
			$C_{11}H_7N_4$	0.06707
			$C_{13}H_9NO$	0.06841
145	191.08337	3.10	$C_{10}H_{11}N_2O_2$	0.08205
			$C_{15}H_{11}$	0.08607

(continued)

APPENDIX II (continued)

No.	ACCURATE MASS	INTENSITY	MOLECULAR FORMULA	ACCURATE MASS
155	184.07415	3.10	$C_9H_{12}O_4$	0.07355
			$C_{10}H_8N_4$	0.07489
			$C_{12}H_{10}NO$	0.07623
159	182.07380	3.1	$C_{11}H_8N_3$	0.07182
			$C_{13}H_{10}O$	0.07316
162	181.08621	4.26	$C_{13}H_{11}N$	0.08915
			$C_8H_{11}N_3O_2$	0.08512
			$C_{10}H_{13}O_3$	0.08646
163	181.06508	7.75	$C_{13}H_9O$	0.06534
			$C_{11}H_7N_3$	0.06399
166	180.08124	8.14	$C_{13}H_{10}N$	0.08132
172	175.09808	7.75	$C_{11}H_{13}NO$	0.09971
			$C_9H_{11}N_4$	0.09837
			$C_8H_{15}O_4$	0.09703
175	174.09206	9.30	$C_{11}H_{12}NO$	0.09188
			$C_9H_{10}N_4$	0.09054
177	173.08412	17.05	$C_{11}H_{11}NO$	0.08406
			$C_9H_9N_4$	0.08272
			$C_8H_{13}O_4$	0.08138
179	172.07624	13.57	$C_{11}H_{10}NO$	0.07623
			$C_9H_8N_4$	0.07489
			$C_8H_{12}O_4$	0.07355
189	168.06941	4.65	$C_{11}H_8N_2$	0.06875
			$C_8H_{10}NO_3$	0.06606

(continued)

APPENDIX II (continued)

No.	ACCURATE MASS	INTENSITY	MOLECULAR FORMULA	ACCURATE MASS
190	167.08735	3.88	$C_{13}H_{11}$	0.08607
			$C_9H_{13}NO_2$	0.09462
			$C_8H_{11}N_2O_2$	0.08205
191	167.07472	6.20	$C_{12}H_9N$	0.07350
			$C_9H_{11}O_3$	0.07081
194	166.06829	4.65	$C_{12}H_8N$ (run #3)	0.06567
			$C_9H_{10}O_3$	0.06299
195	165.07106	11.24	$C_{13}H_9$	0.07042
204	161.08288	34.50	$C_{10}H_{11}NO$ (run #3)	0.08406
			$C_8H_9N_4$	0.08271
			$C_7H_{13}O_4$	0.08138
*206	160.07596	55.81	$C_{10}H_{10}NO$	0.07623
			$C_8H_8N_4$	0.07489
			$C_7H_{12}O_4$	0.07355
*209	159.06784	25.19	$C_{10}H_9NO$ (run #3)	0.06841
			$C_8H_7N_4$	0.06707
			$C_7H_{11}O_4$	0.06573
			$C_{10}H_7O_2$	0.04460
211	158.06094	7.36	$C_{10}H_8NO$	0.06059
			$C_8H_6N_4$	0.05924
			$C_{10}H_6O_2$	0.03677
214	156.08419	3.10	$C_{11}H_{10}N$	0.08132
			$C_7H_{12}N_2O_2$	0.08987
221	154.06525	5.04	$C_{11}H_8N$	0.06567
			$C_8H_{10}O_3$	0.06299

(continued)

APPENDIX II (continued)

NO.	ACCURATE MASS	INTENSITY	MOLECULAR FORMULA	ACCURATE MASS
222	153.06980	7.36	$C_{12}H_9$	0.07042
			$C_7H_9N_2O_2$	0.06640
224	152.06247	8.53	$C_{12}H_8$	0.06260
			$C_7H_8N_2O_2$	0.05857
225	151.05524	5.43	$C_{12}H_7$	0.05477
230	148.07359	5.04	$C_9H_{10}NO$	0.07623
			$C_7H_8N_4$	0.07489
			$C_6H_{12}O_4$	0.07355
233	147.06795	55.81	C_9H_9NO	0.06841
			$C_7H_7N_4$	0.06707
			$C_6H_{11}O_4$	0.06573
236	146.06174	48.45	C_9H_8NO	0.06059
			$C_7H_6N_4$	0.05924
238	145.06485	7.75	$C_{10}H_9O$	0.06534
			$C_8H_7N_3$	0.06399
239	145.05240	21.71	C_9H_7NO	0.05276
			$C_7H_5N_4$	0.05142
242	144.08109	5.04	$C_{10}H_{10}N$	0.08132
243	144.05680	4.65	$C_{10}H_8O$	0.05751
			$C_8H_6N_3$	0.05617
246	143.08451	3.49	$C_{11}H_{11}$	0.086071
			$C_6H_{11}N_2O_2$	0.08205
247	143.07349	5.81	$C_{10}H_9N$	0.07350
249	142.07775	8.91	$C_{11}H_{10}$	0.07825
250	142.06842	3.10	$C_{10}H_8N$	0.06567

(continued)

APPENDIX II (continued)

	ACCURATE MASS	INTENSITY	MOLECULAR FORMULA	ACCURATE MASS
251	141.06982	11.63	$C_{11}H_9$	0.07042
252	141.05707	5.43	$C_{10}H_7N$	0.05785
256	139.05470	7.36	$C_{11}H_7$	0.05477
258	136.08878	4.26	$C_9H_{12}O$	0.88810
261	134.07210	3.10	$C_9H_{10}O$	0.07316
			$C_7H_8N_3$	0.07182
262	134.05796	3.49	$C_5H_{10}O_4$	0.05790
			C_8H_8NO	0.06059
264	133.06360	3.10	$C_7H_7N_3$	0.06399
265	133.05284	25.19	C_8H_7NO	0.05276
267	132.08384	3.10	$C_9H_{10}N$	0.08132
269	132.05830	7.75	C_9H_8O	0.5751
270	132.04422	11.24	C_8H_6NO	0.04494
			$C_6H_4N_4$	0.04360
272	131.08562	5.04	$C_{10}H_{11}$	0.08607
273	131.07322	14.73	C_9H_9N	0.07350
274	131.04952	14.34	C_9H_7O	0.04969
277	130.07799	4.65	$C_{10}H_{10}$	0.07825
278	130.06566	21.71	C_9H_8N	0.06567
279	129.07041	16.28	$C_{10}H_9$	0.07042
282	128.06176	21.32	$C_{10}H_8$	0.06260
283	128.05098	4.65	C_9H_6N	0.05002
290	122.07255	22.09	$C_8H_{10}O$	0.07316
			$C_6H_8N_3$	0.07182
294	121.06544	20.16	C_8H_9O	0.06534

(continued)

APPENDIX II (continued)

NO.	ACCURATE MASS	INTENSITY	MOLECULAR FORMULA	ACCURATE MASS
296	120.09302	5.81	C_9H_{12}	0.09390
297	120.05722	9.30	C_8H_8O	0.05751
302	119.04950	7.75	C_8H_7O	0.04969
308	118.06570	14.34	C_8H_8N	0.06567
310	117.07062	12.02	C_9H_9	0.07042
311	117.05846	25.97	C_8H_7N	0.05785
313	116.06266	9.30	C_9H_8	0.06260
314	116.05010	11.63	C_8H_6N	0.5002
315	115.05523	33.63	C_9H_7	0.5477
332	108.05689	48.06	C_7H_8O	0.05751
			$C_5H_6N_3$	0.5617
336	107.04912	86.43	C_7H_7O	0.04968
337	106.07748	11.63	C_8H_{10}	0.07825
338	106.06450	6.59	C_7H_8N	0.06567
339	106.04098	17.44	C_7H_6O	0.04186
			$C_5H_4N_3$	0.04052
340	105.06997	20.54	C_8H_9	0.07042
341	105.05747	9.30	C_7H_7N	0.05785
342	105.03405	27.91	C_7H_5O	0.03405
344	104.06089	15.12	C_8H_8	0.06260
345	104.04924	19.77	C_7H_6N	0.05002
346	104.02647	3.88	C_7H_4O	0.02621
347	103.05449	17.44	C_8H_7	0.05477
348	103.04131	6.59	C_7H_5N	0.04220
349	102.04714	9.69	C_8H_6	0.04695
369	95.01145	16.67	C_3HN_3O	0.01196

(continued)

APPENDIX II (continued)

NO.	ACCURATE MASS	INTENSITY	MOLECULAR FORMULA	ACCURATE MASS
372	94.04203	37.21	C_6H_6O	0.04186
377	92.06225	33.72	C_7H_8	0.06260
380	91.05496	100.00	C_7H_7	0.05477
382	90.94616	20.16	C_7H_6	0.04695
384	89.03914	28.68	C_7H_5	0.04695
388	86.03638	12.02	$C_4H_6O_2$	0.03678
412	81.06980	10.09	C_6H_9	0.07042
413	81.03404	5.81	C_5H_5O	0.03404
418	79.05372	46.51	C_6H_7	0.05477
420	78.04678	84.11	C_6H_6	0.04695
423	77.03874	84.88	C_6H_5	0.039123
426	76.03120	26.36	C_6H_4	0.03130
429	75.02278	11.63	C_6H_3	0.02347
433	74.01610	12.02	C_6H_2	0.01565
436	73.02928	11.24	$C_3H_5O_2$	0.02895
437	72.02068	18.22	CH_2N_3O	0.01979
			$C_3H_4O_2$	0.02112
445	69.99857	85.00	C_3HO_2	0.99765

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