METABOLITES OF SOME MICROORGANISMS

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A Thesis

by

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ABSTRACT

This thesis is divided into three distinct parts.

PART I.

The sterol and triterpenoid content of the Myxomycetes Physarum flavicomum, P. polycephalum and Badhamia utricularis has been examined, the last only cursorily..

The sterols of the first two organisms have been separated by thin layer and gas liquid chromatography and have been identified by their gas liquid chromatographic retention times, mass spectra, melting points and optical properties. In both organisms the sterols constitute a typical plant sterol mixture of the less common 24β alkylated epimeric type.

Though the triterpenoids of P. flavicomum and P. polycephalum are present in only small amounts, lanosterol has been tentatively identified in both species and 24-dihydrolanosterol in P. flavicomum.

Preliminary experiments have been made to elucidate the biosynthetic mechanism of C24 alkylation of the sterols by incorporating [Me-$^2H_3$] methionine into the diet. The results are complicated by a rather low incorporation of the label resulting in a mixture of labelled and unlabelled sterols, however a possible scheme for C24 alkylation and for the biosynthesis of the sterols from lanosterol, based on this and previous work, is presented.

Using cultures of P. polycephalum with synchronously dividing nuclei, an attempt has been made, using mass

spectrometry, to observe any change in the concentration of sterols or other cell components during the mitotic cycle: some preliminary results are reported.

PART II

The properties of one of the yellow pigments from the Myxomycete B. utricularis have been investigated. The electronic absorption spectrum of this pigment shows a large reversible bathochromic shift on protonation and thus resembles the spectra of pigments previously isolated from P. polycephalum. The spectral shift and also the pK_a and the reaction with sodium borohydride of the pigment have been compared with similar properties of some model polyene Schiff bases.

As the pigment behaves quite differently from the model compounds, it is suggested that either the pigment is not a Schiff base or that if it is its behaviour is modified by electronic or steric effects possibly related to those affecting the properties of the visual pigment rhodopsin and the various pyridoxal phosphate derived enzymes.

PART III

The major pigment of a yellow bacterium, tentatively identified as a Corynebacterium species has been isolated and shown to be the C50 carotenoid decaprenoxanthin.

Two minor pigments have been identified as mono- and diesters of decaprenoxanthin and this is apparently the first time that esters of any C50 carotenoids have been reported.

The DNA guanine plus cytosine content of the bacterium has been shown to be approximately 45% in contrast to other bacterial sources of C50 carotenoids where the guanine plus cytosine content of the DNA is much higher.

ACKNOWLEDGEMENTS

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Thanks are also due to Miss D. Janes who typed the manuscript and to a succession of technical assistants, Mr. R. Bowring, Mr. A. Dupree, Mr. I. Hack, and Mr. E. Gellately, and especially to Mrs. P. Boyle for the isolation of DNA from the bacterial species.

Other persons who have helped through the donation of Myxomycete cultures and authentic samples of chemical compounds and also through assistance in identifying the bacterial species have been mentioned specifically in the text.

To all others who have assisted in any way, particularly members of the Chemistry Department at Memorial University of Newfoundland, I also express my thanks.

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PART I

INTRODUCTION

General

The name "slime moulds" has been applied to the unrelated Myxomycetes or acellular slime moulds, the Acrasiales or cellular slime moulds, the Plasmodiophorales or endoplasmic slime moulds and the Labyrinthulales or net slime moulds. The work reported in this thesis is totally concerned with the chemical and biochemical aspects of three species of Myxomycetes, Physarum polycephalum, P. flavicomum and Badhamia utricularis. The life cycle of the Myxomycetes can be divided into two separate phases, the vegetative and the propagative. The vegetative form is a free-living multinucleate, acellular, mobile mass of protoplasm, the plasmodium, which has the appearance of a large multinucleate single cell. In the propagative phase, spores are formed in a membraneous, non-cellular spore case within which there is often a system of free or netted threads forming a capillitium.

Myxomycetes are widely distributed and can be found almost anywhere where there is dead or decaying vegetable matter and are thus particularly abundant in forested areas, the coloured plasmodia of the order Physarales being commonly found.

The Myxomycetes cannot readily be classified as plants or animals and though this name, first used by Link in

1833, places emphasis on the fungal and hence plant-like characteristics of the class; the alternative name of Mycetozoa (fungous-animals) proposed by Anton de Bary in 1858 suggests a closer relationship to the animal kingdom. Although both names are still in use, Myxomycetes appears to be more generally accepted, but this is possibly due to the fact that most of the work done on the organisms has been by mycologists rather than to any established closer relationship to the fungi than to amoeboid-like animals.

A chemical taxonomic method is potentially of use in defining this relationship more closely, but insufficient work has been done on the chemical composition of these organisms to utilise this procedure. Korn et al. (1) have reported that the unsaturated fatty acids of the Myxomycete Physarum polycephalum follow the same pattern as those in the soil amoeba (Acanthamoeba sp.).

Until fairly recently it proved impossible to cultivate species of the Myxomycetes in axenic culture, but several species (2,3) can now be kept indefinitely in pure culture on a defined or partially-defined medium.

It has also been shown to be possible to induce the nuclei of the whole plasmodium of P. polycephalum to divide synchronously (4). The Myxomycetes thus provide very useful material for the study of important biological processes such as nuclear division and DNA synthesis, for the chemical composition of the plasmodium at any point in the mitotic cycle

can be examined. Potentially such a system which can yield detailed information of changes during normal processes of growth and nuclear division can be invaluable in the study of abnormal cell function as for instance in cancerous tissues.

Sterols in the Myxomycetes

In 1958 Emanuel (5) isolated what he believed to be a novel sterol from the plasmodium of the Myxomycete Physarum polycephalum. The analysis of the sterol, mp 137-138°, closely fitted the formula $C_{30}H_{52}O_3$. The sterol formed a digitonide, gave a rapid reaction in the Liebermann-Burchard test (6), and decolourised a bromine solution. Emanuel failed to acetylate the sterol with acetic anhydride and pyridine and thus inferred that any hydroxyl groups present were at tertiary positions.

The formula would allow for five units of unsaturation, four of these being accounted for by the ABCD rings of the sterol nucleus and the other by a double bond as indicated by the reaction with bromine.

In 1963 Brewer (7) isolated a sterol from the same organism, which also reacted rapidly in the Liebermann-Burchard test and had a melting point of 144.5-146.5°. The infrared spectrum was identical to that of cholesterol except for an additional absorption band at 935 cm^{-1}. Brewer concluded that this was not the same sterol as had been isolated by Emanuel, but did not attempt to identify it further.

While the work reported in this thesis was in progress, Lenfant et al. (8) reported the isolation of a sterol component also from P. polycephalum. Using gas liquid chromatography (glc) and mass spectrometry, it was shown that the sterol comprised of at least seven components, five with molecular weights of 400, 402, 412, 414, 416, and two which were detected by glc only. Together with this information and the glc retention times, it was claimed that in addition to very small amounts of cholesterol (IIa), and brassicasterol (IIIb), there was also present a mixture of five sterols characteristically found in higher plants and algae, a 'phytosterol' mixture. This mixture usually contains campestanol (Ib), campesterol (IIb), stigmastanol (Ia), stigmasterol (IIIa), and β-sitosterol (IIc), or else the C24 epimers of these compounds*.

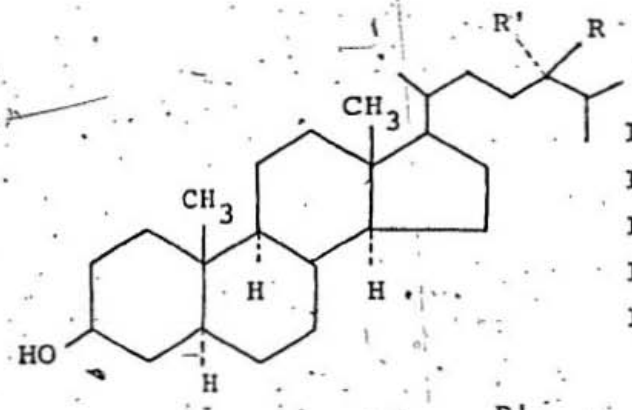
To determine the stereochemistry at C24, Lenfant treated the sterol mixture with ozone, and worked up the products oxidatively to isolate 2-ethyl-3-methylbutanoic acid generated from the side chain of the diunsaturated sterol (stigmasterol or poriferasterol). The circular dichroism curve of this optically active acid was compared with that of a similar product isolated from the ozonolysis of stigmasterol and the two curves were found to have a similar shape. Thus, as the diunsaturated sterol had the α-configuration at C24, it

*Throughout this thesis the trivial names for the sterols will be used in accordance with the list given in Table 1.

Table 1. Systematic* and trivial names of the sterols referred to in the text

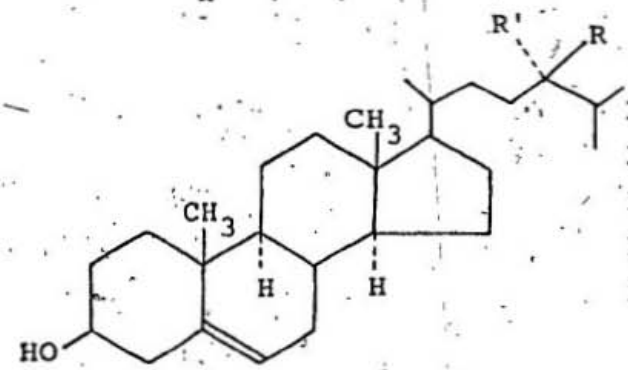
Systematic name	Trivial name
cholestan-3β-ol	cholestanol (Ia)
cholest-5-en-3β-ol	cholesterol (IIa)
24α-methyl-5α-cholestan-3β-ol	campestanol (Ib)
24β-methyl-5α-cholestan-3β-ol	ergostanol (Id)
24α-methylcholest-5-en-3β-ol	campesterol (IIb)
24β-methylcholest-5-en-3β-ol	Δ^5-ergostenol (IId)
24α-ethyl-5α-cholestan-3β-ol	stigmastanol (Ic)
24β-ethyl-5α-cholestan-3β-ol	poriferastanol (Ie)
24α-ethylcholest-5-en-3β-ol	β-sitosterol (IIc)
24β-ethylcholest-5-en-3β-ol	22-dihydroporiferasterol (IIe)
24β-methylcholesta-5,22-dien-3β-ol	brassicasterol (IIIb)
24α-ethylcholesta-5,22-dien-3β-ol	stigmasterol (IIIa)
24β-ethylcholesta-5,22-dien-3β-ol	poriferasterol (IIIc)
24β-methylcholesta-5,7,22-trien-3β-ol	ergosterol (IV)

*The configuration at C24 is stated as α or β, as the more acceptable R and S terminology tends to be somewhat confusing in the description of the stereochemistry of the phytosterols. Thus, β-sitosterol is (24R)-24-ethylcholest-5-en-3β-ol and stigmasterol, because of the introduction of a C22,23 double bond, is (24S)-24-ethylcholest-5-en-3β-ol even though the 24-ethyl group is sterically identical (α) in the two compounds.

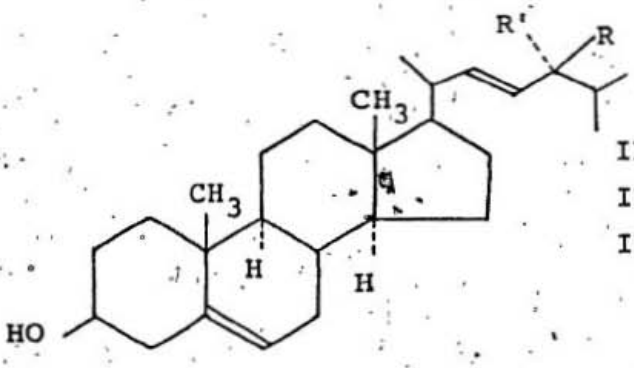


Ia R'=H

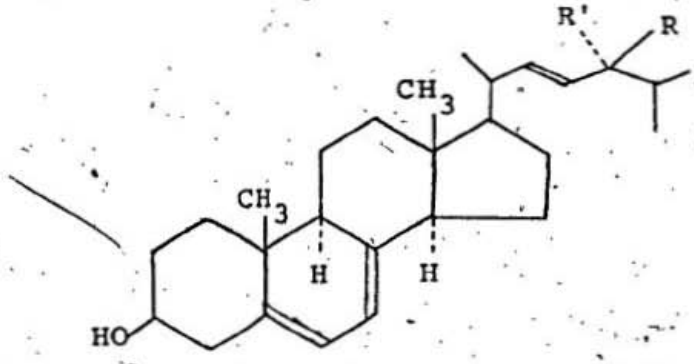
Ia	R'=H	R=H	cholestanol
Ib		$R=CH_3$	campestanol
Ic		$R=C_2H_5$	stigmastanol
Id	R=H	$R'=CH_3$	ergostanol
Ie		$R'=C_2H_5$	poriferastanol



IIa	R'=H	R=H	cholesterol
IIb		$R=CH_3$	campesterol
IIc		$R=C_2H_5$	β-sitosterol
IId	R=H	$R'=CH_3$	Δ^5-ergostenol
IIe		$R'=C_2H_5$	22-dihydroporifer sterol



IIIa	R'=H	$R=C_2H_5$	stigmasterol
IIIb	R=H	$R'=CH_3$	brassicasterol
IIIc		$R'=C_2H_5$	poriferasterol



IV	R=H	$R'=CH_3$	ergosterol

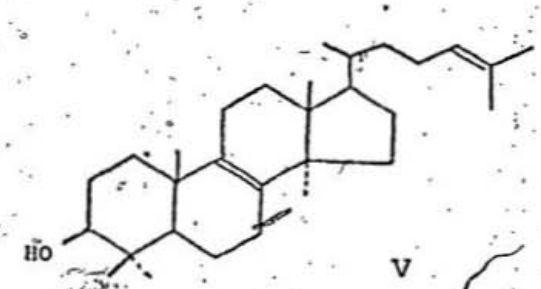
was assumed that the other components of the phytosterol mixture had the same configuration.

It should be noted, however, that brassicasterol, which was identified only by its glc retention time, has the opposite configuration at C24 to stigmasterol. Lenfant did not comment on this.

On the basis of mass spectral and chromatographic data LeStourgeon (9) has suggested that the sterol component of P. flavicomum is a mixture of several closely related sterols, the principle one, which constitutes 70-80% of the total, being stigmasterol. However, as there was no attempt to isolate the individual sterols nor to determine the stereochemistry at C24 in any other way, the identification of stigmasterol can only be considered to be tentative.

Lenfant et al. (8) also isolated the tetracyclic triterpenoid alcohol fraction of P. polycephalum and, though present in only small amounts, the presence of two 4,4'-dimethyl-14-methyl sterols, lanosterol (V) and 24-methylene-dihydrolanosterol (VI), and one 4-methyl sterol, tentatively identified as 4α-methyl-24-ethylcholestenol (VII), was indicated. Lenfant remarked on the uniqueness of finding C24-ethyl sterols together with lanosterol and suggested that this might be a characteristic of Myxomycetes, serving to distinguish them from other classes of organisms.

The occurrence of plant sterols in the Myxomycetes is not necessarily unexpected for, though fungi containing chitin



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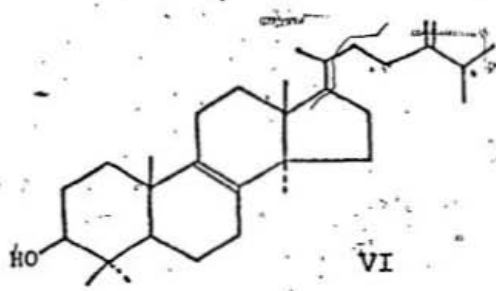

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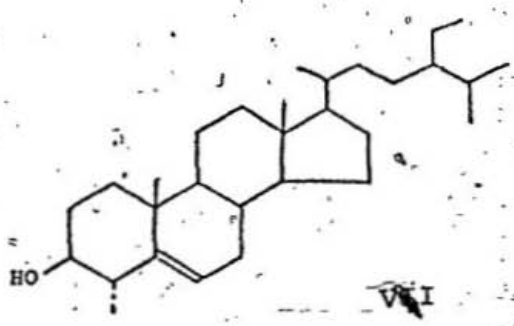

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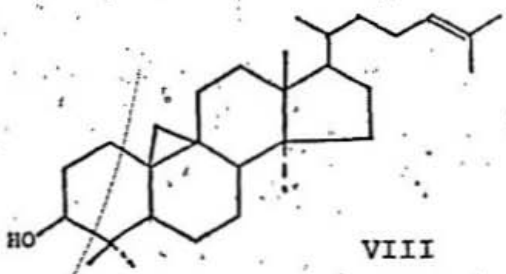

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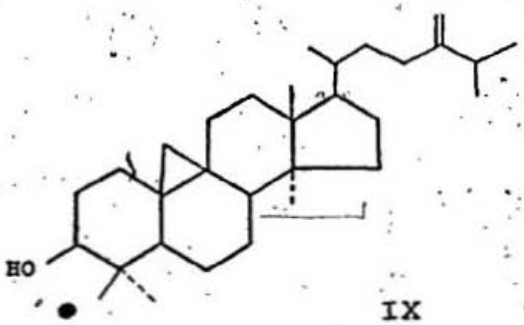

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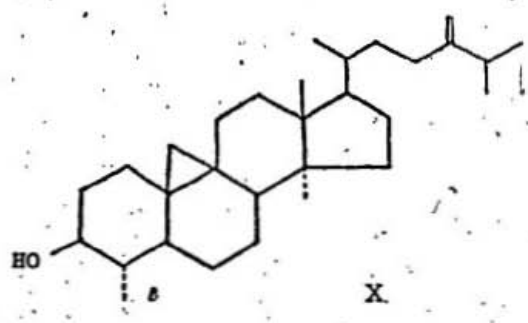

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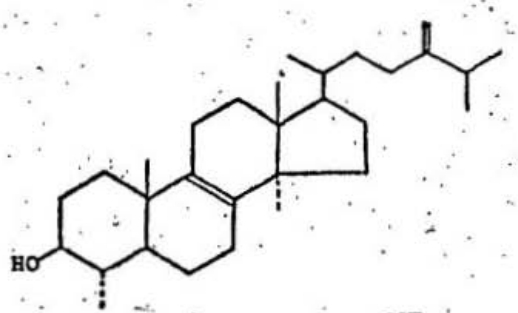

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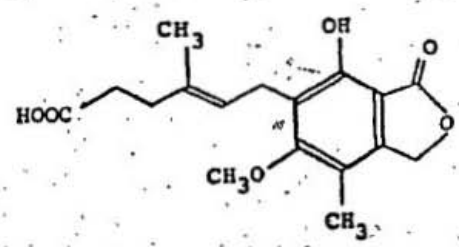


XII

usually contain ergosterol and closely related compounds, fungi which have cellulose walls, such as species of the Saprolegniales and Leptomitales, contain sterols which are commonly found in red or brown algae and in plant pollens (10).

Although chitin has been reported in the capillitium of the Myxomycetes Stemonitis fusca (see Ref. 12) and Hermitrichia serpula (11) a more recent investigation by Goodwin (12) revealed no chitin in any of the species of the Comatrichia genus which casts doubt on the earlier findings. Goodwin did, however, identify cellulose in the stalk, capillitium and spore cases in Comatrichia and Schuster (13) has obtained electron micrographs of the spore wall of Didymium nigripes and has shown that the inner of the two layers gives a cellulose reaction. Dresden also reports that the Myxomycetes, during the resting stage, appear to have cellulose walls which are later absorbed into the growing plasmodium (14).

Biosynthesis of sterols: a brief survey

The initial cyclisation product of squalene, normally through the intermediate 2,3-epoxy squalene, in the biosynthesis of cholesterol in animals is, in all cases studied, the triterpenoid lanosterol (15,16,17). Subsequent conversion of this compound into cholesterol involves reduction at C24, demethylation at C4 and C14 and isomerisation from a Δ^8 to a Δ^5 bond; the order in which these steps is carried out has not been completely determined for any one organism. Recent reviews by Goad (18) and Mulheirn and Ramm (19) have summarised

current knowledge, and emphasise that, though the reaction scheme is generally fairly similar in different organisms, there is a wide variety of individual differences. One possible scheme of reactions which utilises knowledge of the structure of compounds identified singly, or in combination, in different organisms is shown in Fig. 1.

It might be expected that lanosterol would occupy a similar position in the biosynthetic route to other sterols, but it appears that lanosterol is not normally found in either algal or higher plant tissues, being replaced by the isomeric cycloartenol (9, 19-cyclo-9β-lanost-24-en-3β-ol) (VIII) as the phytosterol precursor (20). Lanosterol has only been found in a few species of Euphorbiaceae and then, except in two cases, cycloartenol was also detected (21,22).

Initially it was thought that the first product of cyclisation in plants might be lanosterol with subsequent conversion to cycloartenol (23), but Rees et al. (24) have shown that cycloartenol can arise directly from 2,3-epoxy squalene cyclisation. Investigations have also shown that cycloartenol (i) can be isolated from, and identified in, many plant tissues, (ii) is labelled from acetate and mevalonate, (iii) is converted by plants into phytosterols, (iv) is converted into 24-methylenecycloartanol (IX), a compound often found in plant tissues together with cycloartenol, and this presents quite a large body of evidence favouring its role as an intermediate. The order of the enzymic steps between cycloartenol

and the phytosterols has not been determined for any one organism, though the basic scheme appears to be fairly general and involves, in addition to the steps in the synthesis of cholesterol, opening of the 9β, 19-cyclopropane ring, alkylation at C24 and, in some cases, introduction of a Δ^{22} bond. One possible reaction scheme is given in Fig. 2.

Though cycloartenol is the recognised precursor of the phytosterols, it has been shown that added lanosterol can be metabolised to the same products in Ochromonas malhamensis (25), Nicotiana tabacum (26), and Euphorbia peplus (27). This result has been interpreted as showing a lack of specificity of the C24 alkylating, and the C4 and C14 demethylase enzymes, possibly due to the remoteness of the site of the enzyme action from the site of dissimilarity of the two compounds (lanosterol and cycloartenol).

The origin of lanosterol in some species of Euphorbiaceae and its role, if any, is not known. Ponsinet and Ourisson (28) have shown that the latex of E. helioscopia will incorporate [1-C^{14}] acetate into both cycloartenol and lanosterol, but it is not known whether the lanosterol is formed directly or by opening of the cyclopropane ring of cycloartenol. Certainly the same authors have shown that E. lathyris can metabolise [25-^{14}C] cycloartenol into lanosterol, thus indicating that in this plant there is an enzyme capable of opening the 9β, 19-cyclopropane ring (29).

The relationship of this enzyme to the normal biosynthesis of sterols is not clear. It is possible that the

Fig. 1. A possible route for the conversion of lanosterol into cholesterol (18)

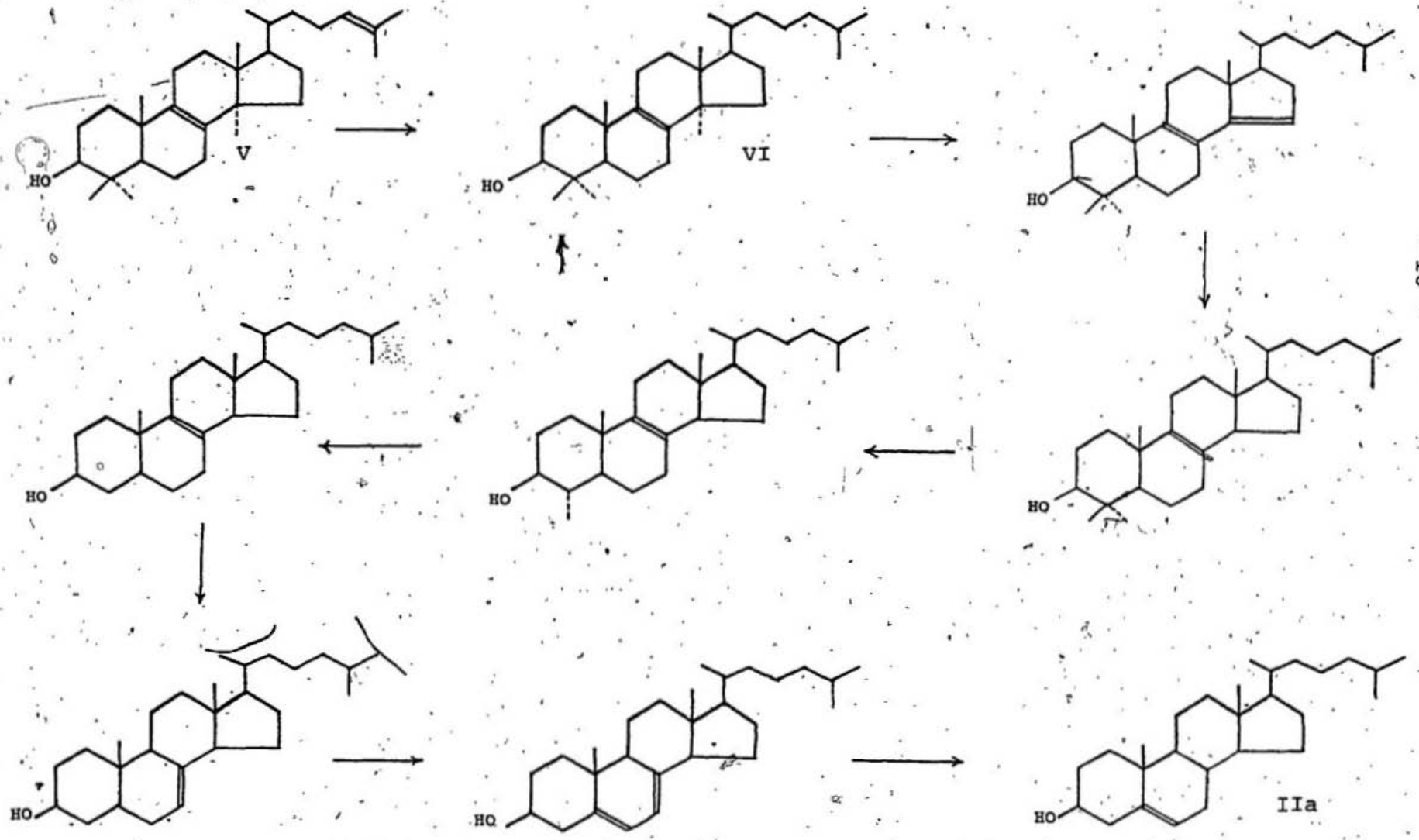
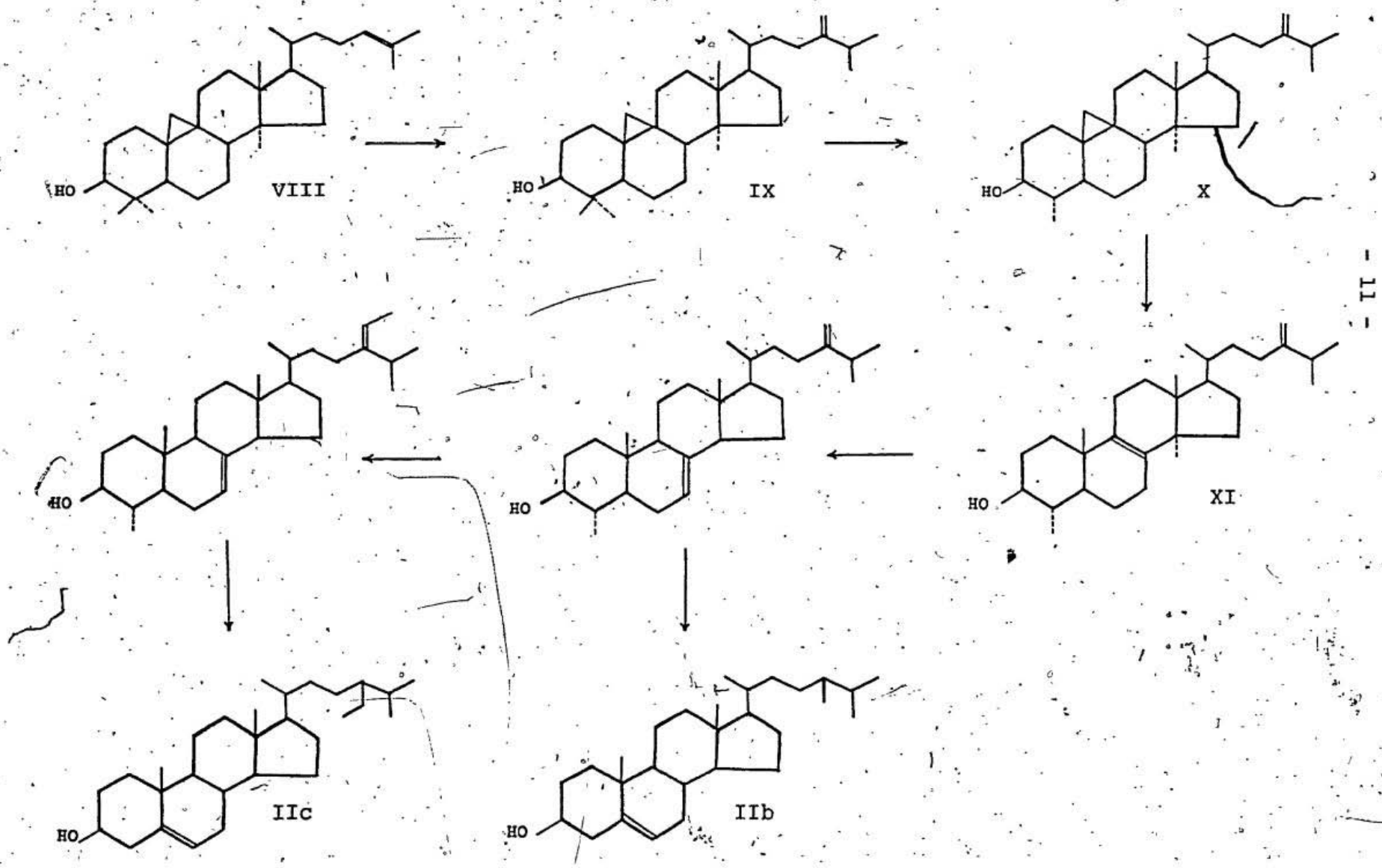


Fig. 2. Hypothetical route for the conversion of cycloartenol into the phytosterols (18)



enzyme may be rather unspecific and may normally operate at a later stage as suggested in Fig. 2. In that scheme the cyclopropane ring of cycloeucalenol (X) is opened to form obtusiofoliol (XI) as the initial ring opened product.

In fungi it appears that the commonly found C24 methylated sterol ergosterol (IV) is formed by way of a lanosterol intermediate and not from cycloartenol. Thus lanosterol has been detected in several fungi, including yeast (30), Aspergillus fumigatus (31) and Saccharomyces cerevisiae (32), where there was no evidence of cycloartenol.

It has been suggested (18) that the C24 alkylation of lanosterol is possibly the first step in ergosterol synthesis in the Phycomycetes and the Basidiomycetes, though in yeasts alkylation appears to be limited to the later stages and thus, as often seems to be the case, the same sterol is synthesised by somewhat different routes in different organisms.

Alkylation at C24

A problem encountered in the biosynthesis of the phytosterols and of ergosterol is the source of the additional carbon atoms at C24 and the mechanism by which this alkylation is effected: in all cases studied the donor of the additional carbon atoms is methionine.

An insight into the biosynthetic route operating in any particular species can be obtained if methionine, deuterium labelled at the S-methyl group, can be, at least partially, substituted for unlabelled methionine in the diet. Thus the

24-methyl group of Δ^5-ergostenol (IId) produced by Chlorella ellipsoidea retains three deuterium atoms from the labelled methionine (33) whereas the 24-methyl group of ergosterol in Neurospora crassa retains only two (34). Poriferasterol synthesized by C. ellipsoidea is labelled by five deuterium atoms in the 24-ethyl group (33) whereas the phytoflagellate Ochromonas malhamensis incorporated only four deuterium atoms into poriferasterol (35).

It had been suggested (36) that the intact methyl group is transferred from methionine to a carbon atom only when an adjacent double bond is activated toward electrophilic attack for example in the formation of mycophenolic acid (XII). In cases when the carbon is not so activated, such as in the formation of the 24-methyl group of ergosterol, it was suggested that a different mechanism resulted in the formation of a 24-methylene intermediate which was then reduced. It is now clear, however, that in certain organisms an intact methyl group can be transferred to a non-activated double bond (with the retention of three deuterium atoms from labelled methionine) and thus at least two distinct enzyme systems are available for C24-methylation.

The additional methylation step required to form the 24-ethyl derivative also takes place by different mechanisms and it has been suggested that the route is through an ethylidene intermediate when only four deuterium atoms are incorporated into the 24-ethyl group (35) but that an intact

methyl group is transferred where five deuterium atoms are retained (33).

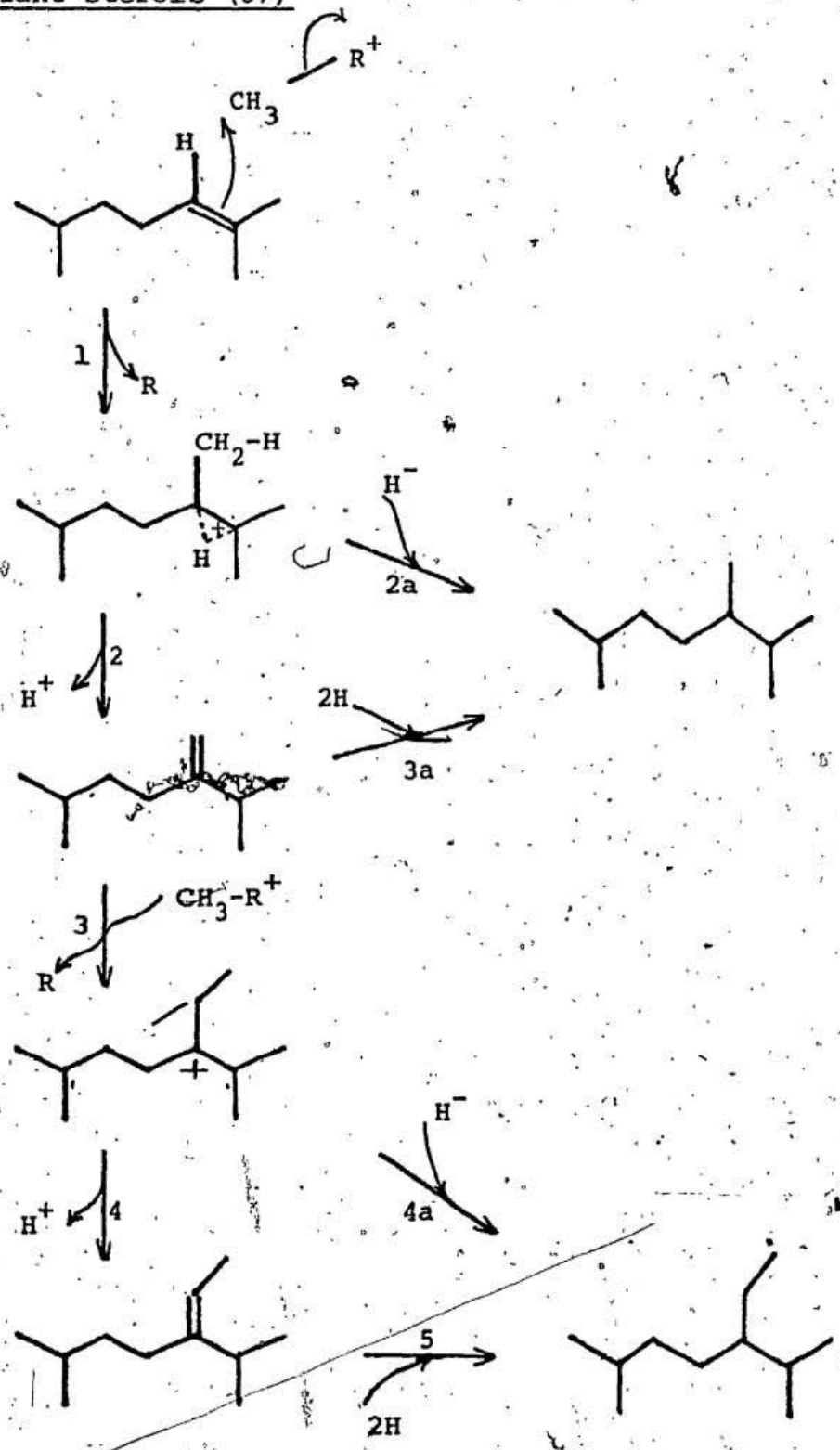
A summary of the possible routes leading to the 24-alkylated sterols has been given (37) (Fig. 3). Using this grid the sterols mentioned above might be synthesised through the routes indicated in Table 2.

Table 2. Possible routes to sterols in different organisms based on the incorporation of deuterium from [Me-$^2H_3$] methionine

Organism	Sterol synthesised	Number of D atoms incorporated	Possible sequence of reactions
N. crassa	ergosterol	2	1,2,3a
C. ellipsoidea	Δ^5-ergosterol	3	1,2a
O. malhamensis	poriferasterol	4	1,2,3,4,5
C. ellipsoidea	poriferasterol	5	1,2,3,4a

From the rather limited data available there is clearly no relationship between (a) the triterpenoid precursor (lanosterol or cycloartenol) and the number of deuterium atoms incorporated from [Me-$^2H_3$] methionine, (b) the triterpenoid precursor and the stereochemistry at C24 of the alkylated sterol or (c) the number of deuterium atoms incorporated and the stereochemistry. As this data can only be collected from rather random investigations of a variety of organisms, it is possible that such regularities might be observed if closely related families or genera are examined.

Fig. 3. Possible routes for C24 alkylation in fungal and plant sterols (37)



DISCUSSION

The sterols of Physarum flavicomum

The total sterol fraction

Column chromatography of the ether soluble component of P. flavicomum gave a white crystalline sterol component which, in the Liebermann-Burchard test (6), gave a green-blue colouration developing to its maximum colour intensity over a period of 0.5 hour (a characteristic of Δ^5 sterols).

The sterol decolourised a bromine solution in carbon tetrachloride and the infrared spectrum showed bands at 1665, 1660 (C=C), and 973 cm^{-1} (Δ^{22}-trans substituted bond in sterol side chain), as well as absorption at 3580 cm^{-1}. There was no ultraviolet absorption in cyclohexane solution above 200 nm indicating the lack of conjugation such as is seen in the common fungal sterol, ergosterol (IV).

Elemental analysis of the sterol (Bernhardt) gave C 83.66%, H 11.51%, O 4.77% (38) which did not agree well with that expected for any pure sterol, and is far lower in oxygen content than the sterol isolated from P. polycephalum by Emanuel (5).

Mass spectrometry of the sterol showed five molecular ions at m/e 400, 402, 412, 414, and 416, whilst after catalytic hydrogenation molecular ions were observed only at m/e 402 and 416. Following acetylation with acetic anhydride and pyridine, molecular ions were only given at m/e 444 and 458, however the

strongest peaks in the spectrum were at m/e 382, 394, and 396 corresponding to the loss of 60 mass units (acetic acid) from the expected molecular ions at m/e 442, 454, and 456. The lack of a molecular ion has been observed in the spectra of 3β-acetoxy-Δ^5-sterols and partially serves to differentiate them from the corresponding 3β-acetoxy-Δ^7-sterols which give relatively strong molecular ions (39). The acetylated mixture was readily hydrogenated to give a product showing molecular ions at m/e 444 and 458 only.

Gas-liquid chromatography (glc) on several columns (four) showed the presence of only three components in the mixture of the sterols and also in the mixture of their acetates, whilst only two components were detected in the reduced sterol mixture and in the reduced acetate mixture. It is known, however, that the silicone stationary phases used will not effectively separate a 3β-Δ^5 unsaturated sterol or its acetate from the corresponding stanol, or its acetate (40). The evidence presented suggested that the mixture contained two saturated stanols (M = 402 and 416), two monounsaturated sterols (M = 400 and 414) and one di-unsaturated derivative (M = 412) and thus appeared to be of the 'phytosterol' type and similar to that isolated by Lenfant from P. polycephalum.

Though the mixture might be tentatively characterised on this evidence, in order to define the stereochemistry at C-24, isolation of pure components or an ozonolysis method similar to that used by Lenfant (8) is required. It was initially decided

to try to isolate pure samples of the major components for this characterisation.

Separation of the phytosterol mixture

Separation of such a mixture into pure components tends to be tedious due to the close structural similarity of the compounds and in many cases may not be worthwhile. However, it was felt that in this case, because of the interest in these organisms and the novel results of Lenfant, that characterisation of the sterols should be made as definite as possible.

Separation of a phytosterol mixture is generally effected in two stages. First, the saturated stanols are separated from the sterols by thin layer chromatography (tlc) on silica gel layers impregnated with silver nitrate. In this system the unsaturated sterols form a π-complex with the silver ion and thus have a lower Rf than the stanols. Following this initial separation, the unsaturated sterol and stanol fractions can be independently separated by glc on one of several possible phases.

In this work this procedure was followed and small amounts of pure sterols were obtained from preparative glc columns. Later work, however, indicated that the initial tlc separation could be utilised to better efficiency and, in addition to the separation of the sterols and stanols, the system was used to separate the diunsaturated sterol (M = 412) from the monounsaturated sterols (M = 400 and 414) by judicious splitting of the rather wide sterol band.

Identification of stanol components

The mass spectrum of the mixture of stanols showed only two molecular ions at m/e 402 and 416, with molecular ions at m/e 444 and 458 in the acetylated mixture. The fragmentation pattern of the mixture was in accordance with that expected for a mixture of the C28 and C29 stanols, campestanol and stigmastanol.

Glc analysis revealed the presence of two components which had retention times identical with campestanol (stanol 1) and stigmastanol (stanol 2). Coinjection with authentic samples of these stanols increased with peak heights and no separation was detected.

From these results it was concluded that stanols 1 and 2 were 24-methyl and 24-ethyl derivatives of cholestanol (Ia), that is either campestanol (Ib) and stigmastanol (Ic) or the C24 epimers, ergostanol (Id) and poriferastanol (Ie).

Unsaturated sterol components

The infrared spectrum of the mixture showed the presence of some unsaturation and suggested the presence of a trans-Δ^{22} bond. Glc separated three components and mass spectrometry detected molecular ions at m/e 400, 412 and 414, with a fragmentation pattern similar to that expected for a mixture of campesterol (IIb), stigmasterol (IIIa) and β-sitosterol (IIc).

The mixture was readily acetylated and the acetate mixture also showed three components on glc. The mass spectrum of this acetylated mixture gave no peaks corresponding to the

expected molecular ions but instead very abundant ions were observed at m/e 382, 394, and 396, corresponding to the loss of acetic acid from each of the three expected molecular ions, again indicative of 3β-acetoxy-Δ^5-sterols (39).

The mixture of sterols, after catalytic hydrogenation, gave the corresponding mixture of stanols which, on a glc analytical column, separated into two components apparently identical with campestanol and stigmastanol, and gave molecular ions at m/e 402 and 416. The mixture of acetates after reduction had molecular ions at m/e 444 and 458, and glc analysis showed only two components.

Efficiency of different phases for preparative glc

As preliminary work to the separation of the mixture of the unsaturated sterols into its components by glc, several phases, each stable at relatively high temperature (350°), were investigated in order to test their efficiency in the separation of an authentic mixture of campesterol, stigmasterol and β-sitosterol and of their acetates. The results indicated that the best phase for the separation of the free sterols was OV101, and for the acetates SE30 with OV101 a close second: the separation of the sterols by OV101 was as efficient as the separation of the acetates.

Though both acetates and trimethylsilyl ether derivatives have been used by various authors (39, 41) to achieve better separation than could be obtained with the free alcohols, the results on OV101 at least partially confirm

those of Grunwald (42) and, in view of the losses of material experienced in forming a derivative and then regenerating the free sterol after chromatography, it seemed preferable to use the sterol mixture without further treatment for the glc separation.

Even using the most efficient phase and varying the operating conditions, including temperature programming, it was not found possible to separate the three known sterols completely by glc, campesterol and stigmasterol in particular tending to overlap. Similarly, the mixture extracted from P. flavicomum was not completely separated, and a typical glc profile, though varying somewhat from batch to batch, is shown in Fig. 4. (For the purpose of future identification the sterols are labelled 1-3 in order of the elution from the column.)

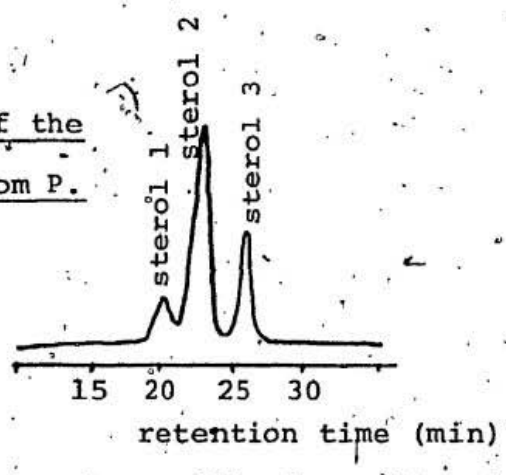


Fig. 4. Part of glc profile of the mixture of sterols from P. flavicomum on OV 101

As can be seen from the glc profile the collection of pure sterol 3 was relatively easy but components 1 and 2 could only be obtained by judicious fraction cutting and pure sterol 1 was obtained in only small amounts, thereby limiting study of this sterol to its mass spectral fragmentation pattern and glc retention time.

An attempt to separate the mixture of sterols by sublimation

An attempt to separate the three sterols by sublimation in a gradient-heated tube failed. Though three distinct bands of crystals were formed, each, when analysed by glc, was seen to contain each of the three sterols in approximately the same relative proportions as the original mixture.

Purification of sterol 2 by tlc

Instrumentation problems, including the unavailability of a chromatograph taking a large enough preparative column and relatively large losses of sterols on the columns (43), made collection of reasonable amounts of samples very tedious and it was decided to investigate the possibility of using tlc more efficiently.

As the unsaturated sterols have a lower Rf than the stanols due to the complexing with the silver ion, it might be expected that diunsaturated sterols would have a lower Rf than the monounsaturated compounds. In fact, in the thin layer system used throughout this work, stigmasterol did have a slightly lower Rf than β-sitosterol (0.291 as opposed to 0.293) and similarly stigmasteryl acetate had a slightly lower Rf (0.920) than β-sitosteryl acetate (0.928). This difference in Rf was insufficient to separate either of the two pairs of compounds into two distinct bands, though it did explain why the band due to the mixture of unsaturated sterol was broader than might have been otherwise expected.

It was found that if the rear third of the sterol band was separated and eluted with ether, the percentage of sterol 2 (as indicated by glc analysis) was greatly increased (for example from 60% of total sterol to 80%). By repeating this procedure twice more the purity could be increased to above 95%, and two or three crystallisations from ether-ethanol gave a product that had none of the other sterols present (shown by glc and mass spectrometry).

This method of purification is rather poor as at each repetition of the thin-layer technique a great deal of the sterol material is rejected (though it can be used in further separations), and thus the actual amount of pure sterol 2 isolated was relatively small, being in the order of 2-3 mg from 100 mg of the starting mixture of sterols.

Although it was not attempted, it should also be possible to work up the leading edge of the sterol band in exactly the same manner thereby obtaining a sample containing the two monounsaturated sterols (sterols 1 and 3) which could then readily be separated by preparative glc.

Identification of unsaturated sterols

Sterol 1.

As the separation from sterol 2 was unsatisfactory, the sterol was never obtained in quantities sufficient for complete characterisation, and only mass spectral and glc data were obtained.

On several glc columns sterol 1 had the same retention time as campesterol and, in addition, the peak height was increased by coinjection with authentic campesterol on each of these columns.

The mass spectrum showed a single molecular ion at m/e 400 and fragmentation ions corresponding to those expected for a 24-methylcholest-5-en-3β-ol; thus ions at m/e 273 formed by the loss of the sterol side chain and at 255 by the loss of the side chain plus water confirmed the presence of a saturated nine carbon side chain (39). Relatively abundant ions at m/e 315 ($M-H_2O-C_5H_7$) and 289 ($M-H_2O-C_7H_9$) were characteristic of a Δ^5 sterol and are not found in the mass spectra of the isomeric Δ^7 sterol (39).

Although these results showed the expected similarity between sterol 1 and campesterol they do not necessarily prove that the two compounds are identical, as neither glc nor mass spectrometry has been shown to differentiate between the two C24 epimers.

Following discussion of the structure of sterols 2 and 3 it will be shown that sterol 1 probably has the 24β configuration (i.e. it is Δ^5-ergostenol).

Sterol 2

Sterol 2 was shown to have retention times identical with those of stigmasterol on several glc columns and coinjection of the two gave no separation. Similar results were obtained for the acetates of the two sterols.

The mass spectrum of the sterol showed a molecular ion at m/e 412, with ions at 273 (M-side chain) and at 255 (M-side chain-H_2O), confirming the presence of a $C_{10}H_{19}$ side chain, which thus contained one unit of unsaturation (39). An abundant ion at m/e 300 is characteristic of Δ^{22} sterols (35).

The acetate of sterol 2 had a spectrum virtually identical with that recorded for stigmasteryl acetate, and was characterised by the lack of a molecular ion and the presence of a very abundant ion at m/e 394 (M-CH_3COOH).

The ir spectra of sterol 2 and of its acetate were virtually identical with those of stigmasterol and its acetate.

The results presented so far indicate that sterol 2 is a 24-ethylcholesta-5,22-dien-3β-ol. In order to distinguish between the two C24 epimers the melting points of the sterol, its acetate and its tetrahydroacetate were compared with the values reported for stigmasterol, poriferasterol and their derivatives, the results showing that sterol 2 is poriferasterol.

Sterol 3

Sterol 3 had glc retention times identical with β-sitosterol, and on four columns coinjection of the two sterols gave a single peak. Similar results were obtained for the acetates.

The mass spectrum showed a molecular ion at m/e 414 and included daughter ions at m/e 329 (M-H_2O-C_5H_7) and 303 (M-H_2O-C_7H_9), these two being characteristic of Δ^5 sterols.

Ions at m/e 273 (M-side chain) and 255 (M-side chain-H_2O) confirmed the presence of a C_{10} saturated side chain.

The mass spectrum of the acetate showed only a very small molecular ion at m/e 456, the base peak being at m/e 396 (M-acetic acid). In both cases the spectra were extremely similar to the corresponding spectra recorded for β-sitosterol and its acetate and, in addition, the infrared speotrum was virtually identical with that recorded for β-sitosterol.

In order to distinguish between the C24 epimers, the melting point of sterol 3 and of its acetate was compared with literature values for β-sitosterol, 22-dihydroporiferasterol and their acetates, the results confirming that sterol 3 is 22-dihydroporiferasterol (IIe).

Optical measurements

As the results presented above indicate the presence of 24-β isomers in P. flavicomum, in contrast to previous work on P. polycephalum (8) it was decided to attempt to obtain confirmation using an optical method similar to that used by Lenfant (8).

The mixture of sterols was ozonolysed and after reductive workup the 2,4-dinitrophenylhydrazone of the 2-ethyl-3-methylbutanal produced from the side chain of sterol 2 was prepared.

As this derivative has only one optically active centre, the rotation of the two enantiomers is equal and opposite

and thus they can be readily distinguished. The preparation of the 2,4-dinitrophenylhydrazone was accomplished only with low yields, however a direct comparison with the product derived from stigmasterol indicated that the two were of opposite configuration. (Though the rotations were opposite they were not quantitatively so, probably due to the small amounts available for the measurement (ca. 14 mg).) As this result confirms the diunsaturated sterol as poriferasterol, the assumption has been made that all the stanols and unsaturated sterols have the same 24-β configuration (this has already been shown to be true for the C24-ethyl cholesterol) and are thus Δ^5-ergostenol, ergostanol, poriferasterol, 22-dihydroporiferasterol and poriferastanol.

It is clear that the sterols extracted from P. flavicomum, although similar to those from P. polycephalum, apparently differ in the stereochemistry at C24.

Triterpenoid fraction of P. flavicomum

As Lenfant had shown the presence of lanosterol (V) in P. polycephalum, the triterpenoid content of P. flavicomum was examined.

The material eluted by benzene from an alumina column was a yellow oil forming 4-6% of the total non-saponifiable lipid. Glc of the fraction showed it to be a rather complex mixture and a preliminary separation was affected by tlc on a silver nitrate impregnated thin layer.

Only a small amount of material was detected in the 4-methyl and 4,4'-dimethyl sterol region, there being a fairly large amount of material which moved with the solvent front (hydrocarbons) and also a small quantity of sterols.

The mass spectrum of the combined 4-methyl and 4,4'-dimethyl sterols showed relatively abundant ions at m/e 426 and 428 together with a less abundant one at m/e 440. Fragmentation ions at m/e 413, 411, 395, and 393 corresponded in relative intensity to those observed in the mass spectrum of a mixture of lanosterol (V) and 24-dihydrolanosterol.

The acetylated mixture showed molecular ions at m/e 468, 470, and 482 with daughter ions at m/e 455, 453, 395, and 393, the latter ions being observed in the spectrum of an acetylated mixture of lanosterol and 24-dihydrolanosterol.

In neither spectrum was there an obvious ion at m/e 286, an ion characteristic of cycloartenol and its acetate (44).

Glc analysis indicated the presence of several components with the two major ones having the same retention times as lanosterol and 24-dihydrolanosterol. A minor component had a retention time slightly greater than lanosterol and might correspond to the molecular ion at m/e 440.

Glc of the acetate mixture showed the two major components to have similar retention times to the acetates of lanosterol and dihydrolanosterol.

Though the presence of lanosterol and its 24-dihydro derivative were not confirmed through isolation and

characterisation of the pure compounds due to the small quantities present, these results, which to an extent support those of Lenfant (8), strongly suggest the presence of these two triterpenoids.

Sterols and triterpenoids of P. polycephalum

In order to see if the observed difference in sterol content between these two closely related Myxomycetes is consistent, a strain of P. polycephalum was examined to characterise its sterol and triterpenoid content.

Much of the work already described for P. flavicomum was repeated though the ozonolysis of the sterol mixture was omitted and the nuclear magnetic resonance spectrum was used to confirm the stereochemistry of sterol 2 at C24. A brief summary of the results is presented.

Stanols

The glc retention times of the components and the mass spectrum of the mixture of the stanols, and of the acetylated mixture, were qualitatively identical with similar information already presented for the stanols of P. flavicomum, though quantitatively the mixture showed relatively more stanol 1.

Unsaturated sterols

Glc and mass spectral data for the mixture and the acetylated product were again qualitatively identical with the data reported for the same fraction from P. flavicomum. It was noted, however, that, though the relative proportions of the

sterols varied somewhat from sample to sample, the relative amount of sterol 2 was consistently lower in this species.

Stereochemistry at C24

Sterol 2 was isolated and purified as previously described. The melting points of the sterol, its acetate and its tetrahydroacetate were again extremely close to the values reported for poriferasterol and its derivatives and a mixed melting point with poriferasterol showed no depression.

Further evidence for the 24-β configuration of sterol 2 was obtained from the nuclear magnetic resonance spectrum. Thompson and Dutky (45) have shown recently that the 100 MHz spectra of the two members of four pairs of C24 epimeric sterols differ slightly in the methyl region; thus, in the range between 81 and 84 Hz, the spectrum of stigmasterol showed a partially resolved doublet, whereas the spectrum of poriferasterol showed only a singlet in this region. Sterol 2 gave a spectrum very unlike that of stigmasterol and extremely similar to that of poriferasterol in this region.

These results clearly show that sterol 2 in this strain of P. polycephalum is poriferasterol and again it is to be expected that the other sterols also have the 24-β configuration at C24.

Triperpenoids of P. polycephalum

Molecular ions were observed in the mass spectrum at m/e 426, 428, and 440, that at 426 being by far the most

intense. Each of the compounds was acetylated, the mass spectrum of the product having molecular ions at m/e 468, 470, and 482. In neither spectrum was there an obvious ion at m/e 286.

Glc showed the presence of four components, lanosterol being the major one. The other components were not identified but 24-dihydrolanosterol was not detected. It was not clear what compound(s) contributed to the small ion peak at m/e 428. In the absence of 24-dihydrolanosterol it might be expected that the compound tentatively identified as 24-ethyl-4α-methylcholestenol by Lenfant would be present, however later experiments involving the incorporation of [Me-$^2H_3$]-methionine into the culture medium did not result in any labelling of this compound as was reported by Lenfant and it appears rather unlikely that this compound is a 24-ethyl triterpenoid.

Sterols of Badhamia utricularis

The sterols extracted from the plasmodium of Badhamia utricularis were also shown to constitute a phytosterol mixture, molecular ions being observed in the mass spectrum at m/e 400, 412, and 414. The ion peaks at 402 and 416 were very small and the stanols, if present at all, can only form a very small percentage of the total sterols.

Glc analysis again showed three peaks with retention times identical with the three sterols obtained from the other two Myxomycete species: no attempt was made to separate the mixture further or to determine the stereochemistry of the component sterols at C24.

Summary of implications of results

It is clear that each of these three closely related species produces a typically plant mixture of sterols and in the case of the two Physarum species the individual sterols have been identified as being the less common 24-β epimeric.

This result contrasts with that of Lenfant et al (8) who claimed to have identified the 24α alkylated sterols in P. polycephalum. It is quite clear that their sterochemical assignment is based on unsound data.

The stereochemistry of the sterols at C24 was supposedly demonstrated by the identity of the circular dichroism (cd) curve of 2-ethyl-3-methylbutanoic acid derived from the side chain of the C29 diunsaturated sterol and that of the corresponding acid obtained from stigmasterol.

The experimental work reported in that paper can be criticised on two important points. Firstly, the reported weight of the derived acid (258 mg) from 328 mg of the sterol mixture is far in excess of the maximum theoretical yield. Secondly, the reported cd curve, with a maximum at 288 nm, cannot be that of a carboxylic acid for though optical rotatory dispersion and circular dichroism curves of asymmetric acids have been reported (46, 47) the Cotton effect observed is related to a weak $n \rightarrow \pi^*$ transition of the carboxyl group at about 215 nm (48). Lenfant's work thus gives no information whatsoever about the stereochemistry of the sterols at C24.

The triterpenoid fraction of both Physarum species had lanosterol as a major component though in P. flavicomum 24-dihydrolanosterol formed approximately 50% of this fraction. A minor component in both species, molecular weight 440, might be identical with a compound of the same molecular weight reported by Lenfant and identified as 24-methylenedihydrolanosterol and a minor peak in the glc analysis has approximately the same retention time as might be expected for this compound, though in the absence of an authentic sample there is no concrete evidence for this.

The accumulated data lends support to Lenfant's suggestion that the Myxomycetes might be unique in utilising lanosterol as the precursor of the 24-ethyl sterols.

The data so far reported gives no information on the biosynthetic route to the phytosterols, except in suggesting that lanosterol is a key intermediate, though it is clear that detailed knowledge of this pathway, in what appears to be a novel system, might be of help in the study of the many intricacies of sterol biosynthesis.

As a first step in the investigation of the biosynthetic route it was decided to investigate the route by which C24 alkylation is effected by attempting to incorporate labelled methionine into the diet of both P. flavicomum and P. polycephalum.

C24 alkylation in P. polycephalum and P. flavicomum

As both species grew normally when additional unlabelled methionine was added to the nutrient medium a similar experiment using [Me-$^2H_3$] methionine was attempted and again growth was normal. Mass spectrometry of the mixture of sterols showed a complex mixture of molecular ions, the spectra obtained from the two moulds being qualitatively similar.

As well as ions corresponding to the usual sterol molecular ions at m/e 400, 402, 412, 414, and 416, other ions indicated the uptake of up to five deuterium atoms into the 24-ethyl sterols. Thus ions at 417, 419, and 421 may be attributed, at least partially, to the addition of five mass units to the usual molecular ions at m/e 412, 414, and 416.

The region m/e 400 to 404 was rather complex due to the contribution of ions formed by the loss of CH_3 and H_2O from the various isotopic forms of the 24-ethyl sterols, and no conclusion could be drawn as to whether two or three deuterium atoms were incorporated into the 24-methyl compounds from this data. The mass spectrum of the acetate, however, which showed molecular ions corresponding to the stanol acetates only, gave molecular ions at m/e 458-463 corresponding to the uptake of up to five deuterium atoms into poriferastanol and at 444-446 indicating the uptake of two deuterium atoms into ergostanol. It is most probable then that both ergostanol and Δ^5-ergostenol are synthesised through a 24-methylene intermediate.

The location of the deuterium atoms is readily shown to be in the side chain for, though there was a complex isotopic

mixture in the 394 to 404 region due to the loss of CH_3 and H_2O from the various isotopic forms of the 24-ethyl sterols, together with the molecular ions of the 24-methyl sterols, the ions at m/e 273 (M-side chain), 271 (M-side chain-2H), 255 (M-side chain-H_2O), and 213 (M-side chain-H_2O-42), which are characteristic of all the three sterols present, were unaccompanied by any abnormal isotope peaks. In addition, the characteristic m/e 369 (M-isopropyl) ion of poriferasterol was accompanied by several isotope peaks, thereby confirming that the side chain isopropyl methyl groups were not derived from labelled methionine, and suggesting that the label is in the 24-ethyl group.

Though the spectra were readily interpreted, the pattern was rather complex because of a contribution due to unlabelled and incompletely labelled sterols and an attempt was made to increase the incorporation of labelled methionine, thereby reducing the quantities of unlabelled sterols in the extract.

Methionine has been identified as an essential amino acid for P. polycephalum (49), and a mixture of amino acids, containing methionine, can be used to replace the normal amino acid source of casein hydrolysate in the medium.

Both organisms were therefore cultivated in a liquid medium with a defined amino acid content, including [Me-$^2H_3$] methionine. The inoculum of plasmodium was kept as small as possible in order to minimise dilution by the preformed, unlabelled sterols from that source.

Growth of both organisms was slow especially for P. polycephalum which had to be grown for two weeks to obtain sufficient plasmodium for sterol extraction. The mass spectra of the isolated sterol mixtures were little better than those already obtained, still showing appreciable quantities of unlabelled compounds in addition to the expected peaks at m/e 417, 419, and 421.

It appears that, to obtain extremely high incorporation, it would be necessary to sub-culture the plasmodia several times into this more closely defined medium, but as the growth rate was very unsatisfactory, this was not attempted.

Hypothetical route to the phytosterols in the Myxomycetes

It seems probable that the initial cyclisation product of 2,3-epoxy squalene oxide in these species of Myxomycetes is lanosterol and not, as might be expected from the phytosterol content, cycloartenol.

It is obvious that the route to the phytosterols from lanosterol cannot be defined but several points are worthy of note.

It appears that there may be some differences between the two Physarum species examined as only one, P. flavicomum, was shown to contain 24-dihydrolanosterol. This result may be a reflection of differing biosynthetic pathways or alternatively may only indicate buildup of a compound that is more rapidly metabolised in P. polycephalum.

The occurrence of 24-dihydrolanosterol is of interest as its presence cannot be fitted into the scheme normally suggested for the biosynthesis of 24-alkyl sterols. Certainly, in the synthesis of sterols which are not alkylated at C24, such as cholesterol, it appears that lanosterol is often reduced to the 24-dihydro derivative as the first step (18) but it is suggested that C24 alkylation by the electrophilic S-adenosylmethionine requires the presence of the $\Delta^{24,25}$ bond and should this be reduced it is difficult to see what structural features might influence the course of alkylation.

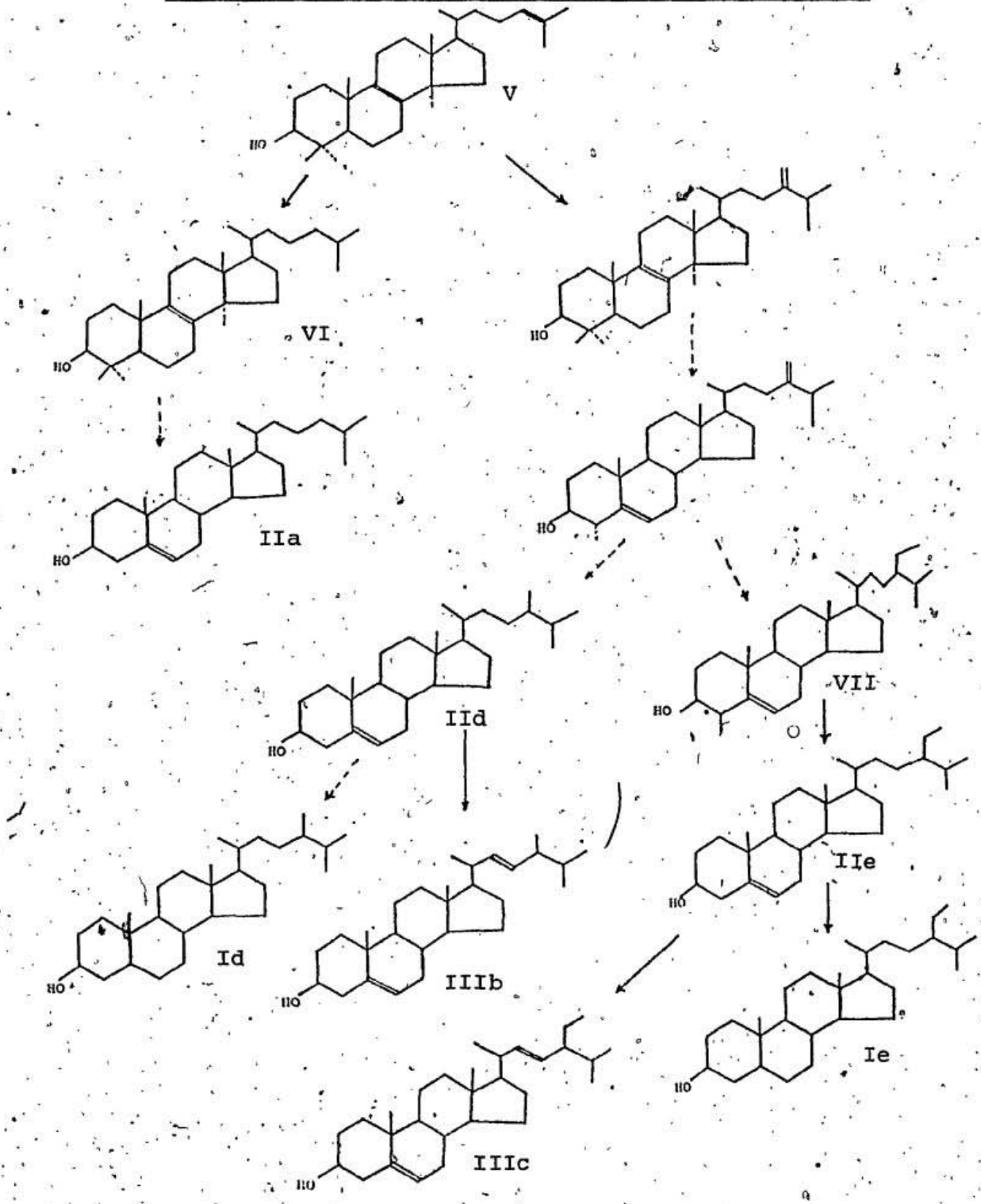
A possible explanation for the presence of dihydrolanosterol is that, following the formation of the cyclised product lanosterol, there is competition at C24 between two reactions with essentially the same biochemical mechanism: alkylation and reduction. The similarity between these two reactions (essentially attack by the electrophile H^+ or CH_3^+) has been suggested by the work of various authors (50,51,52), thus triparanol inhibits both reactions probably by blocking the electrophilic active site on the respective enzymes (53), though it has no effect on any other reaction in sterol biosynthesis. If there is competition of this type lanosterol might be partially reduced to dihydrolanosterol and partially methylated to 24-methylenedihydrolanosterol. In fact, a small amount of a compound with molecular weight 440 (482 in the acetylated mixture) was detected by mass spectrometry in both Physarum species and a small peak in the glc had a retention time similar to that expected for 24-methylenedihydrolanosterol.

Should lanosterol be reduced immediately the product may accumulate, be further metabolised to the phytosterols by a different route or undergo reaction to give sterols with no C24 alkylation. In this regard it is of interest that Lenfant did detect small quantities of cholesterol in the strain of P. polycephalum used, and it is possible that this is synthesized through a typically animal route (Fig. 1).

It is also of interest that Lenfant et al. (8) reported the presence of a triterpenoid alcohol, molecular weight 428, which was tentatively identified as 24-ethyl-4α-methylcholestenol because after incubation of the plasmodium in the presence of [Me-$^2H_3$] methionine the acetate showed a molecular ion at m/e 475 (470 + 5) suggesting that an C24 ethyl compound had been formed. The compound with the same molecular weight in the two species examined in this work was 24-dihydrolanosterol in P. flavicomum, and though it was not identified in P. polycephalum, as no additional isotope peaks were observed after culture with [Me-$^2H_3$] methionine, it was concluded that 24-ethyl-4α-methyl-cholestenol could not be present in appreciable amounts. However, its presence cannot be excluded and this compound can easily be fitted into a hypothetical scheme for sterol biosynthesis (Fig. 5).

This reaction scheme indicates possible routes to the five major sterols, ergostanol (Id), poriferastanol (Ie), Δ^5-ergostenol (IId), 22-dihydroporiferasterol (IIe) and poriferasterol (III), as well as suggesting how cholesterol (IIa)

Fig. 5. A possible scheme for the later stages of sterol biosynthesis in P. polycephalum and P. flavicomum



and brassicasterol (IIIb), which were detected by Lenfant, might be formed. Of the suggested intermediate compounds, 24-dihydrolahosterol has been detected in this work and evidence for the presence of 24-methylenedihydrolanosterol (VI) and 24-ethyl-4α-methylcholestenol (VII) has been presented by Lenfant.

Classification of Myxomycetes

The knowledge of the triterpenoid and sterol content of these species appears to be of little help to the taxonomist attempting to classify the Myxomycetes as plant or animal. The occurrence of a mixture of sterols typically found in higher plants together with the characteristic animal or fungal precursor only serves to emphasise the uniqueness of the organisms.

Further study on the biosynthetic pathways utilised in these organisms is made difficult by the rather complex mixture of sterol products. The ideal situation for further work would be the identification of a species, or a strain of one of the species already examined, in which a single plant sterol is synthesized in relatively large amounts. At present such an ideal system has not been reported, but mass spectrometry, using the rapid technique described later, could possibly locate such an organism.

Function of sterols

It appears to be an exercise in futility to suggest possible functions for the sterols at this time. It is possible

that they are true secondary metabolites being end products of biosynthetic pathways responsible for removing undesirable products from metabolic reactions subject to feedback inhibition, on the other hand it has been shown that such plant sterols may serve as precursors of related components with a possible physiological effect (54). A related compound, Δ^{22}-stigmasten-3β-ol, has been implicated as the cellular aggregation hormone in the non-related cellular slime mould, Dictyostelium discoideum (55).

Sterol content of synchronous cultures of P. polycephalum

Cultures of P. polycephalum were grown in which the nuclei were highly synchronous throughout the plasmodium at the second (MII) and third (MIII) metaphases after being started. It was possible, using phase contrast microscopy and a knowledge of the approximate time required for each step to collect the plasmodium at specified times during the mitotic cycle in order to look at its chemical composition.

The quantity of synchronous plasmodium was limited to about 0.2 g and thus it was not easy to look quantitatively at any particular component, so it was decided to look for a rapid scanning technique that might show changes in concentration of one or more components: mass spectrometry was chosen for this purpose.

It was found that a portion of the wet plasmodium could be introduced into the dipper of the inlet system of the mass spectrometer and a mass spectrum of this sample, after drying, could readily be obtained with ions due to the various sterols being observed when the spectrum was run at 230°.

As no direct quantitative measurement of the individual sterols was obtainable, the relative peak heights of the various ions in the spectra were recorded, all spectra being run under, as far as possible, identical conditions.

Though it is probable that the relative volatilities of the sterols differ and also that the ease with which each forms a molecular ion and the ease with which this, once formed, fragments also varies, the method probably gives an approximate value of the relative concentrations of the sterols and could indicate any change in the concentration of a sterol during the mitotic cycle. Any change located in this way, if considered to be important, could be followed up by alternative quantitative techniques later.

One possible problem in this method might be effects due to any different strengths of binding of a particular sterol within the plasmodial structure at different points in the mitotic cycle; however, it is probable that the drying procedure would disrupt any such binding.

Spectra taken at approximately 20 min time intervals after MII through to MIII showed no significant changes in the relative concentration on any of the five sterols (m/e 400, 402, 412, 414, 416) when the height of each molecular ion was expressed as a percentage of the sum of the peak heights of the five sterol molecular ions. A spectrum of the resting sclerotia was not significantly different from those of the growing plasmodium.

Other cell components in synchronous plasmodia

Though the relative concentrations of the sterols did not alter, other ions in the spectrum did change in relative intensity during the cycle, being undetectable at some stages and quite pronounced at others. Again, there being no basis for a quantitative measurement, the peak height was expressed as a percentage of the sum of the molecular ion peak heights of the sterols.

An ion at m/e 426 (possibly lanosterol) was obvious only in the last hour of the mitotic cycle and immediately after prophase (Fig. 6a).

An ion at m/e 429 increased to a maximum between metaphase and about 3 h after metaphase following which it decreased; the overall change in the relative peak height was about 15 fold (Fig. 6b).

An ion at m/e 448 was first observed immediately before nuclear division was prominent during prophase and decreased rapidly to be undetectable 30 min after metaphase (Fig 6b).

An ion at m/e 365 also showed significant change for, though it was detectable throughout the cycle, it appeared to show an especially high concentration about 6 h after metaphase (Fig. 6b).

The significance of these results cannot at present be determined but it is interesting to note that Rusch et al. (56) reported the occurrence of a relatively thermostable substance

which appears in the plasmodium of P. polycephalum about 2 h before the following metaphase and which, once formed, irrevocably commits the nuclei to division.

No attempt is being made here to use the mass spectral data as a definitive quantitative measure as it is realised that even minor changes in the probe temperature and the time which the sample spends in the spectrometer before the spectrum is run can have an effect on the relative sizes of the ion peaks. However, the results presented are fairly reproducible over three runs and the large increase in the height of the m/e 365 peak after about 6 hours has been observed on five occasions.

Nature of the compound causing the m/e 365 ion

In attempting to follow up the nature of the molecule causing the m/e 365 ion, it was noted that the relative size of the peak to those at m/e 367 and 369 increased if the sample was left in the spectrometer 10 min before running the spectrum. However, on four more occasions the 365 peak was anomalously large at about 5-7 h after metaphase, so it appears that the change is real and not instrumental in origin.

Attempts were made to discover the nature of the m/e 365 peak by obtaining the mass spectrum at increasing temperatures. At 260° the spectrum above m/e 350 was almost totally steroidal with a very small 365 peak but after increasing the temperature to 280-300° the sterol had completely volatilised

off, leaving m/e 365 as the major high mass number ion. It appeared, therefore, that the compound is more polar than the sterols or else the ion is a fragmentation ion of a compound with a molecular weight much greater than the sterols.

An ether soluble fraction of the plasmodium gave a typical sterol spectrum with no obvious ion at m/e 365, however, when the plasmodium was re-extracted with methanol the residue, after evaporation of the solvent, gave a very weak steroidal spectrum at 200-240°, at 250° m/e 365 was one of the strongest ions above m/e 150 and at 300° the ion was close to being the base peak in the spectrum even including the very low mass ions.

It is possible that m/e 365 is not a molecular ion but a fragmentation ion since there are weaker higher mass ions including one at m/e 576 which shows a long series of ions at m/e 576 - 14 n; however, its solubility properties do show that it is far more polar than the sterols.

Figs. 6a and b. The change in concentration of some high mass ions in the mass spectrum of the ether extract of a synchronous plasmodium of P. polycephalum

(Concentration expressed as percentage of total sterol molecular ion peak heights p. 43.)

Fig. 6a. Ion at m/e 365

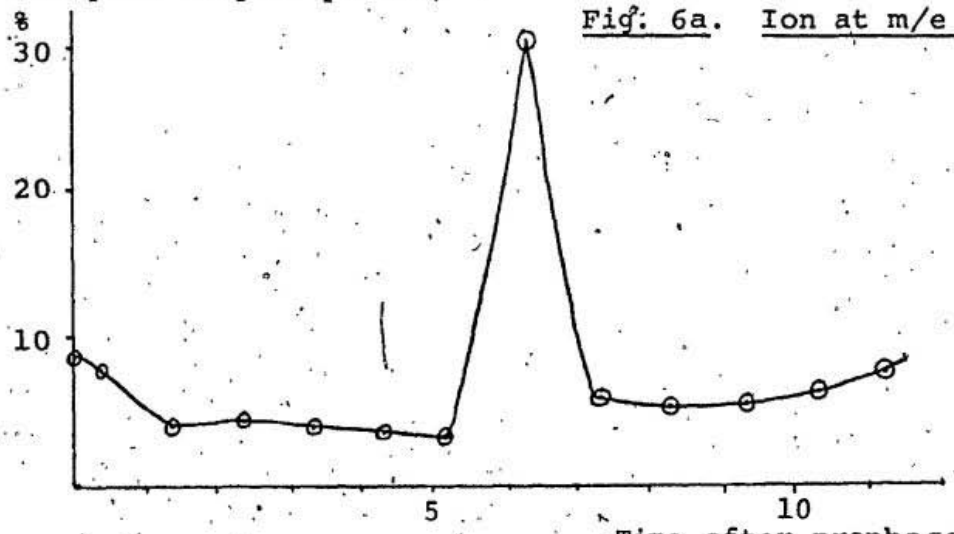
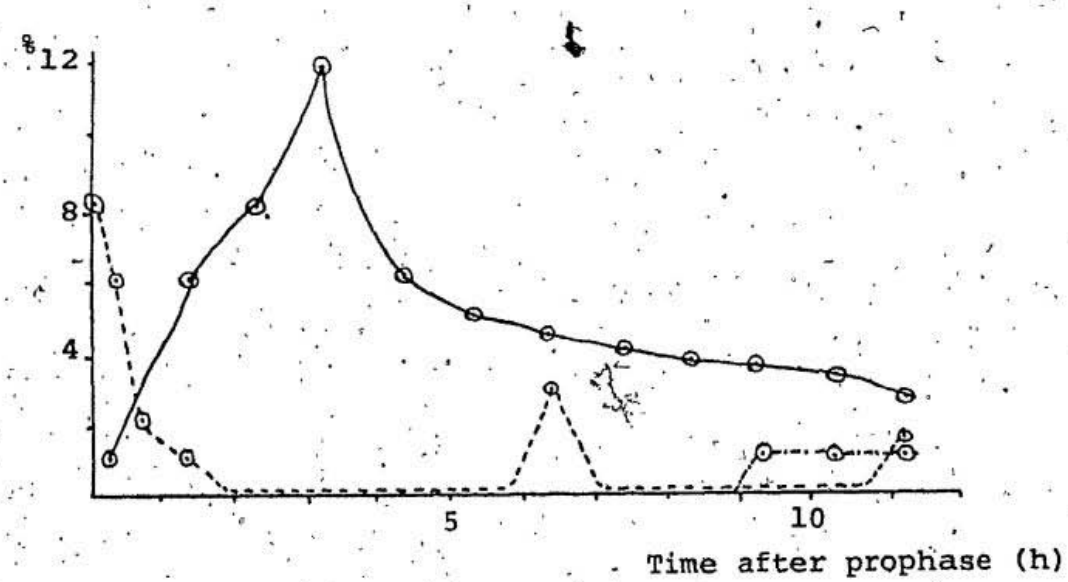


Fig. 6b. Ions at m/e 429 (——), 448 (---)* and 426 (-.-.-)**

*m/e 448 only observed immediately before and after mitosis and at about 6 h after mitosis.

**m/e 426 only observed immediately before nuclear division.



EXPERIMENTAL

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

Ultraviolet /visible absorption spectra were measured using a Perkin-Elmer SP 800 or a P E 202 spectrophotometer.

Optical rotations were determined in benzene solution at the sodium D line on a Perkin Elmer 141 polarimeter.

Mass spectra were recorded on a Hitachi Perkin-Elmer RMU-6E mass spectrometer. The spectra of the sterols are reported only in the upper mass region (above m/e 200) making reference to ions with an abundance of 5% or greater of the base peak. The structure of many of the characteristic ions has been reported by Knights (39).

Nuclear magnetic resonance spectra were determined on a Varian HA100 spectrometer.

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

Gas liquid chromatography (glc)

Glc separation of the sterols was effected on one of two instruments. A Perkin-Elmer PE 881 fitted with a flame ionisation detector (effluent split 4:1 collector:detector) and a 4.5 m x 6.6 mm OD glass column with helium as the carrier gas with a flow rate of 40 cm^3 min^{-1}.

A Varian Aerograph 1520 with a thermal conductivity detector, and a 3.75 m stainless steel column (OD 6.6 mm) was also used. The carrier gas was helium with a flow rate 65 cm^3 min^{-1}; this instrument was fitted with an integrator.

The columns were packed with acid washed, silanised Chromosorb W (100-120 mesh) which was coated with one of the following liquid phases:

3% OV 17

4% OV 101

4% OV 25

3% SE 30.

After a certain amount of experimentation with column temperatures and temperature programming, the operating conditions for all columns were:

column temp. 270°

injector temp. 300°

detector temp. 300°.

Unless otherwise stated all glc data are reported under these conditions.

OV 101 was used for all preparative work when the effluent sterol was condensed in narrow bore glass U-tubes cooled in a Dry Ice bath; the other columns were used for analytical work only.

To compare two sterols, the two were chromatographed separately and were also injected simultaneously onto the column. If the two had the same retention time, when applied

separately on two or more different columns and if when injected together a single symmetrical peak was given, this was used as evidence of their close similarity (probably identical or C24 epimers).

Quantitative measurements on the PE 881 were made by multiplying the peak height by the width at half height; on the Varian Aerograph the integrator was used. These methods both assume that the volatilities of the individual sterols, and the detector response to each, are almost identical, probably valid assumptions (57).

Efficiency of different liquid phases in the separation of a phytosterol mixture by glc

As preliminary work to the separation of the mixture of the Myxomycete sterols into its components several liquid phases, each stable to a relatively high temperature (300°), were investigated in order to test the efficiency of each in the separation of an authentic mixture of campesterol, β-sitosterol and stigmasterol and of their respective acetates (Table 3).

Table 3. Retention times of phytosterols and derivatives on various phases at 270°C

Sterol	OV17*	OV25	SE30	OV101
	**			
cholesterol	1.00 (13.3 min)	1.00 (9.75 min)	1.00 (11.8 min)	1.00 (16.1 min)
campesterol	1.27	1.25	1.35	1.26
stigmasterol	1.44	1.39	1.44	1.43
β-sitosterol	1.64	1.52	1.70	1.69
campesteryl acetate	--	1.63	1.76	1.66
stigmasteryl acetate	--	1.80	1.88	1.80
β-sitosteryl acetate	--	1.96	2.29	2.03

*The three OV phases were used because of the varying percentage of phenyl substitution on the silicone phase (OV101 0%, OV17 50%, OV25 (75%).

**All retention times are given relative to cholesterol for which compound the actual retention time is also given.

Culture conditions for Myxomycetes

I. P. polycephalum and P. flavicomum

Both organisms were normally grown in liquid medium and on agar plates on a partially defined medium. The medium was essentially that developed by Daniel for the culture of P. polycephalum (58) (Table 4).

Table 4. Composition of 1 dm^3 liquid medium for P. polycephalum and P. flavicomum

casein hydrolysate	10 g
dextrose monohydrate	10 g
yeast extract	1.5 g
KH_2PO_4	2 g
(a) Hemin (0.04% in 0.015 M NaOH)	10 cm^3
(b) Supernatant of salt suspension	30 cm^3
citric acid (0.5 M in M NaOH)	27 cm^3
(c) antifoam (AF-60 Canadian General Electric)	2 drops
(d) Wycillin 300 suspension (Penicillin-G Procaine)	30,000 IU
water to	1 dm^3

(a) autoclaved separately

(b) salt suspension

solution A		solution B	
$CaCl_2.2H_2O$	10 g	$FeCl_2.4H_2O$	2 g
$MgSO_4.7H_2O$	10 g	$MnCl_2.4H_2O$	1.4 g
citric acid monohydrate	20 g	$ZnCl_2.7H_2O$	0.56 g
conc. HCl	2 cm^3	water to 100 cm^3	
water to 300 cm^3			

Solutions A and B mixed and made up to 500 cm^3.

(c) Antifoam was added to shake flask cultures in the early work, but was later shown to be unnecessary.

(d) Penicillin was not required providing that extreme care was taken to maintain aseptic conditions.

(a) Shake flask culture

In a typical run six 3 dm^3 Erlenmeyer flasks, each containing 600 cm^3 of the culture medium and stoppered with a cottom wool plug, were autoclaved for 15 min. After cooling, the hemin solution, which had been autoclaved separately, was added aseptically to each flask followed by 20 cm^3 of the plasmodial suspension from the previous 4-day-old culture, the initial cultures having been kindly given by Dr. C. J. Alexopoulous of the University of Texas. The flasks were shaken in the dark in a thermostatically controlled room at about 25° for four days before the plasmodium was harvested. After being removed from the shaker the flasks were allowed to stand until the plasmodium had settled out, then, after decantation of the medium, the plasmodium was centrifuged at 10,000 rpm for 2 min. (The microplasmodia of P. flavicomum tended to be rather gelatinous and, unless the culture was stopped at exactly the right time, settled very slowly if at all.)

For smaller scale cultures, 250 cm^3 flasks, containing 50 cm^3 of medium, were substituted, all other additions being scaled down accordingly. It was found by other workers in the laboratory that the usual hemin concentration tended to cause aggregation of the plasmodium in these smaller flasks and thus the concentration was reduced to 6 cm^3 of standard hemin solution per dm^3 of solution.

(b) Solid medium

Stock cultures of both P. polycephalum and P. flavicomum were maintained in petri dishes on nutrient agar made by adding 1.5% agar to the liquid medium before autoclaving. After allowing the medium to cool, the hemin was added followed by penicillin and, after solidification and inoculation with a small piece of plasmodium from a previous culture, the plates were incubated in the dark at 25° for 1 to 2 weeks before being subcultured.

(c) Medium for methionine and [methyl-$^2H_3$] methionine feeding experiments

(i) Two 50 cm^3 shake flask cultures, one of P. polycephalum and one of P. flavicomum, were set up in the usual manner and to each was added 40 mg of DL-methionine (prepared from homocystine and methyl iodide) which had been sterilised by dry heat at 120° for 1 hour. Incubation followed in the usual manner, both species growing normally.

(ii) [Methyl-$^2H_3$] methionine (prepared from homocystine and $^2H_3$-methyl iodide was substituted for the unlabelled amino acid and growth was again normal.

(d) Defined amino acid medium

(i) The protein source (casein) in the culture medium was replaced by the following mixture of amino acids and ammonium sulphate.

	mg per dm^3
DL-methionine	250
glycine	450
L-arginine hydrochloride	600
DL-alanine	1,200
$(NH_4)_2SO_4$	250

(ii) Labelled [Me-$_2H^3$] methionine replaced the unlabelled compound in medium (d,i) above.

Growth of P. polycephalum and P. flavicomum in both of these media was extremely slow. P. flavicomum was harvested after 1 week but P. polycephalum had to be cultured for 2 weeks until a reasonable amount of growth had taken place.

(e) Synchronous culture of P. polycephalum

P. polycephalum was cultivated by Dr. E. Bullock in this laboratory using a variation of the method described by Mohberg and Rusch (59).

An aliquot (5 cm^3) of the culture stock line, which was maintained in the partially defined liquid medium in shake culture at 26° ± 0.5° and subcultured every 3 days, was added to fresh medium (50 cm^3) and allowed to grow for 20 to 24 h whilst shaking gently. The microplasmodia were centrifuged out, suspended in water and applied to a millipore membrane (Oxoid) which was supported on a Whatman 3 MM filter paper and a stainless steel mesh in a covered steel dish. After 2 h the

microplasmodia had fused and sufficient liquid medium was applied to wet the filter but not the membrane, the medium rising through the membrane pores by capillary action. The dish was agitated in a horizontal plane in a linear fashion at about 25 stokes per minute, so that fresh medium continuously flowed under the paper.

The first metaphase (MI) occurred after 6-7 h, the second (MII) after about 18.25 h and the third (MIII) after about 30 h. Both MII and MIII were highly synchronous, providing that the plasmodium was not too thick, but MI and MIV were much less synchronous.

II. Badhamia utricularis

A thin layer of uncooked oatmeal flakes (Ogilvie) was placed on the bottom of several petri dishes and these were autoclaved at 110° for 15 min. A hot autoclaved solution of 1.5% agar was poured over the oatmeal and allowed to set. A sterile loop was used to transfer a small piece of plasmodium from the previous culture: the original culture was kindly donated by Dr. C. J. Alexopoulous. The plates were kept in the dark at 25° for several days until the yellow plasmodium had covered the surface of the agar.

In larger scale work, 20 cm x 30 cm casserole dishes, fitted with stainless steel lids, replaced the petri dishes, and four pieces of plasmodium were used to inoculate each plate close to each of the four corners.

Extraction of triterpenoids and sterols from P. flavicomum and P. polycephalum

In a typical extraction 4 dm^3 of a 4-day-old culture of either P. polycephalum or P. flavicomum was allowed to settle for 2 or 3 hours and, after decantation of the culture medium, the plasmodium was separated by centrifugation at 10,000 rpm for 2 min. The plasmodium was homogenised with three times its volume of acetone in a Waring blendor for about 30 sec, and the aqueous acetone extract was filtered under suction. The filtrate was concentrated at 35° under reduced pressure until the smell of acetone could no longer be detected, and the resulting suspension was extracted four times by shaking with an equal volume of petroleum ether (35°-60°). (Although ether was a more efficient solvent, it also extracted large amounts of pigment which was required for other work.) The petroleum ether layers were combined and the slightly brown solution was concentrated to one third of its bulk by evaporation under reduced pressure, and then dried over anhydrous sodium sulphate. After drying, the solution was evaporated at 35° under reduced pressure until no more solvent was removed, then the dark brown semi-solid residue was dissolved in warm absolute ethanol and to this solution was added an equal volume of an aqueous solution of sodium hydroxide (20 g in 100 cm^3 water). The resulting solution was left overnight at room temperature, then after dilution by half its volume of water, the unsaponifiable lipid was extracted

into petroleum ether (35^{o}-60^{o}) by shaking three times with half its volume of the fat solvent. The petroleum ether solution was dried over anhydrous sodium sulphate and then evaporated to dryness to give a slightly yellow, semicrystalline solid.

In later work the wet plasmodium, after centrifuging, was freeze dried, 240 g yielding about 12 g dry weight: the dried plasmodium was stored in a vacuum desiccator for several weeks with no visible sign of deterioration.

The dried plasmodium (12 g) was rapidly ground in a mortar (the plasmodium was quite hygroscopic and became very wet if this process was followed too slowly) and refluxed for 2 h with dry ether (200 cm^3). The sterols were readily extracted and, in the absence of water, the ether extracted very little pigment. The ether solution was evaporated to dryness to leave an almost white semi-crystalline solid (ca. 80 mg).

This lipid fraction was then treated with sodium hydroxide solution and the unsaponifiable lipid extracted as previously described.

Extraction of sterols from Badhamia utricularis

The yellow plasmodium (20 g) was scraped from the agar plate and freeze dried. The dry plasmodium (1.1 g) was refluxed with dry ether (15 cm^3) for 2 h when the ether was removed under reduced pressure leaving a pale yellow crystalline solid (11 mg). The mass spectral and glc properties of this crude sterol extract are recorded.

Column chromatography of non-saponifiable lipids from P. flavicomum

The unsaponifiable lipid (203 mg) was dissolved in a small amount of benzene and chromatographed on a 15 x 2 cm neutral alumina (activity 3) column, first with benzene (150 cm^3) and then with 5% ether in benzene (200 cm^3). On evaporation the benzene solution gave a yellow oil (8 mg) which contained the triterpenoids, and the benzene-ether solution a white solid mass of sterols (183 mg).

The lipid fraction of P. polycephalum was chromatographed using the same system (Table 5).

Table 5. Typical yields at different points in the isolation procedure (from 4 dm^3 medium)

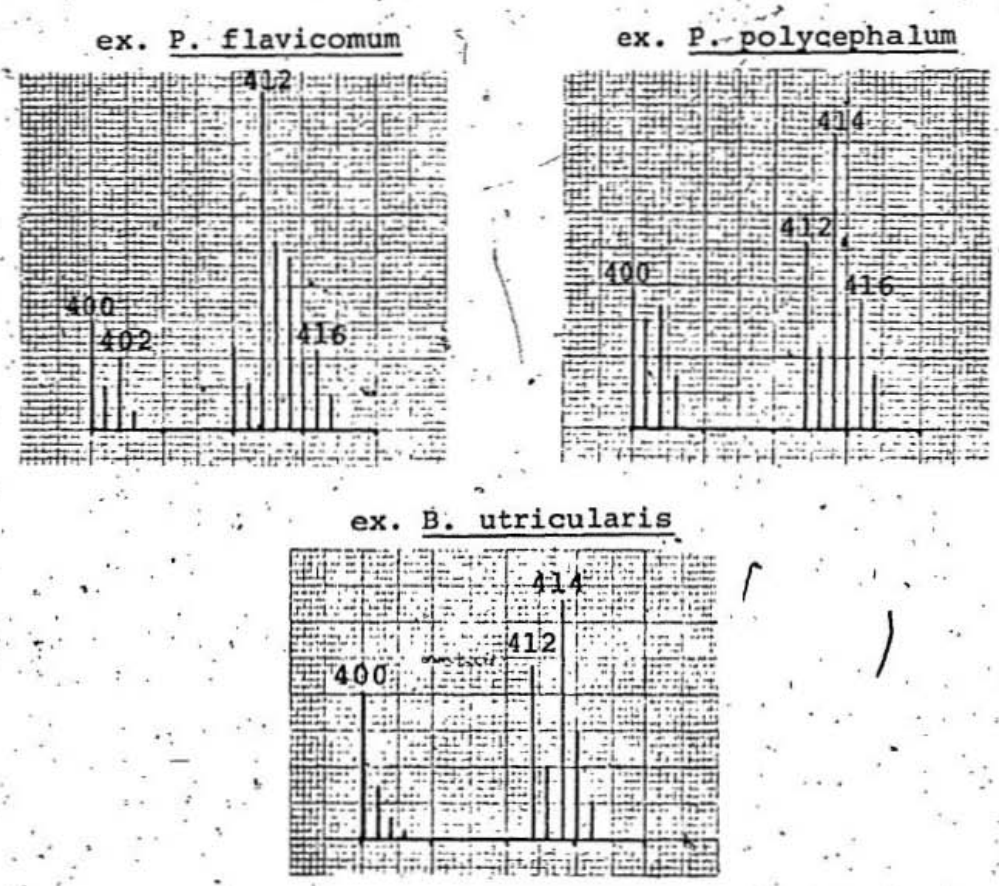
	P. flavicomum	P. polycephalum
Wet wt. plasmodium	125 g (orange-brown, coarse texture)	144 g (yellow-brown, fine texture)
Ether soluble fraction	0.59 g	(not measured)
Non-saponifiable lipid	101 mg	125 mg
Alumina column		
(a) triterpenoids	4 mg	6 mg
(b) sterols	93 mg	108 mg
tlc (a) stanols	7 mg	28 mg
(b) unsaturated sterols	77 mg	71 mg

Mixture of sterols

The sterol mixture from P. polycephalum and P. flavicomum crystallised as white needles from ether-ethanol. The sterol mixture from B. utricularis was only obtained in a crude form.

Mass spectrum: the spectra from m/e 400 to 420 are recorded for the sterol mixture from each of the three species (Fig. 7).

Fig. 7. Mass spectra (from m/e 400 to 420) of the sterol mixtures from P. flavicomum, P. polycephalum and B. utricularis



Liebermann-Burchard reaction (6):

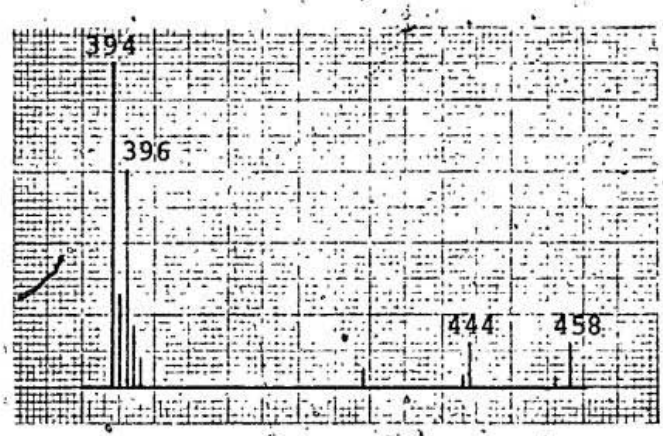
A few crystals of the mixture of sterols (ca. 5 mg) from both P. polycephalum and P. flavicomum were dissolved in glacial acetic acid (1 cm^3). Acetic anhydride (2 cm^3) and concentrated sulphuric acid (0.1 cm^3) were added.

In both cases a green-blue colouration, developing to a maximum intensity in approximately 0.5 h indicated the presence of Δ^5 sterols.

Acetylated mixture of sterols

Mass spectrum: the mass spectrum of the acetylated sterol mixture obtained from P. flavicomum from m/e 390 to 460 is given in Fig. 8. The spectrum of the acetylated sterols from P. polycephalum was qualitatively identical.

Fig. 8. Mass spectrum of the acetylated mixture of sterols from P. flavicomum from m/e 390 to 460



Separation of stanols and unsaturated sterols by thin layer chromatography (tlc)

Silica gel G (30 g; Merck, acc. to Stahl) was shaken vigorously with a solution containing silver nitrate (9.0 g) in water (35 cm^3) and 95% ethanol (35 cm^3). The slurry was spread on 20 x 20 cm^2 glass plates using a Shandon spreader. After air drying for 1 h, the plates were heated to 110° for 0.5 h, then cooled and stored in a vacuum desiccator.

The mixture of sterols was dissolved in the minimum amount of chloroform and applied to the plate with a micro-pipette. The chromatogram was developed using chloroform in a chamber previously saturated with solvent vapour.

After drying of the plate, visualisation of the sterol compounds was achieved by placing a template over the plate and spraying two narrow bands, one approximately 5 cm from each side of the plate, with 50% sulphuric acid. After heating at 110° for 10 min a deep blue colour showed the presence of two separate bands corresponding to the stanols (Rf = 0.34) and the unsaturated sterols (Rf = 0.25).

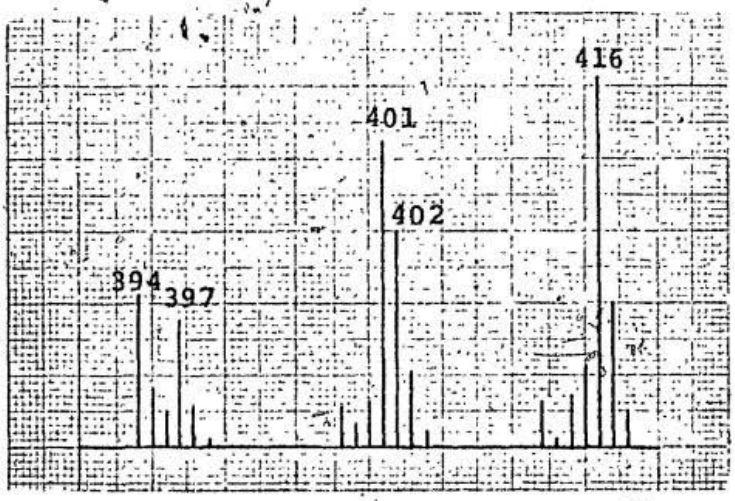
The two bands were scraped from the plate and the sterol compounds eluted from each by refluxing the silica gel with dry ether for 0.5 h. The ether solution was filtered, washed twice with half its volume of water, dried over anhydrous sodium sulphate (2 h) and evaporated to dryness. The mixtures of stanols and unsaturated sterols were separately crystallised from ether-ethanol.

Stanol mixture

Crystallised from ether-ethanol as white needles.

Mass Spectrum: The mass spectra of the mixture of stanols extracted from P. polycephalum and from P. flavicomum were qualitatively identical and is only recorded for P. flavicomum (Fig. 9).

Fig. 9. High mass end region of the mass spectrum of the P. flavicomum stanol mixture



Glc: The relative retention times of the stanols from the two sources were identical, though the relative amounts differed (Table 6).

Table 6. The relative retention times of the components in, and the composition of, the mixture of stanols from P. polycephalum and P. flavicomum together with the relative retention times of campestanol and stigmastanol on the OV 101 column

Stanol	Relative retention time (OV 101)	P. flavicomum	P. polycephalum
stanol 1 (Id)	1.34	20	35
campestanol	1.32	-	-
stanol 2 (Ie)	1.62	80	65
stigmastanol	1.61	-	-

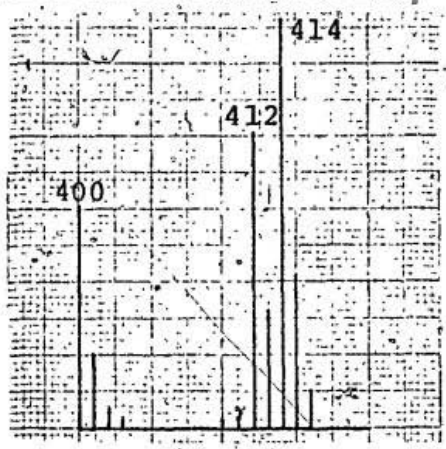
Infrared spectrum: 3560, 3420 (OH); 2910, 2845 (CH); 1455, 1375 (CH); 1125, 1010 (OH); 950, 905, 860 (sterol nucleus) cm^{-1}.

Unsaturated sterol mixture

Crystallised from ether-ethanol as white needles.

Mass spectrum: the spectrum from m/e 400 to 425 for the mixture from P. polycephalum is given in Fig. 10.

Fig. 10. High mass region of mass spectrum of the unsaturated sterols of P. polycephalum



The spectrum of the mixture from P. flavicomum differed only quantitatively.

Glc: The relative retention times of the three sterols and of campesterol, stigmasterol and β-sitosterol are given in Table 7, and of their acetates in Table 8.

Table 7. Relative retention times of the three sterols (in order of elution) from both P. flavicomum and P. polycephalum

	glc liquid phase			
	OV 17	OV 25	SE 30	OV 101
sterol 1	1.29	1.26	1.33	1.24
campesterol	1.27	1.25	1.35	1.26
sterol 2	1.45	1.41	1.44	1.44
stigmasterol	1.44	1.39	1.44	1.43
sterol 3	1.66	1.50	1.71	1.69
β-sitosterol	1.64	1.53	1.70	1.69

Table 8. Relative retention times of the three components of the acetylated mixture of sterols extracted from P. polycephalum and P. flavicomum

	Glc liquid phase		
	OV 25	SE 30	OV 101
sterol 1 acetate	1.63	1.76	1.66
campesteryl acetate	1.65	1.77	1.66
sterol 2 acetate	1.80	1.88	1.80
stigmasteryl acetate	1.82	1.87	1.80
sterol 3 acetate	1.96	2.29	2.05
β-sitosteryl acetate	1.98	2.30	2.03

The relative amounts of the three sterols, calculated from peak areas, are given in Table 9.

Table 9. Relative amounts of three sterols in the mixture extracted from P. flavicomum and P. polycephalum

	% of total unsaturated sterols	
	P. flavicomum	P. polycephalum
sterol 1	10	15
sterol 2	57	39
sterol 3	33	45

The above amounts were somewhat variable but the proportion of sterol 2 was always far greater in P. flavicomum.

Acetylation of the mixture of unsaturated sterols

The mixture of sterols (55 mg) was dissolved in anhydrous pyridine (5 cm^3) and acetic anhydride (1.0 cm^3) was added. The mixture was then allowed to stand overnight at room temperature. (In a second reaction the mixture was refluxed for 1 h.)

Deionised water (10 cm^3) was added to the mixture and the sterol acetates were extracted into ether. The solution was evaporated to dryness and the semi-crystalline solid was chromatographed on neutral alumina (200 mg; Woelm, activity 3). Elution with petroleum ether gave the acetate mixture which crystallised from ether-ethanol as white rectangular plates (40 mg).

Acetylation of the mixture of stanols and of pure samples of sterols 2 and 3

The method described above was used.

Catalytic hydrogenation of the mixture of sterols

The crystalline mixture of sterols (35 mg) was dissolved in ethyl acetate (20 cm^3) and 10% platinum on charcoal (10 mg) was added. The mixture was stirred in the presence of hydrogen at room temperature and atmospheric pressure, the uptake of hydrogen being followed. When no more hydrogen was being absorbed (about 2 h), a sample of the solution was taken and its composition checked by glc which, providing reduction was complete, showed the presence of only two components.

The solution was filtered and evaporated to dryness under reduced pressure at 50°. The reduced product was crystallised from ether-ethanol as white plates (32 mg).

Catalytic hydrogenation of the mixture of sterol acetates

The same method was used as described above except that cyclohexane replaced ethyl acetate as the solvent.

An attempt to separate the mixture of unsaturated sterols by sublimation

The mixture of sterols (ca. 20 mg) was sublimed under reduced pressure (5 mm Hg) for 48 h in a tube heated through a temperature gradient: the maximum temperature in the tube was 120°.

Three quite well separated white crystalline bands formed in the tube but each was shown, by glc, to contain each of the three sterol components in approximately the same relative composition as the original mixture.

Sterol 1 (Δ^5-ergostenol) (IId)

The sterol was separated from the mixture by preparative glc and crystallised from ether-ethanol as white plates. It was apparent from analytical glc and the mass spectrum that the sterol was not completely free from sterol 2.

Mass spectrum: The mass spectrum from the molecular ion (m/e 400) down to m/e 213 is reported in Table 10.

Table 10. Mass spectrum of sterol 1

Structure of ion (39)	Mass number (abundance relative to ions above m/e 200)
M	400 (100)
M-CH_3	385 (39)
M-H_2O	382 (55)
M-(CH_3 + H_2O)	367 (52)
M-(H_2O + 67)	315 (70)
M-(H_2O + 93)	289 (54)
M-(H_2O + 108)	274 (18)
M-(side chain)	273 (41)
M-(H_2O + 121)	261 (20)
M-(side chain + H_2O)	255 (80)
M-(side chain + 27)	246 (12)
M-(side chain + 42)	231 (52)
M-(side chain + 27 + HO)	229 (33)
M-(side chain + 42 + H_2O)	213 (72)

The spectrum was very similar to that reported for campesterol (39).

Glc: The retention times of sterol 1 and of campesterol, relative to cholesterol, are given in Table 8.

Table 11. Relative retention times of sterol 1 and of campesterol on four liquid phases

Sterol	OV 17	OV 25	QV 101	SE 30
cholesterol	1.00	1.00	1.00	1.00
sterol 1	1.27	1.29	1.28	1.37
campesterol	1.28	1.27	1.29	1.35

Thin layer behaviour of β-sitosterol, stigmasterol, β-sitosteryl acetate and stigmasteryl acetate

Stigmasterol, β-sitosterol, stigmasteryl acetate and β-sitosteryl acetate were chromatographed on silver nitrate impregnated silica gel thin layers using the system previously described for the separation of the stanols from the unsaturated sterols. The sterols were detected by charring after spraying with 50% sulphuric acid.

Rf values:

β-sitosterol	0.293
stigmasterol	0.291
β-sitosteryl acetate	0.928
stigmasteryl acetate	0.920

When β-sitosterol and stigmasterol or their respective acetates were co-chromatographed in the same system, separation was incomplete and a rather wide band corresponding to the partially separated mixture was given.

Sterol 2 (Poriferasterol) (IIIc)

Purification:

(i) Initially the sterol was separated by preparative glc on OV 101, the collected sterol being crystallised from ether-ethanol.

(ii) Later work used the thin layer system previously described.

The rear third of the unsaturated sterol band, after visualisation, was scraped off the plate separately, extracted from the silica gel by ether, and analysed by glc to determine the purity of sterol 2 in the sample.

The sample was re-chromatographed, using the same thin layer system, and again the rear third of the band was separated and analysed by glc. This procedure was repeated until the sample was virtually homogeneous when the sterol was crystallised twice from ether-ethanol.

Melting point: The melting point of sterol 2 and of two derivatives is given in Table 12.

Table 12. Melting point of sterol 2 and some derivatives together with literature values for stigmasterol and poriferasterol (64)

Sterol or derivative	Sterol 2 ex P. flavicomum	Sterol 2 ex P. polycephalum	Poriferasterol	Stigmasterol
sterol 2	156°	154-155°	156°	170°
3β-acetoxy sterol	147°	146.5-147°	146.5-147°	144-144.6°
tetrahydro-3β-acetoxy sterol	139-140°	139-140°	140-141°	130-131°

Mass spectrum: Table 13

Table 13. The mass spectra of sterol 2 and its acetate (from P. flavicomum)

Nature of ion (39)	sterol 2 (R = H)	3β-acetoxy sterol 2 (R = CH_3CO)
M	412(62)	-
M-CH_3	397(13)	-
M-ROH	394(17)	394(100)
M-(CH_3 + ROH)	379(10)	379(9)
M-43	369(32)	-
M-(43 + ROH)	351(35)	351(18)
M-98	314(13)	-
	300(50)	-
M-side chain	273(25)	-
M-(side chain + 2H)	271(55)	-
M-(side chain + ROH)	255(100)	255(60)
M-(side chain + ROH + 2H)	253(17)	253(20)
M-(side chain + 42)	231(15)	-
M-(side chain + 27 + RO)	229(15)	-
	-	228(15)
M-(side chain + 42 + RO)	213(24)	213(17)

The spectra are almost identical with those recorded for both stigmasterol and poriferasterol and their respective acetates and also with sterol 2 extracted from P. polycephalum.

Infrared spectrum: absorption maxima in CCl_4 were at 3580, 3430; 2920, 2855; 1665, 1600 (C=C); 1460, 1380; 1120, 1020; 970 (Δ^{22} trans C=C); 960 cm^{-1}.

The spectrum was almost superimposable on the ir spectrum of stigmasterol.

Nuclear magnetic resonance spectrum: Part of the nmr spectrum of sterol 2 from P. polycephalum and of stigmasterol are given in Figs. 11 and 12, (spectra recorded in $CDCl_3$ with tetramethylsilane as internal reference).

Fig. 11. 100 MHz nuclear magnetic resonance spectrum of sterol 2 from P. polycephalum

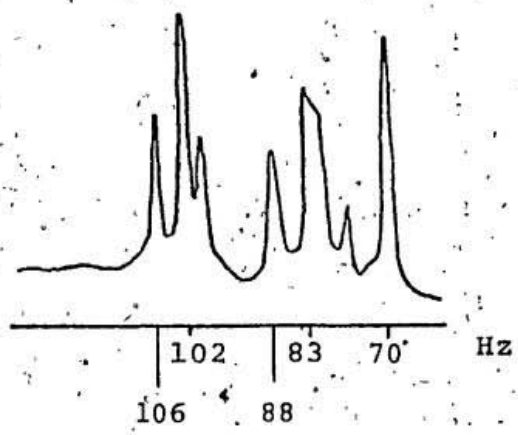
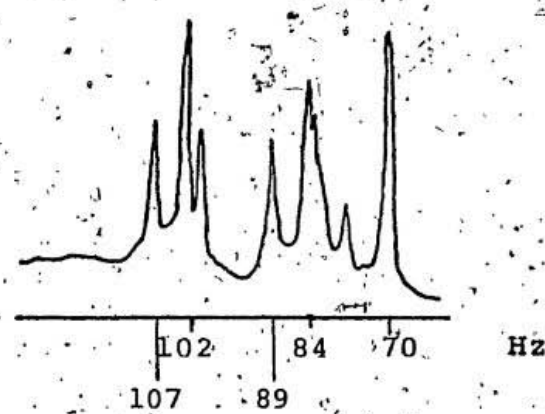


Fig. 12. 100 MHz nuclear magnetic resonance spectrum of stigmasterol



The spectrum of sterol 2 was very similar to that of stigmasterol but differed in showing a single maximum at 83.0 Hz as opposed to a doublet absorption in the spectrum of stigmasterol (49).

Gas liquid chromatography: Table 14. Relative retention times of sterol 2 extracted from both P. flavicomum and P. polycephalum were identical.

Table 14. Glc relative retention times of sterol 2 and stigmasterol and of their acetates

Sterol	OV 17	OV 25	SE 30	OV 101
cholesterol	1.00	1.00	1.00	1.00
sterol 2	1.44	1.39	1.44	1.46
stigmasterol	1.46	1.39	1.45	1.43
sterol 2 acetate	-	1.82	1.90	1.80
stigmasteryl acetate	-	1.80	1.88	1.81

Sterol 3 (22-dihydroporiferasterol) (IIe)

The sterol, extracted from P. flavicomum only, was separated from the mixture by glc on OV 101 and crystallised from ether-ethanol as white needles.

Melting point: 139.5-140°, acetate 142-143°. Lit: 22-dihydroporiferasterol 140°, 22-dihydroporiferasteryl acetate 140-141° (64).

Mass spectrum: Table 15.

Table 15. Mass spectrum of sterol 3 and of its acetate

Nature of ion (25)	sterol 3 (R = H)	3β-acetoxy sterol 3 (R = CH_3CO)
M	414 (100)	-
M-CH_3	399 (46)	-
M-ROH	396 (32)	396 (100)
M-(CH_3 + ROH)	381 (50)	381 (23)
M-(ROH + 67)	329 (63)	-
M-(ROH + 93)	303 (65)	-
	-	288 (25)
M-(ROH + 121)	275 (16)	275 (28)
M-side chain	273 (50)	-
M-(side chain + ROH)	255 (68)	255 (26)
	253 (32)	-
	241 (25)	-
	233 (39)	-
M-(side chain + 42)	231 (50)	-
M-(side chain + 27 + RO)	229 (33)	-
	218 (34)	-
	215 (48)	215 (27)
M-(side chain +42 + ROH)	213 (80)	213 (19)

The spectra were almost identical with those recorded for β-sitosterol and its acetate.

Gas liquid chromatography: Table 16.

Table 16. Relative retention times of sterol 3, β-sitosterol and of their respective acetates

	OV 101	OV 25	SE 30	OV 101
sterol 3	1.64	1.54	1.73	1.69
β-sitosterol	1.64	1.53	1.70	1.69
sterol 3 acetate	-	1.98	2.27	2.03
β-sitosteryl acetate	-	1.96	2.29	2.04

Infrared spectrum: absorption maxima were recorded in CCl_4 solution at 3600; 2925, 2850; 1665, 1600; 1465, 1378; 1125, 1025; 955 cm^{-1}.

The spectrum was almost superimposable on the ir spectrum of β-sitosterol.

Sterol 3 acetate gave maxima in CCl_4 at 2940, 2860; 1725 (C=0, acetate), 1660, 1605; 1465, 1378, 1365; 1250-1200; 1120, 1020; 955, 905 cm^{-1}.

The spectrum was almost identical with that of β-sitosteryl acetate.

Ozonolysis of stigmasterol

Stigmasterol (recrystallised; 190 mg) was dissolved in a warm mixture of methyl acetate (10 cm^3) and methanol (10 cm^3) and then cooled in an ice bath when small, white,

translucent needles crystallised out which, as ozone, generated by a Welsbach ozoniser, was bubbled slowly through the cold, stirred suspension, gradually dissolved.

The state of completion of the reaction was determined by extracting aliquots at different time intervals and determining the composition by tlc on microscope slides using silica gel G and 10% acetone in petroleum ether as the developing solvent. In this system, stigmasterol had an Rf of 0.60 and the product 0.10. This method was rather more sensitive than the quicker method of determining the ability of the solution to decolourise a 1% solution of bromine in carbon tetrachloride. Following completion of ozonolysis the excess ozone was blown off in a stream of nitrogen: the solution remaining was worked up reductively with no attempt being made to isolate the ozonide.

Reduction of ozonide

5% palladium on charcoal (20 mg) was added to the solution of the ozonide, and the mixture was stirred under hydrogen at room temperature and atmospheric pressure for 4 h after which no more hydrogen was taken up. The catalyst was filtered off and ether (50 cm^3) added followed by deionised water (100 cm^3). After shaking and separation of the two layers the aqueous layer was re-extracted with ether (50 cm^3). The ether layers were combined and washed three times with water (30 cm^3), and dried over anhydrous sodium sulphate.

Preparation of 2-ethyl-3-methylbutanal-2,4-dinitrophenylhydrazone

The dried ether solution from above was fractionally distilled, using a 6" Vigreux column, until 5 cm^3 of solution remained.

The ethereal solution was distilled in a Köntes micro apparatus until there was no more distillate at 50°, when the temperature was increased, 2-ethyl-3-methyl-butanal distilling off at about 135°.

The aldehyde was immediately added to a warm solution of 2,4-dinitrophenylhydrazine, prepared according to the method of Fieser (60), and an orange-yellow precipitate formed immediately. After 15 min the suspension was filtered to give yellow crystals of the derivative which were crystallised from 95% ethanol (30 mg).

Further purification was effected by tlc on neutral alumina using benzene as the developing solvent. In this system the 2,4-dinitrophenylhydrazone moved with the solvent front, but a small amount of unreacted 2,4-dinitrophenylhydrazine was removed as a yellow band, Rf 0.2.

The derivative was eluted from the alumina by benzene, the resulting solution evaporated to dryness, and the residue crystallised from 95% ethanol. Yield 15 mg; m.p. 120° (lit. (61) 121°); $[\alpha]_{25}^{D}$ = -6.3° (benzene; 2 mg cm^{-3}); lit. = -5.8° (benzene).

Mass spectrum: Table 17.

Table 17. Mass spectrum of 2-ethyl-3-methylbutanal-2,4-dinitrophenylhydrazone

Mass number	% abundance	Mass number	% abundance
294 (M)	65	152	9
277	3	149	15
265	16	138	18
259	10	135	9
251	29	130	6
235	12	122	9
224	28	103	9
217	20	97	22
206	32	84	41
143	19	83	15
184	18	69	43
183	15	68	26
177	18	55	63
167	21	43	53
164	22	41	100

Ozonolysis of sterol mixture of P. flavicomum and preparation of 2-ethyl-3-methylbutanal -2,4-dinitrophenylhydrazone

The ozonolysis, reduction and subsequent preparation of the 2,4-dinitrophenylhydrazone was repeated using the mixture of sterols (384 mg) extracted from P. flavicomum.

Yield 14 mg; m.p. 119-119.5°; $[\alpha]_{23}^{D}$ = +10.5° (benzene; 2 mg cm^{-3}).

Mass spectrum: this was virtually identical with that of the same compound derived from stigmasterol (loc. cit.).

Triterpenoid fraction

Thin layer chromatography

The material eluted from the alumina column by benzene was chromatographed on silver nitrate impregnated plates using the system previously described for the sterols.

A large amount of material (probably hydrocarbons) moved with the solvent front, and there was a small amount of sterol (Rf 0.25). The triterpenoid region (Rf 0.33-0.44) contained only small quantities of several components and the whole section was extracted by ether to give the triterpenoid component.

Gas liquid chromatography

The triterpenoid fraction was chromatographed on the OV 101 column previously described, though no attempt at separation of the individual components was made.

The retention times of the components were compared with the retention times determined for 24-dihydrolanosterol, lanosterol and cycloartenol and similarly the components of the acetylated mixture were compared with the acetylated triterpenoids.

The retention times of authentic triterpenoids are given in Table 18 and glc profiles of the triterpenoid mixture and its acetylated product from P. flavicomum and P. polycephalum are given in Figs. 13a-d.

Table 18. Retention times of triterpenoids relative to cholesterol on OV 101

Triterpene	Relative retention times
24-dihydrolanosterol	1.44
lanosterol	1.56
cycloartenol	1.74
24-dihydrolanosteryl acetate	1.80
lanosteryl acetate	1.97
cycloartenyl acetate	2.25

Fig. 13. Glc profiles of the triterpenoid fractions and their acetylated products (peaks labelled by relative retention times)

Fig. 13a. P. flavicomum triterpenoids

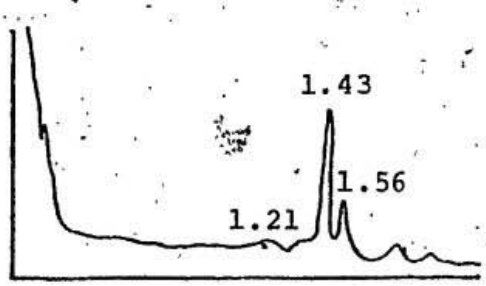


Fig. 13b. P. polycephalum triterpenoids

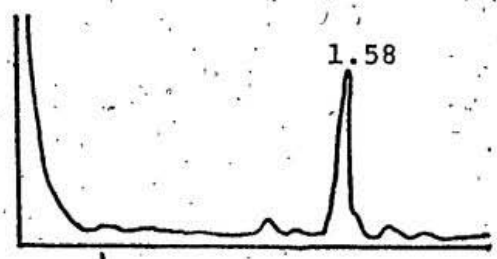


Fig. 13c. P. flavicomum triterpenoid acetates

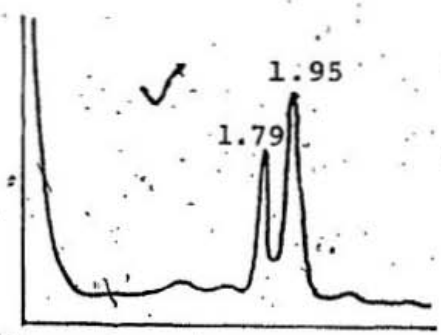
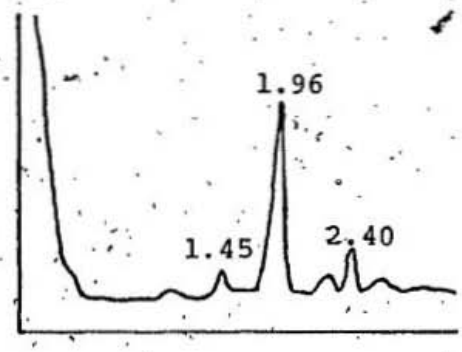


Fig. 13d. P. polycephalum triterpenoid acetates



Mass spectrum: the mass spectra of the triterpenoids from m/e 420-450 are shown in Figs. 14a and 14b, and for the acetates from m/e 390 to 480 in Figs. 14c and 14d.

Fig. 14. Mass spectrum of triterpenoid extracts and their acetylated products

Fig. 14a. P. flavicomum triterpenoids

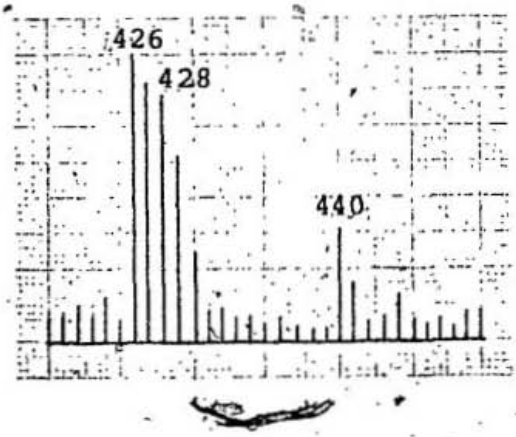


Fig. 14b. P. polycephalum triterpenoids

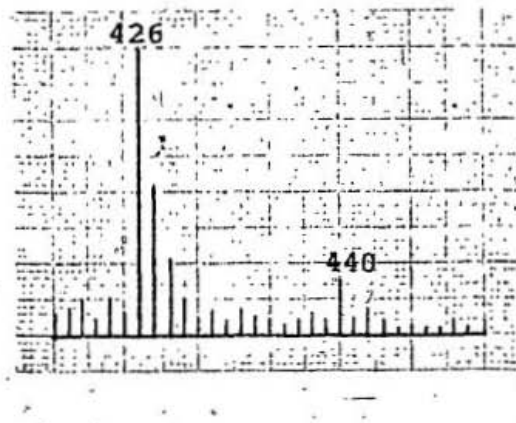
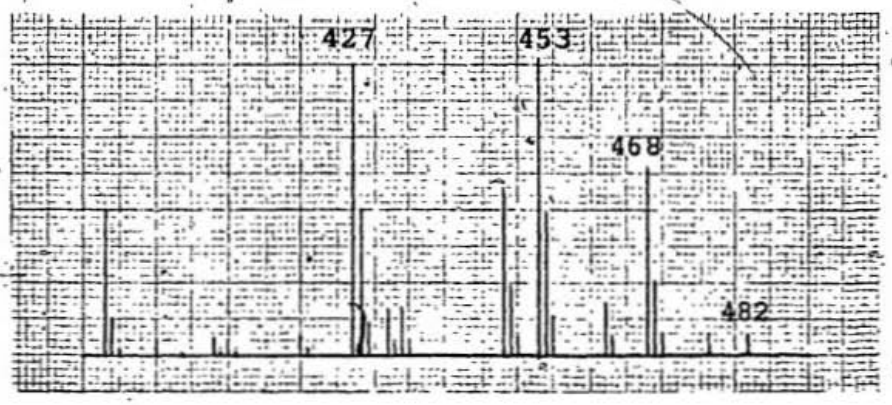


Fig. 14c. P. flavicomum triterpenoid acetates

Fig. 14d. P. polycephalum triterpenoid acetates



Preparation of methionine

The method was almost identical with that used by du Vigneaud et al. (62). A 1 dm^3 three-necked flask was fitted with a dry ice-acetone reflux condenser and an inlet for ammonia gas, the third outlet being stoppered; the flask was placed in a dry ice-acetone bath.

Dried gaseous ammonia was passed through until 60 cm^3 of liquid ammonia had condensed, when the ammonia inlet was disconnected and the flask stoppered removed from the cooling bath and fitted with a magnetic stirrer.

DL-Homocystine (1 g; Sigma) was dissolved in the ammonia and small, freshly cut pieces of sodium were added, one at a time, until the solution retained a permanent blue colouration indicating a slight excess of sodium. Methyl iodide (1.3 g) was added and after 5 min the stirring was discontinued. The condenser was removed and the flask left unstoppered overnight to allow the NH_3 to evaporate spontaneously.

The white residue was dissolved in water (20 cm^3) and the solution was acidified to Congo red using 30% HBr and then filtered. To the filtrate was added pyridine (2 cm^3) and boiling ethanol (3 vols) and the solution was cooled in an ice bath. The white precipate was filtered off and washed with ethanol (3 vols)-water (1-vol).

The solid was dissolved in hot water (12 cm^3) and hot acetone (10 cm^3) was added. The white precipitate was washed with acetone and dried.

Yield 380 mg; m.p. 240-245° decomposed.

Mass spectrum: details are given in Table 16. The spectrum was very similar to that reported by Biemann and McCloskey (63).

Table 19. Mass spectrum of methionine

Mass number	% abundance	Mass number	% abundance
149 (M)	31	83	47
132	15	75	49
131	91	74	37
116	30	61	100
114	16	57	22
104	19	55	34
101	31	47	19
88	14	41	18
87	15		

Preparation of [Me-$^2H_3$] methionine

This was prepared by the method described above for unlabelled methionine with the substitution of $^2H_3$-methyl iodide (1.5 g; Merck, Sharp and Dohme) for methyl iodide.

Yield 340 mg; m.p. 245-260° decomposed.

Mass spectrum: details are given in Table 20.

Table 20. Mass spectrum of [Me-$^2H_3$] methionine

Mass number	% abundance	Mass number	% abundance
152	28	83	55
134	72	78	35
133	35	75	21
117	21	74	43
116	35	64	32
107	25	57	29
101	25	56	100
90	16	55	50
88	18	49	18
		43	25

Crude sterol fraction from Myxomycetes cultured in the presence of [Me-$^2H_3$] methionine

The non-saponifiable lipid was extracted by the usual method but no attempt was made to separate the mixture further.

Mass spectra: The spectra for the sterol mixtures from P. flavicomum and P. polycephalum are recorded in the range m/e 400-430 (Figs. 15a and 15b) and for the acetylated mixtures in the range m/e 400-480 (Figs. 15c and 15d).

Fig. 15a. High mass-end mass spectrum of deuterated sterols from P. flavicomum

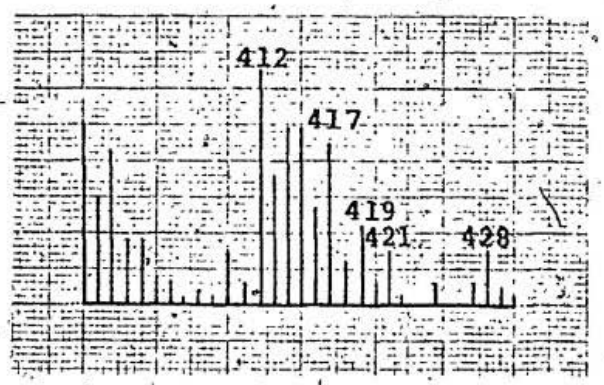


Fig. 15b. High mass-end mass spectrum of deuterated sterols from P. polycephalum

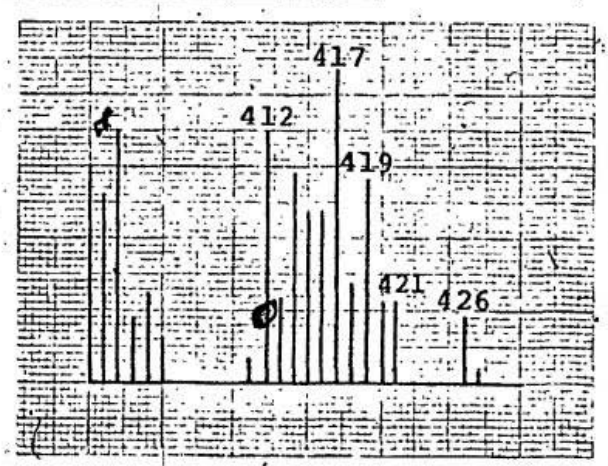


Fig. 15c. High mass-end mass spectrum of acetylated deuterated sterols from P. flavicomum

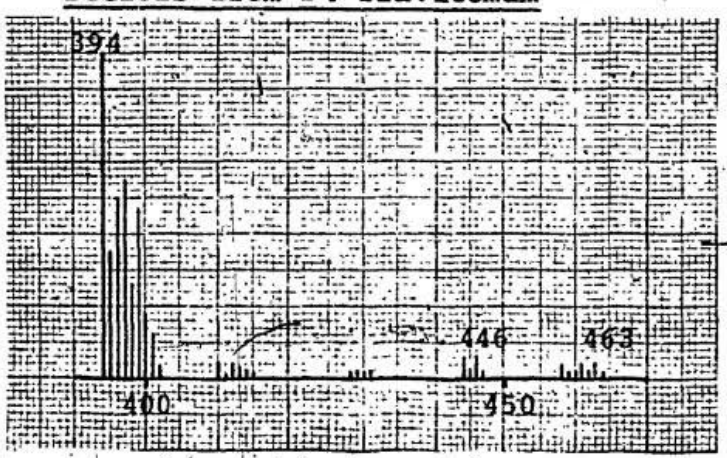
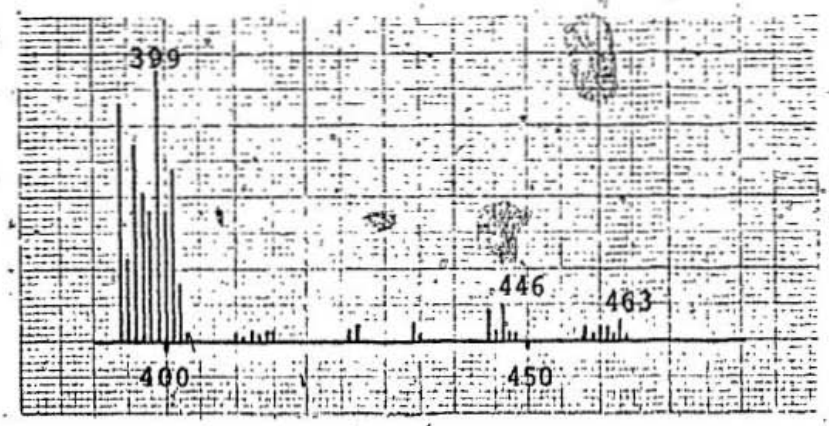


Fig. 15d. High mass-end mass spectrum of acetylated deuterated sterols from P. polycephalum



Changes in the relative concentration of various cell components in synchronous cultures of P. polycephalum

In early experiments different cultures, at known times after prophase, were scraped from the filter paper on which they were cultivated and freeze dried. The freeze dried material (0.20 g) was refluxed with dry ether (50 cm^3) for 2 h, and the ether solution was evaporated to dryness. The mass spectrum of the residue was recorded at 250°.

In later work a small amount of the growing Myxomycete was removed from the edge of the plasmodium at a known time after prophase, and was transferred to the dipper of the direct inlet system of the mass spectrometer. After drying in an oven at 110° for 24 h, the mass spectrum was recorded at 250°.

The relative concentration of each sterol component was expressed as a percentage, being the height of the molecular ion compared with the sum of the five sterol molecular ions (m/e 400, 402, 412, 414, 416): no significant change was observed in the concentration of the sterols between MII and MIII.

The peak heights of some other ions in the high mass-end of the spectrum were expressed in the same way (Figs. 6a-b).

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PART II

INTRODUCTION

It is known that, under appropriate environmental conditions, the plasmodium of the Myxomycete P. polycephalum undergoes sporulation. It has been suggested that light in the 350-500 nm region is a requirement for this process and, as at the same time the plasmodial pigments disappear, the pigments have been implicated as possible photocatalysts for this simple form of differentiation (1,2). More recent work by LeStourgeon has cast some doubt on the role of the plasmodial pigments (3). He has shown that blue light in the region 410-490 nm does not induce cultures of P. flavicomum to sporulate whereas red light (620-720 nm) was twenty-five times as effective as white or yellow light.

The function of the pigments is thus not clear though it is possible that the polyene nature of at least some of them is important in limiting lethal photo-oxidation, a function which has been attributed to the carotenoids of non-photosynthetic bacteria (4,5,6).

Few researchers have attempted to separate and determine the structure of these pigments. Siefritz and Zetzmann (7) suggested that the pigment might be a flavone or a "lyochrome" but gave no convincing evidence to support this hypothesis. On the basis of ultraviolet and infrared spectral evidence, Wolf (8) concluded that the pigments were pteridines, but elemental analysis by Dresden (9) and Brewer (10) has

shown that the nitrogen content is too low for this to be the case.

Dresden (9) and Kuraishi et al. (11) attempted to purify the yellow pigments but none was obtained in a crystalline form even after chromatography on a series of adsorbents.

This early work was limited by the low concentration of pigments in the plasmodium combined with the difficulty of cultivating the organism on a sufficiently large scale.

Brewer et al. (12) reported a method for large scale culture of P. polycephalum in a partially defined liquid medium, thus making available larger quantities of pigment.

Brewer (10) isolated three pigments, A, B, and C, each characterised by its absorption spectrum (Table 1).

Table 1. Absorption maxima of pigments isolated from P. polycephalum (10) (nm)

Pigment	Basic Methanol	Neutral Methanol	Acidic Methanol
A	356 (1100)	356 (1100)	356 (1100)
B	355 (780)	384 (1010)	390 (1190)
C	385 (860)	403 (1230)	418 (1320)

( ) = $E^{1\%}_{1cm}$

Brewer concluded that pigment A was a water soluble hydrochloride containing an amide group, a further basic nitrogen atom and a conjugated hexaene chromophore. Pigment B was identified as a conjugated heptaene containing a carboxyl group and pigment C was similar to pigment B, the spectrum showing a large bathochromic shift on protonation, but with its absorption maxima shifted about 25 nm to longer wavelength. Esterification of pigment C gave a compound with an ultra-violet spectrum similar to pigment A. Daniel (13) later suggested that the pigments showing the large bathochromic shifts on protonation are Schiff bases.

Watson (14) attempted to obtain better purification of the pigments. Using thin layer chromatography the pigments streaked badly in the systems used but pigment A was separated into three separate bands on a column of silicic acid, the most abundant compound having an absorption maximum at 356 nm characteristic of a conjugated hexaene. Infra-red analysis of this pigment suggested the presence of -NH, olefinic CH and a carboxyl group. The highest mass ion in the mass spectrum was at 314 (315 at lower electron voltage) but this was probably not the molecular ion. Oxidation of the pigment by a mixture of permanganate and periodate appeared to give a mixture of oxalic and dimethyl malonic acids.

More recent work (20) has confirmed that pigments showing a bathochromic and hyperchromic shift on protonation can be isolated from both P. polycephalum and P. flavicomum.

Such pigments have been better purified than previously by the use of gel filtration but to date the pure quantities available have been insufficient for elemental analysis.

Evidence for the structure of these pigments is limited, however the spectral shift on protonation and the ready reduction by sodium borohydride to give a polyene derivative (20), together with the reported nitrogen content (ca. 4%) (9,10) does indicate that Daniel's suggestion of polyene derived Schiff bases is quite plausible.

Pigments from other Myxomycete species have received even less attention. Rakoczy (15) suggested that the pigments of P. nudum are the same as those of P. polycephalum following study of the action spectrum of sporulation. Nair and Zabka (16) showed the presence of three pigments in Physarella oblonga, three in P. gyrosum, four in P. polycephalum, and six in Didymium iridis. They concluded that one pigment from P. gyrosum is a flavone and suggested that one pigment in D. iridis and one in P. polycephalum are phenolic in structure.

Leith (17) used electrophoresis to separate four pigments from the red-brown plasmodium of D. nigripes.

Sobels (18) showed that the absorption spectrum of an alcoholic extract of the plasmodium of Badhamia utricularis had two peaks at 335 and 375-380 nm in addition to three minor peaks at 247, 265, and 275 nm. She suggested that the pigments were flavone in nature, though without other supporting evidence.

DISCUSSION

The pigments of Badhamia utricularis were readily extracted by suspending the plasmodium in cold methanol. The initial yellow extract had absorption maxima at 253 and 370 nm together with a very definite shoulder at 335 nm and the spectrum thus closely resembled that reported by Sobels (18). When sodium methoxide was added to the solution the absorption maximum at 370 nm shifted to 356 nm, a result which is opposite to that observed for flavonoid compounds.

After filtration and removal of the solvent, the pigments were separated by gel filtration on Sephadex LH 20. Four coloured bands were eluted from the column by methanol, each of which was purified by further chromatography on Sephadex or, where possible, by thin layer chromatography on silica gel (Table 2). The band first eluted from Sephadex showed spectral properties similar to the pigments, tentatively identified as Schiff bases, reported in P. polycephalum. As work had already commenced in this laboratory on the determination of the structure of a similar pigment in P. flavicomum as well as one in P. polycephalum, it was decided to concentrate on this pigment in order to compare some of its properties with the apparently related Physarum pigments, and thus only very preliminary data is reported for the other three pigments.

Table 2. Absorption spectra of the pigments of B. utricularis in order of elution from Sephadex by methanol

Pigment	Absorption maxima (nm) in methanol	
	Acidic	Basic
1	267 420	238 375
2	(327) 343 359	338 - 358 (broad)
3	(326) 343 (361)	328 - 340 (broad)
4	266 (344) 359 (379)	266 (340) 378

( ) denotes shoulder

Pigment 2

The pigment had a white fluorescence and was very pale yellow in solution. Its absorption spectrum in methanol and in acidic methanol was similar to that of a conjugated aliphatic pentaene (19), though the fine structure was not so pronounced. On addition of base to the solution, the curve flattened and had a broad and ill-defined absorption maximum (Fig. 1).

Pigment 3

This pigment had a stronger yellow colour than pigment 2 and showed an orange fluorescence, though its absorption spectrum in neutral or acidic methanol was also that of pentaene. The absorption maximum was again broadened by base treatment (Fig. 2).

The compound was silylated to give a single less polar compound showing the presence of one hydroxyl group. A solution was evaporated to give a red-brown solid which had a mass spectrum showing ions at m/e 352, 314, and 272 on the highest mass ions. It is not known whether these correspond to the molecular ion and characteristic fragmentation ions.

Pigment 4

Pigment 4 was orange in colour and had a red/orange fluorescence. Chromatography on silica gel thin layers or a repeat of the chromatography on Sephadex at a slower flow rate separated it into four components all with the same absorption spectrum, a broad band at about 380 nm in basic and neutral methanol changing, on acidification, to a polyene type of spectrum similar to that of a conjugated hexaene (19), though without the distinct fine structure (Fig. 3).

Pigment 1

The absorption spectrum (Fig. 4) was similar to that of pigments detected in P. polycephalum (10) and P. flavicomun (20) in showing an absorption maximum in the near ultraviolet (385 nm) which, on acidification, shifted to the visible region (420 nm) and increased in intensity (Table 3). Another absorption maximum of approximately equal intensity at 243 nm also showed a red shift to 267 nm on acidification. This latter band remained in the spectrum after thin layer chromatography of the pigment though its relative intensity to

Fig. 1. Absorption spectrum of pigment 2 in acid methanol (——) and basic methanol (---).

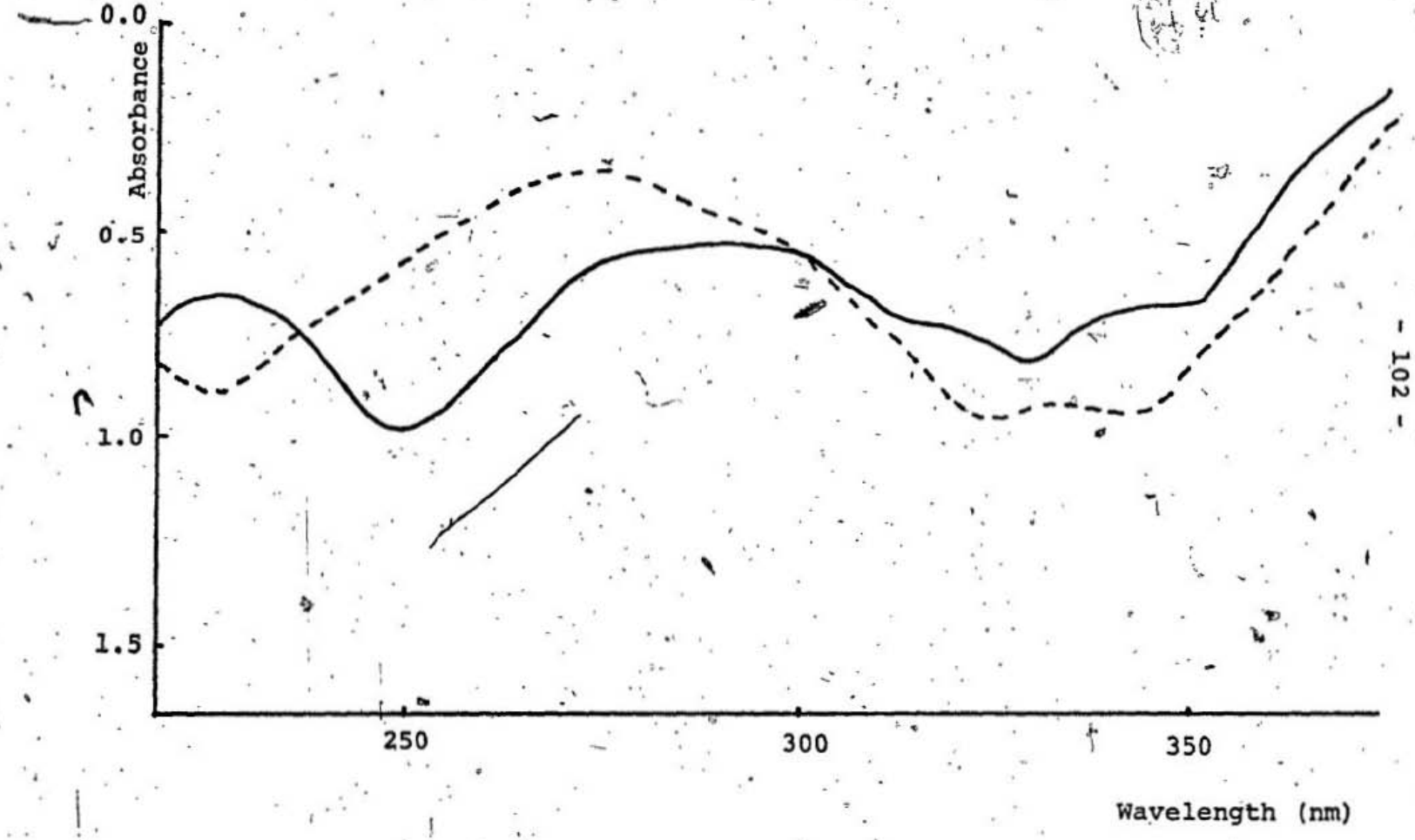
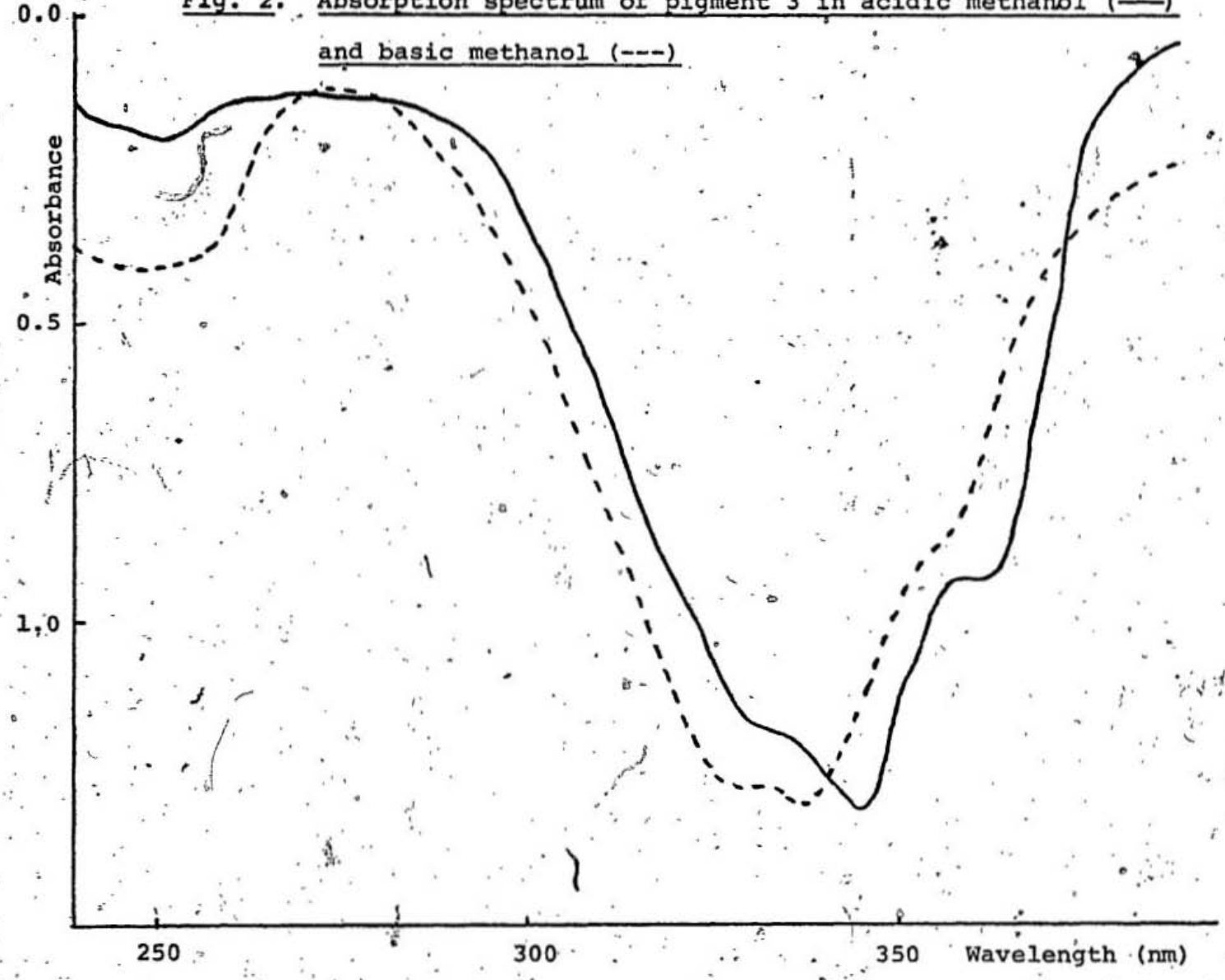


Fig. 2. Absorption spectrum of pigment 3 in acidic methanol (——) and basic methanol (---)



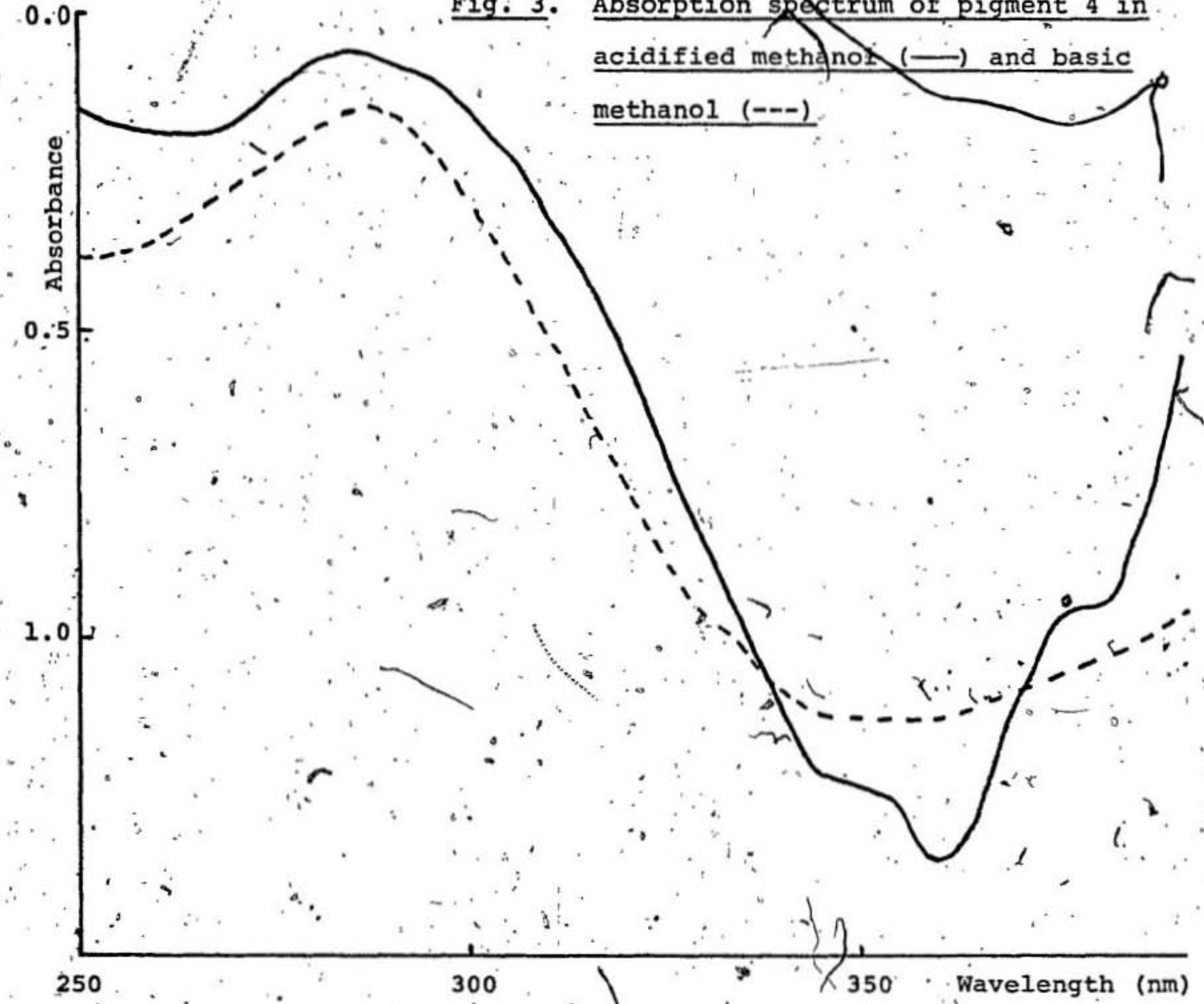
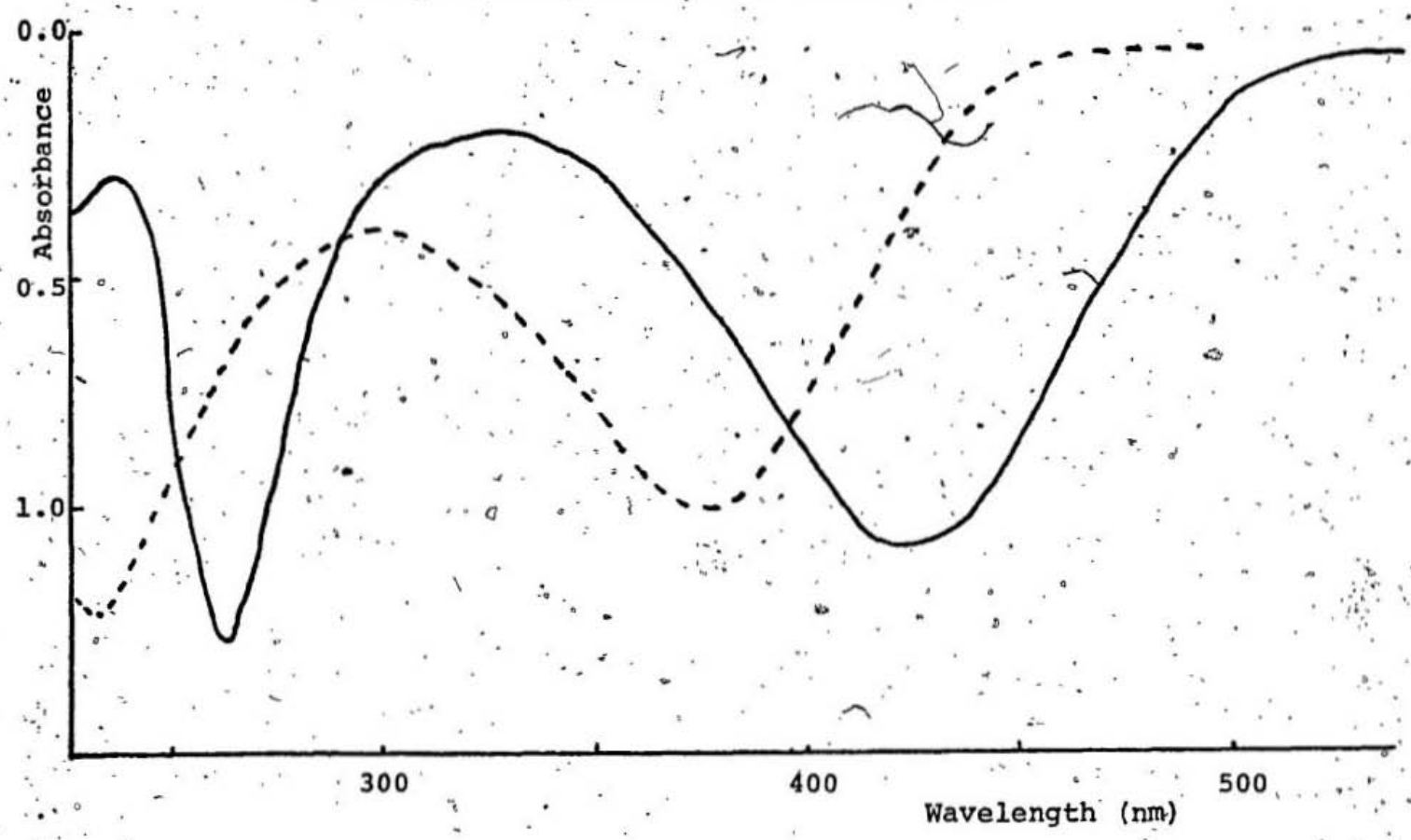


Fig. 3. Absorption spectrum of pigment 4 in acidified methanol (——) and basic methanol (---)

Fig. 4. Absorption spectrum of pigment 1 ("Schiff base") in acidified methanol (——) and in basic methapol (---).



the 375 nm maximum increased; however, as silica gel was shown to degrade the pigment, it is not known whether this ultraviolet maximum is due to an impurity or to a separate, possibly aromatic, chromophore in the pigment.

Table 3. Absorption spectra of related pigments derived from three species of Myxomycetes

Pigment	Absorption spectra in methanol (nm)		
	Basic	Neutral	Acidic
ex *B. utricularis*	238, 375 (100)	245 385	267, 420 (118)
ex *P. polycephalum* (10)	355 (100) 385 (100)	384 403	390 (139) 418 (154)
ex *P. polycephalum* (20)	391 (100)	395	420 (130)
ex *P. flavicomum* (20)	250, 372 (100)	380	286, 412 (121)

( ) relative extinction coefficients

The absorption spectrum of the pigment (Fig. 4) was dependent on the solvent, thus in 10% methanol in water a broad absorption band at 378 nm shifted to 432 nm on protonation with only a 5% increase in extinction. Khalil (20) has shown a similar change with the *P. flavicomum* pigment.

It was initially thought that this pigment could be more easily purified than the Physarum pigments, as it is readily chromatographed on silica gel thin layers or columns, whereas the related Physarum pigments are rather more polar and cannot

be separated in this way. It was soon apparent, however, that chromatography on silica gel caused some degradation of the pigment several yellow bands being formed no matter how many times each "pure" component was rechromatographed. Similarly, when a solution of the pigment in methanol was allowed to stand over silica gel, the absorption spectrum of the solution was changed. The pigment was thus purified only by gel filtration on Sephadex.

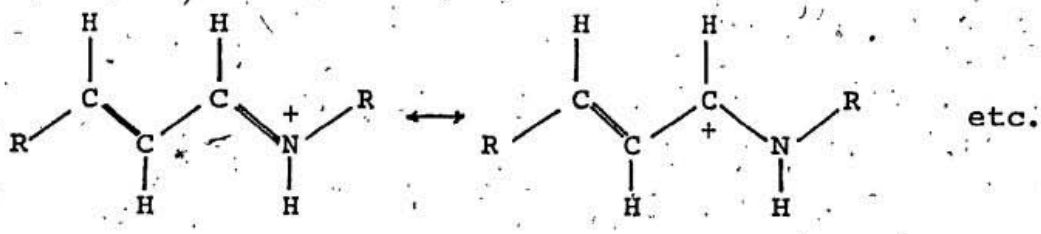
Daniel (13) has suggested that the apparently related pigment from P. polycephalum is a Schiff base and certainly the rather large bathochromic shift and the related hyperchromic effect on protonation, similar to shifts observed with authentic polyene Schiff bases (21,22), tend to support this view. It was therefore decided to prepare some Schiff bases of available, or readily synthesised, polyene aldehydes with the intention of relating the absorption spectra, and the changes on protonation, to the spectra of the Myxomycete pigments.

Electronic absorption spectra of polyene Schiff bases

Various Schiff bases derived from 2,4-hexadienal (sorbaldehyde), 2,4,6-octatrienal, or retinal (Vitamin A aldehyde) together with simple amines and amino acids, were prepared in dilute solution and the ultraviolet or visible spectra determined both before and after protonation. The normal bathochromic shift on protonation of the bases derived from hexadienal was 40-50 nm, from octatrienal 57-62 nm, and from retinal 80-90 nm.

From the position of its absorption maximum it is possible that the B. utricularis pigment 1 is derived from a hexaenal and thus, on the basis of the results from the known Schiff bases, might be expected to show a bathochromic shift of 90-100 nm. The recorded value of 45 nm indicates that it is unlikely that the pigment is a simple polyene Schiff base.

The red shift of Schiff bases on protonation is due to the conversion of the nitrogen atom to the quaternary state (23), and as the positive charge can be formally localised at different positions on the polyene chain, several resonance forms can be written for any protonated polyene Schiff base as compared with one main form for the non-protonated compound. Such charge resonance spectra are found in various dyes and natural products (24).



As the reduced red shift shown by the Badhamia pigment might be accounted for by a change in the ease of the $\pi \rightarrow \pi^*$ transition in either the protonated or non-protonated form, it was thought possible that in Schiff bases formed between retinal and aspartic acid, homoserine and 3-amino-butanoic acid (I, II, III), in which the nitrogen lone pair

could be involved in hydrogen bonding, the interaction of the lone pair with the chromophore might be changed and abnormal shifts on protonation might result. Of the products formed, only retinylidene-3-aminobutanoic acid was abnormal in any respect in showing a bathochromic shift from 374 nm to 440 nm. This shift of 66 nm compares with the more usual value of approximately 80 nm and it is possible that similar hydrogen bonding effects might reduce the shift even further.

An alternative explanation for the relatively small red shift of the pigment on protonation might lie in the various steric and electronic effects related to the environment of a polyene Schiff base chromophore within the complete pigment molecule. Such an effect is observed in the imine formed between retinal and phosphatidylethanolamine (PE) in the visual pigment rhodopsin which absorbs at the remarkably high wavelength of about 500 nm. Part of this red shift from the normal absorption maximum of retinylidene Schiff bases at about 365 nm appears to be due to internal protonation of the nitrogen atom from the phosphoric acid group of PE (25), whilst part might be explained by the twisting of the retinaldehyde molecule about the 11, 12- double bond and thus the actual environment of the chromophore (26,27). The absorption maxima reported for retinal based visual pigments from different species vary between 478 nm and 543 nm (28) reflecting a certain degree of difference in the environment of the retinal moiety. Dartnell and Lythgoe (28) have suggested that transitory

dipoles on the prosthetic group (retinal) could be stabilized by electrostatic interaction with charged groups on the protein and that these dipoles may be located in at least twenty different positions on the chromophore; however, Rosenberg and Krigas (29) have suggested that field or inductive effects due to substituents near the chromophore can affect the positive charge on the nitrogen atom and change the absorption maximum.

Similar bathochromic shifts to those observed on protonation have been recorded when retinylidene-n-butylamine was adsorbed onto silica gel (with available protons at the surface) or when a solution in methanol was cooled to -78° (30). It was suggested that as both adsorption and cooling are conducive to the formation of molecular aggregates this might explain the red shifts.

The absorption maxima of different pyridoxal phosphate (PLP) containing enzymes, in which the PLP is bonded to the ε-amino group of a lysine residue in the protein moiety through a Schiff base link, also show quite a variation in absorption maxima (from 410 to 435 nm), presumably reflecting differences in the nature of the binding (31).

It is quite conceivable that related effects could contribute to the relatively small red shift of the Badhamia pigment on protonation.

As no information is available on the spectra of Schiff bases completely in conjugation from the polyene moiety

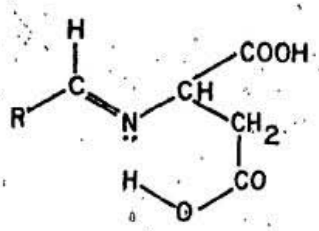
through the azomethine link into the amine derived part of the molecule, an attempt was therefore made to synthesise compounds of this type by reacting retinal with methyl-β-aminocrotonate and cytosine to form compounds IV and V. However, both of these reactions failed, due, no doubt, to the reduced basicity of the nitrogen atom. Ball et al. (21) have also shown that simple amides such as formamide and urea are unreactive under the same conditions.

Retinal was also reacted with allylamine in the hope that the product formed would, as a result of bond migration, contain a completely conjugated system (VI) however the absorption spectrum of the product and the bathochromic shift (80 nm) on protonation were completely normal.

As no conclusions could be drawn about the nature of the pigment by making a comparison of the spectrum and spectral changes with those of various polyene Schiff bases, it was decided to look at other properties of the pigment in order to compare these with simple polyene Schiff bases and with the related pigments from P. polycephalum and P. flavicomum.

pK_a of Schiff's base conjugate acids

The pK_a of retinylidene-t-butylamine and of octatrienylidene-t-butylamine were determined, by a spectroscopic method (32), in 90% methanol and were shown to be 8.6 and 7.4, respectively. These values are in accordance with the expected trend, the pK_a of retinylidene-t-butylamine being



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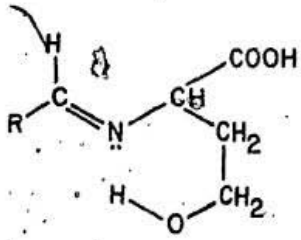

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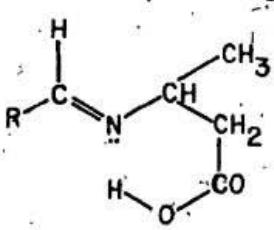

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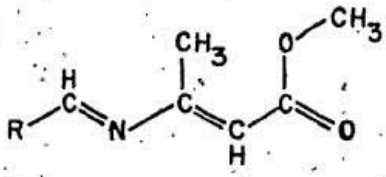

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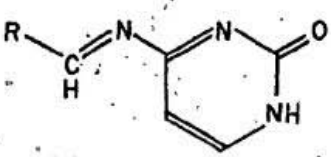

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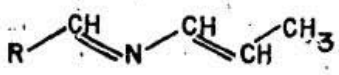

VI

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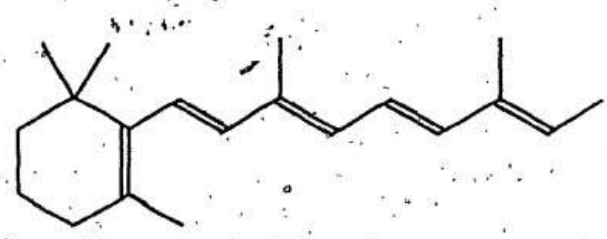


higher due to the inductive effect of the two methyl groups attached to the polyene chain.

The pigments from the Myxomycetes were all sufficiently water soluble to determine the pK_a in aqueous solution. Dresden (9) has shown the pK_a of the P. polycephalum pigment to be 4.7, and on this evidence postulated the presence of a carboxyl group, though he was unable to form either a methyl or an ethyl ester. In aqueous solution the pigment 1 from B. utricularis had a pK_a of 4.0.

It is unlikely that the pK_a determined spectroscopically by Dresden (9) and as reported here relates to the dissociation of a carboxyl group. It is clear that the dissociation of the relevant group radically alters the spectral properties causing a large hypsochromic shift in alkaline conditions, whereas the dissociation of carboxyl groups in conjugation with a polyene chromophore has little effect on the absorption spectrum.

The pK_a of the group is not, however, close to that expected for a simple protonated polyene Schiff base, retinylidene-t-butylamine and octatrienyl-t-butylamine being far more basic. Some of this difference could lie in the nature of the different solvents used, thus Bacarella et al. (33) have shown that the pK_a of several acids change significantly in different concentrations of water-ethanol mixtures. The pK_a of the anilium ion is 4.620 in water, 4.068 in 80% methanol and 4.613 in 95% methanol.

Although this solvent difference makes it impossible to compare directly the dissociation constants of the authentic Schiff bases and the B. utricularis pigment, the magnitude of the differences reported by Bacarella et al. would not account for the differences observed between the pigment and the two authentic Schiff bases. If then the pigment is a polyene derived Schiff base, as Daniel suggested for the apparently related pigment in P. polycephalum (13), the pK_a must be changed by structural features such as the presence of an electron accepting group on the polyene chain. In this vein Cordes' and Jencks (34) have shown that the acid dissociation constants of compounds in a substituted benzilidene-t-butylamine series showed fairly logical changes, basicity being increased by 4-methyl and 4-methoxyl groups and decreased by nitro and halogeno substitution.

Hydrolysis of pigment 1

Treatment of a solution of pigment 1 at room temperature in the dark for 10 hours in both 3 M hydrochloric acid and 3 M potassium hydroxide resulted in no change in the visible spectrum or in the thin layer properties. When a solution in 4 M hydrochloric acid was refluxed for 1 hour in the dark the solution decolourised, the resulting solution having an absorption maximum at 267 nm together with a very weak maximum at 361 nm and it thus appeared probable that more happened to the molecule than the simple hydrolysis of an imine link.

Reaction with sodium borohydride.

It was confirmed that, as reported by other authors (35), polyene Schiff bases are rapidly reduced by sodium borohydride, the site of action, even in the presence of a large excess of the reductant, being only the imine bond. Thus retinylidene-t-butylamine was rapidly decolourised by sodium borohydride giving a compound with an absorption maximum, which was unaffected by acidification, at 328 nm. The mass spectrum showed an increase in the molecular weight from 339 to 341.

A similar rapid reaction is observed when the pigments of both P. polycephalum and P. flavicomum are treated with sodium borohydride (20). In both these cases the product shows a polyene type of spectrum (Table 4).

Table 4. Spectral changes on reduction of P. polycephalum and P. flavicomum "Schiff base" pigments by sodium borohydride in ethanol

	Absorption maxima in basic methanol (nm)	Absorption maxima in methanol after reduction (nm)
P. polycephalum	391	270, 283, 301, 317, 331
P. flavicomum	250, 372	272, 282, 304, 319

The apparently related pigment from B. utricularis was unaffected by treatment with sodium borohydride, there being no change in the absorption spectrum or in the total absorbance of a solution which had been allowed to stand with excess reductant for 0.5 hour. This reaction thus serves to distinguish the Badhamia pigment from those derived from the two Physarum species.

It was interesting to observe, however, that the pigment, after thin layer chromatography on silica gel, was rapidly decolourised by sodium borohydride leaving a solution with an absorption maximum at 235 nm which red shifted to 266 nm in acid together with only very weak absorption at 300, 311 and 343 nm and an obvious inflection at 325 nm.

Still maintaining the assumption that the pigment is a Schiff base, attack by borohydride could be prevented by electronic or steric factors. Thus the lowering of the polarity of the imine link due to an electron with drawing group on the polyene chain could render the bond immune to attack. It should be noted that the presence of such a group is quite compatible with a possible explanation, already advanced, for the low pK_a of the pigment as compared with the pK_a of polyene Schiff bases.

Alternatively, the steric shielding of the imine bond to the borohydride ion might cause this immunity. Such shielding is observed in rhodopsin which is unaffected by borohydride treatment. Only after irradiation to form metarhodopsin II,

when structural changes in the molecule have exposed the imine link, will sodium borohydride effect the expected reduction (36).

It is certainly possible that the Badhamia pigment is related in this way to rhodopsin, for its rapid elution from Sephadex, as compared with the related compound from P. flavicomum from which it readily separates, suggests that it has a much higher molecular weight. Ninhydrin tests for protein have proved inconclusive, however, the brown/blue colour given not being completely characteristic.

Alternative structures for pigment 1

It is recognised that there is, as yet, no definitive evidence for a Schiff base linkage in this type of pigment, the evidence from the bathochromic shift on protonation and from the reaction with sodium borohydride being circumstantial and not completely convincing. However, no alternative chromophore can be postulated at present.

It must certainly not be forgotten that other pigments, with both known and unknown structures, which show a similar bathochromic shift on protonation have been isolated. Thus, abikoviromycin, coumeramycin (VII) and novobiocin (VIII), all of which are antibiotics extracted from Actinomycetes spp. (37), and prodigiosin (IX) from Bacillus prodigiosus (38,39) exhibit considerable bathochromic shifts on protonation (Table 5). It is also worthy of note that both coumeramycin and prodigiosin exhibit a hyperchromic effect on protonation.

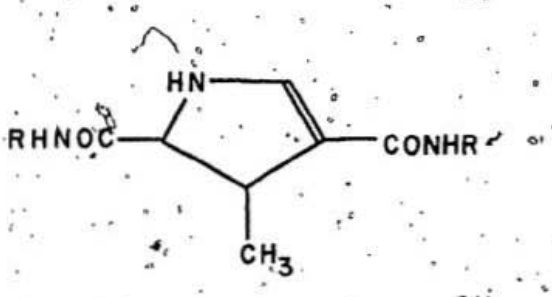
Table 5. Changes in the electronic absorption spectra of some pigments on protonation

Pigment	Absorption maxima in ethanol (nm)	
	Basic	Acidic
abikoviromycin	255, 285	235, 355
coumeramycin	280, 308 ($E^{1\%}_{1cm}$ = 335)	280, 345 ($E^{1\%}_{1cm}$ = 399)
novobiocin	311	344
prodigiosin	468	541

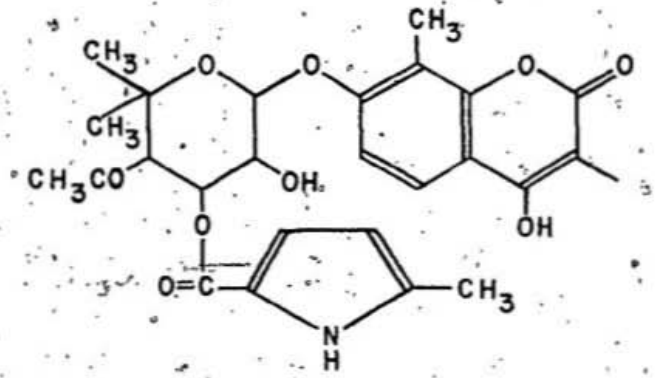
In addition to such naturally occurring pigments, simple compounds are known which exhibit similar properties, thus many pyrrole and pyridine derivatives show a similar bathochromic shift. Trans-2-styrylpyridine with a shift of 32 nm (40) might serve as a model for a polyene derived chromophore.

It is clear that at present the spectral properties of Schiff bases, and in particular conjugated Schiff bases, have been insufficiently investigated to make firm assignments from the available information and Sandorfy has said (41),

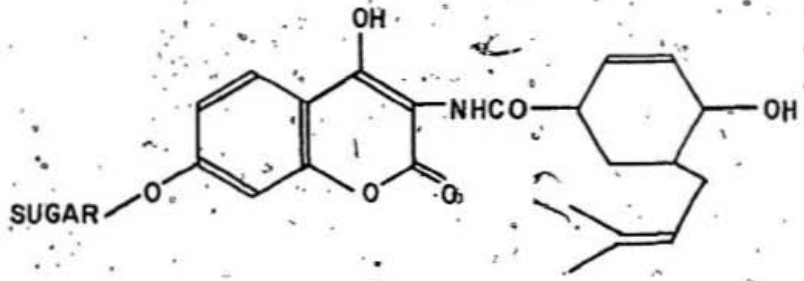
> "We have no definite understanding of the electronic spectra of azomethine compounds or their vibrations. We can only assemble some preliminary results, ask questions and express doubts."


HN
RHNOC
CONHR
CH3

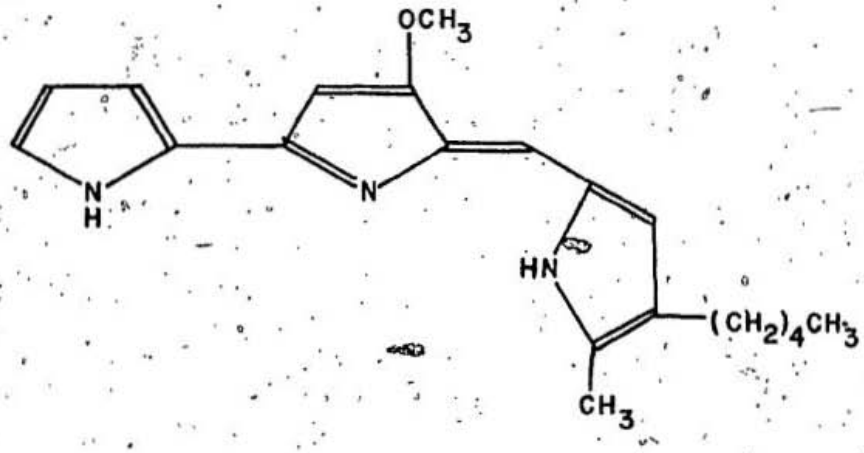
R=


CH3
CH3
O
O
O
O
CH3
CH3CO
OH
OH
O
O=C
CH3
N
H

VII


OH
NHCO
OH
SUGAR
O
O
O

VIII


OCH3
N
H
N
HN
(CH2)4CH3
CH3

XI

Summary

In conclusion there is some circumstantial evidence that the pigments from P. flavicomum and P. polycephalum which show a bathochromic shift on protonation are Schiff bases derived from a polyene aldehyde and an unidentified amine.

The pigment from B. utricularis is chemically somewhat different for, although it is apparently related in having an absorption maximum at approximately the same wavelength which shows bathochromic spectral shift on protonation, there is no evidence for a polyene chromophore nor for an imine linkage. It appears possible that there is a separate chromophore within the pigment which also shows a similar red shift in acid conditions, however, as the purification procedure used was not very rigorous, it is quite possible that this absorption is due to a different, though possible structurally related, compound.

Any proposed structures for these pigments must explain why the bathochromic shift is less than might be expected for a polyene aldehyde derived Schiff base and why the pK_a of the pigments differ radically from the authentic Schiff bases used. They must also explain the difference in the reactivity of the Physarum pigments and the Badhamia pigment toward sodium borohydride.

EXPERIMENTAL

The original culture of *B. utricularis* was kindly donated by Dr. C. J. Alexopoulous, University of Texas. In this culture the slime mould was growing on oatflakes suspended in solid agar and, as attempts to transfer the mould into a semi-defined liquid medium similar to that described on p. 51 failed, subsequent sub-cultures were maintained on the same medium.

Oatflakes (15 g, Ogilvie) were placed in a 20 x 30 cm casserole dish fitted with a close-fitting metal lid. After autoclaving at 124° for 15 min., a hot sterile solution of agar (1.5%) was poured into the dish to completely cover the oatflakes. The dish was then shaken gently to mix the oatflakes into the agar and allowed to cool. The plate was inoculated aseptically by transferring (by means of a flamed wire loop) four small pieces of plasmodium from the previous culture, one close to each corner of the agar surface.

The plates were left in the dark at 25° until the yellow plasmodium had covered the agar surface (1½-2 weeks) when the plasmodium was scraped off.

It was found that if the plasmodium was removed carefully, the plate could be returned to the culture room, and after 4 or 5 days the mould had again covered the surface of the agar, with no sign of contamination, and could be harvested again.

Extraction of pigments

The plasmodium was immediately stirred with ice-cold methanol (5°) and allowed to stand for 24 h. After filtration, the residue was extracted a second time with fresh cold methanol. The combined methanolic extracts were evaporated to dryness at 40° on a rotary evaporator, to leave a yellow oil.

Gel filtration of methanolic extract

Sephadex LH 20 (100 g) was suspended in methanol (500 cm^3) and stirred magnetically for 12 h to allow the gel to expand fully. The suspension was made up into a column (27 x 4 cm), and methanol was slowly passed through until the bed had completely settled and then for at least 3 h afterwards.

The top of the bed was covered with filter paper to prevent its disturbance and the yellow oil obtained above, dissolved in the minimum quantity of methanol, was applied to the column. The column was eluted slowly with methanol (ca. 0.3 cm^3/min) and four distinct pigment bands were separated and eluted (Table 6).

Each of these compounds was further purified by rechromatography on Sephadex LH 20.

Table 6. Properties of pigments eluted from Sephadex LH 20 by methanol

Pigment (in order of elution	Colour	Fluorescence	Absorption max. (nm) (methanol)	
			Base	Acid
1	orange	none	238 375	267 420
2	v. pale yellow	white	338-358	(327) 343 359
3	yellow	orange	328-340	(326) 343 (361)
4	orange	red/orange	266 (340) 378	266 (344) 359 (379)

( ) denotes shoulder

Thin layer chromatography of pigments

Each pigment was chromatographed on silica gel G thin layers. Details are given in Table 7. It was apparent that some degradation of pigment 1 took place on the silica gel when acetic acid was included in the solvent system as each band, after separation from the silica gel, separated into the same components on further chromatography.

Pigment 1

The pigment was separated by repeated chromatography (3x) on Sephadex LH 20 with methanol as the eluting solvent.

The visible absorption spectrum has been reported (Fig. 4).

The infrared spectrum (CCl_4) had a broad absorption 3600-2900 and maxima at 2990, 2910, 2840, 1740, 1625, 1470, 1460, 1380, 1240, and 1170 cm^{-1}.

Column chromatography

Pigment 1 was applied to a silica gel column (60 x 3 cm) in petroleum ether (60-80°)-acetone (1:4) then eluted with the same solvent: the band ran slowly for some time then stopped. Acetone eluted an orange band λ_{max} 268, 367 nm (basic methanol) and methanol a second orange band λ_{max} 269, 368 nm (basic methanol): only the first band showed a red shift to 412 nm on protonation.

Thin layer chromatography (Tlc)

Tlc on silica gel G (Merck, acc. to Stahl) using methanol-chloroform (1:1) gave a single yellow spot Rf 0.85, but with chloroform-methanol-acetic acid (50:15:3) four bands were obtained (Table 7). The major band (band i) when run repeatedly in the same solvent system gave small amounts of (iii) and (iv).

Hydrolysis of pigment 1

(i) To separate portions (5 cm^3) of a solution of pigment 1 in methanol (absorbance ca. 3.0) was added concentrated hydrochloric acid (1.7 cm^3) and 6 M aqueous potassium hydroxide (5 cm^3) to bring the final concentrations to about 3 M. The two solutions were left 10 h in the dark at room temperature. No change in the absorption spectrum of either sample was detected and, after dilution with water and extraction into ether, the pigment remaining gave no additional spots on tlc.

Table 7. Thin layer separation of pigments on silica gel G

Pigment	Solvent	Rf	Absorption maxima (nm) in methanol	
			Base	Acid
1	(a) methanol-chloroform (1:1)	0.85	-	-
	(b) chloroform-methanol-acetic acid (50:15:3)	(i) 0.80 (major)	394	434
		(ii) 0.73	394	434
		(iii) 0.50	388	419 (440)
		(iv) 0.00	370	385
2	methanol-chloroform (1:10)	0.92	-	-
3	methanol-chloroform (1:10)	0.73	337 (broad)	(331) 345 (362)
4	chloroform-methanol-acetic acid (50:15:3)	(i) 0.68	385	(343) 359 (380)
		(ii) 0.51	385	(344) 362 (382)
		(iii) 0.30	385	(343) 359 (380)
		(iv) 0.00	-	---

(ii) A similar solution of pigment 1 (10 cm^3) was made .4 M in hydrochloric acid and refluxed in the dark for 1 h. The solution was almost decolourised and had an absorption maximum at 267 nm together with an indistinct maximum at 361 nm. Tlc on silica gel with chloroform-methanol (1:1) developer gave a pale orange band with orange fluorescence Rf 0.70 and a colourless band, also with orange fluorescence, at Rf 0.96.

Sensitivity of pigment to silica gel

A dilute solution (absorbance ca. 10 in 1 cm cell) of pigment 1 in methanol was prepared. To this solution (10 cm^3) was added silica gel G (100 mg), and the mixture was allowed to stand, with occasional shaking, in the dark at room temperature for 24 h. The absorption spectrum before and after treatment is recorded in Table 8.

Table 8. Absorption spectrum of pigment 1 before and after treatment with silica gel

	Absorption maxima (nm)	
	Basic Methanol	Acidic methanol
before treatment	375	420
after treatment	373	412

Preparation of 2,4-hexadienal and 2,4,6-octatrienal

The method used was essentially that reported by Blout and Fields (42).

Crotonaldehyde (200 cm^3, distilled 100-102°) and acetaldehyde (270 cm^3) were added to a 1 dm^3 flask equipped with a Dry-Ice condenser and the mixture was purged with nitrogen for 0.5 h when the condenser was removed. To the mixture was added piperidine (3 cm^3) and glacial acetic acid (2.1 cm^3), and the mixture immediately turned a brilliant red colour. The mixture was again purged with nitrogen and allowed to stand for 12 h in the dark.

After filtration, the filtrate was extracted with ether (750 cm^3), the ethereal layer being washed four times with water (300 cm^3), filtered, and dried over anhydrous sodium sulphate for 24 h.

After filtration the solution was evaporated on a rotating evaporator to about 200 cm^3. Further distillation at atmospheric pressure and then under reduced pressure afforded three major crude fractions of unchanged crotonaldehyde, hexadienal and octatrienal; the latter two fractions were redistilled under reduced pressure.

Hexadienal

Distilled 54-58° at 5 mm. Yield 14.2 g.

Absorption maximum 271 nm in methanol, ε = 97 500.

Octatrienal

Distilled 118-126° at 5 mm. The distillate failed to crystallise on cooling on a Dry-Ice bath so the crude octatrienal (6.1 g) was added to 5 times its volume of a

saturated solution of sodium hydrogen sulphite. After thorough mixing, the pale yellow precipitate was filtered off, washed three times with 95% ethanol (5 cm^3), with ether (5 cm^3) and lastly with absolute ethanol (5 cm^3).

The hydrogen sulphite derivative was added to a solution of concentrated hydrochloric acid (20 cm^3) and water (250 cm^3) and shaken to effect solution. After being allowed to stand for 0.5 h, the octatrienal was extracted into ether (3 x 50 cm^3) and this solution was washed with water (4 x 50 cm^3) and dried over anhydrous sodium sulphate.

After filtration and removal of the solvent, the residual solid was redistilled (75-80° at 1.5 mm) and the product crystallised from hexane as pale yellow plates, mp 55°. Yield 2.0 g. Absorption maximum 322 nm in methanol, ε = 36 800.

Mass spectrum: m/e 122 (M, 100%), 107 (M-CH_3, 28%), 93 (M-CHO, 28%), 91 (M-HCHO - H, 36%), 79 (M-CO-CH_3, 43%), 77 (M-HCHO-CH_3, 49%).

Infrared spectrum: 1673 (conjugated C=O), 1612 (C=C) cm^{-1} (43).

Preparation of dilute solutions of Schiff bases of hexadienal, octatrienal and retinal

The preparation was essentially that used by Akhtar et al. (21). A dilute solution of the polyene aldehyde (hexadienal, octatrienal or retinal) in methanol was made so that the solution had an optical density of 2.5-3.5 at the absorption maximum. To this solution (2 cm^3) was added a

solution of the amino compound in water (2 cm^3) or, if necessary for solution purposes, methanol (2 cm^3). The amount of the amino compound dissolved was calculated to give an approximately 20 molar excess.

Aqueous sodium hydroxide (2.0 cm^3, 0.1 M) was added and the solution was left to stand in the dark for 2 h when the absorption spectrum was measured. Hydrochloric acid (6 M; 1 or 2 drops) was then added and the absorption spectrum redetermined. The reaction of aspartic acid with retinal was rather slow and the mixture was allowed to stand for 2 days.

The results are shown in Tables 9, 10 and 11.

Table 9. Absorption spectra of Schiff bases derived from hexadienal

Amino compound	Absorption max. in methanol (nm)	Absorption max. in methanol/HCl (nm)
methylamine	266	307
t-butylamine	267	308
cyclohexylamine	268	311
benzylamine	270	315
isoleucine	271	317
threonine	272	315
phenyl alanine	268	317
arginine	272	316
aspartic acid	267	317

Table 10. Absorption spectra of Schiff bases derived from octatrienal

Amino compound	Abs. max. methanol (nm)	Abs. max. methanol /HCl (nm)
methylamine	303 (317)	360
t-butylamine	303 (317)	358-364
cyclohexylamine	304	365
glycine	307-316	367
lysine	303 (317)	365
arginine	313-318	372
aspartic acid	(300) 315	327* v. broad, flat maximum

( ) denotes shoulder

*small bathochromic shift probably due to incomplete reaction

Table 11. Absorption spectra of Schiff bases derived from retinal.

Amino compound	Abs. max. methanol (nm)	Abs. max. methanol /HCl (nm)
methylamine	361	439
t-butylamine	366	441
cyclohexylamine	366	443
benzylamine	367	451
allylamine	369	449
aniline	396	507
glycine	369	450*
asparagine	369	454
phenyl alanine	369	459
aspartic acid	371	459 (after 10 h)
3-amino butyric acid	374	440
homosenine	370	454

*solution rapidly decolourised by addition of excess HCl.

Attempted reaction of retinal with methyl-β-aminocrotonate

(a) No product could be detected when following the general method already described, even after being left for 2 days.

(b) Retinal (5 mg) was dissolved in methanol (10 cm^3) and methyl-β-aminocrotonate (50 mg) was dissolved in the solution. Potassium hydroxide solution (1.0 M, 1 cm^3) was

added and the mixture allowed to stand for 6 h, after which time no product was detected. The solution was refluxed for 3 h and allowed to stand 12 h when the absorption maximum at 375 nm shifted to give a broad band centered at 404 nm on acidification.

No attempt was made to separate what was probably a mixture of retinal and Schiff base product and further reaction did not appear to affect the spectral shift.

Attempted reaction of retinal with cytosine

(a) No product could be detected after following the general method described above, even after standing for 24 h.

(b) Retinal (1 mg) was dissolved in methanol (5 cm^3) and a solution of cytosine (10 mg) in water (5 cm^3), and sodium hydroxide (0.1 M, 2 cm^3) was added. The solution was refluxed for 3 h, and then allowed to stand for 3 days but no product was detected.

(c) Retinal (1 mg) and cytosine (25 mg) were dissolved in tetrahydrofuran (10 cm^3) and allowed to stand for 48 h over molecular sieves. After evaporation to dryness, the spectrum of the residue, when dissolved in methanol, showed no evidence of any reaction.

Preparation of retinylidene-t-butylamine

The method used was identical to that reported by Irving and Leermakers (30). All-trans-retinal (100 mg; Sigma) was dissolved in t-butylamine (5 cm^3) and the solution was

allowed to stand over molecular sieves for 24 h. The solution was decanted and the excess t-butylamine removed on a rotary evaporator, to leave solid retinylidene-t-butylamine.

Mass spectrum: m/e 339 (M), 100%; 324 ($M-CH_3$), 21%; 282 (M-butyl-H), 15%; 268 (M-butyl-H-N), 31%.

Preparation of octatrienylidene-t-butylamine

The method used was identical to that described above using octatrienal (150 mg) and t-butylamine (8 cm^3). Removal of the t-butylamine left a yellow oil which was not purified further.

Mass spectrum: m/e 177 (M), 77%; 162 ($M-CH_3$), 28%; 122, 78%; 121 (M-butyl), 83%; 106 (M-butyl-H-N), 31%.

Sodium borohydride reduction of retinylidene-t-butylamine

Retinylidene-t-butylamine (10 mg) was dissolved in 95% ethanol (5 cm^3) and sodium borohydride (10 mg) was added. 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 (313) 327 nm in methanol.

Mass spectrum: m/e 341 (M); 100%; 326 ($M-CH_3$) 21%, 284 (M-t-butyl-H), 31%, 270 (M-t-butyl-H-N), 26%.

Attempted reduction of pigment 1 by sodium borohydride

A solution of pigment 1 in ethanol (6 cm^3, absorbance 1.8 in 1 cm cell) was treated with sodium borohydride (5 mg). No change in the absorption spectrum nor in

the total absorbance was observed even after 0.5 h, indicating that no reaction had taken place.

Under the same conditions related pigments from P. polycephalum and P. flavicomum and simple Schiff bases derived from retinal and octatrienal were immediately reduced.

Reduction of pigment 1 by sodium borohydride after thin layer chromatography on silica gel

The compound in the major band (band i) obtained when pigment 1 was chromatographed on silica gel was dissolved in methanol. This solution (6 cm^3, absorbance 1.5-1.8 in 1 cm cell) was treated with sodium borohydride (1 mg), the colour being removed instantaneously.

The solution remaining showed no marked absorption maxima though there was evidence of weak absorption at 311, 325 and 343 nm.

Determination of pK_a of retinylidene-t-butylamine

Three stock solutions were prepared:

A benzoic acid (0.679 g) in absolute methanol (500 cm^3)

B sodium benzoate (0.800 g) in absolute methanol (500 cm^3)

C sodium chloride (14.61 g) in deionised water (500 cm^3)

The concentration of the final buffer solutions were: total benzoate 0.01 M, sodium chloride 0.05 M. The NaCl concentration

was relatively high to ensure that the total ion concentration showed little change due to buffer action. The buffer solutions were made up as shown in Table 12, after calculating that the pK_a of benzoic acid in 90% methanol is 7.02 (Interpolated from ref. 33).

Table 12. Volumes of stock solutions used to prepare 50.0 cm^3 benzoate buffer

Vol. solution C = 5.00 cm^3.

pH	Vol. soln. A ml	Vol. soln. B ml
6.0	41.08	3.92
6.5	34.54	10.43
7.0	23.01	21.99
7.02	22.25	22.25
7.2	17.86	27.10
7.34	14.56	30.42
7.5	11.19	33.82
7.7	7.78	37.32
8.0	4.27	40.74
8.04	3.92	41.05
8.14	3.17	41.83
8.5	1.36	43.65
9.0	0.47	44.54
9.5	.15	44.85

A standard solution of retinylidene-t-butylamine in methanol, which, when acidified, had an absorbance of 1.5 in a 1 cm cell at its absorption maximum, was prepared. An aliquot of this solution (5 cm^3) was evaporated to dryness under reduced pressure at 35° and the residue was dissolved in the appropriate buffer solution (5 cm^3). The electronic absorption spectrum was recorded, a series of curves being obtained over a range of pH values (Fig. 5).

The absorbance of each solution at 440 nm was plotted against the pH of the solution, the inflection point being taken as the pK_a of the protonated Schiff base (32,44) (Fig. 7). Although theoretically this plot can be made at any wavelength 440 nm was chosen as there is the largest difference between the extinction coefficients of the protonated and unprotonated species at this wavelength.

The pK_a of retinylidene-t-butylamine was 8.6.

pK_a of octatrienyl-t-butylamine

The pK_a was determined as described above and was shown to be 7.4. Curves showing the change in the ultraviolet spectrum with pH and the change in absorbance at 360 nm with pH are given in Figs. 6 and 8.

pK_a of pigment 1

The pK_a of pigment 1 was determined in aqueous potassium hydrogen phthalate solutions (45), the pH of each being checked by a pH meter. Graphs showing the change in the

visible absorption spectrum with changing pH and the change in absorbance at 421 nm with pH are given in Figs. 9 and 10. The pK_a of the pigment is 4.0.

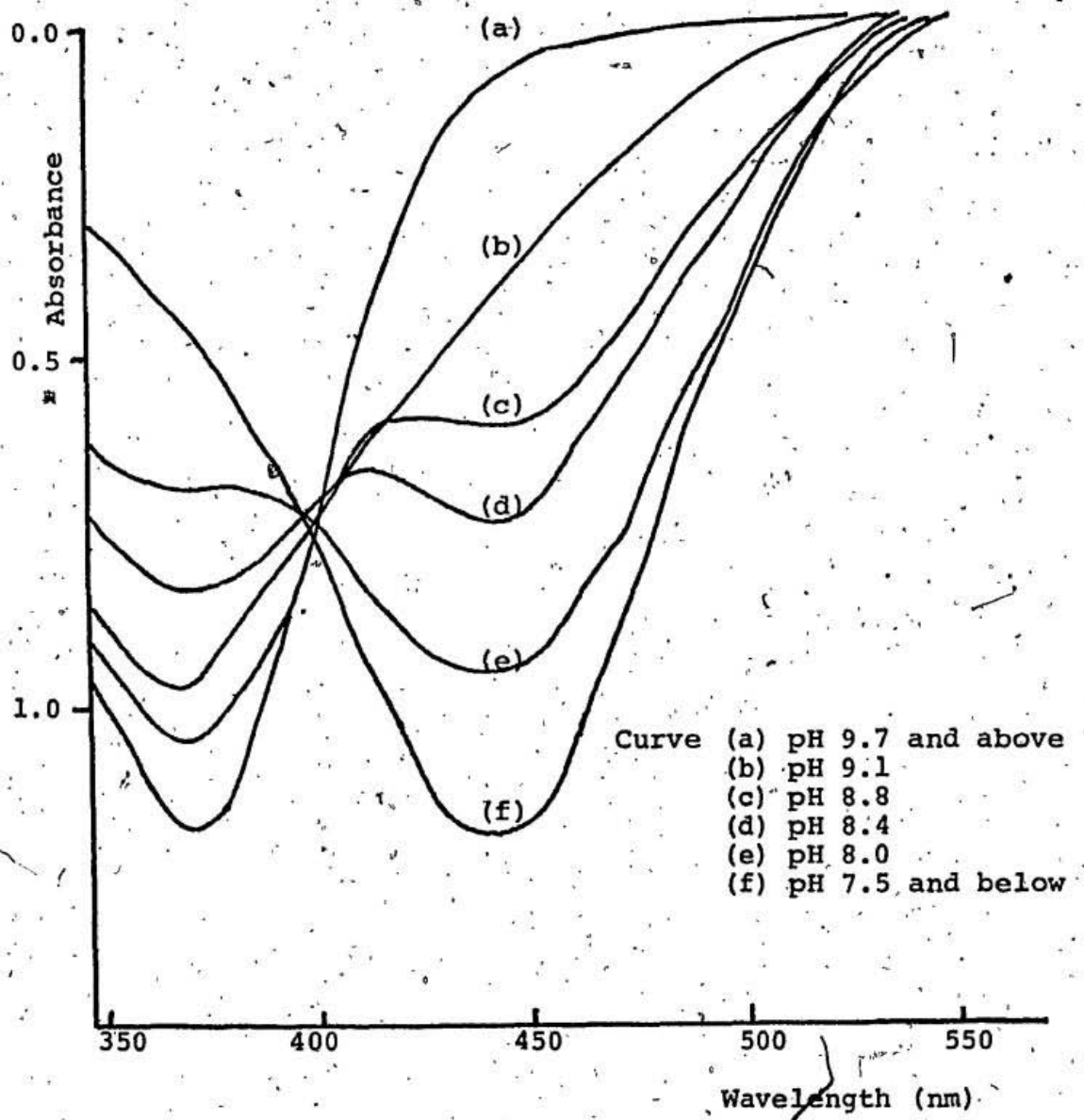


Fig. 5. Effect of pH on the visible absorption spectrum of retinylidene-t-butylamine in 90% methanol

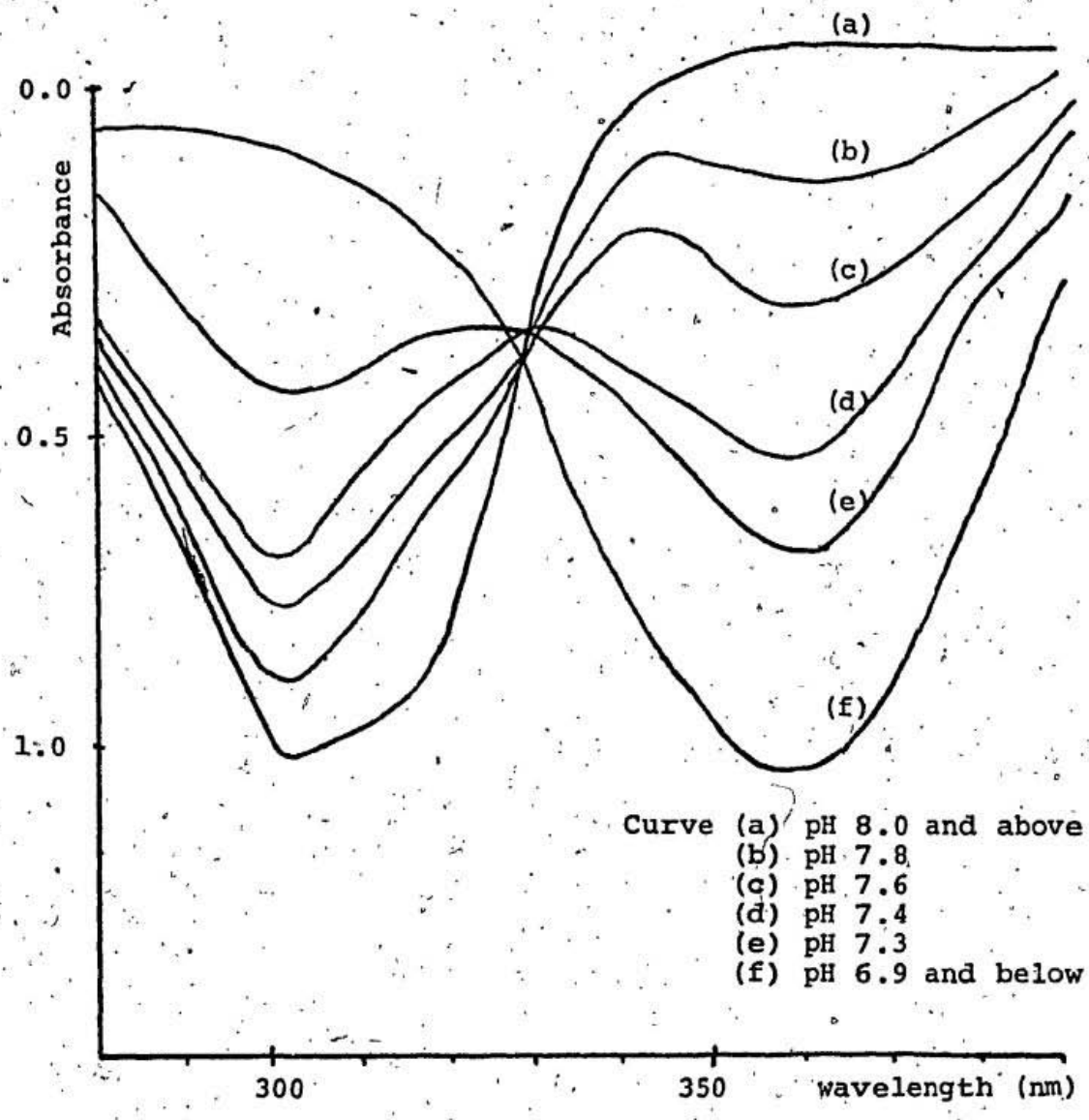


Fig. 6. Effect of pH on the ultraviolet absorption spectrum of octatrienylidene-t-butylamine in 90% methanol.

Fig. 7. Absorbance of retinylidene-t-butylamine at 440 nm in solutions of 90% methanol at different pH.

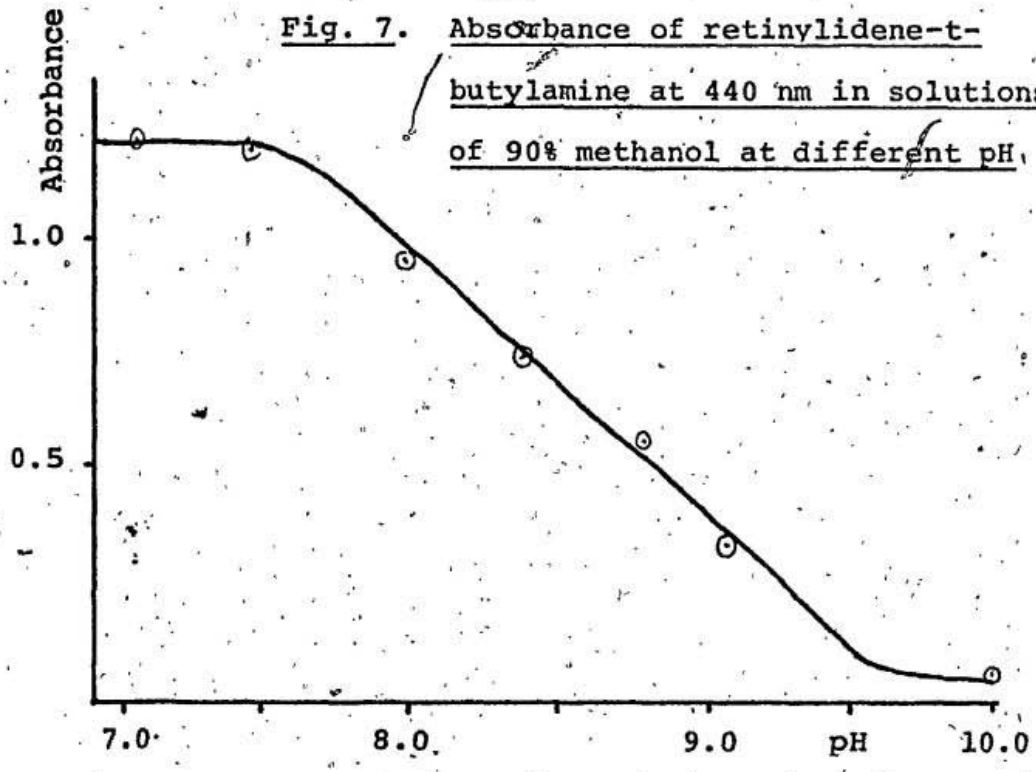


Fig. 8. Absorbance of octatrienyl-t-butylamine at 360 nm in solutions of 90% methanol at different pH

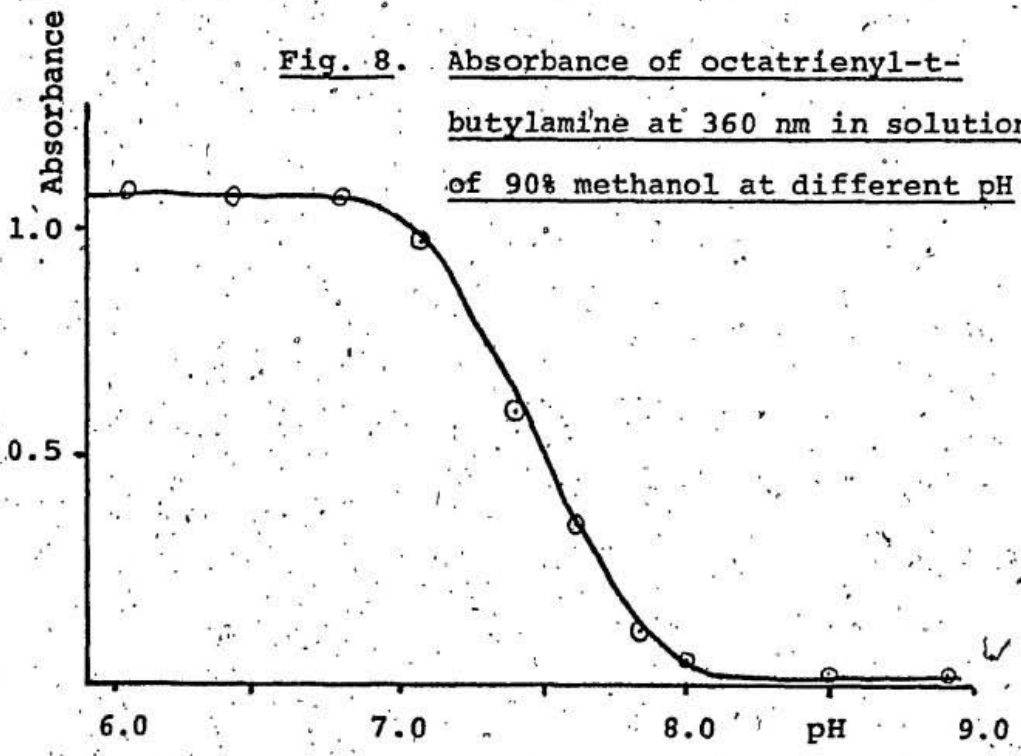


Fig. 9. Effect of pH on the visible absorption spectrum of pigment 1 in aqueous buffers

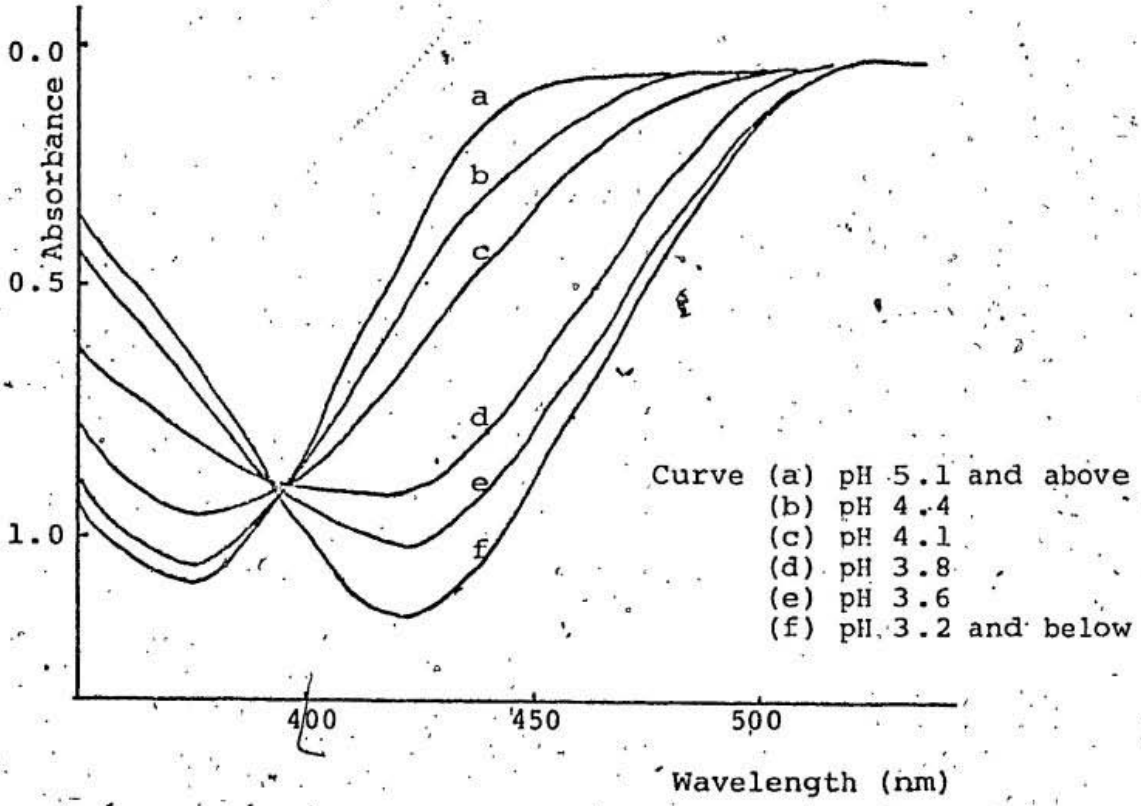
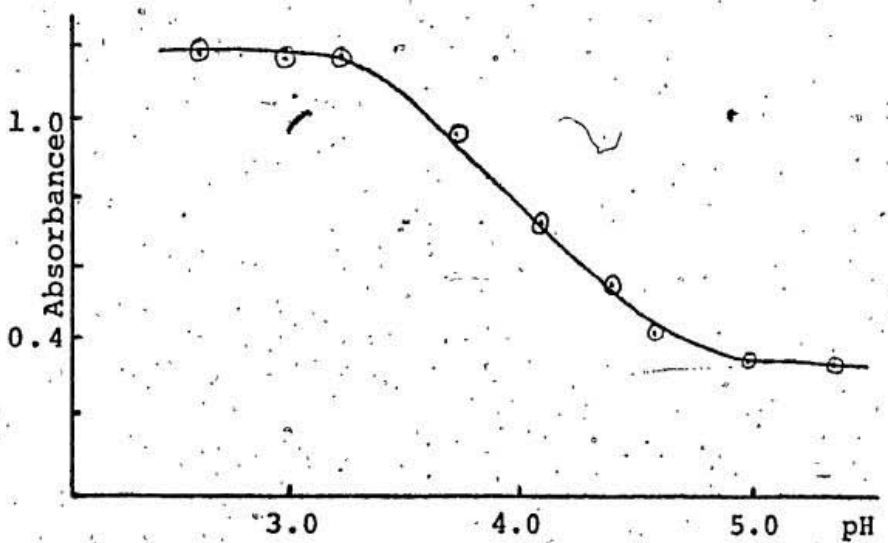


Fig. 10. Absorbance of pigment 1 at 421 nm in aqueous buffer solutions



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PART III

INTRODUCTION

In the course of work on the nature of the pigments of some Myxomycete species, two cultures of Badhamia utricularis were apparently contaminated by a yellow bacterium when purchased. Initially it was thought that the slime mould was growing on a white bacterial species, and that all the extractable pigment was from the yellow Myxomycete, but it was later recognised that B. utricularis had been completely lost from the culture, and examination by Dr. Moskovits of the Memorial University Department of Biology showed that a pure culture of a yellow bacterium was present.

Identification of the bacterium was attempted by Dr. P. Fardy and Miss R. Cross of the Microbiology Unit of the General Hospital, St. John's, who suggested that it was a Corynebacterium species.

In view of the work proceeding on the nature of the yellow pigment (to be discussed later), which appeared to be similar to, or identical with, the major pigment of Flavobacterium dehydrogenans, Professor V. Treccani of the Institute of Microbiology, University of Milan, was asked to compare the culture with different species of Flavobacteria maintained at the institute. Dr. Treccani kindly did this and using biochemical and morphological, including phase contrast micrographic (Fig. 1a-c), data concluded that the yellow culture was possibly

related to F. dehydrogenans, though it was dissimilar in some respects, the points of similarity and difference being shown in Table 1.

Table 1. Comparison of the isolated bacterium with F. dehydrogenans

	F. dehydrogenans VIII (Arnaudi)	Isolated species
Similarities		
	Rods 0.45-0.75 x 1.2 μ	Rods 0.4 x 0.6-1 μ
	Gram positive	Gram positive
	Aerobic	Aerobic
	Catalase positive	Catalase positive
	Nitrites not formed from nitrates	Nitrites not formed from nitrates
	Acid from glucose, maltose	Acid from glucose, maltose
Differences		
	Motile	Non-motile
	Does not hydrolyse gelatin	Hydrolyses gelatin
	Pigment produced only in light	Pigment produced in light and dark

The classification of gram positive bacteria, such as F. dehydrogenans, into the Flavobacteria is not always accepted, indeed Trecani (1) and other authors (2,3) consider that such species are more closely related to the Corynebacteria, and thus, taking this into account, the isolated bacterium will be referred to as a Corynebacterium species.

Fig. 1. Phase contrast micrographic photographs of the development cycle of Corynebacterium sp. (x2800)

Fig. 1a. Time 9h

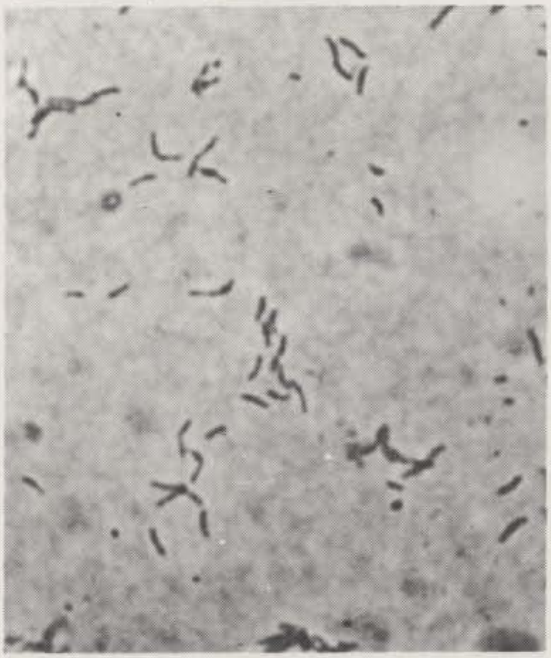


Fig. 1b. Time 16h

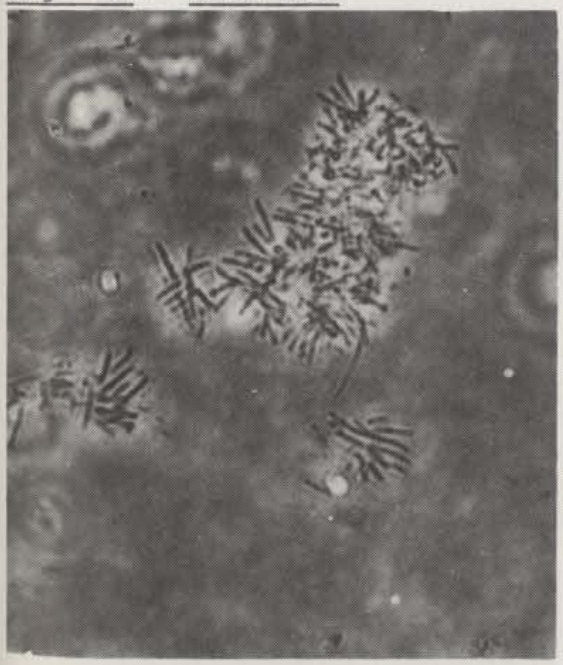
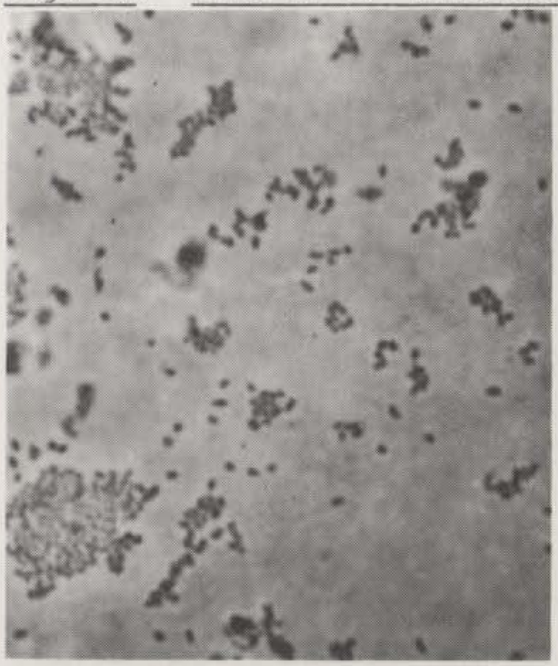


Fig. 1c. Time 24 h. Stationary phase



DISCUSSION

As the culture was initially thought to contain slime mould, it was grown on damp oatflakes, a standard method for the Myxomycetes, and was kept in the light when pigment production was at a maximum. Purification of the pigments from the organism was difficult as pigments from the oatflakes, together with lipids and other contaminants from the same source, had to be removed: use of both column and thin layer chromatography did, however, effect this separation.

In later work the bacterium was grown in a buffered liquid medium containing extract of both yeast and beef and the cells were readily separated from the medium by centrifugation, the pigments being isolated and purified by column and thin layer chromatography.

Column chromatography of a methanolic extract of the Corynebacterium sp., grown in liquid medium, gave five coloured bands. Two very minor components (pigments 1 and 2) were eluted from alumina by petroleum ether indicating that they are probably non-polar carotenes, their visible absorption spectra being close to those expected for β-carotene (pigment 1) and α-carotene (pigment 2). These two pigments were not examined further.

The three major pigments (pigments 3, 4 and 5) were eluted from alumina by petroleum ether/acetone mixtures and each was purified further by additional column chromatography

on alumina. All three had extremely similar visible absorption spectra with maxima at (386), 412, 436, 466 nm in methanol solution (Fig. 2).

These spectra suggested that the three pigments had the same chromophore with nine conjugated double bonds in an aliphatic polyene chain. Thus the carotenoids neurosporene (I), ε-carotene (II), and 5,6,5',6'-tetrahydrolycopene (III), which have a conjugated nonaene chromophore, have very similar spectra (Table 2).

Table 2. Visible absorption spectra of some carotenoids with a conjugated nonaene chromophore

Carotenoid	Absorption maxima	
neurosporene	418, 441.5, 470 (petroleum ether)	(4)
ε-carotene	(394), 416, 439, 470 (petroleum ether)	(5)
5,6,5',6'-tetra-hydrolycopene	413.5, 437.5, 467.5 (hexane)	(6)

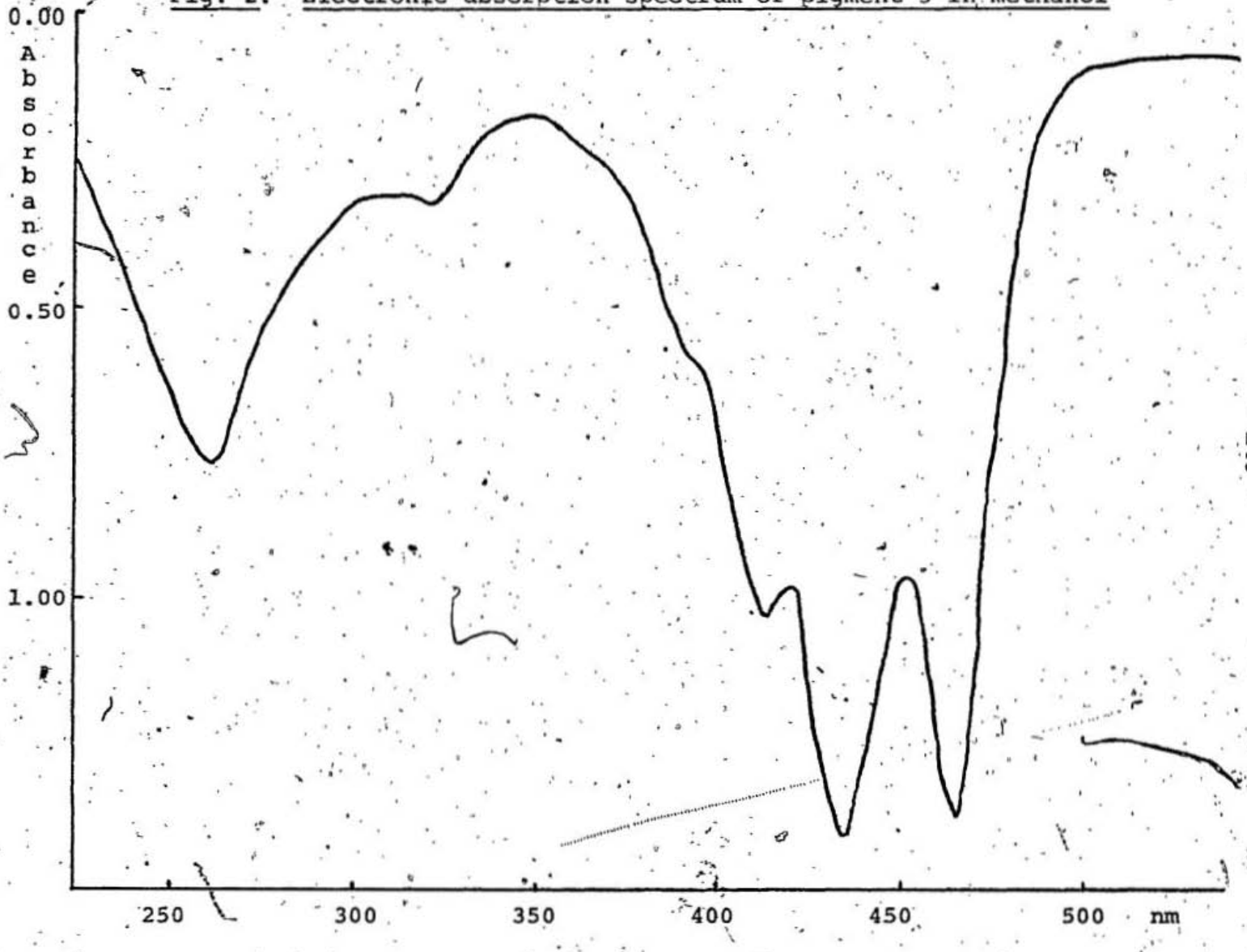
( ) denotes shoulder

The relationship of pigments 3, 4 and 5 was examined by investigating the effect of base on each pigment when both pigments 3 and 4 gave a compound with thin layer behaviour identical with pigment 5 suggesting that both are esters of pigment 5.

Pigment 5

The polyene nature of pigment 5 was confirmed by the rapid decolourisation by hydrogen in the presence of palladium

Fig. 2. Electronic absorption spectrum of pigment 5 in methanol



to give a product with no absorption in the ultraviolet above 200 nm. The infrared spectrum was quite characteristic of non-aromatic carotenoids with absorption maxima at 1600 and 968 cm^{-1}, the latter maximum being typical of the trans disubstituted double bond. Absorption maxima at 3590, 3390 and 1002 cm^{-1} indicated the presence of at least one hydroxl group which was probably allylic.

When treated with acetic anhydride in pyridine, a single monoacetate and a diacetate were formed and it was clear that pigment 5, like isozeaxanthin (I), which under the same conditions also gave a single monoacetate and a diacetate, contained two symmetrical primary or secondary hydroxyl groups.

Pigment 5 gave a single moho and a disilyl ether only, confirming that there was no additional tertiary hydroxyl group available which had not reacted with the acetylating reagent. As expected the silylating reagent had no effect on the diacetate but did produce a single less polar compound with the same visible absorption spectrum as pigment 5 from the monoacetate.

The partition ratio between petroleum ether and 95% methanol can be used quite successfully to determine the number and types of functional groups present in the carotenoid molecule, thus Petracek and Zechmeister have tabulated the partition ratios of carotenoids with different

numbers of keto and hydroxyl groups (7). The partition ratio of pigment 5 was 32/68 which is similar to that expected for a carotenoid with two hydroxyl functions, though the compound is somewhat less polar than the C40 carotenoids investigated by Petracek and Zechmeister.

The relatively high frequency infrared absorption of the hydroxyl group (1002 cm^{-1}) was rather indicative of an allylic hydroxyl group. Such groups in carotenoids can usually be detected by dehydration, using hydrochloric acid in chloroform, when a change in thin layer, and often spectral properties, results (8). Pigment 5, however, gave no elimination product even when treated with hydrogen chloride dissolved in chloroform. Attempted dehydration with phosphorus oxychloride in pyridine did give a new product but on treatment with base this was readily converted back to pigment 5 and was probably a phosphate ester (9).

Allylic alcohols may also be detected through the formation of methyl ethers with hydrochloric acid in methanol (10) but pigment 5 failed to react with this reagent.

Attempts to oxidise an allylic alcohol with manganese dioxide failed, but nickel peroxide oxidation (11) gave both a monocarbonyl and a dicarbonyl product, both of which had unchanged electronic absorption spectra. The

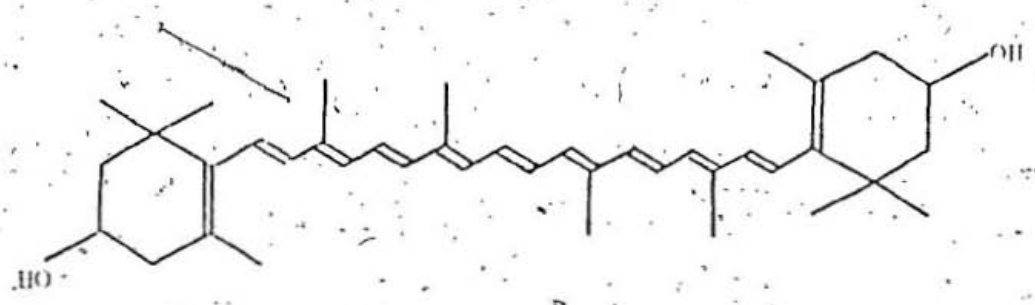
dicarbonyl compound was not silylated but the monocarbonyl compound gave a single silyl ether. It was again apparent that the two symmetrical hydroxyl groups had been oxidised and that, if allylic, they were removed from the main nonaene chromophore.

Treatment of both the oxidised and partially oxidised products with sodium borohydride gave a single product indistinguishable from pigment 5.

Mass spectral information

A review of the mass spectral fragmentation patterns of carotenoids (13) has shown the importance of such data in making structural assignments. Though some schools have modified their spectrometers somewhat and now have little difficulty in obtaining carotenoid spectra, such spectra can be difficult to produce on a routine basis. No spectrum was given on the instrument available, though eventually one was obtained at Liverpool University.

The spectrum showed a strong molecular ion at m/e 704 and although an accurate mass was not obtained it appeared very probable that pigment 5 was a C_{50} carotenoid with the formula $C_{50}H_{72}O_2$.



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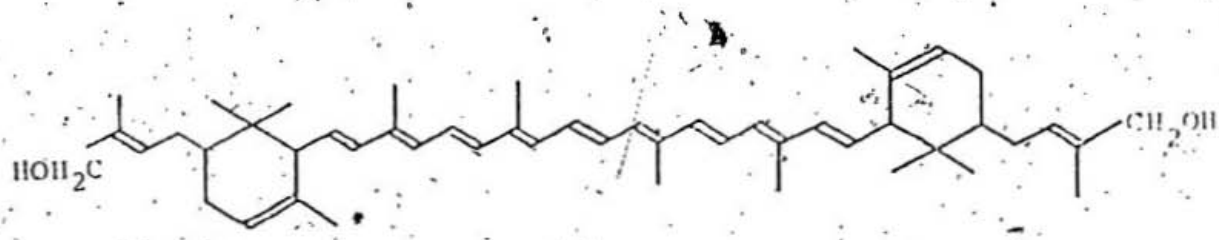

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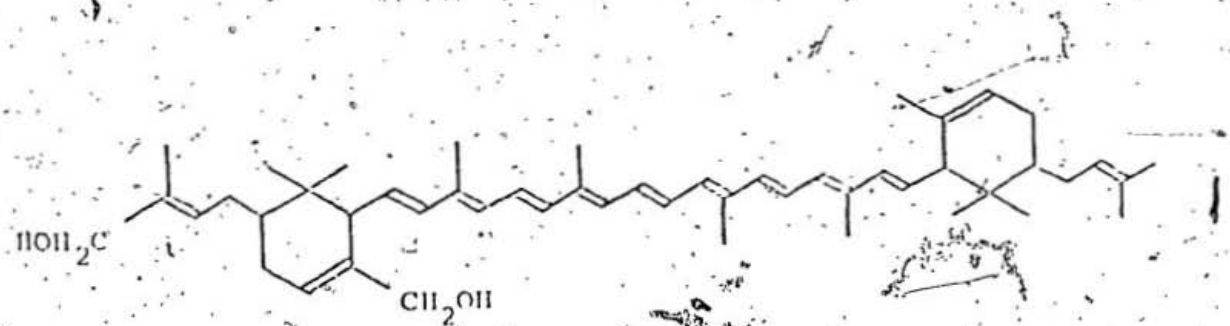

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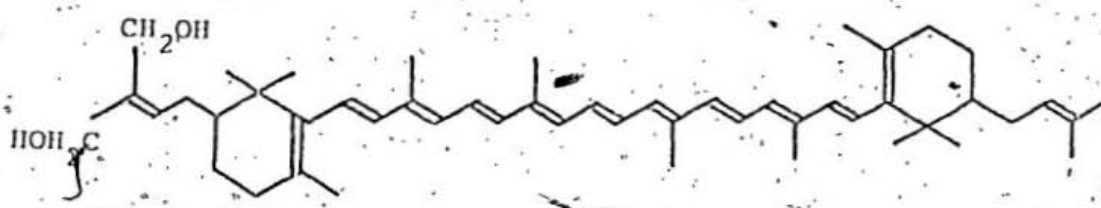

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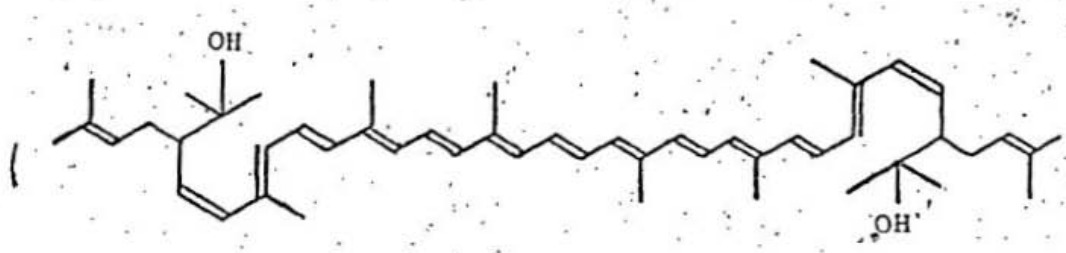

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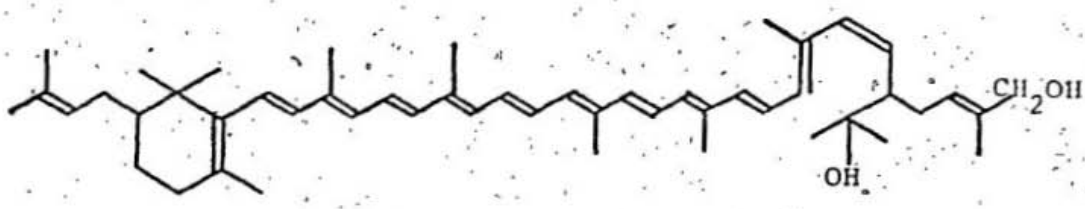


VI

C_{50} carotenoids

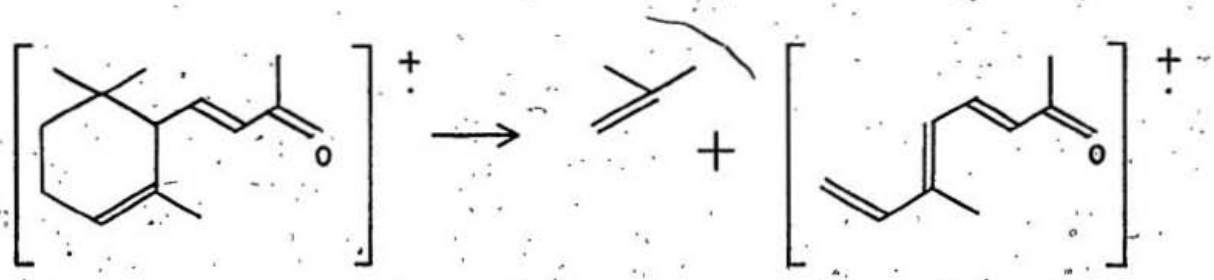
Several C_{50} carotenoids have been characterised since 1966 when the structure of the first such compound 2,2'-(dihydroxymethylbut-2-enyl)-ε-carotene or decaprenoxanthin was determined (5). In all such compounds described to date the additional 10 carbon atoms are in the form of two isopentenyl functions attached to a C_{40} skeleton at C2 and 2'.

Some of these carotenoids have been known for several years and had previously been assigned a C_{40} skeleton, for the C_{50} nature is well hidden, being only revealed by mass spectrometry, an integral technique of carotenoid structural determination only since the middle 1960s (14).

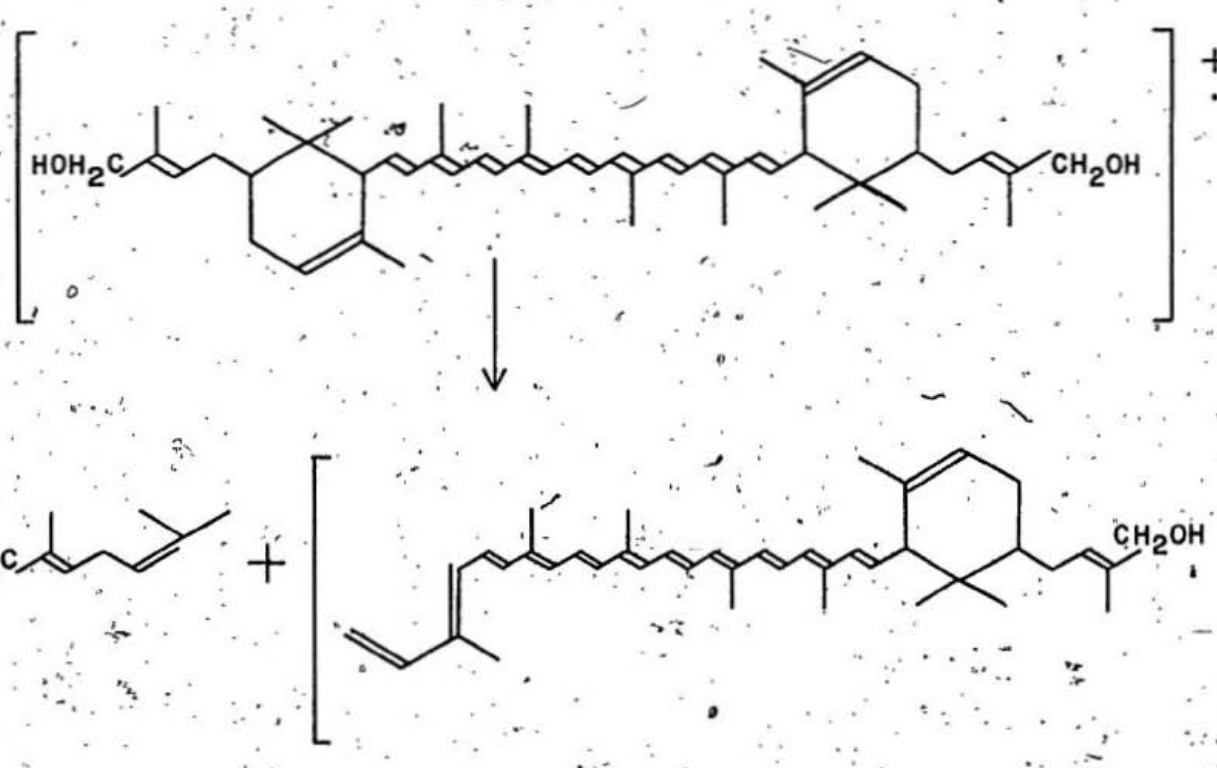
Several dihydroxy C_{50} carotenoids have been fully or partially characterised: decaprenoxanthin (II), the isomeric sarcinaxanthin, tentatively identified as (III), and the three carotenoids P450 (IV), bisanhydrobacteriorubin (V), and 1'-hydroxy-1',2'-dihydro-2-isopentenyl-2'-(hydroxyisopentenyl)-torulene (VI) from Corynebacterium poinsettiae. A further related compound, corynexanthin, is a monoglucoside of decaprenoxanthin (15).

Of these compounds the three from C. poinsettiae are obviously not identical with pigment 5 as each shows absorption at a wavelength greater than ε-carotene. Distinction between decaprenoxanthin, sarcinaxanthin and any other possibly closely related carotenoids can best be made through the mass

spectral fragmentation pattern, in particular that associated with the retro-Diels-Alder reaction characteristic of the α-ionone ring, thus α-ionone (16) and ε-carotene (17) both show a strong characteristic daughter ion due to such fragmentation;



Similarly, decaprenoxanthin gives an ion at m/e 564 due to a similar fragmentation which involves the loss of a 2-(hydroxymethyl-but-2-enyl) function (5).



Mass spectral fragmentation of pigment 5

The mass spectrum of pigment 5 showed a characteristic ion at m/e 564 presumably formed through retro-Diels-Alder fragmentation of an α-ionone ring similarly substituted to that in decaprenoxanthin. Other ions at m/e 686 ($M-H_2O$), 668 ($M-H_2O-H_2O$), 612 (M-92, toluene), 564 (M-140), 472 (M-92-140) were very similar in abundance to those ions observed in the spectrum of decaprenoxanthin (5).

Identity of pigment 5 with decaprenoxanthin

Insufficient quantities of pure pigment were available for the nuclear magnetic resonance spectrum to be obtained and compared with that of decaprenoxanthin and from which a considerable amount of structural information can be deduced (5), however, as the thin layer and spectral properties of an authentic sample of decaprenoxanthin, kindly donated by Dr. R. Berry of the New Mexico State University, Las Cruces, were found to be identical with the properties of pigment 5 and as the melting point of pigment 5 is very close to that reported for decaprenoxanthin (5), there is little doubt that the two compounds are identical.

The abnormal partition coefficient is explained by the addition of two non-polar isoprene units to the dihydroxy-C_{40} carotenoids discussed by Petracek and Zechmeister (7), and the value is identical with that reported for decaprenoxanthin (5).

Pigments 3 and 4

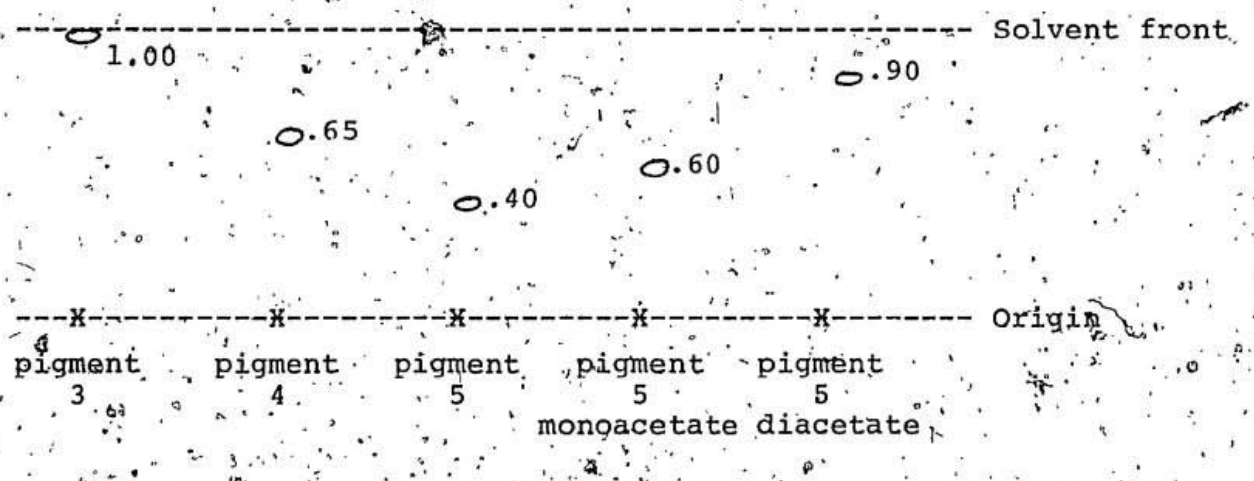
It has previously been suggested that these two pigments are esters of pigment 5. The infrared spectrum of pigment 3 had an absorption maximum at 1735 cm^{-1} (C=0, ester) and none corresponding to a hydroxyl group whereas pigment 4 had absorption maxima at 3570, 3420 cm^{-1} (OH), and at 1730 cm^{-1} (C=0).

Pigment 4 was silylated to give a single product whereas pigment 3 could not be silylated.

It was thus possible that pigment 3 is a diester and pigment 4 a monoester of decaprenoxanthin.

No attempt was made to identify the esterifying acids though, as only single spots were seen on the chromatogram, it is probable that both pigments are homogeneous or that the different esterifying acids are close members of a homologous series. A conclusion as to the size of the acids might be drawn from the Rf values of the naturally occurring esters and the synthetic acetate esters (Fig. 3).

Fig. 3. Thin layer properties of naturally occurring esters and synthetic acetates of decaprenoxanthin



The chromatogram suggested that pigment 3, being less polar than the diacetate, contains a relatively high molecular weight acid; a similar conclusion can be drawn with respect to pigment 4.

Occurrence of C_{50} Carotenoids

To date C_{50} carotenoids have only been found in non-photosynthetic bacteria, especially Gram-positive and aerobic forms. Pigmented, Gram-positive bacteria can be arranged into three natural taxonomic groups - the corynebacteria, the brevibacteria and a group cellulomonas-dehydrogenans-xanthe: each of these groups is characterized by a distinctive range of nucleotide base values (14).

The cellulomonas-dehydrogenans-xanthe group, which has a nucleotide base ratio of 70 - 76.5% guanine plus cytosine, near the upper limit for bacteria (18), contains at least thirty strains including strains of Corynebacterium poinsettiae and C. mediolanum, 14 strains of Flavobacterium dehydrogenans, other unnamed Flavobacteria, Pseudomonas xanthe and various species of Cellulomonas. All the strains of F. dehydrogenans, Cellulomonas and P. xanthe produce a compound closely resembling decaprenoxanthin in chemical properties and which has been definitely identified as decaprenoxanthin by mass spectrometry in many of the strains.

Weeks (14) has noted that C50 carotenoids have normally only been found in Gram-positive bacteria with a high

guanine plus cytosine (GC) ratio in the DNA. The only exception is the Halobacteria, with 60-69% GC, which, though gram-negative, have a cell wall that possibly has much in common with the cell walls of Gram-positive bacteria. However, as the GC content of the Corynebacterium species described here was shown to be only 45%, it seems that a high GC ratio is not related to the production of C50 carotenoids.

In addition, as far as can be determined, this is the first organism in which esters of C50 carotenoid alcohols have been reported though such derivatives of C40 carotenoids are well known.

EXPERIMENTAL

Culture of Corynebacterium species

(a) Water (250 cm^3) was added to oatflakes (12 g) in a 3 dm^3 Erlenmeyer flask. The flask was stoppered with a cotton wool plug and autoclaved at 120°* for 20 min. A suspension of bacterial cells in water (5 cm^3) drained from the oatmeal of the previous 3-day-old culture was used to inoculate the flask, which was then left standing in the light (40 watts at 90 cm) for three days, when the yellow bacterial cells covered the surface of the oatflakes.

(b) An inoculum (10 cm^3) of the previous 2-day-old culture was added to 1 dm^3 of the following medium in a 3 dm^3 Erlenmeyer flask:

bactotryptone (Difco)	4.0 g
beef extract (Bovril)	2.5 g
yeast extract (Difco)	2.5 g
KH_2PO_4	12 g
$Na_2HPO_4 \cdot 7H_2O$	24 g

The flask was shaken in the light (40 watts at 90 cm) for 2 days, after which time the cells were separated by centrifugation at 9,000 rpm for 2 min. and the liquid medium was decanted leaving a yellow mass of cells.

(c) A stock culture of Corynebacterium sp. was retained on nutrient agar plates made by adding 1.5% agar to medium b. After two days growth in the light these plates were

*All temperatures recorded in °Celsius.

transferred to the refrigerator and could be kept at least 3 months before being subcultured.

Extraction of carotenoid pigments

(a) From cells cultured in liquid medium

The yellow cells were collected and stirred briefly with methanol. Nitrogen was then bubbled through the solution to deaerate it, and the mixture left to stand, with occasional shaking, in the refrigerator for 2 days. The suspension was filtered, and the residue resuspended in methanol for an additional two days, when the two methanol extracts were combined, and evaporated to about ¼ volume under reduced pressure at 35°. An equal volume of ether was added and, after shaking, saturated brine was added until the two layers separated. The methanol layer was extracted a second time with an equal volume of ether, when it was virtually colourless, and the two ether layers were combined and evaporated to about one quarter volume at 35° under reduced pressure. After drying over anhydrous sodium sulphate, the ether solution was evaporated to dryness to leave an orange coloured oily residue.

Total carotenoid content estimated spectrophotometrically = 0.46 mg dm^{-3} culture.

(b) From culture on oatflakes

Approximately 100 cm^3 of water was added carefully to each flask which was then gently swirled. The bacterial cells were thereby detached from the surface of the oatflakes and this

suspension was decanted, and centrifuged to leave a yellow mass of cells containing a fairly low percentage of oatflakes. The extraction of the pigments was then effected in the same way as described above.

Total carotenoid content estimated spectrophotometrically = 0.25 mg/flask.

Subsequent purification

The carotenoids extracted from both types of medium were separated and purified in the same way, the only difference arising in the number of coloured bands observed on column chromatography. Those coloured bands derived from pigments in the oatmeal were discarded and will not be discussed further.

Column chromatography

The orange semi-solid residue was dissolved in petroleum ether and chromatographed on a 30 x 2.5 cm alumina (Woelm; activity 3) column, using first petroleum ether (60-80°) and then petroleum ether containing increasing amounts of acetone as the eluting solvent. The pigments obtained from the column are shown in Table 4.

Table 4. Carotenoid pigments of Corynebacterium Species eluted from an alumina (activity 3) column

	Pigment	Electronic absorption spectrum in acetone (nm)	% of carotenoid
petroleum-ether*	1	(427), 454, (480)	0.3
	2	425, 449, 471	0.7
5% acetone in pet. ether	3	421, 442, 473	7
12% acetone in pet. ether	4	417, 441, 471	12
16% acetone in pet. ether	5	421, 440, 470	80

*petroleum ether 60-80°

( ) denote shoulder

Column chromatography of pigments 3 - 5

After the initial separation of the pigments by column chromatography, the solutions of pigments 3, 4 and 5 were evaporated to dryness under reduced pressure at 35°, and each residue was dissolved in the minimum volume of petroleum ether.

Each was chromatographed on a 20 x 1.5 cm column of alumina (Woelm; activity 3), firstly using petroleum ether and then steadily increasing proportions of acetone in petroleum ether, until each pigment was slowly eluted. This required 2% acetone for pigment 3, 8% for pigment 4, and 16% for pigment 5.

Thin layer chromatography (tlc) of pigment 5

Silica gel (30 g; Merck, acc. to Stahl) was shaken with water (35 cm^3) and 95% ethanol (35 cm^3), the resulting slurry being spread on 20 x 20 cm or 5 x 20 cm glass plates using a Shandon spreader. After air drying for 15 min, the plates were heated at 110° for 1 h before being cooled and stored in a desiccator.

The solution of pigment 5, obtained from column chromatography, was evaporated to dryness at 35° under reduced pressure, and the solid was dissolved in the minimum quantity of petroleum ether.

This solution was chromatographed on the silica gel plates using 25% acetone in petroleum ether as the developing solvent. The single coloured band (Rf 0.40) was scraped from the plate, the pigment eluted by several washings with acetone, and the solution, after filtering, was evaporated to dryness.

Pigment 5

Crystallised from dry acetone-petroleum ether as orange-red needles m.p. 149-151° (uncorr). Lit: decaprenoxanthin 153-155°(5).

Absorption spectrum in visible light:

In acetone maxima at (398), 417, 441 ($E^{1\%}_{1cm}$ = 2200, ε = 154,000) and 471 nm, % peak III/II = 97 (Fig. 1). Lit: decaprenoxanthin maxima at (400), 419, 443 ($E^{1\%}_{1cm}$ = 2340, ε = 165,000) and 472 nm, % peak III/II = 101 (5).

The absorption spectrum in the visible region in other solvents is given in Table 5.

Table 5. Electronic absorption spectrum of pigment 5 in different solvents

Solvent	Absorption maxima (nm)
petroleum ether	(391), 412, 435, 466
methanol	(387), 412, 436, 466
chloroform	(400), 423, 447, 478
pyridine	(402), 423, 451, 483
carbon disulphide	(412), 436, 464, 497

Infrared spectrum:

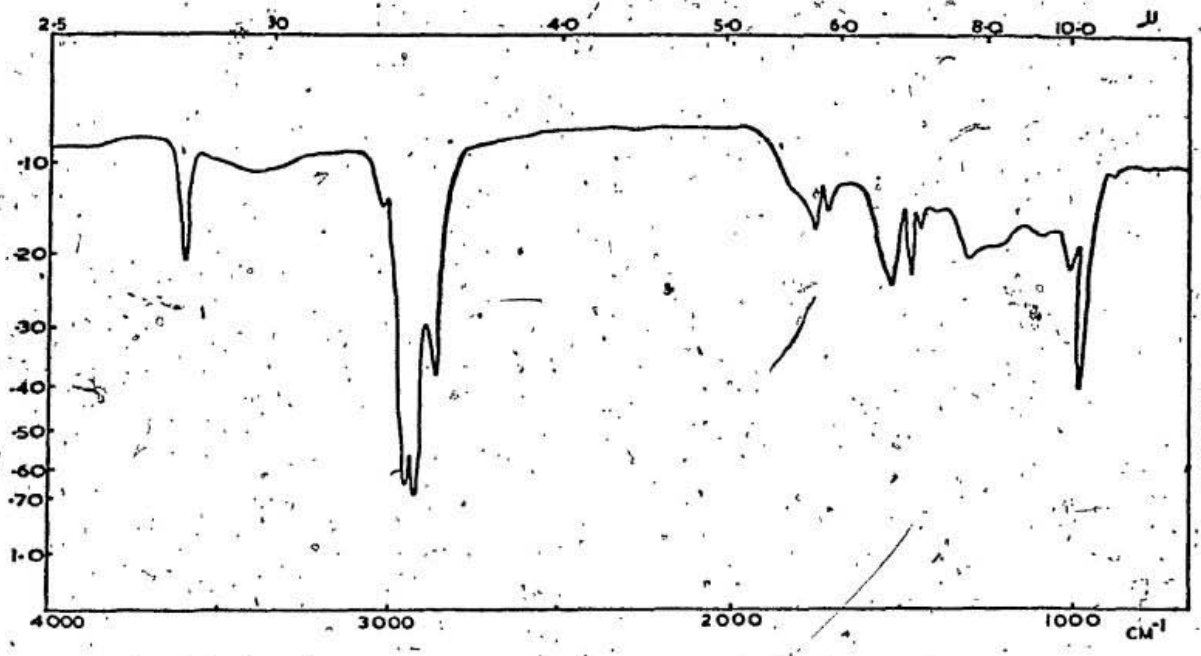
The infrared spectrum in chloroform solution is recorded in Figure 4, abs. max. 3570 (OH); 3015 (conj. CH); 2950 - 2850 (CH); 1665, 1565 (conj. double bonds); 1445 (CH); 1378, 1367 (CH_3, gem CH_3); 1170, 1050, 1003 (prim. allylic OH); 970 (trans disubstituted double bonds) (19) cm^{-1}.

The spectrum is extremely similar to that reported for decaprenoxanthin (5) which was recorded in a KBr pellet.

Mass spectrum:

A spectrum was obtained using an AEI MS-12 instrument with a direct inlet system and a probe temperature of 250°. The spectroscopist remarked on the high stability of the pigment at this temperature!

Fig. 4. Infrared spectrum of pigment 5 in chloroform solution



High mass-end ions were observed at m/e 704 (M^+), 686 ($M-H_2O$), 668 ($M-H_2O-H_2O$), 612 (M-92), 564 (M-140), 472 (M-92-140).

The spectrum was extremely similar to that reported for decaprenoxanthin (5).

Partition ratio of pigment 5:

The partition ratio between petroleum ether and 95% methanol was determined in the usual manner (7), and was 32/68. (Decaprenoxanthin 32/68 (5).)

Hydrogenation of pigment 5

Pigment 5 (0.3 mg) was dissolved in ethyl acetate (5 cm^3) and 10% palladium on charcoal (2 mg) was added. The mixture was stirred magnetically under hydrogen at room temperature and atmospheric pressure for 2 h. After filtration, the ethyl acetate solution was colourless and there was no absorption above 200 nm.

Acetylation of pigment 5

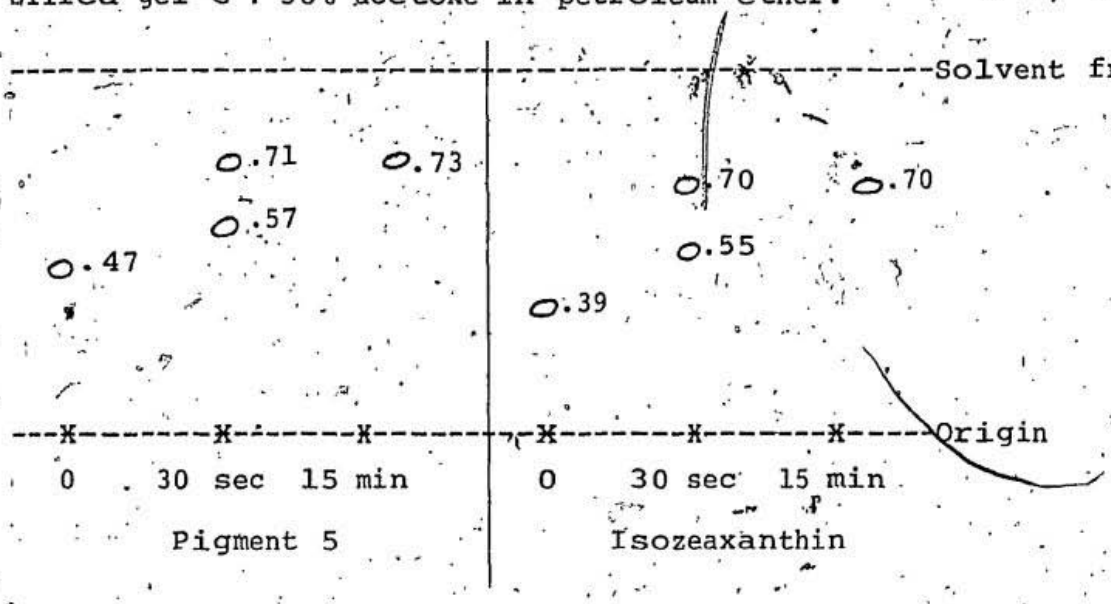
To pigment 5 (0.1 mg) in dry pyridine (1.0 cm^3) was added acetic anhydride (0.2 cm^3). The mixture was allowed to stand at room temperature, the course of acetylation being followed by thin layer chromatography (Fig. 5).

Acetylation of isozeaxanthin

Isozeaxanthin (0.1 mg) was acetylated under the same conditions as described above, the reaction also being followed by tlc (Fig. 6).

Fig. 6. Diagram to show the thin layer properties of pigment 5 and of isozeaxanthin on acetylation

Silica gel G : 30% acetone in petroleum ether.



In another experiment 5 mg of pigment 5 was acetylated, the mixture being left overnight at room temperature when reaction to the diacetate was complete.

Pigment 5 monoacetate

The electronic spectrum in acetone was identical with that of pigment 5.

Pigment 5 diacetate

The electronic spectrum in acetone was identical with that of pigment 5. The ir spectrum in chloroform showed maxima at 3010, 2950, 2915, 2850; 1735 (C=O, unsaturated ester); 1665, 1600 (C=C); 1430, 1375, 1365; 1020 (C-O of acetate); 967 (trans disubstituted C=C) cm^{-1}.

Trimethylsilylation of pigment 5 monoacetate

A spot of pigment 5 monoacetate in petroleum ether was applied to a silica gel thin layer. After the solvent had evaporated, a drop of Trisil (Pierce) was applied to the spot, and after standing for 1 min the excess solvent was evaporated off using a hair dryer.

The plate was chromatographed using 5% acetone in petroleum ether and a single new less polar product (Rf 0.85) was detected.

Attempted trimethylsilation of pigment 5 diacetate

Trimethylsilation of pigment 5 diacetate was attempted using the same method as for the monoacetate. No new coloured products were formed.

Attempted reduction of pigment 5 by sodium borohydride

To pigment 5 (0.13 mg) in 95% ethanol (2 cm^3) was added sodium borohydride (4 mg). After 2 h the solution was diluted to three times its volume with water, saturated with sodium chloride and the coloured component extracted into ether. No new products were detected by tlc and the absorption spectrum was unchanged: recovery 86%.

Attempted allylic dehydration of pigment 5

(a) Pigment 5 (0.09 mg) was treated with 0.05 M HCl in chloroform (2 ml) for 0.5 h. No less polar products were detected by tlc, nor was there any change in the absorption spectrum.

(b) When pigment 5 (0.1 mg) was left in the light dissolved in a saturated solution of HCl(g) in $CHCl_3$ for 1.5 h, no less polar compounds were detected by tlc, nor was there any change in the absorption spectrum.

(c) Pigment 5 (0.15 mg) was dissolved in dry pyridine (2 cm^3) and phosphorus oxychloride (5 mg) was added. The mixture was left overnight and then, as the coloured component could not be extracted into ether, was evaporated to dryness at 50° under reduced pressure. The yellow compound left had an Rf value of 0.00 when chromatographed on silica gel thin layers with acetone as the eluting solvent.

Treatment of the product with 10% KOH in a water-ethanol (1:1) mixture (5 cm^3) overnight at room temperature gave a product which readily extracted into ether and had spectral and thin layer properties identical with pigment 6.

Attempted allylic methylation of pigment 5

When pigment 5 (0.12 mg) was dissolved in methanol (1 cm^3) and 6 M HCl (3 drops) was added no new products were formed after standing for 0.5 h.

Attempted manganese dioxide oxidation of pigment 5

Pigment 5 (0.1 mg) was dissolved in acetone (2 cm^2) and activated MnO_2 (5 mg) was added. The suspension was stirred at room temperature for 2 h, after which time no new products could be detected.

Nickel peroxide oxidation of pigment 5

Nickel peroxide was prepared by the method of Nakagawa et al. (11) and was shown titrimetrically to have an available oxygen content of 3.4×10^{-2} g atom/g nickel peroxide.

Pigment 1 (1.9 mg) was dissolved in a solution of benzene (5 cm^3) and ether (5 cm^3) and nickel peroxide (20 mg) was added. The solution was stirred magnetically at room temperature for 2.5 h then filtered and evaporated to dryness at 35°. Tlc of the residue showed it to contain unchanged pigment 5 together with two less polar products: the mono- (40%) and the dialdehyde (20%) derivatives. Rf values on silica gel/5% acetone in petroleum ether: pigment 5 0.06, mono-aldehyde 0.23, dialdehyde 0.56.

Pigment 5 monoaldehyde

The electronic absorption spectrum in the visible was identical with that of pigment 5.

Reduction of pigment 5 monoaldehyde

Pigment 5 monoaldehyde (ca. 50 µg) was dissolved in ethanol (1 cm^3) and treated with sodium borohydride (2 mg) for 10 min. The product had spectral and thin layer properties identical with pigment 5.

Trimethylsilylation of pigment 5 monoaldehyde

The compound was treated with Trisil on a silica gel thin layer, as described previously, to yield one trimethylsilyl ether (Rf = 0.83 on silica gel/5% acetone in petroleum ether).

Pigment 5 dialdehyde

The electronic absorption spectrum in the visible was identical with that of pigment 5.

Reduction of pigment 5 dialdehyde

The compound (ca. 50 μg) was dissolved in ethanol (1 cm^3) and treated with sodium borohydride (2 mg) for 10 min. The product had the same absorption spectrum and thin layer properties as pigment 5.

Attempted trimethylsilylation of pigment 5 dialdehyde

No trimethylsilyl ethers were formed when the compound was treated with Trisil on a thin layer.

Pigment 3

The electronic absorption spectrum was identical with that of pigment 5. The infrared spectrum included maxima at 3010 (unsat. CH); 1735 (C=O, ester); 1645, 1605 (C=C); and 967 (trans disubstituted C=C) cm^{-1}.

Hydrolysis of pigment 3

Pigment 3 (0.1 mg) was treated with a 10% solution of potassium hydroxide in water-ethanol 1:1 (10 cm^3). After being degassed by nitrogen the solution was left overnight in the dark at room temperature. The yellow product, which was extracted into ether, had the same spectral and thin layer properties as pigment 5 (Fig. 7).

Attempted trimethylsilylation of pigment 3

No new product was formed when pigment 3 was treated with Trisil on a silica gel thin layer.

Pigment 4

Pigment 4 had the same electronic spectrum as pigment 5.

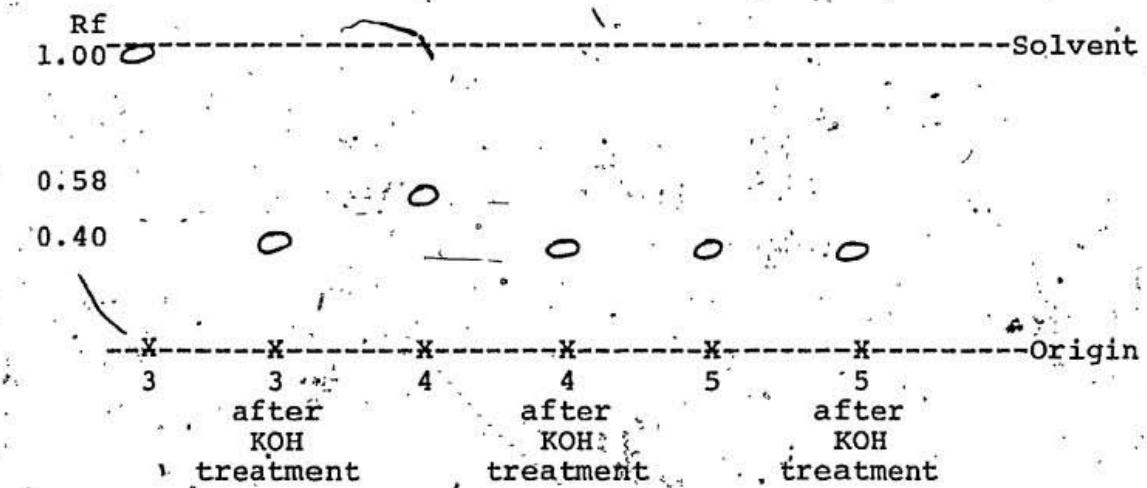
The infrared spectrum in chloroform solution included absorption maxima at 3570, 3420 (OH); 3010 (unsat. CH); 1730 (C=O, ester); 1600, 1595 (C=C); and 964 (trans disub. C=C) cm^{-1}.

Hydrolysis of pigment 4

When treated with potassium hydroxide solution (see above), a yellow compound with spectral and thin layer properties like pigment 5 was given (Fig. 7).

Fig. 7. Thin layer behaviour of pigments 3, 4 and 5 before and after treatment with alcoholic KOH

Thin layer system: silica gel G/22% acetone in petroleum ether (60-80°).



Trimethylsilylation of pigment 4

Pigment 4 was treated with Trisil on a silica gel thin layer and a single trimethylsilyl ether was formed.

Extraction of DNA from E. coli and Corynebacterium species

Cells of E. coli (3 g) and Corynebacterium sp. (3 g), which had been obtained by centrifugation from the culture medium, were extracted by the method used by Marmur (21) in order to obtain the DNA.

Estimation of the melting point of DNA from E. coli and Corynebacterium species

(i) The method of Marmur and Doty (18) was used to determine the melting point (Tm) of the DNA samples. An SP 800 in which the samples could be heated by circulating water from a thermostatically controlled bath was utilised.

The DNA was dissolved in 0.15 M sodium chloride plus 0.015 M sodium citrate and adjusted to approximately pH 7 to give a solution with an absorbance of approximately 1.0 at 260 nm. The absorbance was determined at 25°, at 70° and then at 5° intervals until the intensity of the absorption at 260 nm began to increase. With a second sample an attempt to determine Tm more accurately was made by increasing the temperature in 1°-2° intervals over the melting range and at each temperature allowing ten minutes for temperature equilibration before running the spectrum: a correction was made for a slight temperature drop observed between the heating bath and the cuvette.

At this salt concentration Tm of both samples was too high to make accurate measurements.

(ii) The concentration of salts was lowered to 0.015 M sodium chloride and 0.0015 M sodium citrate, and the Tm determined in the same way as described above. At this concentration the Tm was 72° for DNA from E. coli and 69.5° for the Corynebacterium DNA (Figs. 8a and 8b).

The Tm for the E. coli DNA is what would be expected from the data of Marmur and Doty in this salt concentration and, making the assumption that the E. coli DNA guanine plus cytosine (GC) conteht is 50% as recorded by Marmur and Doty, use of their graphs permits a value of approximately 45% to be assigned to the GC content of the Corynebacterium DNA.

Fig. 8. Change in absorbance at 260 nm with temperature for DNA extracted from E. coli (Strain B, wild type) (Fig. 8a) and Corynebacterium sp. (Fig. 8b)

Fig. 8a

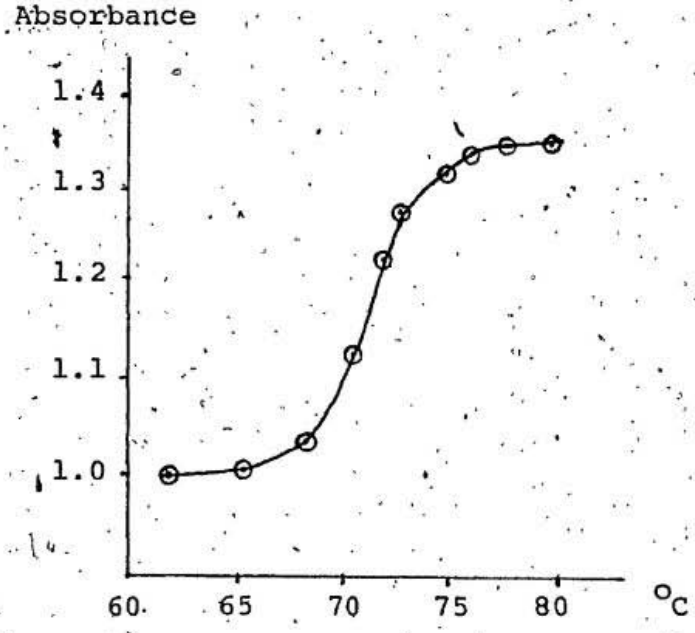
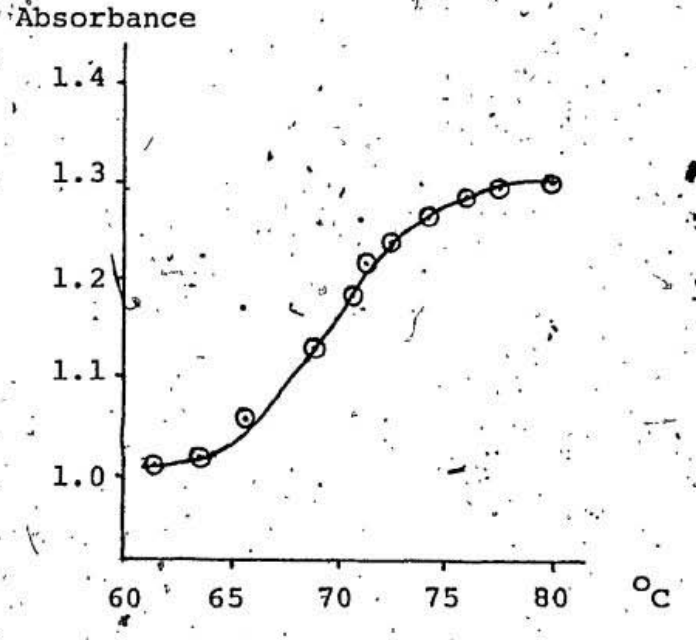


Fig. 8b



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