A STUDY OF THE "INDICATOR" PIGMENT ISOLATED FROM THE SLIME MOULD PHYSARUM FLAVICOMUM

JUDITH MARGARET HILLIER


CENTRE FOR NFLD. STUDIES
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by

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ABSTRACT

The slime mould Physarum flavicomum exhibits synchronous nuclear divisions and has been widely used to study the biochemical changes which take place on differentiation. One example of this, sporulation, has been shown to require light and the plasmodial pigments have been implicated as possible photocatalysts or photoreceptors. In order to fully understand this process it is necessary to know the chemical structure of the pigments. One pigment shows changes in absorption maxima with variations in the pH of the solution and has been called the "indicator" pigment. It is with this pigment that the present investigation is concerned.

The pigment was isolated and purified using a modification of the method employed by Khalil(23). Elemental analyses gave empirical formulae of $C_{12.1}H_{13.1}O_5N$ for the protonated pigment and $C_{11.2}H_{16.5}O_{7.2}N\ Na$ and $C_{12.3}H_{13.9}O_{7.7}N\ Na_{2.5}$ for the sodium salt of the pigment. The n.m.r. spectrum agrees with a high degree of unsaturation in the pigment molecule.

The presence of the tetraene, identified by Khalil, was confirmed by oxidation experiments. Hydrolysis indicated the presence of a carbohydrate, possibly sorbose, and basic compounds which were isolated, by several methods, as their hydrochlorides, but were not identified.

Pigment isolated from frozen plasmodia had a molecular mass of approximately 1880, determined by oxidation techniques, and a low $E^{1\%}_{1cm}$ value of 200. This pigment yielded five amino

acids on hydrolysis, two of which were tentatively identified as glycine and alanine. There was also evidence, on hydrolysis, of an amino sugar which was possibly glucosamine. It is suggested that this pigment contains a peptide.

Khalil proposed a tetraene conjugated to a 2,4-pyranone system to be the chromophore of the pigment. In order to investigate this possibility, compounds were synthesised by reacting triacetic acid lactone at the 3 and 6α positions. One of the products, 4-hydroxy-6-(4-(2-furyl)buta-2,4-dienyl)-2H-pyran-2-one, had the same absorption maximum as the pigment but the hyperchromic, bathochromic shifts it displayed on acidification were not as great as those shown by the pigment. However, it does represent the best model system for the pigment available at the present time.

ACKNOWLEDGEMENTS

I would like to thank Dr. E. Bullock for the help and advice he has given during my course of study. I acknowledge the receipt of a fellowship from Memorial University of Newfoundland which made the investigation possible. Also, I am grateful to my husband for the encouragement he has given to me during this work.

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PART I

INTRODUCTION

The Myxomycetes or plasmodial slime moulds are fungus like organisms which have a unique life cycle (1). The assimilative phase is a free-living, acellular, multinucleate plasmodium which exibits naturally synchronous nuclear divisions. Thus, these systems are ideal for the study of biochemical changes which take place during mitosis. In the reproductive stage the organism forms fruiting bodies, which in turn produce spores. Sporulation thus takes place, and this simple process has been very widely studied in order to obtain a more comprehensive picture of the nature of differentiation.

Sporulation can be initiated by starvation followed by exposure to light, although factors such as temperature, pH and the age of the plasmodium have been found to affect the degree of spore formation. The necessity of light for induction of sporulation in pigmented plasmodia was demonstrated by Gray in 1938 (2). Non-pigmented plasmodia do not appear to have this light requirement (3). The influence of the wavelength of the radiation on the efficiency of sporulation was studied by Gray in 1953 (4) and by Daniel and Rusch in 1962 (5), the most effective region for Physarum polycephalum being 350nm to 500nm.

During sporulation the plasmodial pigments gradually disappear, and this disappearance is accompanied by an increase in the colouration of the spores. The colour of these

spore pigments can be yellow, orange, brown or black. The plasmodial pigments have been implicated as possible photoreceptors or photocatalysts (6), although Gray and Alexopoulos (7) suggest that their function is the screening of the organism from harmful radiation. Daniel (8) suggested that the mitochondria are the primary light receptors, but did not give evidence to support this view.

The slime moulds which have been most extensively studied are those which can be grown in cultures in the laboratory. Daniel and Rusch in 1961 (9) grew axenic cultures of P. polycephalum on a semi-defined medium and the medium was fully defined by Daniel et al. in 1963 (10). The culture of Physarum flavicomum was achieved by Gray in 1961 (11) and by Ross and Sunshine in 1965 (12). The large scale culture of P. polycephalum was investigated by Brewer et al. in 1964 (13). Because of its comparative ease of growth, P. polycephalum has been more extensively studied than other members of the Physarum genus.

Interest in the yellow pigments of P. polycephalum dates back to 1889 when Zopf (14) extracted the pigments and classified them as lipochromes, since they were fat soluble.

Siefriz and Zetzmann in 1935 (15) investigated the colour changes of the plasmodia of P. polycephalum associated with changes in acidity of the medium and noticed the "indicator" properties of the pigments. In 1953, Gray (4) measured the absorption spectrum of a pigment extract over the pH range 2.9 to 8.4.

An alumina column has been used by Wolf (16) to isolate two pigments from a methanol extract of the* plasmodium. One of these pigments had absorption maxima at 245 and 380nm in neutral methanol, 260-80 and 420nm in acidic methanol and 265 and 380nm in basic methanol. The infrared spectrum had absorptions at 3400, 1600, 1440, 1120 and 800cm^{-1}. These pigments were identified as pteridines but Wolf did not support this identification with chemical evidence. Dresden (17) disagreed with this conclusion and suggested that the pigments were peptide-like. He isolated the* pigment which had the indicator properties, using paper chromatography, from a mixture containing three other pigments. It was found to behave as an organic acid with a $pK_a = 4.7$. The visible absorption spectrum had a maximum, in methanol, at 390nm which shifted to 410nm on acidification. Elemental analysis gave values, for the purest samples, of C, 55.59% H, 7.18% N, 3.12% and Fe, 1.10%. Sulphur and phosphorus were not detected. Acid hydrolysis gave six amino acids and, on this evidence, Dresden suggested that the pigment was a peptide. More recently Kuraishi (18) found that the three main* pigments were interconvertible and were neither pteridines nor peptides, although he did not suggest alternative chemical structures.

Brewer, in 1965 (19), obtained three* purified pigments, two of which showed changes in their absorption maxima on acidification. These two pigments were designated B and C. Pigment B, a nitrogen containing polyene, was insoluble in

*Pigments isolated from P. polycephalum.

water but soluble in aqueous alkali. It was suggested to have a zwitterion structure, but this was not substantiated. The infrared absorption spectrum of B showed the presence of olefinic and amine or hydroxyl groups but did not indicate carbonyl or aromatic functions. The absorption maxima of the compound were at 355, 384 and 390nm in basic, neutral and acid media respectively. The shift on acidification was hyperchromic. Pigment C (absorption maximum 385nm in basic solution, 403nm in neutral solution and 418nm in acidic solution with an increase in intensity) was considered to be a breakdown product of pigment B obtained during the isolation procedure. Although elemental analyses were obtained for these two compounds, Brewer was unable to determine the molecular weights. These compounds did not fall into any recognisable group of naturally occurring polyenes.

In 1966, Daniel (8) isolated several pigments, one of which had a pH dependent absorption maximum and extinction coefficient (380-90nm in basic solution and 415nm in acidic media). From chemical reactions leading to the loss of absorption, he suggested that a carbonyl group was an essential part of the chromophore. Pigments of the Schiff's base type have the same spectral changes as this plasmodial pigment and so Daniel considered that the pigment could contain an azomethine function. He noted, however, that solubility factors did not substantiate this proposal.

Nair and Zabka (20) separated six pigments from P. poly-

cephalum by paper chromatography and reported the presence of a phenolic component.

Continuing Brewer's work (19), Watson, in 1970 (21), was able to separate "pure" pigment A into three fractions. From ultraviolet absorption data, he concluded that they were trans-polyenes containing carbonyl and amine functions. Even after acetylation, he was unable to determine the molecular mass of the compound by mass spectroscopy and the only information obtained from a nuclear magnetic resonance spectrum was the presence of a gem-dimethyl group in one of the fractions, A-1. Further conclusions concerning the chemical nature of the pigment were not made because mutation of the mould made it impossible to repeat the isolation of the pigment.

LeStourgeon, in 1970 (22), isolated two pigments from P. flavicomum by methanol extraction followed by chromatography on an alumina column. The absorption maximum of the pigment was at 420nm, however, if younger plasmodia were used, only one pigment could be isolated and this had an absorption maximum at 420nm with shoulders at 430, 455 and 480nm. This suggests that chemical modifications may take place during growth and that pigment extraction must be performed on plasmodia of a specific age if consistent results are to be obtained.

Khalil reported in 1975 (23) the extraction and purification of pigments from both P. polycephalum and P. flavicomum. Previous workers had extracted the plasmodia with aqueous acetone, and, after removal of the acetone, precipitated the

pigment by acidification. After washing with water, the pigment was dissolved and purified by chromatography on various adsorbants. Preliminary isolation work by Baker and Bullock (24) in this laboratory using the above method had given inconsistent results, often with appreciable decomposition on the chromatographic column employed. A milder isolation technique was required. Khalil investigated several procedures, and found the most satisfactory (for P. flavicomum) to be aqueous extraction followed by lyophilisation. After dissolving the solid in aqueous methanol and removing insoluble material by filtration, preliminary purification was achieved by chromatography on a column of Sephadex LH 20. Four pigment fractions were separated and pigment from fraction 2 (absorption maximum 380nm in neutral solution, 418nm in acidic solution) was further separated into two components using chromatography on a column of Sephadex G 10. One of the components, pigment A-2, showed the pH dependent absorption maximum and extinction coefficient. Similar procedures were used for the isolation of pigments from P. polyecphalum.

Khalil concentrated on the pigments which showed the indicator properties. Elemental analyses of the P. flavicomum pigment gave high ash residues and this suggested that the pigment, in the native state, is a salt. The presence of nitrogen in the molecule was not confirmed. On protonation, this compound became much less soluble in water and precipitated. After investigating the possible presence of a Schiff's base,

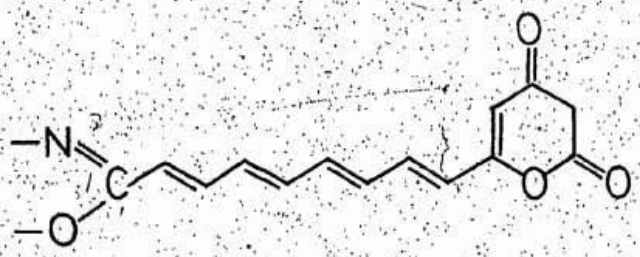
Khalil rejected this suggestion for three reasons. Firstly, electrophoresis of the pigment on cellulose acetate in acidic media did not produce the migration towards the cathode expected of Schiff's base compounds. Secondly, the pK_a of the pigment from P.flavicomum was determined as 3.4, whereas the pK_a of Schiff's base compounds are usually in the range 7-9. Finally, the resonance Raman spectrum of the pigment, whilst indicating the presence of a C=N group, showed that this group was non-basic and a function similar to that of an oxazole was suggested.

As an alternative to the Schiff's base, a 2,4-pyranone system, conjugated with a polyene was proposed as the function exhibiting the bathochromic, hyperchromic shift on acidification. Oxidation experiments confirmed the presence of a tetraene, by the isolation and identification of 2,4,6,8-decatetraene-1,10-dioic acid. Catalytic hydrogenation of the pigment gave a compound having a chromophore similar to that of 4-hydroxy-6-methyl-2H-pyran-2-one (triacetic acid lactone). The infrared spectrum of the pigment was consistent with these results showing the presence of carbonyl and olefinic functions. Sufficient pigment was not available to record a proton magnetic resonance spectrum. A high resolution mass spectrum, while not giving a molecular ion, gave several ions consistent with a polyene function to which carbonyl and nitrogen containing functions are attached.

Although the presence of a pyrone moiety was not proved in this work, several pyrone containing compounds have indeed

been isolated from fungi. The structures and sources of some of these are given in Table 1. Polyene systems have also been isolated from fungi, in the form of carotenoids and macrocyclic antibiotics. The macrocyclic antibiotics (some examples of which are given in Table 2) often contain lactone functions and are highly oxygenated. This type of system cannot be ruled out on the basis of present evidence.

This thesis is concerned with the indicator pigment isolated from P. flavicomum. The partial structure (I) suggested by



(I)

Khalil is further investigated.

TABLE 1

NATURALLY OCCURING PYRONE SYSTEMS

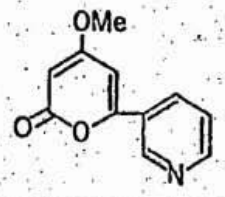
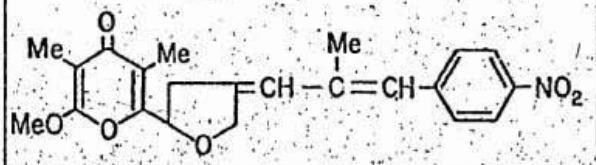
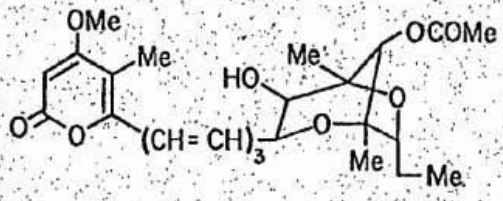
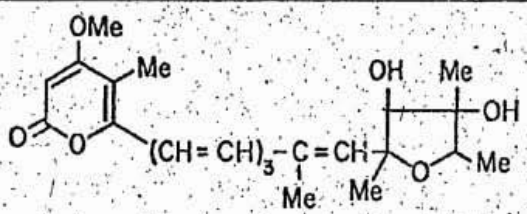
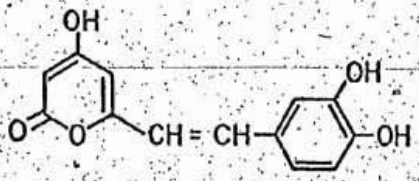
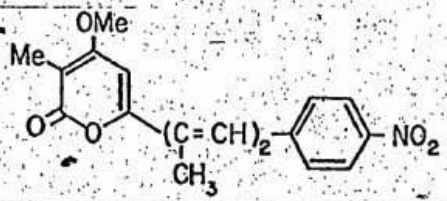
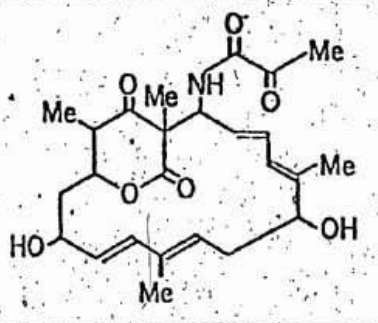
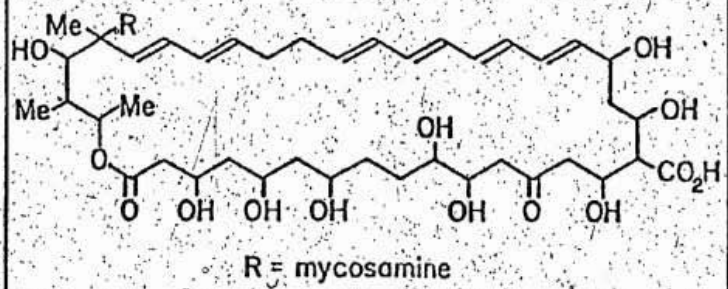
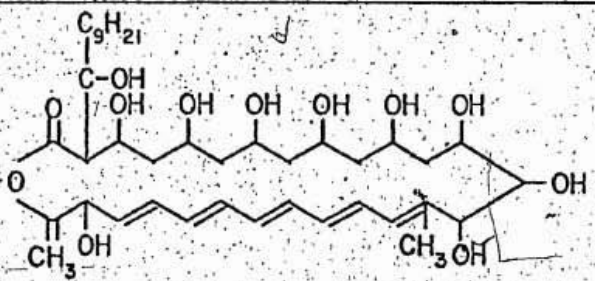
NAME	STRUCTURE	SOURCE	REF
Anibine (II)		Aniba fragrans	25
Aureothin (III)		Streptomyces thioluteus	26
Aurovertin B (IV)		Calcarisporium arbuscula	27
Citreoviridin (V)		Penicillium species	28
Hispidin (VI)		Polyporus hispidus P.Schweinitzii	29 30
Luteoreticulin (VII)		Streptomyces luteoreticuli	31

TABLE 2

POLYENE MACROLIDE ANTIBIOTICS

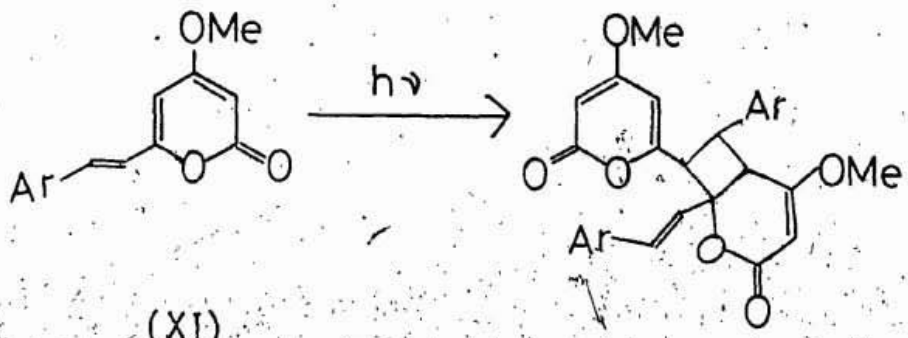
NAME	STRUCTURE	SOURCE	REF
Lankacidin (Bundlin A) (VIII)		Streptomyces species	32
Nystatin (IX)	 R = mycosamine	Streptomyces noursei	33
Fungichromin (X)		Streptomyces cellulesae	34

RESULTS AND DISCUSSION

i) Isolation and preparation of the pigment

The methods of pigment isolation and purification used by previous workers have varied widely. The technique used by Khalil (23) successfully separated and purified all the pigments present in the slime mould P. flavicomum. However, in this work, only one pigment, the indicator pigment, was studied. This pigment is the only one present which is readily soluble in water and so extraction of the plasmodia with water gives a solution containing most of the indicator pigment, some protein and inorganic salts. Precipitation of most of the protein, followed by chromatography on Sephadex columns using aqueous alcoholic solvents gives pure pigment. This technique is even milder than that employed by Khalil and degradation is thus avoided. Although the solutions were not examined for colourless impurities, it is probable that any other compounds would be removed by passage through the three chromatographic columns employed here.

It has been shown by Bartle et al. (35) that methoxyhispidin (XI) undergoes photodimerisation to form (XII) and so care was taken to exclude light at all stages of isolation, purification and storage of the pigment. It was stated that 4-hydroxy-pyran-2-ones form dimers less readily than 4-methoxy-pyran-2-ones, but any possible decomposition is to be avoided.



(XI) (XII)

Ar = 3,4-dihydroxyphenyl-

The indicator pigment isolated here shows the same spectroscopic properties as pigment A-2 isolated by Khalil (23), λ_{max} (80% aqueous methanol) 380nm showing a bathochromic shift to 415nm on acidification. The shift is accompanied by an approximately 40% increase in intensity. In acidic solutions slight shoulders are visible at 400 and 425nm. The pigment also exhibits an absorption maximum at 260nm, which is more intense in water than in alcoholic solvents.

The spectrum of the pigment isolated from frozen plasmodia exhibits the same properties in the visible region of the spectrum, but the maximum at 260nm is more intense. This pigment has an $E^{1\%}_{1cm}$ * of 300 compared to the values determined by previous workers of 2080 (23) for P.flavicomum, 1500 (17) and 1010(19) for P.polycephalum. Since the chromophore appears to be unchanged and further chromatography suggested

* $E^{1\%}_{1cm}$ values were measured at 380nm.

that the pigment was indeed pure, it is suggested that, in this case, the pigment has a higher molecular weight than previous samples. A higher molecular weight is also supported by the faster rate of passage of this pigment through the Sephadex chromatographic columns. If this molecule is larger, either some degradation has been avoided during isolation or, on storing, another moiety has become attached to the pigment molecule.

ii) Preparation of the protonated form of the pigment

Previous analyses of the pigment, obtained by mild isolation techniques, had been performed on the native pigment which, because of the high ash content remaining after combustion, was thought to be a salt (23). Although flame tests indicate that sodium is the main cation, the presence of other cations, such as potassium and calcium, cannot be excluded. In order to obtain an estimate of the molecular weight, it is essential to know the elemental composition of the compound.

It had been found by Brewer (19) that acidification of aqueous solutions precipitated the pigment, which elemental analysis showed contained C,H,O and N only. While it has been shown here that hydrochloric acid does not degrade the chromophore at room temperature, it is possible that another part of the molecule could be hydrolysed. Ion exchange methods, which would protonate the pigment without direct addition of acid, were investigated.

Sephadex LH20 gives sharper pigment bands than Sephadex G 10

chromatographic columns and it was anticipated that protonation using this would enable the protonated pigment to be easily separated from any unprotonated pigment. However the use of a Sephadex LH20 column was the least successful of the methods tried. Since the dextran of this form of Sephadex is methylated, it does not retain a sufficient number of protons, even when the column is washed with only a small volume of solvent prior to pigment application. The use of Sephadex G 10, which has free hydroxyl groups, was more successful. Protonated pigment was formed, could be separated from non-protonated pigment and isolated. However, only a small volume of aqueous ethanol could be used to wash the column prior to application of the pigment and this could lead to contamination of the solutions with chloride ions. Since these ions would be removed on washing the solid pigment with water, this did not present a serious drawback, but the presence of chloride ions was undesirable. A disadvantage of the use of Sephadex columns is the small quantity of material which can be applied to a column at any one time.

The cation exchange resin, Dowex (50W-8X), was found to be the most satisfactory material for the formation of the protonated pigment since larger quantities of pigment could be used and a higher degree of protonation was achieved. However, it was necessary to use very pure pigment since any contaminating pigments could not be separated on the column. Column chromatography on Sephadex LH20 after protonation indicated that

no decomposition had occurred on the ion exchange column.

It was not possible to determine the exact degree of protonation by directly examining fractions collected from the columns since in dilute aqueous solutions ($< 1 \times 10^{-4}$M) the pigment, which has a $pK_a = 3.4$ (23), is more than 90% dissociated. Figure 1 shows the absorption spectra of the protonated pigment in various solvents. Those solutions which showed a significant degree of protonation when their absorption spectra were determined in 95% aqueous ethanol were combined. Methanol was used as the spectroscopic solvent to check that the solid pigment, formed on concentration, was completely protonated.

The two analyses, obtained from different samples, were consistent with each other and give empirical formulae $C_{12}H_{13}O_{4.8}N$ and $C_{12.1}H_{13.1}O_5N$. No other analysis of the protonated pigment of P. flavicomum has been determined, but the values obtained here are similar to those of Brewer for pigment B of P. polycephalum, C 64.97%, H 6.03%, O 25.04%, N 3.77% (19). The second of the analyses obtained here accounts for 99.73% of the total weight and so excludes the presence of other atoms such as phosphorus, sulphur, iron (17) and chloride (19). The empirical mass of the second analysis is 252.9. If the molecule contains two or three nitrogen atoms the molecular mass would be 505.8 or 758.7 respectively. Since the high resolution mass spectrum gave peaks up to m/e 409 (23), the molecular formula must be at least twice the empirical formula.

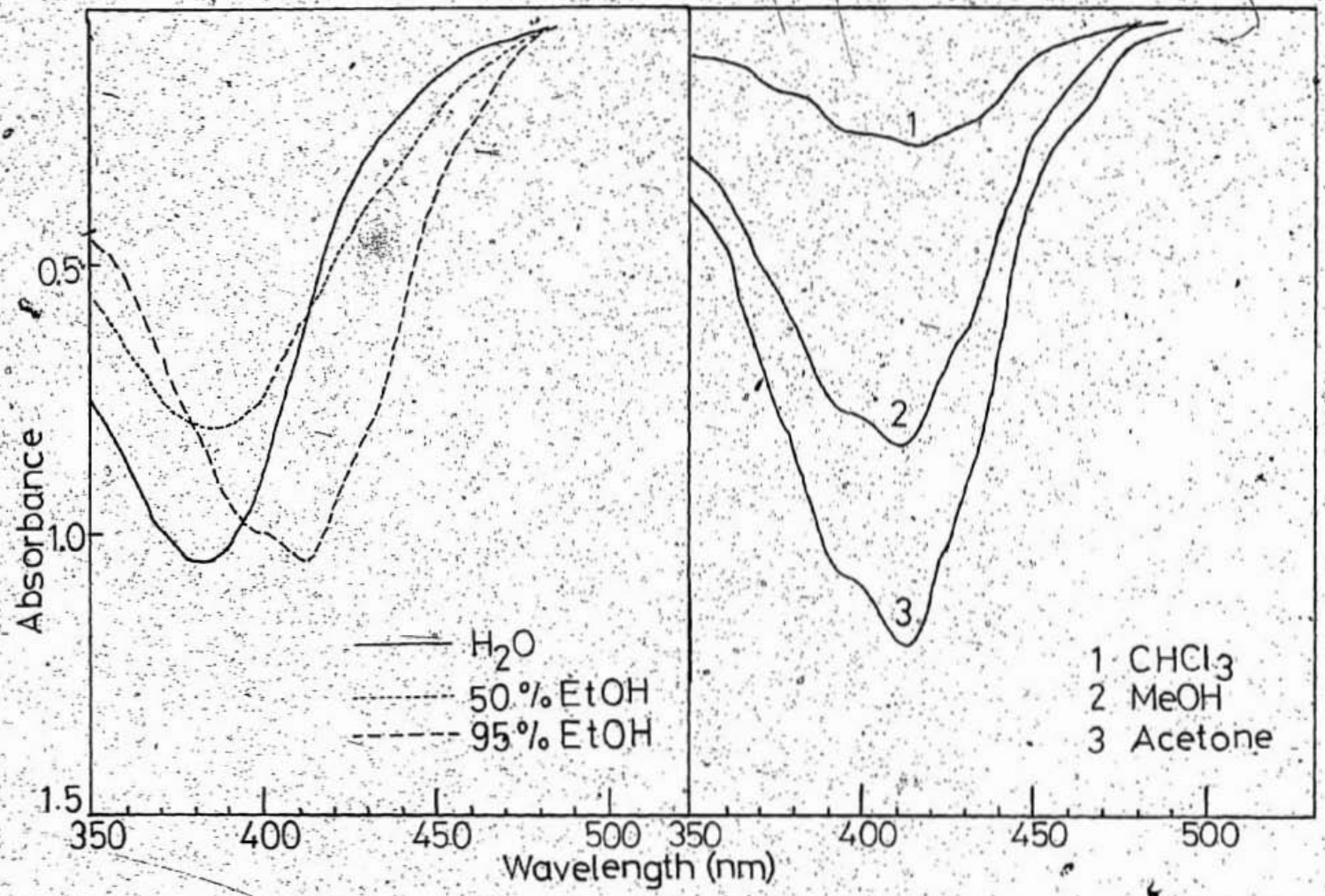


Figure 1. Absorption spectrum of protonated pigment in six solvents.

iii) Preparation of the sodium salt of the pigment

It is desirable, when determining the structure of a compound, to know the molecular formula. The pigment, as isolated, is a salt and elemental analysis of this, by determining the ratio of the other elements to the cation, could help define the molecular mass. Since the major ion present is sodium, the pigment was completely converted to the sodium salt of the pigment. Formation of the protonated form removed all metal cations and was followed by ion exchange chromatography to replace the protons by sodium ions.

The Dowex cation exchange column, in the sodium form, readily replaced most of the hydrogen ions. In order that dissociation of the protonated pigment should not be confused with formation of the sodium salt, spectra to check the degree of reaction were determined in methanol. The salt formed was purified by column chromatography before analysis was performed, since some decomposition could have taken place. It was found, however, that there was very little deterioration in the quality of the pigment. Elemental analysis of this sample gave an empirical formula of $C_{11.2}H_{16.5}N\,Na$. If oxygen is the only other element present, the empirical formula would be $C_{11.2}H_{16.5}O_{7.2}N\,Na$. The presence of the sodium does not make it possible to determine if any impurity is present which would lead to ash formation.

It was noted, however, that while this sample had λ_{max} (CH_3OH) 385nm shifting to 414nm on acidification, addition of

sodium hydroxide, although changing the maximum back to 385nm, did not exactly reproduce the original curve (Fig. 2). The Dowex ion exchange resin is a strong acid and it was felt that, since the pigment is also a fairly strong acid, exchange was not complete. Use of a weaker cation exchange resin, CM-Sephadex C 50, which did give complete exchange when dilute pigment solutions were used, upheld this view. Elemental analysis of a pigment sodium salt prepared from this column gave an empirical formula $C_{12.3}H_{13.9}O_{7.7}N$, which, if the remainder is sodium, becomes $C_{12.3}H_{13.9}O_{7.7}N\,Na_{2.5}$. The relatively high hydrogen and oxygen content of the sodium salt, compared to the protonated form, could be due to water of crystallisation.

It was shown by Khalil (23) that determination of the pK_a of the pigment by a spectroscopic method at 380 and 416nm, gave values of 3.4 and 2 respectively. Since all of the curves did not pass through an isosbestic point, the latter value was considered to be either lower than the true value for the pigment or representative of a second, more acidic functional group present in the molecule. That two acidic groups exist in the molecule is supported by the above analyses. One sodium ion, intimately connected with the part of the chromophore giving the bathochromic shift on acidification, is readily replaced by a proton, whereas another, which produces only small spectral changes, is less readily replaced. 2-Acetyl-4-hydroxy-6-methyl-2H-pyran-2-one (dehydroacetic acid) and

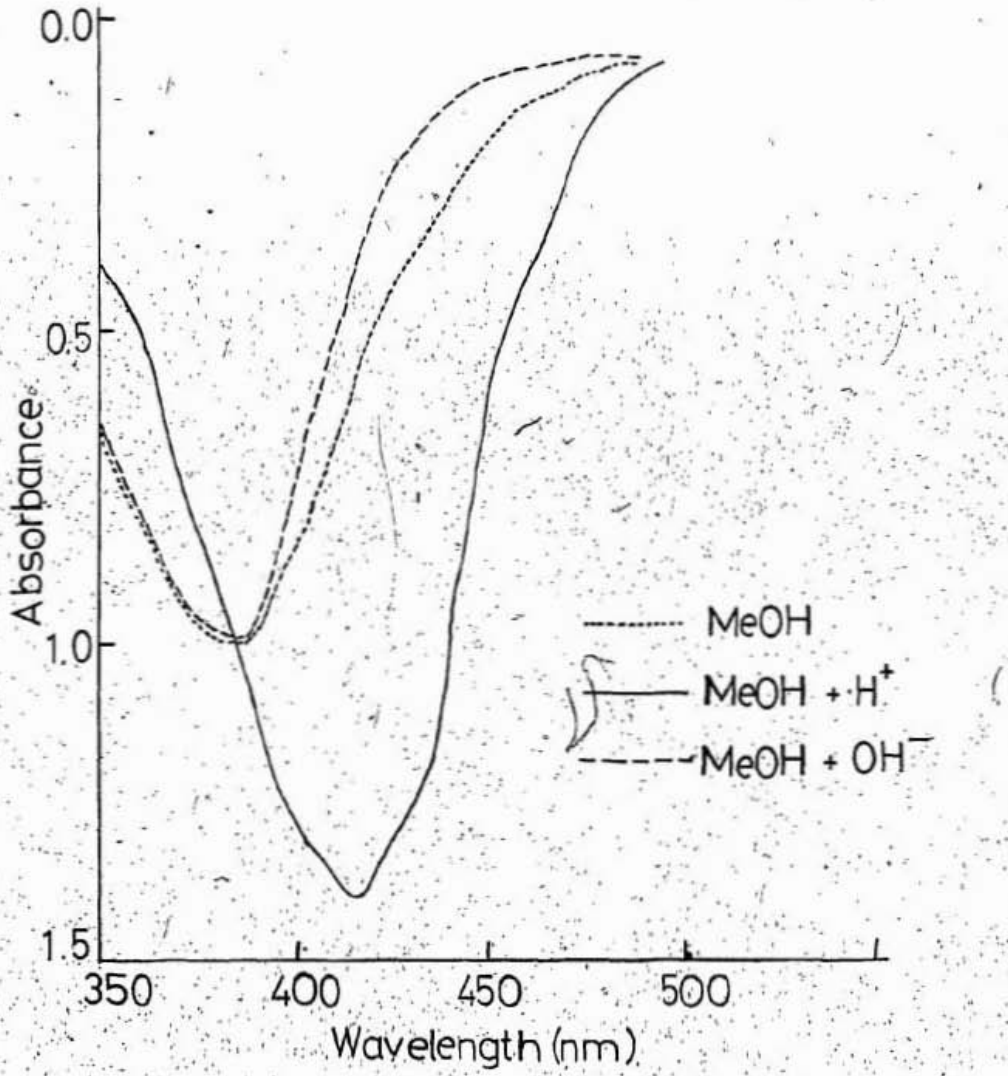
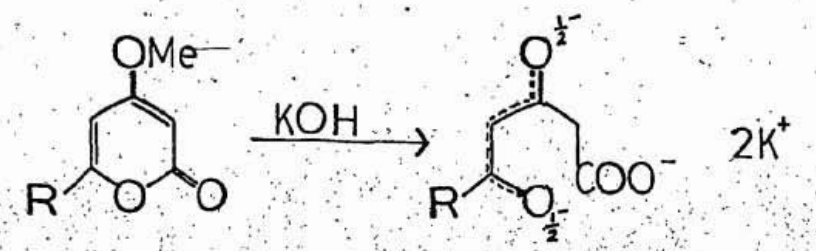


Figure 2. Absorption spectrum of the sodium salt of the pigment from the Dowex cation exchange column.

4-hydroxy-6-methyl-2H-pyran-2-one (triacetic acid lactone) readily form monosodium salts. Studies of the alkaline hydrolysis of 4-methoxy-pyran-2-ones (36) have suggested that potassium salts initially isolated in these reactions can be represented by :-



It is unlikely, however, that this ion, which necessitates the opening of the lactone ring, would be formed under the mild conditions employed here to form the pigment salt.

Investigation of the kinetics of protonation should give an indication of the nature of the reaction taking place. Following the intensity change at 408nm with time should lead to this information. A stopped-flow apparatus, used for measuring rate constants up to approximately $k = 10^5 mole^{-1} dm^3 sec^{-1}$, was unable to follow the increase in intensity produced on mixing pigment and acid solutions, because the reaction was too rapid. It is known that protonation of oxygen is usually very rapid and may sometimes reach diffusion control. This confirms the presence of a very acidic group in the molecule. However, a much slower reaction was visible. This showed a decrease in intensity over a period of time of approximately 20 seconds, and pseudo first order kinetics are

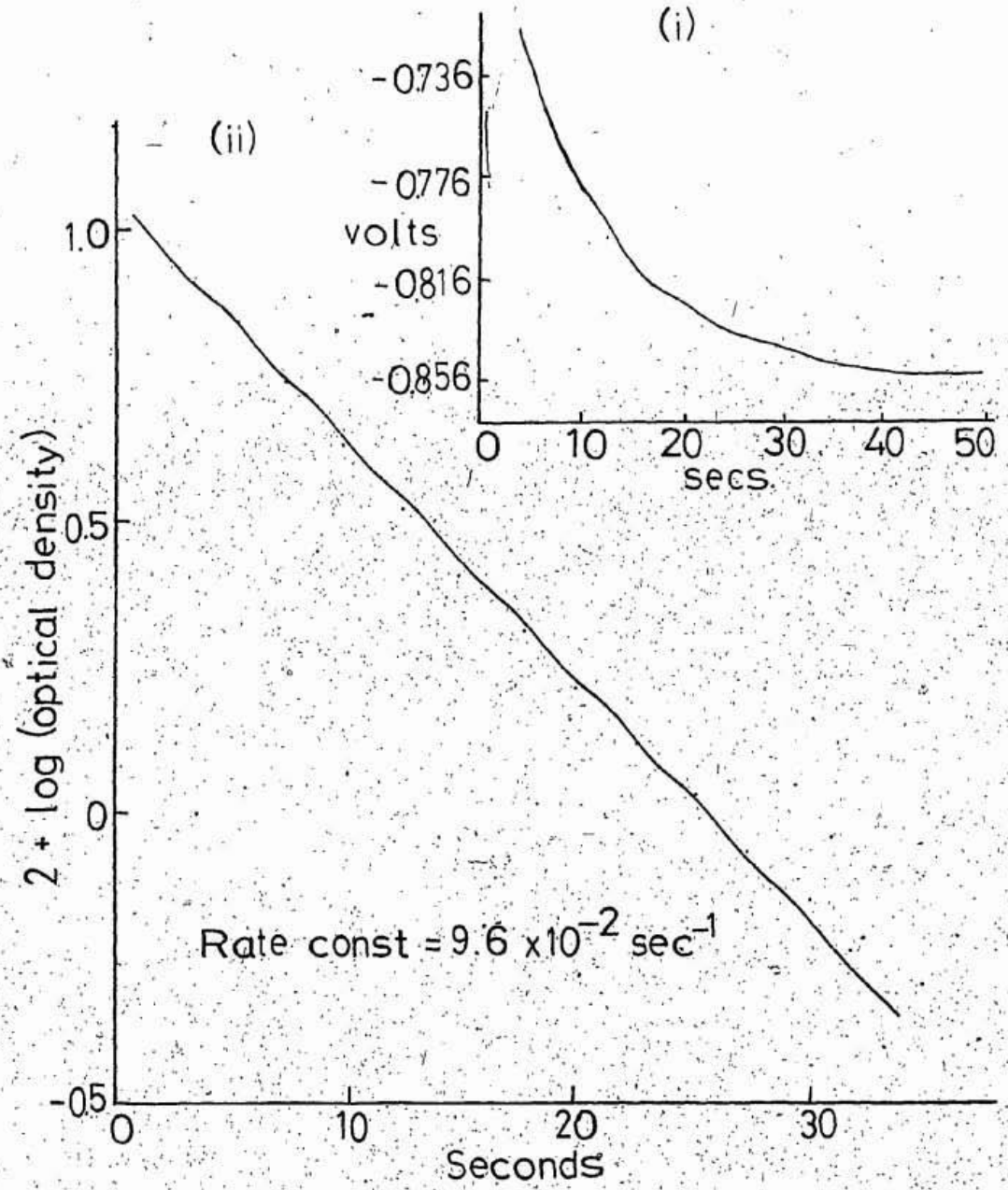


Figure 3. Kinetics of pigment protonation.

(i) Oscilloscope trace from stopped-flow apparatus.

(ii) First order kinetic plot from trace (i).

obeyed for this reaction (Fig. 3). To explain these observations it is suggested that initial protonation is either followed by a rearrangement or a second slower protonation, resulting in a slight decrease in intensity at 408nm.

When an attempt was made to reproduce these results at a later date, no reaction of this type was visible. Since the pigment in this second experiment came from frozen plasmodia, it is possible that the group causing the decrease in intensity is blocked by the additional function in the molecule (as suggested by the low $E^{1\%}_{1cm}$ value determined for this pigment).

It was found that the absorption at 408nm, of a solution of pigment from frozen plasmodia at pH = 3 (that is an equilibrium mixture of the protonated and ionised pigment) was not affected by changes in temperature. Thus, it was not possible to use simple relaxation studies (temperature-jump apparatus) to follow the fast protonation.

iv) Nuclear magnetic resonance spectrum of the pigment

The pigment, as isolated, is very soluble in aqueous solvents but only slightly soluble in non-polar solvents. For this reason the n.m.r. spectrum was recorded in D_2O. This has the disadvantage that polar protons which are easily exchanged (e.g. -OH and $-NH_2$) cannot be detected.

It can be seen (Fig. 4) that olefinic and aromatic type hydrogens are present (τ = 4.7 - 3.0 and 2.8 respectively). The peak at τ = 2.0 could be produced by protons in a hetero-aromatic system and the peaks at τ = 6.6 and 6.2 could represent the presence of hydrogen atoms on carbon atoms which are

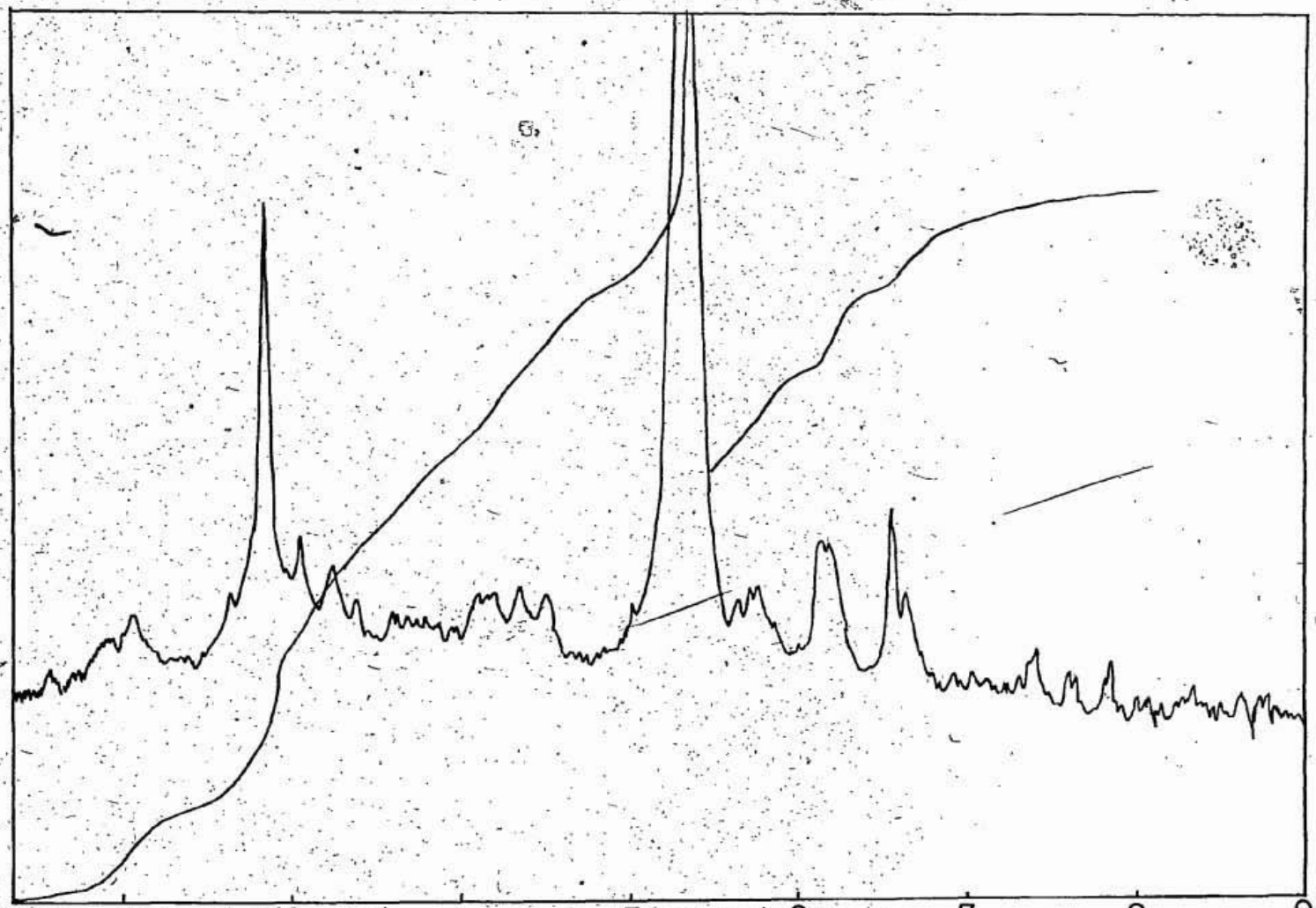


Figure 4. N.m.r. spectrum of the pigment in D_2O

adjacent to nitrogen or oxygen. A similar peak is obtained at τ = 6.18 in the spectrum of 4-methoxy-6-methyl-2H-pyran-2-one, using chloroform as solvent. Apart from small peaks, which are not readily distinguished from the base line, at τ = 7.4 and 7.85, no peaks representing aliphatic protons are present at τ values greater than 6.7.

Since the peaks are broad, the splittings are not easily distinguished and the coupling constants cannot be determined. The situation is not clarified by expansion of the scale. Integration of these peaks gives the approximate hydrogen ratios shown in Table 3.

TABLE 3

Relative intensity of the hydrogen atoms in the n.m.r. spectrum of the pigment

τ	2.0	2.8	3.0–4.7	5.7	6.2	6.6
relative intensity	2	3	7	2	2	1

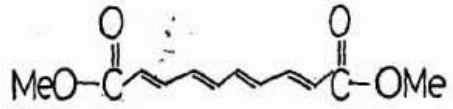
These relative intensities suggest a high degree of unsaturation, which agrees with the empirical formula already determined.

v) Oxidation of the pigment

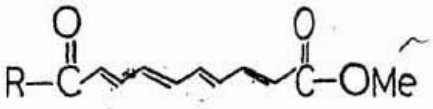
(a) Sodium metaperiodate

Sodium metaperiodate has been used previously(23) to oxidise the pigment. Then, the reaction was allowed to continue until no yellow colouration of the pigment remained, which, under the conditions used, took 48–88 hours. A tetraene diacid ester, trans dimethyl 2,4,6,8-decatetraene-1,10-

dioate (XIII), was isolated and identified, primarily by ultraviolet and mass spectroscopy, as being the major product of an ether extract of the oxidation mixture which had been treated with diazomethane.



(XIII)

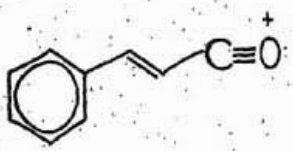


(XIV)

Another compound found in the ether extract, having the same chromophore and a similar mass spectrum was suggested to be (XIV), but the nature of the R group was not identified.

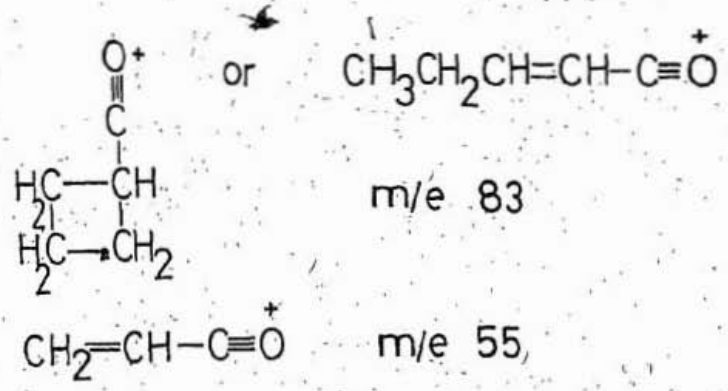
To investigate further the functional groups attached to the polyene chromophore, shorter oxidation times have been employed here. The aqueous phase remaining after ether extraction was also investigated. The reaction was terminated when the ultraviolet spectrum began to show peaks corresponding to the presence of the polyene function at 345, 331 and 320nm. The mass spectrum of the solid from the untreated ether extract at 290° has a molecular ion 194, which corresponds to 2,4,6,8-decatetraene-1,10-dioic acid (XV). The peak at m/e 176 (M-18) corresponds to loss of water. The ion at m/e 131 can be rationalised in several ways, one of which is loss of water, isomerisation of one double bond, followed by loss of a hydrogen atom and carbon dioxide.

m/e 131



Loss of carbon monoxide gives m/e 103 which is followed by loss of acetylene to give m/e 77. A detailed discussion of the routes of breakdown of the dimethyl ester (XIII), many of which are applicable to the free acid, is given by Khalil (23).

The mass spectrum at a lower temperature shows peaks with m/e values greater than 194, suggesting that a larger, more volatile compound is present. The most significant of the higher mass peaks is m/e 299. While many of the peaks in this spectrum are representative of the polyene spectrum, the m/e 131 peak which is the base peak of the spectrum of (XIII), is insignificant. That the untreated ether extracts do not vary significantly from the extracts treated with diazomethane would seem to indicate that no hydroxyl or acidic functions are present. Large peaks at m/e values 43, 57 and 71 are given by alkanes (RCH_2^+) or aliphatic aldehydes or ketones from structures of the type $R\text{-}C\equiv O^+$, whereas the peaks at m/e 55 and 83 could arise from an unsaturated ketone or a cyclic ketone. Suggested structures for these are : -



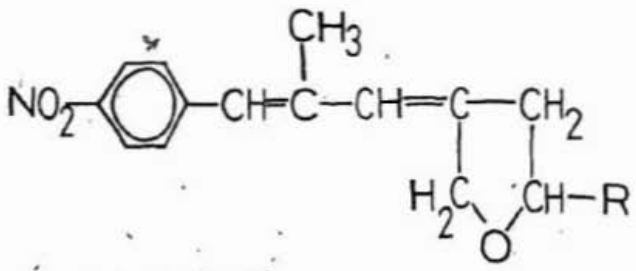
A compound containing a carbonyl group was isolated from the aqueous solution as the 2,4-dinitrophenylhydrazone. The molecular ion of the mass spectrum was m/e 294 giving the carbonyl compound a molecular mass of 114. The same product was formed when another pigment from P. flavicomum was treated in a similar manner (37). Many of the major peaks can be explained in terms of breakdown of a 2,4-dinitrophenylhydrazone and as such are not diagnostic in determining the nature of the carbonyl compound.

Spot tests using the aqueous reaction mixture indicated that the carbonyl compound was fairly readily oxidised and it is suggested that the carbonyl group is an aldehyde. It is surprising that a positive test is given with 2,3,5-triphenyltetrazolium chloride, since the presence of a carbohydrate would not be expected after periodate reaction. A deoxy furanose, having no adjacent hydroxyl groups, could be present. However, no other carbohydrate tests were conclusive because of the interference of periodate with the reagents. An attempt to isolate the products by extraction into chloroform was unsuccessful since some periodate was also extracted into this solvent.

(b) Hydrogen peroxide

Alkaline and neutral hydrogen peroxide are widely used oxidising agents. The alkaline peroxide oxidation of aureothin (III, ref. 26) was found to give two compounds, aureothinic acid (XVI) and aureothinic ketone (XVII), a neutral compound. Thus, while the pyrone ring was degraded

part of the structure remained intact.



R = CO_2H (XVI)

R = $-\overset{\overset{O}{\|}}{C}-CH_2CH_3$ (XVII)

Pigment oxidation, using both neutral and alkaline hydrogen peroxide, gave a compound having a polyene ultraviolet spectrum with peaks at 345, 330 and 320nm. Material ether extracted from the oxidation mixture gave a high temperature mass spectrum characteristic of (XV). A more volatile component was found to have highest mass at m/e 355. Using extraction techniques two compounds could be separated, one acidic, the other neutral or basic. The acidic compound was mainly (XV) while the basic/neutral compound gave highest peak at m/e 399 with other prominent high mass peaks at m/e 299 and 199. The absorption spectrum of this compound has very small peaks at 316 and 350nm with no absorption at higher wavelengths. The polyene chromophore is not present. It is possible that trace metal impurities have acted as catalysts for the oxidative degradation of the chromophore. These trace metals may originate from the glass reaction vessel. Again insufficient material was obtained for infrared or n.m.r. spectra. Although

most spot tests were negative, the accuracy of these tests was limited since only a small quantity of material was available.

The mass spectrum of this material is very similar to that obtained from the ether extract of the sodium metaperiodate reaction, showing that both these oxidative techniques give similar products. Lack of material precluded any further structure determination of these products.

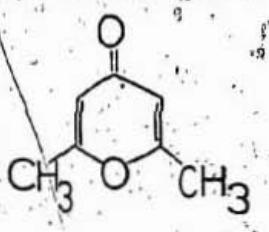
vi) Pigment hydrolysis

Khalil (23) investigated the ether extracts which were obtained from both acid and alkaline hydrolyses but the aqueous solutions remaining were studied only to a limited extent. It was suggested that acid hydrolysis solutions contained a carbonyl compound, a sugar (positive tests with 2,4-dinitrophenylhydrazine and 2,3,5-triphenyltetrazolium chloride respectively) and a phenol (λ_{max} (CH_3OH) 258nm).

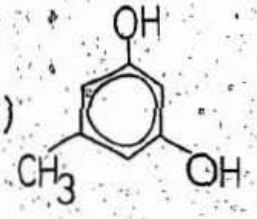
(a) Spot tests on aqueous solutions after ether extraction

Contrary to previous work, no reaction with 2,4-dinitrophenylhydrazine was seen here and there was no change in colouration on the addition of ferric chloride. Dehydroacetic acid (XVIII) produces 2,6-dimethylpyran-2-one (XIX) on treatment with hydrochloric acid (38) and orcinol (XX) on reaction with sodium hydroxide (39). Both of these reactions

(XIX)



(XX)



involve ring opening at the lactone followed by decarboxylation and ring closure. If dehydroacetic acid, or a derivative of dehydroacetic acid, was present in the pigment positive tests for carbonyl and phenolic compounds would be expected.

Decolourisation of potassium permanganate and bromine solutions could be caused by the presence of polyene in unreacted or partially reacted pigment not extracted into ether. While reactions with aniline phthalate and 2,3,5-triphenyltetrazolium chloride were positive, no reaction was given with the Molisch reagent. Thus no conclusions regarding the presence of a carbohydrate molecule could be reached, but this was further investigated by paper chromatography. Hydrolysis solutions from pigment isolated by the method of Khalil (23) showed no reaction with ninhydrin, whereas pigment from frozen plasmodia gave a purple colouration with ninhydrin. The nature of the compounds giving this reaction was again investigated by paper chromatography.

(b) Paper chromatography

Paper chromatography has been widely used for the identification of sugars and many solvents and locating reagents have been used. Chromatograms of the hydrolysis mixture were run and tested for the presence of sugars. In each solvent system a spot could be located which appeared dark under ultraviolet light after treatment with fluorescein, gradually became grey/mauve on treatment with neutral silver nitrate and produced a white spot which gradually became

darker than the background with an alkaline silver nitrate reagent. Sugars are expected to quickly produce brown spots on treatment with neutral or alkaline silver nitrate. In solvent systems 1 and 2, Table 4, the spot which gave the positive sugar tests had the same R_f value as the spot which gave positive reactions with silver nitrate. Since this spot appeared to contain both sugar and another function, positive identification of the sugar is difficult. However, the positive reaction with aniline phthalate in solvent system 4 was at a higher R_f (0.44) than the spot which reacted strongly with silver nitrate (R_f = 0.3). Alkaline silver nitrate did produce a slightly darker spot at R_f = 0.44. Diphenylamine:aniline: phosphoric acid (40) and p-anisidine:periodate (41) reagents give specific colour reactions with sugars and using these reagents the sugar present here is tentatively suggested to be sorbose. The possibility that this spot is the hydrochloride of an amino sugar cannot be ignored.

In all chromatograms the most prominent spot was the one which was visible after spraying with fluorescein and gave characteristic reactions with neutral and alkaline silver nitrate. Since purines are revealed as white or yellow spots and pyrimidines as mauve or grey/mauve spots with neutral silver nitrate (42) and are often combined with a sugar molecule, the possibility that these types of compound might be present was investigated. However treatment of the chromatogram with acidic mercury (II) nitrate and ammonium sulphide did not reveal any purine bases in the hydrolysis mixture.

TABLE 4

Location of sugars on paper chromatograms

Solvent system	Locating reagent	Colour of reaction	R_f
1) 2-methyl-propan-2-ol: butan-2-one: water:ammonium hydroxide 4:3:2:1	a) p-ansidine/ periodate	red/purple fluorescent	0.45
	b) p-aminophenol	brown/ yellow	0.44
	c) malonic acid	slight fluorescence	0.47
	d) aniline phthalate	pale brown	0.46
	e) diphenylamine/ aniline/phosphoric acid	grey/green- lighter than background	0.45
2) butan-1-ol: pyridine: water 45:25:40	p-ansidine/ periodate	no reaction with p-ansidine, darker than background with periodate	0.37
3) butan-1-ol: acetic acid: water 10:1:3	diphenylamine/ aniline/ phosphoric acid	pale yellow	0.06
4) butan-1-ol: pyridine: water 9:5:4	aniline phthalate	orange/ brown	0.44

While cytidine and uridine did not have the same R_f value as the compound in the hydrolysis mixture, the presence of a pyrimidine remains possible.

After elution of this spot the absorption spectrum was recorded (λ_{max} (H_2O) 260nm) and was unchanged on addition of acid or base. The highest peak in the mass spectrum was at m/e 135, although this was not present in all spectra recorded. Uracil has an absorption maximum at 262nm, unchanged by addition of acid or base, and has a formula mass of 112. Another prominent peak in the mass spectrum of uracil is at m/e 69 (43), also present in the mass spectrum of the eluted compound. However, coincidence of peaks does not occur at lower m/e values. It was found that chloride ions were present in the eluate, so it is possible that the reactions with silver nitrate were only reactions of these chloride ions. That this compound is basic and exists as the hydrochloride can be seen by the presence of large peaks at m/e 36 and 38 in the mass spectrum. Thus, while the compound isolated here is not uracil, it could be a derivative of uracil. This could be verified by extensive chromatography of uracil derivatives.

Since the aqueous solutions from hydrolysis of the pigment isolated from frozen plasmodia had given a positive ninhydrin reaction, these were analysed for the presence of amino acids. After chromatography in two solvent systems, (butan-1-ol:acetic acid:water) and (methanol:pyridine:water), five compounds could be located using ninhydrin. A typical chromatogram is shown in Fig. 5. Three of the compounds were tentatively

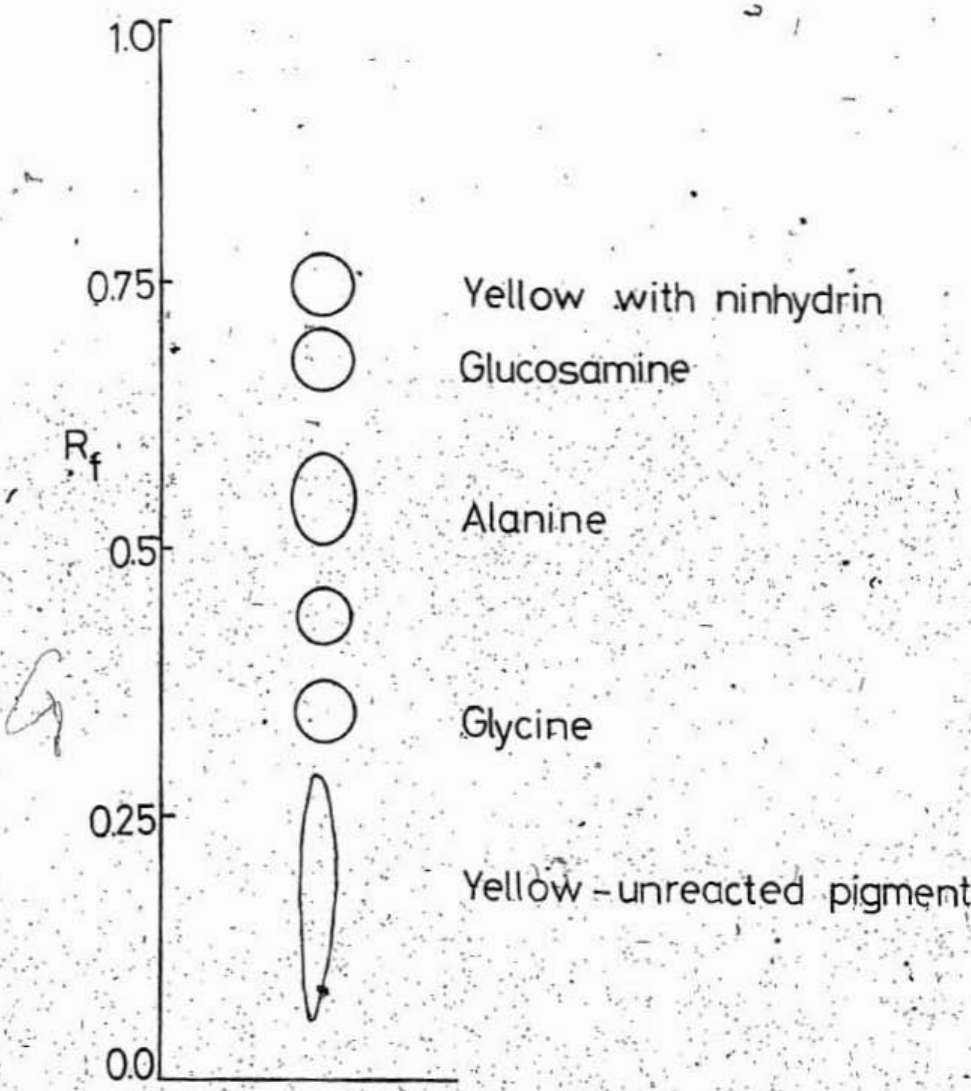


Figure 5. Paper chromatogram of hydrolysis mixture of pigment from frozen plasmodia (methanol: pyridine:water 80:4:20).

identified as glycine, alanine and glucosamine. Another compound formed a yellow spot with ninhydrin, but comparison with standards showed that it was neither proline nor hydroxyproline.

Pigment from frozen plasmodia which was subjected to further column chromatography using Sephadex LH 20 was also found to contain the same amino acids on hydrolysis. Since a limited number of amino acids were detected and these were consistent for several different pigment samples, they must be joined to the pigment and are not a random association of peptide with the pigment. It is therefore suggested that a peptide is, in this instance, bonded to the pigment giving this sample a low $E^{1\%}_{1cm}$ value.

(c) Column chromatography

Sephadex and other dextran chromatographic columns have been used to desalt mixtures of biological origin. A Sephadex column was employed here to remove extraneous inorganic ions from the aqueous solution. However, fractions containing chloride ions were quickly removed from the column, showing that they formed part of a larger molecule such as an amine hydrochloride. Most of these fractions had absorption maxima near 260nm and their mass spectra had highest peaks at m/e 112 or 82. In these respects they were similar to the compound eluted from paper chromatograms. However, one fraction (fraction A) from each hydrolysis had absorption and mass spectra indicating the compound to be pyridine hydrochloride (λ_{max} (H_2O) 245,255, 262nm; mass spectrum m/e 79 and 52). The eluted solid was

shown not to be pyridine hydrochloride by paper chromatography using propanol:concentrated hydrochloric acid as solvent. Pryidine hydrochloride had R_f = .0.4, whereas the eluted compound had R_f = 0.1. It is possible that both the chromophore and mass spectrum could be due to a triene, octa-2,4,6-triene having λ_{max} (hexane) 254,262,274nm (44). However, the addition of an amine group to a triene molecule would move the absorption maxima to longer wavelengths.

(d) Distillation of alkaline solutions

Another method used to separate this hydrochloride from the remainder of the products was the distillation of alkaline solutions. The absorption spectrum of the distillate was the same as that of fraction A above and the base peak of the mass spectrum was m/e 79. Since there are also peaks at higher mass values a substituted pyridine could be present, although any substituent could not be polar since this would change the position of the absorption maxima. An alkyl substituted pyridine would be expected to have a significant peak at m/e 93 but this is not observed in this spectrum.

The distillate obtained from the pigment of frozen plasmodia gave rise to a mass spectrum in which major peaks were 14 mass units apart, suggesting an alkane. This series, m/e 41,55,69,83,97,111,125,139,153,167,181,195 and 209 is characteristic of aliphatic nitriles, alkenes, oxazoles, carbazoles and cycloalkanes (45). Since the pigment contains nitrogen either an aliphatic nitrile, oxazole or carbazole is considered the most likely possibility. In the mass spectrum

the highest mass recorded was m/e 219. Khalil (23) suggested, on the basis of a resonance Raman spectrum, that an oxazole type system was present in the pigment.

(e) Desalting of alkaline hydrolysis mixture

After acidification, alkaline hydrolysis solutions contain sodium chloride. The presence of inorganic ions is undesirable and in many cases it has been found necessary to remove them before analysis. The pyridine method of Saini (46) has been used for desalting urine prior to sugar determinations. It was found here that removal of all the pyridine hydrochloride by precipitation was difficult. Any other amines or basic compounds would also have been precipitated when using this method. The mass spectrum indicated that some pyridine hydrochloride did remain.

Since many of the compounds obtained from these hydrolyses are very similar, separation and hence identification remains difficult.

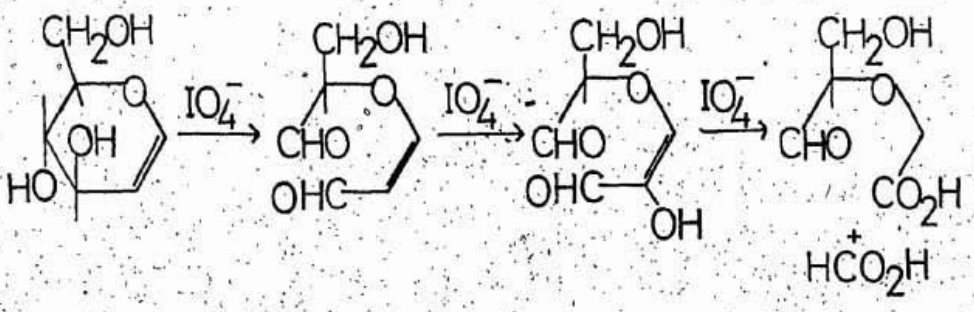
vii) Determination of the molecular weight of the pigment from oxidation reactions

Khalil (23) isolated and identified 2,4,6,8-decatetraene-1,10-dioic acid (XV) after sodium metaperiodate oxidation of the pigment. The quantity of (XV) produced during a reaction can be accurately determined from the absorbance of this compound in the region 320-350nm. Assuming that one molecule of pigment contains one polyene moiety and that the reaction is quantitative, determination of the quantity of polyene produced from a known weight of pigment can be used to give a value for the

molecular mass of the pigment. Since the acid (XV) is insoluble in water (pH = 7), a Beer-Lambert Law plot was determined in alkaline solution (Fig. 6)

(a) Sodium metaperiodate

Under the reaction conditions used, sodium metaperiodate has absorption in the region 320-350nm and so absorbances for solutions of this compound were determined at 331 and 348nm in order that corrections could be made in determinations of the polyene acid (XV) concentration. After 50 hours there was little absorbance attributable to the polyene acid. A further experiment showed that decomposition of the polyene (XV) took place under these conditions. Although sodium metaperiodate does not usually react with carbon double bonds, there is some available evidence that periodate can react with a double bond under certain conditions. The unsaturated sugar D-glycal has been shown to react by the following route (47a) :-



It has been shown that methylene groups adjacent to carboxylic acid functions are susceptible to oxidation (47b). By analogy to the above reaction the polyene function could react in the following manner :-

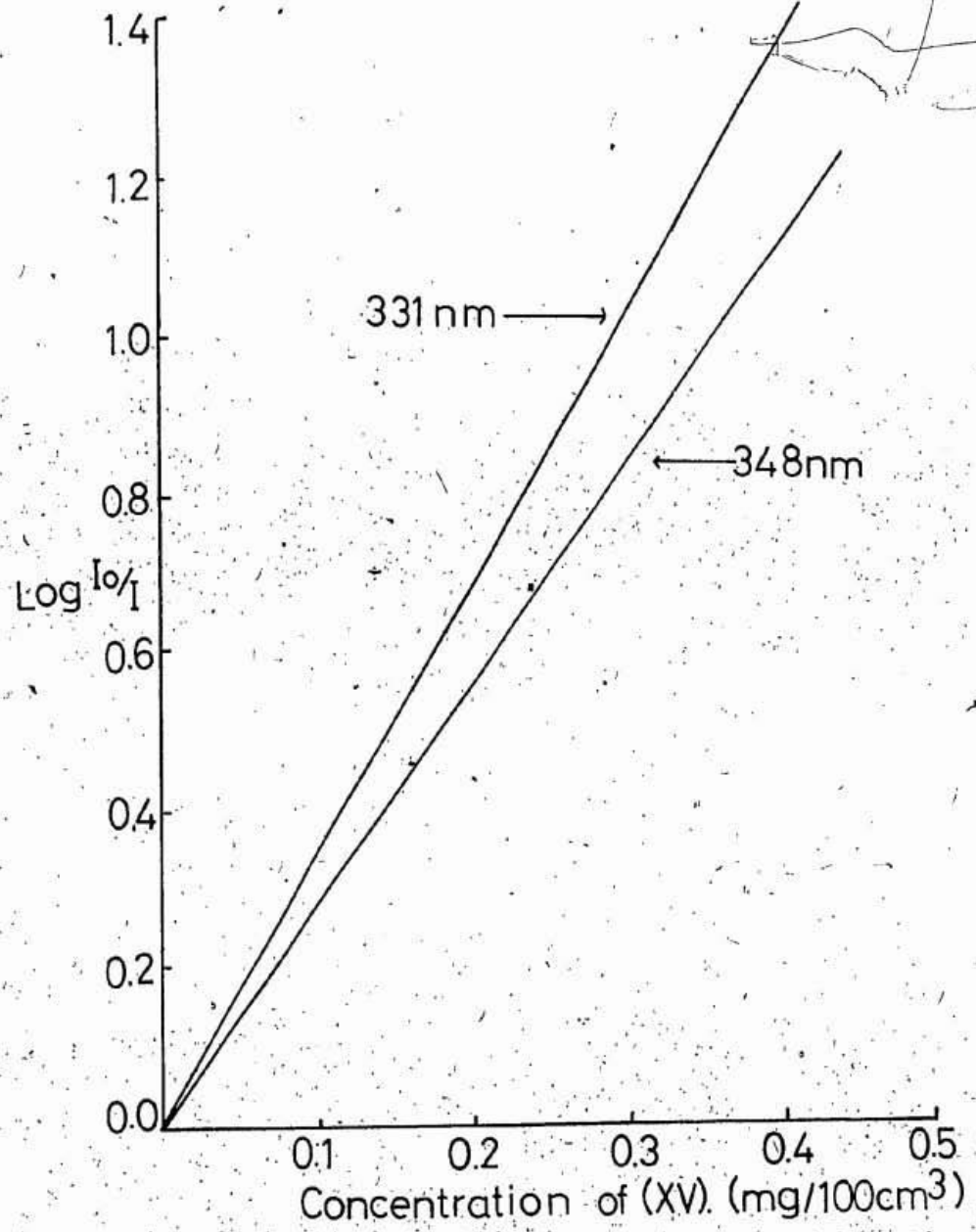
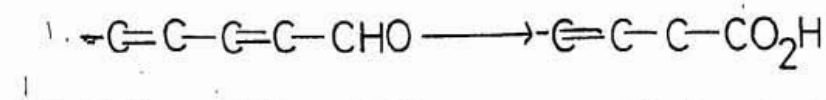


Figure 6. Beer-Lambert Law plot for 2,4,6,8-deca-tetraene-1,10-dioic acid.



Oxidation of the methylene group could then lead to further degradation of the polyene.

(b) Hydrogen peroxide

The use of hydrogen peroxide as an oxidising agent is preferred to sodium metaperiodate, since, at concentrations necessary to produce a fairly fast rate of reaction it has no absorption in the range 330-348nm. Although some degradation of the polyene did occur, it could be minimised by the use of a quartz vessel, but was not reduced by the dilution of the hydrogen peroxide. As mentioned previously, it is suggested that this degradation is catalysed by metal ions from the vessel.

Definite peaks at 331 and 348nm were formed in all three experiments and values for the molecular mass calculated from the most intense absorbances at 331 and 348nm, consistent with the spectrum of (XV), were found to be 2930, 2690 and 1880. These are average values for the molecular masses calculated at the two wavelengths.

The last of these values is considered to be the most accurate, since here reaction was most complete (as measured by the absorbance at 380nm) and the time interval for this reaction (6½ hours) was shortest, so least decomposition of the polyene had taken place. Since decomposition of the polyene could not be completely prevented, these values must be regarded as maximum values for the molecular mass.

All pigment used in these oxidation experiments was iso-

lated from frozen plasmodia and since this pigment has been shown to contain five amino acids, a molecular mass of 1880 is not unreasonable.

viii) Summary

The indicator pigment was isolated from the slime mould P. flavicomum using a modification of the method employed by Khalil (23). Elemental analyses of this pigment gave empirical formulae of $C_{12.1}H_{13.1}O_5N$ for the protonated pigment and $C_{11.2}H_{16.5}O_{7.2}N\,Na$ and $C_{12.3}H_{13.9}O_{7.7}N\,Na_{2.5}$ for the sodium salt of the pigment. Support for two hydrogen atoms being replaceable by sodium atoms is given by the pK_a measurements of Khalil, absorption spectra and kinetic data determined in this work. The n.m.r. spectrum of the pigment agrees with a high degree of unsaturation being present in the molecule.

Oxidation experiments using sodium metaperiodate and hydrogen peroxide confirmed the presence of the tetraene identified by Khalil but were unable to supply further information as to the nature of the groups at either end of the polyene moiety. A water soluble compound of molecular mass 114 was isolated as the 2,4-dinitrophenylhydrazone, but was not identified.

Hydrolysis experiments indicated the presence of a carbohydrate molecule, possibly sorbose, and basic compounds. These basic compounds were isolated as the hydrochlorides using several techniques and their absorption and mass spectra determined. It is suggested that one of these is a substituted

pyridine. This could possibly be linked to the sugar to form a pyridine nucleoside. No phosphate was detected at any time. As the hydrochlorides were not very volatile it was difficult to determine their molecular mass. It was thought that these basic compounds might be amines of low molecular mass (possibly 112). These amines are probably volatile and hence difficult to isolate. Since only small quantities of material were available, identification was not possible.

Pigment isolated from frozen plasmodia had a high molecular mass. This was suspected from the low $E^{1\%}_{1cm}$ value (200) determined and confirmed by molecular mass determinations from oxidation experiments as being approximately 1880. Hydrolysis of this pigment gave five compounds which could be detected using ninhydrin, of which three were tentatively identified, using paper chromatography, as glycine, alanine and glucosamine. It is suggested that this pigment contains a peptide.

Since none of these experimental results disagree with the structure (I) proposed by Khalil, the presence of a pyrone ring still remains a possibility. It has been shown here (Part II) that a tetraene conjugated to the 6α position of triacetic acid lactone has the correct absorption maximum, but does not show exactly the same spectral changes on acidification as the pigment. ^{13}C n.m.r. has been used with success in determining the structure of aurovertin B (IV; ref.27) and in studying the biosynthesis of aureothin (III; ref.48). Since both these systems contain pyrone and polyene functions, it is possible that this technique

could be successfully applied to this pigment to provide information concerning the carbon skeleton, which has so far been unobtainable from chemical degradation experiments.

EXPERIMENTAL

Ultraviolet spectra were measured on a Perkin-Elmer 202 spectrophotometer and infrared spectra were recorded on a Perkin-Elmer 237B grating spectrophotometer. Nuclear magnetic resonance spectra were recorded at 30° with tetramethylsilane as an internal standard on a Varian EM-360 spectrometer.

Mass spectra were obtained on a RMU-6E mass spectrometer at 70eV (unless otherwise stated) and samples were introduced directly into the ion source.

Melting points were determined on a flat top Fisher-Johns melting point apparatus, and are uncorrected.

Elemental analyses were performed by Alfred Bernhardt Mikroanalytisches Laboratorium.

All solvents were distilled prior to use.

i) Culture of Physarum flavicomum

The slime mould, P.flavicomum, was grown in 500cm^3 liquid shake flask cultures. The composition of the liquid medium was that used by Khalil (23) which contained quinic acid. This acid was added to improve pigment yield. After four days growth the plasmodia were harvested and the pigment extracted. In the latter part of this work plasmodia, which had been frozen after four days growth, were used.

ii) Pigment isolation and purification

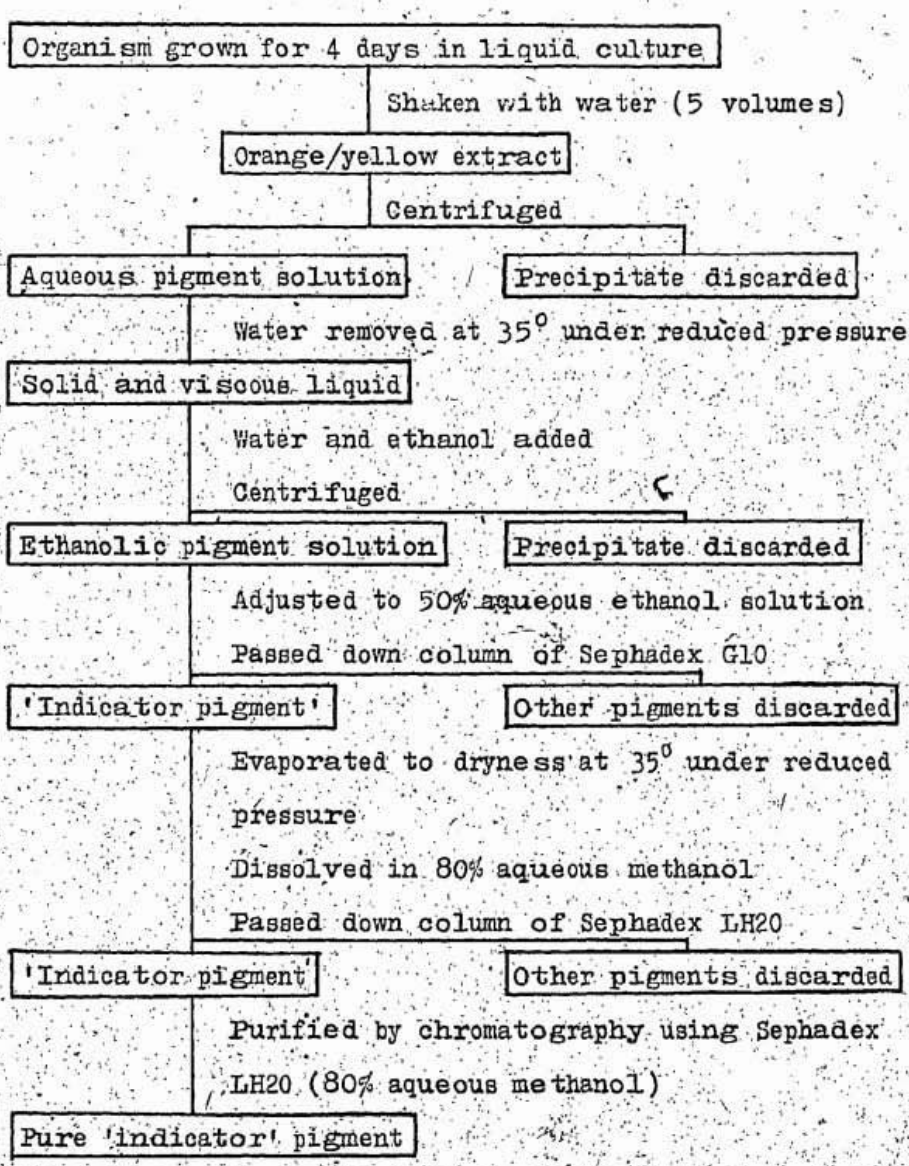
The procedures used here (49) to isolate and purify the pigment were based on the methods employed by Khalil (23). To isolate the water soluble pigments, the plasmodium (Physarum flavicomum) was shaken repeatedly with water (5 x 150cm^3) and

after each extraction the solid was spun down in a centrifuge (approximately 2000 r.p.m. for 10 minutes). As much water as possible was then removed by rotary evaporation under reduced pressure. Addition of a small volume of ethanol to the extract was found to reduce frothing. Sufficient water ($20cm^3$) was then added to just dissolve the remaining solid and viscous liquid in order to render it homogenous. Ethanol ($80cm^3$) was added and the precipitated protein removed by centrifuging. Water was then added to the supernatant liquid to give a 50% aqueous ethanol solution, which was applied to a Sephadex G10 chromatography column (30cm x 5cm). Fractions were collected from the column and tested by taking a visible spectrum. Those fractions which showed bathochromic shifts on acidification (380nm to 410nm) were combined, evaporated to dryness under reduced pressure, dissolved in 80% aqueous methanol and purified by chromatography on a column of Sephadex LH20 (23cm x 5cm).

Since the pigment decomposed slightly on storage, prior to use it was further purified by chromatography on Sephadex LH20 (9cm x 4.5cm) using 80% aqueous methanol as solvent. In order to prevent decomposition of the pigment in solution, the temperature at which solvents were removed under reduced pressure was maintained at approximately at 35°. Light was excluded from the pigment whenever possible to prevent photodecomposition. The complete procedure is outlined in Chart 1.

CHART 1

Extraction and purification of 'indicator' pigment from *P. flavicomum*



iii) Preparation of the protonated pigment

The protonated form of the 'indicator' pigment could be prepared by passing the extracted pigment down a proton exchange column. Three types of material were used to prepare the columns, which were :-

(a) Sephadex LH20

(b) Sephadex G 10

(c) cationic exchange resin (Dowex 50W-8X).

All glassware used in these experiments was washed with nitric acid (2M), rinsed thoroughly with distilled water and oven dried.

(a) Sephadex LH20

Three procedures were used with this material :-

(1) A Sephadex LH20 column (23cm x 5cm) was washed with hydrochloric acid (0.05M) in 80% aqueous methanol ($500cm^3$), followed by an equal volume of 80% aqueous methanol to remove excess hydrochloric acid from the column. The pigment solution was then applied to the column and the fractions examined by UV spectroscopy.

(2) The column, after treatment with acidic aqueous methanol, was washed with a smaller volume of aqueous methanol ($10cm^3$) before application of the pigment solution.

(3) The column was treated with 80% aqueous methanol (0.05M in HCl; $150 cm^3$), then 80% aqueous methanol (0.1M in HCl; $200cm^3$) and washed with 80% aqueous methanol ($10cm^3$) prior to the application of the pigment solution.

(b) Sephadex G 10

A Sephadex G 10 column (30cm x 5cm) was treated with 50% aqueous ethanol (0.05M in HCl; 350cm^3), followed by 50% aqueous ethanol (10cm^3). The pigment solution was then applied to the column and fractions collected as before.

(c) Cationic exchange resin (Dowex 50W-8X)

A column of resin (Dowex 50W-8X; 12cm x 4.5cm) was treated with sulphuric acid (2M) to convert the resin to the protonated form. The resin was washed with water, to remove the acid, and then with 50% aqueous ethanol. A 50% aqueous ethanol solution of the pigment was applied to the column and allowed to pass slowly through (1cm^3/minute).

After each of these preparative techniques, 2 or 3 drops of eluant were dissolved in 95% ethanol and the visible spectrum determined. Hydrochloric acid (2M, 2 drops) was added to the pigment solution in the cell (1cm) to check the degree of protonation. An increase in the intensity of absorption at 412nm indicated incomplete protonation. Addition of sodium hydroxide (2M, 4 drops) shifted the absorption maximum to 380nm and served as an additional check of pigment purity by the observation of relative intensities at the two wavelengths.

The protonated pigment solution was concentrated by evaporation of the solvent under reduced pressure. On cooling, an orange/red microcrystalline precipitate was observed and this was spun down in a centrifuge. The supernatant was decanted, the solid washed twice with cold water (0°, 2cm^3) and spun down after each washing. The pigment was then dried under vacuum, at room temperature, for 15 hours.

Elemental analysis of the protonated pigment gave :-

Sample	%C	%H	%O	%N	Residue
1	54.88	4.96	29.04	5.37	4.16
2	57.38	5.15	31.69	5.51	-

iv) Preparation of the sodium salt of the pigment

The pigment was converted to the protonated form using the cation exchange column (Dowex 50W-8X). Two ion exchange methods were then successfully used to convert this pigment to the sodium salt. The columns were made with :-

(a) cation exchange resin

(b) Sephadex cation exchanger.

(a) *Cation exchange resin*

The resin column (Dowex 50W-8X, 9cm x 4cm) was washed with aqueous sodium chloride solution (4M) until the eluate was no longer acidic, and then with distilled water until all the chloride ions had been removed. The water was replaced by 80% aqueous methanol and the protonated pigment, in this solvent, applied to the column. The sodium salt of the pigment so formed, was purified by chromatography on a column of Sephadex LH20 (9cm x 4.5cm) using 80% aqueous methanol as solvent.

(b) Sephadex cation exchanger

Protonated pigment solution was applied to a column of CM Sephadex C-50 (11.5cm x 3.5cm) using 50% aqueous ethanol as solvent. Solutions containing the sodium salt, as indicated by an absorption at 380nm in the UV spectrum, were combined and the solvent removed under reduced pressure.

The pigment was washed twice with cold absolute ethanol ($5cm^3$), the solid being spun down in a centrifuge after each washing, and then dried under vacuum at room temperature. Elemental analysis gave :-

Sample	%C	%H	%N	%O	%Na
1	44.27	5.44	4.82	*	7.54
2	41.57	3.89	3.84	34.79	*

* Sufficient pigment was not available for these determinations.

v) Kinetics of pigment protonation

Pigment isolated from fresh plasmodia by the method of Khalil (23) was used in these experiments. The protonation reactions were followed using a stopped-flow apparatus in which a pigment solution (optical density at 380nm = 1.07, 1cm cell, H_2O solvent) was rapidly mixed with an equal volume of hydrochloric acid (0.02M) at 25°. A reaction rate could be followed by measuring the change of absorption at 408nm, (Fig.3).

This experiment was repeated at a later date with a pigment sample isolated from frozen plasmodia by the method described on page 44. However, in this case, no reaction could be observed.

vi) Temperature dependence of the equilibrium reaction between the pigment salt and the protonated pigment

Pigment was dissolved in water ($10cm^3$) and the solution adjusted to pH 3 by the addition of hydrochloric acid (0.02M, $0.5cm^3$). The optical density at 380nm was 0.95 in a 1cm cell.

The solution was placed in a thermostatted ultraviolet cell and the absorbance at 410nm was recorded over the temperature range 15-27°. A Unicam SP500 spectrophotometer linked to a chart recorder was used for these measurements. No change in absorption was noted. The pigment used in this experiment was obtained from frozen plasmodia using the isolation method described on page 44.

vii) Nuclear magnetic resonance spectrum of the pigment

Pigment was dissolved in D_2O to give a saturated solution. Tetramethylsilane was used as an external standard. This spectrum is recorded in Fig. 4, p. 23.

viii) Oxidation of the pigment

Two reagents were used to oxidise the indicator pigment,

(a) sodium metaperiodate

(b) hydrogen peroxide.

(a) Sodium metaperiodate

Pigment (10mg) was dissolved in 50% aqueous methanol ($10cm^3$) and sodium metaperiodate (100mg) added. The solution was mixed thoroughly and kept in the dark at room temperature. The reaction was followed spectrophotometrically, by observing the decrease of the absorption maximum at 380nm. After 1½ hours, half of the reaction mixture was removed, acidified with sulphuric acid (2M) and extracted with ether ($100cm^3$). The ether layer was washed to neutrality and evaporated to dryness. The remainder of the reaction mixture was treated in a similar manner after a further 1½ hours. Ultraviolet spectra were recorded of both ether extracts and the

remaining aqueous solutions. The aqueous phase showed only the absorption of sodium metaperiodate (λ_{max} 222nm). An aliquot of each ether extract was added to methanol and the ultraviolet spectra determined. Ether extract, 1½ hours λ_{max} 317-21(sh),331,345nm; $\lambda^{OH^-}_{max}$ 311-13(sh), 328,344nm; ether extract,3 hours λ_{max}315-20(sh), 331,345nm; $\lambda^{OH^-}_{max}$ 312,327,344nm.

(1) Solid from the ether extracts

Neither of the solid materials remaining after removal of the ether melted below 290°. The mass spectrum of the solid from the first ether extract was recorded at 238° and 290°. At 238° peaks were observed at m/e 299(7%),255(6%), 243(5%),240(6%),239(11%),227(10%),225(6%),217(5%),211(5%), 207(5%),206(5%),205(5%),199(16%),197(5%),195(5%),193(8%), 191(10%),185(8%),183(7%),181(7%),179(8%),177(8%),169(8%), 167(17%),165(10%),163(9%),155(11%),154(8%),153(14%),152(6%), 151(12%),150(9%),149(64%),141(14%),140(6%),139(16%),138(8%), 137(19%),136(8%),135(12%),129(6%),127(15%),126(9%),125(29%), 124(10%),123(27%),122(6%),121(13%),119(6%),115(10%),113(26%), 112(16%),111(45%),110(16%),109(35%),108(6%),107(14%),105(14%), 101(29%),100(29%),99(27%),98(15%),97(70%),96(24%),95(49%), 93(12%),91(19%),85(10%),84(21%),83(73%),82(24%),81(49%), 79(11%),77(9%),73(30%),71(31%),70(37%),69(22%),68(12%), 67(29%),66(10%),58(13%),57(64%),56(86%),55(92%),45(91%), 44(35%),43(100%),42(10%),41(80%),29(36%),17(20%). Other small peaks (less than 5%) are visible at m/e 300,301,317, 337,343,355,368,369,371,386,400 and 412.

At 290° peaks were observed at m/e 194(10%),176(7%),

149(12%),131(9%),111(8%),105(8%),103(10%),97(12%),95(8%), 91(9%),85(11%),83(15%),81(9%),77(12%),71(15%),69(18%),57(28%), 55(19%),44(100%),43(24%),41(19%),36(13%),31(15%),29(11%), 17(50%).

Ethereal solutions of the material extracted into ether were treated with a solution of diazomethane in ether. The diazomethane was prepared in the usual manner from N-methyl-N-nitroso-p-toluenesulphonamide (Diazald, ALDRICH). Mass spectra were recorded of the solids remaining after removal of the solvent. These spectra did not differ greatly from the mass spectrum of the untreated ether extract, although in these spectra, recorded at 120°, the base peak was at m/e 57 and the relative intensities of the peaks at m/e 71 and 85 had increased significantly. The solid materials did not melt below 290°. Methanolic solutions of both these solids decolourised acidified potassium permanganate solution and bromine dissolved in carbon tetrachloride.

(2) Aqueous phase

The two aqueous solutions gave similar reactions in all cases. Spot tests performed on the aqueous solutions gave positive reactions with the following reagents. Blank reactions were also performed on solutions of sodium iodate and sodium metaperiodate. Many tests (not listed) were inconclusive because the solutions of these salts reacted with the reagents.

(a) Acidified and neutral potassium permanganate:- slowly turned brown.

(b) 2,4-dinitrophenylhydrazine:- immediate yellow precipitate.

(c) Bromine in carbon tetrachloride:- slowly decolourised.

(d) Acidified sodium dichromate:- became blue/green on warming.

(e) 2,3,5-triphenyltetrazolium chloride:- formed a red precipitate on warming.

The 2,3,5-triphenyltetrazolium chloride reagent was prepared according to Stahl(50), all other reagents were prepared according to Vogel (51).

Preparation of the 2,4-dinitrophenylhydrazine derivative

The precipitate formed by the addition of a solution of 2,4-dinitrophenylhydrazine to the aqueous phase was removed by centrifuging; washed with water and dried; m.p. 185-90° (decomp.). Thin layer chromatography (silica GF 254),using *benzene:ethyl acetate (95:5) as solvent*, indicated that one major and two minor products had been formed. The mass spectrum of the major product was determined (189°), m/e 294(22%), 202(18%),183(85%),181(31%),168(13%),165(15%), 153(12%),149(29%),137(9%),110(9%),107(15%),92(12%),91(40%), 83(9%),77(19%),76(16%),75(51%),74(29%),64(20%),63(22%), 61(14%),57(13%),52(18%),50(12%),44(10%),43(11%),41(13%), 38(23%),36(52%),30(100%).

(b) Hydrogen peroxide

(1) Alkaline hydrogen peroxide

Pigment (10mg) was dissolved in ethanol (1cm^3). To this solution, hydrogen peroxide (30%,1cm^3) and ethanolic potassium

hydroxide solution (10%, 0.8cm^3) were added. The mixture was kept in the dark at room temperature and stirred continuously. After 6½ hours the ultraviolet spectrum of the reaction mixture was recorded: λ_{max}(95% C_2H_5OH) 208, 300(sh), 313, 329, 345, 380(sh)nm, $\lambda^{H^+}_{max}$ 210, 260(broad), 307(sh), 309-12(sh), 334, 350, 410nm. The solution was acidified with hydrochloric acid (6M, 0.5cm^3) and extracted with ether (5x10cm^3). The yellow ether extract was washed with water (20cm^3) and evaporated to dryness. The residue consisted of a solid, which did not melt below 300°, and an oil. The mass spectrum of this mixture was recorded at two temperatures, 178° and 270°.

270°; m/e 194(30%), 183(9%), 176(19%), 154(9%), 149(10%), 148(12%), 138(9%), 131(22%), 107(12%), 105(12%), 103(25%), 91(12%), 84(12%), 83(21%), 79(12%), 78(10%), 77(24%), 69(10%), 57(10%), 55(19%), 51(16%), 44(21%), 43(100%), 42(17%), 41(13%), 36(17%), 39(13%), 31(29%), 29(13%), 27(18%), 17(15%).

178°; m/e 355(4%), 300(3%), 299(16%), 285(3%), 255(7%), 253(4%), 244(4%), 239(4%), 227(14%), 226(5%), 225(9%), 199(23%), 185(4%), 183(8%), 171(6%), 169(4%), 168(5%), 167(8%), 155(12%), 154(13%), 153(17%), 152(16%), 149(19%), 142(5%), 141(5%), 139(5%), 138(10%), 136(7%), 129(7%), 127(7%), 125(21%), 123(7%), 121(7%), 113(10%), 111(11%), 107(57%), 101(26%), 100(25%), 99(14%), 98(13%), 97(20%), 95(13%), 91(28%); 87(19%), 85(47%), 84(11%), 83(24%), 82(11%), 81(15%), 77(11%), 73(22%), 71(40%), 70(14%), 69(27%), 67(13%), 60(19%), 57(100%), 56(48%), 55(44%), 46(14%), 45(86%), 44(60%), 43(56%), 42(16%), 41(40%), 39(12%), 29(35%), 27(20%).

An aliquot of the ether extract was added to methanol and

the ultraviolet spectrum recorded, λ_{max} 315-20(sh), 328,344, 370-90(sh)nm.

The absorption spectrum (in water) of the aqueous phase after ether extraction showed a maximum at 255-60nm. This aqueous solution was not investigated further.

The material isolated in the ether extract was separated into neutral and acidic components using the following procedure. The solid was dissolved in ether and shaken with sodium bicarbonate solution (0.5M, 3 x 10cm^3). The ethereal solution was then washed with water (2 x 10cm^3) and evaporated to dryness, leaving an orange/yellow solid, the basic ether extract. The sodium bicarbonate solution was acidified with hydrochloric acid (6M) and extracted with ether (3 x 15cm^3). This ether extract was washed with water and evaporated to dryness leaving a pale yellow oil, the acidic ether extract.

Paper chromatography (Whatman No.1, descending) using 1-butanol:pyridine:water (45:25:40) as solvent, showed that both of the extracted materials contained a single component. The basic ether extract gave one strongly fluorescent spot at R_f= 0.9 and the acidic ether extract gave a yellow streak from R_f= 0.5 to 0.2. No other spots were located when the chromatograms were sprayed with bromophenol blue or alkaline silver nitrate solutions.

The ultraviolet spectra were recorded in methanol; basic ether extract λ_{max} 210-20, shoulders at 271,290,316,350nm; acidic ether extract λ_{max} 315-20(sh),330,347nm.

The mass spectra were determined. At 275° the compound obtained from the acidic ether extract was very similar to that of the original mixture (270°), however, at lower temperatures the spectrum had some features of the 178° spectrum, indicating that complete separation had not been achieved. The mass spectrum of the material from the basic ether extract was as follows :- (270°) m/e 355(3%),299(11%),255(5%),253(3%), 227(12%),226(4%),225(6%),219(7%),199(18%),183(3%),171(3%), 169(3%),167(5%),155(9%),154(7%),153(12%),149(21%),141(5%), 127(7%),125(19%),113(9%),111(7%),101(24%),100(25%),99(13%), 97(11%),95(10%),85(46%),83(15%),71(35%),69(13%),57(100%), 56(49%),55(20%),45(62%),43(30%),41(27%),29(16%). At 20eV a peak was visible at m/e 399.

No reaction was observed with either extract when treated with the following reagents:-2,4-dinitrophenylhydrazine, Benedict's reagent, ammoniacal silver nitrate and neutral ferric chloride (see Vogel(51) for the composition of all the reagents used here). Acidified sodium dichromate solution slowly became blue on warming and acidified potassium permanganate slowly became brown. Ninhydrin gave a positive reaction only with the acidic ether extract.

(2) Neutral hydrogen peroxide

Pigment (10mg) was dissolved in water (10cm^3) and hydrogen peroxide (30%, 0.5cm^3) was added. Thereaction mixture was placed in the dark at room temperature. After 22 hours an aliquot (5cm^3) was removed and extracted with ether (60cm^3). The aqueous phase was then acidified and again extracted with

ether ($60cm^3$). The remainder of the reaction mixture was left in the dark at room temperature. After a further 10 hours this was extracted, acidified and extracted again with ether in the same manner. The ultraviolet spectra of the ether extracted materials were recorded in methanol; neutral ether extract λ_{max} 208,260,330(sh),350(sh)nm, acid ether extract λ_{max} (i) 230(sh),312-19(sh),329,345,400(sh), (ii) 220(sh),315-17(sh),329,345nm. The peak at 329nm was more intense with respect to the 345nm peak in the acid ether extract (ii) than that in the first acid ether extract.

Mass spectra were recorded at 140° for these three extracted materials. They were all very similar to that obtained from the low temperature mass spectrum of the ether extracted material from the alkaline oxidation using hydrogen peroxide.

ix) Hydrolysis of the pigment

The pigment was hydrolysed using (a) hydrochloric acid and (b) sodium hydroxide.

(a) Hydrochloric acid

Pigment (10mg) was dissolved in water ($10cm^3$) and hydrochloric acid (2M,$1cm^3$) added. The absorption spectrum was determined and then the mixture was placed in the dark at 0° for 16 hours. No changes were visible when the absorption spectrum was again determined after this time interval. The above reaction mixture was then warmed to room temperature and kept at this temperature in the dark for 24 hours. The absorption spectrum was determined in water and again no changes were visible.

Acid hydrolyses were carried out under reflux conditions and a typical example is described. Pigment (12mg) was dissolved in water ($10cm^3$) and hydrochloric acid (6M, $5cm^3$) added. The mixture was refluxed for 24 hours. After cooling, the reaction mixture was extracted with ether (4 x $20cm^3$). The ether extract was not investigated further.

(b) Sodium hydroxide

A typical alkaline hydrolysis is described. Pigment (10mg) was dissolved in water ($10cm^3$) and sodium hydroxide (2M, $10cm^3$) added. The mixture was refluxed for 24 hours, acidified with hydrochloric acid and extracted with ether (4 x $20cm^3$). The ether extract was not investigated further.

Analysis of the aqueous hydrolysis solutions after ether extraction

The following methods were employed to determine the components of the aqueous phase after ether extraction :-

(i) spot tests
(ii) paper chromatography
(iii) column chromatography
(iv) distillation of alkaline solutions
(v) desalting of the alkaline hydrolysis mixture.

(i) Spot tests

The triphenyltetrazolium chloride reagent was prepared by the method of Stahl (50). All other reagents were prepared and reactions performed according to Vogel (51).

(ii) Paper chromatography

Whatman paper (3MM), prewashed with 50% aqueous ethanol,

was used for preparative chromatography and Whatman No.1 paper used on all other occasions. In all cases the descending technique was used. The locating reagents were prepared according to Sherma and Zweig (52) unless otherwise stated.

(a) Acid hydrolysis

When 2-methylpropan-2-ol:butan-2-one:water:ammonia (4:3:2:1) was used as solvent, spots were detected at R_f values of 0,0.12 and 0.45 using fluorescein as locating reagent. The spot at R_f= 0.45 remained white when sprayed with fluorescein and appeared dark under ultraviolet light. This spot was eluted with water and removal of the water left a whitish solid which did not melt below 300°: λ_{max} (H_2O) 260nm, mass spectrum 225° m/e 149(7%),135(11%),112(30%),82(7%),80(7%),72(7%),69(20%), 60(17%),57(9%),55(7%),45(46%),44(100%),43(24%),42(15%),41(11%), 37(100%),35(75%). Very large peaks also exist at m/e 38,36, 17 and 16.

When butan-1-ol:pyridine:water (9:5:4) was used as solvent three spots were visible (using alkaline silver nitrate as locating reagent) at R_f values 0.28,0.3 and 0.44. The spot at R_f= 0.28 remained white when sprayed with fluorescein and was dark when viewed under ultraviolet light. Elution of this spot with water gave a solid when the solvent was removed. This solid had properties similar to those of the previously eluted solid, but the absorption spectrum, in this case, had more structure, λ_{max} (H_2O) 256,260(sh)nm. Although the spectra were similar, no peak at m/e 135 was visible in this mass spectrum and peaks at m/e 79 and 56 were more prominent here.

(b) Alkaline hydrolysis

Using 2-methyl-propan-2-ol:butan-2-one:water:ammonia (4:3:2:1) as solvent, a chromatogram was developed of the aqueous solution from the alkaline hydrolysis. In addition to a spot at R_f= 0.45, a spot at R_f= 0.38 could be detected using alkaline silver nitrate. The spot at R_f= 0.45 was eluted with water. Removal of the solvent gave a white solid which did not melt below 300°; λ_{max} (H_2O) 262nm. The mass spectrum (220°) was very similar to that of the compound eluted from the chromatogram of the acid hydrolysis mixture, but here the peak at m/e 135 did not appear until the spectrum was recorded at a higher temperature, 300°.

(iii) Column chromatography

The aqueous solution from the acid or alkaline hydrolysis was applied to a column of Sephadex G 10 (7.0 x 2.5cm). Water was used as eluant. Fractions were collected, tested for the presence of chloride ions and the absorption spectra recorded. The water was then removed under reduced pressure.

(a) Acid hydrolysis

Fraction 1. White solid, m.p. 300°, contains some chloride, λ_{max} (H_2O) 195nm, mass spectrum (150°) m/e 82,80,44, with large peaks at m/e 35,36,37 and 38.

Fraction 2. (contains much chloride ion) Yellow solid and some oil, λ_{max} (H_2O) 250(sh),256,260(sh)nm, mass spectrum (150°) m/e 82,80,79,55,52,44,39, with large peaks at m/e 35,36,37 and 38. (258°) m/e 79,52,44.

Fraction 3. Yellow solid, λ_{max} (H_2O) 260nm, mass spectrum m/e (150°) 82,80,44,38,37,36,35, (250°) 112,69,44,42,

38,36,35,17.

(b) Alkaline hydrolysis

Fraction 2. (contains chloride ions) λ_{max} (H_2O) 249(sh), 254,260(sh), $\lambda_{max}^{OH^-}$ 243,249,255,262nm, mass spectrum (150°) m/e 80(7%),79(100%),78(14%),52(72%),51(24%), 50(13%),39(9%),38(31%),36(92%),17(71%).

Other fractions. λ_{max} (H_2O) 256-60nm.

(c) Acid hydrolysis (pigment from frozen plasmodia)

Pigment from frozen plasmodia was treated in the same manner as above, after acid hydrolysis. The fraction from the Sephadex column which contained chloride ions was subjected to further chromatography on a Sephadex G10 column (6 x 1.5cm) using water as solvent. Fractions were collected and examined.

Fraction 3. (contains some chloride ions) λ_{max} (H_2O) 262nm, mass spectrum (250°) 113(7%),112(64%),101(11%), 100(20%),85(7%),74(10%),73(13%),69(43%),68(10%),56(10%), 55(25%),45(100%),44(55%),43(23%),42(32%),41(12%), 40(10%),38(43%),36(31%),31(12%),29(22%),27(23%),19(14%), 17(10%).

Fraction 4. contained most of the chloride ions and had the same mass spectrum as the previous acid hydrolysis fraction 3.

(iv) Distillation of alkaline solutions

The pigment was hydrolysed using sodium hydroxide and, after acidification, extracted with ether. Sodium hydroxide (2M) was added until the solution was alkaline and the mixture heated until approximately half of the solvent had

distilled over. To the distillate hydrochloric acid was added (2M, 1 drop) and the solvent removed. The solid remaining did not melt below 300°. λ_{max} (H_2O) 250(sh),256,262(sh)nm. Mass spectrum (238°) m/e 185(4%),149(4%),141(4%),101(9%), 96(4%),86(35%),83(7%),81(4%),80(22%),79(100%),78(42%),75(7%), 71(11%),70(7%),69(7%),58(27%),57(17%),56(7%),55(10%),53(29%), 52(75%),51(87%),50(50%),49(9%),44(12%),43(12%),42(7%),41(12%), 39(35%),38(60%),37(12%),36(100%),35(29%),30(21%),27(21%), 26(26%),17(100%),16(17%).

The experiment was repeated using pigment from frozen plasmodia. The absorption spectrum of the distillate was recorded, λ_{max} (H_2O) 260nm. The distillate was extracted with ether and the ether extract evaporated to dryness. A very small volume of yellow oil remained, λ_{max} (CH_3OH) 260nm. Mass spectrum (150°) m/e 219(30%),211(10%),209(11%),207(11%), 205(11%),203(10%),199(10%),197(13%),195(15%),193(15%),191(13%), 183(17%),181(19%),179(18%),177(13%),175(10%),169(18%),167(26%), 165(28%),163(20%),161(11%),155(19%),153(34%),152(17%),151(33%), 150(13%),149(45%),141(30%),139(45%),138(23%),137(36%),136(16%), 135(25%),127(32%),126(13%),125(75%),124(30%),123(38%),122(15%), 121(19%),113(40%),112(23%),111(98%),110(40%),109(47%),108(17%), 100(12%),99(49%),98(26%),97(100%),96(53%),95(63%),94(24%), 93(13%),91(19%),85(70%),84(34%),83(68%),82(38%),81(60%),80(15%), 79(21%),76(12%),71(74%),70(28%),69(36%),67(34%),57(85%),56(36%), 55(41%),43(30%),41(51%),39(12%),17(22%).

The experiment was repeated using acid hydrolyses of the pigment and similar results were obtained.

(v) Desalting of the alkaline hydrolysis mixture

After alkaline hydrolysis, the solution was acidified and extracted with ether. The aqueous solution was concentrated and sodium hydroxide added until the solution was only just acid (pH 6). The solution was desalted using pyridine by the method of Saini(46). The mass spectrum of the solid obtained was determined, (255°) m/e 149(5%), 140(5%),125(5%), 112(8%),101(5%),88(6%),87(20%),86(7%),79(32%),78(9%),73(10%), 69(8%),60(34%),59(29%),58(17%),57(10%),56(5%),55(5%),51(8%), 46(16%),45(21%),44(71%),43(32%),42(13%),41(12%),38(34%), 36(100%),31(12%),30(21%),29(13%),27(12%). Also present were very large peaks at m/e 17 and 16 (200 and 99% respectively).

x) Determination of the molecular weight of the pigment from oxidation reactions

(a) Determination of the absorbance of 2,4,6,8-decatetraene-1,10-dioic acid (XV) at varying concentrations in aqueous solutions

2,4,6,8-decatetraene-1,10-dioic acid (10.36mg) was dissolved in sodium hydroxide (1.0M, 1.5cm^3) and the solution made up to 100cm^3 with water. The absorbance of this solution was measured at 331 and 348nm at various dilutions. The results are shown in Fig. 6, p. 39.

(b) Determination of the absorbance of sodium metaperiodate at varying concentrations in aqueous solutions

Sodium hydroxide (1.0M, 6cm^3) was added to a sodium metaperiodate solution (9.37 x 10^{-2}M, 6cm^3) and the mixture made up to 50cm^3 with water. The absorbance of this solution and dilutions of this solution was measured at 331 and 348nm.

(c) Reaction of the pigment with sodium metaperiodate

(1) Pigment (11.6lmg) was dissolved in water ($5cm^3$) and sodium metaperiodate solution ($9.37 \times 10^{-2}M$, $6cm^3$) added. The reaction mixture was allowed to stand in the dark, at room temperature, until the yellow colouration had disappeared (approx. 50 hours). Sodium hydroxide (1M, $0.5cm^3$) was added and the reaction mixture made up to $50cm^3$ with water. The absorbances of this and dilutions of this solution were measured at 331 and 348nm. After correcting for the absorbance of the sodium metaperiodate, the amount of 2,4,6,8-decatetraene-1,10-dioic acid formed during the reaction was calculated.

The reaction mixture was acidified with hydrochloric acid (2M) and extracted with ether ($5 \times 10cm^3$). The ether extract was washed with water ($20cm^3$) and the ether then removed under reduced pressure. The solid remaining was dissolved in sodium hydroxide (1M, $0.25cm^3$) and the solution made up to $50cm^3$ with water. The ultraviolet spectrum was determined in water, λ_{max} 195-205,263-5(broad),310-20,331(sh)nm.

The ultraviolet spectrum of the aqueous solution, after ether extraction, was determined λ_{max} 197,253-68(shallow and broad)nm.

(2) Sodium metaperiodate ($9.37 \times 10^{-2}M$, $0.1cm^3$) was added to an aqueous solution of the pigment (absorbance = 1.17 at 380nm, $10cm^3$) and the reaction followed spectrophotometrically. Under these conditions,only shoulders appeared at 330 and 350nm. The change in absorbance at 350nm with time is given in Table 5.

TABLE 5.

Reaction of pigment solution with sodium metaperiodate

Time (hours)	Absorbance (at 350nm)
0	1.28
3.0	1.12
6.75	0.98
23.25	0.63
27.0	0.58
30.0	0.54
49.0	0.43

(d) Reaction of 2,4,6,8-decatetraene-1,10-dioic acid (XV) with sodium metaperiodate

To an aqueous, alkaline solution of compound (XV) (0.4mg, 10cm^3), sodium metaperiodate solution (9.37×10^{-2}M, 0.1cm^3) was added. The absorption spectrum of the reaction mixture was recorded over a time interval of two days. Decomposition of (XV) was followed by measuring the decrease in the intensity of absorption at 330nm, Table 6.

TABLE 6.

Time (hours)	Absorbance (at 330nm)
0	1.32
3.0	1.15
5.25	1.06
19.75	0.58
24.0	0.50
44.0	0.375

(e) Reaction of 2,4,6,8-decatetraene-1,10-dioic acid (XV) with hydrogen peroxide

The reaction of (XV) with hydrogen peroxide was followed by measuring changes in the absorption spectrum.

Compound (XV , 2mg) was dissolved in sodium hydroxide (2M, $0.2cm^3$) and water added to give a total volume of $50cm^3$. Appropriate dilutions of this solution were used for these experiments. In each case, a solution of (XV , $10cm^3$) was placed in the vessel and hydrogen peroxide (30%, $0.5cm^3$) added. Three types of reaction vessel were used:-

(1) Pyrex glass flask

(2) Nalgene tube

(3) quartz flask.

The reactions were carried out in the dark at room temperature. The results are given in Table 7, Fig. 7.

TABLE 7.

Reaction of (XV) with hydrogen peroxide

Pyrex flask		Nalgene tube		Quartz flask	
Time (hr)	Absorbance (330nm)	Time (hr)	Absorbance (330nm)	Time (hr)	Absorbance (330nm)
0	1.37	0	1.33	0	1.25
3.0	1.29	3.0	1.28	17.5	1.10
5.5	1.19	19.0	1.07	21.5	1.09
21.0	0.79	23.5	1.05	25.5	1.05
25.5	0.69	44.0	0.80	40.5	0.94
48.5	0.33	48.0	0.76	45.5	0.89
5 days	*			48.5	0.885

* No maxima visible.

The reaction was repeated using a quartz vessel and hydrogen peroxide at a lower concentration (3%, $0.5cm^3$). The rate of polyene decomposition was not decreased.

(f) Reaction of pigment with hydrogen peroxide

Pigment (10.5mg) was dissolved in water ($9cm^3$) and

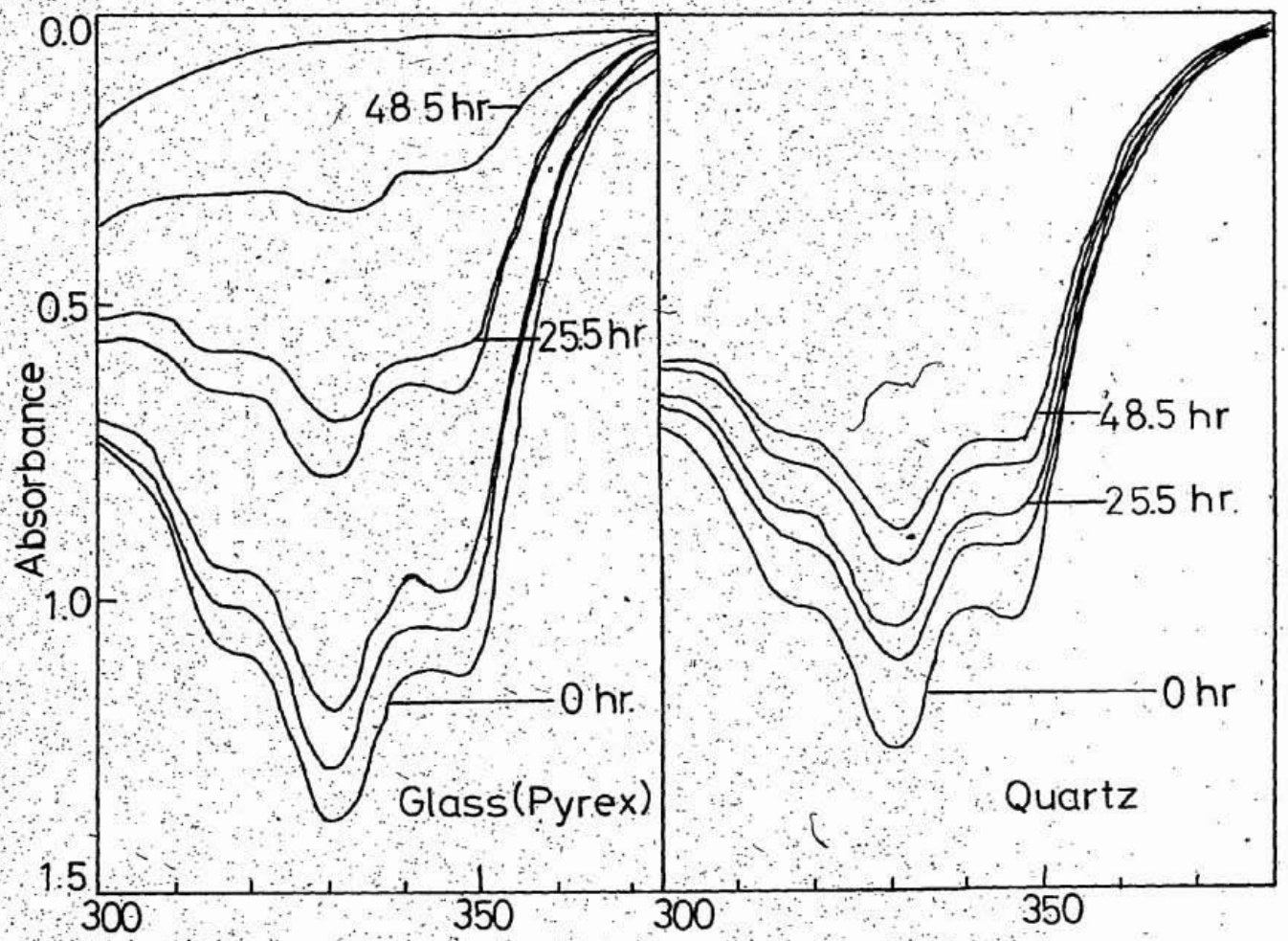


Figure 7. Reaction of (XV) with hydrogen peroxide

hydrogen peroxide added (30%, $1cm^3$). The reaction mixture was kept in the dark at room temperature. All reactions of the pigment with hydrogen peroxide were carried out in the quartz vessel. An aliquot of the reaction mixture ($0.5cm^3$) was removed and diluted, with water, to $10cm^3$. A portion of this solution ($4cm^3$) was diluted to $10cm^3$ and the absorption spectrum recorded. Thereafter, aliquots ($0.5cm^3$) were removed at intervals, diluted to $10cm^3$ and the absorption spectrum recorded, Table 8.

TABLE 8.

Reaction of pigment with hydrogen peroxide (30%, $1cm^3$)

Time (hr)	Absorbance		
	380nm	348nm	331nm
*0	0.70	0.54	0.40
3.25	1.40	1.32	1.01
5.75	1.08	1.245	1.02
7.75	0.86	1.205	1.06
9.0	0.76	1.16	1.06
10.0	0.67	1.13	1.03
19.0	0.34	0.96	0.99

The reaction was repeated using pigment (11.3mg) dissolved in water ($8cm^3$) to which a larger volume of hydrogen peroxide (30%, $2cm^3$) was added. Initially, and after one hour, aliquots ($0.5cm^3$) were removed, diluted 40 times and the ultraviolet spectra recorded. At subsequent time intervals, aliquots ($0.5cm^3$) were removed, diluted to $10cm^3$ and the absorption spectra recorded, Table 9, Figure 8.

*Initially the reaction mixture was diluted to measure the absorbance.

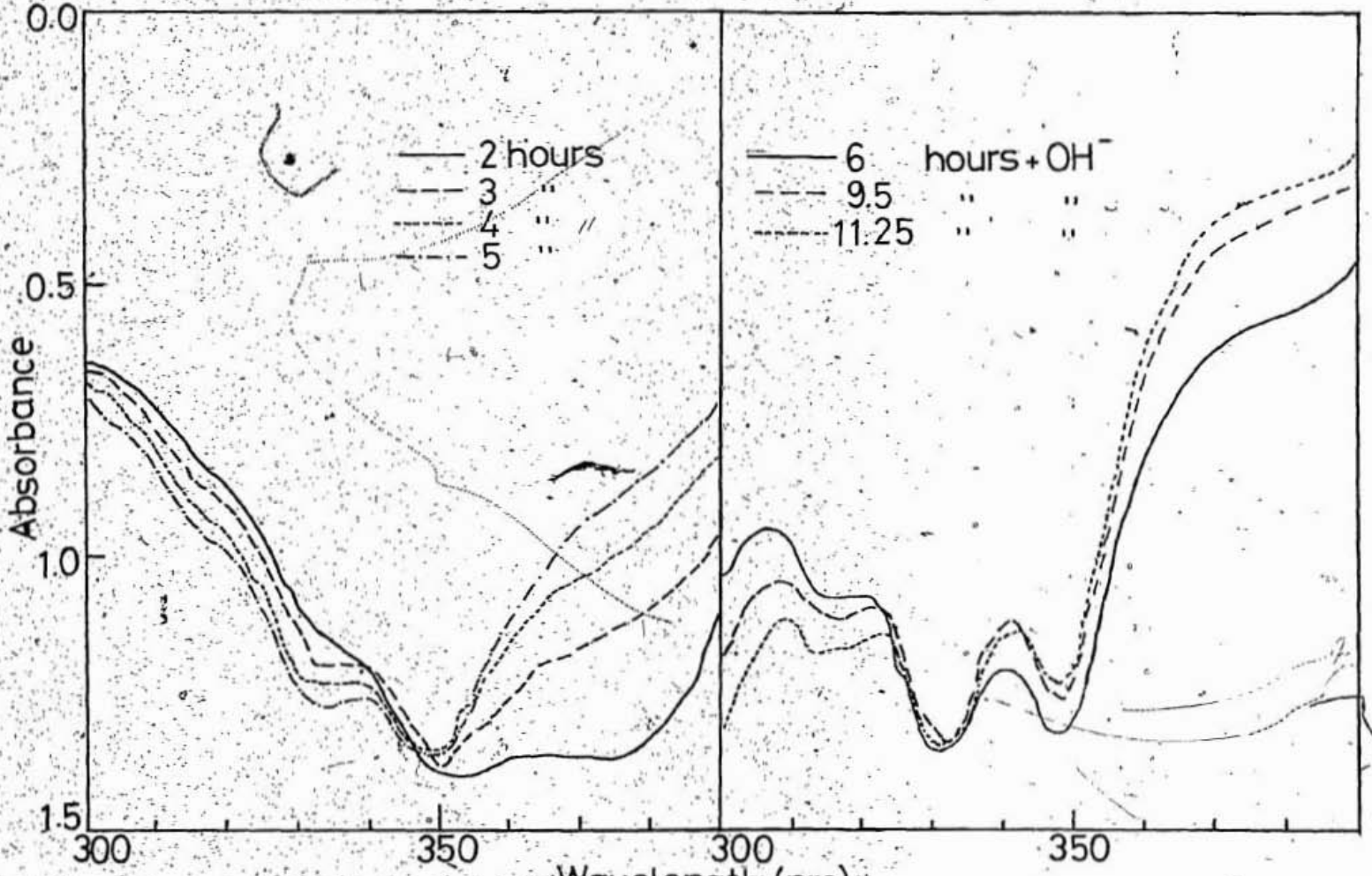


Figure 8. Reaction of pigment with hydrogen peroxide (30%, 2cm^3)

TABLE 9.

Reaction of pigment with hydrogen peroxide (30%, 2cm^3)

Time (hr)	Absorbance		
	380nm	348nm	331nm
0	0.90	0.68	0.48
1.0	0.79	0.69	0.53
2.0	1.33	1.39	1.11
3.0	1.10	1.35	1.15
4.0	0.95	1.38	1.22
5.0	0.85(0.66)*	1.38(1.41)	1.25(1.42)
6.0	0.70(0.55)	1.30(1.32)	1.23(1.35)
7.0	0.60(0.39)	1.27(1.30)	1.22(1.38)
9.5	0.45(0.37)	1.20(1.25)	1.18(1.33)
11.25	0.37(0.32)	1.17(1.23)	1.18(1.35)

*Absorbances given in parentheses are of solutions to which sodium hydroxide (2M, 1 drop) was added.

The concentration of the hydrogen peroxide was again increased (30%, 5cm^3) in a third experiment with the pigment solution. Aliquots were removed (1cm^3), diluted to 100cm^3 and the absorbance measured, Table 10.

TABLE 10.

Reaction of pigment with hydrogen peroxide (30%, 5cm^3)

Time (hr)	Absorbance		
	380nm	348nm	331nm
0	0.435	0.33	0.23
1.5	0.325	0.35	0.30
2.5	0.27	0.37	0.33
3.5	0.17(0.14)*	0.33(0.35)	0.32(0.38)
5.0	0.14(0.10)	0.32(0.33)	0.32(0.38)
6.5	0.12(0.10)	0.33(0.36)	0.35(0.41)

*Absorbances given in parentheses are of solutions to which sodium hydroxide (2M, 1 drop) was added.

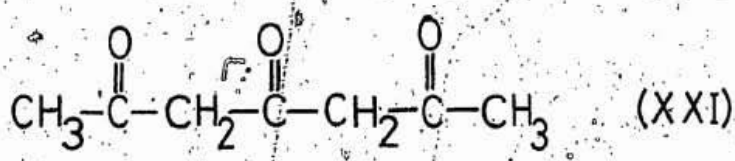
To pigment solution (absorbance at 380nm = 1.49, 1cm cell; 10cm^3), hydrogen peroxide at a lower concentration (0.3%, 0.5cm^3) was added. The resulting reaction was very slow (some pigment remaining unreacted after 4 days) and no peaks at 331 or 348nm were visible at any time.

Part II SYNTHESIS OF MODEL COMPOUNDS

INTRODUCTION

The structure (I), suggested by Khalil to be the chromophore of the pigment, is degraded by acid and alkali hydrolysis and also by oxidation and reduction. This high reactivity towards reagents normally used to degrade and hence identify large molecules would make the moiety difficult to isolate. This difficulty has been found by workers when determining the structures such as aureothin (III, ref. 26). An investigation of model compounds was undertaken in order to study systems similar to (I). The aim of this work was to prepare a model system having "indicator" properties similar to the pigment, which would show a shift of absorption maximum from 380 to 410nm on acidification.

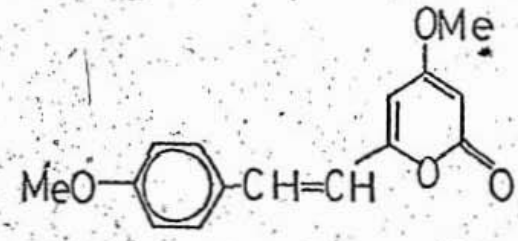
It has been suggested that many aromatic natural products are derived from head to tail condensation of acetic acid units via β-polycarbonyl intermediates (53). Observation that some natural products, for example orcinol (XX) and the triketide diacetylacetone (XXI), can be formed from dehydroacetic acid (54) suggested that 2,4-pyranone systems can be regarded as protected β-polycarbonyl compounds. The instability of unprotected β-polycarbonyl compounds has lead to a wide investigation of the synthesis and properties of 2,4-pyranone systems.



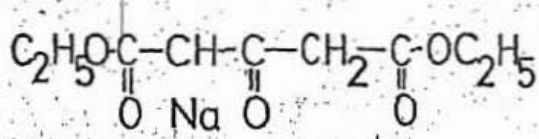
(XXI)

Synthesis of some 4-hydroxy-2-pyrones has been achieved by condensing aromatic aldehydes (30,55,56,57), acyl chlorides (58,59) or alkyl chlorides (60,61) with the pyrone, or by condensing an acid chloride with substituted 3-oxo-penta-1,5-dicarboxylic acids and then cyclising the products (62). An alternative procedure, used by Suzuki et al. (63), involved the reaction of Grignard reagents with ethyl methylmalonyl chloride, which on further treatment and cyclisation yielded alkyl substituted 4-hydroxy-2-pyrones. These compounds were then suitable for the synthesis of luteoroticulin (VII), citreoviridin (V) and aureothin (III).

Yangonin (XXII) has been synthesised by condensation of

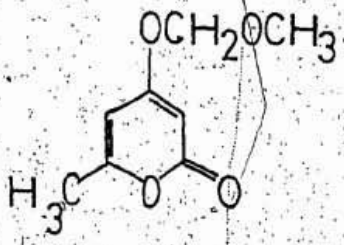


(XXII)

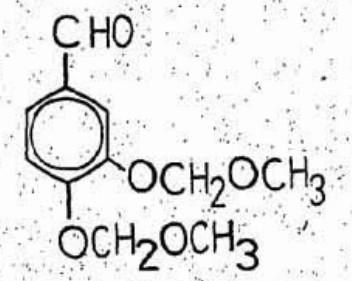


(XXIII)

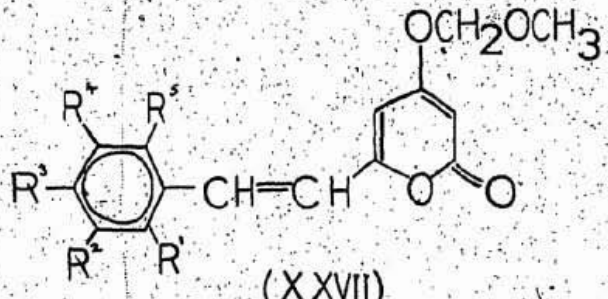
p-methoxycinnamoyl chloride with (XXIII), followed by cyclisation and then ether formation (64,65). Bu'Lock and Smith (57) prepared yangonin by refluxing 4-methoxy-6-methyl-2H-pyran-2-one (XXIV) and p-methoxybenzaldehyde in the presence of magnesium methoxide. Hispidin (VI) was synthesised in a similar manner by the condensation of either (XXIV) or (XXV) with (XXVI), followed by hydrolysis of the ether with sulphuric acid (56). Using



(XXV)



(XXVI)

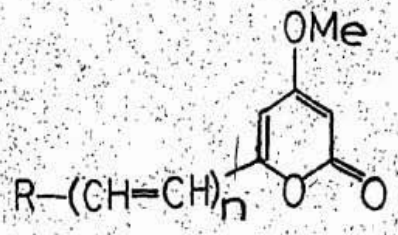


(XXVII)

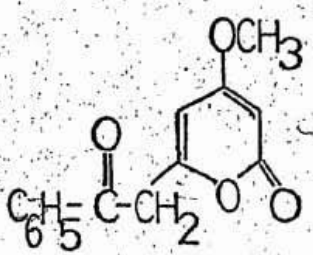
this method, twenty four substituted 6-styryl-2-pyrones (XXVII) were prepared by Edwards and Mir in 1967 (66).

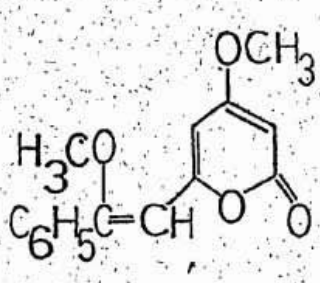
Further reactions of (XXIV) have been reported by Mir, Ahmad and Razaq (67), who prepared the following series of compounds.

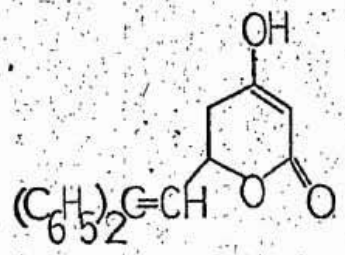
R	n
phenyl	2
2-furyl	2
2-thienyl	1
2-furyl	1
2-pyridyl	1
3-pyridyl	1



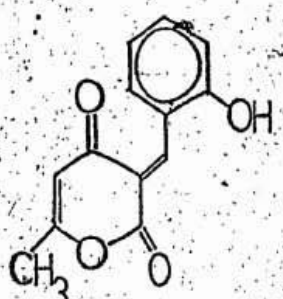
Wachter and Harris (61) have reported reactions of triacetic acid lactone (XXVIII) at the 6α position in liquid ammonia. Treatment with alkali amides yielded the dianion which reacted with alkyl halides and benzophenone to form derivatives, three of which are shown below.



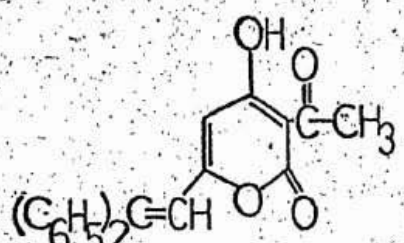




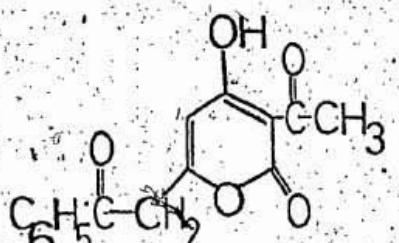
Formation of (XXIX) in low yield was reported by Scott et al. (68) from the reaction of triacetic acid lactone with salicylaldehyde in liquid ammonia. No absorption data were recorded.



(XXIX)



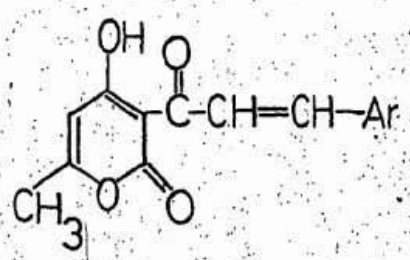
(XXX)



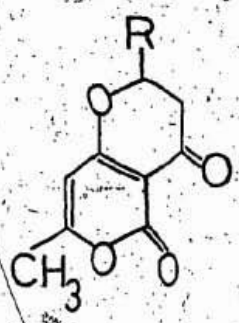
(XXXI)

Reactions at the 6α position of dehydroacetic acid (XVIII) in liquid ammonia were reported in 1968 (60) and the preparation of (XXX) and (XXXI) are two of the syntheses described. Base catalysed condensation of the acetyl function of dehydroacetic acid with aromatic aldehydes (using piperidine as the

base) gave substituted 3-cinnamoyl-4-hydroxy-6-methyl-2-pyrones (XXXII, ref. 55). However, aliphatic aldehydes, under the same conditions, cyclised to form dipyrones (XXXIII, ref. 69).

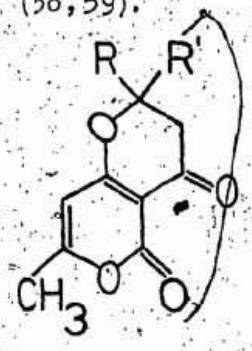


(XXXII)

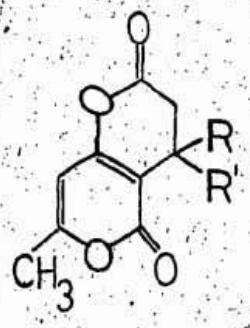


(XXXIII)

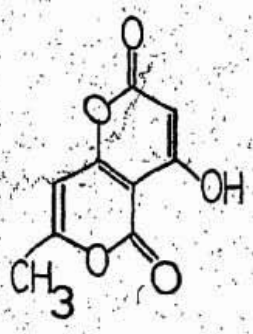
Triacetic acid lactone, when reacted with α,β-unsaturated acyl chlorides, also formed cyclic products (XXXIV) and (XXXV) (58,59).



(XXXIV)



(XXXV)



(XXXVI)

The product of the reaction of malonyl chloride with triacetic acid lactone was reported to be (XXXVI) (70).

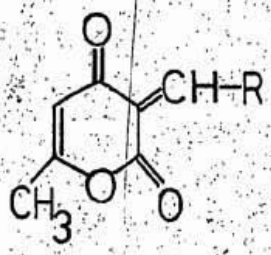
Many other derivatives of dehydroacetic acid and triacetic acid lactone have been prepared and some examples of these can be found in reviews by Gren (38), Mors et al. (36) and Harris-

et al. (39). Those reactions mentioned above are representative of the general types of conditions employed in these types of syntheses.

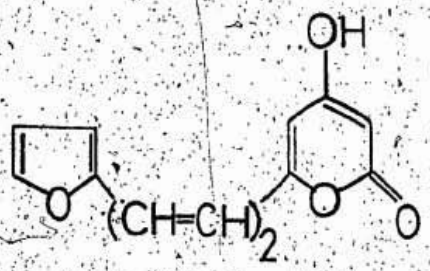
In most of the reported cases, even when 4-hydroxypyrones were prepared, no information was given concerning changes in absorption maxima on the addition of acids or bases. The absorption maxima of some pyrones are given in Table 11.

In this work, attempts have been made to prepare derivatives of triacetic acid lactone, (XXXVII) and (XXXVIII), in order to study the spectral shifts that occur with changes in the acidity of the medium.

R= aliphatic



(XXXVII)



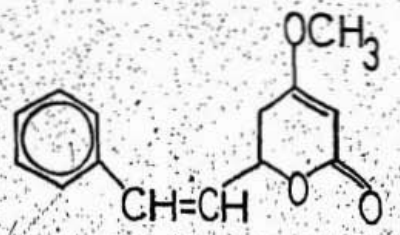
(XXXVIII)

TABLE 11

Absorption maxima of some pyrones

Name	Compound	λ_{max}(EtOH)* (nm)	log ε	Ref.
Anibine	II	315	4.09	24
Aureothin	III	346	4.27	25
Aurovertin B	IV	372	4.54	26
Citreoviridin	V	388	4.68	27
Helipyrone	XXXIX	294	4.16	74
Hispidin	VI	367	4.38	28
Kawain	XL	291	3.04	36
Yangonin	XXII	257	4.48	36
4-methoxy-pyran-2-one	XXIV	280	3.76	57
2-methoxy-pyran-4-one	XLI	240	4.17	57
Dehydroacetic acid	XVIII	310	4.03	71

*longest wavelength only given.



(XL)

RESULTS AND DISCUSSION

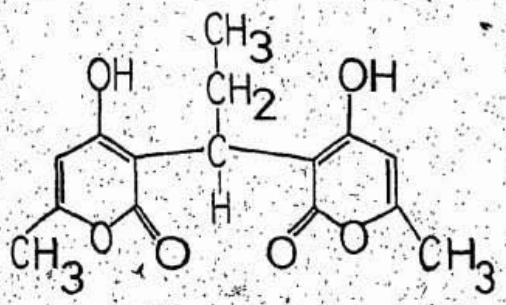
Triacetic acid lactone was first prepared by Collie in 1891 (72) by deacetylating dehydroacetic acid. The properties of the product obtained in this laboratory are in good agreement with those obtained by previous workers (70).

i) Reactions of triacetic acid lactone at C3

The Knoevenagel type condensation of triacetic acid lactone with benzaldehyde, propanal and butenal did not give compounds of the type expected (XXXVII). The ultraviolet spectra showed that no further conjugation had been introduced into the molecule, since the absorption maxima (288, 289 and 285nm respectively) were approximately the same as that of triacetic acid lactone itself (280nm), this value being typical for a pyran-2-one system. However, the product obtained with propanal (XLII) did show a slight bathochromic shift on acidification (7nm) and this was accompanied by an increase in intensity ($\log \epsilon = 4.26 \rightarrow 4.32$). These properties are also inherent to the pigment, although both increases in wavelength and intensity were much smaller than those shown by the pigment.

The 6H singlet (2 x CH_3 at C6) at $\tau = 7.8$ in the n.m.r. spectrum of the compound from the propanal reaction (XLII) indicates that two pyrone functions are combined with one propanal molecule (3H(t) $\tau = 9.15(CH_3)$, 2H(m) $\tau = 7.8(CH_2)$, 1H(t) $\tau = 5.99(CH)$). The C5 proton ($\tau = 4.0$) of the pyrone ring is a singlet, the coupling with the hydrogen at C3 having

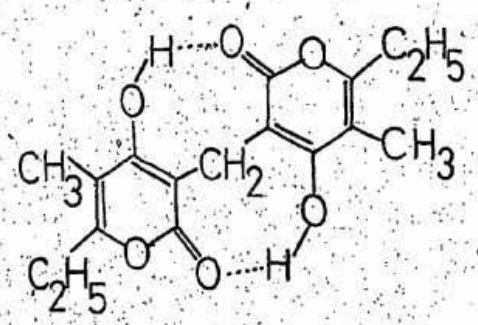
been removed, showing that substitution has taken place at C3. Compound (XLII), 3:3'-di-(4-hydroxy-6-methyl-pyran-2-one)-1-propane has the structure :-



(XLII)

The mass spectrum has the molecular ion at m/e 292 which corresponds to the formula of $C_{15}H_{16}O_6$ of (XLII). Other major ions are present at m/e 263 (M-CH_2CH_3), 221 (M-71, either -C_2H_5 -C_2H_2O; or -CO, -C_2H_3O), 205 (M-87, -C_2H_5, -CH_3, -C_2H_3O), 179 (221-C_2H_2O), 166 (M-127, 127= protonated triacetic acid lactone), 151 (179-CO), 137 (179 -C_2H_2O).

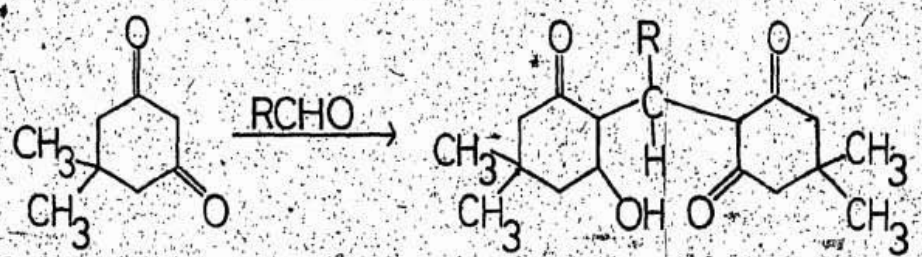
The infrared spectrum has a band consistent with intramolecularly hydrogen bonded 4-hydroxy-2-pyrones (73,74) at 1680cm^{-1} (C=O), and a band consistent with alkene groups at 1620cm^{-1}. A broad band at 3400-2600cm^{-1}, and the absence of bands above 3500cm^{-1}, shows that the 4-hydroxy groups are completely hydrogen bonded. This type of structure has been assigned to helipyrone (XXXIX) which is isolated from Helichrysum italicum (74) and prepared by the condensation of 4-hydroxy-5-methyl-6-ethyl-2H-pyran-2-one with formaldehyde, (75).



(XXXIX)

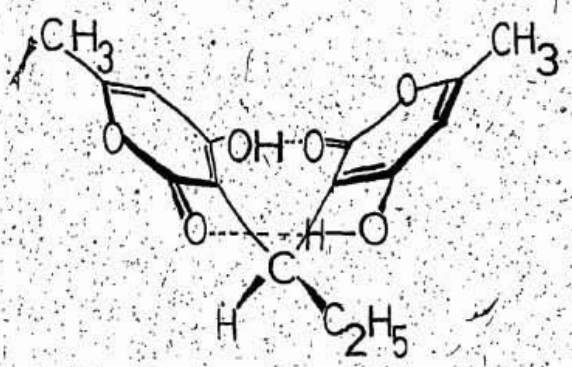
A similar reaction has been reported by Henbest and Jones (76) between 5:6-dihydro-4-hydroxy-6-phenylpyran-2-one and formaldehyde giving 3:3'-methylene-di(5:6 dihydro-4-hydroxy-6-phenylpyran-2-one). The same product was obtained even in the absence of a catalyst. It was found here, that, even when an excess of aldehyde was used and the lactone slowly added to a cold reaction mixture, no product formed from the condensation of one aldehyde molecule with one lactone molecule.

5:5-dimethyldihydroresorcinol (XLIII) also forms adducts of this type (XLIV) with aldehydes using piperidine as the catalyst (76).



(XLIII) (XLIV)

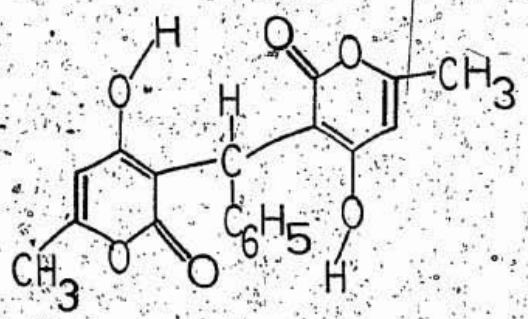
The structure of (XLII) can be more accurately represented by :-



Molecular models (Dreiding) show that the presence of the ethyl group does not sterically hinder hydrogen bonding betweenthe hydroxyl and carbonyl groups.

The compound formed from the reaction between benzaldehyde and triacetic acid lactone, 3:3'-di-(4-Hydroxy-6-methylpyran-2-one)phenyl methane (XLV) has the same type of structure as (XLII). The n.m.r. spectrum is again consistent with two pyrone molecules linked to one aldehyde function (6H singlet $\tau = 7.76$ ($2 \times CH_3$) and the broadened 5H singlet at $\tau = 2.83$ (aromatic protons)). Reaction has again occurred at the 3 position of triacetic acid lactone since the hydrogen at C5 is uncoupled ($\tau = 4.0$). The singlet at $\tau = 4.3$ is derived from the hydrogen at the carbon bridge. The hydroxyl groups are hydrogen bonded (broad singlet $\tau = -0.3$). The signal at $\tau = 6.57$ is probably due to water impurities.

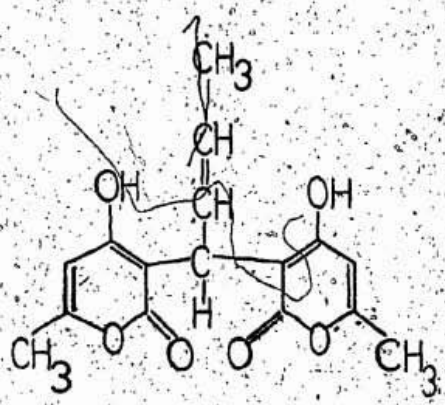
The signals at τ = -0.3 and 6.57 both disappear when the solution is shaken with D_2O. The broad band 3400-2500cm^{-1} in the infrared spectrum also indicates that hydrogen bonding is complete. No free hydroxyl band appears in the infrared spectrum on dilution, showing that the hydrogen bonding is intramolecular. The structure of compound (XLV) consistent with these results is shown below :-



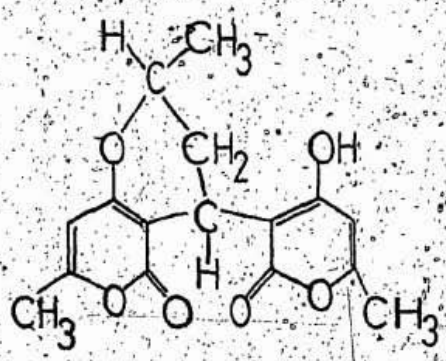
(XLV)

The mass spectrum has a molecular ion m/e 340, which agrees with the above formula and the other peaks are also consistent with structure (XLV). This compound has been previously prepared by Douglas and Money (78) from triacetic acid lactone and benzaldehyde in pyridine with added acetic acid and piperidine. The data given here are in good agreement with those reported. However, the procedure used by Douglas and Money yielded only 20mg of product from triacetic acid lactone (3g) and so the procedure used here is a more viable synthetic method for the preparation of (XLV) and other derivatives from aromatic and saturated aliphatic aldehydes.

The product of the reaction of butenal with triacetic acid lactone, while similar to (XLII) and (XLV), shows some differences in both the n.m.r. and infrared spectra. The mass spectrum of the product shows the molecular ion to be m/e 304, which corresponds to the expected formula $C_{16}H_{16}O_6$. Two possible structures (XLVI) and (XLVII) can be drawn to represent the product. Structure (XLVI) would be the product expected of a reaction analogous to that of benzaldehyde or propanal, whereas (XLVII) is derived from a further intramolecular alkylation of the hydroxyl group at the 4 position.



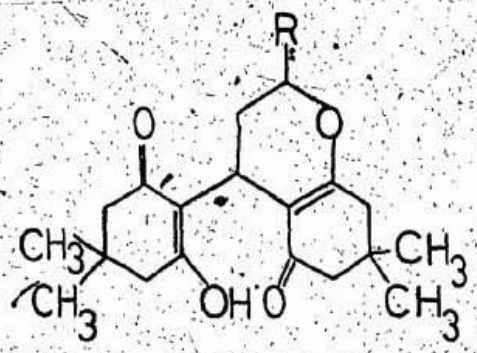
(XLVI)



(XLVII)

The n.m.r. spectrum indicates that the two C6 methyl groups (τ = 7.83) and the two hydrogens at C5 of the pyrone ring (τ = 4.02 and 4.24) are non-equivalent. The doublet of multiplets, of one hydrogen intensity at τ 4.68 is consistent with that expected for the hydrogen on the carbon of the cyclic ether linkage of (XLVII). Signals consistent with the two

hydrogen atoms of the alkene function in (XLVI) are not observed. Only one hydrogen bonded hydrogen could be detected (1H at $\tau = -1.10$, which was removed by shaking the solution with D_2O). The infrared spectrum does not have the broad hydrogen bonded band shown by compounds (XLII) and (XLV). On dilution, the bands at 3650 and 3400cm^{-1} are replaced by a band at 3650cm^{-1}, indicating that the hydrogen bonding is predominantly intermolecular. Horning and Horning (77) suggested that the product of the reaction between (XLIII) and α,β-unsaturated aldehydes has the structure (XLVIII), although no evidence was given to support this.



(XLVIII)

The evidence available suggests that the product of the reaction between butenal and triacetic acid lactone is (XLVII).

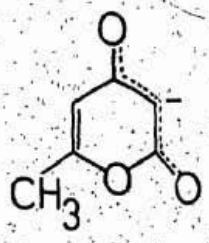
ii) Reaction of the acetyl group of dehydroacetic acid

Although the reaction conditions used with dehydroacetic acid and propanal were those used by Wiley et al. (55) to prepare derivatives of dehydroacetic acid with aromatic aldehydes, no product could be identified in this case. Most of the dehydroacetic acid remained unreacted. However, the react-

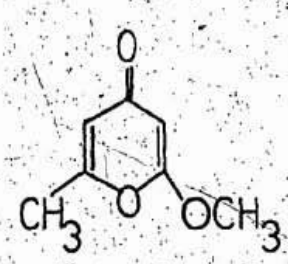
ions of aliphatic aldehydes which have been reported (69). I have given rise to cyclic products (XXXIII) which would not be expected to show changes of absorption with changing acidity.

iii) Methylation of triacetic acid lactone

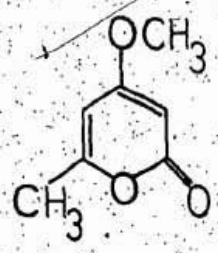
Activation of the methyl group at C6 of triacetic acid lactone, either by enolisation or ionisation, is necessary for reaction at this position. Under acidic conditions the stable pyrylium ion is formed and reaction does not take place at C6. In basic conditions the ionisation of the 4-hydroxyl group is favoured because it is more acidic than the 2-hydroxyl group (70). Conversion of triacetic acid lactone to its methyl ether prevents the formation of the enolate ion (XLIX) and reduces the reactivity of C3 towards electrophilic attack. Reaction under basic conditions then takes place at the methyl group at C6.



(XLIX)



(XLI)



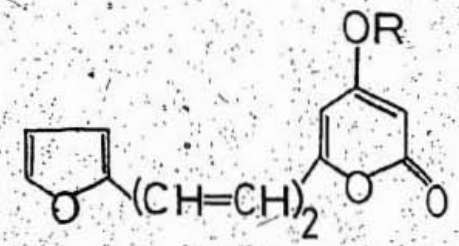
(XXIV)

Treatment of triacetic acid lactone with diazomethane has been shown (57,78) to yield both the 2-methoxypyran-4-one (XLI) and the 4-methoxypyran-2-one (XXIV). However, methylation with dimethyl sulphate forms only the 4-methoxy compound (57). The absorption maximum found here for the methylation product (280nm) agrees with the formation of only (XXIV).

The carbonyl absorptions in the infrared spectrum (1740 and 1715cm^{-1}) are also consistent with a pyran-2-one system (57).

iv) Reaction of 4-methoxy-6-methyl-2H-pyran-2-one (XXIV) at the 6 position

The product of the reaction between 3(2-furyl)-propenal and (XXIV) was established by Mir et al. to be 4-methoxy-6-(4-(2-furyl)buta-2,4-dienyl)2H-pyran-2-one (L, ref.67).



R = CH_3 (L)
R = H (LI)

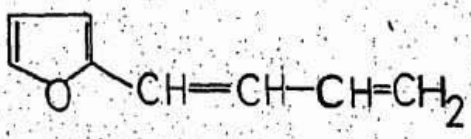
The yield obtained by Mir was much higher than that obtained here, although the slightly lower melting point quoted (195-7° compared to 199-202°) could indicate that a material of lower purity was obtained by these workers. The infrared spectra are in good agreement, in both cases the presence of a band at approximately 990cm^{-1} indicating the presence of trans double bonds. The n.m.r. spectrum, while showing clearly the OCH_3 and the C3 and C5 hydrogens of the pyrone ring at τ = 6.22, 4.59 and 4.19 respectively, is complex in the region τ = 4.1 to 2.5 because of overlap of the olefinic and furan hydrogen peaks. The ultraviolet absorption maximum found here differs from the value quoted by 24nm (360nm (lit.) and 384nm), however, the solvent used in the literature is not stated.

v) Demethylation of 4-methoxypyran-2-ones

While many hydroxy pyrones have been methylated, very few examples of the removal of the methyl group of methoxy pyrones have been reported. Aureothin (III) was demethylated using hydrochloric acid by Hirata et al.(26). However, it was not found possible to demethylate isoaureothin (the 4-methoxy-pyran-2-one isomer) using this method. The presence of the furan ring in the compound (L) precludes the use of acids for ether cleavage. The method of Prey(80) used for the demethylation of methyl ethers when mild conditions are necessary, failed to cleave the ether in this case.

The method of Yamamura et al. (81) gave good results with (XXIV), although separation of product and unchanged reactant proved difficult. In order to improve the separation of the products in the ethyl acetate extract of the reaction between aluminium chloride and (L) a Sephadex column was employed. Care was taken to exclude light from all solutions and the solid material. If this precaution was not taken, decomposition was seen to take place, a black compound being formed on prolonged exposure. It is known that pyran-2-one compounds undergo photochemical reactions in the solid state (35).

The absorption maximum of (L) and (LI) at 384nm is very similar to that of the pigment. The 1-(2-furyl)buta-2,4-diene chromophore (LII) has been shown by Van Reijendam (82) to have an absorptiom maximum only 2nm different from that of trans octa-1,3,5,7-tetraene (306 and 304nm respectively). Thus the compound (LI) is a very good model system for a tetraene



(LII)

conjugated to the 6 position of a pyran-2-one ring as in structure (I).

The absorption maxima of the pigment and (LI) are in very good agreement. However, it is unlikely that this exact chromophore is present in the pigment molecule since compound (LI) does not show a large hyperchromic, bathochromic shift on acidification. A slight modification of this molecule may be all that is required, but this as yet has not been shown. Dehydroacetic acid shows greater changes, both in intensity and position of absorption maximum with changes in the pH of the medium, than does triacetic acid lactone, (Fig. 9). A compound formed from the reaction of 3-(2-furyl)propenal at the 6 α position of dehydroacetic acid would be expected to show spectral changes more in agreement with those shown by the pigment. This compound would, however, be expected to have an absorption maximum at approximately 15-20nm longer wavelength than (LI). The absorption maxima of the compounds formed by Harris et al. (60) at the 6 α position of dehydroacetic acid are not given. Compound (LI), while not exactly duplicating the properties of the pigment chromophore, is probably the best model system available at the present time.

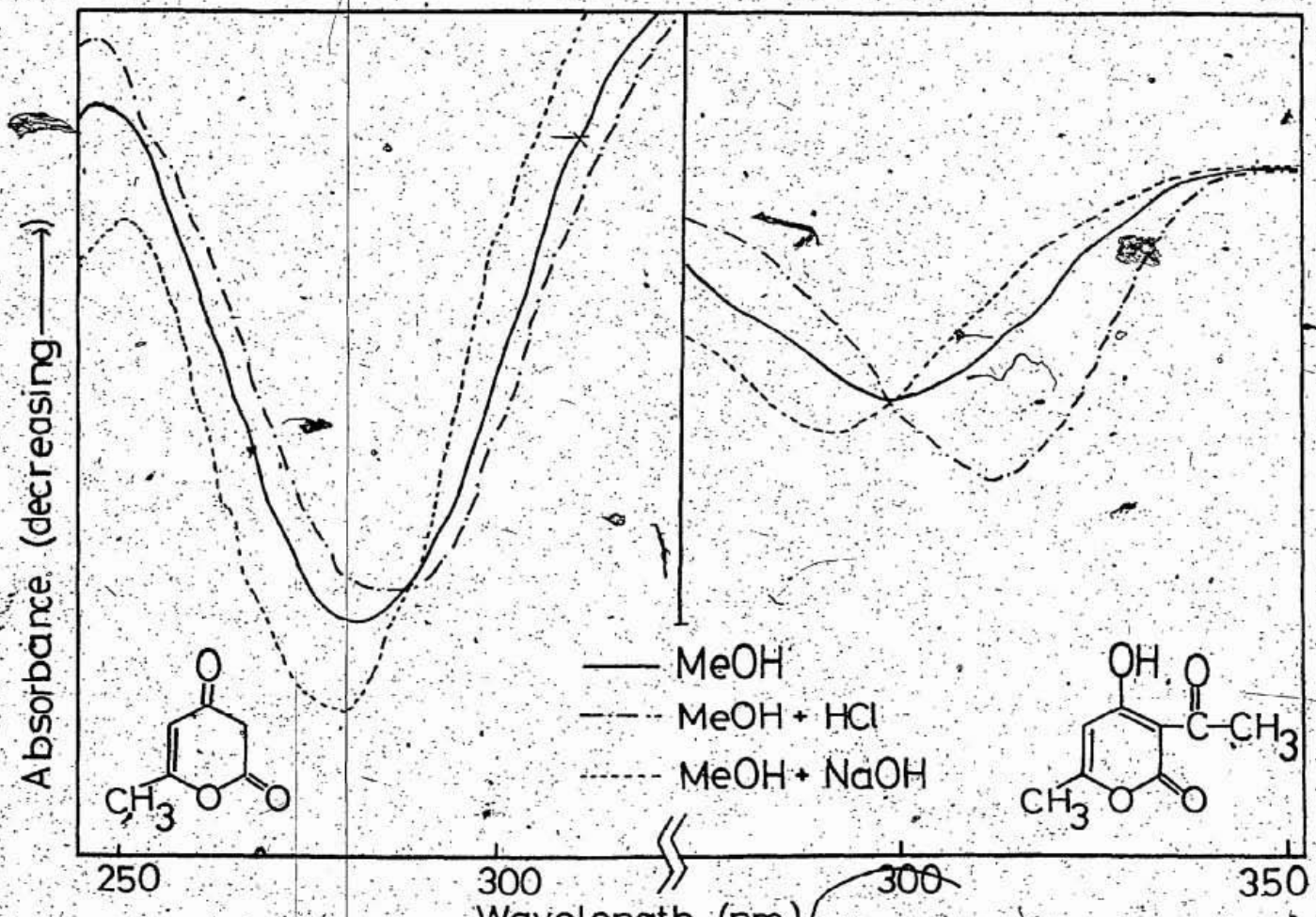
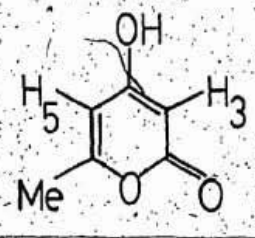


Figure 9. Absorption spectra of dehydroacetic acid and triacetic acid lactone

EXPERIMENTAL

i) Preparation of triacetic acid lactone (XXVIII)

Deacetylation of dehydroacetic acid (ALDRICH) (XVIII) was carried out by the method of Mir et al. (67). (XVIII) (20g) was heated with 90% sulphuric acid (60g, 34cm^3) at 130° on an oil bath until a drop of reaction mixture failed to produce a precipitate when added to cold water. After cooling, the solution was poured into cold water (80cm^3) and the mixture further cooled in ice. Recrystallisation of the solid from acetonitrile gave (XXVIII) in 80% yield, m.p. 190-191°. U.v. (CH_3OH): 212, 280nm (log ϵ 4.34, 3.93); n.m.r. ($(CD_3)_2CO$) τ: multiplet (1H) 4.13 (H_5), doublet (J = 2 Hz) 4.73 (H_3), singlet (3H) 7.85 p.p.m. (C-CH_3); mass spectrum (200°) m/e 126 (parent), 111, 98, 85, 84, 69(base peak), 55, 43, 42, 41, 39.



(XXVIII)

ii) Reactions of triacetic acid lactone at C3

The procedure used was based on the methods of Horning and Horning (77) and Wiley et al. (55).

a) Reaction of triacetic acid lactone with benzaldehyde

Benzaldehyde (BAKER) was redistilled before use. Benzaldehyde (0.02 mole) and (XXVIII) (0.02 mole) were dissolved in 50% aqueous ethanol (30cm^3). Piperidine (10 drops) was added and the mixture refluxed for ten minutes. On cooling, a pale

pink oil separated. Further separation was achieved by the addition of water and then the upper aqueous layer was decanted. Addition of a small volume of methanol to the oil produced a solid which was recrystallised from methanol to give white crystals of compound (XLV) (1.8g), m.p. 210-15°. U.v. (CH_3OH) 212, 288, 300(shoulder)nm (log ϵ 4.51, 4.18, 4.06); ($CH_3OH + H^+$) 206, 295nm (log ϵ 4.43, 4.30); i.r. ($CHCl_3$):3400-2500, 1680, 1620, 1570cm^{-1}; n.m.r. ($CDCl_3$) τ: singlet (6H) 7.76 (2 x C-CH_3), singlet(½H) 6.57, singlet (1H) 4.30 (bridging hydrogen), singlet (2H) 4.00 (H_5 pyrone), singlet (5H) 2.83 (aromatic), broad (1H) -0.3p.p.m. (hydroxyl); mass spectrum m/e 340 (parent),255,214, 213(base peak),185,171,144,130,126,102,98,85,77,69,43. Analysis: Calculated for $C_{19}H_{16}O_6$: C, 67.06; H,4.71: Found: C, 66.66; H, 5.38. (Fig. 10 is i.r. of (XLV)).

b) Reaction of triacetic acid lactone with propanal

Propanal (EASTMAN) was used without further purification. The procedure used was similar to that used with benzaldehyde in a) above. After cooling the reaction mixture, water was added dropwise until the solution became cloudy. The crystals which formed on cooling in ice were washed with aqueous ethanol and recrystallised from methanol to give compound (XLII) (2.4g), m.p. 189-191°. U.v. (CH_3OH) 205,214(shoulder); 289nm (log ϵ 4.51, 4.46, 4.26); ($CH_3OH + H^+$) 205, 295nm (log ϵ 4.45, 4.32); i.r. ($CHCl_3$): 3300-2500, 1680,1620, 1575cm^{-1}; n.m.r. ($CDCl_3$) τ: triplet (J = 7 Hz) (3H) 9.15 (CH_3-CH_2-), multiplet (2H) 7.85 (CH_3-CH_2-CH-), singlet (6H) 7.8 (2 x C-CH_3), triplet (J = 8 Hz).

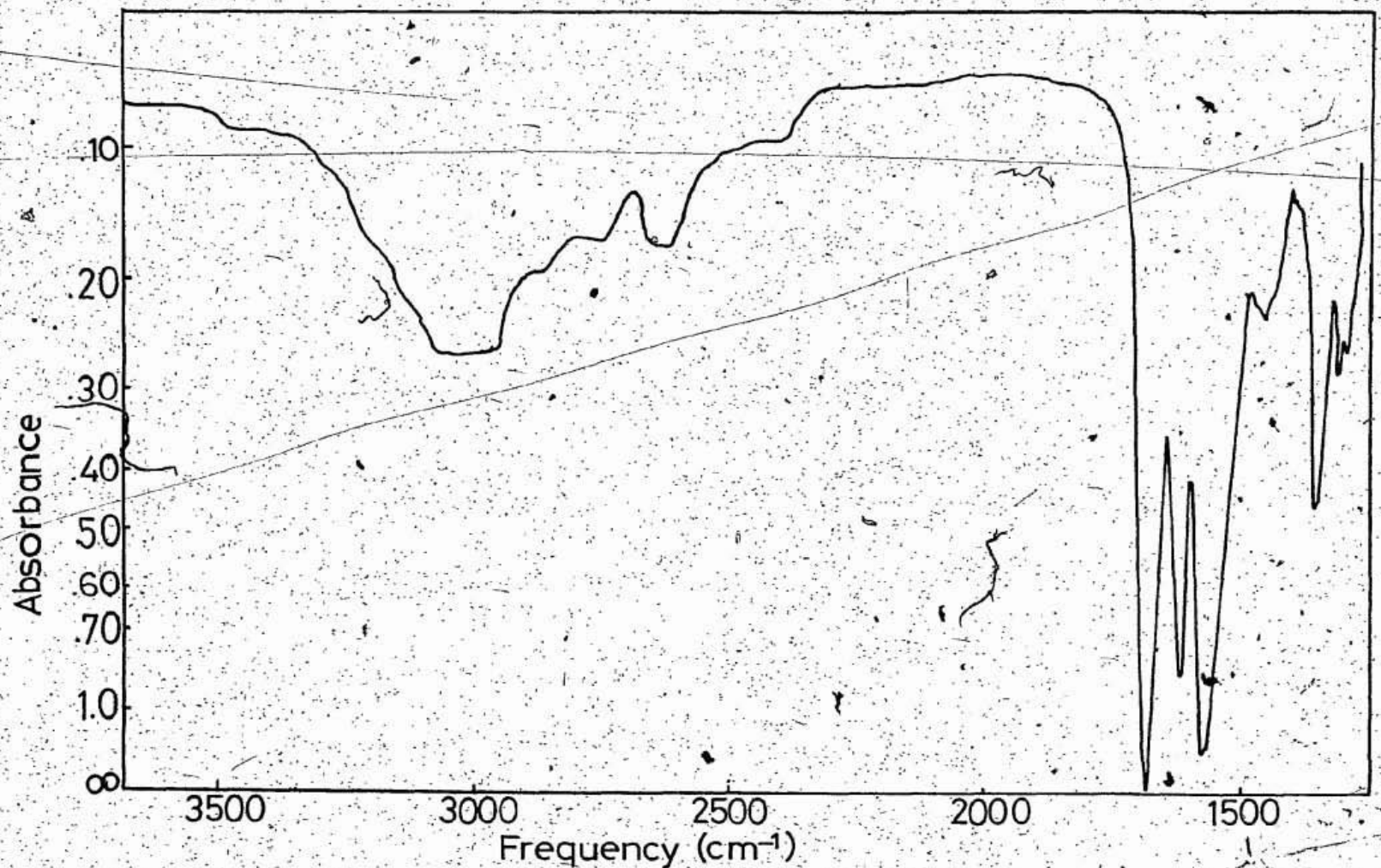


Figure 10. Infrared spectrum of compound (XLV)

(1H) 5.99 (CH-CH_2), singlet (2H) 4.08 (H_5 pyrone), broad (2H) -1.3p.p.m. (hydroxyl); mass spectrum m/e 292(parent),263,221 (base peak), 205, 203,179,166,151,140,137,126,98,85,82,69,54, 43,39. (Fig. 11 n.m.r. and Fig. 12 u.v. of (XLII)).

Analysis: Calculated for $C_{15}H_{16}O_6$: C, 61.64; H, 5.50: Found: C, 61.62; H, 5.63.

The above reaction was repeated. Propanal ($3cm^3$, > 0.04 mole) and piperidine (10 drops) were mixed and placed in an ice bath. Triacetic acid lactone (0.02 mole) in 50% aqueous ethanol ($35cm^3$) was gradually added to the mixture, which was maintained at 0° and stirred continuously. A precipitate slowly formed and this was removed three times during the reaction, after 3, 19 and 24 hours, and washed with 50% aqueous ethanol. This solid was identified as compound (XLII) by ultraviolet spectroscopy and a mixed melting point determination.

c) Reaction of triacetic acid lactone with butenal

Butenal was redistilled before use (b.p. 102-4°). The procedure used was the same as that for benzaldehyde above. After addition of water to the cooled reaction mixture, a yellow oil separated. After removal of the aqueous layer, the oil was dissolved in chloroform. Removal of the chloroform by evaporation under reduced pressure gave an oil which slowly solidified and was recrystallised from methanol to give compound (XLVII) (0.5g), m.p. 218-20°. U.v. (CH_3OH) 214, 285nm (log ε 4.50, 4.26); ($CH_3OH + H^+$) 207, 290nm (log ε 4.46, 4.22); i.r. ($CHCl_3$): 3650, 3400, 1700, 1655, 1595cm^{-1};

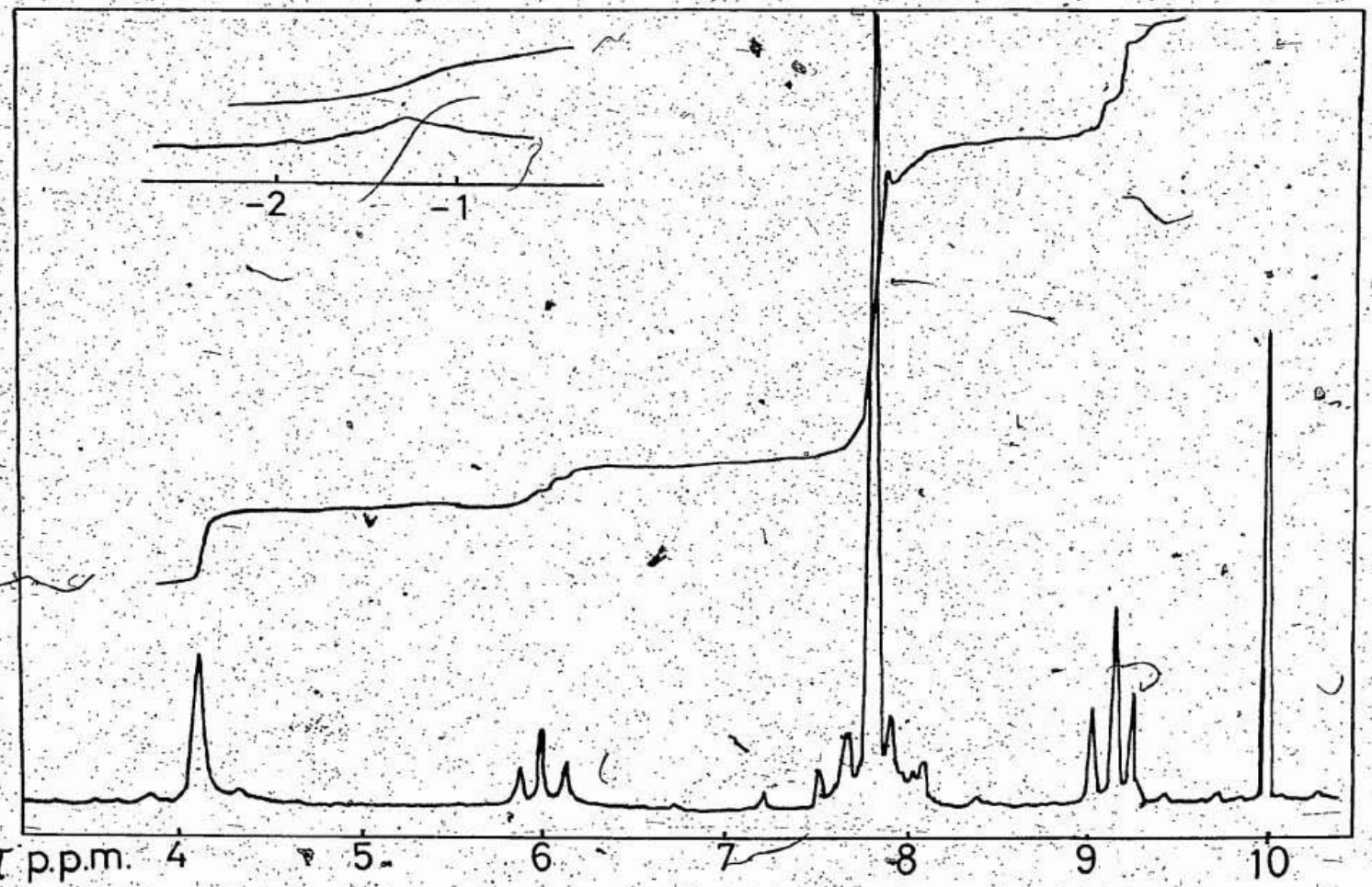


Figure 11. N.m.r. spectrum of compound (XLII)

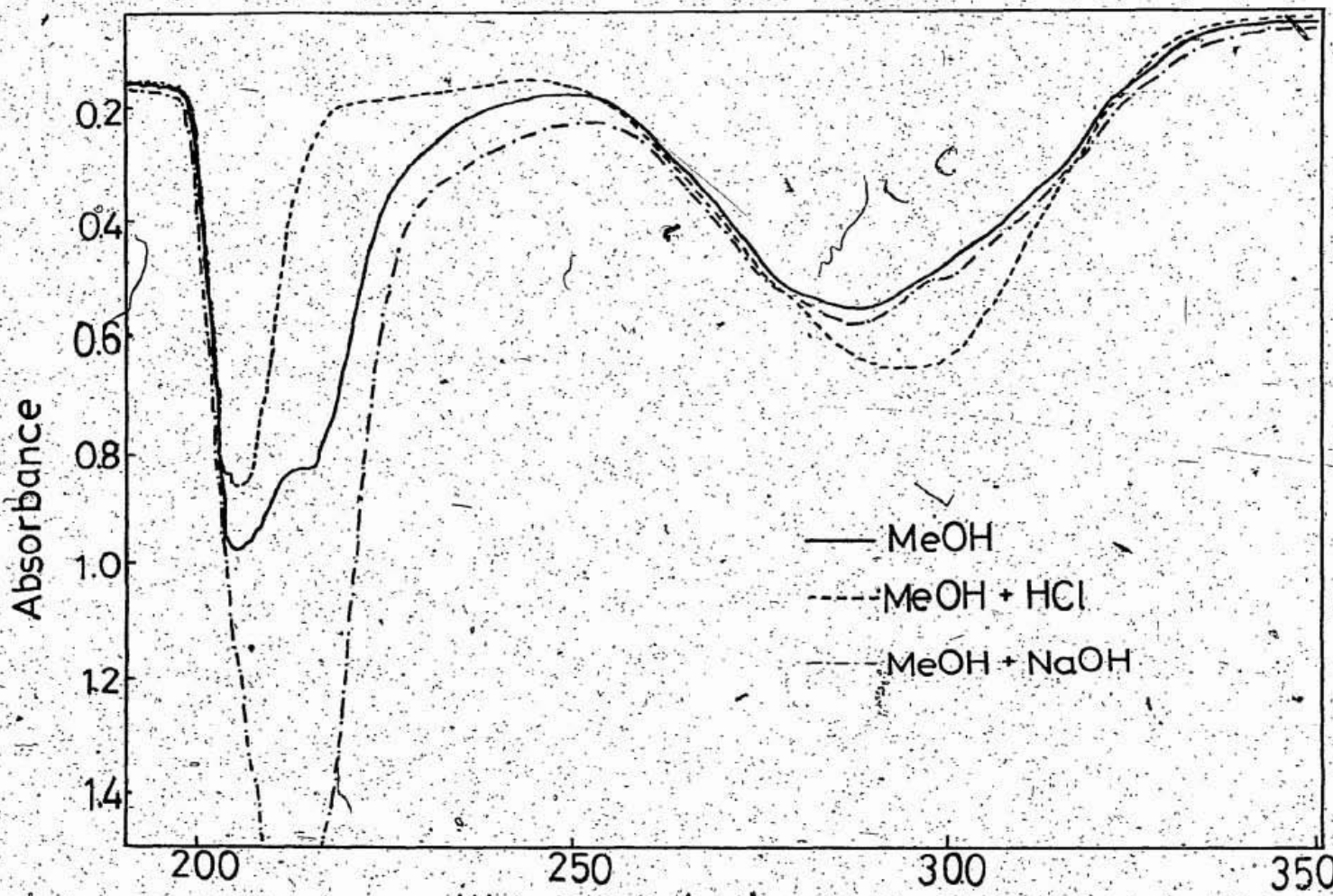


Figure 12. Absorption spectrum of compound (XLII)

n.m.r. ($CDCl_3$ + DMSO-d_6) τ: doublet (J = 6 Hz) (3H) 8.78 (CH_3-CH), doublet (J = 2 Hz) (6H) 7.83 (2 x C-CH_3), triplet of multiplets (3H) 7.2 (-CH_2- and -CH-), doublet of multiplets (J = 12 Hz) (1H) 4.68 (O-CH-), singlet (1H) 4.24 (H_5 pyrone), singlet (1H) 4.02 (H_5 pyrone), singlet (1H) -1.10 p.p.m. (hydroxyl); mass spectrum (250°) m/e 304 (parent), 246, 178, 165, 153, 152, 124, 110, 109, 85, 69, 68 (base peak), 53, 43, 40, 39.

(Fig. 13 is u.v. spectrum of (XLVII)).

Analysis: Calculated for $C_{16}H_{16}O_6$: C, 63.19; H, 5.26: Found: C, 63.33; H, 5.27.

iii) Reaction of dehydroacetic acid with propanal

Dehydroacetic acid (XVII) (0.02 mole) and propanal (0.02 mole) were dissolved in chloroform (25cm^3). Piperidine (10 drops) was added and the mixture refluxed for 8 hours. During this time the water produced was collected in a Barrett type distilling receiver and the chloroform returned to the reaction mixture. After 8 hours, chloroform (10cm^3) was removed by distillation and the remainder allowed to evaporate slowly. The crystals which formed were collected and shown, by ultra-violet spectroscopy and mixed melting point, to be (XVII) (2g). The mass spectrum of the remaining yellow oil was recorded. This showed that the oil was mainly dehydroacetic acid, although at 272° the maximum m/e value was 417. The n.m.r. spectrum was consistent with the above information, showing dehydroacetic acid to be the major constituent.

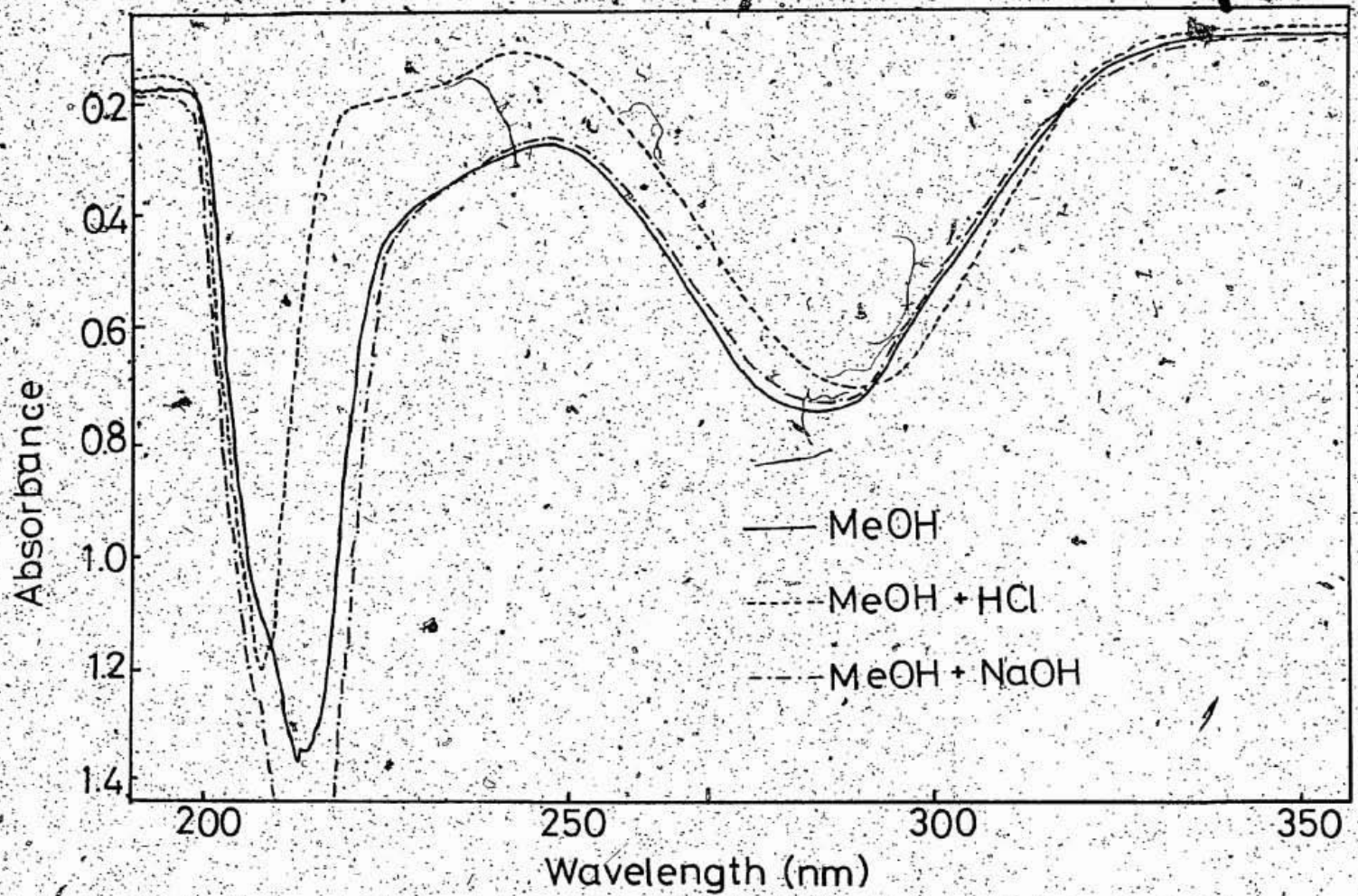


Figure 13. Absorption spectrum of compound (XLVII)

iv) Preparation of 4-methoxy-6-methyl-2H-pyran-2-one (XXIV)

Dimethyl sulphate (10g) was added dropwise to a refluxing vigorously stirred solution of (XXVIII)(10g) in dry acetone ($300cm^3$) containing potassium carbonate (50g). The mixture was refluxed for 15 hours and, after cooling, the solid was removed by filtration and washed with dry acetone. The washings and filtrate were combined and evaporated to dryness. The solid remaining was repeatedly extracted with petroleum ether (60-80°) under reflux conditions. The extract, on cooling gave needle shaped crystals which were recrystallised from petroleum ether (60-80°) to give compound (XXIV) (6.5g) m.p. 86-88°. U.v. (CH_3OH)207, 280nm (log ε 4.41, 4.13): i.r. ($CHCl_3$) 1740(shoulder) 1715, 1650cm^{-1}: n.m.r. ($CDCl_3$) τ: singlet (3H) 7.76 ($C-CH_3$), singlet (3H) 6.18 ($O-CH_3$), doublet (J = 2 Hz) (1H) 4.52 (H_3), doublet (J = 2 Hz) (1H) 4.20 p.p.m.(H_5): mass spectrum m/e 140 (parent ion and base peak), 125,97,112,83,82,69,53,44,43, 41,39.

v) Demethylation of 4-methoxy-6-methyl-2H-pyran-2-one (XXIV)

a) Pyridine hydrochloride

The method used was based on that of Prey (80). Fusion of (XXIV) (100mg) with pyridine hydrochloride (200mg) at 210° for one hour produced a black tar which was treated with water (2 x $5cm^3$) and filtered. The residue and solid remaining in the flask were extracted with ether ($50cm^3$) and then chloroform ($50cm^3$). The ether extract yielded yellow crystals and a yellow oil (20mg) when evaporated to dryness. The crystals were

identified, by melting point and ultraviolet spectrum, as unreacted starting material. The yellow oil was not identified. No identifiable product was obtained from the chloroform extract.

b) Aluminium chloride

The procedure employed was that used by Yamamura et al. (81) to demethylate 6-acetyl-4-methoxy-2H-pyran-2-one. Anhydrous aluminium chloride (FISHER) (400mg) and (XXIV) (100mg) were added to tetrachloroethane (1.5cm^3). After reaction, the mixture was poured into hydrochloric acid at 0° and extracted with chloroform (2 x 20cm^3) and then ethyl acetate (5 x 20cm^3). Removal of the ethyl acetate gave a pale yellow solid which was recrystallised from ethanol to give triacetic acid lactone (48mg). The melting point, ultraviolet spectrum and mass spectrum were identical with that of authentic material. The eluate from column chromatography of the chloroform extract afforded a dark solid which, from the ultraviolet spectrum, was shown to contain some product and some starting material.

vi) Reaction of triacetic acid lactone (XXVIII) at the 6-position: preparation of 4-methoxy-6-(4-(2-furyl)buta-2,4-dienyl)-2H-pyran-2-one from the 4-methoxy-pyran-2-one (XXIV)

The procedure used was that employed by Mir et al. (67). 3(2-Furyl)-propenal (0.02 mole) (ALDRICH), used without further purification, and (XXIV) (0.02 mole) were dissolved in dry methanol (30cm^3) containing magnesium methoxide (1.8g). The

mixture was refluxed for 6 hours. After cooling, the solvent was removed under reduced pressure and the remaining solid dissolved in chloroform. The chloroform solution was shaken with acetic acid (1M, 15cm^3) and then with sodium hydrogen carbonate solution (1M, 30cm^3). After washing with water, the chloroform solution was dried (Na_2SO_4) and passed down a column of neutral alumina. Unless a dilute chloroform solution was used, difficulty was experienced with filtration and the mixture would only pass very slowly down the column. The eluate was evaporated to dryness to give a yellow solid. This was recrystallised from methanol to give compound (L) (0.9g) m.p. 199-202° (Lit. 195-197°). U.v. (CH_3OH) 226, 287, 384nm (log ε 6.44, 6.21, 6.64); i.r. ($CHCl_3$) 1710, 1640, 1620, 1550cm^{-1}, n.m.r. ($CDCl_3$) τ: singlet (3H) 6.22 (O-CH_3), doublet (J = 2 Hz) (1H) 4.59 (H_3 pyrone), doublet (J = 2 Hz) (1H) 4.19 (H_5 pyrone), broad singlet (½H) 4.08, broad singlet (½H) 3.84, doublet (J = 1 Hz) (2H) 3.64, multiplet (1½H) 3.43, broad singlet (½H) 3.28, multiplet (1H) 2.78-3.08, broad singlet (1H) 2.62 p.p.m.: mass spectrum (192°) 244(parent ion and base peak),216,187,173,125, 124, 115,107,91,69,65,59,39. (Fig. 14 n.m.r. of (L)).
Analysis : Calculated for $C_{12}H_{12}O_4$: C, 68.84; H, 4.92: Found: C, 68.01; H, 4.91.

vii) <u>Demethylation of 4-methoxy-6-(4-(2-furyl)buta-2,4-dienyl)-2H-pyran-2-one (L)</u>

Aluminium chloride (400mg) and (L) (50mg) were dissolved in tetrachloro-ethane (3cm^3). The reaction procedure was the

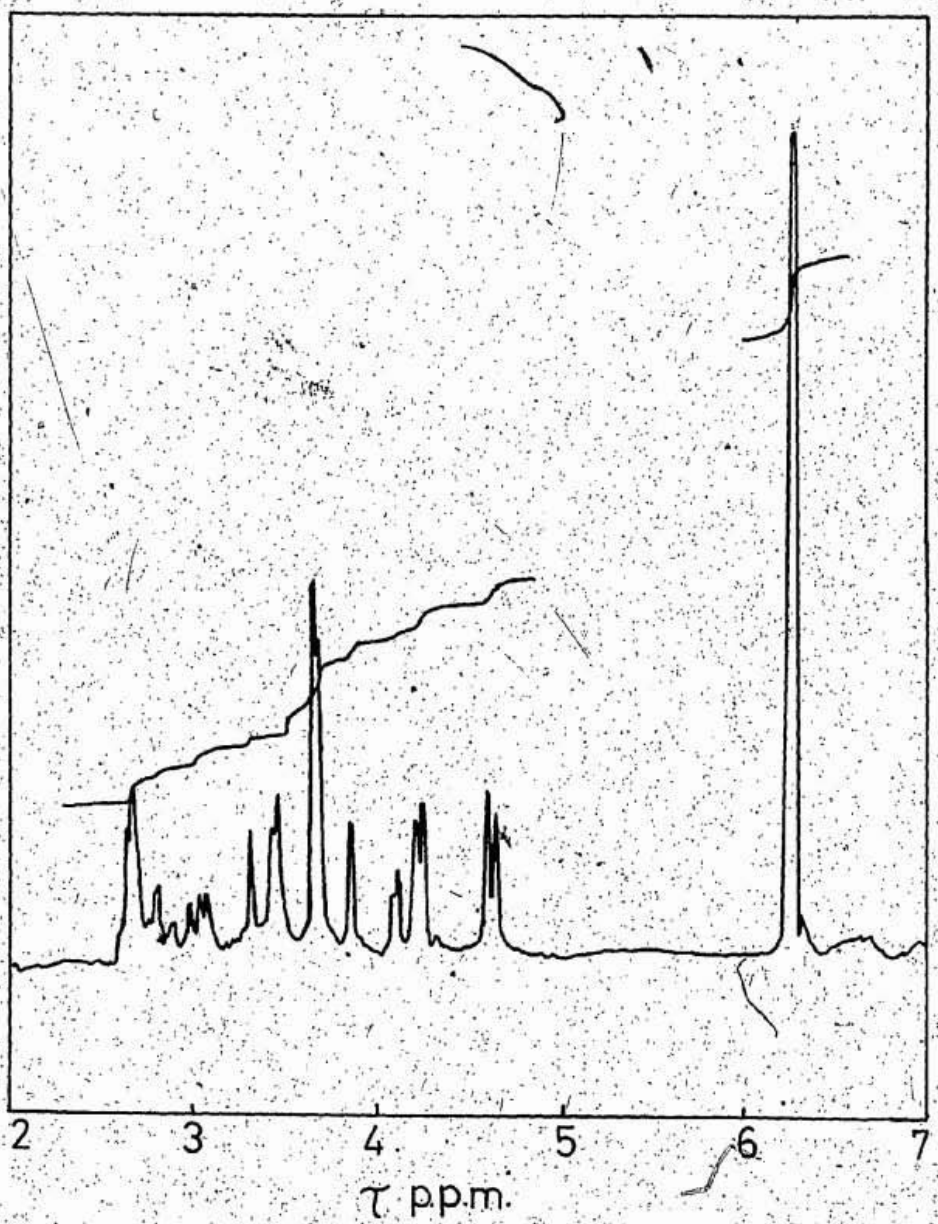


Figure 14. N.m.r. spectrum of compound (L).

same as that used above with compound (XXIV). The ethyl acetate extract gave a solid and a yellow oil. This mixture was washed with petroleum ether (60-80°) to give a solid (10mg), m.p. 120-130°decomp. No separation could be achieved using thin layer chromatography. The solid was dissolved in 80% aqueous methanol and applied to a column of Sephadex G 10 (6cm x 0.5cm). Fractions of the eluate were collected and examined by ultraviolet spectroscopy. Those fractions which showed a shift in absorption maximum on acidification were combined and the solvent removed to give compound (LI) (4mg), m.p. 115-7°. U.v. (CH_3OH) 230, 280, 383nm; ($CH_3OH + H^+$) 225, 275, 396nm: mass spectrum m/e 230(parent),161,149,111,97,85,71,57(base peak),44,43: n.m.r. ($(CD_3)_2CO$) -no peak at τ= 6.22. (Fig. 15 u.v. of (LI)).

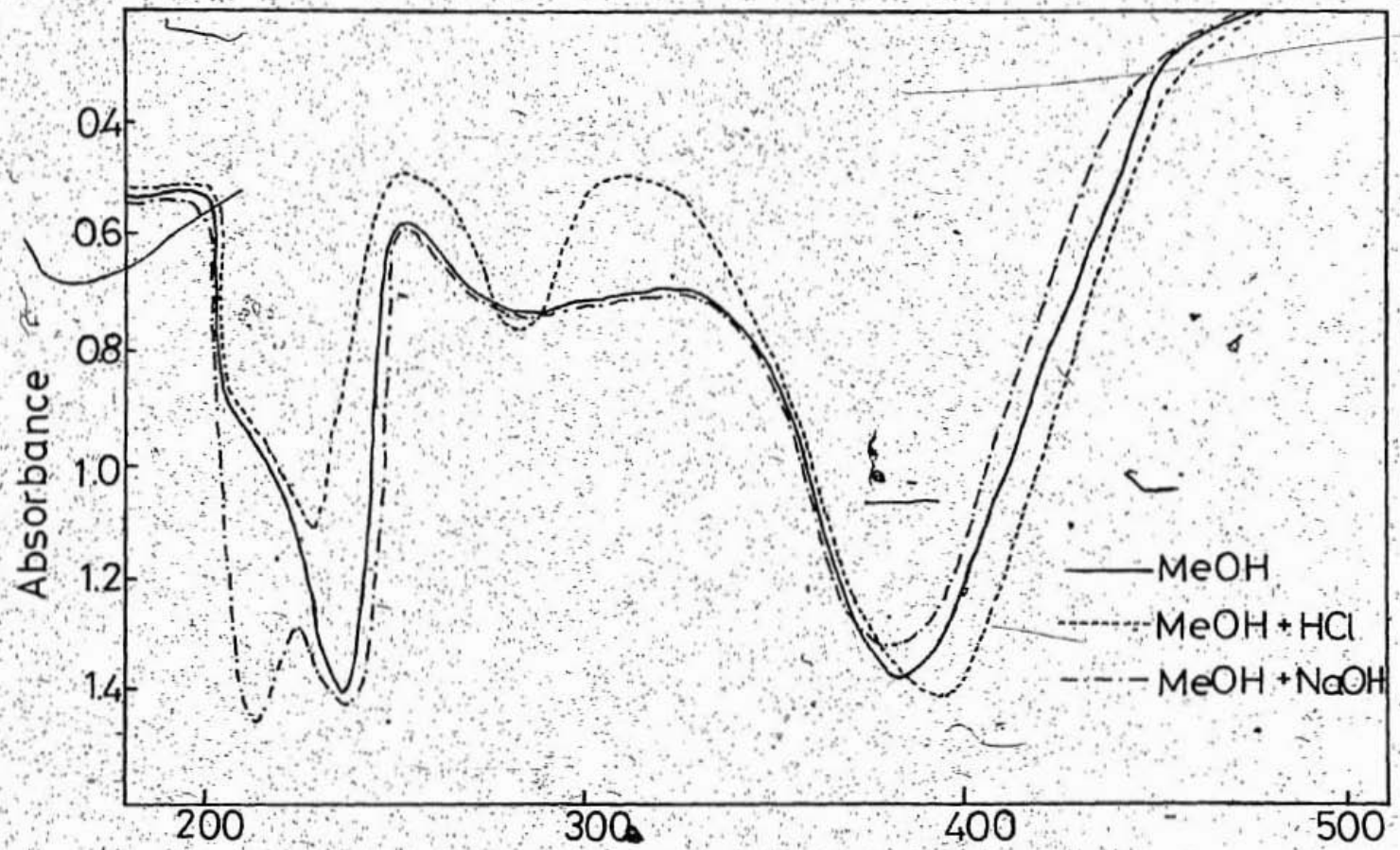


Figure 15. Absorption spectrum of compound (LI)

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