PHOTOLUMINESCENCE

STUDIES ON STEROIDAL SUBSTANCES

By

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SUMMARY

The literature is briefly reviewed on current fluorimetric methods for the analysis of steroidal substances. The trend in the development of these methods is indicated with particular interest on sensitivity and selectivity. Fluorescence quenching, complex formation with metals, and chemical transformation are briefly investigated in search of selective reactions with substituents on the steroid skeleton. Chemical transformation under controlled conditions is shown to be a promising tool. The application of trichloroacetic acid as a reagent for locating a wide range of steroids on thin layer plates is shown to give a rapid indication of its selectivity and approximate sensitivity. A specific and sensitive fluorimetric method for determination of digoxin in solution and its application to tablet preparations is described. This method based on a reaction with trichloroacetic acid can be used for digoxin determination in the presence of digitoxin, gitoxin and ouabain. Physiological steroids are also shown not to interfere with the reaction under the conditions described. 17α-methyltestosterone which is readily available and reacts rapidly with trichloroacetic acid on thin layer plates is used in a detailed study of the trichloroacetic acid reaction. A hydro-oxo-phenanthrene is isolated from the reaction. The structure of the product which has an aromatic ring-C and is dehydrogenated at the C6-C7 is established from its physical properties and a comparison of these with synthetic hydro-oxo-phenanthrenes. A new route to 2, 3, 4, 12-tetrahydro-12-methyl-2-oxophenanthrene is described and some aspects of steroid rearrangement reported. Absorption and fluorescence study of the hydro-oxo-phenanthrene chromophore in polar and non-polar solvents at room temperature is discussed. Fluorescence is shown to be due to enolisation in strongly acidic solvents.

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To

Mama, Papa and Vicky

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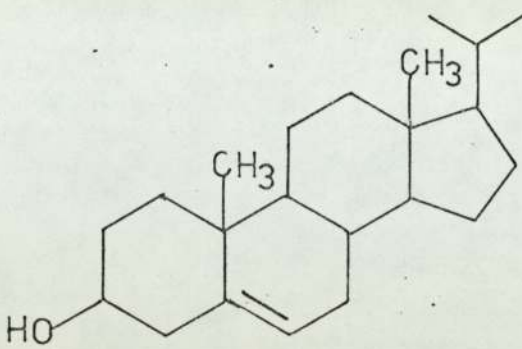
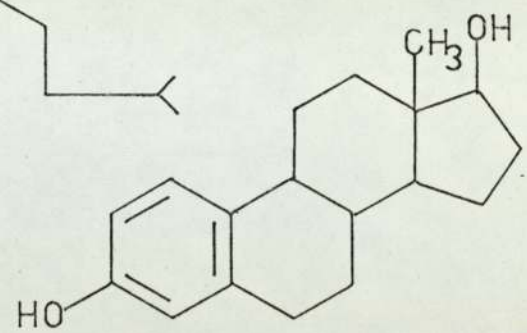
INTRODUCTION

The steroids form an important group of medicinal substances which may be divided into five important classes on physiological and structural basis: cholesterol, oestrogens, adrenal steroids and androgens, bile acids, and cardioactive steroids. Only the oestrogens possess an aromatic ring and phenolic substitution, which results in ultraviolet absorption and native fluorescence. The clinical importance of many steroids has led to a considerable effort to determine their presence in small amounts in biological fluids. Methods available usually involve extraction and some partial purification followed by application of a colour reaction under various conditions which permit quantitative spectroscopic estimation. For example, the Liebermann-Burchard colour reaction with chloroform acetic anhydride and sulphuric acid has been used to estimate 'blood' cholesterol, whilst other methods such as the Zimmerman reaction with m-dinitrobenzene have been used to estimate 17-ketosteroids. These methods may, however, indicate the presence of a particular chromophore but only rarely are they diagnostic of a specific compound. A review on the fluorescence of steroids prepared by Braunsberg and James[1] indicating that the best conditions for developing the fluorescence of a given steroid must be arrived at empirically supports this view.

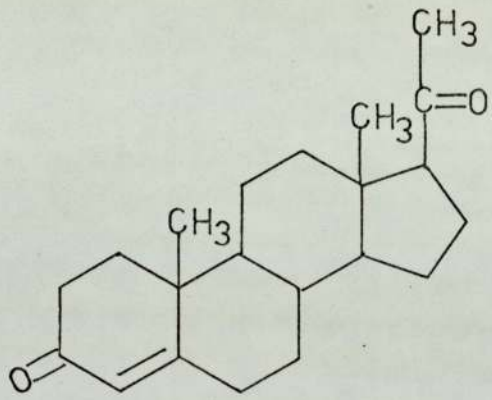
Currently, fluorescence is used routinely for the assay of physiological steroids and steroidal drugs.[2] Most reported methods are based on fluorophors formed in strong acid or alkali[3-13].



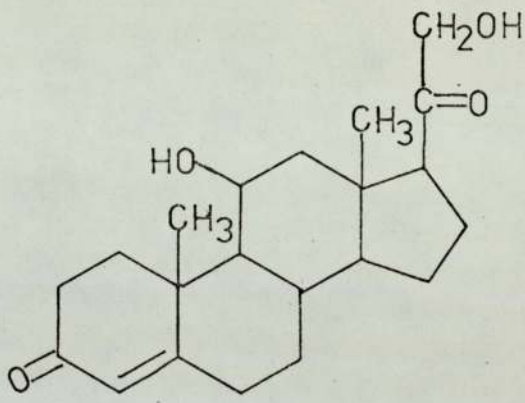
I Cholesterol



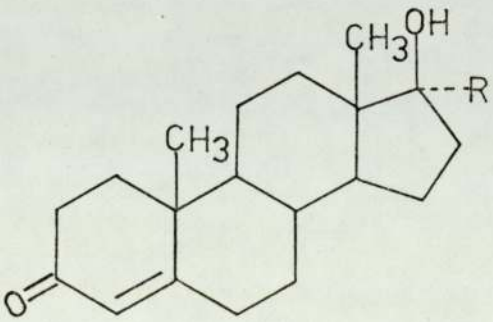
II β-Oestradiol



III Progesterone

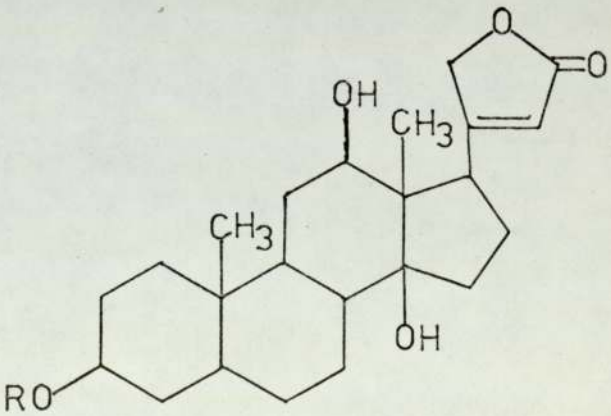


IV Corticosterone



V R=H, Testosterone

(a) R=CH_3, 17α-Methyl Testosterone



VI R=H, Digoxigenin

(a) R=$C_{18}H_{31}O_9$, Digoxin

Fig. 1. Some Common Steroids

Fluorescence Produced in Strong Acid

Many fluorescence procedures have been described for oestrogen assay based on solvent extraction followed by treatment with sulphuric or phosphoric acid to yield stable visible fluorescence[2,3]. Most of these reports were largely concerned with appropriate instrumentation and with optimal conditions for forming the fluorophor. The absorption spectra of the acid products of oestrogens exhibit broad maxima ranging from 410nm to 450nm and the resulting fluorescence is also a broad band with a peak near 500nm. In selected cases, detailed chemistry of the reaction of some oestrogens in moderately concentrated sulphuric acid has been reported[14]. The extensive aromatisation of the steroid skeleton is postulated with particular emphasis on disproportionation and hydride transfer. Complete identification of all the reaction products was not, however, possible and little is provided in terms of proof of the structures suggested. On the other hand, a systematic study of the luminescence of oestrogens by Weinreb and Werner[15] has yielded useful information on the true luminescence behaviour of these compounds. The photophysical parameters reported would indicate routine analysis of oestrogens on the basis of their native luminescence to be possible.

Reactions of strong sulphuric or phosphoric acid with adrenal steroids possessing an 11-hydroxy group and some dehydrotestosterones yield characteristic fluorescent derivatives, except for aldosterone. The characteristics of the resulting fluorophors are 470 to 475nm excitation and 520 to 530nm emission[16,17]. Steroid drugs such as prednisone and prednisolone do not yield fluorescence although cholesterol and oestrogens showed weak fluorescence.

Jensen[18], and later Wells, Katzung and Meyers[19] described a useful reagent for the fluorimetric analysis of cardioactive steroids. The technique is based on the fluorimetric measurement of dehydration products of the cardioactive steroids resulting from the reactions of the glycosides with concentrated hydrochloric acid, methanol, ascorbic acid and dilute aqueous hydrogen peroxide. The application of this method to automated unit dose analysis of digoxin and digitoxin tablets is also documented[20]. The method, however, lacks specificity for the individual cardiac glycosides.

Fluorescence Produced in Alkali

Bush and Sandberg[21] showed that Δ^4-3 ketosteroids on paper chromatograms exhibit an orange fluorescence under UV light after spraying with 10% NaOH and drying. Ableson and Bondy[22] were able to produce the same sort of fluorescence by carrying out the reaction in potassium tert-butoxide dissolved in tert-butyl alcohol. All the Δ^4-3-ketosteroids investigated yielded specific products with an absorption maximum about 385nm and emission maximum about 580nm. Its specificity for $\alpha\beta$-unsaturated ketosteroids was demonstrated by cortisone, hydrocortisone, corticosterone, progesterone and aldosterone, whilst most other types of steroids did not show any fluorescence.

Investigations on the effect of nucleotide coenzymes on the excitability of steroid molecules in the presence of 5% NaOH showed a very strong greenish-yellow fluorescence[23]. The fluorescenc was much stronger with NAD than with $NADH_2$, NADP or $NADPH_2$. Since the absorption maximum of Δ^4-3-ketosteroids is about 240nm, and that of NAD, adenosine, and nicotinamide are about 260nm, it was thought that both the steroid and nucleotide chromophores could be excited at the

same energy level. As these Δ^4-3-ketosteroids fluoresce in the presence of NaOH alone, it was suggested that the presence of NAD, energy absorbed by the nucleotide may be transferred to the steroid molecule with the result that the excited state appears more quickly. The presence of the whole coenzyme molecule is however essential for the reaction as nicotinamide, adenine, adenosine or ATP do not give the reaction. The reaction was found to be transferable into solution with excitation maximum at 370nm and emission maxima at 460nm (from $NADH_2$), and at 580nm (characteristic of the complex).

Protein Fluorescence and steroid protein binding

Interest in fluorescence quenching of proteins by steroids stems from a physiological origin rather than an analytical one. Albumin was one of the first proteins to be studied in terms of steroid binding. Being the main constituent of blood it has tremendous capacity for binding a great number of substances and was formerly thought to play a predominant if not a unique role in the transport of steroids. This capacity is so great that it can never be saturated even at steroid concentrations far above the physiological range[24]. For this reason the determination of the number of sites available is hindered by the limited solubility of the free steroids in the aqueous medium. Also for the same reason, it is impossible to know if the different free steroids are fixed or not in the same site of the albumin molecule, as they do not compete with each other within the limits of analytical concentrations. However, the binding affinity of albumin for steroids is comparatively low as the complexes formed dissociate very easily. A simple passage through a column of Sephadex is sufficient to release bound cortisol[24] or testosterone[25], nearly completely. Therefore, the

combination with albumin does not seem to interfere much, either with biological activity or with their metabolic transformations.

Serum albumin from different species can be easily isolated in a pure state, most of its physico-chemical properties are well known and it has been possible to determine precisely the apparent association constants for various steroids. These values appear to vary according to the origin of the albumin and the nature of the steroid. In the case of oestrogens, oestradiol and oestrone seem to have the highest association constants and oestriol the lowest one. Progesterone and testosterone are relatively strongly bound. As demonstrated by Sandberg,[26] binding affinity decreases with polarity.

Quantitative aspects of the perturbation of UV spectra of some Δ^4-3-ketosteroids and human serum albumin (HSA) was reported by Ryan and Gibbs[27]. They demonstrated the occurrence of a blue shift in case of several Δ^4-3-ketosteroids and, in addition, a red shift in case of progesterone[28]. The blue shift has been attributed to perturbation of the steroid bands and the red shift to perturbation of aromatic protein bands. The latter was also observed by Attallah and Lata in their studies on the steroid-induced fluorescence quenching on bovine serum albumin (BSA).[29] Little information is available on such steroid-protein interaction studies and less so on their analytical applications. While it may be convenient to correlate steroid binding with changes in the intrinsic tryptophanyl or tyrosyl fluorescence of proteins, binding may not always result in alteration of fluorescence. However, quenching of the native fluorescence of L-tyrosine and L-tryptophan has been observed in the presence of steroids.

Enzymatic Measurement of Steroids

The 3α-hydroxysteroid dehydrogenase isolated from P. testosterone catalyses NAD-linked stereospecific, interconversion of an A/B trans or A/B cis-3-ketosteroid and the corresponding alcohols[30,31]. The equilibrium of the reaction

$$\text{(steroid alcohol)} + NAD^+ \rightleftharpoons \text{steroid ketone} + NADH + H^+$$

shifts to the right on using a ketone trapping agent such as hydrazine. Iwata and Yamaski[32] used this reaction to estimate bile acids in blood spectrophotometrically and their technique was later adapted by Turnberg and Anthony-Mote[33] for the determination of bile acids in bile. Application of this reaction for fluorimetric estimation of serum total bile acids was later developed by Murphy and others[34]. The developments following the isolation of this enzyme marks a turning point in the search for selective methods for steroid analysis.

The purpose for this short literature review was not to draw a detailed picture of the developments of various methods for steroid analysis, nor to describe all the important investigations which have been published on this matter. It was only intended to present a few characteristic features in the development of current methods in steroid analysis and to depict several outstanding facts which may throw some light on the nature and complexity of steroid reactions.

In order to determine the parameters essential to selective steroid analysis, a number of methods were used:

i) Fluorescence quenching.

ii) Complex formation with metals.

iii) Chemical transformation.

The relative merits of each method used are discussed and all contributions made are compared with existing literature methods. Investi-

gations were done on steroids lacking in UV absorption and $\alpha\beta$-unsaturated ketones which do not seem to have received as much attention in the literature as those steroids with an aromatic or phenolic ring substitution.

2. METHODS FOR STEROID ANALYSIS

a) Fluorescence Quenching

The known phenomenon of fluorescence quenching shows that excited molecules which are formed by absorption of photons can undergo reactions which are comparable to or faster than emission of fluorescence. Dissolved solutes can result in decreased fluorescence by at least two types of chemical quenching processes:

Collisional Quenching - This results from a diffusion controlled process in which a molecule of a quencher interacts with an excited molecule of the potentially fluorescing substance. Interaction results in the dissipation of excitation energy not by fluorescence but by transfer of energy to the quenching molecule. A simplified mechanism can be written to describe the situation:

$$F + h\nu \longrightarrow F^* \quad \ldots\ldots\ldots\ldots \text{(i)}$$

$$F^* \longrightarrow F + h\nu^1 \quad \ldots\ldots\ldots\ldots\ldots \text{(ii)}$$

$$F^* + Q \longrightarrow F + Q \quad \ldots\ldots\ldots\ldots \text{(iii)}$$

The fluorescent molecule F absorbs a photon to give F*, the lowest excited singlet state. F* can either emit a photon, $h\nu^1$, and return to the ground state, or it may interact with the quencher molecule, Q, thereby losing excitation and return to the ground state without emission of fluorescence. This mechanism is described by the Stern-Volmer law[35]:

$$(fo/f) - 1 = kC_Q \quad \ldots\ldots\ldots\ldots\ldots \text{(iv)}$$

fo = fluorescence intensity in the absence of quencher;

f = fluorescence intensity at the concentration, C_Q, of quencher;

k = proportionality constant.

Static Quenching - Here, complex formation occurs between a potentially fluorescing molecule in the ground state and a quencher

molecule. If the complexed form of the potentially fluorescing molecule has different spectral characteristics than the free form, it may not undergo excitation or may be excited to a lesser degree than the non-complexed species. The reduction in fluorescence caused by this type of quenching is thus dictated by the stability constant of the complex and the concentration of quenching agent.

The four fundamental aromatic aminoacids which are present in proteins - phenylalanine, tyrosine, histidine and tryptophan are moderate π-electron donors and rather poor electron acceptors. Isenberg and Szent-Györgyi[36] showed that out of these four aminoacids, tryptophan is the only one which forms an electron transfer complex with riboflavin. The easy formation of such a complex with 5-hydroxy-tryptamine and lysergic acid confirmed the hypothesis that the transferred electron was derived from the π-electron pool of the indole system. Molecular orbital calculations available on adenine and guanine also show that they should be moderately good donors and rather restricted in their electron acceptor properties. Investigation of steroid fluorescence quenching of HSA, and a few selected nucleic acid bases and their derivatives which possess intense native fluorescence, was undertaken. It was hoped that specificity in the quenching of fluorescence could be correlated with steroid structure.

The quenching of fluorescence of a solution of HSA, UV absorbance 0.34 at 280nm (calculated $E^{1\%}_{1cm}$ 5.23 on the basis of molecular weight of 65,000) was investigated with a number of steroids as substrate. The use of such a high concentration of HSA was thought warrantable as the fluorescence produced on excitation at 280nm changed linearly with dilution. The results are expressed in the form of the Stern-Volmer equation (table 1). The parameters fo and f represent the

heights of the intensity of emission of HSA at 330nm in the absence and in the presence of steroid, respectively.

Table 1

Conc^{n}. Steroid	Relative Fluorescence $^{fo}/f$							
µg/ml.	1	2	3	4	5	6	7	8
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
4	1.02	1.03	1.02	1.02	1.01	1.02	1.01	1.02
8	1.05	1.05	1.03	1.05	1.04	1.06	1.02	1.08
12	1.07	1.06	1.03	1.06	1.07	1.07	1.03	1.12
16	1.08	1.07	1.02	1.08	1.07	1.07	1.05	1.13
20	1.07	1.06	1.02	1.08	1.07	1.07	1.06	1.12
40	1.06	1.03	1.02	1.07	1.04	1.04	1.06	1.11
80	1.03	0.97	1.01	1.04	1.03	1.02	1.02	1.04
120	1.02	0.91	1.01	1.04	1.02	1.00	1.00	0.99
160	1.01	0.89	1.00	1.03	1.02	0.98	0.97	0.94
200	1.01	0.86	1.00	1.02	0.98	0.99	0.95	0.93

1. 5α-Androstan-17β-ol-3-one.
2. 5α-Androstan-17α-olethyl-3β,17β-diol.
3. 5α-Androstan-3α-ol-17-one.
4. 5α-Androstan-3β,17β-diol.
5. 5-Androsten-3β-ol,17-one.
6. 4-Androsten-3,17-dione.
7. 4-Androsten-3,11,17-trione.
8. 4-Androsten-17β-ol,3-one.

It was shown by Udenfriend and Saltzman[37] that with the exception of thymine at pH 10, the naturally occuring pyrimidines and their derivatives do not fluoresce in aqueous solution at room temperature although it was later demonstrated that they fluoresce at $77^{o}k$[38]. Nucleotides of adenine and guanine have appreciable fluorescence at acid pH with quantum efficiency of 0.003 and 0.007 respectively, at 390nm. Their fluorescence forms the basis for a simple and sensitive method for determining their content in nucleic acids. Some derivatives of nucleic acid bases are intensely fluorescent. 8-Azaguanine and 2,6 diaminopurine are intensely fluorescent at basic pH 13.0 and neutral pH 7.0, respectively. Guanine, guanosine, 2,6-diaminopurine and 8-azaguanine were examined for fluorescence quenching on titration with steroids. Similar results were obtained as in the case of HSA and no fluorescence quenching was observed at submicrogram steroid concentrations. The reaction was, therefore, abandoned.

b) Complex Formation Reactions with Metals

In the formation of oxygen containing ligands hydroxy groups and carbonyl groups offer potential sites for coordination. Ketosteroids containing hydroxy groups would, therefore, be potentially capable of forming complexes with certain metals. A study undertaken by Eger and others[39] demonstrates that the hydroxyacetone group of the 17β-side chain of corticosteroids is able to form complexes with cobalt and probably also with other metals of the first transition series. Since their values for the complex formation constants were low they concluded that the complex dissociates to a large extent in aqueous medium but becomes more stable with a decrease in water concentration. It was not however, intended that any efforts be directed at attempting to produce fluorescent transition metal complexes

with steroids as transition metal chelates are normally non-fluorescent. Most of these species are paramagnetic because of unfilled outer d-subshells with unpaired electron on the metal ion; often the metallic ion is reduced. Few transition metal ion chelates are known to fluoresce in solution. Tetracyanoplatinate (II) fluoresces weakly in solution[40] the emission spectrum being a rather broad band and its appearance is suggestive of a $\pi^* \rightarrow \pi$ transition. The species is diamagnetic just like all the other platinum (II) complexes.

The trivalent rare-earth ions in the middle of the series have for a long time been known to fluoresce under ultraviolet excitation. The sharp fluorescence transitions of these ions correspond to the absorption lines of the shielded 4f shells. Although Sm^{3+} and Dy^{3+} ions show weak fluorescence, that of Pr^{3+} and Nd^{3+} ions is extremely rare. The outstanding exceptions are the anhydrous chlorides which show bright fluorescence for the whole rare earth group. The fluorescence characteristics of the rare earth chelates is rather attractive as emission may occur from one of a few sources:

(i) from the excited ligand part of the molecule, perturbed by a metal ion,

(ii) from the excited metal ion perturbed by the ligand,

(iii) due to the intramolecular energy transfer in the chelates i.e. absorption of energy takes place in the organic part of the chelate and is transferred to the bound central metal ion (on indirect excitation of the metal ion) which in turn may undergo radiative transition consequently giving rise to characteristic emission of the metal ion.

McCarthy and Winefordner[41] studied the conditions under which various aromatic carbonyl compounds selectively sensitized the emission

of terbium, europium and dysprosium. Excitation of their system was done at the wavelength of maximum absorption for the phosphorescence transition of the carbonyl compound and emission measured at the wavelength corresponding to the rare earth ion emission line. The intramolecular energy transfer from excited organic compounds to rare earth ions[42-44], on grounds of selectivity and efficiency of energy transfer, has a wide scope for analytical utility.

Attempts were made at the sensitization of the fluorescence emission of Eu^{3+}, Dy^{3+} and Tb^{3+} in acetic anhydride using $\alpha\beta$-unsaturated ketosteroids containing the 17-hydroxyacetone side chain as well as those without side chain substitution. The excitation wavelength was kept well within the n-π* absorption region for the steroids (300nm - 360nm), but no sensitization of the emission of the rare earths was observed with any of the steroids examined.

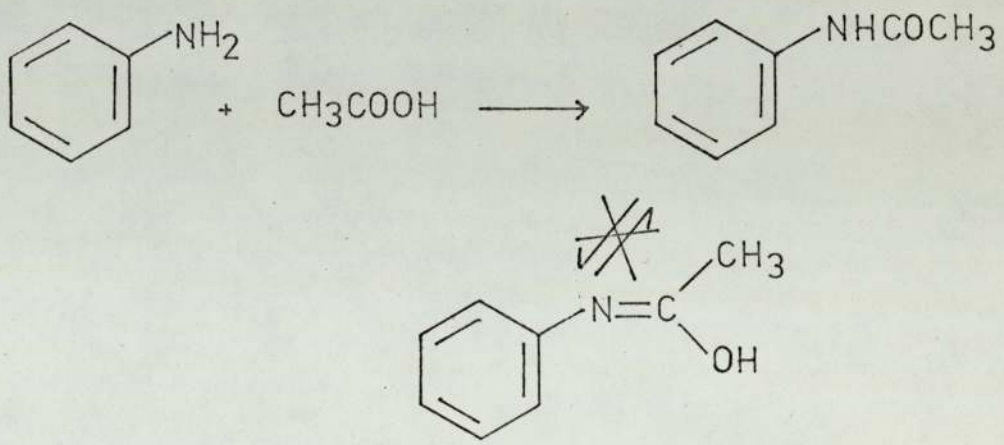
c) Chemical Transformation of the Steroid Skeleton

O-methylaniline was introduced by Hultman[45] as an analytical reagent for the measurement of oligosaccharides. He suggested that aldohexoses react with o-methylaniline in acetic acid to form an equilibrium mixture of glycosamines and the corresponding Schiff base with the resulting chromogen exhibiting an absorption maxima at 630nm. The reaction was further developed by Goodwin[44] for the determination of hexoses and pentoses using aniline and its alkyl derivatives. He found that only primary and secondary aromatic amines reacted with hexose and pentoses to yield a chromogen and that the absorption maxima of these chromogens ranged from 330 - 630nm. The ease of formation of these chromogens prompted similar work to begin on the 17-hydroxyacetone side chain. Preliminary investigations indicated that hydrocortisone in glacial acetic acid (HOAC) reacted

with aniline producing a chromogen with an absorption and emission maxima at 355 and 440nm respectively. However, it was observed that aniline in HOAC left at room temperature became progressively yellow on standing. The colour change was monitored spectrophotometrically between 250 - 450nm as a function of time.

The spectra (fig. 2) show that there is initial formation of a chromophore A at 325nm which decreases in intensity with time as a second chromophore B appears at 385nm. The reaction appears to have reached completion after 24hr. when chromophore A has completely disappeared and B has fully formed.

Intuitively, it was suggested that the appearance of chromophore A was due to a reaction of aniline with HOAC to form acetanilide which then enolized under acid conditions to extend the conjugation.



Acetanilide in HOAC even after 24hr exhibited UV absorption similar to that in ethanol. Further attempts to effect enolization with concentrated hydrochloric and perchloric acids were unsuccessful; the UV absorption observed was still that of acetanilide alone.

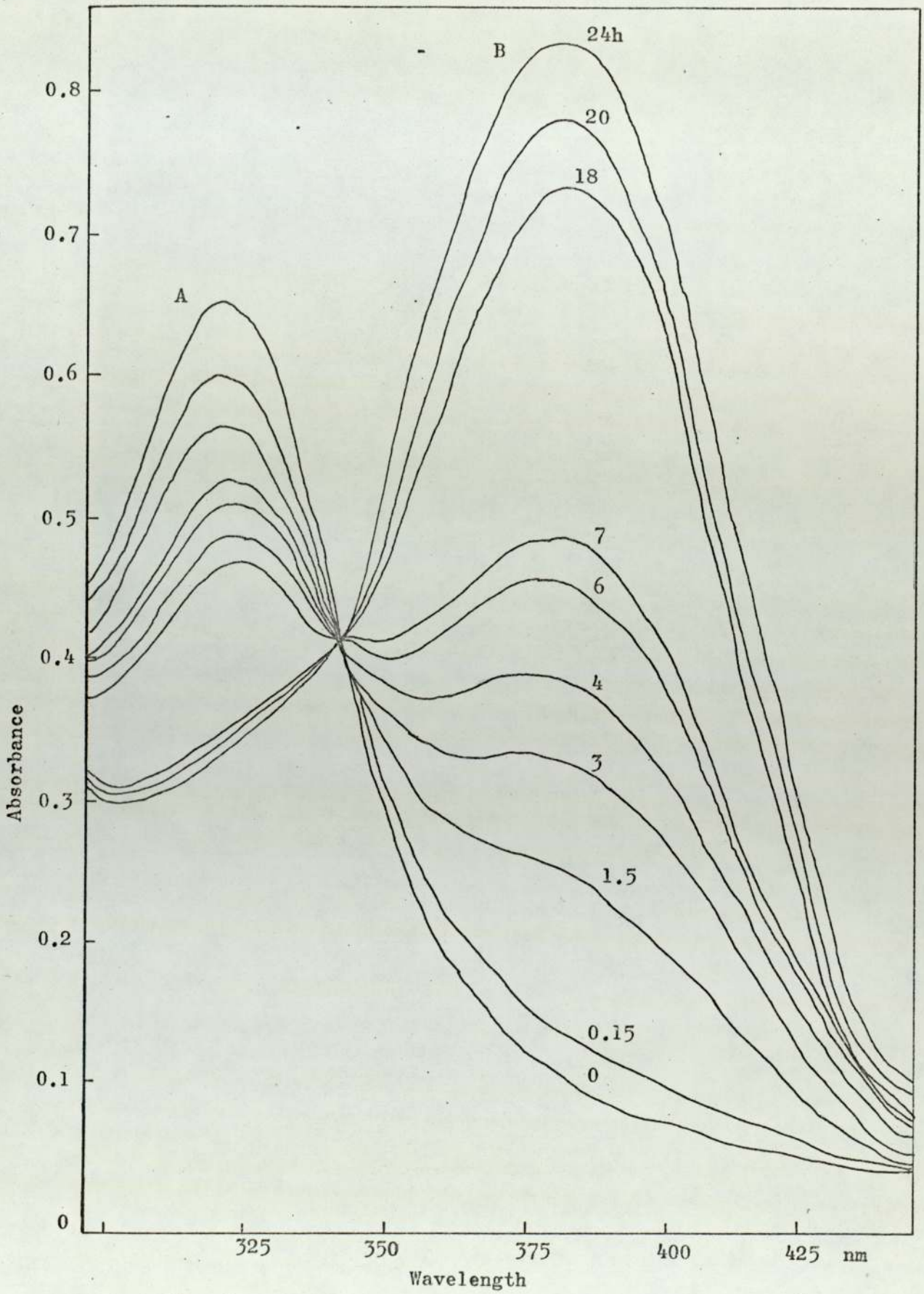


Fig. 2. Absorption characteristics of Aniline (conc. $^{M}/400$) time dependent spectra in glacial acetic Acid over 24 hours.

Many factors influenced the behaviour of aniline and its derivatives in HOAC. A summary of these factors observed in the course of the study is as follows:

(i) The concentration of aniline in solution was varied as the amount of product formed (equated to the final intensity of absorption of chromophore B) was observed. There was little change observed on reducing the aniline concentration from M/100 to M/400. This would seem to suggest that the amount of product formed is probably very small and, therefore, largely uninfluenced by aniline concentration. The reaction was, however, time dependent and with M/400 aniline concentration it took 24hr to reach completion.

(ii) The spectrum of aniline in HOAC showed a small peak about 255nm which would indicate the presence of some anilinium cation species. Since HOAC does not transmit in the UV below 250nm, another aromatic amine, 2-aminonaphthalene, possessing a chromophore with a longer wavelength of absorption was investigated. There was a notable increase in the intensity of the spectrum in HOAC at 275nm and the appearance of a peak at 311nm as compared with the spectrum of this compound in ethanol. These observations are consistent with the presence of 2-naphthylamonium cation species in solution. Therefore, aniline and its derivatives are weakly protonated in HOAC.

(iii) The reaction with aniline was found to be both oxygen and light independent as reactions done under nitrogen and in the absence of light gave the same chromophores as outlined above. The reaction was, however, affected by pH, temperature, and dilution changes. Chromophore A disappears on dilution, acidification with mineral acid or addition of base, and on heating. Chromophore B is also destroyed by pH changes although it is stable to heat. HOAC ($ka = 1.8 \times 10^{-5}$)

provides excellent conditions for the reaction to occur. When propionic acid was substituted for HOAC, although it did form, there was a significant reduction in the chromogen, whilst on substitution with formic acid no chromogen was formed at all. Mineral acids also failed to produce the chromogen which would suggest the reaction is strongly pH dependent.

(iv) The presence and position of alkyl groups on aniline influences the spectrum of the reaction products. Steric hinderance of the amino group by methyl substitution at 2 and 6 positions prevents the reaction from taking place. Substitution by a -COOH group at the para position increases the rate of the reaction and the intensity of chromophore B. The presence of another basic center in the aromatic ring prevents the reaction. Aliphatic amines are not substrates for this reaction. Effect of substitution on the reactivity of aniline in HOAC is outlined in table 2.

The nature of the reaction of these aromatic amines in HOAC is not clear. Whatever reaction might be taking place it seems the participation of the lone pair of electrons on the nitrogen in the π-electron system of the benzene chromophore is essential. The lack of success in isolating any products and the dependence of the reaction on temperature pH and reaction time would support the suggestion that a charge transfer type reaction is taking place. Contact charge transfer spectra can occur without the existence of a stabilized ground state of the complex.[47] The existence of a charge-transfer spectrum in a solution of two components cannot, therefore, be taken as evidence for the formation of a complex between them; and if a complex is formed, the appearance of a charge-transfer spectrum gives no evidence that the charge transfer plays a dominant or even a significant part in the ground state stabilization.

Table 2

Compound	max Chromophore A	max Chromophore B	Absorbance (after 24hr)
Aniline	325nm	385nm	0.58
2-Me	320	360	0.72
2,6-Me_2	-	-	-
4-Me	325	390	1.04
2-OMe	335	400	0.98
3-OMe	325	345 + 390	1.38 + 1.24
4-OMe	330	400	0.66
4-COOH	340	405	1.74
4-COOEt	335	405	1.74
2-NH_2	327	375	0.88
N-Me	-	-	-
N-Me_2	-	-	-
Phenylhydrazine	-	-	-
Indoline	340	410	1.54
1,2,3,4-Tetrahydro-quinoline	310	380	0.40
3-Aminopyridine	295	340	0.10
4-Aminopyridine	-	-	-
2-Aminopyridine	-	-	-
Pyridine	-	-	-
Benzylamine	-	-	-

\- No reaction

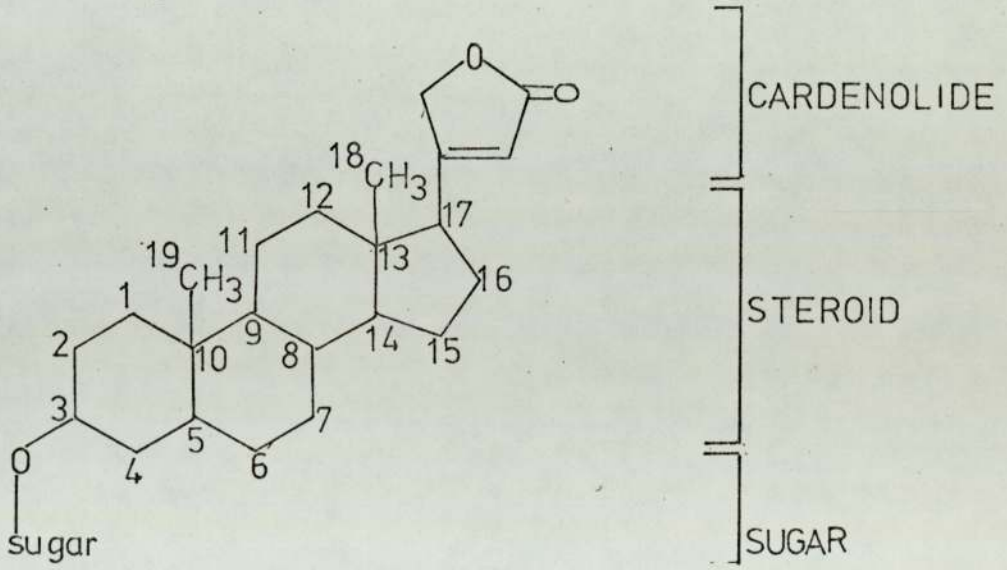
The amines (in table 2) with chromophores A + B in HOAC on reaction with steroids with 17-hydroxyacetone side chain gave fluorescent products at sub-microgram concentrations. The fluorophors were, however, unstable and their emission intensities decreased with time.

Steroid fluorescence analysis has depended to a very large extent on the chemical transformation of the steroid skeleton using concentrated mineral acids. The nature of the reacions which take place still remains unknown and the numerous reaction products difficult to isolate[48,49]. The use of moderately strong organic acids for steroid analysis has remained relatively unexplored. It was felt that the use of organic acids could reduce the number of reaction products and aid in the production of more specific fluorophors for individual steroids under controlled conditions.

3. SELECTIVE ACID-INDUCED FLUORESCENCE OF DIGOXIN

3.1 Introduction

The cardiac glycosides form an important group of steroidal glycosides capable, in very small amounts, of stimulating the heart muscle. A well known source is the foxglove, Digitalis purpurea, the active principles of which are glycosides of digoxigenin, digitoxigenin and gitoxigenin and afford these aglycones on acid hydrolysis. The free aglycones or genins are only weakly active. The corresponding glycosides digoxin (Vla), digitoxin (Vll), and gitoxin (VIII) are all triosides of comparable high potency. These glycosides are of outstanding value in the treatment of heart disease because they exert a selective action upon the organ: they increase the contractibility of



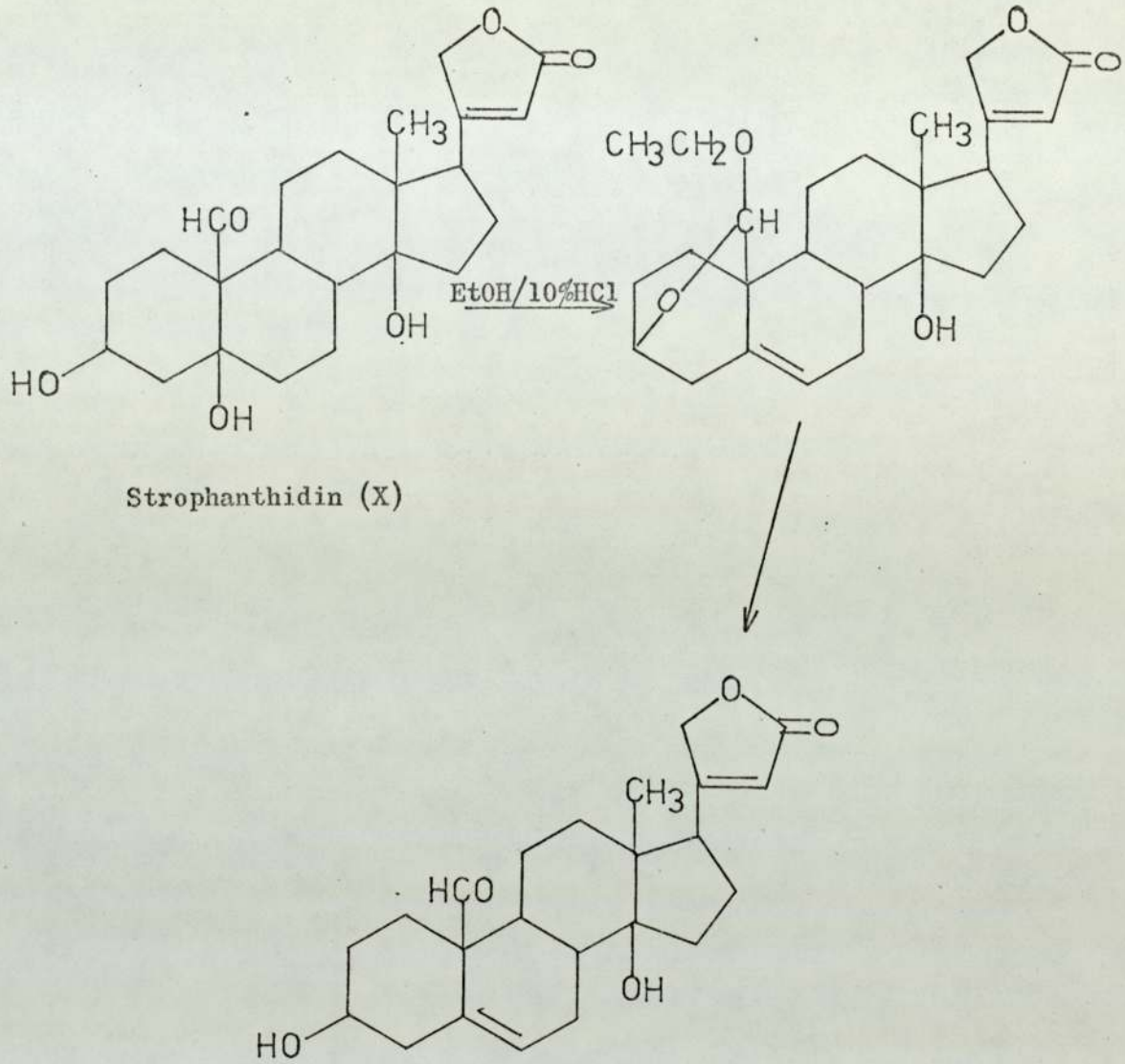
Name	OH position
Digoxin Vla	14, 12
Digitoxin Vll	14
Gitoxin Vlll	14, 16
Ouabain lX	14, 1, 5, 11, 19

Fig. 3. The structure of some cardiac glycosides.

the heart muscle, diminish the heart rate, and increase cardiac efficiency. Cardiac glycosides have a selective affinity for cardiac tissue and whether given orally or by intravenous injection the amount taken up by heart muscle is 10 - 40 times that taken up by all other tissues and organs.[50]

Common to all the glycosides in Fig. 3 are: an $\alpha\beta$-unsaturated five-membered lactone ring, a 14β-hydroxyl group and a trisaccharide sugar moiety at position 3. The sugar residue may be of several types (cymarose, digitoxose, glucose, rhamnose, sarmantose and xylose). Much of the early work to elucidate the structure of these glycosides was done with strophanthidin (X) which proved an unfortunate choice.[51] Strophanthidin has a tertiary hydroxyl group at C - 5 and an angular aldehyde group which enable it to undergo a number of dehydration, rearrangement and lactone formation reactions not possible with any of the glycosides in Fig. 3, except ouabain (1X), with which it has some structural similarities. The reaction of HCl with strophanthidin (Fig. 4), however, demonstrates that the reaction products are dependent on the extent of substitution of the steroid ring as well as on the reagent constitution.

Of the cardiac glycosides, digoxin has received most attention because it is probably the most widely used and it has of late been a subject of much controversy with respect to its biopharmaceutical evaluation.[52] The majority of methods reported for quantitative determination of cardiac glycosides are colorimetric,[53-55] fluorimetric,[18,19,56-59] or radioimmunoassay.[60,61] None of the colorimetric procedures exhibit the necessary sensitivity or selectivity for the detection or estimation of these glycosides at submicrogram levels. The radioimmunoassay for digoxin reported by Parker and



Strophanthidin (X)

Anhydrostrophanthidin

Fig. 4 Reaction of Strophanthidin with HCl.

others,[60] although highly sensitive, requires a whole working day to complete the measurement of blood and urine digoxin samples. The method also lacks delectivity for digoxin and when digitoxin is present in a concentration three times that of digoxin, it produces an equal effect to digoxin. Recently, Bogulaski and Shwartz[61] in developing a column radioimmunoassay method for the determination of digitoxin also noted that digoxin cross-reacted significantly with

the antisera they used, hence interfering with digitoxin estimation. From their data they conclude that the antiserum cannot discriminate very well between the various cardiac glycosides implying the need to know which drug has been prescribed prior to assay. Since the cardiac glycosides are sometimes administered in admixture and are of non-equivalent potency, it is of both analytical and therapeutic interest to develop selective methods for the estimation of the individual glycosides. Fluorimetric methods were, therefore, investigated.

The cardenolides possess a common chromophore, the $\alpha\beta$-unsaturated five-membered lactone ring, with characteristic absorption at 217nm ($\log \varepsilon$ 4.22).[51] Existing fluorimetric methods usually depend on the dehydration of the cardenolides by treatment with strong acids and oxidising agents to form products with extended conjugation which fluoresce under ultraviolet irradiation. Sciarini and Salter[56] described a fluorimetric method for digitalis estimation using hot sulphuric acid. This method was superseded by Jensen's hydrochloric acid methods for digitoxigenin[18] and gitoxigenin[57]. Wells et al,[19] describes a modification of Jensen's methods in which he used hydrogen peroxide, ascorbic acid, and methanol to enhance the fluorescence intensity. Detailed discussion of the influence of time, temperature, and reagent concentrations on the excitation and emission properties of the fluorophors as well as the possible mechanisms of fluorescence were described. Although difficulties were encountered in their attempts to apply this method to assay extracts of the glycosides from biological fluids, it still is probably the most widely used method for routine fluorimetric estimation of cardiac glycosides. Jakovljevick[59] reported a method

for simultaneous determination of digitoxin and digoxin based on a mixture of acetic anhydride, acetyl chloride and trifluoroacetic acid to produce fluorophors which were partly resolved using a combination of filters. From the photophysical data obtained it was suggested that the reagent reacted with digitoxin to produce, in a low yield, a fluorophor of a substituted 3,4-benzpyrene type and with digoxin to produce a mixture of both chrysene and 3,4-benzpyrene type. No further reports on the fluorophors have so far been cited in the literature.

It would seem reasonable to suggest that the successful development of a fluorimetric method with total selectivity for a particular cardiac glycoside will, of necessity, depend on a reaction taking place on the steroid ring to form a product with unique photophysical properties. Following previous reports by Smith[62,63] that dehydration products of digitoxin on reacting with HCl were dependent on the acid concentration, it was felt that careful selection of acid as well as acid concentration are necessary pre-requisites to making attempts to develop a selective method for a particular glycoside. The work described here which has led to a development of the specific fluorimetric determination of digoxin has also been described elsewhere.[64]

3.2. Thin-layer Chromatographic Evaluation of Steroids

There are no colour spot tests specific for steroid molecules. Several different groups of compounds frequently present in biological fluids, tissues and crude extracts may give positive reactions similar to steroids when treated with the same reagent. However, when steroids are available as pure substances, certain chemical structures present in the molecule can be responsible for a positive test when carried

out under specified conditions.

The reactions available for the chromatographic detection of steroidal substances show a very large range of sensitivities. A review in 1969 by Lisboa[65] quotes over eighty colorimetric reagents for the detection of steroidal compounds. There are reactions which develop a very definite and stable colour almost immediately after applying the spray reagent (tetrazolium salt reaction).[66] Others give colour spots which disappear very easily or change colour when left standing for a few minutes (Iodine - KI reaction).[66] In some of the reactions which require heating, the control of temperature is extremely critical (phosphomolybdic reactions).[67] There are reactions which develop very light colours unless the concentration of the steroid is increased (sulphuric acid).[68,69] Some reactions give a positive or a negative result when a given steroid is present in amounts above or below the limits of detection, while others give a variety of colours depending on the actual specific structure of the compound in question[70,71], or depending on the concentration of the steroid (Iodine - KI reaction).

As part of a programme into the development of sensitive and possibly specific luminescence methods for the determination of steroidal substances in biological fluids, evaluation of some of the colorimetric reagents for this purpose was undertaken. It was found that an assessment of a reagent's potential for quantitative analysis could be obtained by using it as a spray on the plates. Examination of the fluorescence produced gave a rapid indication of the reagent's specificity and approximate sensitivity.

Prompted by the report that α-hydroxyketones can be detected by reagents containing m-, and p-phenylenediamines in acetic acid[72], these amines and also aniline and o-phenylenediamine were used for the

detection of steroidal compounds. It was considered that these reagents might be reactive with corticosteroids. Table 3, summarises the results obtained by spraying over twenty-five steroidal substances with the amines in either glacial acetic acid (HOAC) or trichloroacetic acid (TCA). With the steroids at 0.4μg levels only aniline and p-phenylene diamine (PPD) in (HOAC) produced detectable chromophores. Aniline developed fluorescent spots with four of the compounds and PPD gave rise to non-fluorescent brown spots with nine compounds. The reaction of PPD is seen to be structure specific. The colour is obtained only with those steroids which possess an unsaturated conjugated carbonyl 4-en-3-one, system in ring A. On using the same amines in TCA the specificity is lost and both aniline and PPD detect roughly the same number of steroids (10 - 13). On attempting to apply the HOAC system to the estimation of steroids in solution it was found that both the fluorescence produced with aniline and the absorption with PPD was not quantitative and insensitive at levels below 1μg of steroid. It has been suggested by Frerejacques and de Graeve[73] that a mixture of chloramine-T(CLAT) in TCA produces stable fluorescent products with cardiac glycosides on tlc plates. The use of this reagent for the detection of other steroids or for the estimation of either group of compounds in solution has not been reported. The scope of this reagent was widely examined and the results are summarised in Table 4. The role of the acid was determined by a prior examination of the effect of TCA, HOAC, mono- and dichloroacetic acids, (MCA and DCA), in the absence of CLAT. It can be seen, Table 4, that all four acids produce fluorescence in the absence of CLAT with many of the steroids. The TCA reacts with more of the compounds than the other

STEROIDAL COMPOUND	REAGENT			
Applied at 0.4μg on to T.L. silica plate, sprayed with reagent, baked for 5 min. at 105°; visualised under 366nm., (+)ve signs indicate visual estimate of fluorescence	1% Aniline in 25% aqueous		1% PPD in 25% aqueous	
	HOAC	TCA	HOAC	TCA
. 5α-Androstan-17β-ol, 3-one	-	-	-	-
. 5α-Androstan-17α-methyl-3β,17β-diol	-	+	-	++
. 5α-Androstan-3α-ol, 17-one(Androsterone)	-	-	-	-
. 5α-Androstan-3β, 17β-diol.	-	-	-	-
. 5-Androsten-3β-ol, 17-one(Dehydroepiandosterone)	-	+	-	++
. 4-Androsten-3,17-dione	-	-	++	-
. 4-Androsten-3,11,17-trione	-	-	++	-
. 4-Androsten-17β-ol, 3-one(Testosterone)	-	-	++	-
. 4-Androsten-17α-methyl, 17β-ol, 3-one	++	+++	++	++
. 5-Androsten-17α-methyl, 3β, 17β-diol	-	+	-	-
. 5α-Pregnan-3,20-dione	-	-	-	-
. 5α-Pregnan-3β-ol 20-one	-	-	-	-
. 5β-Pregnan-3α, 20α- diol. (Pregnanediol)	-	-	-	+
. 5β-Pregnan-3α, 6α-diol, 20-one	-	+	-	-
. 4-Pregnen-3,20-dione(Progesterone)	-	-	++	-
. 4-Pregnen-17α, 21-diol, 3,11,20-trione(Cortisone)	++	+	++	-
. 4-Pregnen-11α-ol, 3,20-dione	-	-	++	-
. 4-Pregnen-21-ol-3,20-dione	-	-	++	-
. 4-Pregnen-3,11,20-trione	-	+	++	-
. 5-Pregnen-3β, 20βdiol.	-	+	-	++
. 5-Pregnen-3β, 17α-diol; 20-one	-	+	-	++
. 5-Pregnen-3β-ol, 20-one	-	+	-	++
. Digoxin	-	++	-	++
. Digitoxin	++	++	-	++
. Gitoxin	++	++	-	++
. Ouabain	-	-	-	-
. Cholesterol	-	+	-	++

No reaction was obtained with o- and m-phenylenediamines in either HOAC or TCA.	Fluorescent spots +	Fluorescent spots ++	Non fluorescent spots -

Table 3.

Steroid numbered as in Table 3.

REAGENT- sprayed onto steroid at 5-10µg on T.L. silica plate, then baked at 120° for 5 min. and visualised under 350nm. *Substances not detected below 0.4µg level.

	HOAC	HOAc/CLAT	MCA	MCA/CLAT	DCA	DCA/CLAT	TCA	TCA/CLAT
1	-	-	-	-	-	-	-	-
2	-	++	+	++	+	+++	+	+++
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	+++	-	+++	-	+++	++	+++
6	-	+++	-	+++	-	++	-	+++*
7	-	-	-	-	-	-	-	-
8	-	+++	-	+++	-	+++	+	+++*
9	+++	+++	+++	+++	+++	+++	+++	+++
10	+++	+++	+++	+++	+++	+++	+++	+++
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	+++
14	-	-	-	-	-	-	-	-
15	-	++	-	+++	-	+++	-	+++
16	-	-	-	-	-	-	-	-
17	-	-	-	-	-	++	-	+++
18	+	++	+	+++	-	+++	+	+++*
19	+	+	+	+	+	+	++	++
20	+	++	+	++	++	++	++	+++
21	-	-	-	-	-	-	++	++
22	-	+++	-	++	+	+++	+	+++
23	+	+++	-	++	++	+++	+	+++
24	++	+++	++	+++	++	+++	++	+++
25	++	+++	++	+++	++	+++	+++	+++
26	-	+	-	++	-	++	-	++*
27	+	++	+	++	++	+++	+++	+++

Table 4.

three acids. The presence of CLAT with acids, particularly with TCA, appears to increase the range of detection in terms of the number of compounds which react. The results show that a mixture of TCA/CLAT may be used as a general purpose spray reagent for the detection of steroidal compounds at 5 - 10μg levels. At levels of 1 - 5μg the number of steroids detected is reduced by four and below these levels intense fluorescence is observed from only the cardiac glycosides digoxin, digitoxin and gitoxin. With the exception of 4-androsten-17α-methyl, 17β-ol, 3-one and 5-Androsten-17α-methyl, 3β, 17β-diol, only six other steroids showed a weak fluorescence at this level. From these observations it may safely be concluded that a solution of p-phenylenediamine in acetic acid can be used as a spray reagent for locating αβ-unsaturated steroidal ketones without interference from other steroids and that a mixture of chloramine-T and trichloroacetic acid can be used as a general purpose reagent for the location of steroidal substances on tlc plates. The possibility of the transfer of cardiac glycoside - TCA fluorescence on tlc plates into solution was subsequently investigated.

3.3 The Specific Fluorimetric Determination of Digoxin

Mechanism of the Reaction

The determination is based on the formation of a fluorophor upon heating digoxin with TCA in ethanol at 130°C for 40 minutes. Excitation and emission spectra of the fluorophor in ethanol are shown Fig. 5. Maximum excitation occurs at 340nm and emission at 420nm. The mechanism of the reaction is not clear but work is still under way to isolate and characterize the fluorophor. Preliminary examination of a crude extract of the reaction by IR spectroscopy, however, shows

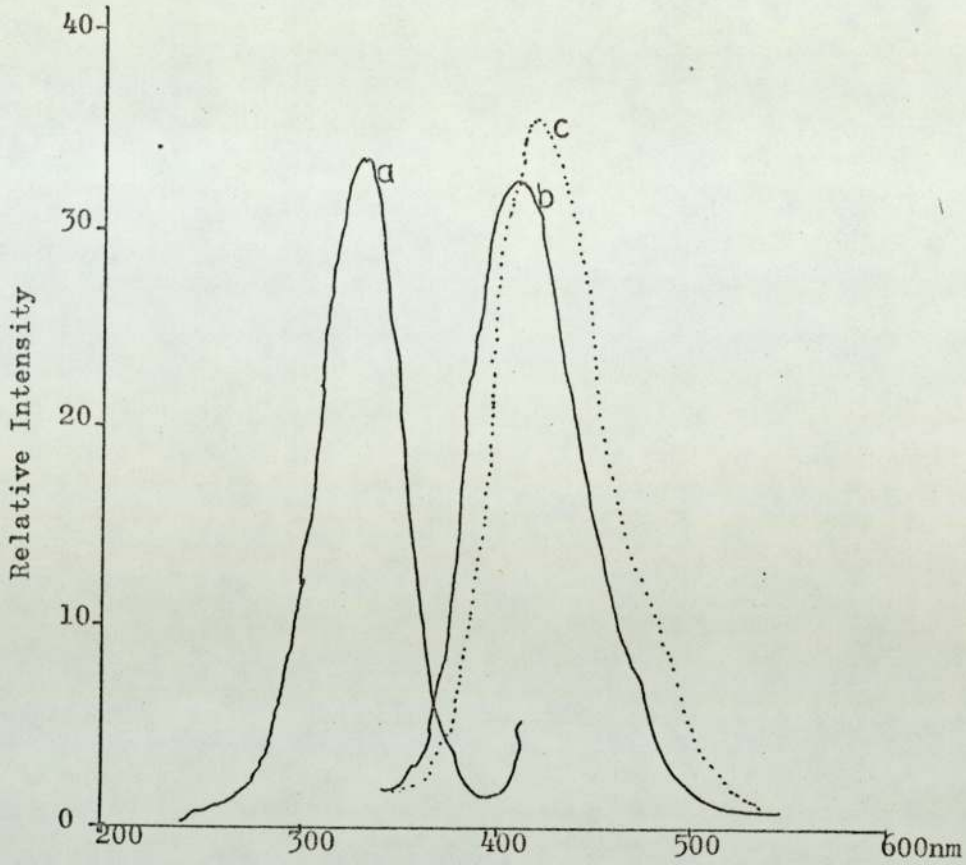


Fig. 5 The excitation and emission spectra of digoxin fluorophor in ethanol.

a = excitation

b = emission

c = corrected emission

that the carbonyl frequency of the cardenolide at 1720cm^{-1} remains relatively unchanged. It is known that the treatment of digoxigenin with hydrochloric acid yields two anhydro derivatives[62,63] Fig. 6, which have been further reacted to produce fluorophors.[19] The excitation (350nm) and emission (490mm) properties of such fluorophors are different from those observed for the digoxin-trichloroacetic acid reaction. Therefore the formation of the fluorophor in the reaction described here may well involve a more complex reaction than a mere dehydration.

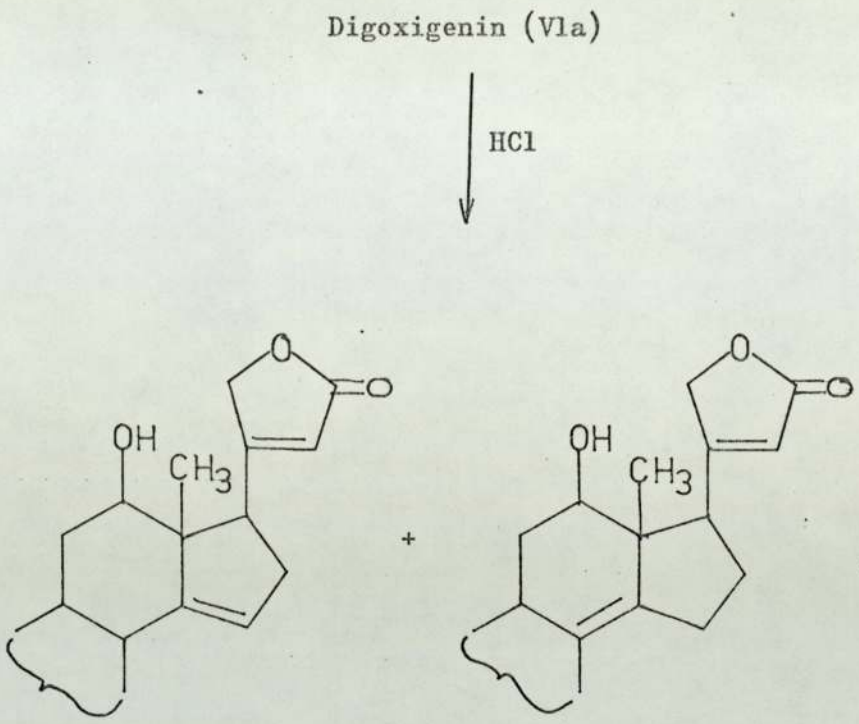


Fig. 6. Reaction of Digoxigenin with HCl

Sensitivity and Reproducibility of the Fluorimetric Determination

The determination of the precise amount of digoxin in tablets is of obvious interest to the pharmaceutical industry, as these tablets are of low dosage (50-250μg per tablet) and high potency. As such they require unit dose analysis as a control on the homogeneity of the product. Clinically, digoxin is normally given in oral doses ranging from 50-1500μg daily. Since the therapeutic dose is very close to the toxic dose methods of high sensitivity are required for the study of the pharmacokinetic behaviour of this drug. The method of Oliver et al[60] on the radioimmunoassay of digoxin is sufficiently sensitive but it suffers from the drawback that a whole working day is needed to complete the assay. On the basis of the sensitivity and

rapidity of the method described here, it seems feasible to investigate its application to direct analysis of digoxin extracts from urine and other biological fluids.

The concentration range of digoxin showing linearity with fluorescence was 0.02 - 1.0μg/ml Fig. 7, and the lowest limit of detection was 0.008μg/ml. A secondary filter of sodium nitrite solution (1% $^{w}/_{v}$) at a fixed position in the path was chosen to eliminate the Raman scatter peak for ethanol which appeared at about 410nm, distorting the emission spectrum. Loss of intensity caused by the filter was about 5%.

Three samples of 250μg tablets and one of 62.5μg were obtained from three different manufacturers and assayed as described. Their digoxin content ranged from 98.6 - 101% of the labelled amount. The standard deviation of duplicate determinations within one assay was ± 0.361% indicating good reporducibility.

Selectivity

The fluorophor is produced as a result of a reaction specific to the steroid ring, as the aglycone gives identical results to digoxin taking into account the difference in molecular weights. The reaction product is characterized by a U.V. absorption maximum in ethanol at 290nm with a shoulder at 320nm. Reaction products of digitoxin and gitoxin have U.V. absorption maxima at 280nm and 335nm, respectively (Fig. 8). Their genins exhibit similar absorption characteristics. Ouabain octahydrate, however, exhibits no U.V. absorption above 250nm after the reaction with trichloroacetic acid. Under the conditions described here for the determination, digitoxin, gitoxin, and ouabain do not interfere with digoxin estimation when present in concentrations ten times that of digoxin (Fig. 9). Other steroids,

including oestrogens and some androgens listed (Table 5) were also found not to interefere.

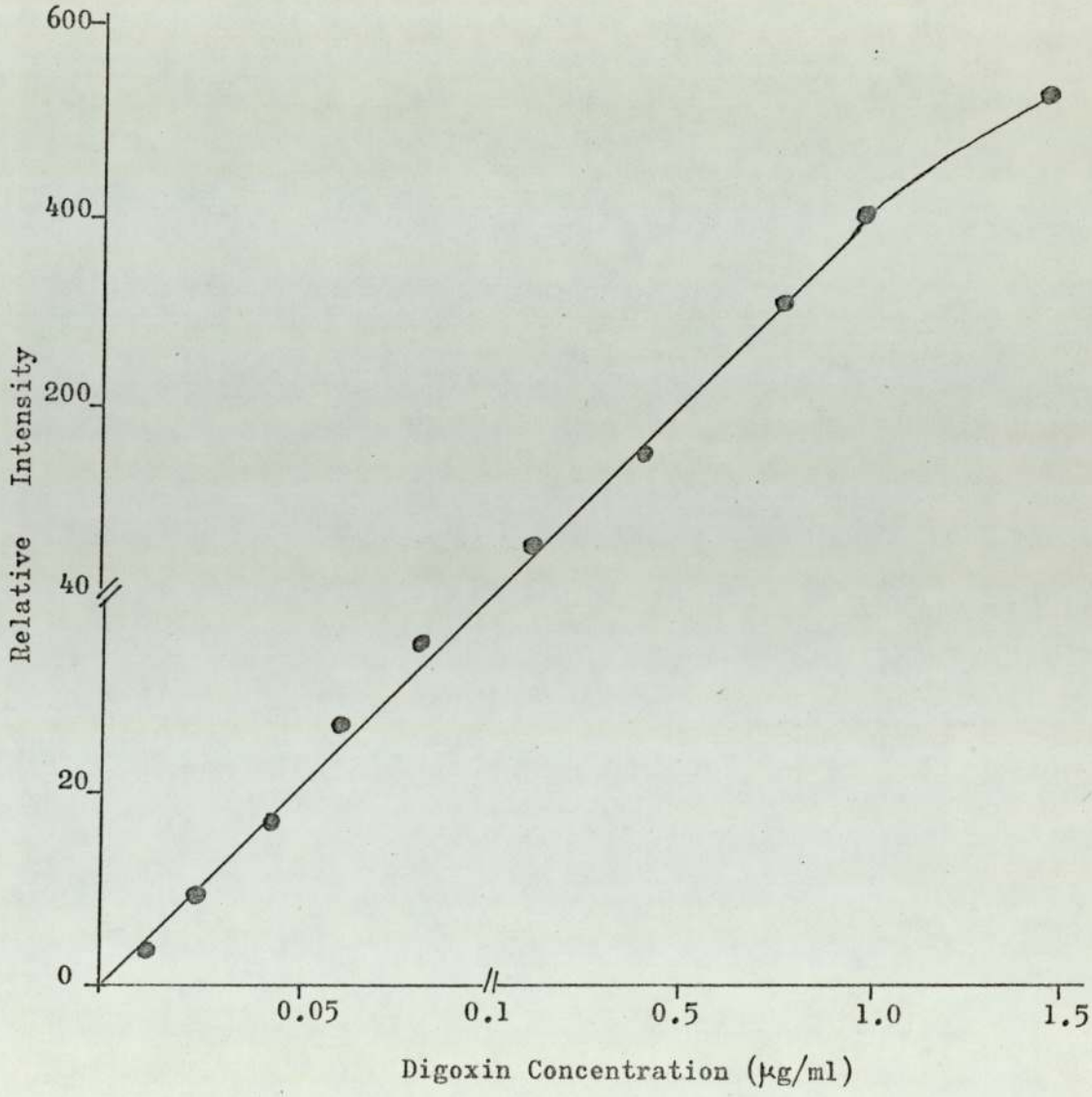


Fig. 7 Calibration curve showing the intensity of fluorescence as a function of digoxin concentration.

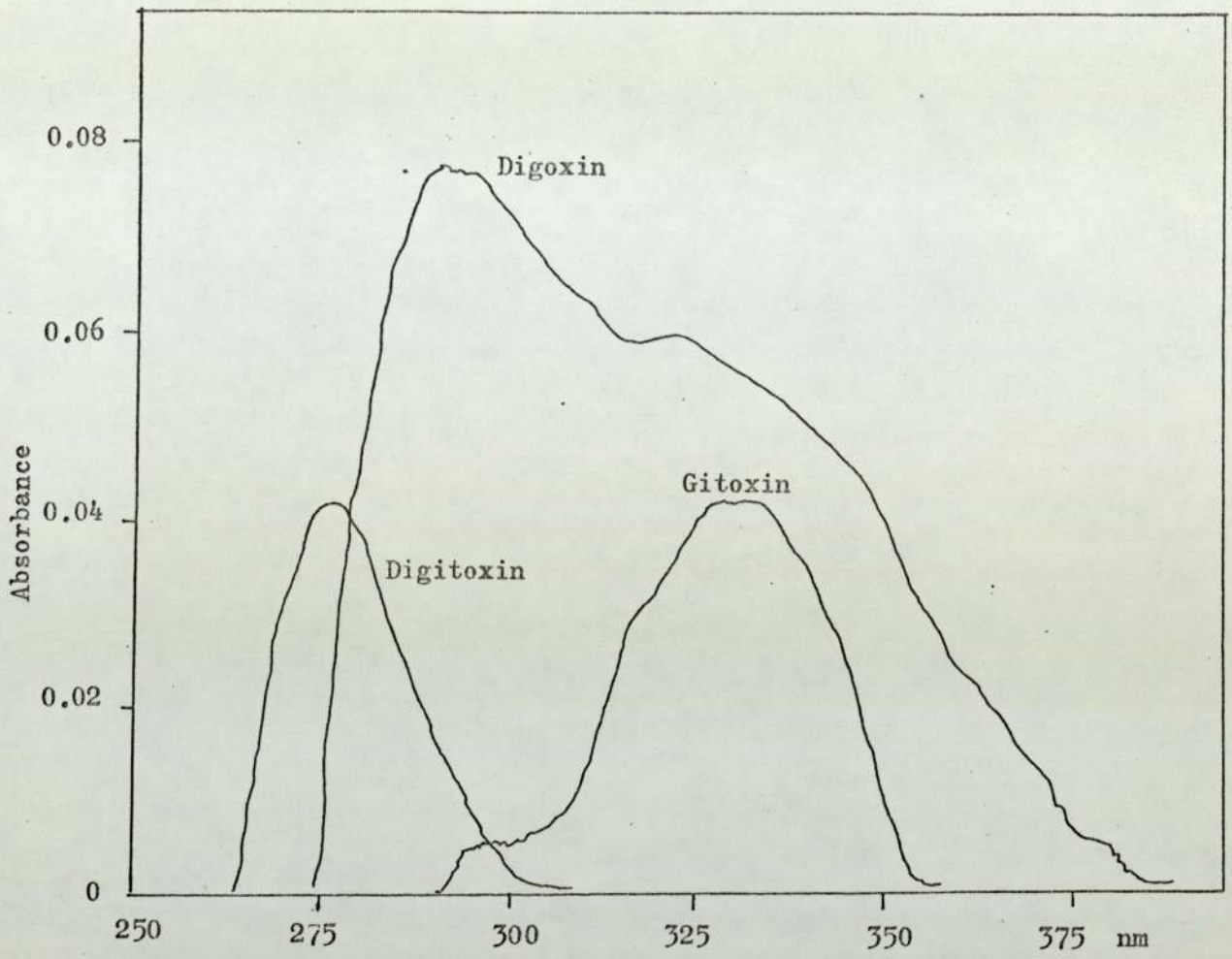


Fig. 8 Absorption characteristics of digoxin, digitoxin and gitoxin, all at 0.4μg/ml, after reaction with TCA.

3.4. Optimal Reaction Conditions for the Assay

The reaction takes about 3 hours to be completed when carried out on a steam bath at 98 - 100°C. At lower temperatures there is no significant reaction. Raising the reaction temperature to 130°C enables the maximum fluorescence to be attained in only 30 minutes. Prolonged heating leads to a gradual decay of fluorescence with time (Fig. 10). A reaction time of 30-45 minutes is optimal under the conditions described for the assay.

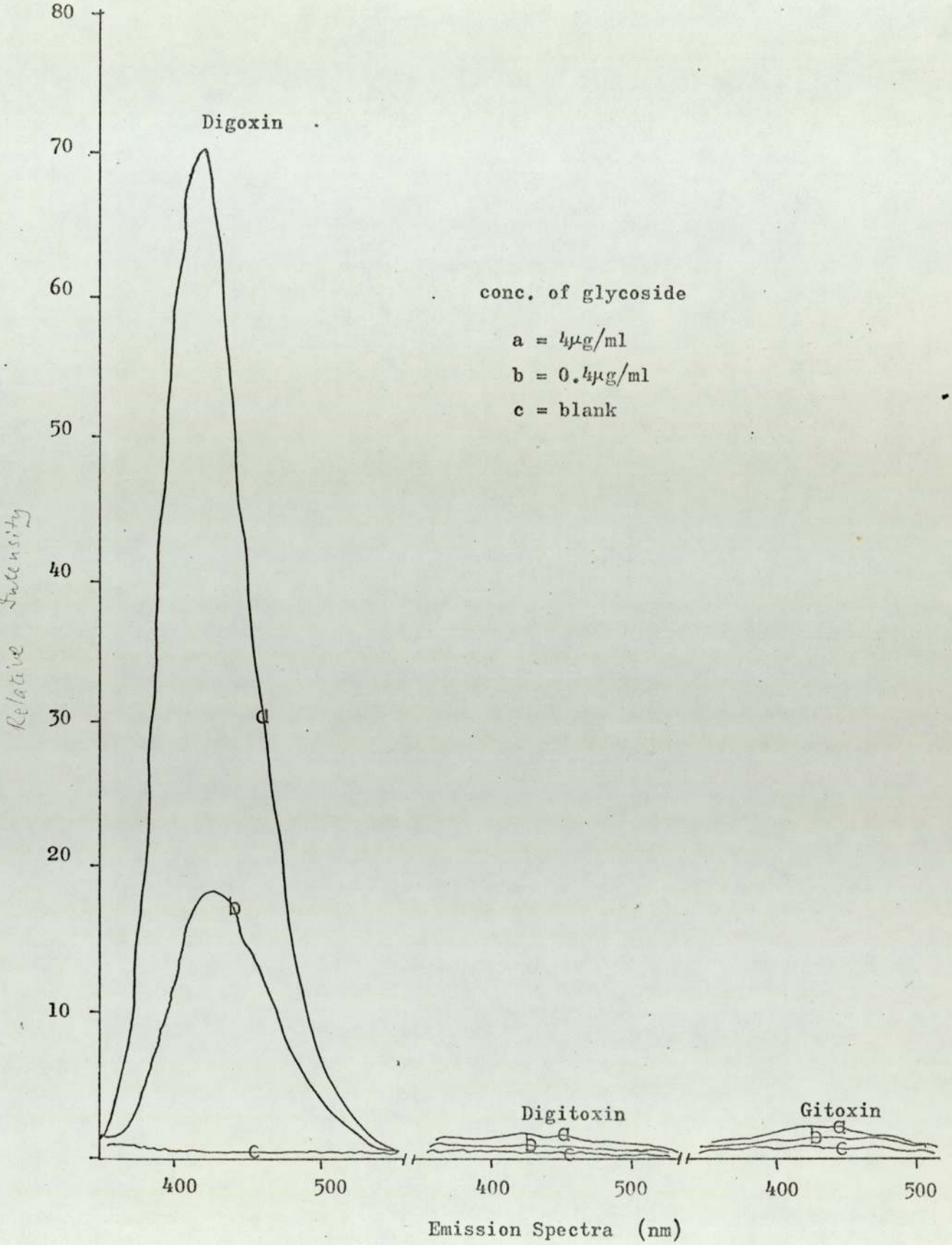


Fig. 9 The specificity of the digoxin assay. Relative intensity of emission spectra of digoxin, digitoxin & gitoxin.

Table 5 Specificity of the Digoxin Assay Method

Steroids & Glycosides	Concentration µg/ml	Fluorescence Intensity	Relative Intensity %
Digoxin	0.4	16.0	100.0
	4.0	189.0	100.0
Gitoxin	0.4	0.7	4.4
	4.0	1.0	0.6
Digitoxin	0.4	0.8	5.0
	4.0	2.5	1.3
Ouabain	0.4	0	0
	4.0	0	0
4-Androsten-17β-ol,3-one	4.0	0	0
4-Androsten-17α-methyl,17β-ol,3-one	"	0	0
5-Androsten-17α-methyl,17β-ol,3-one	"	0	0
4-Pregnen-17α,21-diol,3,11,20-trione	"	0	0
1,3,5(10)Estratriene-3-ol,17-one	"	0	0
1,3,5,(10) Estratriene-3,17β-diol	"	0	0
1,3,5(10) Estratriene-3,16,17-triol	"	0	0
1,3,5,(10) Estratriene-17α-ethyl-3, 17β-diol,3-methyl ether	"	0	0

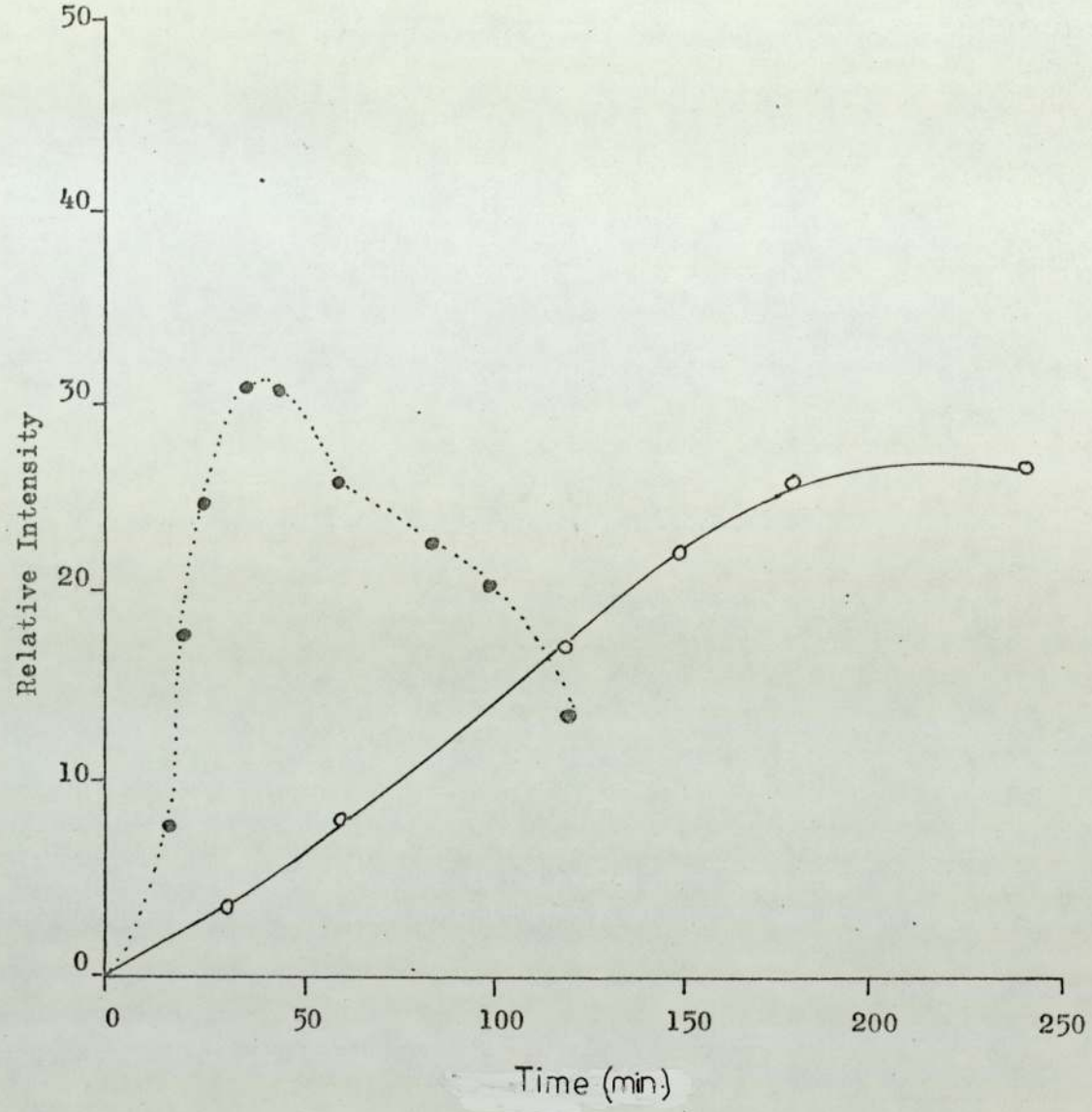


Fig. 10. Effect of temperature and reaction time on the intensity of fluorescence of digoxin (0.4μg/ml) in solution.
(o) 98-100°C, (•) 130 ± 1°C.

The concentration of trichloroacetic acid in the reaction mixture influences the development of fluorescence remarkably (Fig. 11). Though the fluorescence intensity obtained with concentrations of trichloroacetic acid below 10% was large, it was not possible to obtain reproducible results for assay purposes. There was little change in intensity between 10% and 25% concentrations and reproducible results were easily obtained. Between 25% and 40% concentrations the intensity

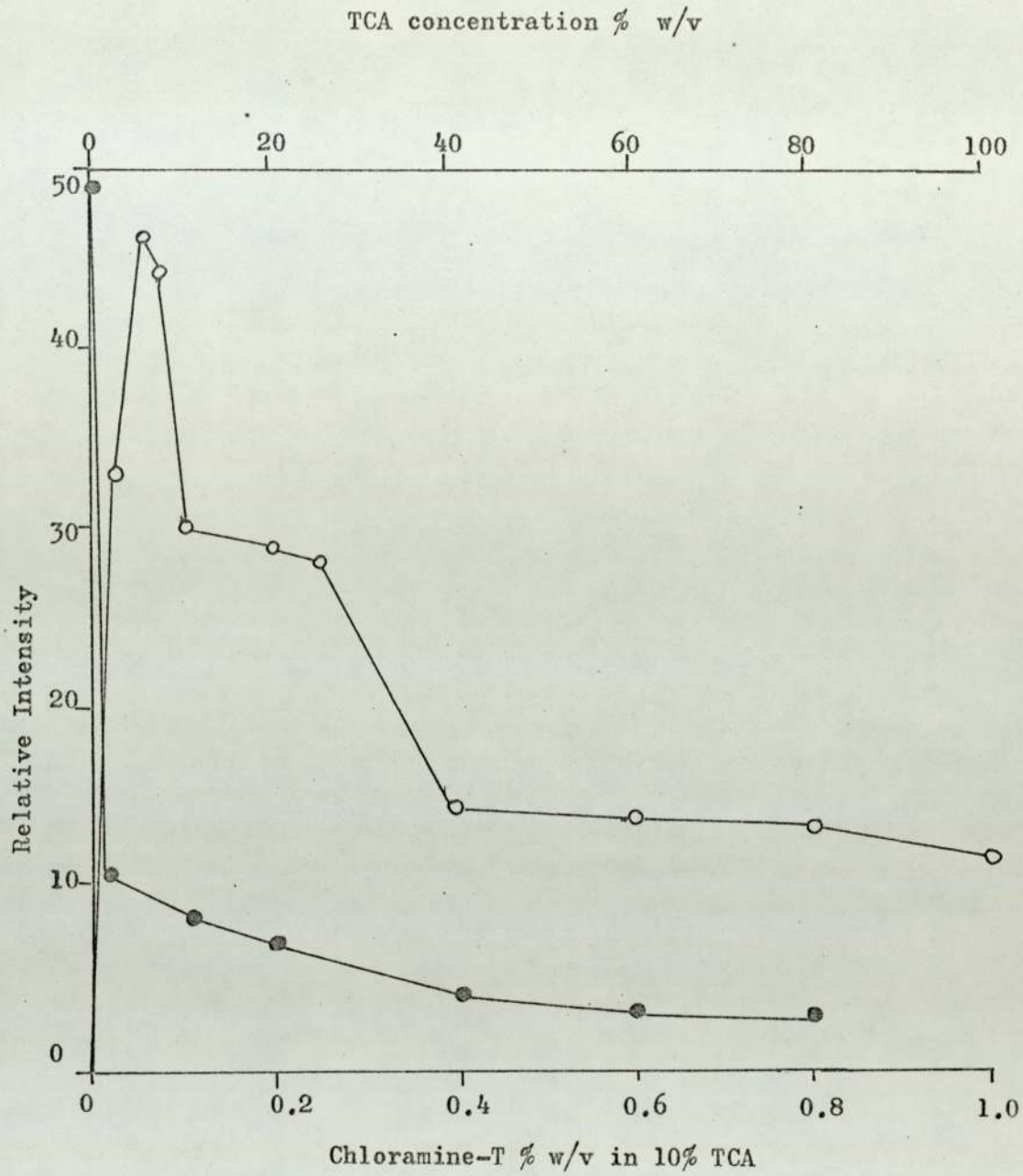


Fig. 11. Effect of trichloroacetic acid and of Chloramine-T concentrations on the development of fluorescence, 0.4μg of digoxin per ml.

(0) Trichloroacetic acid

(●) Chloramine-T in trichloroacetic acid.

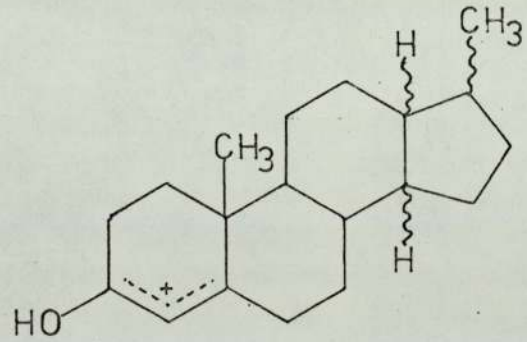
dropped by a factor of about 2 and then gradually levelled off. A concentration of 10-25% was chosen for the reagent on the basis of sensitivity and reproducibility.

A direct application of the chloramine-T-trichloroacetic acid reagent reported by Frerejacques and de Graeve to the determination of digoxin in solution was attempted. The fluorophor was readily produced in the same manner as described except that the emission occurred at a slightly longer wavelength (430nm), the excitation wavelength remaining the same (340nm); the fluorescence intensity was, however, reduced by a factor of 5.0 as compared with the digoxin-trichloroacetic acid reaction. The role of chloramine-T in the reaction was therefore investigated. It was clearly demonstrated that Chloramine-T, even when present in concentrations as little as 0.01% partly quenched the fluorescence of the digoxin fluorophor (Fig. 11). Although it seems feasible to use this reagent to assay digoxin in solution, Chloramine-T appears to serve no useful purpose in the reaction and only reduces the sensitivity.

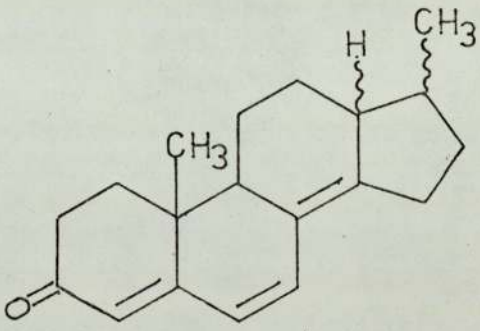
4. ON THE CHEMISTRY OF HYDRO-OXO-PHENANTHRENES

4.1 Introduction

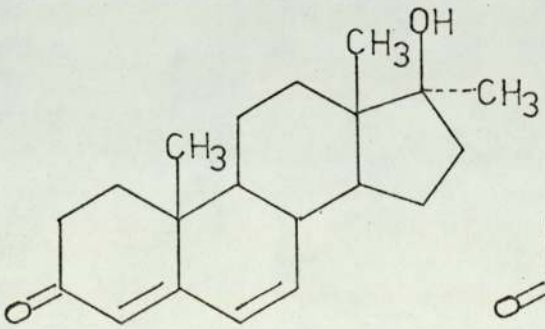
Some recent reports on reactions of steroids with strong mineral acids include attempts to isolate and identify the reaction products[14, 17,48,49,92]. In the majority of the cases structures are merely assigned on the basis of observed chromophores. The colorimetric determination of testosterone by a reaction with strong acids depends upon dehydration with Wagner-Meerwein migration of the 18-methyl group to C-17, followed by a combination of oxidation and protonation leading to chromophoric species of various types[49], such as (X1) and (X11).



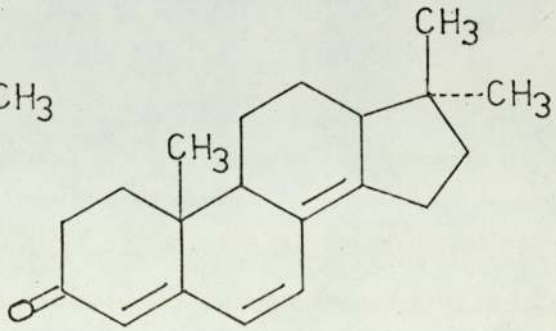
X1



X11



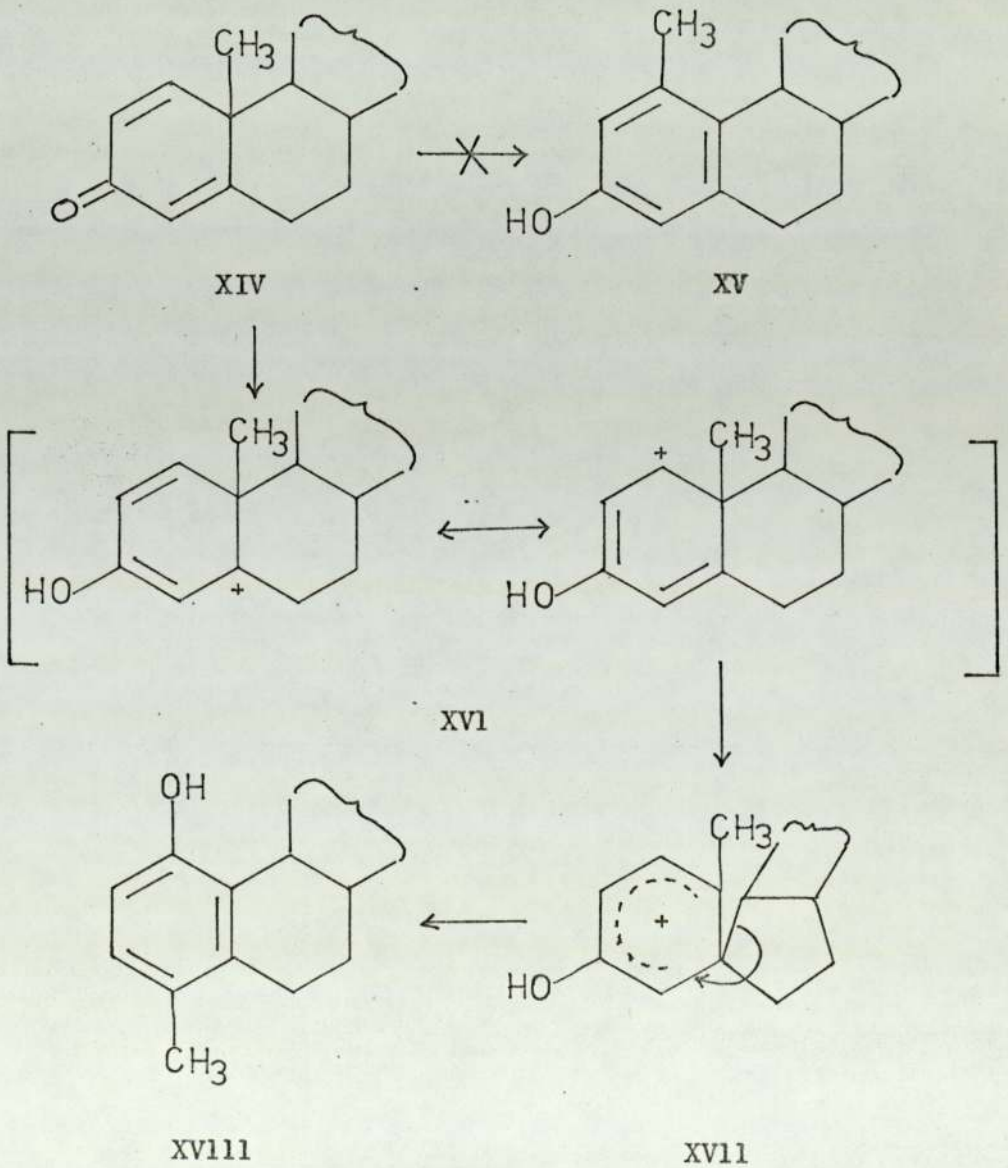
X111



X11a

6,7-Dehydro-17 -methyltestosterone (X111) and similar compounds, rearranged with dehydration in 62% sulphuric acid to give a fluorescent solution which afforded 17 , 17 -dimethyl-18-norandrosta-4,6,8(14)-trien-3-one (X11a). Fluorescence is attributed to the protonated trienone[17].

Aromatisation of the steroid ring in commonly occurring steroids usually requires vigorous reaction conditions and the yields are often very low. Although selenium and palladium charcoal are used as standard reagents at high temperatures[93], the high potential quinones are also widely used, especially in the synthesis of phenanthrenes[74].

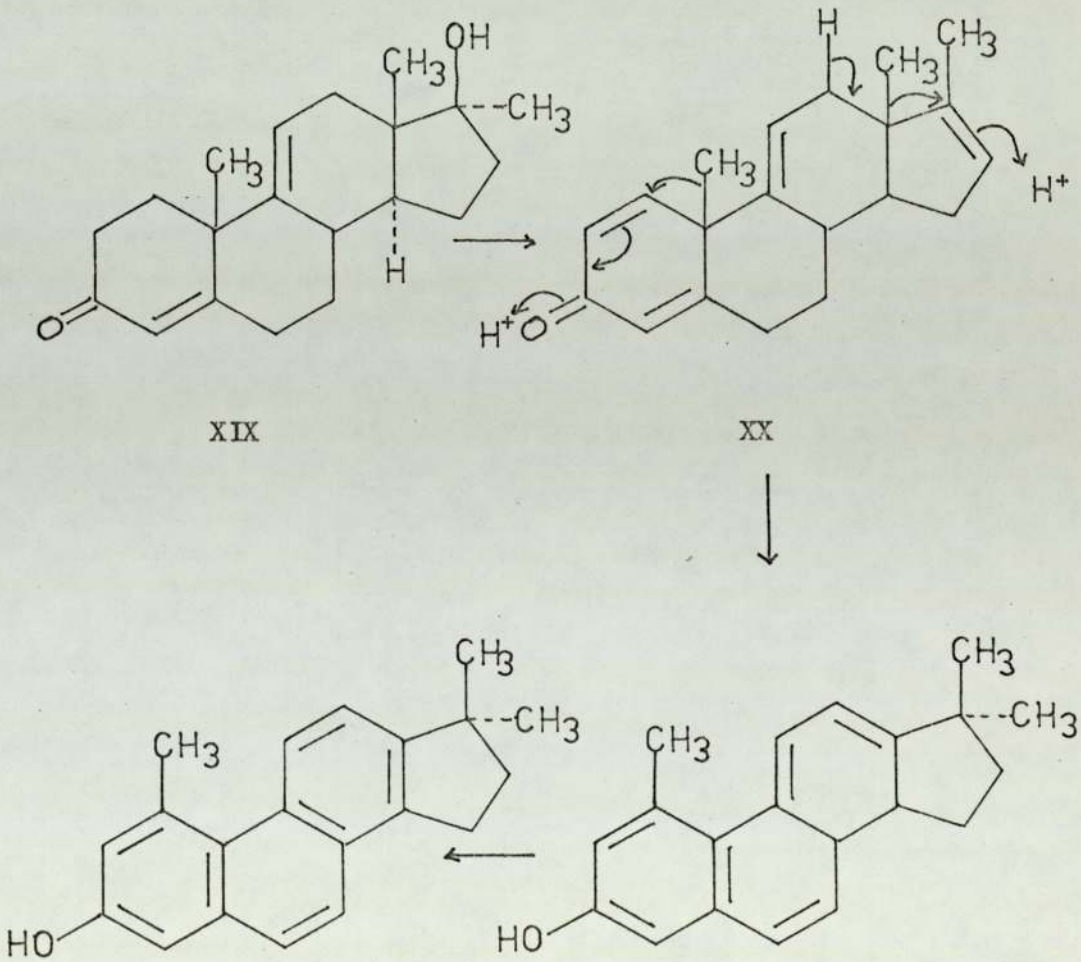


The rearrangement of 1,4-dienones (XIV) occurs to give "para" (1-hydroxy-4-methyl)phenols[75], and not a product corresponding to angular methyl rearrangement (XV). This is envisaged as occurring through the sequence of reactions outlined. It requires an initial attack by a proton or (CH_3CO^+) on the ketonic oxygen atom to give the mesomeric carbonium ion (XVI) which undergoes a 1,2-migration of the C-9 to give a spiranic intermediate (XVII). A second 1,2-shift affords the product (XVIII). A -ring aromatisation is easily effected with acetic anhydride and sulphuric acid.

Dehydrogenation of 9(11)-dehydro-17 -methyl testosterone (XIX) with dichlorodicyano-p-quinone (DDQ) in an acidic catalyst gave the cyclopentenophenanthrene (XXI)[74]. Examination of the resulting mixture showed that 1,4,6,9(11),16-pentaen-3-one (XX) was a likely intermediate. Its formation by acid catalysed dehydration at C-17 is most unusual but it is suggested that the presence of a $\Delta^{9(11)}$ - unsaturation avoids the usual compression of the 18-methyl group. Such compression would seem to exist when an 11-β hydrogen is present and is suggested as the driving force for the 18-methyl migration to C-17, normally observed when tertiary alcohols are dehydrated. The aromatisation is postulated to comprise of a trienone-phenol rearrangement in rings A and B and acid catalysed migration of the 18-methyl group as shown. A final dehydrogenation step in ring C completes the generation of the phenanthrene derivative (XXI).

The anthrasteroid rearrangement, Fig. 12, is unique in the respect that it transforms the normal cyclopentanophenanthrenoid ring system to cyclopentanoanthracenoid skeleton. The nature of the rearrangement is in many ways similar to the dienol-phenol rearrangement as both rings A and B are also involved here.

Treatment of dehydroergosterol (XXll) in chloroform with hydrogen chloride yields anthraergostapentaene (XXlV) as the final rearrangement product[76]. Under controlled conditions, the intermediate product (XXlll) can be isolated[77] and subsequently converted to (XXlV). Therefore, it is clear that the initial dehydration of ergosterol is followed by successive double bond isomerization to give (XXlll) which finally undergoes skeletal rearrangement.



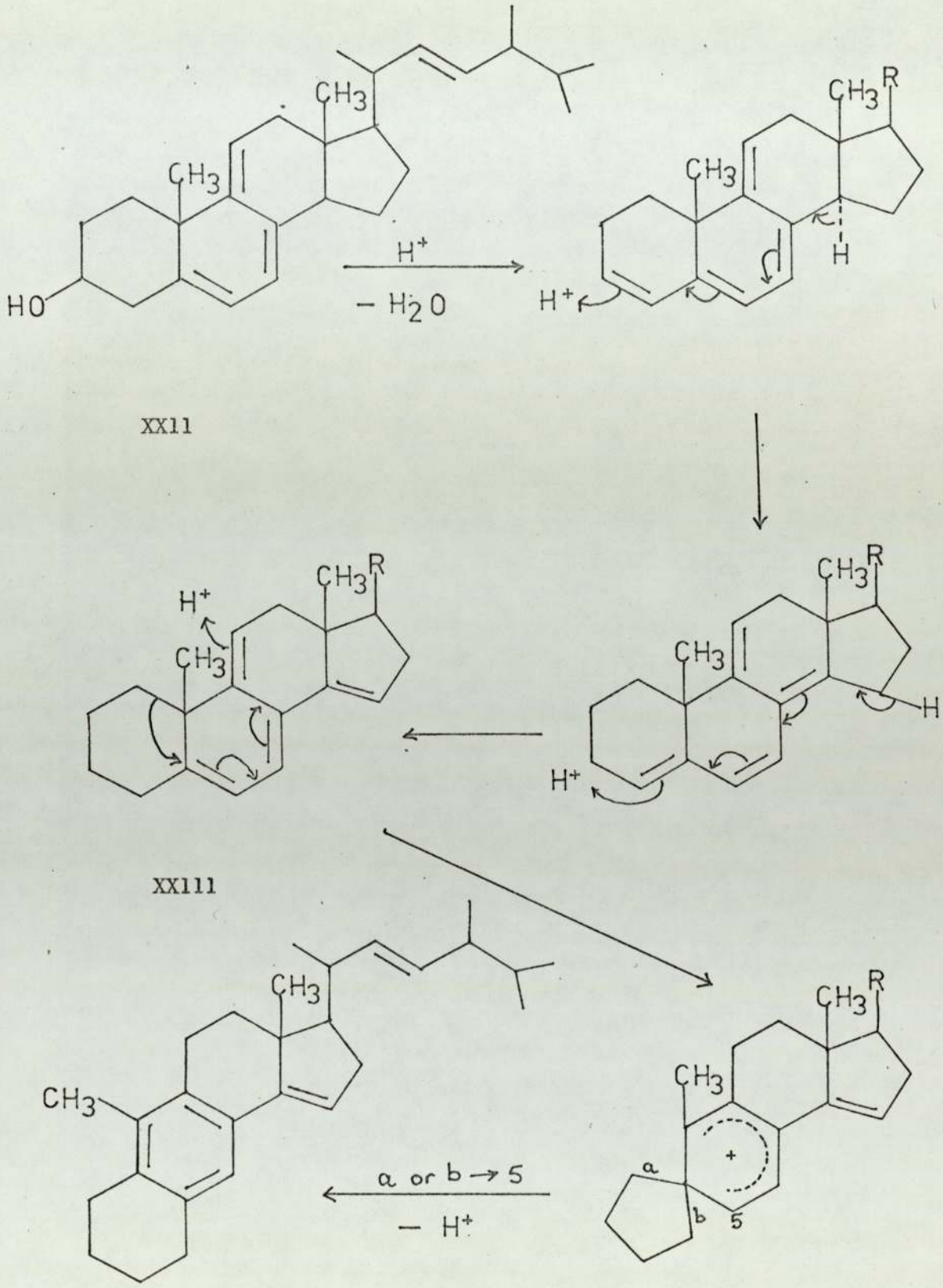
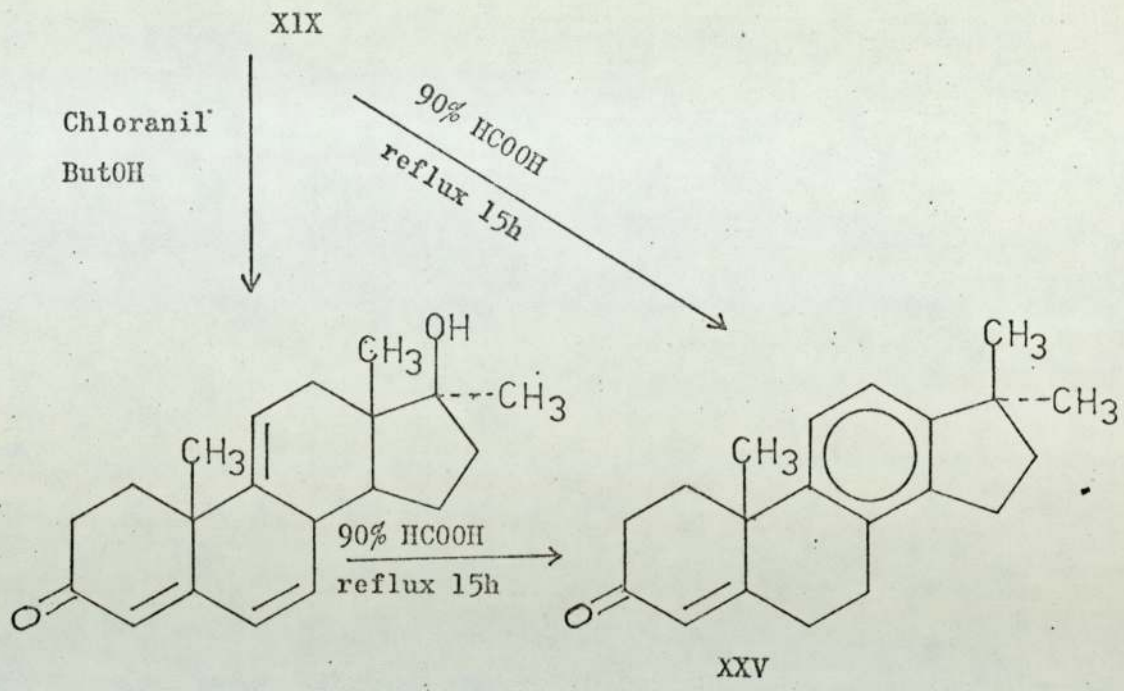

CH3
CH3
HO
H+
- H2O
CH3
R
CH3
H
H+
XXII
CH3
R
CH3
H
H+
CH3
R
H+
CH3
XXIII
CH3
CH3
a or b → 5
- H+
CH3
R
CH3
a
b
5
+
XXIV

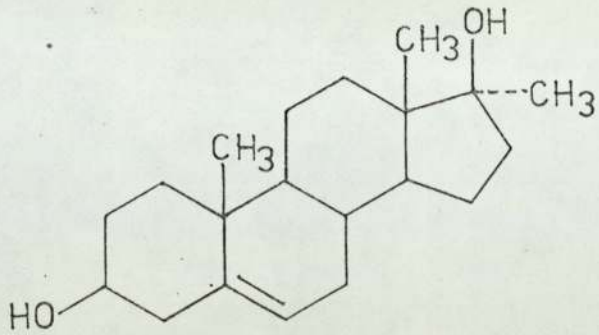
Fig. 12



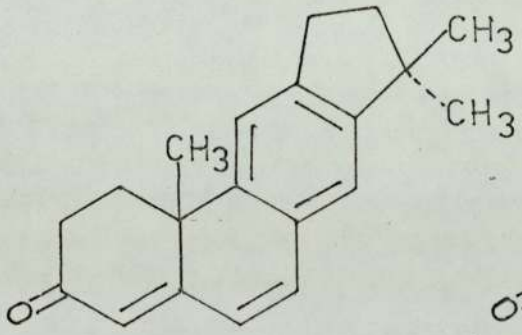
Dehydration of steroidal tertiary alcohols with re-arrangement has been used for selective ring-C aromatisation of steroids[78-80]. Heating (XIX) in 90% formic acid yielded a product identified as (XXV) which was also made more conveniently via a different route[80].

4.2 Aromatisation of 17-methyl testosterone into a hydro-oxo-phenanthrene

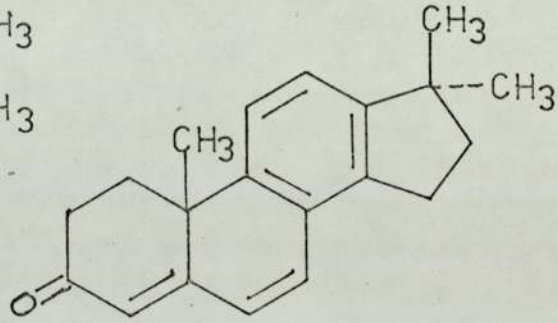
In the course of development of a new fluorimetric method for the quantitative estimation of digoxin (VIa), it was observed that, of the 25 steroids subjected to the TCA reaction, digoxigenin (VI), 5-Androsten-17α-methyl-3β,17β-diol (XXVI) and 17α-methyltestosterone (Va) reacted most readily. The reactivity and availability of (Va) prompted a detailed examination of the reaction an account of which has also been given elsewhere[81].



XXVl



XXVll



XXVlll

A solution of (Va) in TCA and aqueous ethanol on heating under reflux gave rise to a product with fluorescence on tlc and UV absorption at 366nm. The reaction was terminated when the intensity of absorption reached a maximum. A brown glass was isolated which on column chromatography was separated into six fractions of differing Rf values. Only one fraction had the intense UV absorption at 366nm. This was rechromatographed to give yellow prisms of a compound m.p. 154 - 155°C, $C_{20}H_{22}O$. The UV absorption (table 5) indicates the presence of an extended conjugated chromophore. The presence of a

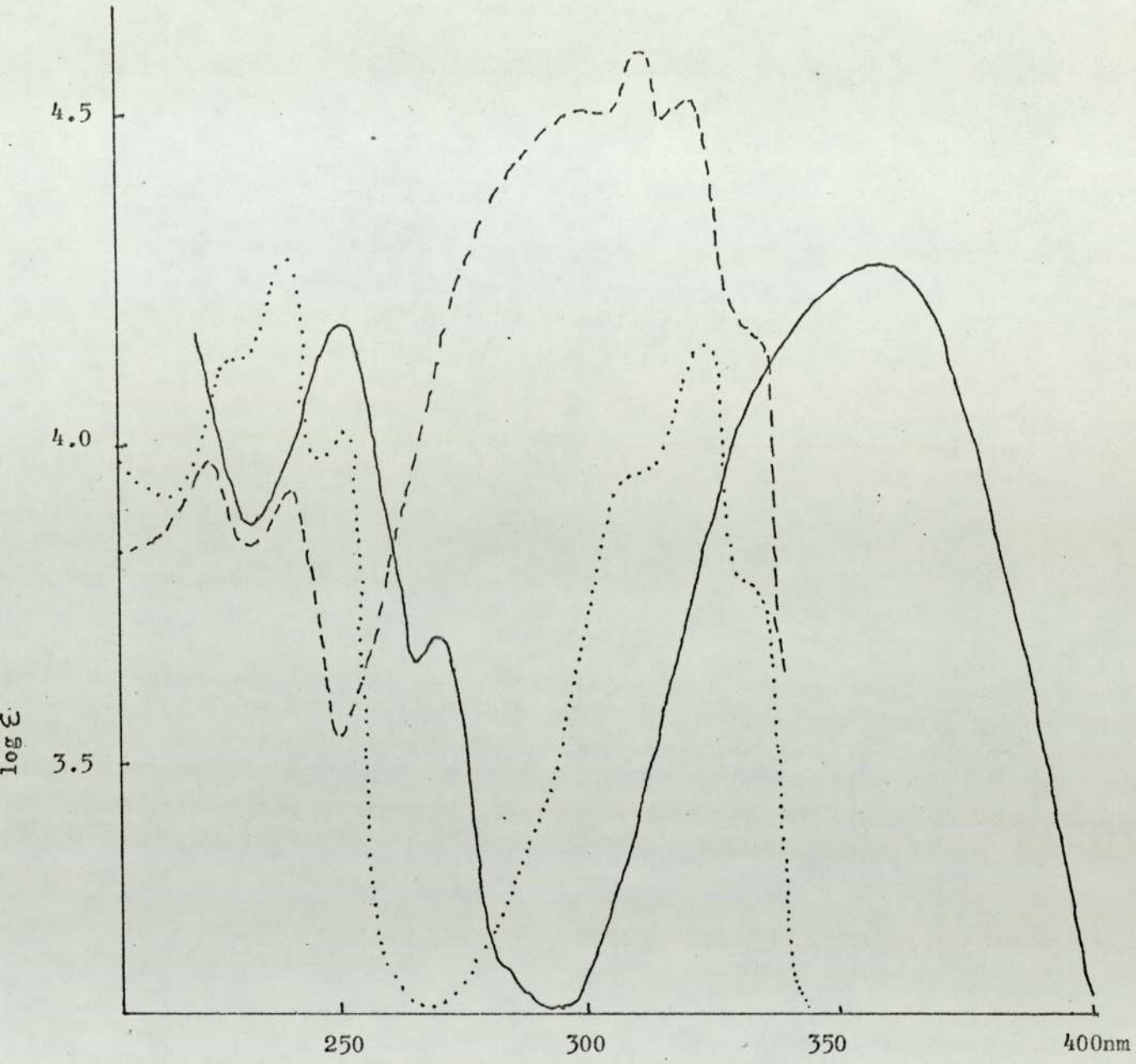


Fig. 13.

——— hydro-oxo-phenanthrene (XXVlll)

····· (XXVlll) after $NaBH_4$ reduction

- - - - - - trans-1-phenylbutadiene

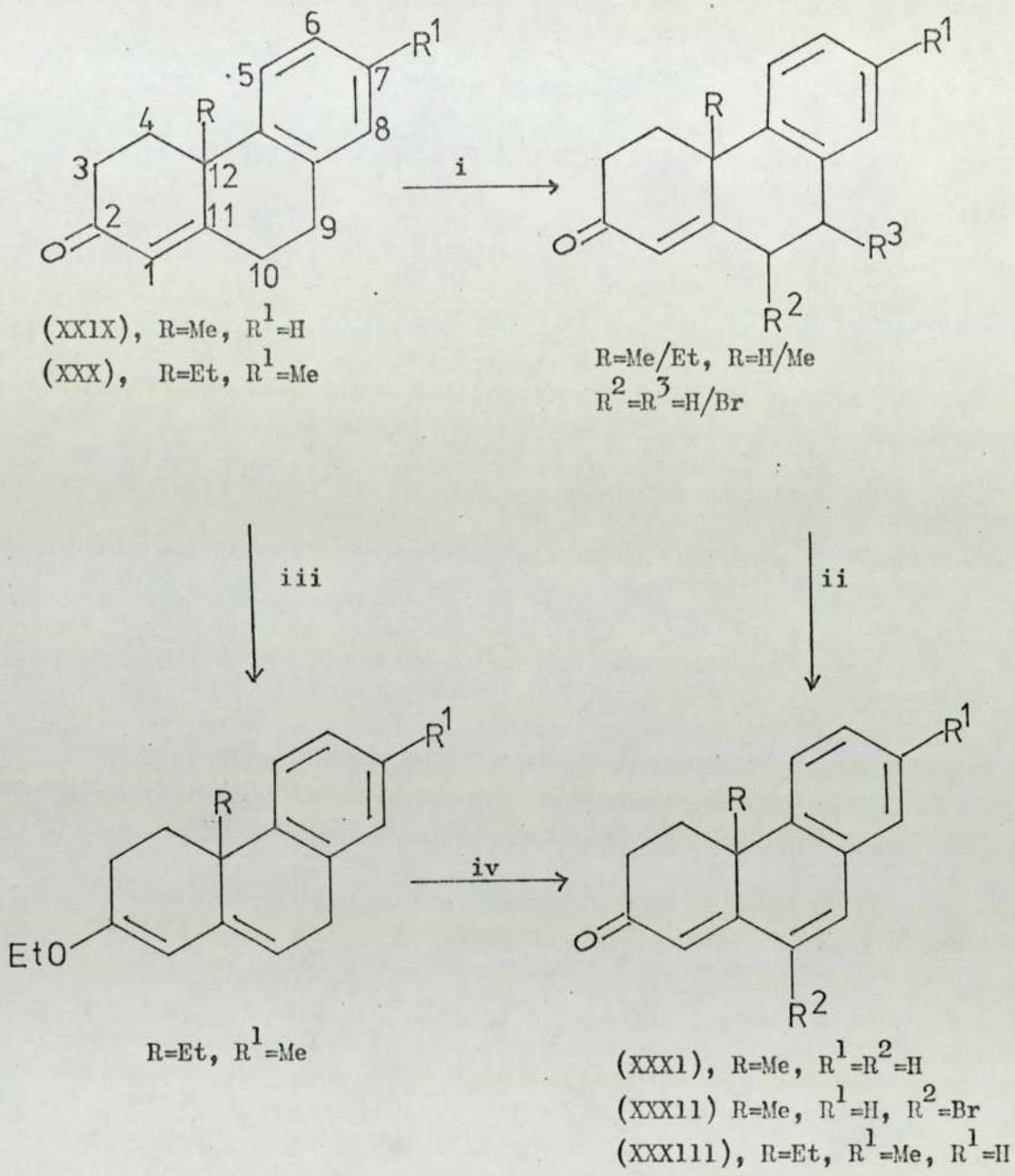
keto C = O group in the conjugation with this chromophore was suspected from the intense $1655cm^{-1}$ IR absorption band in the product. This was established as well as the identity of the chromophore by the sodium borohydride reduction of the product. A solution was obtained whose UV absorption closely resembled that of trans-1-phenylbutadiene[82] (fig. 13). The presence of alkene and aryl systems in the product was also indicated from the intense absorption bands in the C = C stretching and C - H out of plane deformation regions of the IR spectrum. The benzene ring could be seen to be either 1,2,4,5 or 1,2,3,4 tetra-substituted from the presence of intense absorptions at $830cm^{-1}$ and $880cm^{-1}$. The PMR spectrum of the product in $CDCl_3$ showed the presence of two aromatic singlets at 2.85τ and 2.90τ adjacent to a chloroform impurity peak at 2.73τ. This prompted the suggestion of 15(14→12)-abeo-steroid (XXV11) with an aromatic ring-C as a possible structure of the product[64]. High resolution PMR re-examination of the product in ethanol showed a clear AB quartet with doublets centered around 2.80τ and 2.95τ with a JAB value of 9.0Hz. This clearly established the presence of a 1,2,3,4 tetrasubstituted benzene ring in the product whose structure is therefore given by (XXV111).

The mechanism of the rearrangement was found to be structure and reagent specific. Thus testosterone (V) under the same conditions as 17α-methyltestosterone (Va) did not yield any product analogous to (XXV111). The 6,7-dehydro-17 -methyltestosterone (X111) gave the same product, (XXV111), as (Va). The presence of a 17-methyl as well as a 17-hydroxy group is clearly required for the aromatisation of ring C. This can only proceed after the 1,2-shift of the C-13 angular methyl group has occurred on to C-17. This is an example of a well documented acid-catalysed 1,2-alkyl migration in steroid compounds[85].

The aromatisation, however, is not a case of general acid catalysis. Substitution of HOAC or HCl for TCA did not give any of the product (XXVlll) from either (Va) or (Xlll). It was also found that if the reaction of TCA on either (Va) or (Xlll) was carried out under nitrogen, in the absence of air, then (XXVlll) was not formed. Therefore, both TCA and oxygen are required for the aromatisation of ring C in either compound. It may be also true to say that this is required for the dehydrogenation of (Va) at C_6-C_7 prior to ring C aromatisation. A further requirement in the TCA reaction is the presence of a hydroxylic solvent. Using dry methoxyethane and TCA did not produce the product (XXVlll). This last condition may be necessary to produce the required acid conditions for the 1,2-alkyl migration to take place. Though this appears to be the first recorded trichloroacetic acid mediated hydrogen transfer in steroids, it would be of little value to postulate a specific mechanism until the reactions of this acid under varied conditions and using a wider range of substrates have been studied.

4.3 A New Route to Hydro-oxo-phenanthrenes

In order to confirm the conclusions drawn from the spectral data of the product isolated from the TCA reaction (XXVlll), two hydro-oxo-phenanthrenes were synthesized to serve as model compounds. Attempts to prepare the compound, (XXXI), by annelation of 1-methyl-2-naphthol with methyl vinyl ketone were unsuccessful. Low yield of the desired product could be seen to be present from its UV absorption but it could not be isolated from the mixture obtained in this reaction. Consequently, a new route was developed as shown in Scheme 1.



Scheme 1

Reagents:

i, N-Bromosuccinimide

ii, Li_2CO_3, LiBr

iii, Triethyl orthoformate + pyridine HCl

iv, DDQ/acetone

The known hexahydro-oxo-phenanthrene (XXIX) was brominated and the mixed mono- and dibromo- products, without prior separation, were dehydrobrominated. The major product isolated was the required tetrahydro-oxo-phenanthrene together with a small amount of a material assigned the structure (XXXII). The low yield of (XXXII) prevented a full examination of its properties. Its UV absorption, (table 5), showed it to have the same chromophore as (XXVIII) and (XXXI), and its molecular formula determined from an accurate mass spectrometric molecular weight was found to be $C_{15}H_{13}BrO$. Structure (XXXII) is assigned on the basis that a monobromo-product could only arise from a 9,10-dibromo intermediate by loss of a mole of HBr on dehydrobromination. It is interesting to note that benzylic and allylic bromination are competing processes on the same molecule. It is taken that the 9, or benzylic bromine atom would be eliminated in preference to the 10-bromine atom in the dehydrobromination reaction.

The preparation of (XXXI) by direct dehydrogenation of (XXIX) with DDQ resulted in difficultly separable mixtures and was abandoned. However, conversion of (XXX) to the enol ether, followed by dehydrogenation under controlled conditions,[87] afforded (XXXIII) in a satisfactory yield.

Table 5

*Compound	λ^1 max	(Log ε)	λ^2 max	(log ε)
XXVIII	243	(4.18)	357	(4.18)
XXXI	240	(4.22)	350	(4.18)
XXXII	250	-	365	-
XXXIII	238	(4.23)	350	(4.22)

* Spectra of all compounds obtained in ethanol.

From (table 5), it can be seen that the UV absorptions of all the compounds listed are almost identical and so in agreement of the proposed chromophore being their common structural unit. All the compounds show the intense $1660cm^{-1}$ absorption found to be due to the conjugated carbonyl system and the $880-900cm^{-1}$ found due to alkene C-H out of plane deformation. The PMR spectra of XXVlll, XXXl and XXXlll possess the alkene AB quartets and the -C=C-CO- singlets which are discussed in a greater detail (4.4).

4.4 Proton Magnetic Resonance Studies

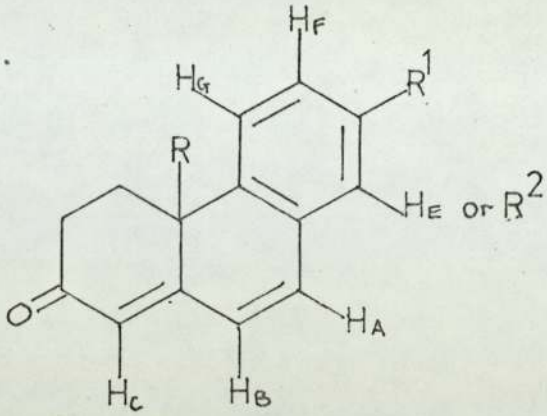


Fig. 14

Table 6

PMR Data $\tau(CDCl_3)$ for Substituted Hydro-oxo-phenanthrenes

COMPOUND	C_1-H_C	C_{10}-H_B	C9-H_A	C_6-H_F	C_5-H_G	C8-H_E	J_{AB} Hz	J_{FG} Hz	J_{EF} Hz
XXV111	4.15	3.65	3.10	2.95	2.80		9.0	9.0	
XXX1	4.19	3.70	3.30	(2.90	-	2.65)	10.0	10.0	
XXX111	4.14	3.65	3.32	2.92	2.86	2.98	10.0	10.0	
XXX1V	4.10	3.60	3.30	3.16	2.75	3.10	9.0	9.0	2.50

The PMR spectrum of 17α-methyltestosterone (Va), Fig. 15 shows a singlet for the C_4-H at 4.30 . The 18-methyl resonance is at 9.10τ while the 19- and 17-methyls appear as a single peak further down field at 8.80 due to the deshielding effects of the Δ^4-3-oxo and 17-β-hydroxy functions[88]. The product isolated from the reaction of (Va) with TCA,(XXV111), shows a much more complex PMR spectrum Fig. 16 & 17. It is named using the phenanthrene rather than steroid nomenclature and its PMR data is compared here with that of model hydro-oxo-phenanthrenes (fig. 18-20).

The C_1-H_C of XXVlll at 4.15τ (corresponding to the C_4-H at 4.19τ of Va) is comparatively further deshielded due to the extension of the carbonyl conjugation and the presence of an aromatic centre in the molecule (fig. 14). The olefinic quartet with doublets centered at 3.10 and 3.65τ with J_{AB} of 9.0 Hz is attributed to the C_{15}-CH=CH-system (C_9-H_A and C_{10}-H_B, respectively). Also, the aromatic quartet with doublets centered at 2.80 and 2.95τ, J_{FG} 9.0 Hz is assigned to C_5-H_G and C_6-H_F, respectively, as it is considered that the electronic effects due to the carbonyl would cause H_G to be deshielded more than H_F (fig.14). These assigments are consistent with previous PMR studies of some ring-C aromatic diterpenoids[89,90]. The benzylic and alkane methylene absorptions of the cyclopentane ring are seen as triplets at 7.05 and 8.0τ, respectively, with a mutual coupling constant of 7.0 Hz. A 3H singlet at 8.56τ is due to the 12-methyl which is by comparison more deshielded than the methyl at the A/B ring junction (8.80τ) of the starting material (Va). The D-ring geminal methyls are observed as 3H singlets at 8.61 and 8.66τ (fig. 16).

The relevant data of the 7-substituted and 7,8-disubstituted compounds reported above is presented in table 6. The 7,8-disubstituted compound (XXVlll) examined shows a marked deshielding of the C_9-H_A signal (3.10τ) from the position observed for the unsubstituted or 7-substituted compounds (3.30 - 3.32τ). However, it was not possible to assign relative contributions of the C7 and C8 substituents to the deshielding. Assignment of meta-coupling constants, J_{EF}, is made for XXXlV where R^1 is a methoxy group (fig. 14). Assignment was not possible with (XXXlll) where R^1 is replaced by a methyl group presumably due to signal overlap.

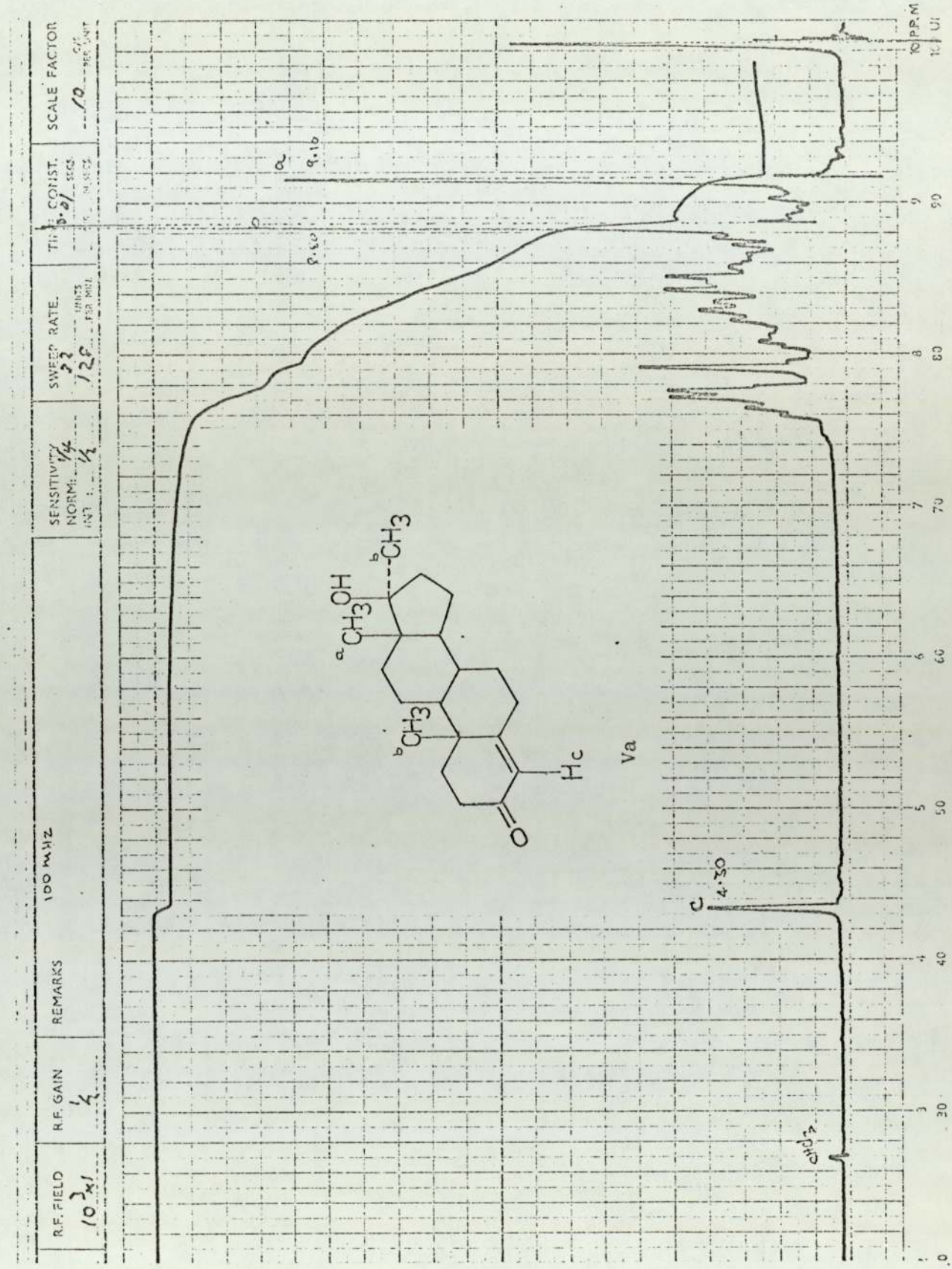

R.F. FIELD
R.F. GAIN
REMARKS
100 MHz
SENSITIVITY
NORM.
INT.
SWEEP RATE
TIME CONST.
SCALE FACTOR
Va
PPM

Fig. 15.

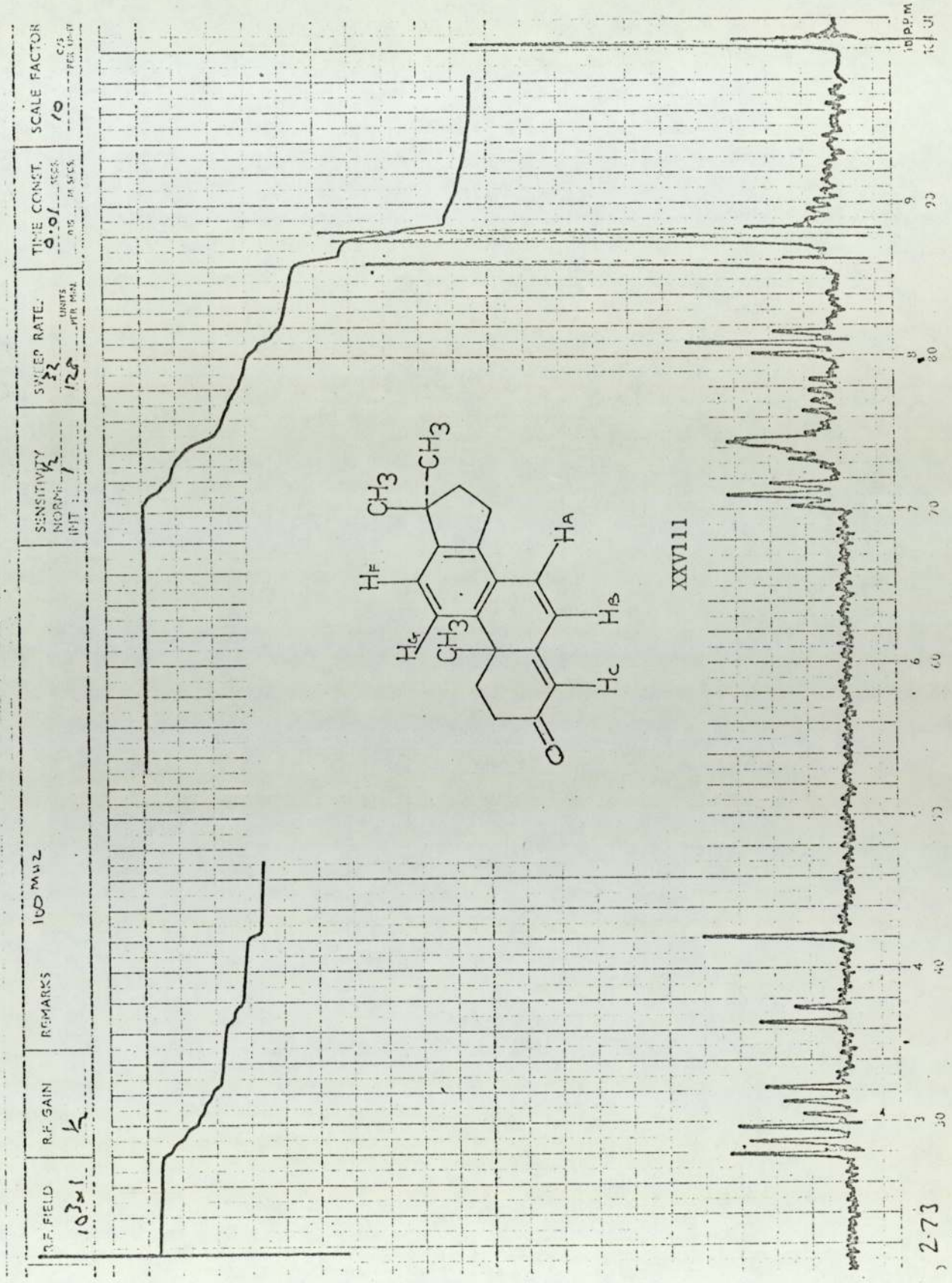

R.F. FIELD
R.F. GAIN
REMARKS
100 MHZ
SENSITIVITY
SWEEP RATE
TIME CONST.
SCALE FACTOR
XXVIII
H_A
H_B
H_C
H_E
H_F
CH_3
CH_3
CH_3
O
10 P.P.M.
2.73

Fig. 16.

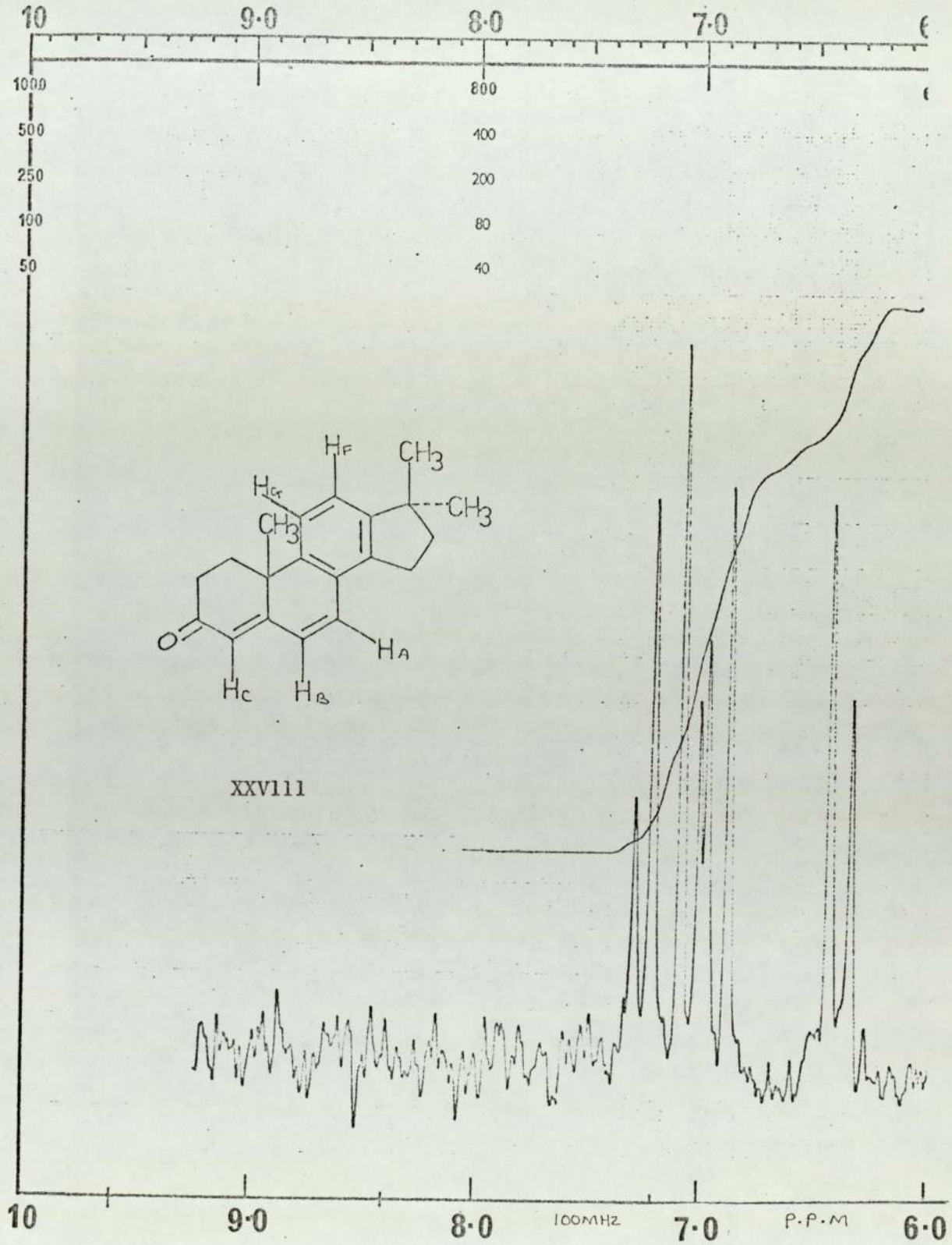


Fig. 17.

Spectrum in EtOH showing the aromatic and olefinic quartets.

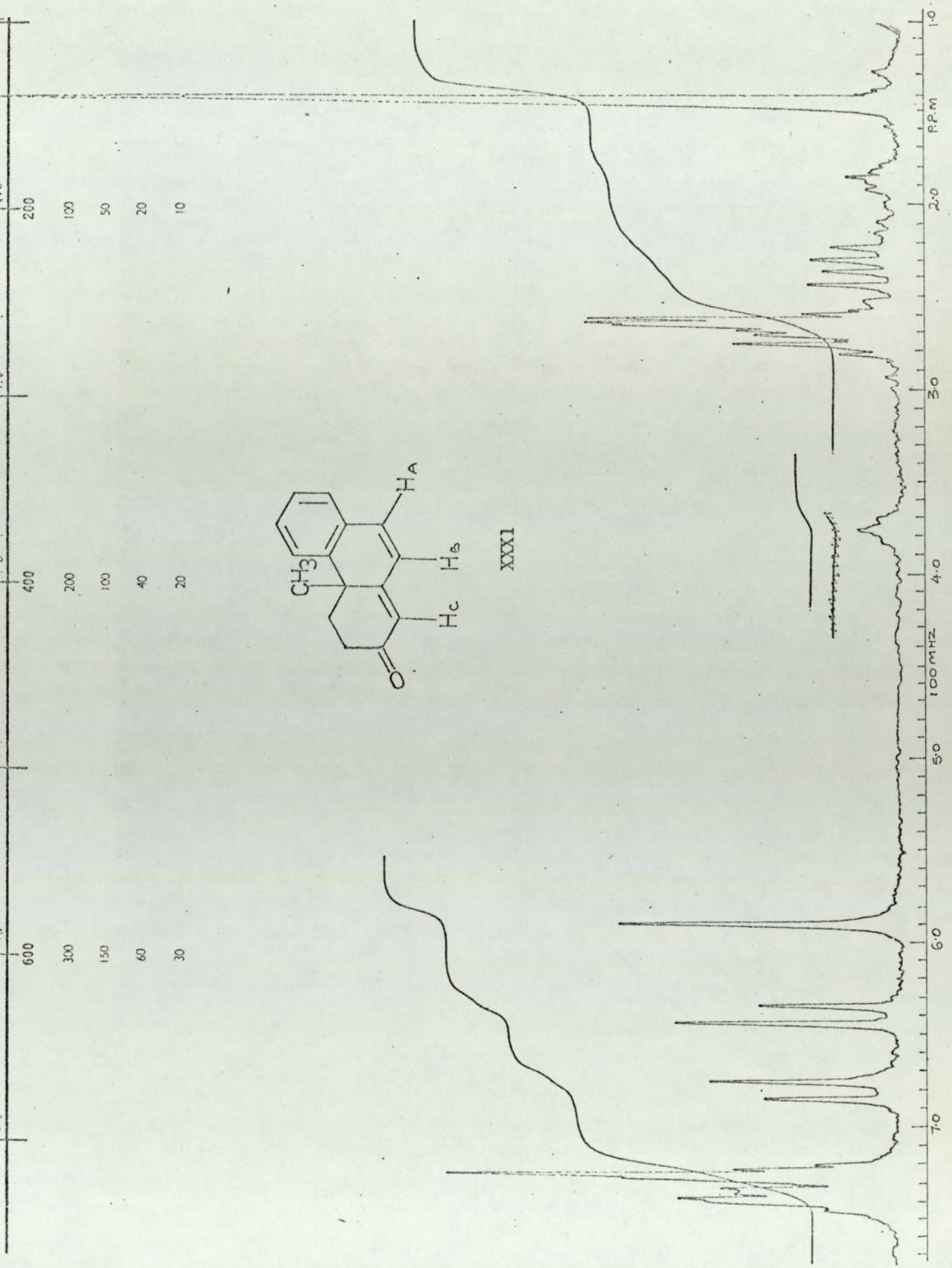

600
300
150
60
30
400
200
100
40
20
200
100
50
20
10
H_A
H_B
H_C
CH_3
O
XXXI
7·0
6·0
5·0
4·0
3·0
2·0
1·0
100 MHz
P.P.M

Fig. 18.

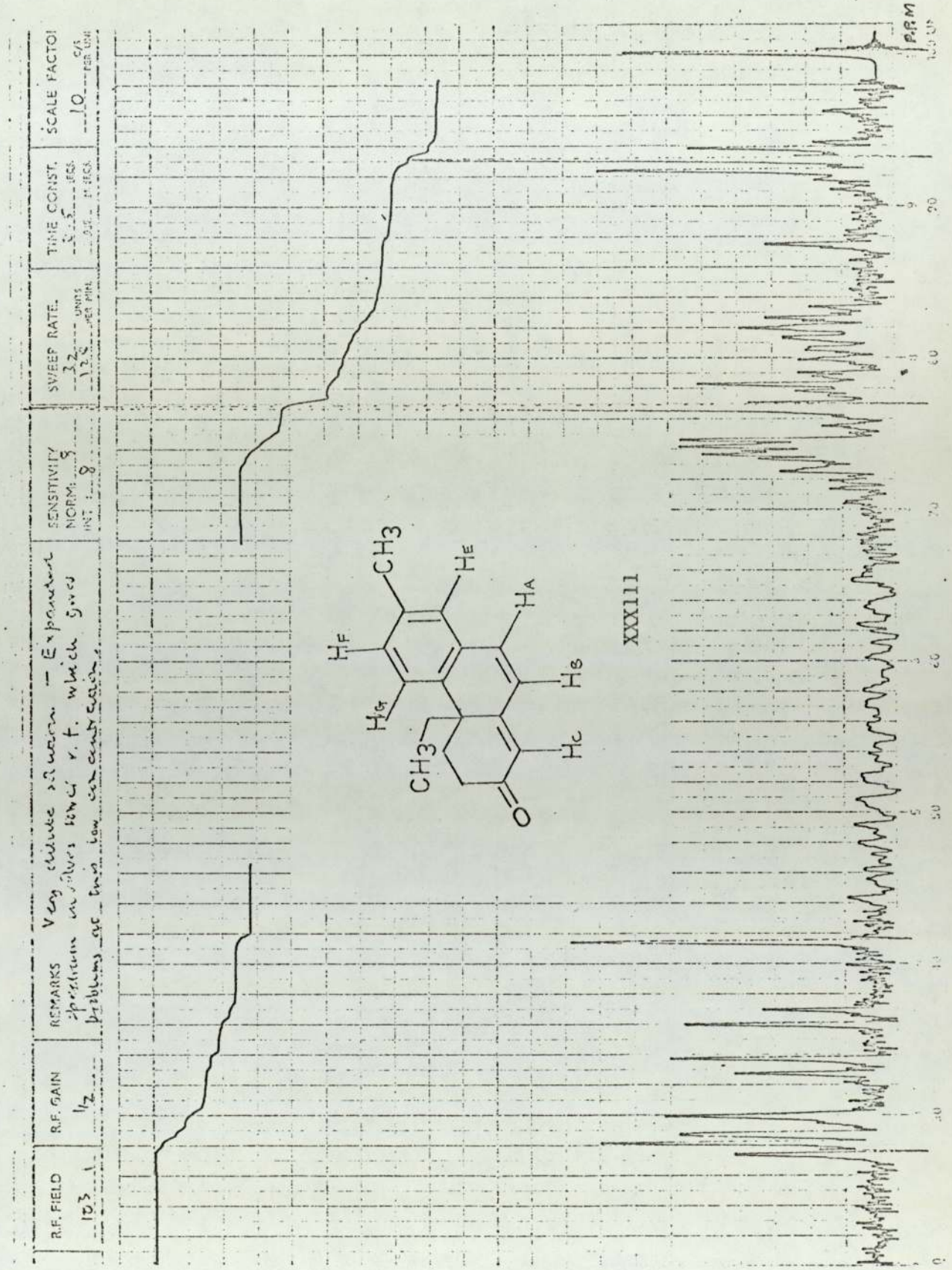

R.F. FIELD
R.F. GAIN
REMARKS
SENSITIVITY
SWEEP RATE
TIME CONST.
SCALE FACTOR
PPM
CH3
HE
HA
HB
HC
HF
HG
CH3
O
XXXIII

Fig. 19.

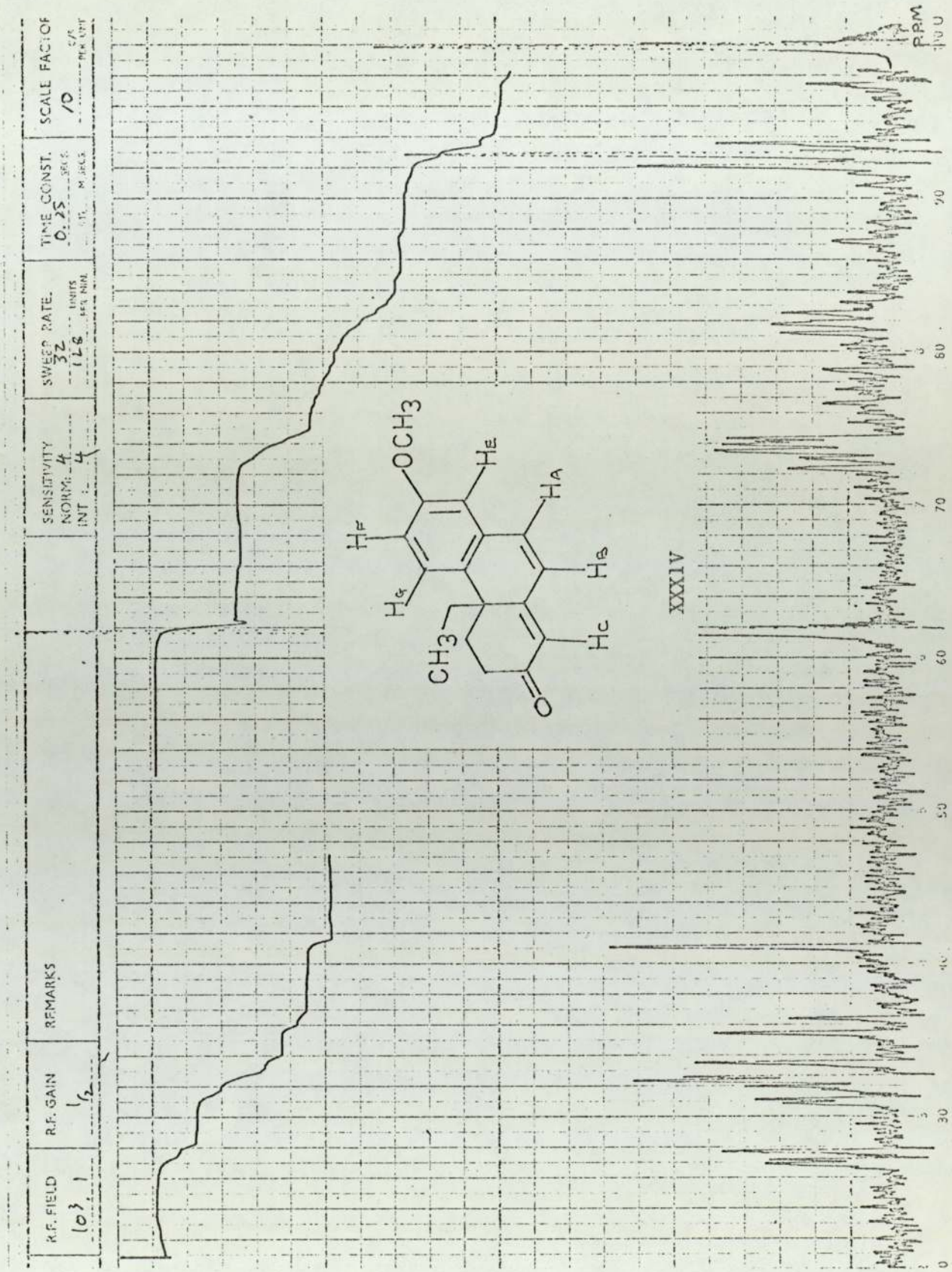

R.F. FIELD
R.F. GAIN
REMARKS
SENSITIVITY
SWEEP RATE
TIME CONST.
SCALE FACTOR
OCH3
CH3
XXXIV
PPM

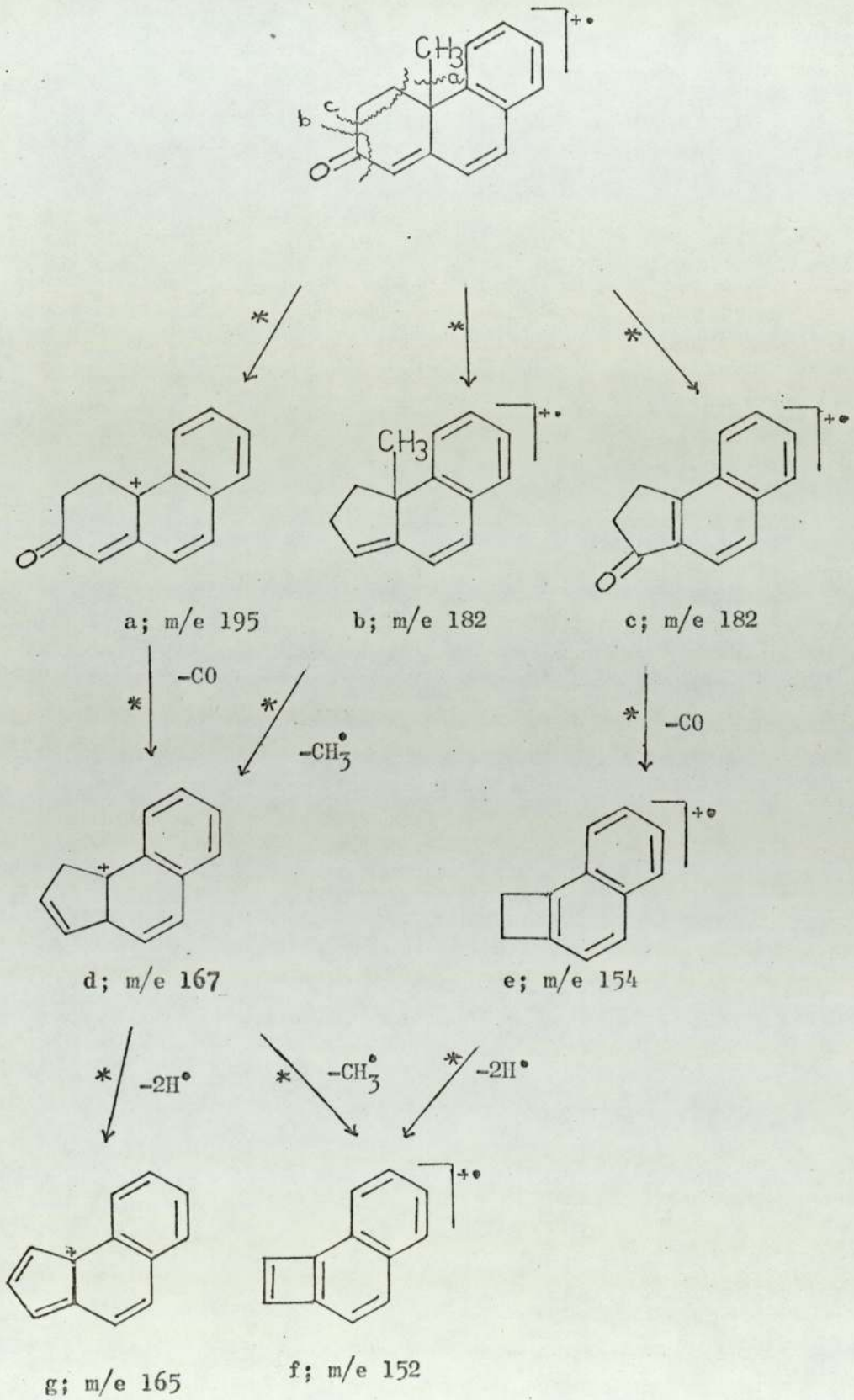
Fig. 20.

4.5 Mass Spectra Studies

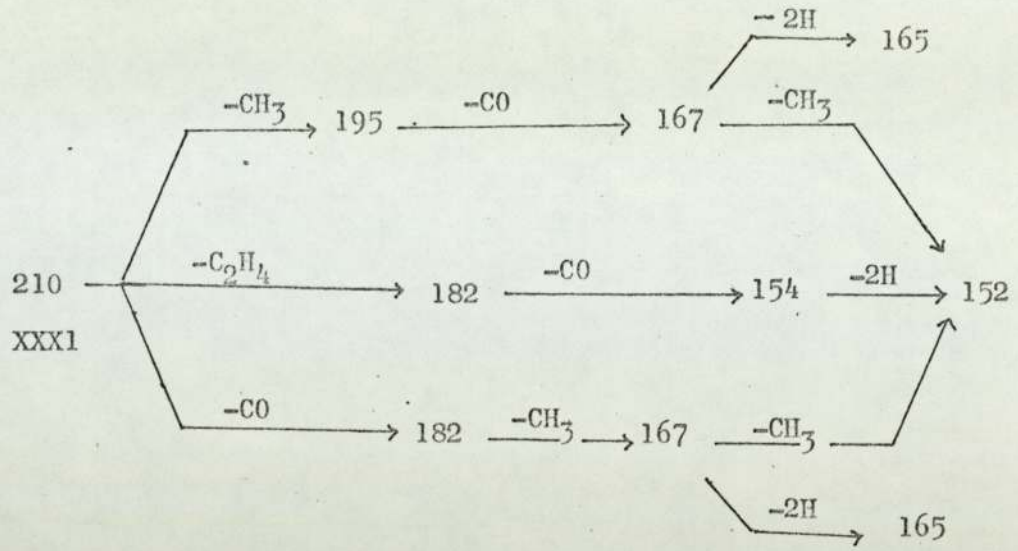
The simplest member of this series studied is the hydro-oxo-phenanthrene (XXXl). In these mass spectra fig. 21-24, the common peaks are at $(M - \dot{R})$ and $(M - R^{\bullet} - 28)$, as well as peaks in the lower mass region at $^{m}/e$ 152,166 and 179. The almost identical spectral patterns in the lower mass range reflects the presence of a similar structural relationship in these compounds. Several interesting fragmentations are revealed by high resolution mass measurements and metastable determinations of all the major peaks of the hydro-oxo-phenanthrenes and the results for (XXXl) are summarised (Table 7). Specific structures are assigned, scheme 2, in an attempt to rationalise the interpretation of the spectra and may be regarded as formal representations only. The fragmentation pattern is compared across the hydro-oxo-phenanthrene group and with cinnamylidene compounds possessing the hydro-oxo-phenanthrene chromophore.

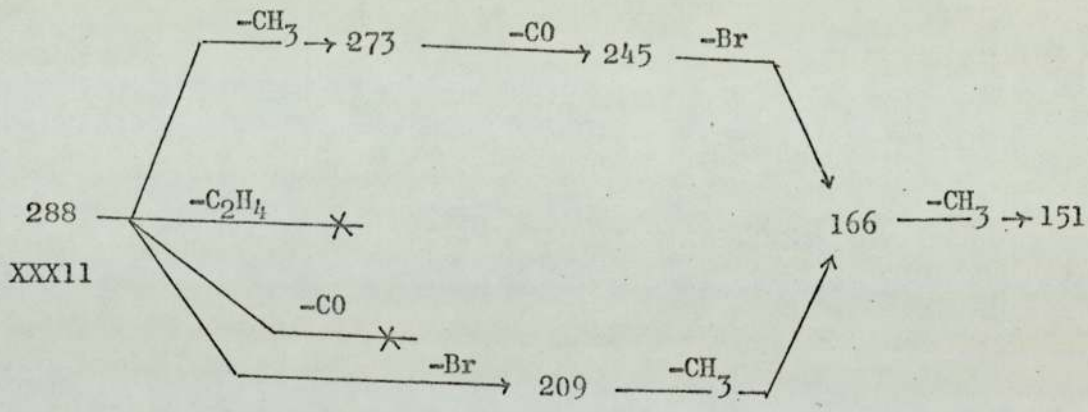
Table 7 High Resolution Mass Measurements

Hydro-oxo-phenanthrene XXXl		Calcd.	Obs.
m/e 210	$C_{15}H_{14}O$	210.105234	210.104459
m/e 182	$C_{13}H_{10}O$	182.073124	182.073161
	$C_{14}H_{14}$	182.109321	182.109545
m/e 167	$C_{13}H_{11}$	167.086044	167.086071
m/e 154	$C_{12}H_{10}$	154.077498	154.078247
m/e 152	$C_{12}H_{8}$	152.062597	152.062597
Metastables			
210	195	181.07	181.0
167	165	163.02	163.0
182	167	153.24	153.0
154	152	150.02	150.0
195	167	143.02	143.0
167	152	138.35	138.3
182	154	130.31	130.2

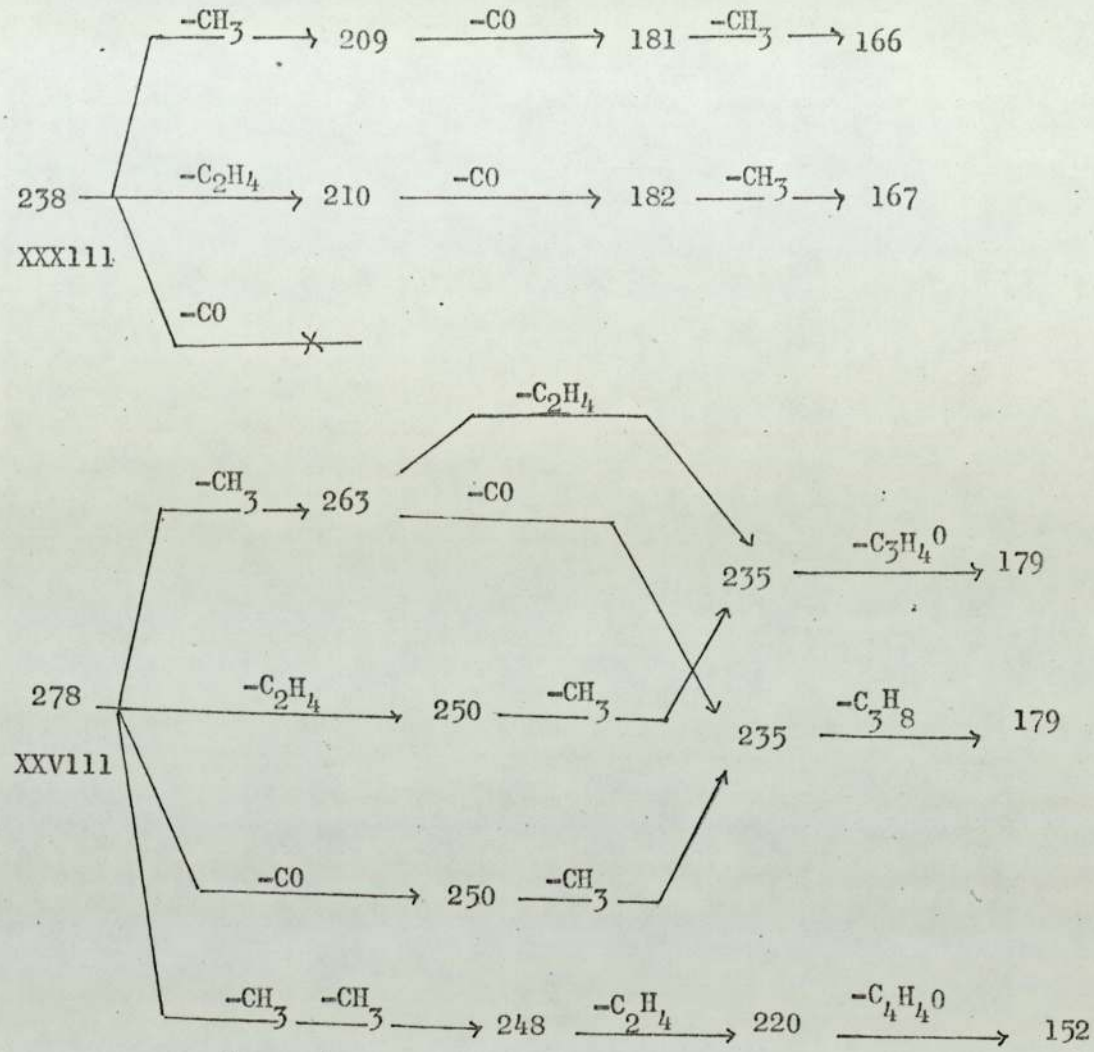

CH3
a
b
c
a; m/e 195
b; m/e 182
c; m/e 182
-CO
-CH3
d; m/e 167
e; m/e 154
-2H
g; m/e 165
f; m/e 152

SCHEME 2





The fragmentation pattern of XXX1 is seen to follow three pathways. In agreement with previous observations on the fragmentation of octahydrophenanthrenes,[91] the peak at m/e 195, (M - 15), is due to the expulsion of the angular methyl group. Fragments b and c of mass m/e 182 indicate losses of both carbon monoxide and ethylene from ring A. Elimination of a methyl radical from b gives the most abundant fragment d, m/e 167 which could undergo a further loss of a methyl radical to give the stable aromatic ion, f, m/e 152. Alternatively, d loses hydrogen to form a stable cation, g, m/e 165. Expulsion of carbon monoxide from c, however, accounts for the formation of e, m/e 154 which on losing hydrogen forms the stable ion f. The observed



losses are consistent with similar studies on high resolution mass spectra of some saturated cyclic ketones[94]. Characteristic peaks at m/e 152 and 165 in the lower mass region of this compound have also been observed in some alkyl phenanthrenes and aporphine alkaloids.[95] Although their structure remains unestablished, aromatised stable ions may be expected through extensive reorganisation of the molecule.

Introduction of bromine into (XXX1) alters the fragmentation pattern appreciably. Initial losses from (XXX1) are methyl and bromine radicals in preference to carbon monoxide and ethylene

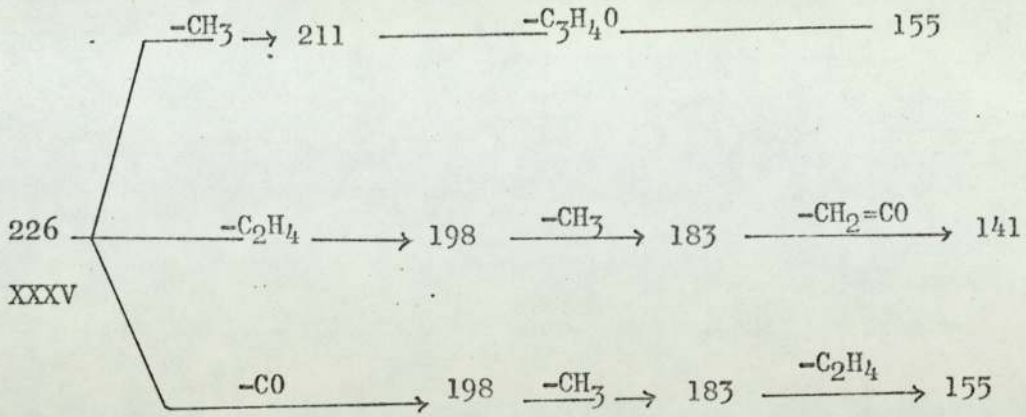
elimination. $(M - Br^{\bullet})$ gives the most abundant fragment at m/e 209 which may be envisaged to eliminate CH_3CO^+ to afford another abundant fragment m/e 166. On the other hand, $(M - CH_3^{\bullet})$gives m/e 273 which then loses carbon monoxide to form a weak intensity fragment m/e 245, extrusion of bromine from which affords the abundant fragment m/e 166. Loss of a methyl radical from this probably affords an aromatic cation m/e 151.

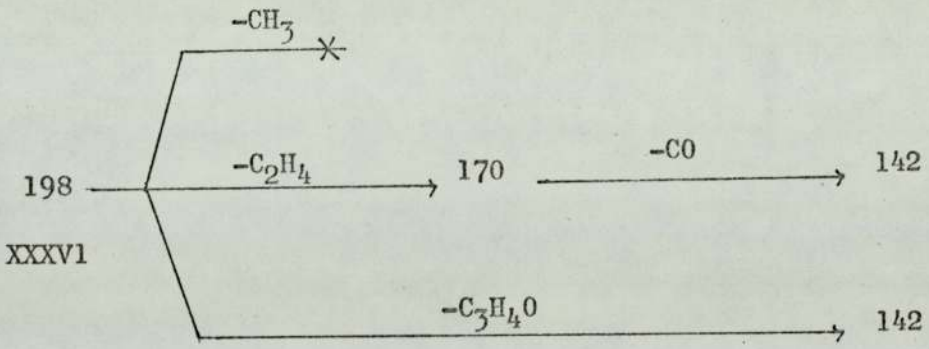
Enzell[96] noted that substituents in the aromatic ring of octahydrophenanthrenes had little influence on their fragmentation pathways. This is shown to be so by the similarities between the fragmentation of the parent compound XXXl and the disubstituted hydro-oxo-phenanthrene XXVlll. The difference manifested by the mono-substituted hydro-oxo-phenanthrene XXXlll is attributed to the effect of the C_{12} - ethyl group, the lack of initial elimination of which appears to prevent the loss of carbon monoxide from the molecular ion. $(M - C_2H_5^{\bullet})$ gives the most abundant fragment m/e 209which then eliminates carbon monoxide to give an abundant cation at m/e 181. Loss of a methyl radical from the latter affords a presumably stable aromatic fragment at m/e 166. Consecutive losses of ethylene and carbon monoxide from the molecular ion affords m/e 182 which may also lose a methyl radical to form a stable cation at m/e 167.

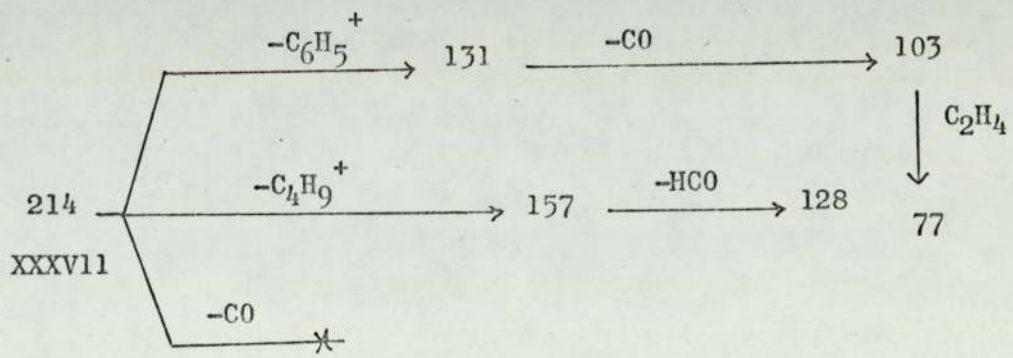
The fragmentation of XXVlll is essentially similar to that of XXXl but the presence of a cyclopentane ring in the molecule of the former affords an extra fragmentation pathway. An (M - 15) loss occurs initially to give m/e 263 as the base peak. Subsequent loss of carbon monoxide from the latter affords the abundant cation m/e 235 $(C_{19}H_{22})$. The presence of an abundant ion at m/e 250$(C_{19}H_{22})$ indicates the m/e 235 fragment also arises from $(M - CO - CH_3^{\bullet})$. Expulsion of an olefinic (C_3H_8) fragment from the latter may easily afford an aromatic cation m/e 179. Similar losses of ethylene and the angular methyl from M affords an m/e 235 $(C_{17}H_{15}O)$ which may then eliminate C_3H_4O to form a

stable cation m/e 179. Elimination of ethylene for this particular pathway would have to involve the cyclopentane ring. The (M - 30) loss yielding m/e 248 ($C_{18}H_{16}O$) can only be envisaged to occur through a concerted loss of two methyl radicals, possibly involving the angular methyl and one other methyls on the cyclopentane ring. Subsequent losses of ethylene and carbon monoxide would yield m/e 220, ($C_{17}H_{16}$) and ($C_{16}H_{12}O$). The latter probably eliminates C_4H_4O to afford a low abundance fragment at m/e 152. A series of fragments at m/e 219, 221, 222 might be accounted for in terms of hydrogen expulsion rearrangement and possibly cyclization to form stable cations.

Of the cinnamylidene derivatives studied, fig. 25 - 27, 2-cinnamylidene-6-methylcyclohexanone (XXXV) fragmentation provides the most useful information for comparison with the oxo-phenanthrene series above. This is not surprising as, structurally, it approximates the hydro-oxo-phenanthrene chromophore. Concurrent elimination of ethylene and carbon monoxide from the molecular ion yields m/e 198, ($C_{15}H_{18}$) and ($C_{14}H_{14}O$) which then lose methyl radicals to afford m/e 183, ($C_{14}H_{15}$) and ($C_{13}H_{11}O$). Elimination of ethylene and ketene from these fragments, respectively, gives m/e 155 and m/e 141 aromatic cations. Initial loss of a methyl radical from the molecular ion, however, yields m/e 211 which could conceivably lose C_3H_4O to form the m/e 155 cation. Fragmentation of cinnamylidene-t-butyl ketone (XXXVII) is directed by the tert-butyl group and has little in common with the oxo-phenanthrene series. Similarly, the fragmentation of 2-cinnamylidene cyclopentanone which lacks a methyl substituent in the cyclopentanone ring bears little relation to the oxo-phenanthrenes in its fragmentation pattern. (M - C_2H_4 - CO) appears to be the major fragmentation pathway.


226
XXXV
-CH3
211
-C3H4O
155
-C2H4
198
-CH3
183
-CH2=CO
141
-CO
198
-CH3
183
-C2H4
155


198
XXXV1
-CH3
-C2H4
170
-CO
142
-C3H4O
142


214
XXXV11
-C6H5+
131
-CO
103
C2H4
77
-C4H9+
157
-HCO
128
-CO

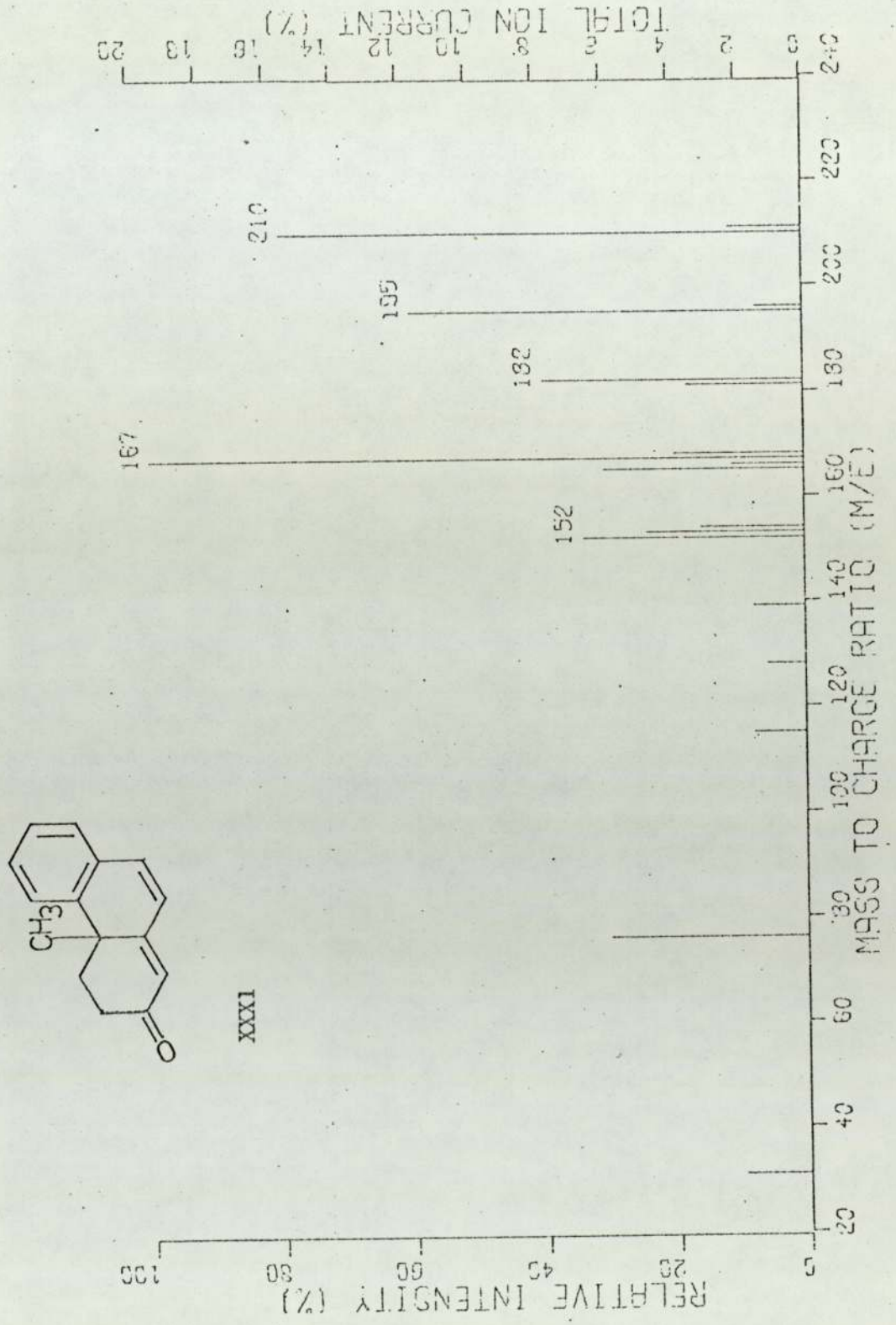

TOTAL ION CURRENT (%)
0 2 4 6 8 10 12 14 16 18 20
MASS TO CHARGE RATIO (M/E)
20 40 60 80 100 120 140 160 180 200 220 240
RELATIVE INTENSITY (%)
0 20 40 60 80 100
167
152
132
195
210
CH3
O
XXXI

Fig. 21.

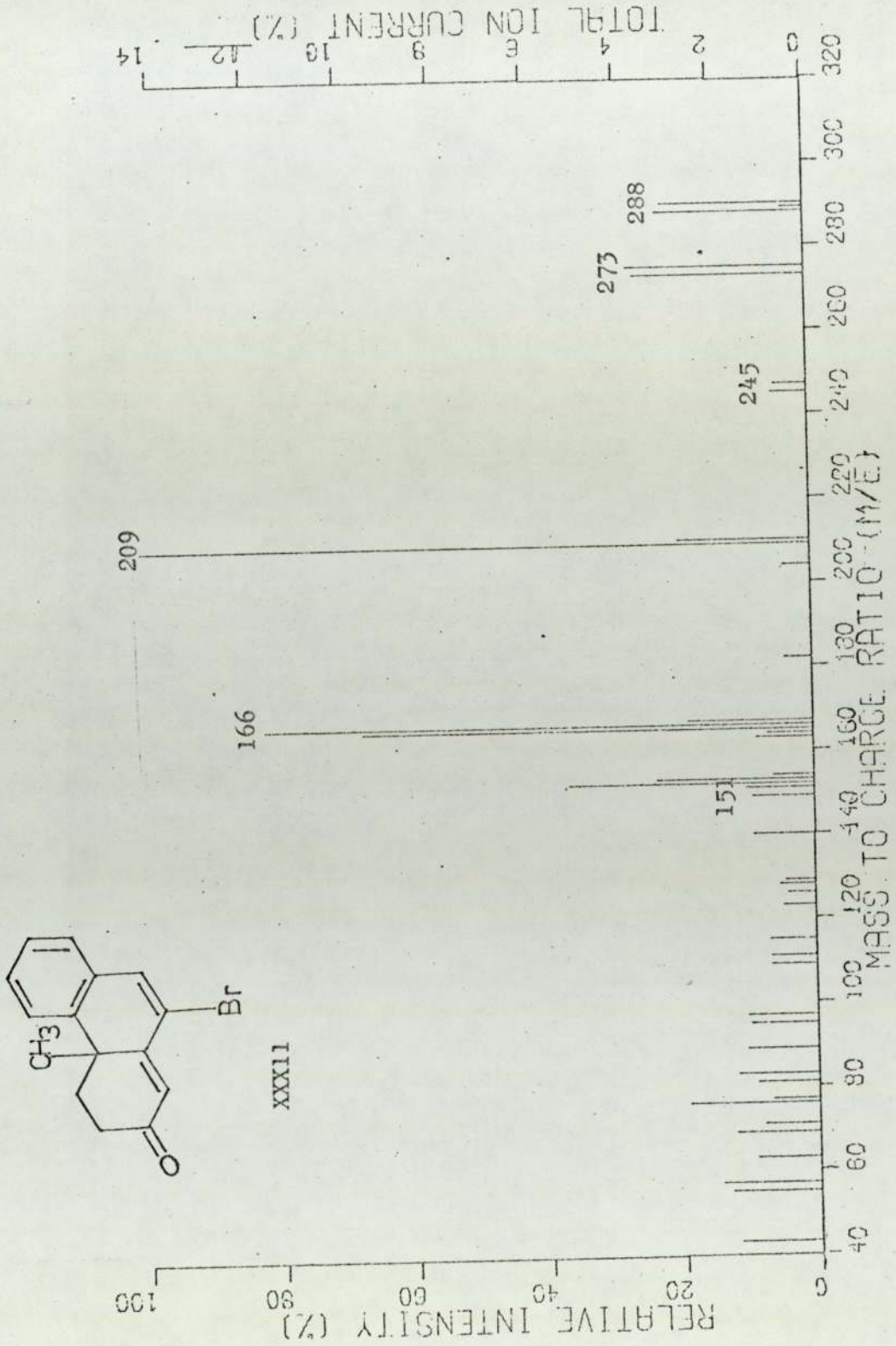
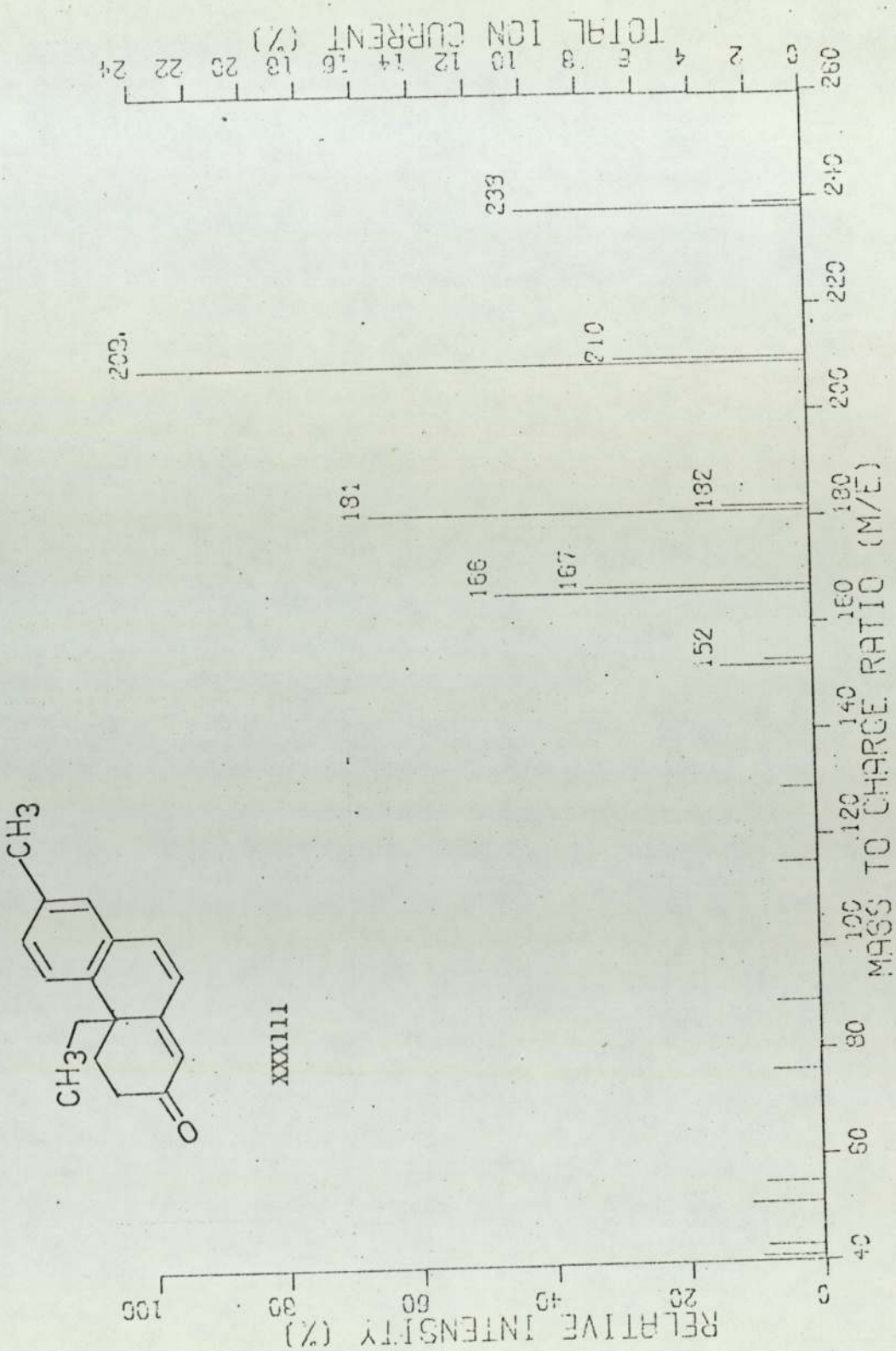

TOTAL ION CURRENT (%)
0
2
4
6
8
10
12
14
MASS TO CHARGE RATIO (M/E)
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
RELATIVE INTENSITY (%)
0
20
40
60
80
100
209
166
151
245
273
288
Br
CH3
O
XXXII

Fig. 22.



XXXIII

Fig. 23.

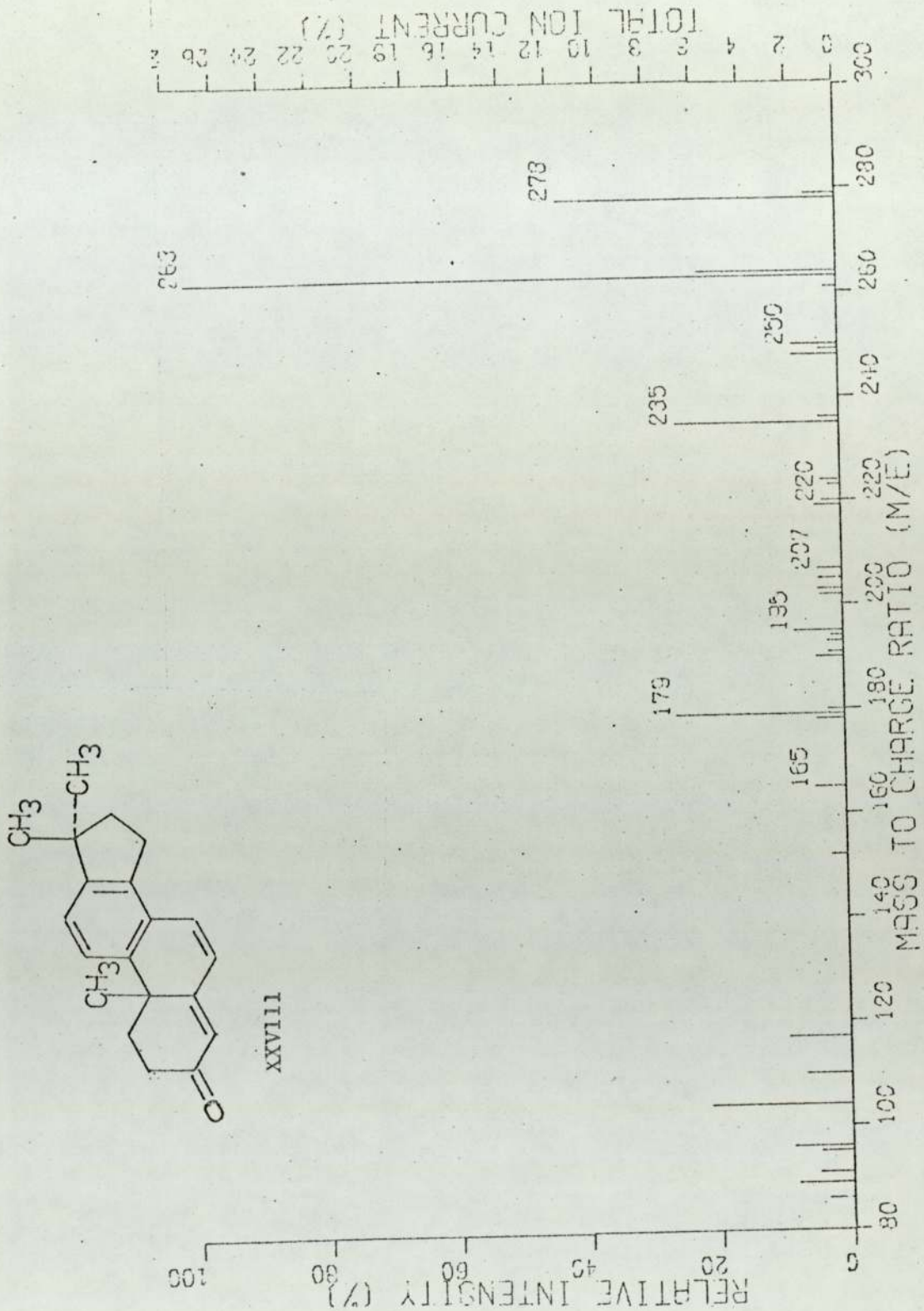

TOTAL ION CURRENT (%)
MASS TO CHARGE RATIO (M/E)
RELATIVE INTENSITY (%)
263
273
250
235
179
165
135
CH3
CH3
CH3
O
XXVIII

Fig. 24.

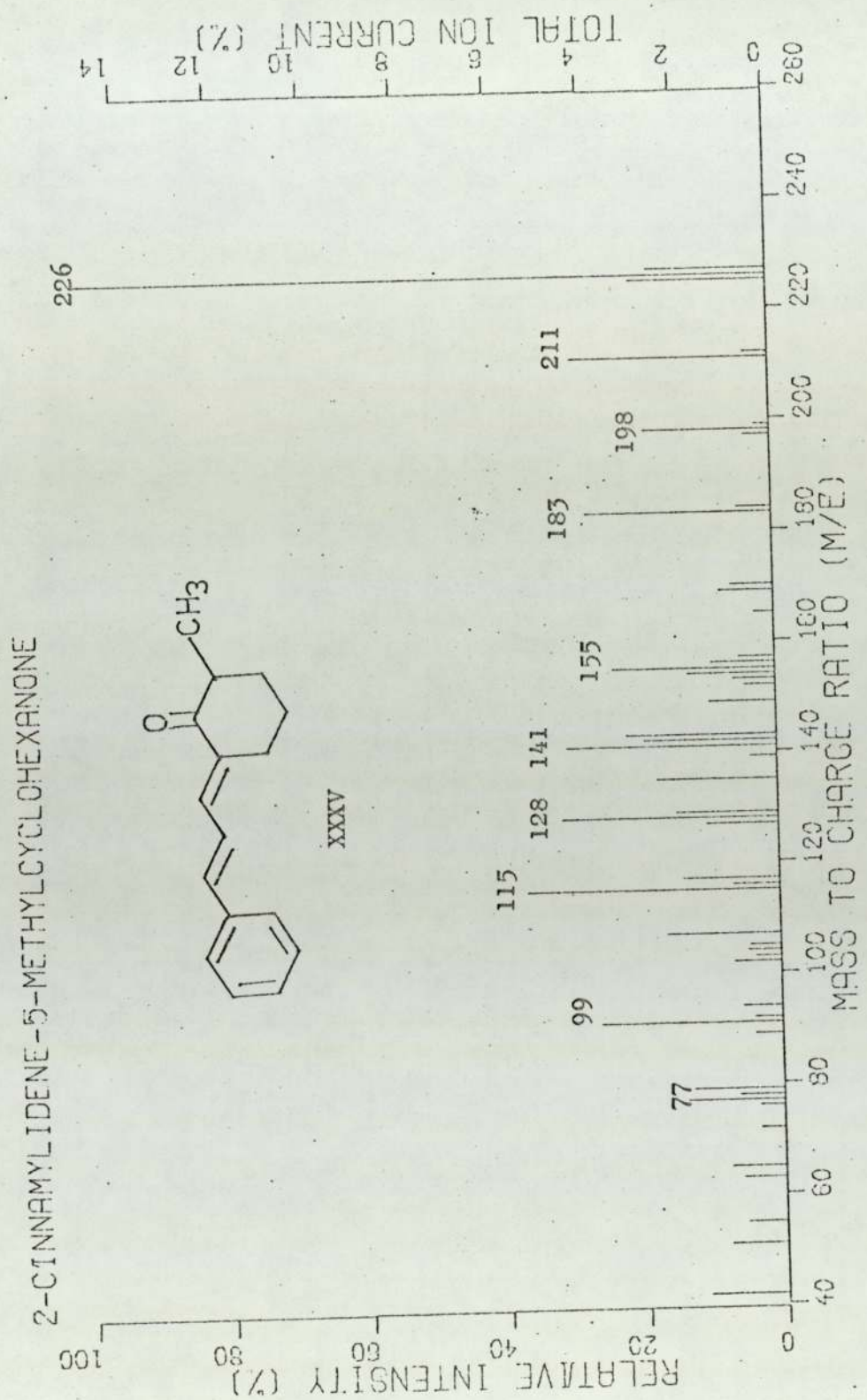

2-CINNAMYLIDENE-5-METHYLCYCLOHEXANONE
XXXV
CH3
O
RELATIVE INTENSITY (%)
MASS TO CHARGE RATIO (M/E)
TOTAL ION CURRENT (%)
226
211
198
183
155
141
128
115
99
77

Fig. 25.

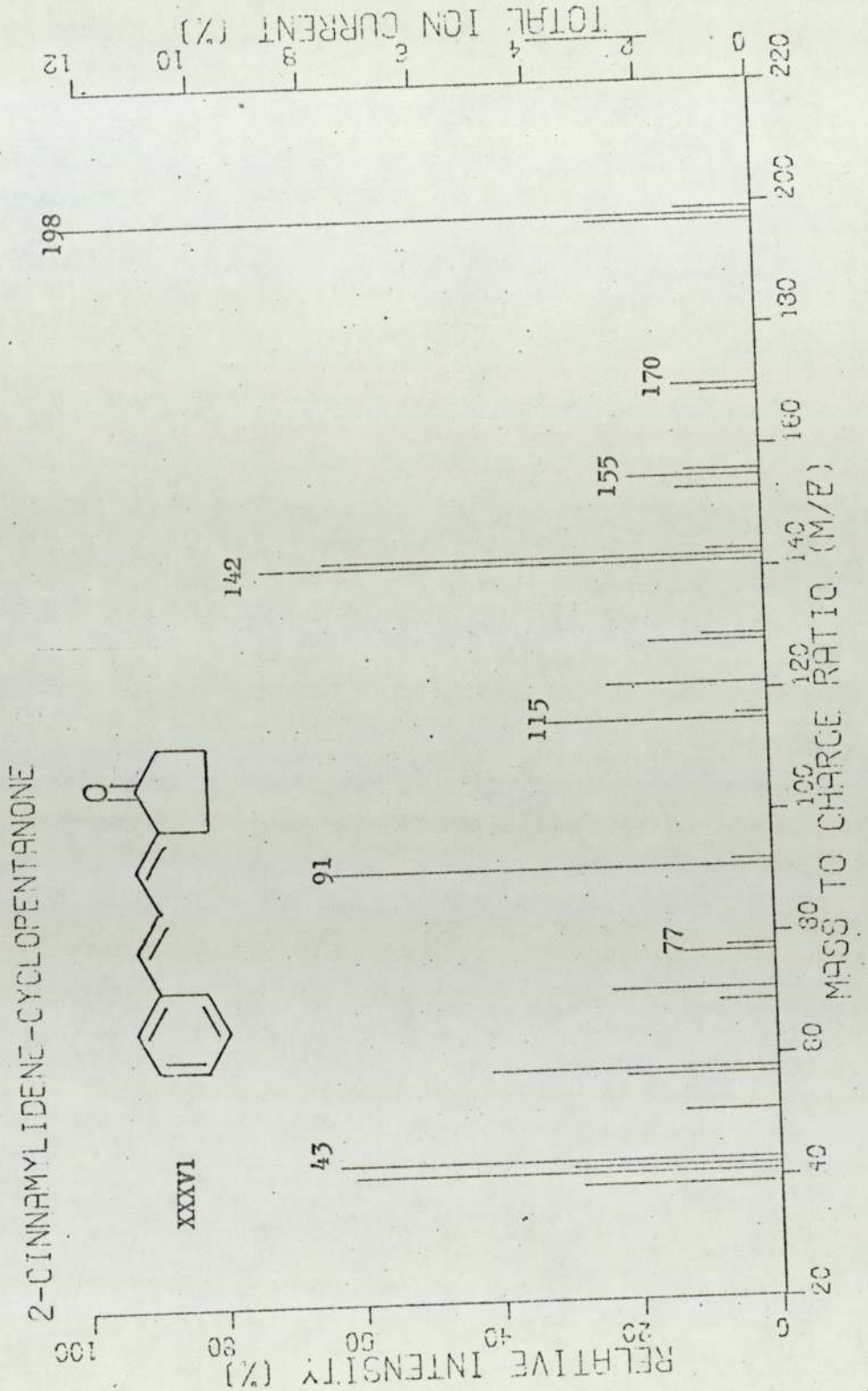

2-CINNAMYLIDENE-CYCLOPENTANONE
XXXVI
198
142
155
170
115
91
77
43
MASS TO CHARGE RATIO (M/E)
RELATIVE INTENSITY (%)
TOTAL ION CURRENT (%)

Fig. 26.

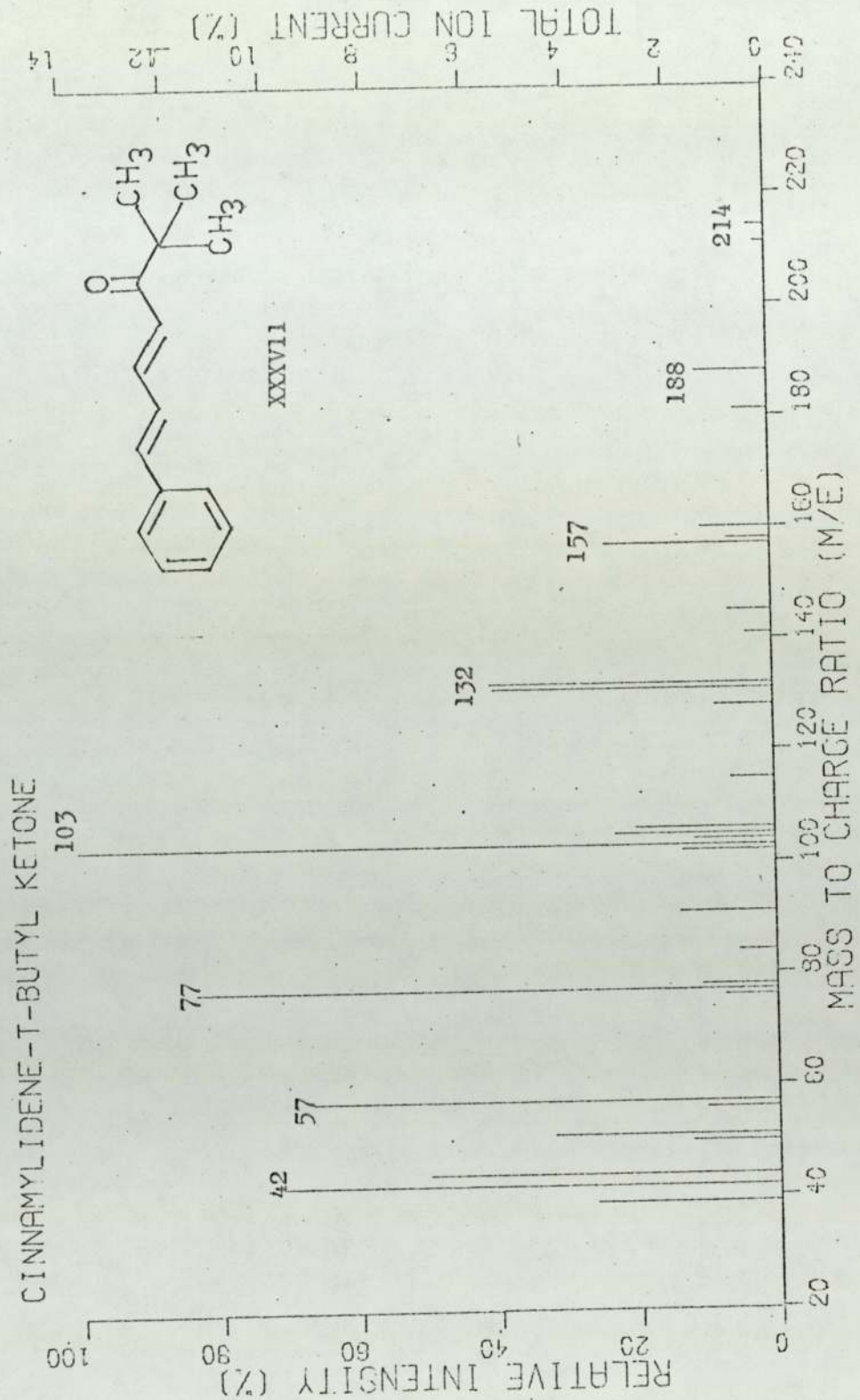

CINNAMYLIDENE-T-BUTYL KETONE
XXXVII
RELATIVE INTENSITY (%)
MASS TO CHARGE RATIO (M/E)
TOTAL ION CURRENT (%)
103
77
57
42
132
157
188
214

Fig. 27.

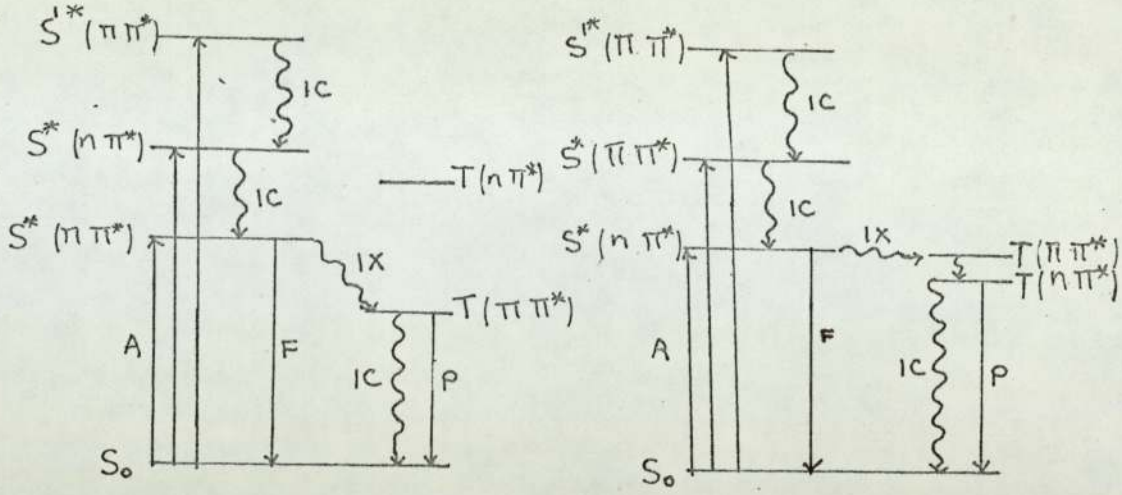
5. ABSORPTION AND FLUORESCENCE STUDY OF THE HYDRO-OXO-PHENANTHRENE CHROMOPHORE.

5.1 Introduction

Aromatic molecules possessing carbonyl groups with n electrons participating in the aromatic structure may show $n\pi^*$ transitions at lower frequencies than any of the $\pi\pi^*$ transitions. These transitional characteristics are due to the n orbitals in the ground state of the molecule, which are higher in energy than the occupied π orbitals. Such generalizations are, however, solvent dependent. In condensed phases solvents are often very polar. The positive end of a dipole exerts an attractive force on n electrons in a solute molecule, thus lowering their potential energy, as commonly exemplified by hydrogen bonding. This has the effect of lowering the energy of the n orbital relative to that of the π orbitals, which being more symmetrically bound by the molecule as a whole, do not experience this electrostatic interaction to a considerable extent. The excited $n\pi^*$ and $\pi\pi^*$ states are, however, frequently more polar than is the ground state of the molecule. Interactions of the excited states of the aromatic molecule with a polar solvent tend to lower the energies of the excited states. Those with n electrons, the effect of a polar solvent is generally more pronounced on the energy of ground state n orbitals than on the n orbital excited states. As a result, the more polar the solvent, the greater is the difference in energy between the highest occupied n orbital in the ground state and the excited states of the molecule.[109] On the other hand, since the ground state π orbitals are almost unaffected by solvent polarity, increasing the polarity of the solvent results in a decrease in the energy difference between the highest occupied π orbital of the ground state and the excited states of the molecule. Therefore, in polar solvents $n\pi^*$ transitions are shifted

to higher frequencies (shorter wavelengths), whereas $\pi\pi^*$ transitions are shifted to lower frequencies (longer wavelengths). If the solvent becomes polar enough the lowest $\pi\pi^*$ transition may overtake the $n\pi^*$ transition and become the lowest energy band in the spectrum.[109] In the extreme case of solvent-n electron interaction, a co-ordinate covalent σ bond is formed and the energy of the $n\pi^*$ transition which has now become a $\sigma\pi^*$ transition is so great that it appears in the vacuum UV region.

Only a few reports [106,15,107,108] on $n\pi^*$ fluorescence at room temperature have been cited in the literature. The presence of a hetero-atom in an aromatic molecule often induces a lowest excited singlet state that is $n\pi^*$ in nature, except when n electrons are involved in bonding. Fluorescence from this state is not only rare, but also extremely weak when it does occur; it is of the order 10^2 to 10^3 times lower in efficiency than $\pi\pi^*$ fluorescence.[125] This is a consequence of symmetry forbiddenness of transitions between $n\pi^*$ state and the ground state, which result in a long lifetime and allow intersystem crossing to compete effectively with fluorescence. The rarity of $n\pi^*$ fluorescence is due to the efficiency of intersystem crossing between $n\pi^*$ singlet and $n\pi^*$ and $\pi\pi^*$ triplet states. If the $n\pi^*$ triplet state is lowest in energy fig. 28b, phosphorescence is possible but very difficult to observe because of combined symmetry forbiddenness and spin forbiddenness of the $n\pi^*$ triplet state to the ground state transition which allows singlet-triplet internal conversion to compete effectively with phosphorescence. On the other hand, if the $\pi\pi^*$ triplet state is lowest in energy, fig. 28a, which is often due to the greater separation between $\pi\pi^*$ singlet and triplet than between $n\pi^*$ singlet and triplet states, then phosphorescence is likely to occur.



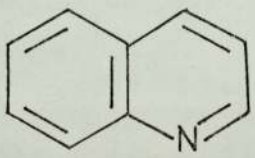
(a) ππ* Excited state Lowest in Energy

(b) nπ* Excited State Lowest in Energy

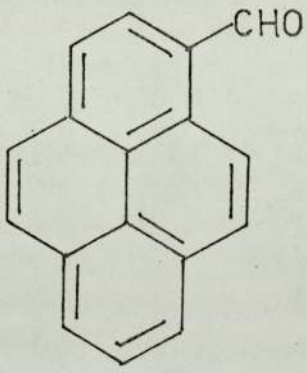
Fig. 28. Comparison of electronic processes for molecules having lowest nπ* and ππ* states: A, absorption; IC, internal conversion; IX, intersystem crossing; P, phosphorescence; F, Fluorescence; S*, excited singlet state; T, triplet state; So, ground state.

It would appear well established[110-112] that almost complete reversal of the excited state energy levels occurs in certain aromatic molecules which are sensitive to moderate changes in solvent polarity and hydrogen bonding. For example, it is reported that the fluorescence intensity of quinoline in solvents benzene, ethanol and water is enhanced in the ratio 1:50:1000, respectively. The authors conclude that the lowest nπ* state in benzene is replaced by a nearby ππ* state in water.[110] Singlet state inversion has also been shown to be a property of pyrene-3-aldehyde[111] and acridine[112] whose fluorescence behaviour is similar

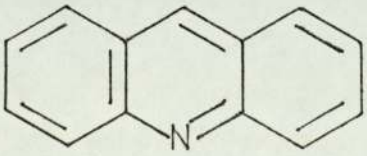
to quinoline fig. 29. In contrast, room temperature fluorescence observed from 6-keto-oestradiol (fig. 29) is not as well established. It is suggested that the molecule is excited to the $n\pi^*$ level. Then it undergoes internal conversion to $\pi\pi^*$ singlet state, from which fluorescence is observed. This is claimed to be a more efficient process than $n\pi^*$ triplet conversion for this molecule.



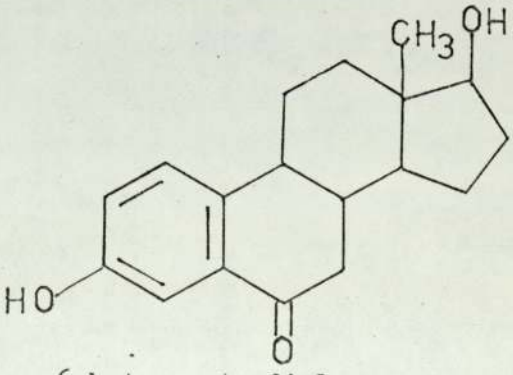
Quinoline



Pyrene-3-aldehyde



Acridine



6-keto-oestradiol

Fig. 29. Some compounds which exhibit the property of singlet state inversion.

Rusakowicz and others[113] in their study of aromatic carbonyl compounds in polar hydrogen bonding solvents suggest that to obtain inversion of $n\pi^*$ and $\pi\pi^*$ states, strong hydrogen bonding solvents are required which may even lead to protonation of the carbonyl oxygen. Their observations on the fluorescence and phosphorescence of a range of aromatic carbonyl compounds in 85% H_3PO_4 are shown to be consistent with a model they postulate for carbonyl solvation in which they suggest the excited state to possess the following characteristics: (i) have $n\pi^*$ character in non-polar and weakly bonding solvents; (ii) exhibit enhanced $\pi\pi^*$ character in very polar hydrogen bonding solvents; and (iii) become a protonated $\pi\pi^*$ state in strongly acidic media.

The absorption and fluorescence characteristics of the oxo-phenanthrene chromophore under study was investigated in the light of this model.

5.2 Absorption studies in Hydrocarbon and Polar Solvents

When the carbonyl group is conjugated with an ethylenic bond, with an aromatic group, or both, the $n\pi^*$ band of the carbonyl group will be at lower energies as a result of interaction mainly with the lowest antibonding π-orbital of the unsaturated hydrocarbon group. The hydro-oxo-phenanthrene, XXXI, shows (fig 30) three bands in the UV region. The intense band at about 350nm is probably due to $\pi\pi^*$ and $n\pi^*$ absorption of the conjugated chromophore. Of similar intensity, the band at 240nm is interpreted as a $\pi\pi^*$ which has the character of an intramolecular charge transfer (CT) band.[114] The most intense band at about 200nm is probably a perturbed local excitation band corresponding to the benzene ^1L_a band. The $n\pi^*$ transition of this oxo-phenanthrene is not seen as an isolated band. When a carbonyl group is conjugated with a polyene system, both $n\pi^*$ and $\pi\pi^*$ bands shift to longer wavelengths as the length of the conjugated system increases. However, the energy of the

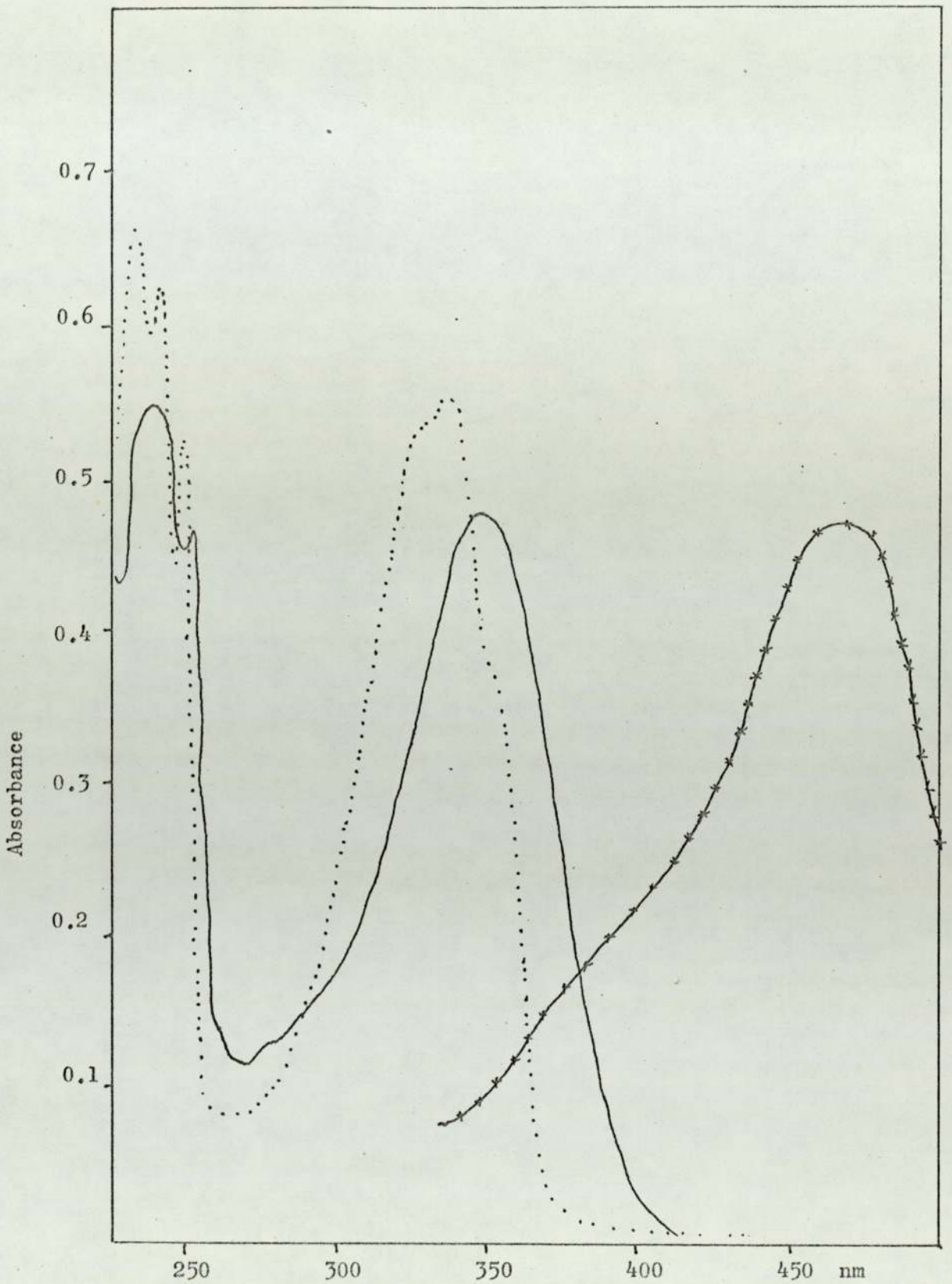


Fig. 30. Absorption characteristics of the oxophenanthrene XXXl in

——— EtOH

····· n Hexane

-x-x-x- 88% H_3PO_4

$n\pi^*$ band does not decrease as rapidly as does the energy of the $\pi\pi^*$ band. The $\pi\pi^*$ band energy is lowered by raising of the donor orbital and lowering of the acceptor orbital.[115] The conjugated system of the oxophenanthrene chromophore under study is extended enough for the $n\pi^*$ band to be swamped under the more intense $\pi\pi^*$ band. On changing from ethanol to hexane both the CT and long wavelength absorption bands show fine structure and are accompanied by solvent shifts of 8nm and 13nm, respectively, towards shorter wavelengths. Using phosphoric acid as solvent, however, causes a change in the chromophore resulting in the formation of a new absorption band in the visible region (fig. 30). This may possibly be due to enolization.

The absorption characteristics of the oxo-phenanthrenes in ethanol (table 5) are largely similar, except for slight changes in fine structure, in hexane. This may be due to the different substitution. Although the cinnamylidene compounds (XXXV-XXXVll) have the same chromophore and exhibit similar absorption features, they differ considerably from the latter in position and intensity of absorption as well as fine structure of both the CT and the long wavelength absorption bands e.g. (fig. 31 for XXXVll). The observed differences may be attributed to the lack of rigidity and planarity of these compounds as compared to the oxo-phenanthrenes.[126] It is interesting to note that XXXVll is the only compound with the oxo-phenanthrene chromophore studied here which shows an enhanced $n\pi^*$ (360nm) absorption band (ε =2100). The long wavelength absorption band at 305nm, considerably shorter than that of other cinnamylidene derivatives XXXVl and XXXV (both at 335nm). The bulky t-butyl group on XXXVll presumably twists the carbonyl group out of the plane of conjugation, resulting in a poor orbital overlap between the carbonyl and the rest of the chromophore. This is partly supported by the fact that the UV spectrum of the latter in hexane is also remarkably

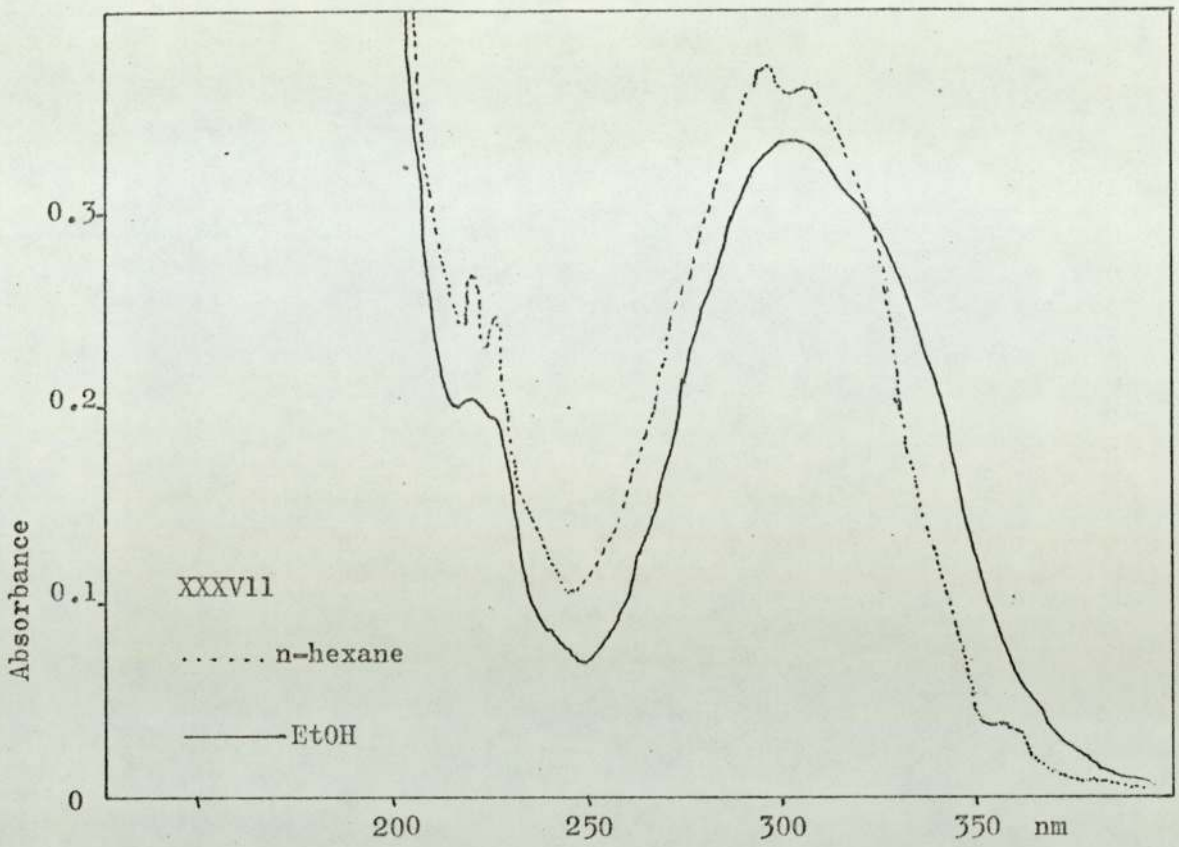

0.3
0.2
0.1
0
Absorbance
XXXV11
..... n-hexane
——— EtOH
200
250
300
350
nm

Fig. 31.

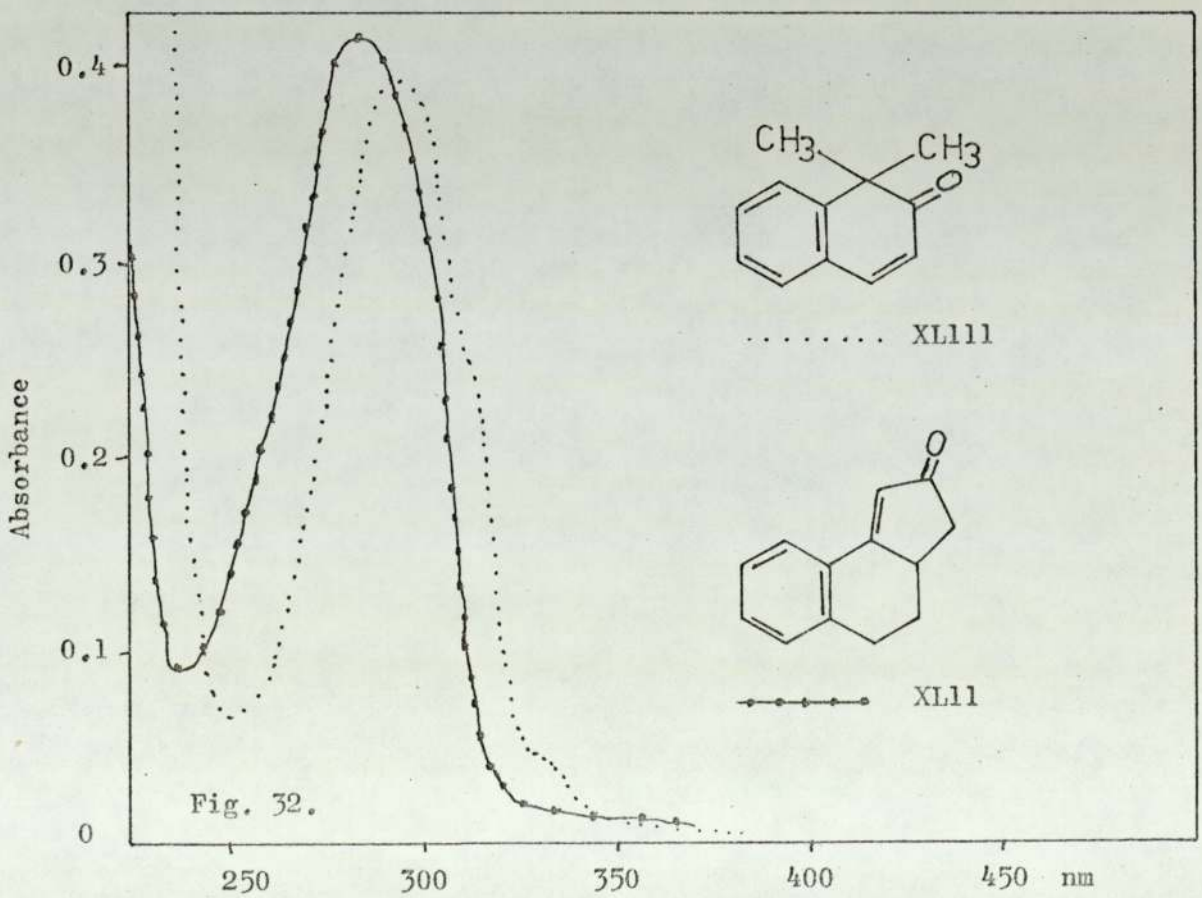

0.4
0.3
0.2
0.1
0
Absorbance
CH3
CH3
O
..... XL111
O
XL11
250
300
350
400
450
nm

Fig. 32.

similar, both in position of absorption and fine structure, with that of trans-1-phenyl-butadiene except for the $n\pi^*$ shoulder. Also the t-butyl group may be implicated in shifting the $n\pi^*$ transition towards longer wavelengths. This would seem to be supported by the fact that the keto-dihydronaphthalene (XL111) with geminal methyl substitution adjacent to the carbonyl function shows an $n\pi^*$ absorption band in n-hexane whereas the related similarly absorbing cyclopenteno derivative (XL11) does not (fig. 32).

5.3 Room Temperature Fluorescence Studies in Acidic Media

Thin layer chromatography evaluation of all the ketones studied was done on silica and it revealed that only the hydro-oxo-phenanthrenes and the keto-dihydro-naphthalenes showed appreciable fluorescence. All the benzylidene and cinnamylidene compounds run were found to be non-fluorescent. Attempts were made to transfer the fluorescence observed on thin layer plates into solution. All the compounds studied, including the oxo-phenanthrenes and keto-dehydronaphthalenes were found to be non-fluorescent at all concentrations in both hexane and ethanol. However, on using both 88% orthophosphoric acid and 60% perchloric acid all the compounds with the oxo-phenanthrene chromophore and the keto-dehydro-naphthalene XL11 exhibited fluorescence which was found to be structure and pH dependent.

The parent hydro-oxo-phenanthrene XXX1, was studied in detail to evaluate the effect of pH on both the absorption and emission characteristics of the fluorescent species. It is suggested that in strongly acidic solvent enolization of the chromophore occurs and that it is the enol form that exhibits fluorescence. The suggestion is consistent with the observed disappearance of fluorescence on neutralization of an acidic solution of XXX1 followed by ether extraction and subsequent quantitative

recovery of the ketonic species. Also gradually decreasing the pH of an ethanolic solution of XXXl results in a shift of the 350nm peak towards the visible region (fig. 33). The fluorescence intensity correlates closely with polarity . The excitation and emission spectra of the fluorescent species occurs at 465nm and 530nm (fig. 34).

Fluorescence efficiencies were evaluated from the comparison of the intensities at the wavelength of maximum emission with respect to the emission intensity of the reference compound. The concentration of the samples was carefully adjusted to give the same absorbance as the reference compound at the exciting wavelength chosen. Great care was taken to ensure that the concentration of the samples was well below 10^{-5} mol/lit to avoid re-absorption of the fluorescence emission[117] in the 1cm cell at the right angle illumination viewing geometry. No differences were observed on the intensity on degassing solutions. Corrections were not made for the refractive index of the solutions. The reproducibility of duplicate determinations in the fluorescence quantum yield of these compounds is about ± 3% and all the reported emission spectra are corrected.

Table 8.

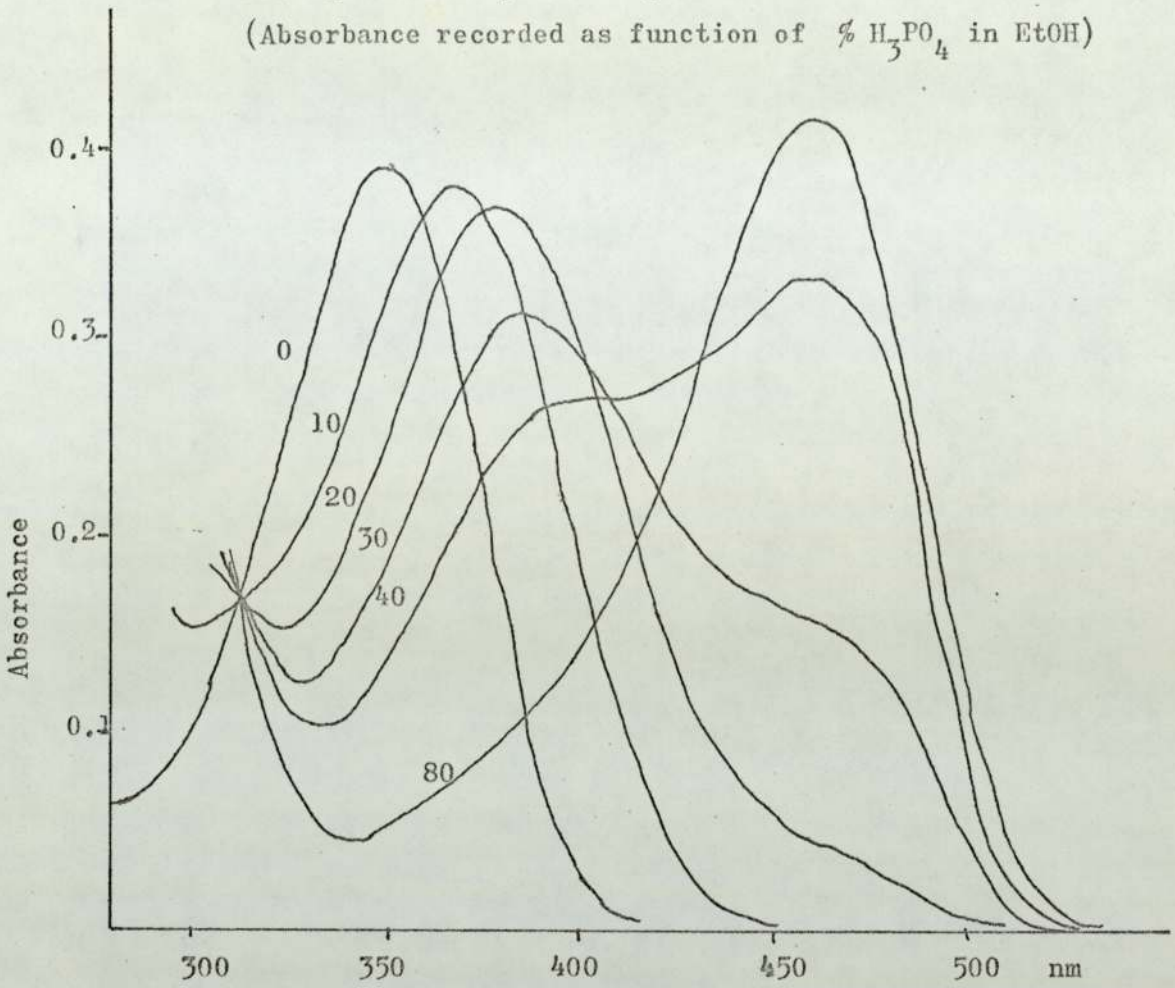
Fluorescence Data in 88% H_3PO_4 at Room Temperature.

Compound		Excitation	Emission	Quantum Yield
Oxo-phenanthrene	XXXl	465 nm	530 nm	0.33[a]
"	XXXlll	470	550	0.017[a]
"	XXVlll	465	535	0.0015[a]
Cinnamylidene Compound	XXXV	370	420	0.041[b]
"	XXXVl	380	430	0.041[b]
"	XXXVll	370	420	0.032[b]
Keto-dihydro-naphthalene	XLll	340	470	0.020[b]

[a] Quantum yields (Φ_f) are relative to fluorescein (Φ_f = 0.87)[118] in 0.1N NaOH.

[b] Quantum yield (Φ_f) are relative to quinine sulphate (Φ_f= 0.55)[119] with excitation at 366nm.

Enolization of Hydro-oxo-phenanthrene XXXl
(Absorbance recorded as function of % H_3PO_4 in EtOH)



Rel. Fluorescence of 10^{-5}M solution of XXXl as a function of conc. H_3PO_4 in EtOH.

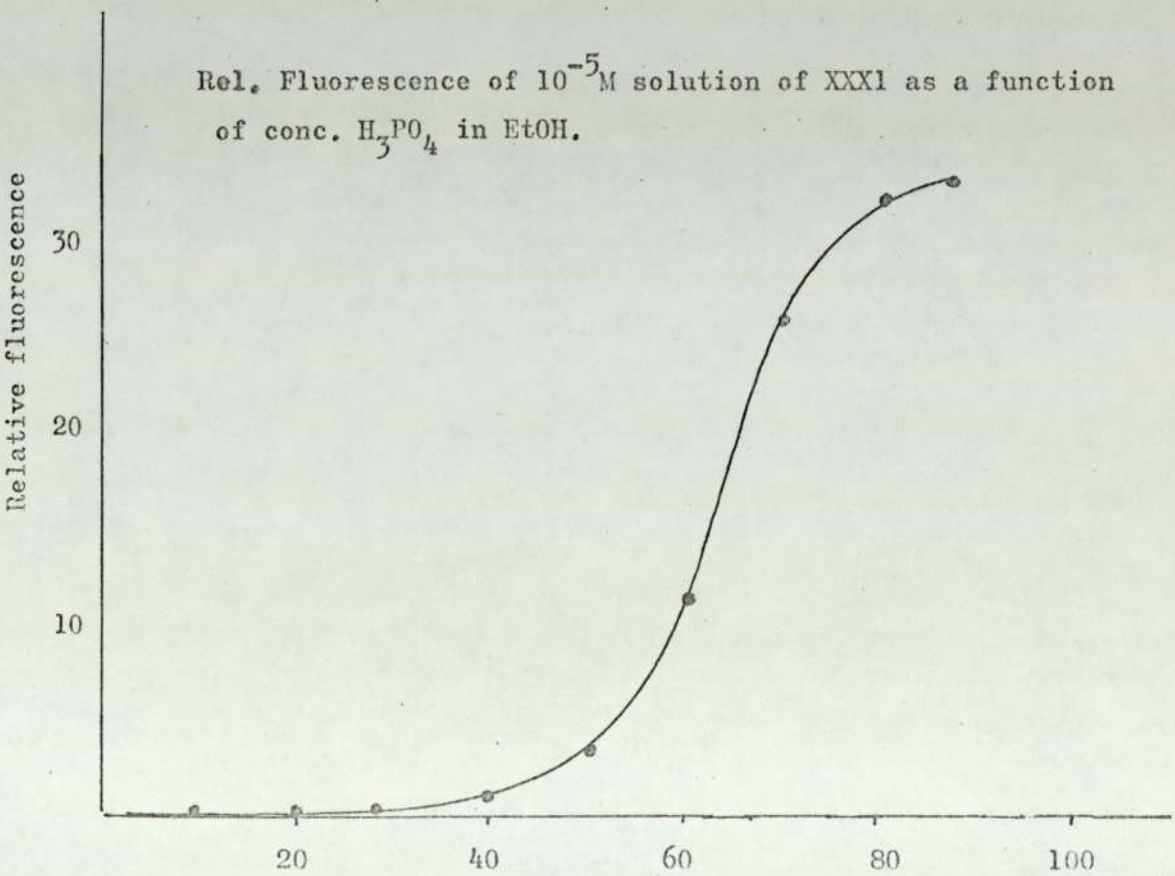


Fig. 33. % H_3PO_4

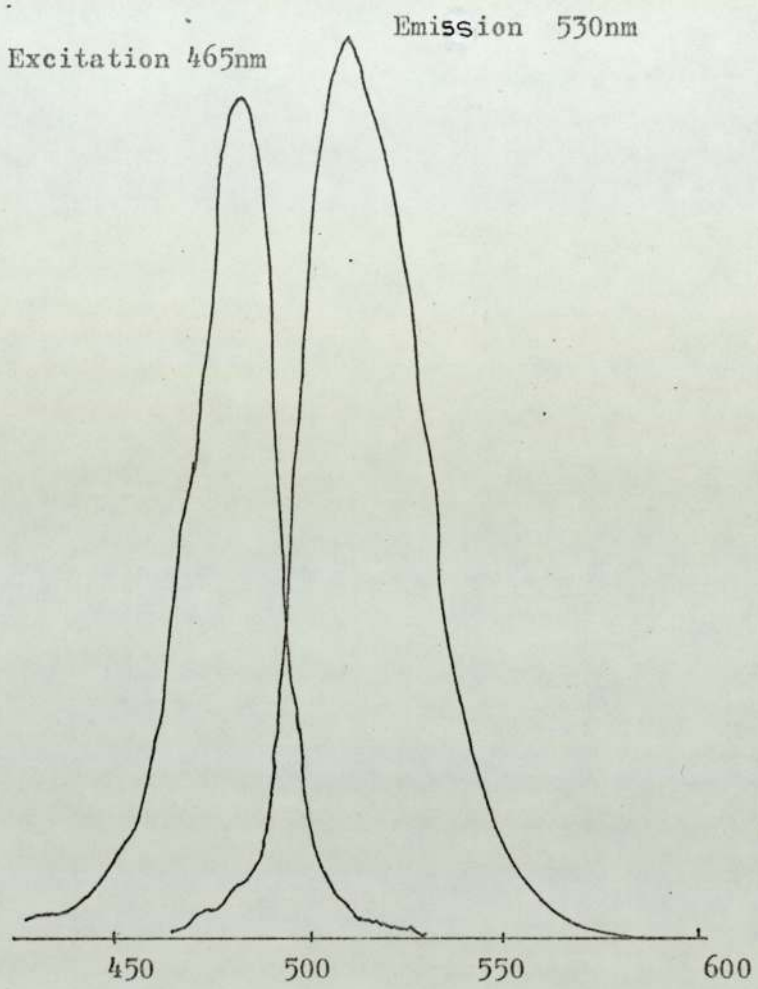


Fig. 34. Excitation and Emission Spectra of the oxo-phenanthrene XXXl in H_3PO_4 88%

The compound XXXl is the most intensely fluorescent compound in acid and it has a high quantum yield of 0.33. It is thought that the decrease in quantum efficiency observed for both XXXlll and more so for XXVlll is consistent with substitution on the aromatic ring at the 7 and 8 positions. The quantum yield of the disubstituted compound XXVlll has decreased by a factor of nearly 200 as compared with the parent compound.

The cinnamylidene compounds fluoresce at shorter wavelengths than the oxo-phenanthrenes. Their quantum yields are also comparatively lower by a factor of 10. Their fluorescence is also attributed to possible enolization of the chromophore under acid conditions. The tertiary-butyl derivative XXXVll would not, however, be expected to enolize under acid conditions. Its fluorescence may be attributed to originate from the $\pi\pi^*$ protonated species which was also shown to be reversible on neutralizing, or due to a chemical reaction.

5.4 Ionization Potentials

a) Practical Considerations

Ionization potential (IP) of a molecule may be defined as the minimum energy required to remove an electron from the highest occupied molecular orbital. Only particles of at least this energy are able to produce ions. Upon increasing the energy of the bombarding particle it may happen that the molecular ion MA^+ dissociates into fragments of which at least one is an ion M^+. The minimum energy required to do this is referred to as the "appearance potential of M^+ (AP) whose determination is closely related to the determination of IPs[127]. Once the ion is produced its abundance varies with the electron (or photon) energy and a curve may be drawn of the ion abundance vs electron (or photon)energy. It is generally called the ionization efficiency curve, and has a specific shape characteristic of the type of process involved.

It is obvious that IPs are closely related to the chemical nature of the molecule as well as the structure and the thermodynamic characteristics.

Four general techniques may be used for the determination of ionization potentials:

(i) UV spectroscopy,

(ii) Electron impact,

(iii) Photon impact,

(iv) Ion impact.

UV spectroscopy has been used for some time to deduce ionization potentials[120,121] but it yields only approximate values, useful when no other methods are available. Electron impact was considered the method of choice.

The ionizing efficiency curve of the ion under investigation is obtained by plotting the ion current as a function of the electron energy. This being done with sufficient accuracy, generally yields an S-shaped curve with a more or less asymptotic onset and the tendency to level off between 10-20V above onset. The problem of determining the ionization potential then remains that of interpreting the efficiency curve. Whatever method is applied to the treatment of the ionization curve, it is necessary to have a suitable calibration of the electron energy scale. The electron accelerating potential reading on a meter is not generally representative of the actual energy of the electrons for a number of reasons. There is practically always contact and surface potentials, caused by molecules adsorbed on the different electrodes of the electrode gun, which have to be taken into account. The potential lines into the ionization box depend very much on the field penetration from the ion accelerating system[122], as well as from the electron collector and from the field disturbance by the repeller plate voltage.

Both the temperature of the filament and the pressure of the sample are important owing to the sensitivity of the detecting device. One of the simplest ways to eliminate these difficulties is to use as a reference standard a calibrating gas of which the IP is accurately known; generally it is a rare gas, Ar, Kr or Xe, and is admitted into the mass spectrometer simultaneously with the sample at such a pressure that the ion being studied and the reference ion give about the same current intensity. The ionization curves for both ions are then taken, one after the other, from which the differences in ionization potentials are deduced.

A modification of the widely used Honigs method which was proposed by Lossing et al[123] was used for the IP determinations. It gave reproducible results, more so since the shapes of the ionization efficiency curves being compared are not too different. The pressure was adjusted to get comparable ion intensities at 50V, and a log plot of peak heights as percentages of the abundance at 50V, against the electron energy yielded parallel curves near the onset.

b) Ionization of the carbonyl group

The ionization potentials of these conjugated ketones were determined in order to find out whether the observed fluorescence could be more closely correlated with structure. Electron impact studies on formaldehyde showed that it has three ionization potentials[124], 10.8 ± 0.1, 11.8 ± 0.2 and 13.1 ± 0.2 eV. These are attributed to the removal of one electron from the non-bonding p orbital, the bonding π orbital, and the C-O bonding σ orbital, respectively. The first ionization potentials of the compounds shown (Fig. 35-37) are attributed to the removal of electron from the nonbonding p orbitals. The rigid and planar oxophenanthrenes have low IPs as expected while the floppy and less conjugated chromophores have higher IPs but there seems to be no specific relation between IPs and absorption or fluorescence in this series of compounds.

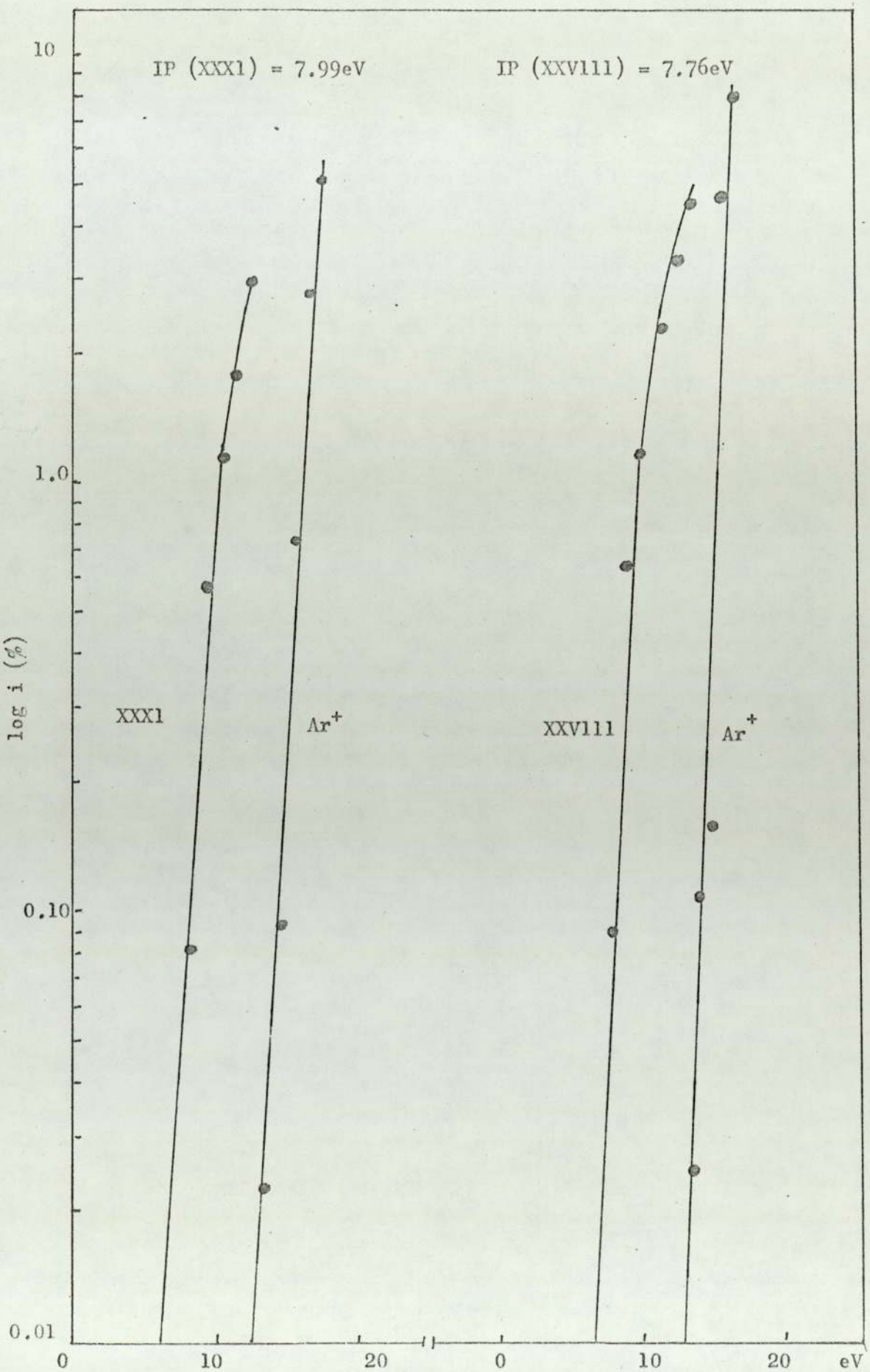

IP (XXX1) = 7.99eV
IP (XXV111) = 7.76eV
10
1.0
0.10
0.01
log i (%)
XXX1
Ar+
XXV111
Ar+
0
10
20
0
10
20
eV

Fig. 35.

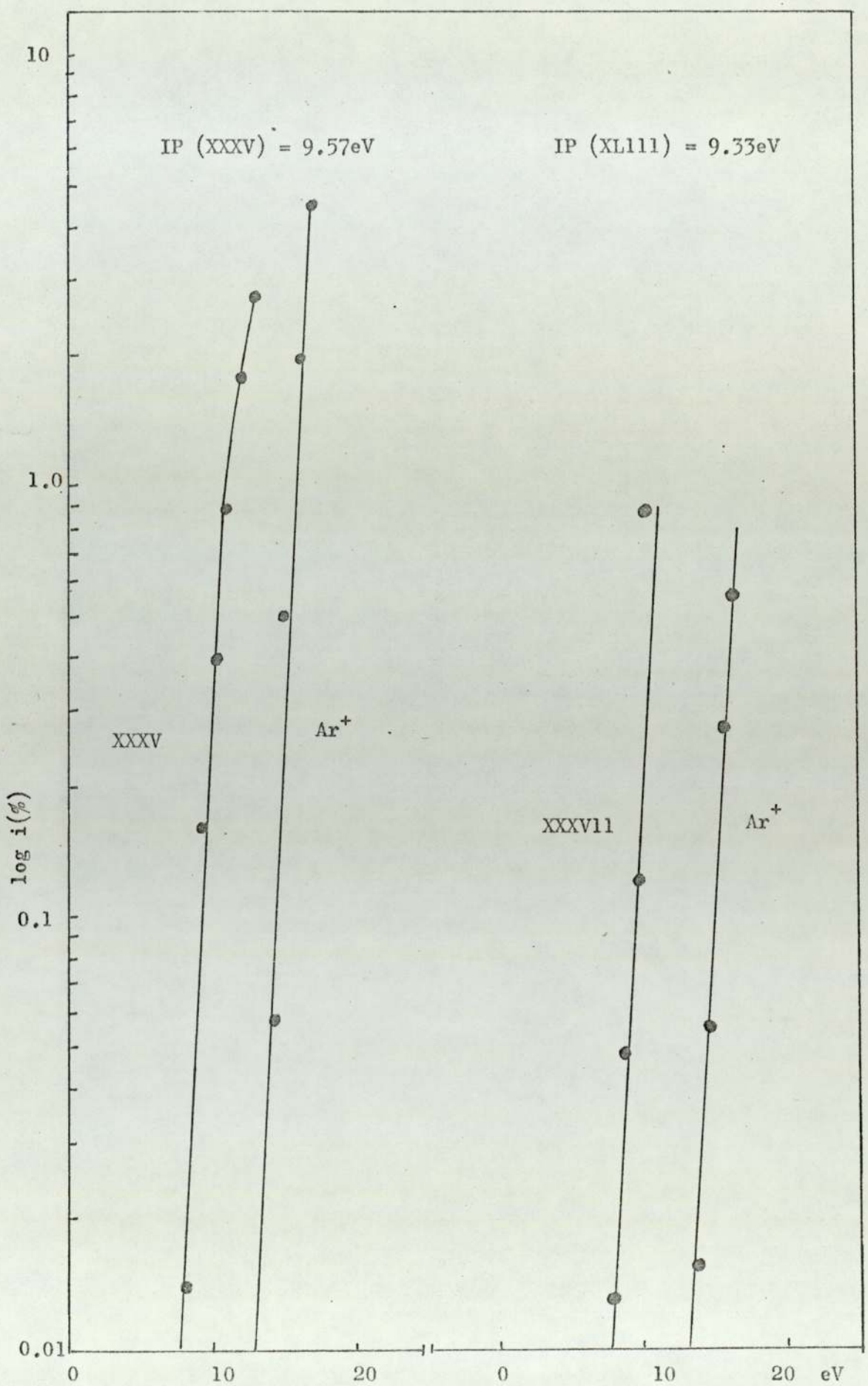

10
1.0
0.1
0.01
log i(%)
IP (XXXV) = 9.57eV
IP (XL111) = 9.33eV
XXXV
Ar+
XXXV11
Ar+
0
10
20
0
10
20
eV

Fig. 36

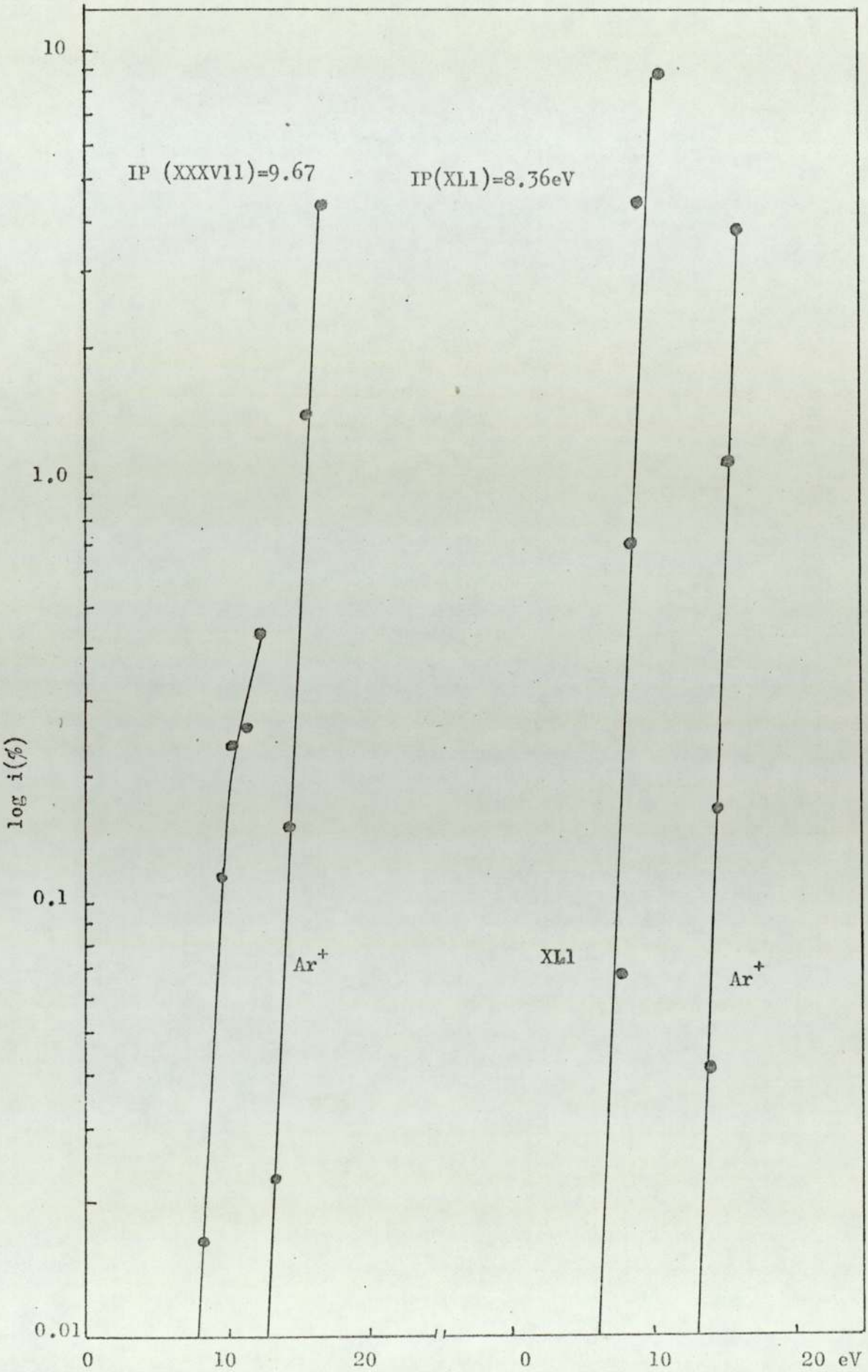

10
1.0
0.1
0.01
log i(%)
IP (XXXV11)=9.67
IP(XL1)=8.36eV
Ar+
XL1
Ar+
0
10
20
0
10
20 eV

Fig. 37

EXPERIMENTAL

Melting Points

An electrothermal apparatus was used and all the reported values are uncorrected.

Titrations

Fluorescence quenching titrations were done using an "Agla" micrometer syringe (Wellcome Foundation Ltd.)

Refractive Indices

These were obtained using an Abbé type refractometer, Bellingham and Stanley Ltd., London.

pH Measurements

These were determined using a Corning-Eel pH meter, Model 10, at 22°C.

I.R. Spectra

Routine I.R. measurements were done on a unicam SP 200 spectrophotometer.

UV Measurements

UV spectra were obtained on a Beckmann Acta V spectrophotometer using a 0.2mm slit width selection at 450nm on program.

Fluorescence Equipment and Accessories

Fluorescence spectra were obtained with an Aminco-Bowman Spectrophotofluorimeter (Am. Instr. Comp.) equipped with Hanovia 200W mercury/Xenon and 250W Xenon lamps and a Hewlett-Packard X/Y recorder. The recommended slit arrangement No. 3 (3233232) was used throughout with sensitivity set at maximum, unless otherwise indicated. A 1cm quartz cell equipped with a stopper was used for all excitation and emission spectra.

Mass Measurements and Ionization Potential Determinations.

All mass spectra were obtained from an A.E.I. MS 9 spectrometer

employing direct sample insertion into an ion source (temp. 200-250°C) and an ionizing potential of 70 eV.

Ionization potentials were determined by admitting argon into the mass spectrometer simultaneously with the sample at such a pressure that the ion being studied gave the same current intensity. The ionization curves of both ions were taken, one after the other, from which the differences in ionization potentials were deduced.

PMR Spectra

These were run on Perkin Elmer R14 and Varian HA 100D instruments at 33° and 37°C respectively. $CDCl_3$ was used as a solvent throughout unless otherwise stated.

Chemicals and Solvents

All chemicals used were of analytical or pure grade quality and were checked for chromatographic homogeneity and melting points; liquids were re-distilled prior to use. Solvents for fluorescence work were doubly distilled and also checked for fluorescence prior to use.

6.1 Fluorescence Quenching of Proteins, nucleic acid bases and their derivatives

1.0ml of 0.10μM/ml solution of HSA in 25% ethanolic phosphate buffer pH 7.4 0.02 was titrated with a solution of steroid prepared similarly and the fluorescence read at 330nm on excitation at 280nm. Corrections were made for dilution. The heterocycles below were similarly investigated in concentrations ranging from 4-20μg/ml:

Compound	Medium	Excitation	Emission
Guanine	pH 11.0 N/1 NH_4OH	275 nm	350 nm
Guanosine	pH 1.0 N/10 H_2SO_4	285	390
2,6-Diaminopurine	pH 7.0 Phosphate Buffer	300	360
8-Azaguanine	pH 13.0 N/10 NaOH	290	360

6.2 Attempts at the Sensitization of the Emission of Eu^{3+}, Tb^{3+} and Dy^{3+} in solution by Steroidal ketones

The rare earth oxides were dissolved in nitric acid and evaporated to dryness on a hot plate. The rare earth nitrate was then dissolved in acetic anhydride. Concentrations of $5x10^{-2}$M solutions of the rare earths were made up and diluted as required. The steroids (cortisone, hydrocortisone, corticosterone and testosterone) were made up at concentrations of 2mg/ml also in acetic anhydride and used to titrate 1ml aliquots of rare earth solutions while their emission intensities were recorded. Emissions for Eu^{3+}, Tb^{3+} and Dy^{3+} were measured at 610nm, 550nm and 580nm respectively.

6.3 Chromogenic Reaction of Aniline in Glacial Acetic Acid (HOAC)

i) Effect of aniline concentration

Solutions of m/100 and m/400 of aniline were allowed to stand and changes in colour were monitored spectrophotometrically for 2 days, following the intensity of chromophore B. The reaction for the m/400 solution was completed after 24hrs. Fig. 2, and for the m/100 solution after 35 hours.

ii) Effect of Oxygen and Light

Air was removed from an m/400 solution of aniline in HOAC using a mercury vapour pump and liquid nitrogen. The sample in a strong pyrex ampoule was frozen by immersing in liquid nitrogen and then allowed to thaw up slowly while connected to the vacuum pump. The process was repeated till all the air was removed and the frozen contents of the ampoule sealed under vacuum. One ampoule was stored under normal light conditions and another in the dark for 1 week. They both gave the chromogenic reaction described.

iii) Effect of pH

An m/40 solution of aniline in (HOAC) was diluted ten-fold with:

0.1 M HCl and

0.1 M NaOH.

Both dilutions did not develop the A or B chromophores when left standing for 24hr. With NaOH there was no change in the UV spectrum (λmax 280nm) but on acidification with HCl the 280nm peak shifted to 255nm which is characteristic of benzenoid absorption.

The UV of acetanilide m/100 in EtOH, Conc. HCl, and 71% perchloric acid were recorded. They all looked remarkably similar with λmax at about 240nm.

iv) Effect of Heat on the formation and Stability of Chromophores A & B

An m/100 solution of aniline in HOAC heated at 100°C for 30 min. showed no trace of chromophore A when examined spectrophotometrically. Chromophore B had reached a steady absorption intensity which did not change with time on standing. Continued heating for one hour had no effect on the stability or intensity of B. Cooling the solution and allowing it to stand for 24hrs caused no further changes.

v) Attempts at isolation of reaction products

500mg of aniline was dissolved in 20ml of HOAC and allowed to stand overnight. The solution was made alkaline with NaOH (5%) to pH 12.0 before extracting with 5x10mls of ether. Removal of the ether under reduced pressure gave a product with UV and IR spectra indentical with aniline.

vi) Attempts at semi-quantitative comparison of the chromophores of various aniline derivatives

An m/200 solution in HOAC of each amine was made and the absorption recorded over 24hr. The position of absorption of the designated A and

B chromophores as well as the 24hr. absorption intensity of the B peak for each compound was also recorded as shown in table 2.

6.4 The Specific Fluorimetric Determination of Digoxin

(i) Calibration procedure

Digoxin B.P. (1mg) was weighed out and dissolved in ethanol (80%) to make 100ml of solution. After serial dilution 1ml aliquots containing 0.1 to 2.0μg were pipetted into 5ml volumetric flasks. To each flask 1ml of TCA reagent (trichloroacetic acid 10% $^{w}/v$ in 80% ethanol) was added making a reaction mixture of 2ml total in volume. A reagent blank without digoxin was similarly set up. The flasks were transferred to an oil-bath maintained at $130^{o}C \pm 1^{o}$ and heated over a period of 40 minutes before cooling to $4^{o}C$ and the contents made up with ethanol to 5ml. The solutions were read in the fluorimeter within 20 minutes of being made up to volume. There was, however, no significant change in intensity on recording the fluorescence after 24 hours. The relative intensity was found to be linear with digoxin concentration between 0.02 and 1.0μg/ml.

(ii) The Effect of Temperature and Reaction Time

A study of the effect of temperature on the development of the fluorescence as a function of time was carried out using a single reaction concentration of digoxin (0.4μg/ml). The reaction mixtures were set up as described and heated on an oil bath at $130^{o}C + 1^{o}$ for 15, 30, 60, 90 and 120 minute periods, made up to volume, and read in the fluorimeter. Other solutions were similarly prepared and heated at a lower temperature on a water bath 98°-$100^{o}C$ for 30, 60, 120, 180 and 240 minute periods and their fluorescence recorded. Below these temperatures no significant reaction was observed.

(iii) The Effect of Concentration of Trichloroacetic Acid.

Using 1ml aliquots of a 0.4μg/ml solution of digoxin reactions were carried out as described with concentrations of TCA ranging from 0 to 100%^{w}/v. The fluorescence was recorded as a function of TCA concentration.

(iv) The Effect of Chloramine-T on Development of Fluorescence.

The digoxin solutions 0.4μg/ml were reacted as described except that the TCA reagent contained chloramine-T in a concentration ranging from 0.01% to 0.8%^{w}/v. The fluorescence intensity was then recorded as a function of chloramine-T concentration.

(v) The Determination of Digoxin in Tablets.

One tablet containing approximately 250μg of digoxin was placed in a 250ml volumetric flask, pulverised with a glass rod and 50mls of ethanol added. The flask was stoppered and shaken mechanically for 30 minutes. The mixture was then filtered through a sintered glass filter and after several washings with alcohol made up to 250ml. 1ml of this solution was treated as described. Tablets stated to contain 62.5μg of digoxin were similarly shaken up with 5mls of ethanol and the filtrate and washings made up to 50ml. The solutions were read on the fluorimeter and the amount of digoxin present in μg/ml of solution read from the calibration curve.

6.5 Synthesis of Hydro-oxo-phenanthrenes

(i) 7,8-(1,1-Dimethylcyclopenteno)-2,3,4,12-tetrahydro-12-methyl-2-oxophenanthrene, XXVlll.

A solution of 4-androsten-17α-methyl-17β-ol-3-one (1.0g) in 5.0ml of 10% trichloroacetic acid - 80% aqueous ethanol was heated on an oil bath at 130° for 3 hours using an air condenser. The brown oily residue was taken up in ether, washed with 2.5% NaOH and water and dried (Na_2SO_4).

The ether was removed at 60°C under reduced pressure to give a clear glass (600mg). This was taken up in minimum ether and chromatographed on neutral alumina (activity 1, 300g packed in a 4.0cm i.d. column). Elution was effected with petroleum ether (60-80°C), pet-ether-diethyl ether (3:;), pet-ether-ether (1:1), and ether, in that order. Aliquots (50ml) were collected and examined by UV and tlc (silica and C_6H_6:$CHCl_3$, 9:1). Six substances with R_f 0, 0.13, 0.24, 0.34, 0.45, 0.75 were detected. The major component, R_f 0.45 was the only one with intense UV absorption at 240nm and 350nm. The absorptions of the other componnets did not differ substantially from that of the starting material. The aliquots with the 350nm absorption were poded, evaporated under reduced pressure and re-chromatographed under the same conditions to give (65mg) of the oxophenanthrene, XXVill, as pale yellow needles m.p. 154-155°C, ex.pet-ether.

IR (KBr) 1655, 1615, 1600, 1575, 880, 830 Cm^{-1}

MS $C_{20}H_{22}O$ requires M^+ 278.1668

found M^+ 278.1670

Analysis: $C_{20}H_{22}O$ requires C, 86.36 ; H, 7.97

found C, 86.11 ; H, 8.07

(ii) <u>2,3,4,12-Tetrahydro-12-methyl-2-oxo-phenanthrene, XXXl</u>.

a) Attempts were made at preparing this oxophenanthrene from 1-methyl-2-naphthol[97] (5g) and methyl vinyl ketone (7.0g) to which (5.5g) of p-toluenesulphonic acid hydrate in 80ml of glacial acetic acid[84] was added. After shaking vigorously, the solution was allowed to stand at room temperature under nitrogen for 11 hours. The reaction mixture was poured into 500ml of water, partially neutralized with 10% sodium bicarbonate solution and dried ($MgSO_4$), the chloroform was evaporated leaving a dark oil. The latter was extracted with ten 100ml portions of hot petroleum ether (B.P. 60-80°C) and the cooled combined extracts

chromatographed on a celite-silica gel column, starting with petroleum ether and gradually increasing the polarity to 9:3 petroleum ether-ether ratio. All the fractions obtained contained very small amounts of the title compound in admixture with the starting material. Attempts to remove the starting material by alkali extraction (10% Na_2CO_3 and 5% NaOH) proved unsuccessful as the oxophenanthrene was also alkali soluble. The reaction was, therefore, abandoned.

b) Dehydrogenation

The ketone, 2,3,4,9,10,12-hexahydro-12-methyl-2-oxo-phenanthrene[86], XXIX, was made from 2-tetralone[98] and 4-chlorobutan-2-one[99]. Anhydrous HCl was bubbled for a few minutes into a solution of XXIX (1g) and DDQ (0.8g) in dioxane (20ml). The mixture was kept for 30 minutes at room temperature, the precipitated hydroquinone filtered off and the filtrate diluted with ether. The ether solution was washed with 1% NaOH solution, water, and then dried (Na_2SO_4). The solvent was evaporated and the oily residue (0.9g) did not crystalize from petroleum ether or petroleum ether-ether mixture. UV revealed the presence of XXVIII but the resolution of the residue was unsuccessful and the method was abandoned.

c) Bromination

The ketone XXIX, (1.0g) was heated under nitrogen with stirring under reflux in CCl_4(25.0ml) containing NBS(0.67g) and $(phCO)_2O$ (0.010g). After 40min. the mixture was cooled and the succinimide filtered off and washed with CCl_4. The filtrate and the washings were combined and taken to dryness under reduced pressure. The residual oil (1.10g) on tlc (silica, C_6H_6:$CHCl_3$, 9:1) resolved into four components of which one was starting material. The mixture was passed down a column of neutral alumina in petroleum ether: ether, 9:1, and used in the next stage.

d) Dehydrobromination

The above bromo products were boiled under reflux, under nitrogen, in dry DMF (25.0ml) containing Li_2CO_3 (0.118g) and LiBr (0.139g). After 3 hours the mixture was cooled, filtered and poured into water which was ether extracted. The ethereal extract was dried (Na_2SO_4) and taken to dryness under reduced pressure to yield an oil (300mg). Column chromatography of this oil on neutral alumina gave a product which on tlc showed the presence of two components. The oil was separated into two components by preparative tlc (silica, C_6H_6 : $CHCl_3$, 9:1). The major component, XXXl, (200mg, 20% yield based on XXlX) was obtained as pale yellow needles m.p. 98-99°C, ex-pet. ether (Lit.[84] 97-98°C).

IR (KBr) 1650, 1610, 1580, 1560, 880, 755 cm^{-1}.

PMR $\tau(CDCl_3)$, 8.61(s, 3H angular Me), 4.19(s, 1H Cl-H), 3.30, 3.70 (AB quartet C9-H, C10-H respectively, J=10Hz), 2.90 - 2.65 (m, 4H, Aryl).

MS $C_{15}H_{14}O$ requires M^+ 210.1052

found M^+ 210.1045

Analysis for $C_{15}H_{14}O$ requires C, 85.75; H, 6.72

found C, 85.50; H, 6.66

(ii) 2,3,4,12-Tetrahydro-10-bromo-12-methyl-2-oxo-phenanthrene, XXXll.

The minor (4.0mg) component from above, m.p. 123-125°C.

MS $C_{15}H_{13}BrO$ requires M^+ 288.014

found M^+ 288.015

IR (KBr) 1655, 1640, 1610, 815, 765 cm^{-1}.

(iii) 2,3,4,12-Tetrahydro-7-methyl-12-ethyl-2-oxo-phenanthrene XXXlll.

A solution of 2,3,4,9,10,12-hexahydro-7-methyl-12-ethyl-2-oxo-phenanthrene[100], XXX, in dry benzene (10.0ml), ethyl orthoformate (1 ml), and pyridine hydrochloride (10.0mg) was heated under reflux, under nitrogen, for 10 hours. The solution was poured into ice-cooled 2N aq. Na_2CO_3 and the product ether

extracted. The extract was water washed, dried (Na_2SO_4) and evaporated under reduced pressure to yield the enol ether (100mg). The crude ether in 95% aq. acetone (13.0ml) was treated with DDQ (96mg in 7ml of 95% aq. acetone) and stirred for 10 minutes. The mixture was poured onto ice-water and the product ether extracted. The extract was washed with 2N Na_2CO_3,H_2O, dried (Na_2SO_4) and evaporated under reduced pressure to dryness. The amber oil left behind was chromatographed (preparative tlc, silica C_6H_6:$CHCl_3$, 9:1) to afford XXXlll, (20mg) m.p. 69-70°, ex-pet. ether.

IR (KBr) 1650, 1603, 1570, 1565, 905, 815 cm^{-1}

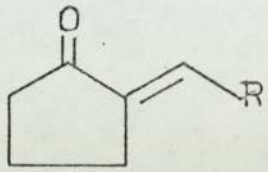
PMR τ($CDCl_3$), 9.30 (t, 3H, Me of angular Et, J=8.0Hz); 7.68(s, 3H, aryl C7-Me); 4.14(s, 1H, C1-H); 3.32, 3.65 (AB quartet, C9-H, C10-H, respectively, J=10.0Hz); 2.86, 2.92(quartet, C5-H, C6-H, J=10Hz); 2.98,(C8-H).

MS $C_{17}H_{18}O$ requires M^+ 238.135

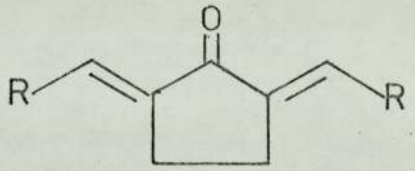
found M^+ 238.135

6.6 Synthesis of Benzylidene and Cinnamylidene Derivatives

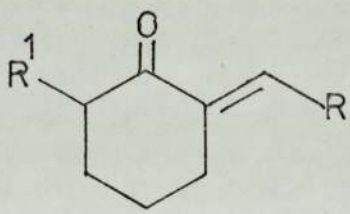
The disubstituted cyclic derivatives were synthesized from the corresponding aldehyde by the method of Johnson[101] while the mono-substituted derivatives were made via the enamine as described by Birkofer and others[102]. All the compounds were shown to be pure by determination of melting points and elemental analysis.



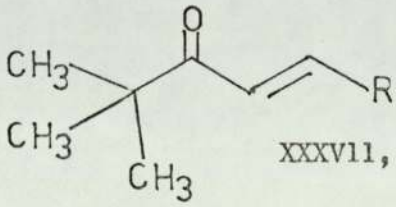
		M.Pt.	(M.Pt.Lit.)
XXXVIII,	R=Ph	68.5°	(68°)
XXXVI ,	R=CH.CH.Ph	102-103°	



XXXIX,	R=Ph	190°	(192°)[102]
XL,	R=CH.CH.Ph	224°	(225-226°)



XLI	R=Ph; R^1=CH_3	61-62°	(56-60°)[101]
XXXV	R=CH.CH.Ph; R^1=CH_3	105-107°	



XXXVII, R=CH.CH.Ph

Liquid, distilled at 158-160°C, 0.2mm Hg[101]

20° = 1.5795

6.7 Preparation of Keto-Dihydro-Naphthalene Derivatives :

(a) $\Delta^{1,1}$-2′-keto-3,4-dihydro-1,2-Cyclopentenonaphthalene, (XL11).

(i) 2-Bromo-1-tetralone was prepared by the method of Wilds and Johnson[105] from 1-tetralone and was used without purification for the next stage.

(ii) The bromo-tetralone was converted to the sodio-acetoacetate ester which was decarboxylated to yield the title compound.

(b) 1,1-Dimethyl-2-keto-1,2-dihydronaphthalene, (XL111), was prepared by bromination[104] and dehydrobromination of 1,1;dimethyl-2-tetralone[103] to afford the desired product on distilled at 105-110° (4mm Hg), n Δ^{20} = 1.5910

Lit· 146-150° (18mm Hg), n Δ^{23} = 1.5915

7. APPENDIX

Extinction Coefficients

Compounds		λ max (nm)	log ε
Hydro-oxo-phenanthrenes:	XXVlll	EtOH	
		357	4.18
		243	4.18
		Hexane	
		334	4.16
		237	4.21
		H_3PO_4	
		480	4.35
	XXXl	EtOH	
		350	4.18
		240	4.22
		Hexane	
		337	4.18
		232	4.26
		H_3PO_4	
		463	4.23
	XXXlll	EtOH	
		350	4.22
		238	4.23
		Hexane	
		342	4.22
		338	4.34
		H_3PO_4	
		480	4.19
	XXXlV	EtOH	
		365	3.85
		325	3.99
		265	3.92
		248	3.48
		Hexane	
		335	4.04
		325	4.03
		265	3.98
		250	4.03
		242	4.13
		H_3PO_4	
		475	4.10
		405	4.02

Compounds			λ max (nm)	log ε
Cinnamylidene Compounds:				
	XXXV	EtOH		
			335	4.48
			230	4.05
		Hexane		
			322	4.50
			225	3.90
		H_3PO_4		
			390	4.42
	XXXVl	EtOH		
			335	4.47
			232	3.97
		Hexane		
			322	4.51
			230	3.87
		H_3PO_4		
			430	4.23
	XXXVll	EtOH		
			305	4.21
			218	4.02
		Hexane		
			305	4.18
			360	3.33
			218	4.04
		H_3PO_4		
			430	3.49
			320	4.07
	XL	EtOH		
			407	4.70
			260	4.35
		Hexane		
			380	4.69
			260	4.21
		H_3PO_4		
			595	4.84
Benzylidene Compounds				
	XLl	EtOH		
			280	4.18

Compounds		λ max(nm)	log ε
		Hexane	
		272	4.15
		H_3PO_4	
		365	3.54
		295	4.08
	XXXV111	EtOH	
		295	4.41
		222	4.05
		Hexane	
		290	4.38
		220	4.08
		H_3PO_4	
		328	4.29
	XXX1X	EtOH	
		320	4.33
		237	4.31
		Hexane	
		315	4.38
		237	4.30
		H_3PO_4	
		465	4.52
<u>keto-dihydro-naphthalenes</u>:			
	XL11	EtOH	
		286	4.41
		222	4.17
		Hexane	
		272	4.39
		215	4.21
		H_3PO_4	
		338	4.21
	XL111	EtOH	
		307	4.05
		230	4.17
		Hexane	
		330	3.02
		300	3.70
		229	3.94
		H_3PO_4 350	3.94

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