

A thesis

entitled

"THE OCCURRENCE OF PENTACYCLIC TRITERPENES IN THE GREEN RIVER SHALE"

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A C K N O W L E D G E M E N T S

I wish to express my gratitude to Dr. G. Eglinton for his close interest and guidance throughout the work of this thesis.

My appreciation is also due to W. Henderson* of the Organic Geochemistry Unit for repeating his original work in order to collaborate with me in the isolation and separation of the total lipid fraction of the Green River shale.

I should also like to thank Miss F. Greene for technical assistance, Mrs. F. Lawrie and Miss A. Roberts for the infra-red spectra, Miss H. Humphrys and Miss J. Malcolm for the mass spectra and Dr. A. McCormick for help in interpreting them.

My sincere gratitude is also due to Miss I. Graham, Miss E. Clark and Miss M. Shaw for their typing.

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*W. Henderson, Ph.D. Thesis, Glasgow, 1968.

A B S T R A C T

This thesis investigates both the occurrence of pentacyclic triterpene hydrocarbons in the Green River shale and pertinent analytical topics.

The introduction gives a concise summary of the scope, implications and limitations of organic geochemistry. The biogenesis, biological occurrence and known organic geochemistry of triterpene hydrocarbons are also reviewed.

The discussion describes the collection and characterisation of standard triterpene hydrocarbons necessary as an analytical aid, including an investigation into the use of osmylation as a method for detecting unsaturation in a triterpene. The development of a micro-sublimation block as a further assistance in the accurate characterisation of triterpenes is also described. The distribution of naturally occurring pentacyclic triterpenoids is presented both in tabular and text form in an attempt to clarify possible chemotaxonomic relationships of triterpene hydrocarbons isolated from geological environments. A description of the isolation, separation and fractionation procedures applied to the total lipid fraction of the Green River shale is followed by a discussion of the mass spectral cracking patterns of triterpenes.

Several steranes/triterpanes and one tetraterpane were isolated from Fraction 1 (see flow diagram). Confirmation (based on mass spectral evidence) of the occurrence of cholestane, sitostane, ergostane, gammacerane and carotane can be made. In addition, another triterpane, tentatively identified as moretane, has been isolated.

A series of hydrocarbons, postulated alkenyl benzenes, was isolated from Fraction 2. Such compounds have previously been found in petroleum, but have not been isolated from sediments. In addition, an unsaturated triterpene hydrocarbon, as yet unidentified, was also isolated from this fraction. The presence of this compound supports the hypothesis that saturated triterpenes present in sediments may be formed by reduction of the corresponding alkene, itself formed by dehydration of the biologically common oxygenated triterpenoids.

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INTRODUCTION

Organic Geochemistry

Two quite different chemical approaches are used to investigate the origin of terrestrial life and to estimate the time of its first appearance.

Certain experiments, simulating prebiotic conditions by treating gaseous mixtures of methane, water and ammonia (the presumed primitive atmosphere of the Earth¹) with ionising radiation² or an electric discharge^{3,4,5,6}, show that a wide variety of small molecules of biological significance can be formed by abiogenic means. The range of organic compounds which have been synthesised includes amino acids^{2,3,4,5,6}, sugars², nucleic-acid bases^{7,8,9,10,11}, alkanes^{12,13} and carboxylic acids¹⁴, all of which are constituents of contemporary organisms. Feasible laboratory techniques and conditions, whereby the simple monomers formed abiotically can polymerise to form the biologically essential polypeptides, polysaccharides and polynucleotides have still to be devised. Only then can the formation of a molecularly complex yet biologically simple self-replicating system be investigated.

However, a different primitive atmosphere has been postulated by Matthews and Moser¹⁵. The authors suggest that only methane and ammonia were present in the atmosphere, to the complete exclusion of water. It was found that in the laboratory the action of a spark-discharge mechanism on this binary mixture produced a tar which was shown by analysis to contain several peptides. Only on strong hydrolysis were amino acids liberated.

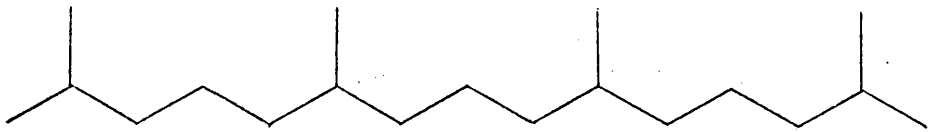
The geological approach utilizes the record in ancient sediments provided by the morphological remains of organisms whereas the geochemical approach is an examination of the chemical nature of the indigenous organic matter^{16,17,18,19}. The latter aspect of this approach is given the name organic geochemistry, a broad definition of which would be the study of the organic matter in geological situations. The object of this more specific approach is to deduce information pertaining to the chemical and biological evolution by an organic geochemical analysis of sediments which have undergone minimal metamorphosis. More succinctly, an answer is required to the question, "what is the oldest sediment in which the presence of organic compounds of biogenic origin can be detected?"

The organic compounds, whose presence in sediments is regarded as meaningful, are known as biological markers. They must satisfy two main provisos; they must be stable over geological time and they must be incapable of having been formed in significant amounts by simple abiogenic processes, such as the primitive atmosphere experiments described above. Alkanes^{20,26}, long chain fatty acids^{21,22,23,24} and porphyrins²⁵ have been used as biological markers since they possess biogenically conferred characteristics which make their isolation from a sediment meaningful to the organic geochemist.

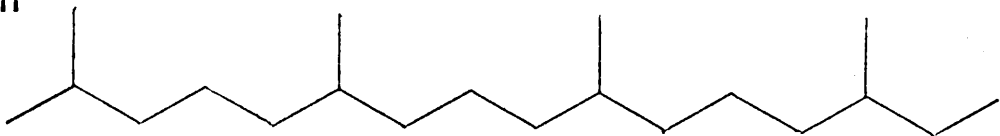
A biological marker should therefore be of biogenic derivation and should have a high structural specificity which, of course, arises from their enzyme-catalysed biological synthesis. If such a molecule possessed asymmetric centres, optical rotation would result. However, the optical activity of a molecule may be destroyed by diagenetically induced isomerisation under the effects of elevated temperature and pressure. Conversely, optical activity may be created by the action of a micro-organism acting stereospecifically on a racemic mixture of an organic compound during deposition of a sediment, resulting in a residue of one of the enantiomers. Also, biological markers should be of a size and complexity such that the probability of their being formed abiotically is negligible. The more complicated the structure of a molecule, the less likely is its formation by the random, non-directed processes associated with abiogenic syntheses.

Thus the acyclic isoprenoid hydrocarbons^{26,27}, the steroid and triterpenoid hydrocarbons^{28,29,30,31}, the isoprenoid fatty acids^{23,24} and the porphyrins²⁵ are suitable as biological markers. Hydrocarbons persist as biological markers, despite the abiogenic synthesis of certain types by Ponnamperna¹³. The synthetic hydrocarbons were seen by gas-liquid chromatography to have a continuous distribution unlike naturally occurring hydrocarbons which have been shown to have a periodic carbon number distribution. The hydrocarbons formed appeared to contain no normal or branched alkanes and were shown chemically to be highly unsaturated. In the Fischer Tropsch conversion of carbon monoxide and hydrogen into alkanes and alkanes etc. the distribution of the alkanes differs from the distribution of the alkanes in sediments²⁰. In the opinion of Marten et al³², a preponderance of normal alkanes suggests a biogenic origin.

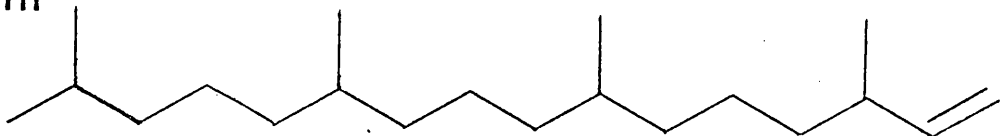
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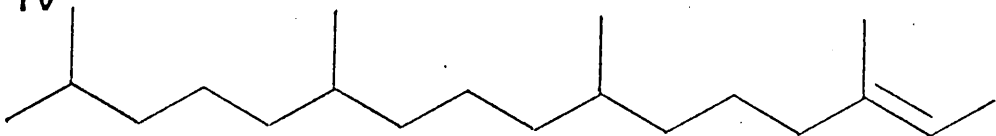
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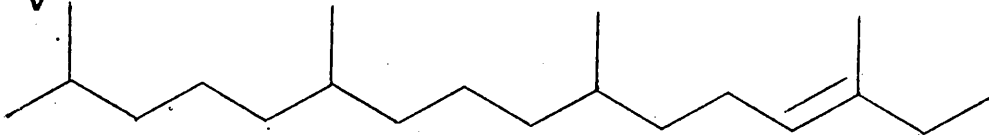
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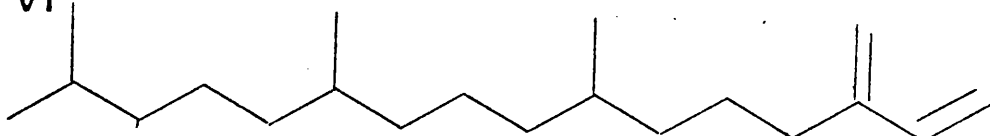
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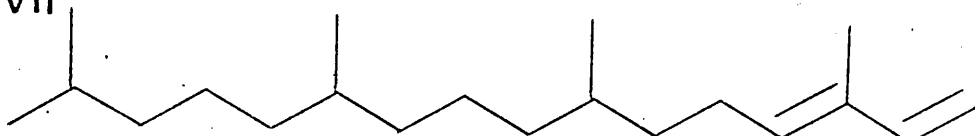
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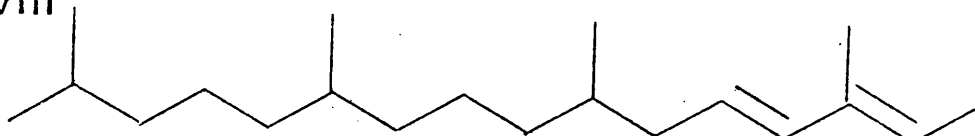
VI



VII



VIII



The use of the isoprenoid hydrocarbons pristane (I) (2,6,10,14 - tetramethylpentadecane) and phytane (II) (2,6,10,14 - tetramethylhexadecane) as biological markers would, superficially, appear rather incongruous since there is only one reference to the author's knowledge which records the isolation of pristane from a Recent sediment. There is no recorded occurrence of phytane. This leads to a strong presumption that pristane, phytane and the lower molecular weight isoprenoid hydrocarbons isolated from geological samples are formed during diagenesis by the degradation of the phytyl side chain of chlorophyll³⁴. Johns et al³³ have suggested that the C₁₉ (I), C₁₈ and C₁₆ isoprenoid hydrocarbons result from cleavage of the double bonds in the phytanes, (III, IV and V), which are formed in turn during sediment compaction. Other possible sources of geological pristane have been suggested, namely by decarboxylation of the naturally occurring, though rare, phytanic acid^{35,36,37,38,39}, or by the actual presence of discrete pristane at the time of deposition of the sediment, as a result of its isolation from a number of marine sources⁴⁰. The C₂₀ isoprenoid hydrocarbon, phytane, is not a common constituent of living organisms yet the doubly unsaturated phytadienes (VI, VII and VIII) have been isolated by Blumer and Thomas from zooplankton⁴¹. These could, quite foreseeably, give rise to phytane by the as yet unsubstantiated process of geological reduction.

As further evidence for the appearance of pristane and phytane only during diagenesis, it should be noted that neither pristane nor phytane have been isolated from the Brown Coal of Germany⁴² ($\sim 30 \times 10^6$ years), a Recent sediment which is relatively unaltered and which consists of plant debris. However, in the Green River shale, which has undergone somewhat more extensive metamorphosis, pristane and phytane predominate the branched/cyclic alkane fraction²⁶.

Long chain fatty acids, amongst other types of compounds, have been shown to form in nature by the polymerisation of acetate units utilizing an acetyl co-enzyme A starter molecule with chain extension by malonyl co-enzyme A⁴³. This results in the fatty acid molecule having an even number of carbon atoms. It is postulated that plant n-alkanes are formed by decarboxylation of the fatty acids, resulting in an odd number of carbon atoms in the hydrocarbon⁸⁸. This is held to account for the fact that normal alkanes isolated from a living plant normally show an odd/even carbon number preference^{44,45}. The odd/even preference is retained in recent sediments such as the Green River shale²⁰ but older sediments do not in general, show this preference⁴⁶. Johns et al³³, amongst others, take this as an indication that the odd/even carbon number preference of normal alkanes is unsatisfactory as a mark of a biological origin for the saturated hydrocarbons in older sediments.

It should be noted, however, that the characteristics necessary for a molecule to be a biological marker are not possessed by all the biologically important molecules. For example, amino acids cannot be used, since they have been shown to form in the primitive atmosphere experiments mentioned above.

Organic molecules are known to undergo structural changes after deposition by the processes of maturation and diagenesis. One method of investigating the molecular changes is to compare the structures of certain compounds or the distribution of classes of compounds in a sediment or fossil with the structure and distribution of contemporary biological compounds. Such a correlation has been made by Mair⁴⁷ who postulates that the naturally occurring terpenoids could have been geochemically altered to produce most of the hydrocarbons containing benzene and cyclohexane rings found in crude petroleum (and sediments).

Another quite different approach to the same problem involves the simulation of diagenesis under laboratory conditions (geogenesis). Douglas et al⁴⁸ have carried out such a process on a sample of Green River shale (Eocene, 60×10^6 years). By investigating the difference in alkane and alkene distribution after pyrolysis at 500°C , the authors suggest the alkenes and alkanes are in all probability geogenetically related, with the important proviso that their results may be transferred to reactions at much lower temperatures over long periods of geological time.

Eglinton et al,²⁶ suggested that the presence of pristane and phytane, the C_{19} and C_{20} acyclic isoprenoid hydrocarbons, in the Green River shale of Colorado and the Nonesuch shale (Precambrian, 1×10^9 years) of Michigan might be considered as evidence of a biological origin for these sediments. A survey, more comprehensive in both time scale and in type of organic deposit, has been carried out by Johns et al,³³. The authors isolated, along with other compounds, isoprenoid alkanes from the Soudan shale of Minnesota (2.7×10^9 years), the Antrim shale from Michigan (265×10^6 years); the San Joaquin oil (30×10^6 years), the Abbot Rock oil (3×10^6 years) and the Nonesuch Seep oil (1×10^9 years).

The occurrence and isolation, but not the identification of three biogenic steranes and one biogenic pentacyclic triterpene in the Green River shale and in the Soudan shale is reported by Burlingame et al²⁸. The difficulty of unequivocal identification is a general problem in the analysis of steroid

and triterpenoid compounds isolated from sediments, since the quantity available of any one component is of the order of nanograms (10^{-9} gram). This means that without repeated collections and concentration of a particular compound, the only analytical technique readily available is mass spectrometry. However, as is illustrated by Karliner and Djerassi^{49,50}, the mass spectra of triterpene hydrocarbons within the same structural group are very similar. Definite identification by mass spectrometry is therefore difficult and generally involves a direct comparison of the unknown mass spectrum to that of a known, pure compound³¹.

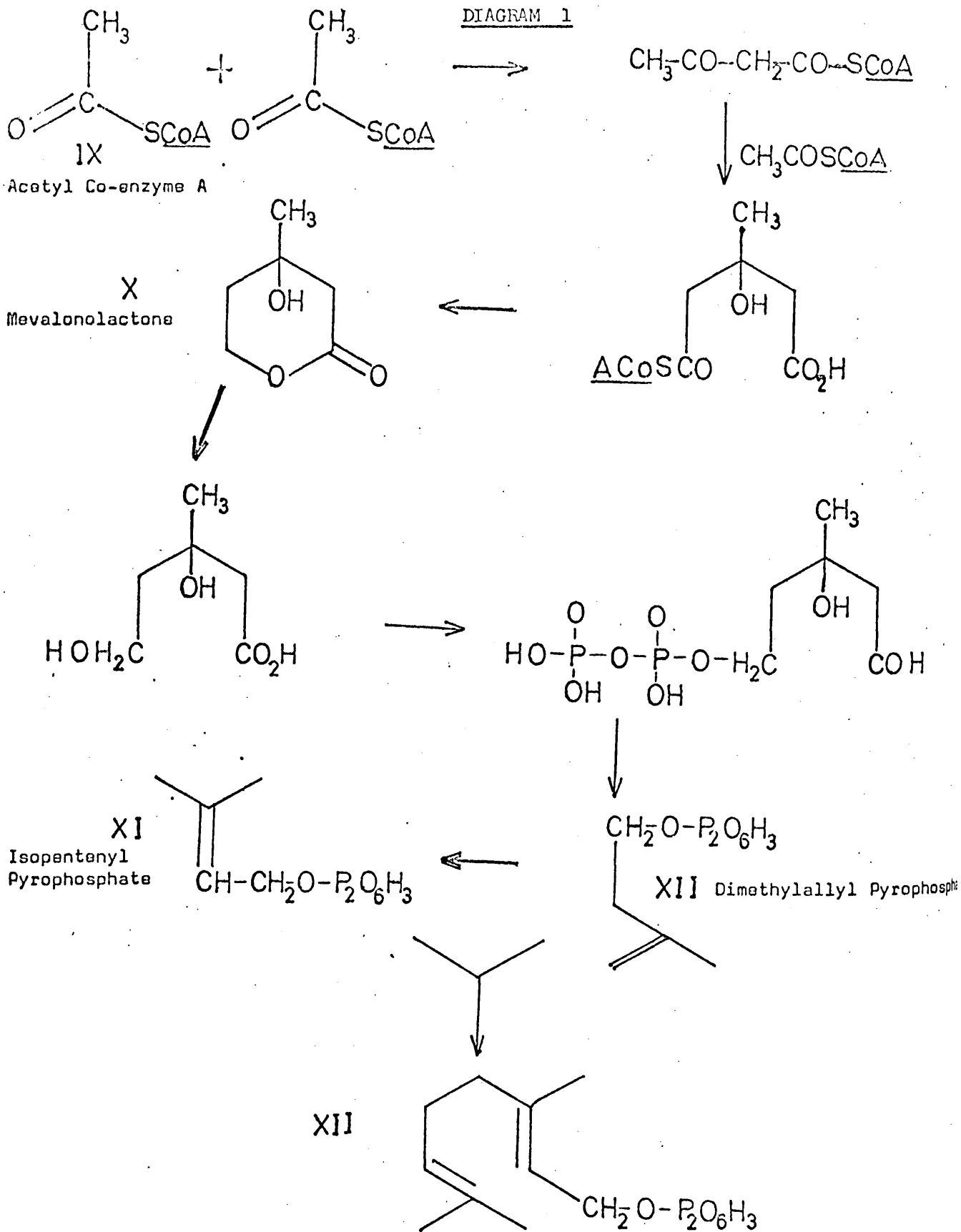
Improved techniques for the detection and identification of organic molecules in sediments enable the analysis of relatively minor components. The explanation of the presence of such entities is generally uncertain.

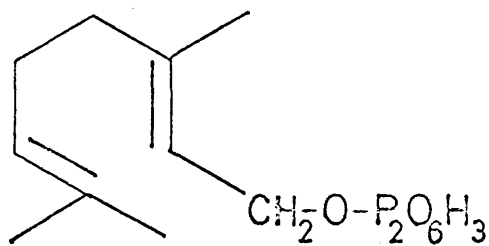
In the Fig Tree shale (3.2×10^9 years) aliphatic hydrocarbons are present in very low concentrations (0.003-0.15 ppm)⁵¹ c.f. (18-400 ppm)³³. Insufficient data exists to ascertain whether or not the hydrocarbons date from deposition but preliminary treatment of the sample precludes any possibility of the hydrocarbon being surface contaminant. However, entities have been found in the Fig Tree shale having the morphology of fossil bacteria and possibly algae; consequently the hydrocarbons may result from bacteria or algae.

Pristane is present in the sea in relatively large amounts³³, suggesting that it could be present in sediments during deposition and need not only appear during diagenesis. Thus pristane may occur in sediments from two biogenic sources, either by degradation of chlorophyll or by its inclusion as a specific entity. In addition, although pristane has never been known to occur abiotically, certain branched hydrocarbons,^{86,51} including 2-methylbutane⁸⁷ and other isoprenoid hydrocarbons, can be synthesised by Fischer-Tropsch processes⁵².

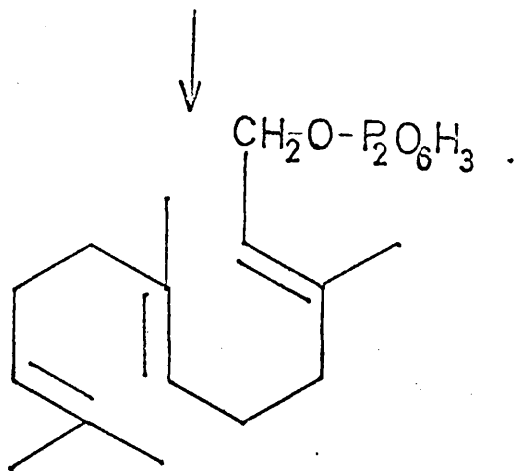
A discussion on the role of contamination and metamorphism and on whether hydrocarbons are indigenous in Pre-Cambrian sediments is given by Hoering⁵³. Similar questions were raised by Oro^{89,90} when, in an analysis of meteorites for aliphatic hydrocarbons, he found that carbonaceous chondrites gave the same aliphatic hydrocarbon chromatographic patterns as naturally occurring mixtures of terrestrial isoprenoid and other aliphatic hydrocarbons. Ornan, another meteorite, contained no isoprenoid hydrocarbons whereas Orgueil and the non-carbonaceous chondrites generally

DIAGRAM 1

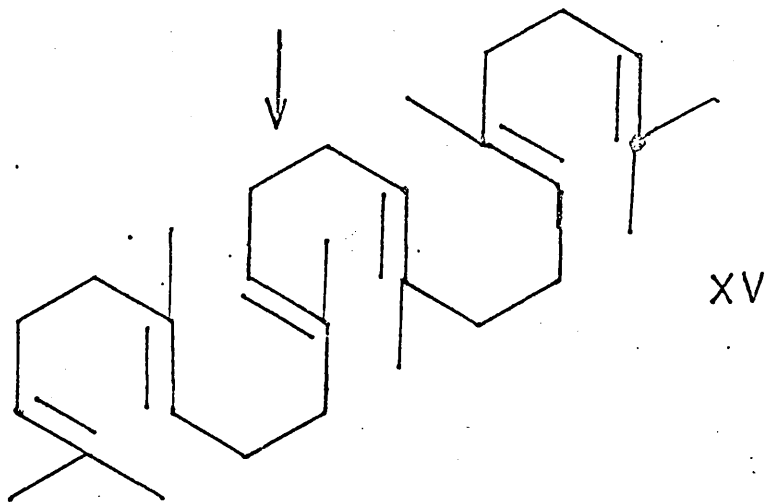




XIII
Geranyl Pyrophosphate



XIV
Farnesyl Pyrophosphate



XV
Squalene

contained but little. The conclusion reached was that more work was required to decide whether or not the hydrocarbons were in fact a result of terrestrial biological contamination.

Obviously doubts such as these must be resolved before any attempt is made at a total organic analysis of a lunar sample with the purpose, along with others, of deciding whether life as it is known on earth, namely, based on the carbon atom, has ever existed on the moon.

Triterpenes - Genesis

As stated above, a biological marker is only of value when there is the maximum possible probability of it having had a biogenic origin. Pentacyclic triterpenes have a high structural specificity and their size and complexity is such that the possibility of their abiotic formation is negligible. The biogenesis of terpenes was originally explained in terms of the isoprene rule⁵⁴ which simply invoked the concept of regular polymerisation of 2-methyl-but-2-ene units to produce the required length of acyclic polyisoprenoid. However, Ruzicka and Jeger⁵⁵, found that the structure assigned to lanosterol, if it were correct, could not possibly be accounted for by the isoprene rule. This led to the formulation of the biogenetic isoprene rule by Woodward and Block⁵⁷, and by Ruzicka⁵⁶, which in effect is merely an extension of the isoprene rule. Cyclisation of an acyclic precursor by means of antiplanar 1 : 2 additions and associated 1 : 2 eliminations, is preceded by conformational folding of the polyisoprenoid and may or may not be followed by 1 : 2 rearrangements involving hydride and/or methyl shifts. The biogenetic isoprene rule is based on the enzyme catalysed condensation of acetate derived units⁵⁸

The biogenesis of triterpenes is explained by a preliminary condensation of three acetyl co-enzyme A molecules (IX), which form, after cyclisation, mevalonolactone (X). Basic hydrolysis of this ester produces two free hydroxyl groups which are successively esterified by inorganic pyrophosphate. These excellent leaving groups facilitate the formation of isopentenyl pyrophosphate (XI), some of which by acid catalysed isomerisation produces dimethylallyl pyrophosphate (XII).

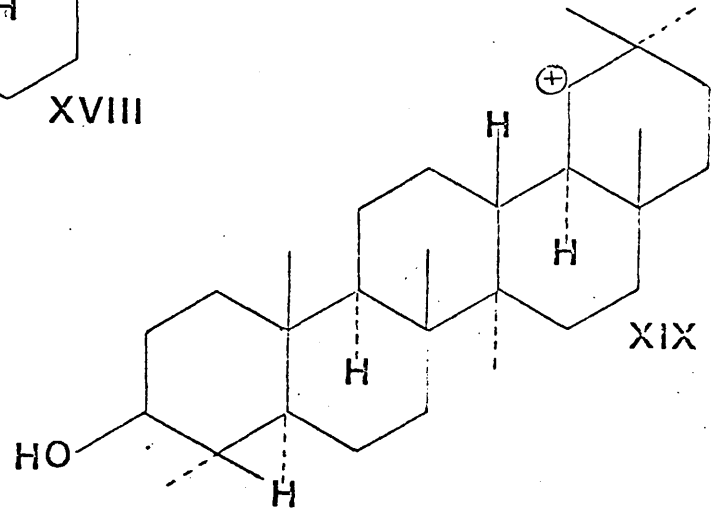
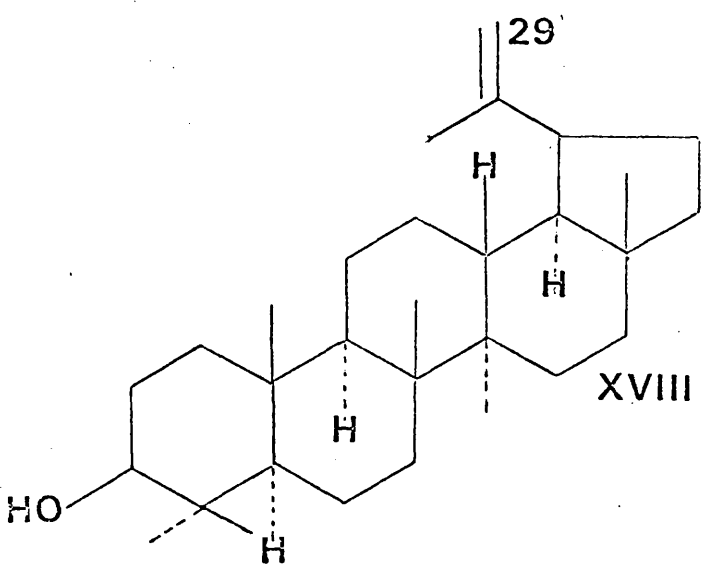
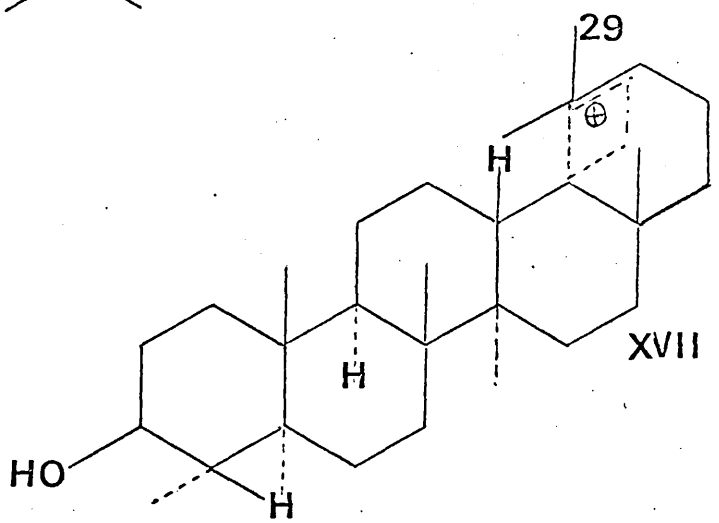
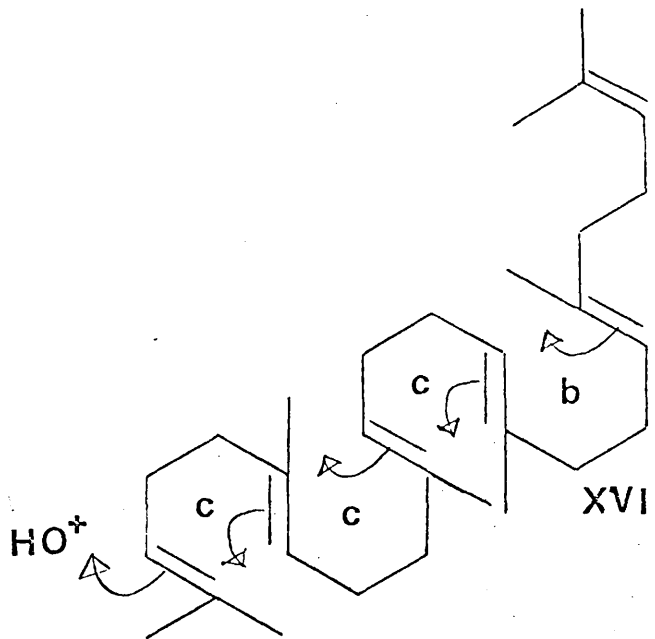
The structural isomers are postulated to then condense to produce geranyl pyrophosphate (XIII), which on further condensation results in farnesyl pyrophosphate (XIV). The last stage, involving a quite different tail to tail coupling of two farnesyl units has been extensively

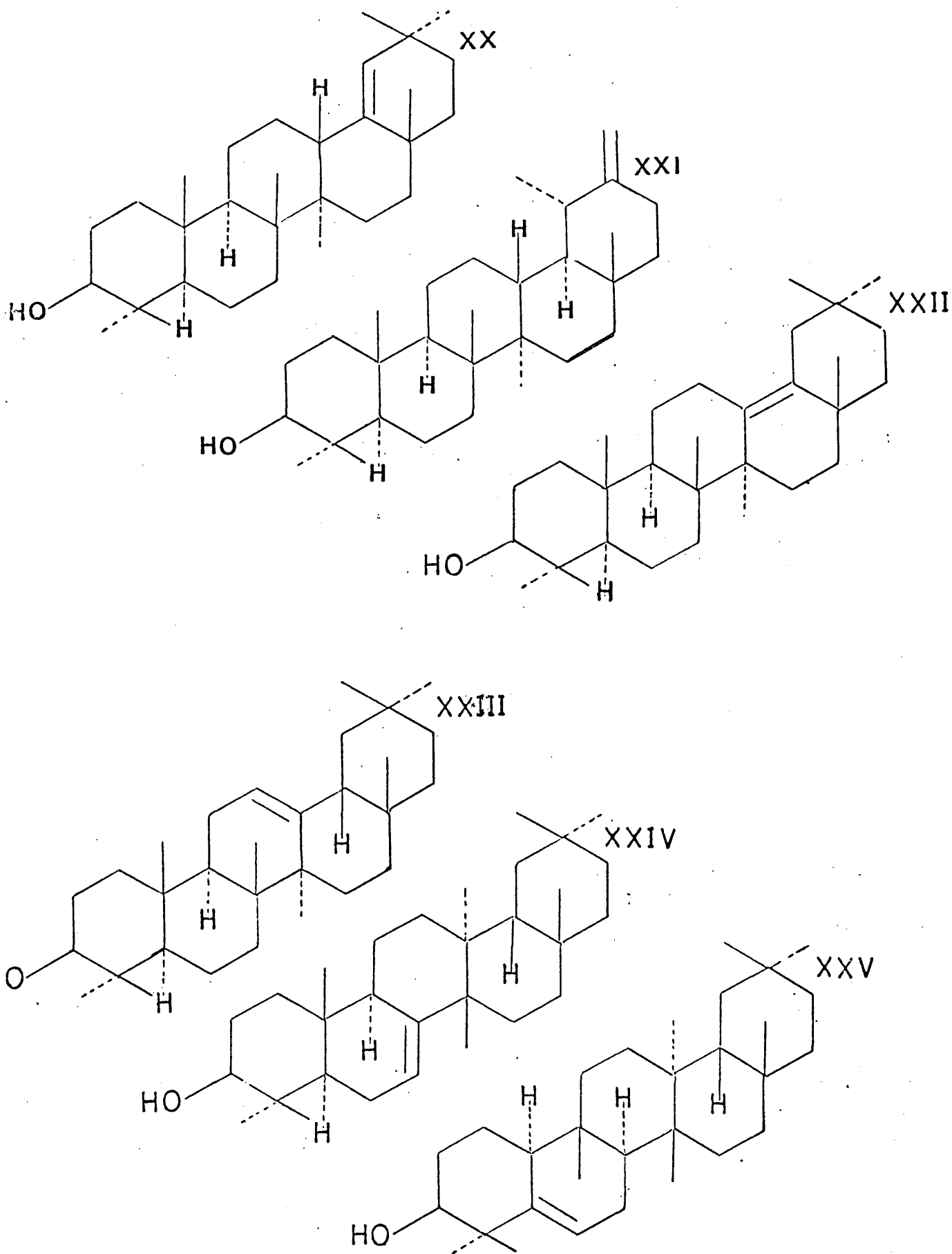
studied by Cornforth et al⁵⁹, and results in the formation of the acyclic triterpene squalene (XV) (Diagram 1). Additional references pertinent to the development of poly-isoprenoid biogenetic theory and dealing with the biosynthetic verification of the theory by incorporating radioactive tracer molecules in the metabolic pathways are given by Clayton⁴³ in a comprehensive two-part article.

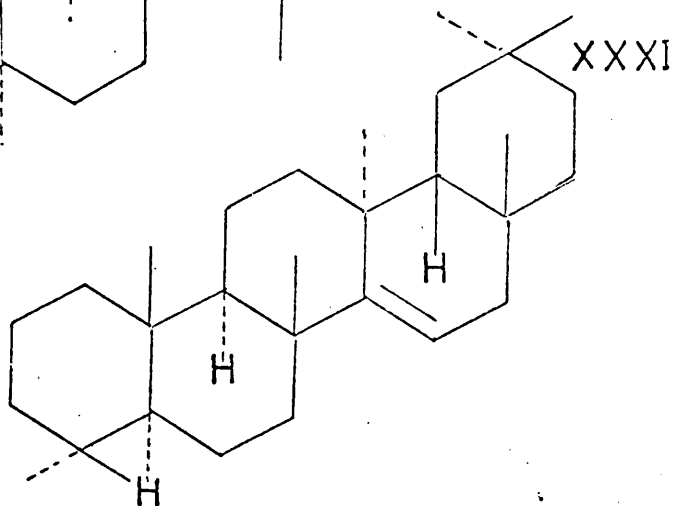
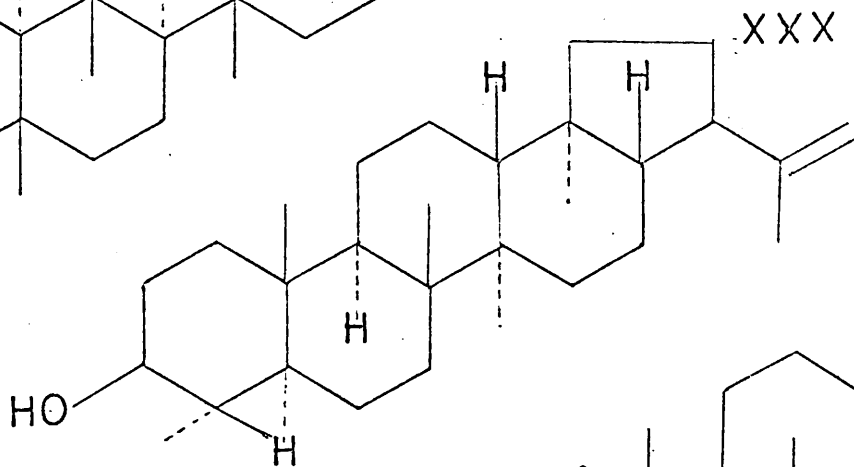
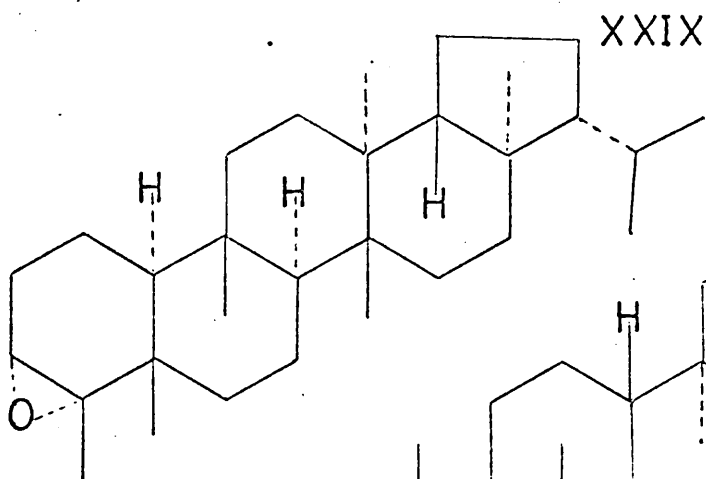
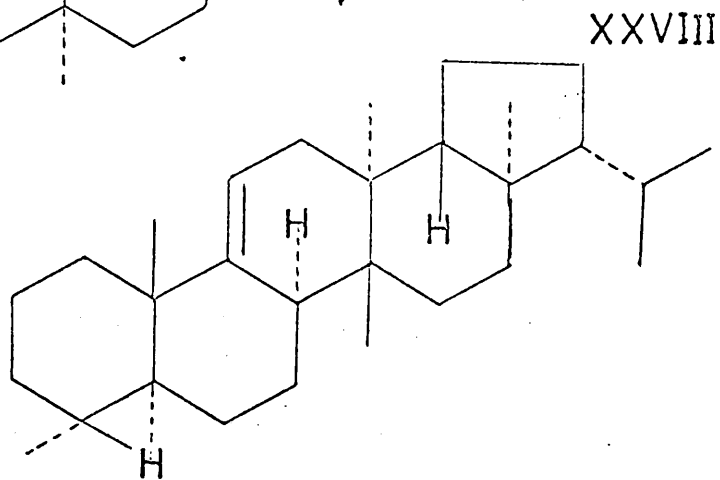
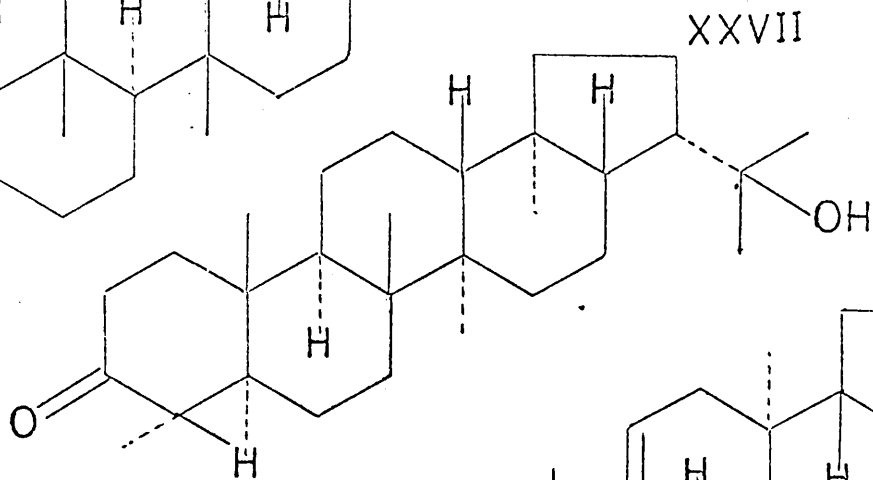
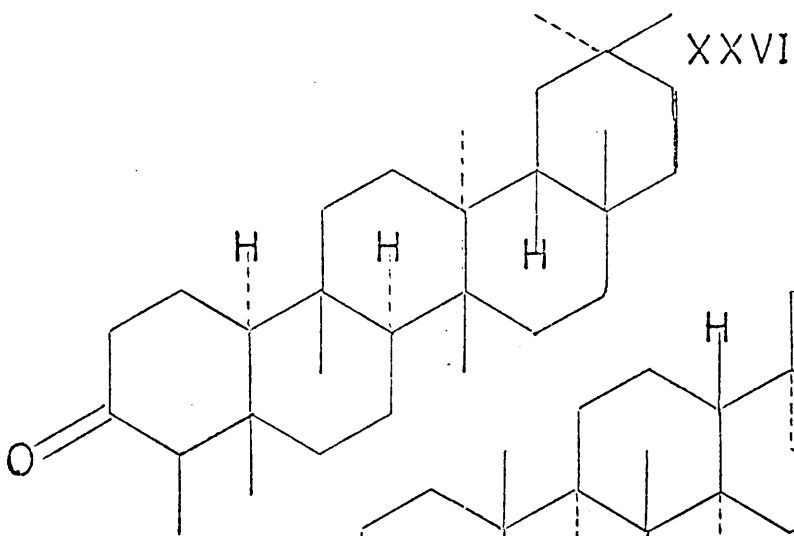
Comparatively little work has been carried out on the biosynthesis of pentacyclic triterpenes. However, labelled β -amyrin has been produced from labelled sodium mevalonate in the pea⁶⁰, whilst Arigoni has achieved similar success in the formation of soyasapogenol D by the sprouting soya bean⁶¹. The pattern of labelling found in those cases is in agreement with Eschenmoser's theory of the stereochemistry of the oxidative cyclisation of squalene to triterpenes⁶². According to the theory squalene is folded into a series of rings possessing incipient chair or boat conformations and attack by the equivalent of HO^+ then initiates a concerted process leading to the triterpene with associated elimination of a proton. When the conformation of the squalene acyclic precursor is in a chair, chair, chair, boat form (XVV) (Diagram 2), the intermediate (XVIII) can result which by elimination of a proton results in lupeol (XVIII). If, however, concerted ring E enlargement occurs to give the ion (XIX) followed by loss of a proton from C-18, germanicol (XX) results. Further concerted Wagner-Meerwein shifts, beginning with the ion (XIX) lead to taraxasterol (XXI), δ -amyrin (XXII), β -amyrin (XXIII), multifuorenol (XXIV), glutinol (XXV) and friedelin (XXVI).

A different pattern of conformational folding of squalene can occur on the enzyme surface where ring closure occurs to produce a chair, chair, chair, chair series which leads to the hopane ring system, exemplified by hydroxyhopanone (XXVII). This is the simplest, biogenetically speaking, of the pentacyclic triterpenes and leads, by a series of non-concerted rearrangements to the carbon skeletons of fernene (XXVIII) and adiantoxide (XXIX). Should ring E be in a boat conformation the cyclic product is the 21α (H)-hopane derivative moretenol (XXX). Using the same ring system fernene and adiantoxide can be explained by a concerted cyclisation mechanism.

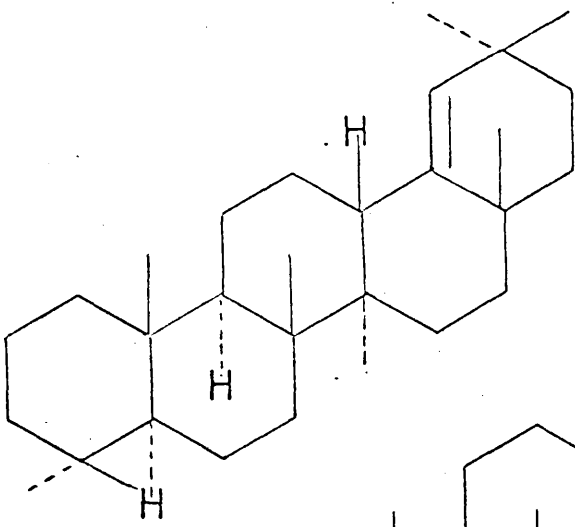
The occurrence in nature of triterpenoid hydrocarbons raises an interesting biogenetic point. Are they formed directly from squalene by a cyclisation



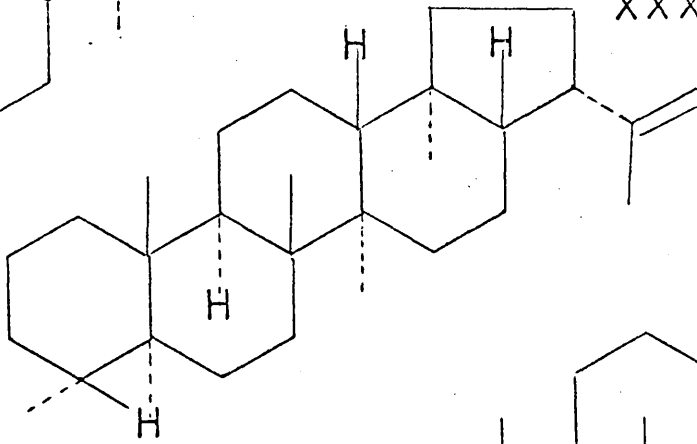




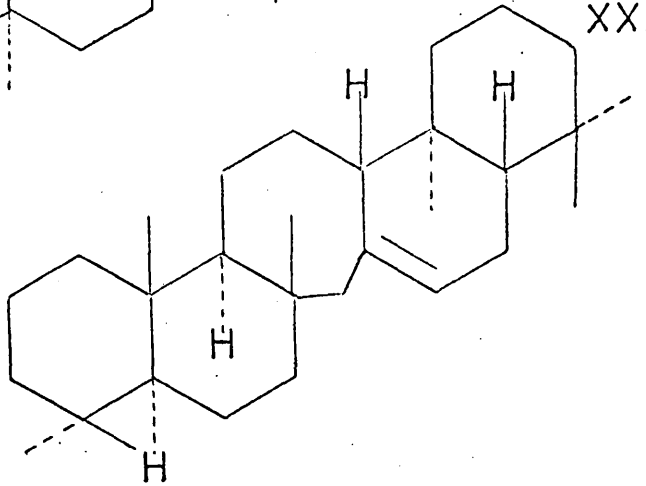
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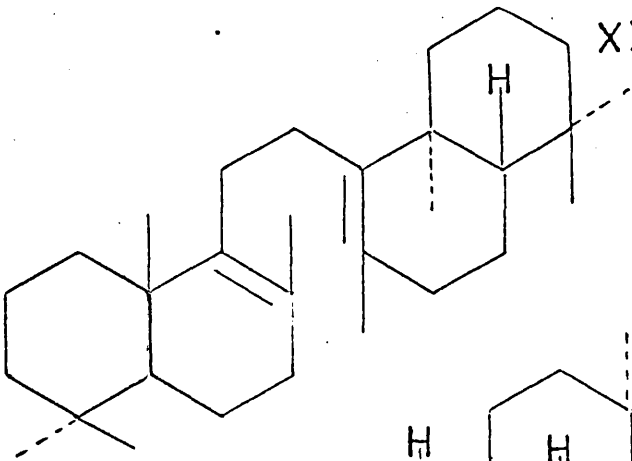
XXXIII



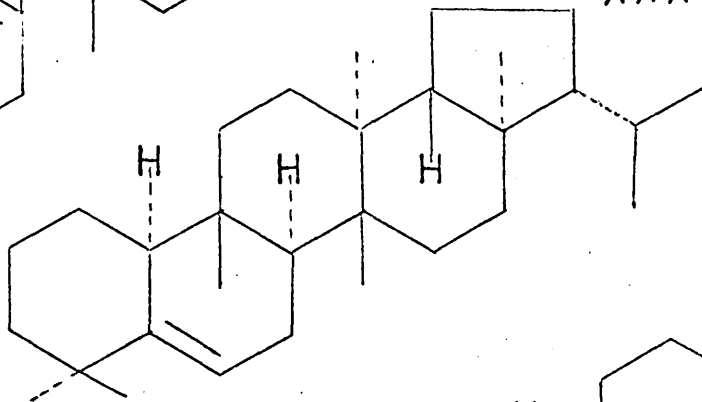
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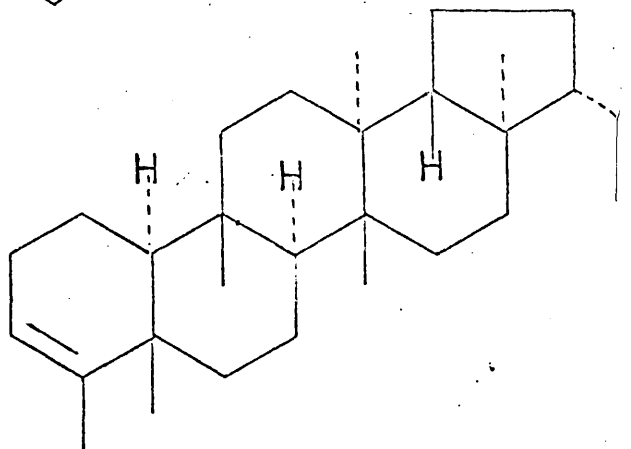
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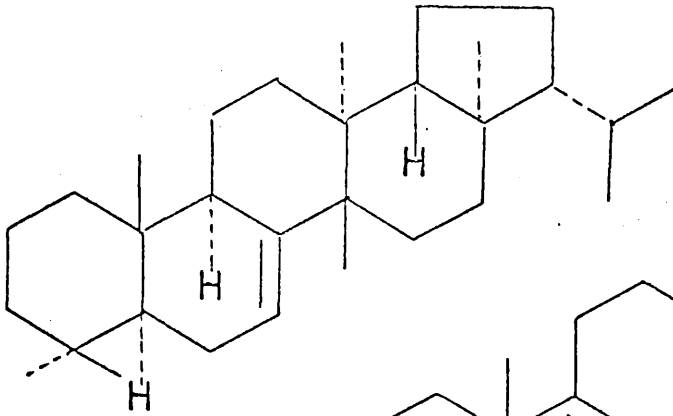
XXXVI



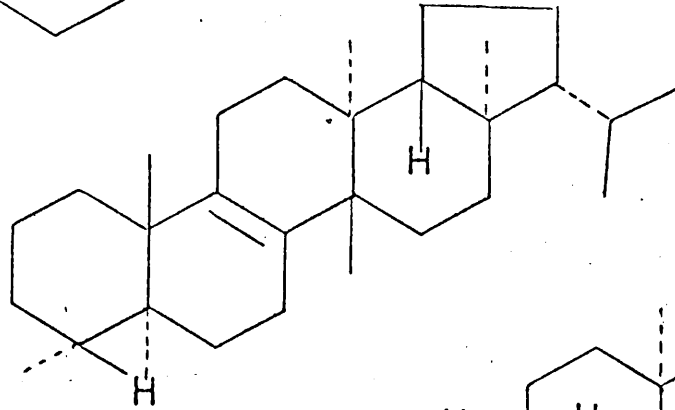
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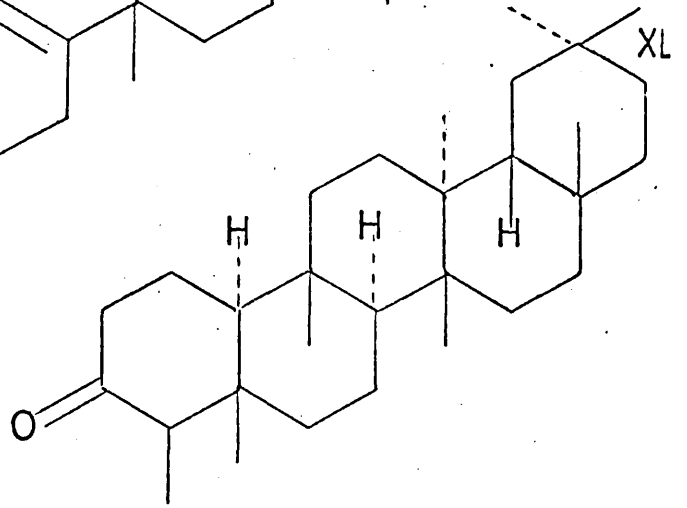
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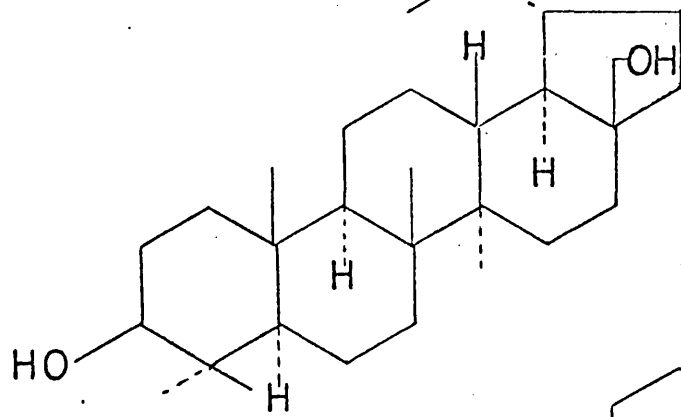
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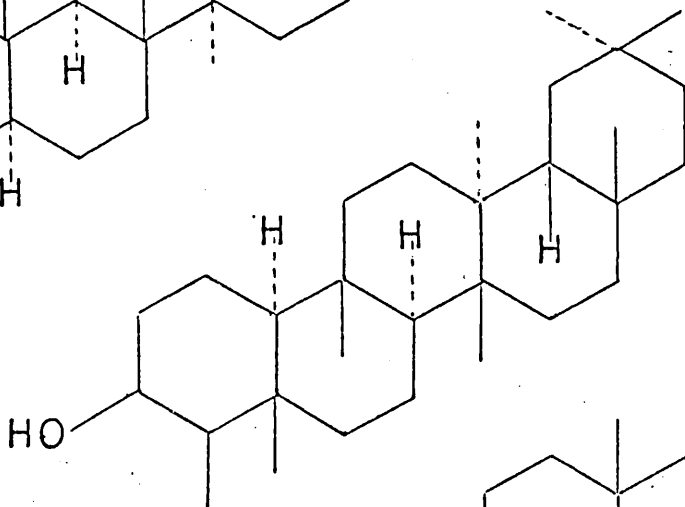
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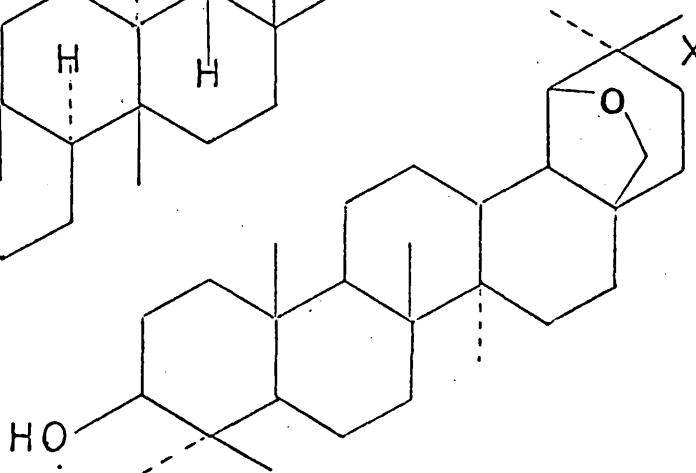
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XLI

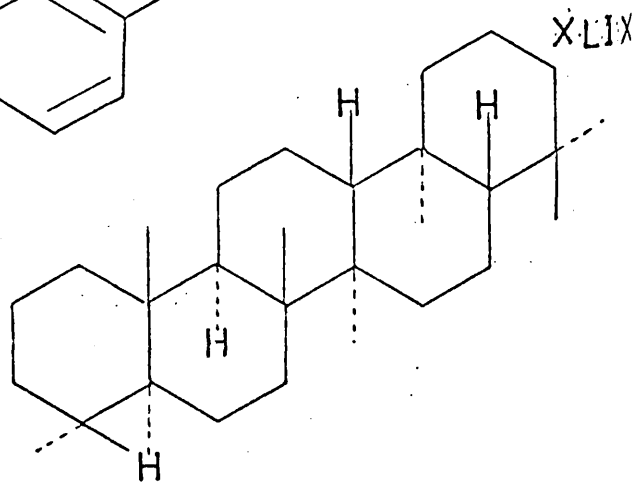
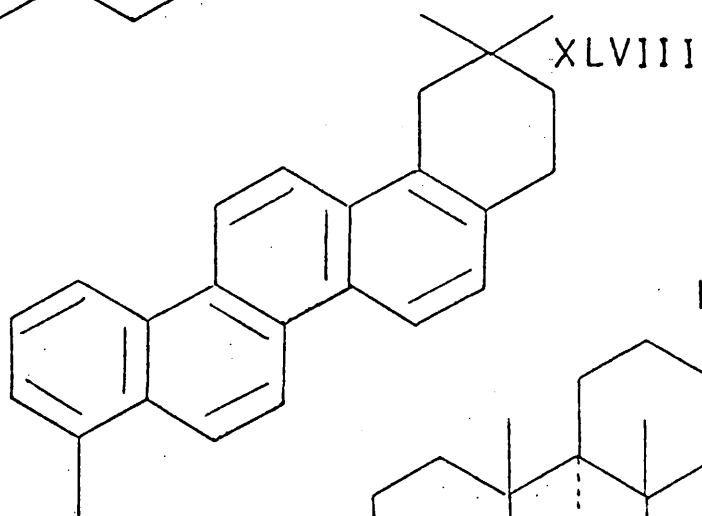
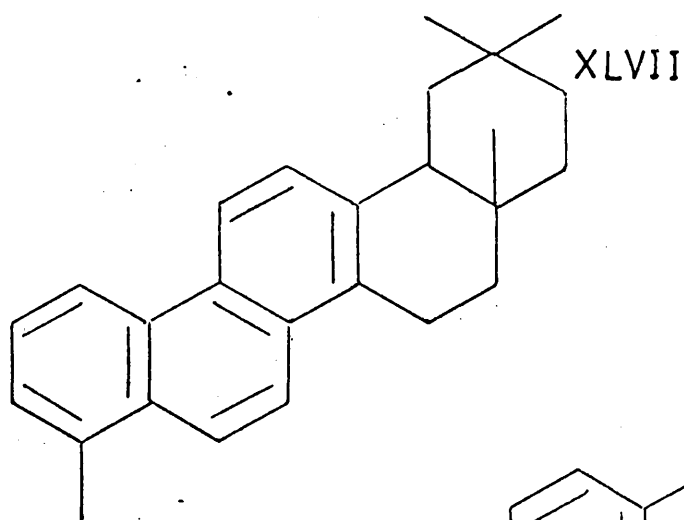
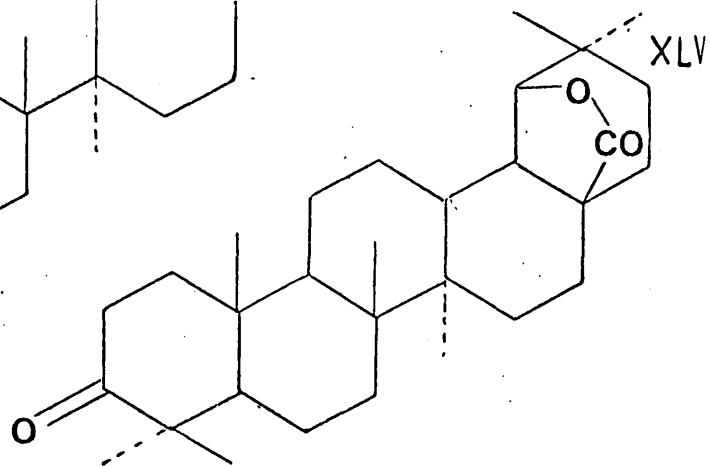
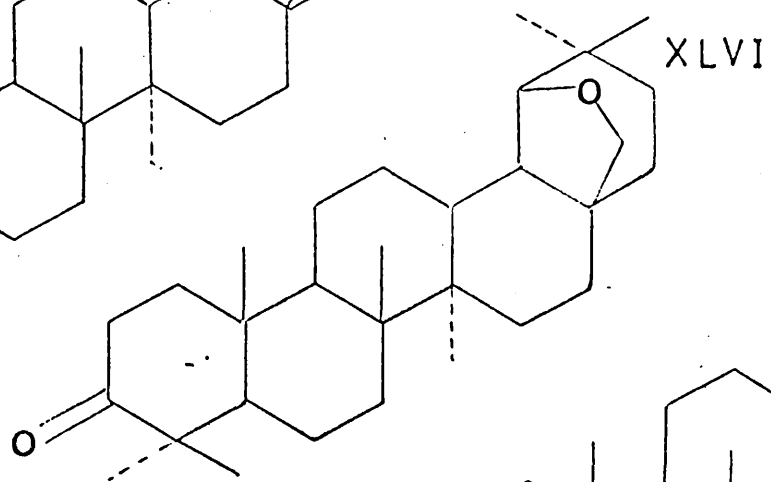
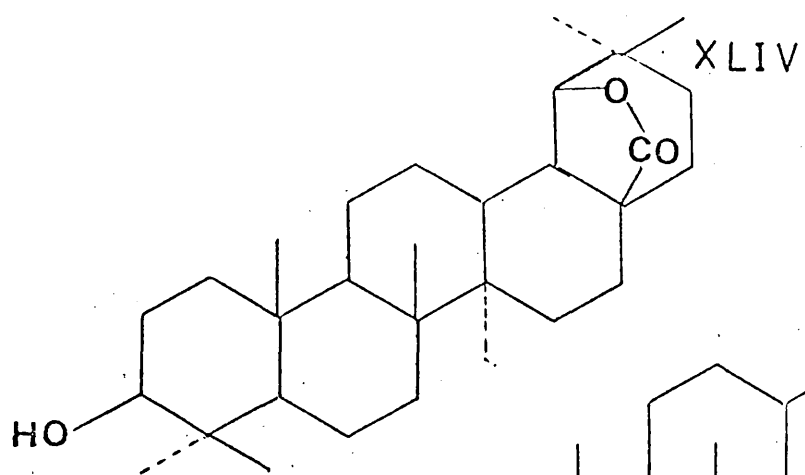


XLII



XLIII





process initiated by H^+ or do they result from the reduction of the corresponding 3β alcohols? Recent results by Barton and Moss⁶³ on the enzymic oxygenation of lanosta - 8, 24-diene to lanosterol have offered experimental evidence for the possibility of an H^+ initiated cyclisation. The pentacyclic division of triterpenes may be further subdivided into seventeen systems on the basis of structural differences in the carbon skeleton. This biogenetic classification, employed by McCrindle and Overton⁶⁵, takes into account both the position and orientation of the methyl substituents (Table 1).

Triterpenes - Occurrence

Oxygenation, a feature of nearly all naturally occurring pentacyclic triterpenes is thought to occur in the course of biosynthesis after the initial oxidative cyclisation and associated rearrangements. In a review article on pentacyclic triterpenes, Halsall and Aplin⁶⁴ note that the more sophisticated techniques of separation and analysis now being applied should allow the isolation and characterisation of compounds present in quantities much less than had hitherto been necessary. For the same reason, the authors think that an investigation of more highly oxygenated species and an overall more systematic examination of plants is now possible.

The structures of all the known naturally occurring pentacyclic triterpenes are given in a review article by McCrindle and Overton⁶⁵. An earlier review⁶⁴ names the plant families from which the pentacyclic triterpenes have been isolated. The occurrence of steroids and triterpenes in nature is briefly summarized below. (see Page 18).

Triterpene hydrocarbons occur only rarely in nature and have been isolated from only two plant families. Bruun⁶⁶ isolated a triterpene hydrocarbon from the lichen Cladonia deformis Hoffm. The triterpene, of the β -amyrin group, was shown to be taraxarene (XXXI) which differs from its stereoisomer germanicene (XXXII) by having a cis C, D ring junction instead of trans. The rhizomes of Polypodium vulgare⁶⁷ were found to contain 0.4% of a mixture of three distinct triterpene hydrocarbons, two of which, 9 (11) fernene (XXVIII) and 22 (29) Hopene (XXXIII) are known to have a rearranged hopane and a hopane skeleton respectively. The third hydrocarbon, serratene (XXXIV), is of particular note since it is the first triterpene hydrocarbon isolated which does not possess a hopane or rearranged hopane skeleton. The authors suggest that serratene originates from an onoceradiene (XXXV) precursor, itself formed by cyclisation of

squalene starting from both ends of the molecule. The methanol extract of the fern leaves, Dryopteris crassirhizoma NAKAI (Aspidiaceae), was found^{68,69} to contain two triterpene hydrocarbons, fernene (XXVIII) and diploptene (XXXIII). No less than five triterpene hydrocarbons, all with structures explicable by the same biogenetic scheme, have been isolated⁷⁰, inter alia, from the fern Adiantum monochlamys EATON (Adiantaceae). The compounds present were identified as adianene (XXXVI), filicene (XXXVII), 7-fernene (XXXVIII), iso-fernene (XXXIX) and diploptene (XXXIII).

Triterpenes - Organic Geochemistry

It is an accepted fact that biological compounds possess optical activity by virtue of their enzyme-catalysed stereospecific biosynthesis. Mair⁴⁷ and Zahn et al⁷¹ have associated the optical activity of geo-biochemical detrital material found in petroleum distillates and coal respectively with saturated pentacyclic hydrocarbons. Several authors^{72,73,74} have suggested that these polycyclic hydrocarbons may be steranes and triterpanes derived, under the conditions of diagenesis, from naturally occurring steroids and triterpenoids. This postulate appears quite feasible when viewed in relation to the common *in vitro* reduction of oxygenated triterpenoids as a method of structural elucidation⁷⁵. However, it appears that very young sediments may contain a large amount of oxygenated triterpenes, although very few such sediments have been examined for these compounds. Sorm and co-workers⁷⁶ have identified a wide range of triterpenoids in Brown Coal. Some of these compounds are known to occur naturally (friedelin (XL), betulin (XLI)) in cork and birch bark⁷⁷ respectively. Others, such as oxyallobetulin and allobetulone have only been found in lignite^{76,78}. These results suggest that certain oxygenated triterpenes are in fact structurally altered in a geological environment whilst others remain unchanged.

According to Ikan and McLean⁷⁸, lignite is generally regarded as an intermediate in the alteration of peat to coal and is believed to be a precursor of cannel coal. Their examination of peat produced only one identified triterpenoid structure, friedelan-3 β -ol (XLII), whereas lignite was shown to contain betulin (XLI), allobetulin (XLIII), oxyallobetulin (XLIV), allobetulone (XLVI) and oxyallobetulone (XLV). The subsequent stage in the geological sequence produced cannel coal which has not been found to contain any triterpenoids. This sequence would suggest an initial proliferation of triterpenoids, followed by

their alteration to other compounds possessing non-triterpenoid characteristics. The latter type of compound may well be the partially or fully aromatised pentacyclic carbon skeletons of the triterpenoids. Jarolim et al⁷⁶ have isolated from Brown Coal two such compounds of the allobetulin type and one hydrocarbon, 1, 2, 3, 4, 4a, 5, 6, 14b - octahydro - 2, 2, 4a, 9 - tetramethylpicene (XLVII).

Barton et al⁷⁹ have identified triterpenoids in petroleum and Carruthers and Cook⁸⁰ have isolated such compounds from crude oil. The partially aromatised pentacyclic compound 1, 2, 3, 4 -tetrahydro-2, 2, 9, - trimethylpicene (XLVIII) has been isolated from a crude oil by Carruthers and Watkins⁸¹.

The occurrence of saturated triterpene hydrocarbon (triterpanes) in petroleum distillates has been shown by Hills and Whitehead⁸². Identification of the hydrocarbons has not yet been completed but it appears that two compounds isolated are C₃₀ pentacyclic triterpenes, one is a nortriterpene and one is a trisnortriterpene. The same authors, in conjunction with Anders et al³¹ have identified an optically active triterpane, gammacerane (XLIX), in Colorado shale oil bitumen. The isolation of this compound was initially carried out by Cummings and Robinson³⁰. In a preliminary report, Burlingame et al²⁸ describe the isolation of three steranes and one triterpane from the Green River shale, although these compounds were not obtained in a pure state.

In a hydrocarbon analysis of certain samples from the Scottish Carboniferous Formation (250-300 x 10⁶ years), Maxwell⁸³ isolated two pentacyclic triterpanes, one a nortriterpene of molecular formula C₂₉ H₅₀, the other a triterpene of formula C₃₀ H₅₂.

The discovery of fully saturated triterpene hydrocarbons in sediments has led to the search for unsaturated triterpene hydrocarbons since their direct formation from oxygenated precursors is quite feasible. Douglas et al⁴⁸ suggest that the formation of alk-1-enes under pyrolysis, may involve, *inter alia*, the dehydration of an alcohol or the decarboxylation of an acid or an ester. The authors suggest that the effect of pyrolysis on certain compounds under laboratory conditions can be directly correlated with the diagenetic alterations undergone by the same type of compounds. It would appear feasible that more complex molecules (e.g. triterpenes) are altered in the same way.

The isolation of olefins from the Green River shales by Iida et al⁸⁴ and by Henderson⁸⁵ may be taken as a tentative verification of the validity of the geogenetic approach and suggests that an investigation of the Green River shale directed at the isolation of unsaturated triterpene hydrocarbons may produce further evidence.

The geo-genetic mechanisms of alkene formation may well explain the presence of unsaturated triterpenes in sediments, but it must be remembered that there may be several naturally occurring unsaturated triterpenes which have not yet been isolated or, alternatively, biogenetic pathways may have altered in certain systems so that molecules naturally occurring at the time of deposition are now absent from living systems.

TABLE 1

BIOGENETIC CLASSIFICATION OF TRITERPENOID STRUCTURES

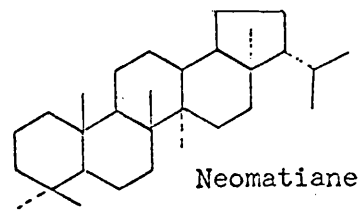
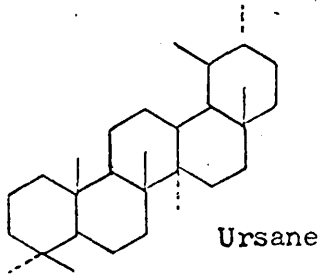
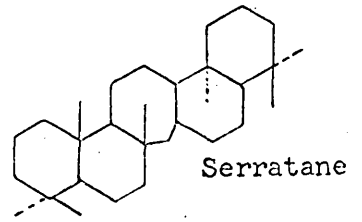
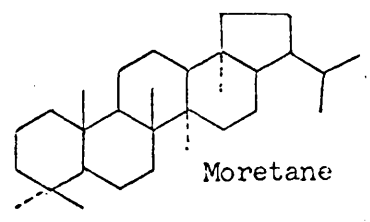
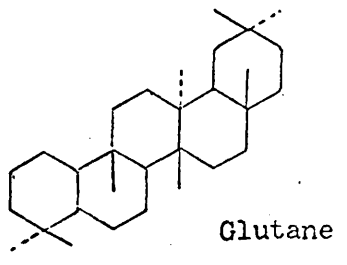
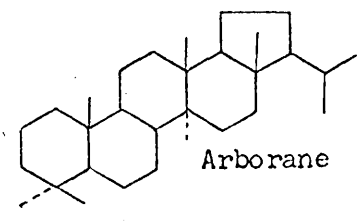
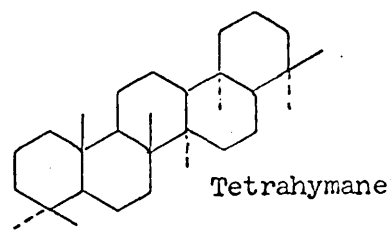
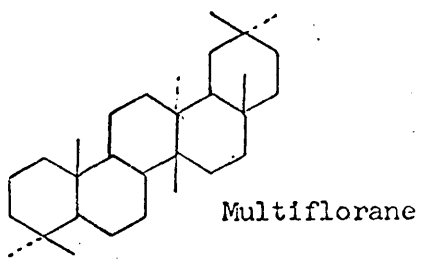
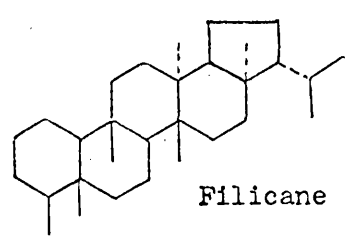
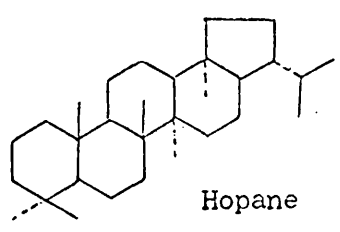
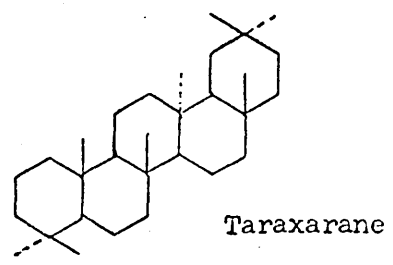
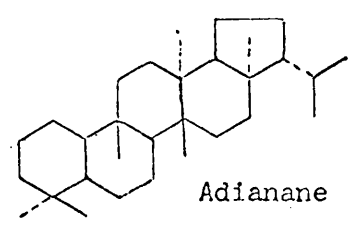
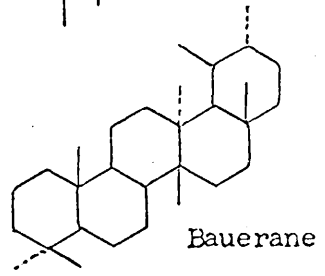
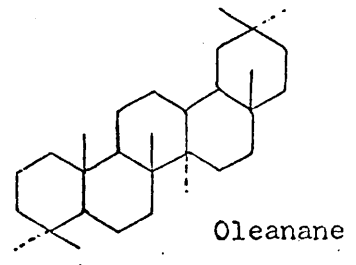
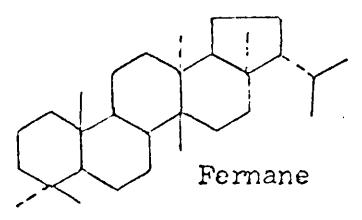
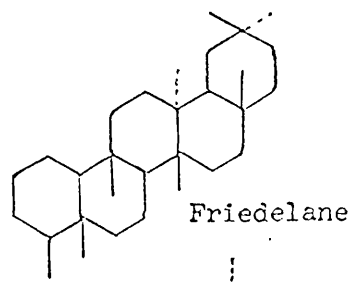
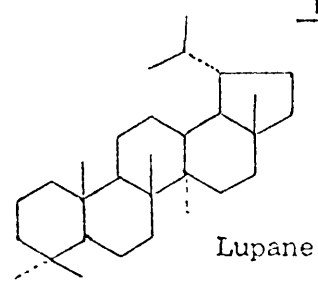


Table 2

Collection of Standard Triterpenes

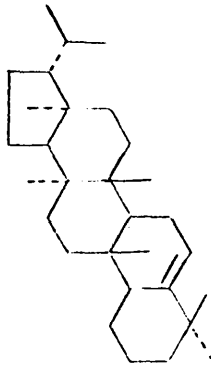
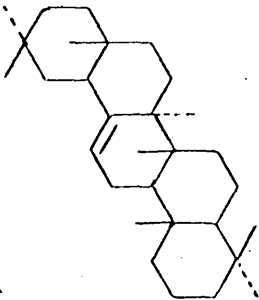
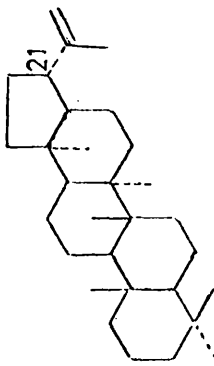
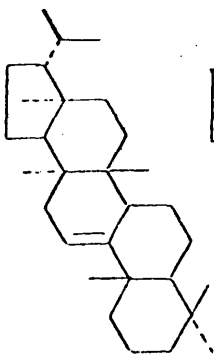
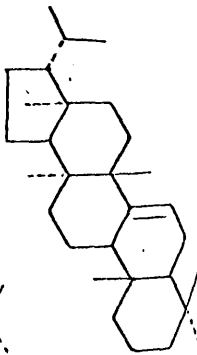
Compound (a)	Structure	Source	Optical Rotation (b)	Melting Point (b)	Rf. Value (c)	Retention Time (d)
			(°)	(°C)		(mins)
adianene		A.	+51°	190.5 - 191.5	0.30	18.29
β-amyrane II		E.	+91.8°	161 - 162	0.81	8.55
diploptene		A. B.	+61°	210 - 211	0.13	26.40
fernene		A. B.	-16.5°	170 - 171	0.82	15.73
7-fernene		A.	-27°	208.5 - 209.5	0.47	21.00

Table 2 (Continued)

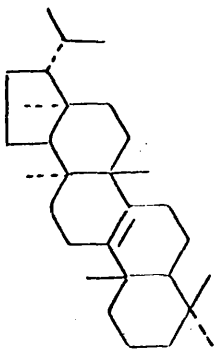
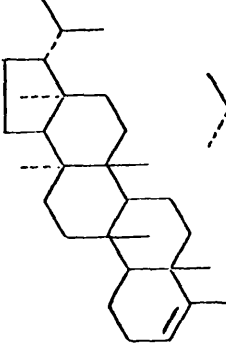
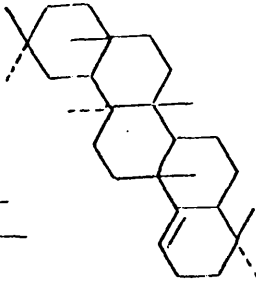
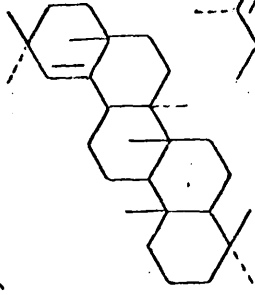
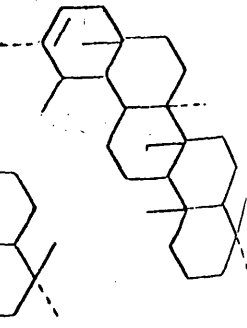
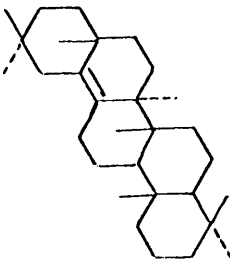
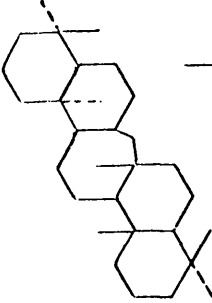
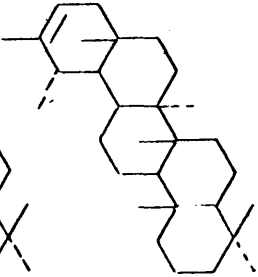
Compound (a)	Structure	Source	Optical Rotation ^(b)	Melting Point (°C)	Rf. Value (c)	Retention Time (mins) (d)
6.		A.	+15.2°	184 - 185	0.84	13.63
7.		A. B.	+50°	228.5 - 229.5	0.20	25.95
8.		C.	+53°	261 - 264	0.20	8.70
9.		D.	+5°	168 - 169	0.37	9.30
10.		E.	+102°	224.5 - 225.5	0.47	19.22

Table 2 (Continued)

Compound (a)	Structure	Source	Optical Rotation (b)	Melting Point (c)	Rf. Value	Retention Time (d)
			(°)	(°C)		(mins)
11. α -lupene		E.	+30.2°	163	0.25	5.86 *
12. lup20(30)ene		D.	+28°	162 - 164	0.30	11.40
13. γ -lupene		E.	-12.2°	187 - 191	0.43	16.52
14. moretene		F.	+24°	202 - 204	0.15	15.89
15. olean(12)ene		D.	+94°	159 - 161	0.82	9.01

Table 2 (Continued)

Compound (a)	Structure	Source	Optical Rotation ^(b)	Melting Point ^(b) (°C)	Rf. Value ^(c)	Retention Time ^(d)
16.		D.	-50°	184 - 185	0.84	8.40
17.		B.	-13.4°	237 - 239	0.67	20.71
18.		E.	+48° to +50°	181 - 182	0.47	16.35
cholestane					0.87	0.00 (17.0 after solvent)

Sources

- A. - H. Ageta, Showa College of Pharmacy, Tokyo, Japan.
 B. - G. Berti, University of Pisa, Italy.
 C. - G. Eglinton, University of Bristol, England.
 D. - W. Laurie, University of Strathclyde, Glasgow, Scotland.
 E. - T. Halsall, University of Oxford, England.
 F. - M. Galbraith, University of Sydney, Australia.

Notes

- a) Arranged in alphabetical order.
 b) Literature or supplied value.
 c) Rf. value obtained on Silica Gel t.l.c., impregnated with 5% AgNO₃ by weight. Developer, 100% n-hexane. Visualizer, 50% H₂SO₄ with charring.
 d) Retention times w.r.t. cholestane. Obtained using a 10' x 1/16", 3% SE 30 column, run isothermally at 250°C with an N₂ pressure of 30 p.s.i. Except for *, obtained a 1% SE 30, otherwise identical.

DISCUSSION

Collection and Characterisation of Standard Triterpenes

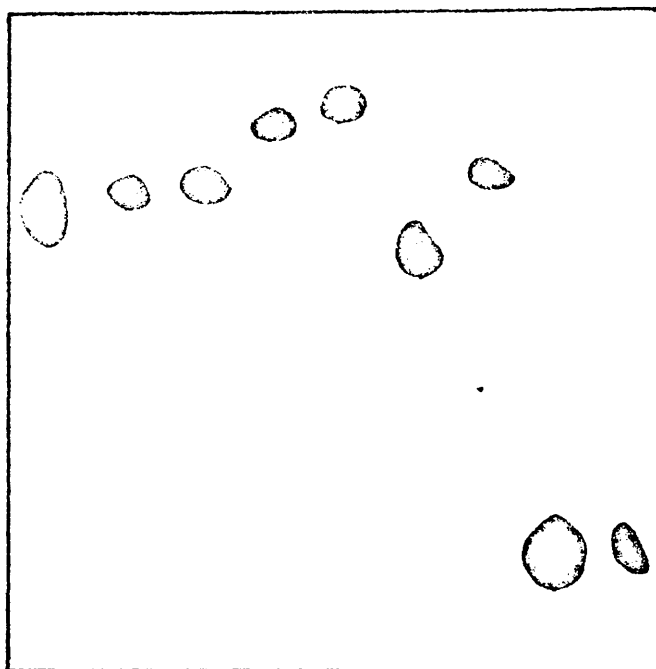
The direct comparison of the physical data of an unidentified triterpene to that of a standard triterpene is an essential feature in the procedure leading to identification of the compound. Close structural similarities between members of each pentacyclic triterpene system, together with a lack of any distinguishing functionality, result in the mass spectrum of each being not fully characteristic except by direct comparison to that of a standard. Fernene (4) and isofernene (6) have similar mass spectra (m.s. 1. & 2), but the former has a 9 : 11, the latter an 8 : 9 double bond. Differing only in stereochemistry, diploptene (3) (C₂₁ - β - isopropenyl) and moretene (14) (C₂₁ - α - isopropenyl) also have mass spectra closely resembling each other (m.s. 3 & 4). Since mass spectral analysis is the prime identification method applicable to the small quantities of pure compounds obtainable from geological samples, a comprehensive collection of standard triterpenes is required for comparison; each being fully characterised by mass and infra-red spectroscopy, melting point and optical rotation.

The standard triterpene hydrocarbons (Table 2) were either isolated directly from living systems or else synthesised from naturally occurring triterpenoid structures. Characterisation of the reference compounds was by their R_f values on silver nitrate impregnated silica gel chromatoplates, their micro infra-red spectra, gas-liquid chromatographs (g.l.c.) retention times, and mass spectra. Literature values for the melting points and optical rotations of the standards are also included in the table.

It is seen that the R_f values cover a wide range, the highest being approximately equal to that of an n-alkane, the lowest approaching that of anthracene. The values obtained are obviously related to the position of a double bond in the molecule, high R_f values being recorded when the molecule contains a highly double bond. Such a relationship between the location of unsaturation and chromatographic behaviour has been demonstrated for the g.l.c. retention times of members of the hopane-zeorinane group⁹¹. The results obtained, in the present work, for the retention times of isofernene (6) and fernene (4) confirm Ikekawa's finding that a tetrasubstituted double bond shortens the retention time more than a trisubstituted double bond does (13.65 and 15.75 minutes respectively).

CHROMATOPLATES

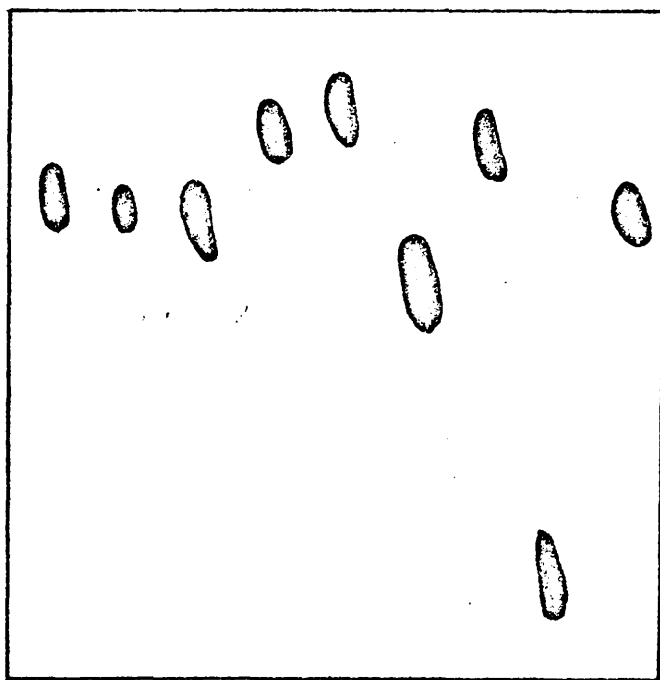
No. 1



5% $\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$

(a) (b) (c) (d) (e) (f) (g) (h) (i)

No. 2



10% $\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$

(a) (b) (c) (d) (e) (f) (g) (h) (j)

Developing solvent:- 100% n-hexane.

Visualizer :- No.1 ; 50% rhodamine/fluorescein.

No.2 ; 50% H_2SO_4 and charring.

(a) C_{17} -1-ene (b) Ψ - taraxastene (c) lupene-1

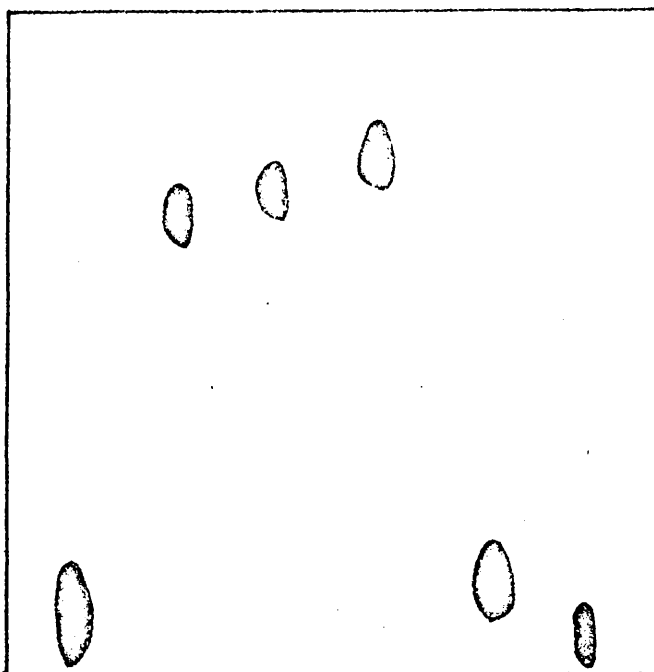
(d) iso-fernene (e) n- $\text{C}_{22}, \text{C}_{24}$ (f) diploptene (g) fernene

(h) anthracene (i) Green River fraction 3

(j) Green River fraction 2.

CHROMATOPLATES

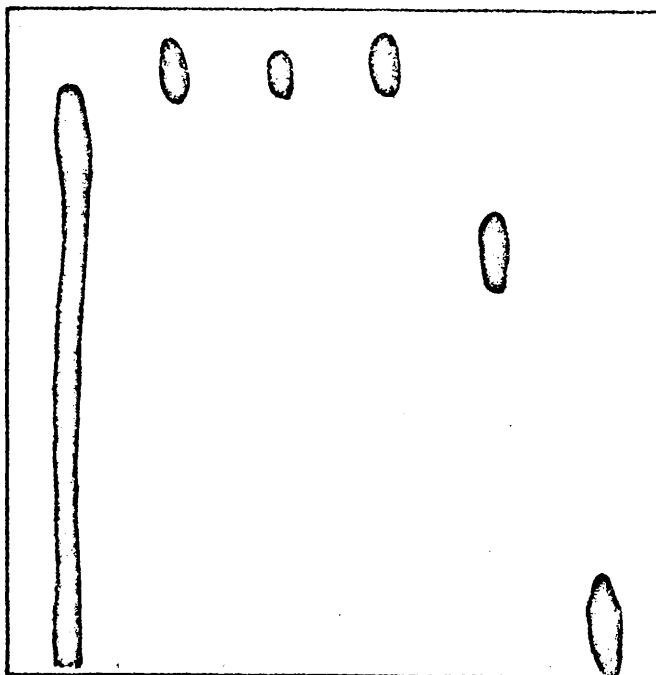
No. 3



10% mercuric acetate

(a) (b) (c) (d) (e) (f)

No. 4



10% mercuric acetate

(a) (b) (c) (d) (e) (f)

Developing solvent:- No. 3 ; 100% n-hexane.
 No. 4 ; 95:5 hexane:methanol.

Visualizer :- 50% H₂SO₄ and charring.
 (a) C₁₇-1-ene (b) γ -taraxastene (c) iso-fernene
 (d) n-C₂₂, C₂₄ (e) anthracene (f) diploptene.

It would appear that thin layer chromatography (t.l.c.) Rf. values could also be supplied to structure elucidation as Ikekawa et al suggest for the g.l.c. retention times⁹¹. The diversity of the Rf. values obtained carries the implication that the conventional method of separating saturated from unsaturated molecules, argentative t.l.c., was not applicable to pentacyclic triterpene hydrocarbons.

Since the presence or absence of unsaturation is a useful basic criterion in the analysis of complex organic mixtures, a suitable method of chromatographic separation, sensitive to hindered double bonds, is desired. It was reasoned that, since AgNO_3 t.l.c. was ineffective for steric reasons, a smaller complexing ion could forseably approach the unsaturation sufficiently for a π -complex to form and thus effect the required separation. The mercuric ion, Hg^{++} (radius 1.10\AA c.f. to $\text{Ag}^+1.26\text{\AA}$) was thought to have the greatest possibilities and consequently silica gel t.l.c. plates, impregnated with 5% and 10% mercuric nitrate by weight were produced. Selected standards were chromatographed on these plates, the developing solvent being 100% n-hexane and the results obtained are shown in the diagrams (chromatoplates 1 & 2). It is seen that differential complex formation does occur but the separation achieved is no better than that on AgNO_3 t.l.c. and the triterpenes containing hindered double bonds are not resolvable from the alkanes.

Two further attempts at the chromatographic separation were made using mercuric ion derivatives. The first of these employed mercuric acetate impregnated t.l.c. plates with 100% n-hexane as developing solvent, the second attempted to invoke the use of methoxy-mercuri-acetate complexes of the olefins. The t.l.c. plates were made in the accepted fashion, the silica gel being impregnated with 10% mercuric acetate. The first chromatoplate (chromatoplate 3) shows that mercuric acetate is capable of complexing π -bonds but the required resolution of hindered double bond triterpenes from alkanes is not obtained. The second plate (chromatoplate 4) was developed in 95 : 5 hexane : methanol. It was realised that the 5% of polar solvent would raise the Rf. values considerably but it was hoped that the effect of the methoxy-mercuri-acetate would more than counteract this. However, the chromatoplate shows that the increased polarity of the developing solvent has the controlling effect and no separation was achieved.

It was also thought that better separation on AgNO_3 t.l.c. might be achieved if the chromatoplate were developed at a low temperature. The results obtained with a plate developed in 100% n-hexane at -10°C differed only slightly from those obtained at room temperature.

An examination of Ikekawa's results⁹¹ clearly shows the relationship between the position and steric hindrance of the double bond and the consequent g.l.c. retention time of the compound. Although the location of the double bond in a mono-unsaturated triterpene may be tentatively determined by reference to the g.l.c. retention time and the t.l.c. Rf. value as shown above, the specific position can only be determined by the unequivocal identification of the compound. As has been mentioned previously, the identification relies to a great extent on the comparison of the unknown compound's mass spectrum to that of a standard, but this method becomes invalid when two triterpenes of differing structure have similar mass spectrum. (e.g. α -lupene (II), m.s. 5 and diploptene (3) m.s. 3).

Location of Unsaturation by the Introduction of Oxygenation

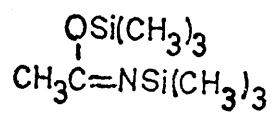
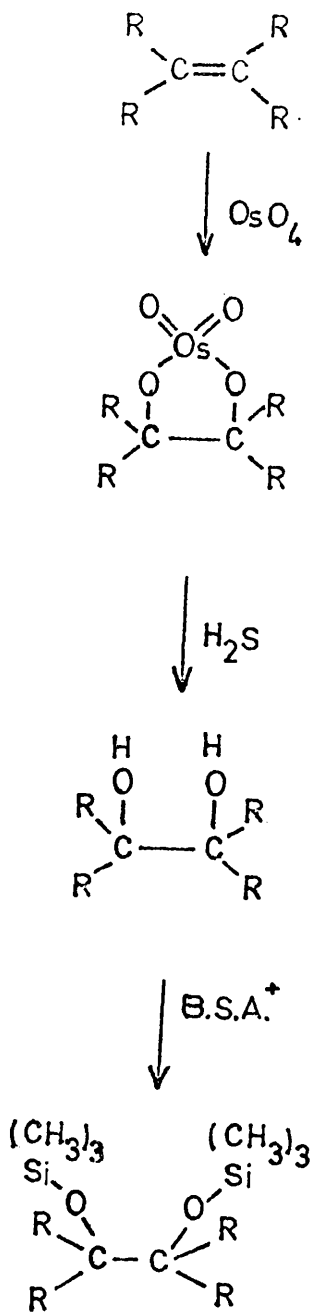
The localisation of a double bond in a molecule by analysis of the mass spectrum of the compound after oxygenation of the unsaturation has been demonstrated by Wolff et al⁹², and by Aplin and Coles⁹³. The latter authors describe a method for locating the position of unsaturation by examination of the mass spectral cracking pattern induced by introduction of an epoxide group.

A similar principle, but employing an alternative oxygen function, was applied to the triterpenes, α -lupene and diploptene, in an attempt to achieve a clear distinction between them using mass spectrometry alone. Such a process had previously been used by this group to locate unsaturation in long chain fatty acids⁹⁴. In view of this, it was decided that osmylation of the triterpene to produce a diol, followed by formation of the trimethylsilylether, would give a product, the mass spectrum of which would be meaningful with regard to the position of the double bond.

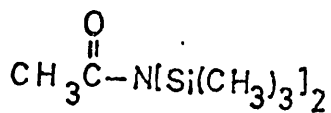
Osmylation of a Diterpene Hydrocarbon

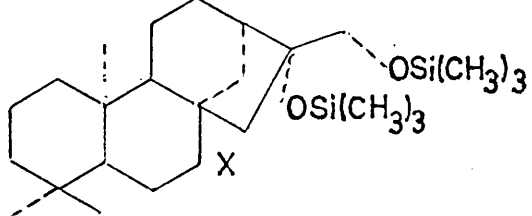
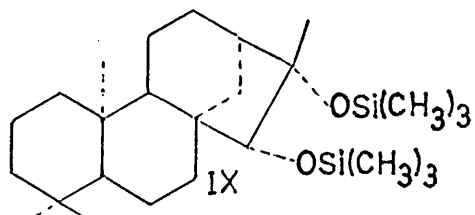
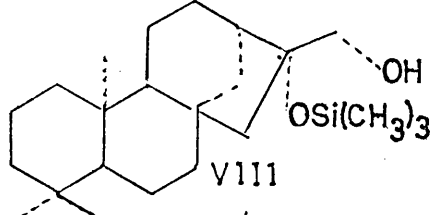
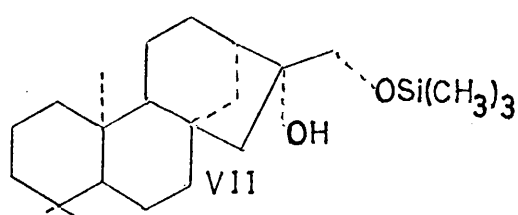
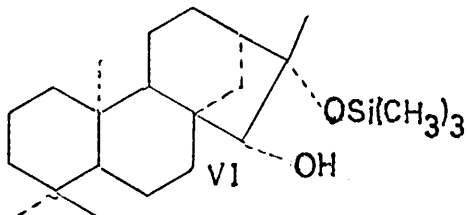
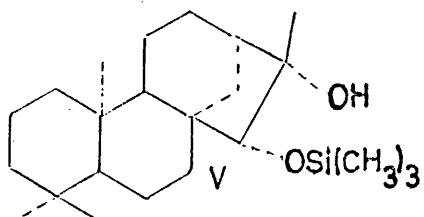
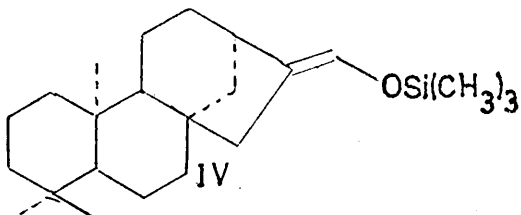
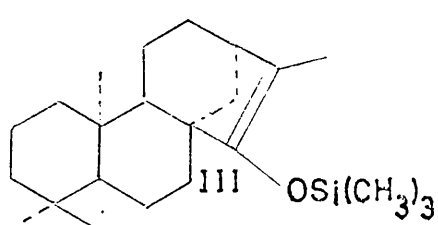
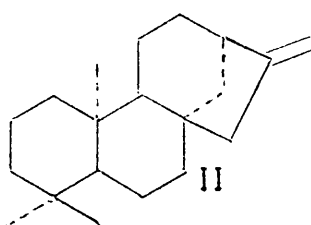
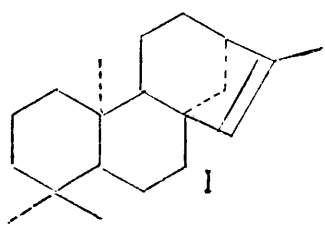
The osmylation method employed was essentially a scaled down version of that used by Briggs for diterpenes⁹⁵. In this procedure, osmium

T.M.Si. ETHER FORMATION.



or

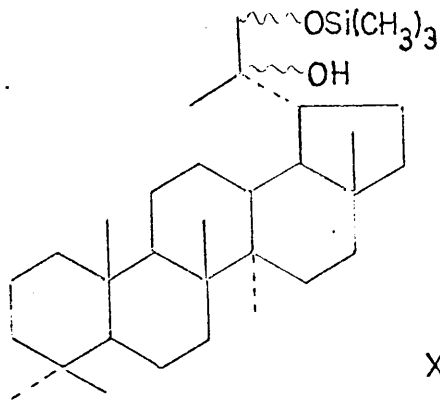




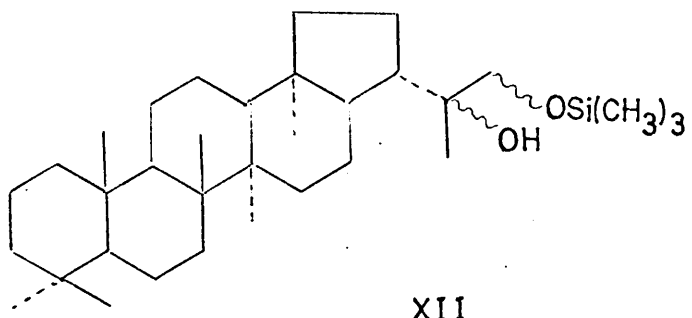
tetroxide in ether is added to a solution of the diterpene in ether and left overnight. The ether is then evaporated and the total residue dissolved in benzene, prior to bubbling hydrogen sulphide gas through the solution to hydrolyse the osmate ester. This causes a black precipitate to form which is removed after centrifuging. The trimethylsilylether is then formed by adding a small volume of *N,N* bistrimethylsilylacetamide⁹⁶ (Diagram 3). The procedure described was initially used in the present work with a diterpene, iso-kaurene, which was adapted as a suitable control compound. However, the iso-kaurene was found by g.l.c. to contain a major impurity, shown by Appleton⁹⁷ to be an isomer, kaurene. Despite this complication, the g.c.m.s. analysis demonstrated that four types of compound were formed, during osmylation, in appreciable quantities. The starting material and the major impurity have the structures (I) and (II). The g.l.c. trace is shown, (g.c.l.).

The products of lowest molecular weight (approximately 270) were not assigned a structure. It was initially thought that the compounds were merely unreacted hydrocarbons but the mass spectral cracking patterns were completely different from those of the starting material (M.W. = 272). The mass spectrum of a product having a molecular weight of 360 was obtained. This was assigned the possible structures, (III) or (IV), obtainable by dehydration of the mono-ol, mono-T.M.Si. ether. Iso-kaurene and kaurene would each give only one such product since a removeable hydrogen α to the hydrogen is required. The major g.l.c. peaks were found by mass spectroscopy to represent compounds of molecular weight 378. These compounds are thought to be the mono-ol, mono-T.M.Si. ether, i.e. vicinal diols in which only one hydroxyl group forms an ether linkage. Both kaurene and iso-kaurene would each give two such compounds and in fact four peaks on the g.l.c. traces give the expected molecular weight. (V, VI, VII, & VIII). A small amount of the vicinal di-ether (M.W. = 450) was formed (IX or X). As only one such peak was obtained, it cannot be determined whether kaurene or iso-kaurene is the parent compound.

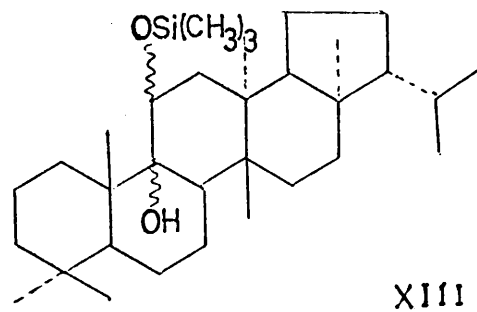
In all the cases described, steric considerations dictate that the osmate ester and all subsequent oxygen functions are attached on the α -face. In retrospect, preparative t.l.c. purification of the supposedly pure starting material would have simplified the g.c.m.s. analysis, as would preparative t.l.c. separation of the components of the reaction mixture



XI



XII



XIII

prior to analysis. However, this was a useful control experiment as it demonstrated:-

- a) that the method is suitable for scaling down to 100 microgram quantities or possibly less.
- b) that the major product was the mono-ol, mono-T.M.Si ether. (V or VI). Steric hinderance would appear to prevent the formation of the di-ether (IX) or (X) to any great extent. The same consideration would appear to favour (V) as the structure of the major product.

Osmylation of Triterpene Hydrocarbons

The triterpene α -lupene, diploptene and fernene were then treated in the same way, the latter being included merely to investigate whether the osmylation technique was applicable to a hindered double bond.

Both α -lupene (11) and diploptene (3) were shown by g.c.m.s. to form predominantly the mono-ol, mono-T.M.Si. ether (M.W. = 516) (XI and XII) respectively. Steric consideration suggest, as with isokaurene, that the hydroxyl is tertiary and the silyl ether is secondary. Fernene, reacted in the same way, gave a major g.l.c. peak, confirmed by g.c.m.s., corresponding to the unreacted starting material (4). A minor peak did give a molecular weight of 516 on g.c.m.s. analysis, corresponding to the mono-ol, mono-ether (XIII).

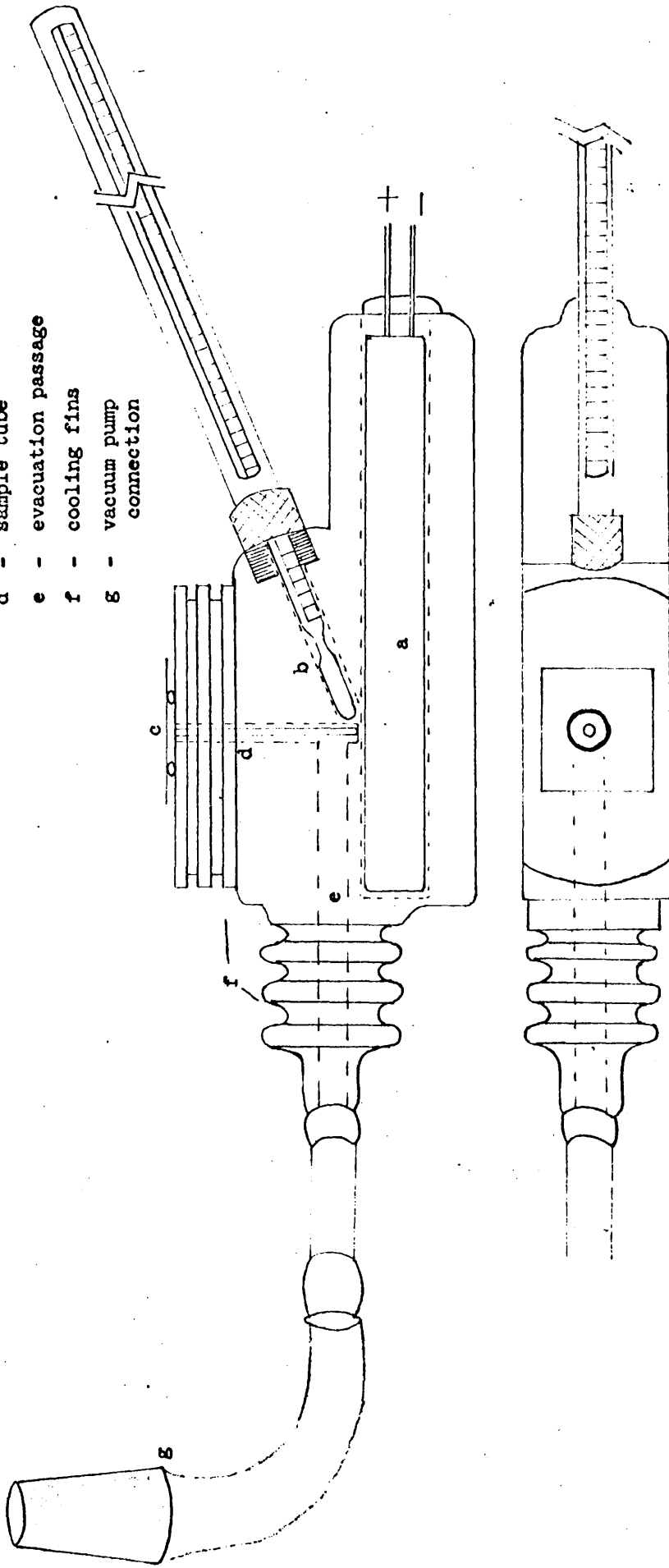
α -lupene and diploptene, which give closely similar mass spectra, formed products of the same molecular weight after osmylation. The cracking patterns however are quite different. (m.s.6 and 7 respectively). The mass spectrum of diploptene mono-ol, mono-T.M.Si. ether, (m.s.7.) showed a large loss of 103, corresponding to $\text{CH}_2 - \text{O} - \text{Si}(\text{CH}_3)_3$, followed by a large loss of 18, explained by the remaining hydroxyl coming off as water. α -lupene, however, showed an initial loss of 90, $\text{H} - \text{O} - \text{Si}(\text{CH}_3)_3$, followed by loss of 15, a methyl group from one of several possible positions. (m.s.6.). This demonstrates the application of introduced functionality to differentiate between two similar triterpene hydrocarbons using mass spectroscopy.

Purification of Triterpene Hydrocarbons by Micro-Sublimation

A full characterisation of triterpene hydrocarbons requires an accurate

MICRO-SUBLIMATION BLOCK

- a - heater
- b - thermometer
- c - microscope slide
- d - sample tube
- e - evacuation passage
- f - cooling fins
- g - vacuum pump connection



Bores

- a - 0.375
- b - 0.170
- c - 0.125
- d - 0.0625
- e - 0.25

melting point to be quoted for the pure compound. However, the use of preparative g.l.c. in the separation and purification procedures leading to the isolation of an unknown triterpene from a complex mixture, results in the presence of impurities, such as the g.l.c. stationary phase, in the sample. Since such impurities would render the melting point inaccurate, a method was required for the preliminary purification of the material by sublimation.

As commercially available sublimation units require quantities of material greatly in excess of that isolable from a geological sample, a unit was designed and manufactured in the department. (Details in the experimental section). Cholestane was selected as a test material since it has a molecular weight (372) close to that of a triterpene (410) and it was also readily available. A standard solution of cholestane was made up and aliquots injected into short (1") capillary tubes when required. After evaporation of the solvent, the tube was placed in the sublimation block, a seal formed by placing a glass slide over the "O"-ring, and a vacuum applied. When a vacuum, of typically 0.025 mm. Hg, was reached, the sublimation unit was isolated and the heating started. Raising the temperature from room temperature to 200°C over a period of 30 minutes was found to give an acceptable sublimation return. Such a return was evaluated by comparison of the g.l.c. peak areas obtained from equal sample volumes before and after sublimation.

It was found that the sublimation of a large quantity (1.5 mgs.) of cholestane returned only 38% of that amount. This appears to arise from the small condensation area becoming covered in crystals and the vapour proceeding to condense on alternative surfaces. A quantity of 10µg was found to give a return of 70%. (g.c.2.). Thus 10µg. was seen to be a suitable quantity of material for sublimation. The successful sublimation of β-amylene II was also carried out on 10µg. With quantities as low as 1µg., crystals were seen to sublime but in this case the crystals forming on condensation were too small to be seen clearly under the magnification available on the sub-micro melting point apparatus.

TABLE 3 - PENTACYCLIC TRITERPENOIDS IN THE PLANT KINGDOM

DIVISION.	CLASS/ORDER.	COMMENTS.	SKELETAL SYSTEMS.	REFERENCES.
Angiosperms.	Monocots. Dicots.	abundant steroids & pentacyclic triterpenoids.	oleanane, ursane, hopane, arborane, lupane, moretane.	100, 102, 99, 105, 106, 111.
Gymnosperms.	Gnetales. Conferales. Ginkgales. Cycadales. Pteridospermales.	no pentacyclic triterpenoids isolated.	-	99
Pteridophyta.	Filicales. Equisetales. Lycopodiales. Psilotales.	several triterpenoids including hydracarboids. triterpenoids.	hopane, serratane. -	68, 69, 67, 70, 110. 99
Bryophyta.	Musci. Hepaticae.	triterpenoids.	taraxarane, oleanane.	99
Thallophyta.	Lichens. Fungi. Algae. Bacteria.	triterpenoids. sterols, lanosterols and tetracyclic triterpenoids. sterols, triterpenoids. no sterols or triterpenoids.	hopane, ursane, oleanane. - hopane, ursane, oleanane. -	103, 104, 99. 100, 101. 109. 100.

Distribution of Naturally Occurring Pentacyclic Triterpenes.

Pentacyclic triterpenes occur widely in the phylogenetically highest plant division, Angiospermophyta, which is made up of contemporary flowering plants. This division first appeared in the Cretaceous period ($\sim 70 \times 10^6$ years) of geological time. Gymnosperma, which includes divisions Pteridospermophyta and Coniferophyta⁹⁸, appear to produce few pentacyclic triterpenes⁹⁹. Bryophyta (mosses) and Pteridophyta (ferns), two phylogenetically lower members of the plant kingdom produce both pentacyclic triterpenes and phytosterols¹⁰⁰. The occurrence of pentacyclic triterpenes in Thallophyta is limited to the lichen and algae classes: the fungi have, at present, yielded only tetracyclic triterpenes such as eburicoic acid¹⁰¹.

Brieskorn¹⁰², in a review of the characteristics and distribution of pentacyclic triterpenes in the plant kingdom, concludes that only 47 out of 300 families of higher plants contain pentacyclic triterpenes. The author continues by noting that all 47 appear in the Monocotyledon and Dicotyledon classes of the angiosperms and that a correlation can be drawn between the evolution of the dicotyledons and that of the α -amyrin triterpene group from the β -amyrin, lupeol and germanicol groups.

Developments since this review appeared in 1956 illustrate the difficulties associated with a chemotaxonomic survey of geological samples employing triterpenes as indicators. Not only has doubt been cast on the phylogenetic order (for example the position of the order Apetales in the dicotyledons), but also pentacyclic triterpenes have been found in phyla far removed from the Angiospermophyta discussed by Brieskorn. The lichens of Thallophyta have been found to produce several pentacyclic triterpenes,^{103,104} and one pentacyclic triterpene hydrocarbon¹⁰³. The division Pterophyta produces several pentacyclic triterpene-hydrocarbons^{67,68,69,70}, and with the lichens are, as far as is known, unique in this way. Thus the distribution of pentacyclic triterpenes in the plant kingdom is more extensive than originally thought and obviously more information on the topic has yet to be obtained.

The abundant yet uneven distribution of such compounds in the plant kingdom would appear to suggest a method for the correlation of geological sediments with the plant families occluded during deposition and hence provide information regarding the depositional environment of a particular

sediment. For the identification of pentacyclic triterpenes obtained from a geological sample to provide any information pertaining to depositional conditions, the following assumptions must be valid:

- a) Biological processes have not essentially changed during the intervening geological period.
- b) Changes in the carbon skeleton do not occur during geogenesis. Any molecular changes are confined to chemical functions, e.g. reduction, decarboxylation.

The difficulties associated with the chemotaxonomy of triterpenes are apparent when one considers that angiosperms have been shown to contain triterpenes predominantly of the oleanane and ursane systems⁹⁹, but also of the hopane^{105,106}, arborane¹⁰⁶ and lupane¹⁰² systems. Phyllogenetically lower plants (lichen, ferns, masses), however, contain not only triterpenes of the hopane system but also of the ursane, oleanane and gammacerane systems. It is apparent that a clear distinction cannot be made between the triterpene systems produced by different plant divisions. However, organisms in general do appear to differ in the specific compounds they make and also in the proportions of the same compounds they synthesize, thus a geological sediment of a reasonably homogeneous composition could forseebly yield information on its genesis, provided the triterpene chemotaxonomy was known.

Green River Shale - Origin and Possible Occurrence of Triterpenes

The Green River basin in Colorado and Wyoming was occupied by a vast shallow lake during much of middle Eocene time¹⁰⁷. It was the site for accumulation of fine, evenly bedded oil shales.

W.H. Bradley¹⁰⁸, in a search for contemporary examples similar to this Eocene lacustrine environment, found four lakes producing a kind of organic coze which is judged to be a modern analogue of the precursors of oil shale. The ooze in all four is predominantly algal, almost entirely in the form of minute fecal pellets and does not decay in a warm, wet oxidising environment. The four lakes characteristically have a dense mat of vegetation along the shores that filters out most of the clastic

material and also have an essentially fluid ooze to a limited depth. This creates conditions markedly different from most Temperate Zone lakes, in that the latter support the rapid degradation of organic matter by supporting anaerobic bacteria in the closely compacted organic debris on the lake-bed.

Gas liquid chromatograms of the branched/cyclic alkane and alkene fractions of the Green River shale total lipid extract show a large number of components in the sterane/triterpane region. The presence of steranes is explicable by the common occurrence of steroids in algae¹⁰⁹, but the presence of triterpenes in the sediment suggests that either the presence of triterpenes in algae is more common than at present thought or other classes of plant e.g. lichens, which contain triterpenes to a greater extent^{103,104}, played a greater part in deposition than originally thought.

It thus appears feasible to identify specific triterpenes, presumed to be indigenous, in a geological sample, to correlate them to a class or species of plant by chemical taxonomy and, if the preferred environment of the plant is known, to obtain information of the depositional environment.

ISOLATION AND SEPARATION OF THE TOTAL LIPID FRACTION OF GREEN RIVER SHALE :

Preliminary Separation

Isolation of the total organic soluble matter from a sample of Green River shale was effected by the ultra-sonication of the powdered rock in a polar solvent system. Accepted procedures for the fractionation of the total organic extract into classes of compounds, usually involving alumina t.l.c. and 5A⁰ molecular sieving, could not be employed in this work on account of two factors:-

- i) Diverse chromatographic behaviour in the various olefinic triterpene hydrocarbon standards.
- ii) The unknown effect of olefinic unsaturation, in a rigid cyclic system, on a 5A⁰ sieving process.

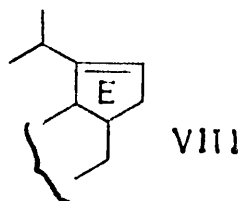
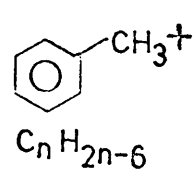
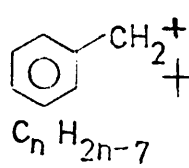
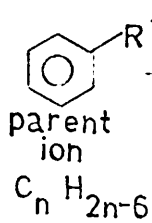
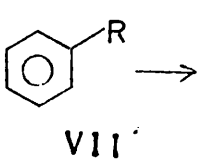
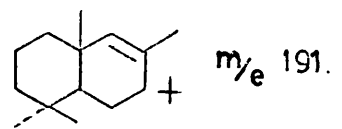
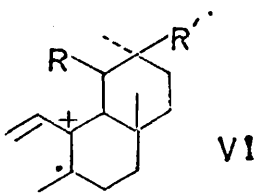
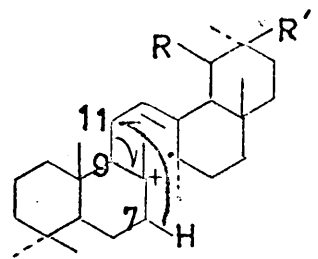
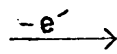
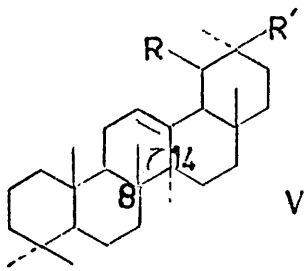
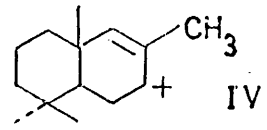
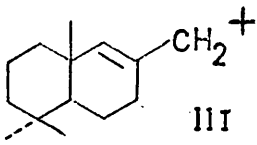
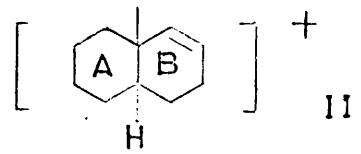
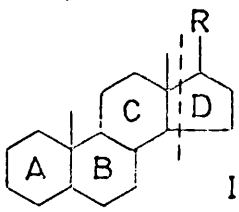
The procedure adopted, after the ultrasonic extraction of the total organic-soluble material (2.2g), required gradient-elution of the extract from an alumina column, followed by preparative thin layer chromatography (t.l.c.) on silica coated chromatoplates impregnated with silver nitrate.

The partial separation achieved by column chromatography was demonstrated by t.l.c. monitoring. Preparative AgNO_3 t.l.c. enabled the eluted hydrocarbon fractions to be further separated on the basis of molecular unsaturation.

The initial hydrocarbon fraction eluted from the column by hexane (22 mg.) showed infra-red absorption typical of fully saturated molecules (i.r.1.). The ultra-violet spectrum however (u.v.1.), although largely typical of saturated hydrocarbons suggested, by traces of absorption at 270 - 280 μ , the possible presence of alkyl or alkenyl substituted benzenes. The succeeding chromatographic fraction (101 mg.), eluted from the column by benzene, produced an infra-red spectrum showing absorption characteristic of unsaturation, predominately aromatic (3100 - 3000, 1625 - 1575, 1520 - 1490, 870, 810 and 680 cm^{-1}). This interpretation was confirmed by the ultra-violet absorption which showed maxima occurring at 200 and 235 μ . (i.r.2, u.v.2.). Two further column eluates were collected and both were shown by infra-red and ultra-violet spectroscopy to contain compounds possessing extensive oxygenation, both ketonic and hydroxylic (i.r.3, u.v.3.).

The hexane eluate was shown by the Rf values obtained on t.l.c. to contain both saturated and unsaturated material suggesting that the column chromatography had not given as definite a separation as at first thought. Perhaps the collection of smaller fractions together with monitoring employing more concentrated aliquots would realise the desired separation. However, further separation of a 122 mg. aliquot of this fraction was achieved by repeated preparative t.l.c. Three fractions were thus obtained from the hexane eluate, comprising (1) the non-polar saturated material (85 mg.) (2) the less polar unsaturated (24 mg.) and (3) the more polar unsaturated material (1 mg.).

Fraction (1) was investigated for the presence of unsaturated triterpene hydrocarbons since in the light of results obtained for the appropriate standards, the chromatographic behaviour of certain olefinic triterpenes is comparable to that of the corresponding saturated molecules. The analytical g.l.c. of this fraction showed it to be a highly complex mixture possessing two outstanding components, shown to be pristane and phytane¹¹², along with several prominent peaks in the C-30 region.



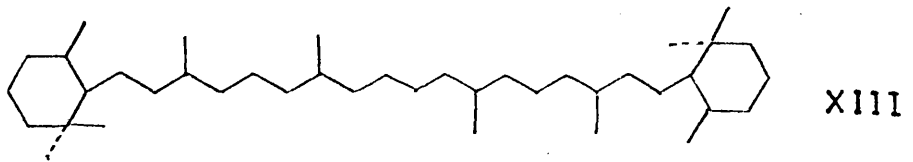
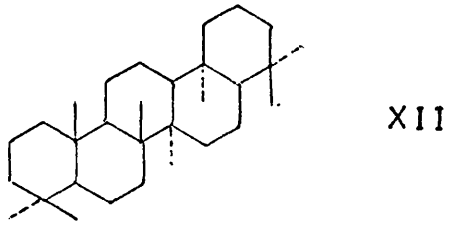
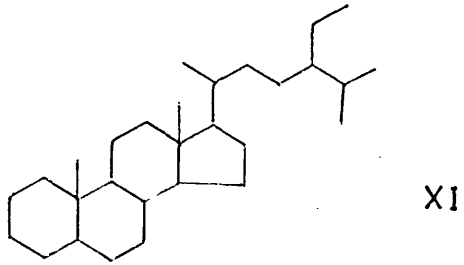
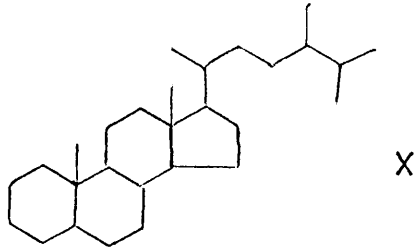
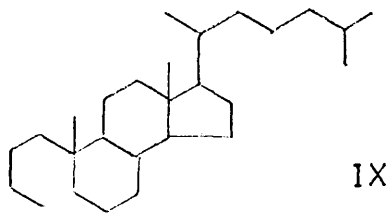
Mass Spectral Analysis

Since the identification of isolated compounds was to be based primarily on the mass spectra obtained by g.c.m.s. analysis, a prior knowledge of the expected mass spectra was obtained from the literature.

The mass spectra of steroidal (C-27) hydrocarbons have been extensively investigated and are pertinent to this investigation since the structures of steranes are such that only small differences in chromatographic behaviour between steranes, triterpenes and hindered triterpenes might be expected. Elimination of the side chain plus 42 mass units is one of the most common features in the mass spectra of C-17 substituted steranes. Biemann¹¹³ summarises the various processes invoked to rationalise this fragmentation, but Budzikiewicz et al¹¹⁴ conclude that fragmentation I is most likely to be operative. The cleavage shown results in a fragment at m/e 217. Elision of ring D also occurs without hydrogen transfer affording a fragment of m/e 218. The formation of the fragment of m/e 149 corresponding to the structure II is also discussed by Budzikiewicz et al¹¹⁴.

The mass spectra of steroidal olefins are also discussed, but only of those formed during mass spectral investigation by thermally induced 1, 2 elimination of alcohol or acetate groups.

Pentacyclic triterpene hydrocarbons however, have a distinctive cracking pattern quite different from that of steranes. Djerassi and co-workers^{49,50}, note the frequency with which an intense fragment at m/e 191 occurs in the mass spectra of such compounds. The absence of this prominent fragment is not conclusive proof of the absence of a pentacyclic triterpene hydrocarbon since the formation of the fragment at m/e 191 merely requires rings A and B of the system to be saturated and unsubstituted. Confusion may still arise from the mass spectral interpretation, as friedelane (C-30) and some of its derivatives show fragments at m/e 149, 217 and 259 which are all associated with steranes¹¹⁵. Also fragments at m/e 149, 217 and 218, prominent in the mass spectra of cholestane and other steranes are of no consequence in the mass spectrum of lanostane (C-~~30~~) which produces prominent fragments at m/e 274, 259 and 190.



Further confusion would appear to result from Karliner and Djerassi's⁵⁰ mass spectral studies on two pentacyclic triterpenes, ¹²oleanene and ¹²ursene which reveal a base peak in both cases of m/e 218, a typical sterane fragment. However, a fragment does appear at m/e 191, corresponding to (III) or (IV), thus showing a triterpene to be present. The authors reason that (IV) is the energetically more favourable fragment and give the cleavage mechanism for its formation. (V). The fragment at m/e 218 is quite different from that obtained for a sterane and the authors state one resonance form to be (VI).

COLLECTION OF INDIVIDUAL COMPONENTS

Fraction 1. - Saturated Alkanes, Steranes and Triterpanes.

Collection of certain individual components of Fraction 1, selected on the criteria of their being represented on the g.l.c. trace by major, well-resolved peaks in the C-30 region, was achieved by preparative g.l.c. In all, sixteen fractions were collected and eleven tentatively identified from their micro infra-red and mass spectrometric data. Of the eleven, eight were thought to be pertinent to this investigation. (g.c.3.). Five isolated components, by examination of their respective mass spectral cracking patterns were thought to be steranes. The molecular ions obtained for three (327, 386, 400) corresponded to the steranes cholestane, (IX), ergostane (X), and sitostane (XI) respectively. The mass spectrum of the supposed cholestane, although showing the presence of small amounts of impurities was very similar to that of an authentic sample. Further support for the assignation was the close similarity between the g.l.c. retention times of the isolated fraction and authentic cholestane taken on a capillary column. Authentic samples of ergostane and sitostane could not be obtained thus preventing direct comparisons of the isolated components. The two remaining steroidal molecules were not assigned a structure but appear to have molecular weights of 360 and 458 respectively, the mass spectral cracking patterns of each suggesting they possessed a steroidal carbon skeleton. The micro infra-red spectra of all five fractions merely showed the absence of any unsaturation or other functionality.

Two saturated terpenoids, one a C-30 compound, the other a C-40, were tentatively assigned the structures of gammacerane (XII) and carotane (XIII) on the basis of their mass spectra. The micro infra-red spectra again showed

the presence of saturated, unsubstituted molecules. Comparison of the mass and infra-red spectra of each with those of authentic samples illustrated a close agreement. However, it must be emphasised that the mass and i.r. spectra of saturated polycyclic hydrocarbons often do not differ from those of other compounds in the same class.

Yet another component was assigned a triterpenoid structure on the basis of mass spectral evidence - a C-31 saturated hydrocarbon of molecular weight 428. In addition a component was found to have a typical triterpenoid mass spectrum, although it had a molecular ion two mass units less than that of saturated hydrocarbon (412). The implication that this component was an unsaturated triterpene hydrocarbon was not fully substantiated by infra-red spectroscopy, as the absorption possessed only a trace of that arising from unsaturation ($\sim 3020 \text{ cm}^{-1}$; 1650 cm^{-1}). The mass spectrum in addition to having a base peak at m/e 191 also showed large fragments corresponding to the loss of 15 (methyl) and 43 (iso-propyl) mass units from the parent ion. The mass spectrum is virtually identical to that of a standard, moretane, except of course the parent ion (M), (M-15) and (M-43) all occur at 2 mass units below that of the saturated standard. The corresponding unsaturated standard, moretane, does not exhibit a similar mass spectral cracking pattern. The above considerations suggest the possibility of erroneous counting in the low resolution, high molecular weight part of the mass spectrum but conclusive proof of identity could be obtained employing the methods discussed later when sufficient material becomes available. (see m.s. 8, 9 and 10).

Whilst preparative g.l.c. collections were largely confined to pure components as shown by the g.l.c. trace, some impurities were present in certain components. Since the micro infra-red spectra were recorded prior to g.c.m.s. analysis, the absorption spectra cannot be regarded as fully characteristic in the appropriate cases where impurities occur.

The infra-red spectrum of fraction (2) (i.r.4.) of the hexane eluate showed that the concentration of the unsaturated material, compared to that shown by the i.r. of the total hexane eluate, had greatly increased and was seen to be predominately aromatic in nature. Further information regarding the type of unsaturation present was provided by n.m.r. and u.v. spectrometry. The ultra-violet absorption (u.v.4.) (220, 270, 273 and $280 \text{ m}\mu$) is characteristic of alkyl or alkenyl benzenes. The absorption

at 270 μ is very similar to that obtained for alkyl and cyclo-alkyl benzenes isolated from Recent marine sediments¹¹⁶. The n.m.r. spectrum showed the presence on only a limited number of aromatic protons (2.9 - 3.2 τ) in comparison to methylene ($\sim 8.75 \tau$) and methyl (9.15 τ) protons. Similarly limited was the presence of methine (7.7 τ) protons. No olefinic proton signals are resolved, which suggests that the aromatic nuclei are highly substituted with long aliphatic side chains which are in turn highly substituted. Since there are very few methine groups present, the aliphatic substitution would appear to be tertiary.

Fraction 2. - Postulated Alkenyl Aromatics

Although no olefinic triterpenes were thought to be present in this fraction, the structures of the aromatic compounds were adjudged to be of sufficient interest for further analysis. Consequently ten individual components were isolated and collected by preparative g.l.c., prior to obtaining micro infra-red and mass spectra. (g.c.4.). Several components demonstrated infra-red spectra with intense aromatic absorption (e.g. component iii, 3100, 3020, 1605, 1409, 955 and 885 cm^{-1}); however, one component (component xvii) exhibited unsaturation of a purely olefinic nature (> 3000 , 1610, 975 and 855 cm^{-1}), suggesting a trans or possibly tri-substituted double bond. (i.r. 5 and 6 respectively). An attempt to record the micro u.v. spectra of certain components failed because insufficient material was available. The i.r. and n.m.r. data presented above suggest the aromatic entities could be alkyl, alkenyl or cyclo-alkyl substituted benzenes. The occurrence of alkyl benzenes in petroleum is mentioned by Hood¹¹⁷ and of alkenylbenzenes in sediments by Meinschein¹¹⁶.

A discussion on the mass spectra of alkyl benzenes is made by Hood as part of a mass spectral study of the molecular structure of petroleum. The author states that typical petroleum alkyl benzenes do not contain two or more medium-to-long alkyl substitutes on the ring but instead have one long chain and several methyl groups attached to the ring. The mass spectral analysis used to substantiate this statement show that the cracking pattern for any long chain alkyl benzene is as depicted in VII. Mono, di, tri and tetra alkyl benzenes all show outstanding peaks in their mass spectra at $m/e C_n H_{2n-7}$, C_n corresponding to the sum of the number of atoms in the ring, the methyl substituents and the residual methylene of the long chain substituent.

The aromatic compounds isolated in this investigation were found by g.c.m.s. analysis to contain five double bond equivalents, corresponding to a benzene ring (confirmed by i.r. and u.v. absorption) together with either an olefinic double bond, possibly in conjunction with the ring or a cyclo-alkyl substituent. An examination of the mass spectrum of component iii (m.s. 11) illustrates the cracking pattern described by Hood. The parent ion occurs at m/e 216 ($C_{16}H_{24}$), the base peak m/e 105, which if assigned to the major fragment of the series C_nH_{2n-7} , shows the aromatic nucleus to be di-substituted with one methyl group. Component VA ($C_{20}H_{32}$) on the other hand, although showing a fragment at m/e 105, has a very much more intense fragment at m/e 119 showing the aromatic ring to be tri-substituted with two methyl groups and one long chain alkyl substituent. (m.s.12).

The micro i.r. cell available for this work allowed the use of only CCl_4 solutions, consequently the substitution pattern on the aromatic rings could only be determined by repeat infra-red spectra, recorded in a non-absorbing solvent (CS_2). However, the total fraction (2) showed in its infra-red absorption an aromatic substitution pattern of 1 : 3 : 5 and/or 1 : 3 (i.r.4).

The compounds isolated were thought to be alkenyl benzenes, rather than cyclo-alkyl benzenes for the following reasons:-

- (i) The mass spectral base peaks typically belong to the C_nH_{2n-7} series (91, 105, 119 etc.).
- (ii) The m/e 120 peak is large as it is both an isotope peak of 119 and a rearrangement peak. To produce this latter (rearrangement) peak a side chain of at least three carbon atoms is required with a hydrogen on the third carbon atom.
- (iii) The parent ions are reasonably large suggesting a considerable stability.
- (iv) The double bond equivalents and the i.r. absorption data support the presence of a double bond.

One apparently contradictory fact arises, namely, that too many alkyl ions are produced in the 55, 69, 83 series. However, a double bond in a side chain would help to stabilise any ion formed and could cause the more intense peaks which are observed. Component xvii of this predominately aromatic fraction demonstrated a mass spectral cracking pattern quite different from that of the alkenyl benzenes. The molecular ion was found to occur at m/e 410, corresponding to that of a mono-unsaturated triterpene hydrocarbon. (m.s.13). The base peak occurred at m/e 135, an ion which is present in the spectra of the majority of triterpene hydrocarbons but which only attains comparable prominence in the mass spectrum of δ' -lupene. Other prominent fragments, associated with triterpenes, occur at m/e 161, 175, 191 and 231, in addition to fragments due to loss of a methyl group (M-15, m/e 395) and loss of an iso-propyl group (M-43, m/e 367). The latter fragment is uniquely intense, comparable intensity again only being attained in the spectra of δ -lupene which possesses structure (13) in the table of standards. However, δ -lupene has a base peak at m/e 341 (M-69) which does not appear at all in the mass spectrum of component xvii.

The micro infra-red absorption of the component is attributable to a trisubstituted double bond (1675, 1610 and 855 cm^{-1}).

Thus component xvii is thought to be a pentacyclic triterpene hydrocarbon possessing no unsaturation or substitution in rings A and B, a trisubstituted double bond and one iso-propyl group. The partial structure (VIII) would explain the infra-red absorption, and the large (M-43) peak in the mass spectrum could be explained by the stability provided by the olefinic bond, to the resulting ion.

Fraction A - Long Chain Alkanes

The least polar fraction (fraction A), isolated by preparative t.l.c., of the benzene eluate was thought to be interesting as the g.l.c. was unusual for a Green River hydrocarbon fraction in not having a wide molecular weight/boiling point distribution. Instead, a group of peaks appeared in the C-30 region of the trace. G.c.m.s. analysis of the

components showed them to be six saturated normal hydrocarbons ranging from n-C₂₈ to n-C₃₃ inclusive.

The difficulties associated with structure determinations of pentacyclic triterpenes by mass and infra-red spectroscopy along have been made obvious in this work. Close structural similarities in many cases cause very similar mass and i.r. spectra to be obtained. Further characterisation of the isolated and standard compounds employing a variety of techniques would greatly facilitate the conclusive structure determination which is required. To this end, several techniques are being developed in the group including capillary g.l.c. and g.c.m.s., micro zone-melting, micro n.m.r. and micro O.R.D. measurements. In addition, the techniques of micro-sublimation and the introduction of functionality to an olefinic triterpene on a micro-scale have been developed in the course of this research. The former technique is required to remove impurities such as g.l.c. stationary phase from isolated fractions prior to further characterisation, the latter is used to produce a more characteristic mass spectrum for any particular olefinic triterpene. The application of such techniques will obviously aid the characterisation of compounds available only in microgram quantities.

EXPERIMENTAL

The general methods of isolation and analysis are discussed, followed by an account of the specific experimental procedure.

General Methods

Thin-layer Chromatography (t.l.c.)

Silica gel (Kieselgel G-E Merck) impregnated with 10% by weight silver nitrate was used as adsorbent¹¹⁸. The glass plates were coated with a slurry of the adsorbent in distilled water on a motorised t.l.c. spreader (Baird & Tatlock, London). After air drying, the plates were activated at 120° for approximately one hour and stored in a desiccator over self-indicating silica gel granules. Storage, without requiring reactivation, was limited to one day. Light was excluded from the desiccator. Preparative plates were developed with ethyl acetate prior to activation and samples were loaded by means of a 50 or 100 μ l. Hamilton syringe. Preparative plates (20 x 20 cm) had an adsorbent coating of 1 m.m. thickness. n-hexane was used for development unless otherwise stated. Detection was achieved by spraying with 50% sulphuric acid followed by charring at 200°C (for analytical plates) or by spraying with a 0.2% solution of dichloro-fluorescein or rhodamine G in ethanol and viewing under u.v. light (254 m μ). Recovery of the sample was achieved by ether extraction of the adsorbent material in an extraction funnel fitted with a defatted cotton plug and a layer of neutral alumina to retain the dye.

Infra-red Absorption Spectroscopy (i.r.)

Routine spectra were recorded on a Perkin-Elmer 257 grating spectrometer (accuracy ± 5 cm⁻¹ above 2000 cm⁻¹ and ± 2 cm⁻¹ below 2000 cm⁻¹). Fractions isolated by preparative g.l.c. were dissolved in CCl₄ (~ 4 μ l) and the spectra recorded (on ~ 40 μ g) on the P.E. 257 instrument using a micro-cell (0.5m.m; capacity ~ 2.5 μ l). A beam condenser was used in these cases.

Ultra-violet Absorption Spectroscopy (u.v.)

The u.v. spectra were recorded on a Unicam SP 800 spectrophotometer. The slit width was set at the minimum 0.02 m.m. with normal programme energy. Routine spectra were recorded employing a 10 m.m. cell but for individual

components a.u.v. micro-cell (R.I.I.C. u.v. 01) was used (1 m.m.; $\sim 20 \mu\text{l}$). All spectra were run in Spectrosol n-hexane after the high concentration of aromatics in distilled Analar n-hexane was noticed (u.v.5 and 6).

Gas-liquid Chromatography (g.l.c.)

Analytical : Perkin-Elmer F.11 instruments, each equipped with a hydrogen flame ionisation detector, were used for analytical g.l.c. Stainless steel columns, 1/8 in. (6 or 10 ft. in length) or 1/16 in. (10 ft. in length) in diameter were employed unless otherwise stated. Nitrogen was used as carrier gas at flow rates of 20 - 30 ml./min. The liquid phases were: 1% and 3% SE-30 (Applied Science); 3% OV-1 (Applied Science). Linear temperature programming was usually from 100 - 250 $^{\circ}$ or 300 $^{\circ}$ at 4 $^{\circ}$ /min. The supports used were Gas Chrom P (100 - 120 mesh, acid washed and silanized, Applied Science) and Gas Chrom Q (60 - 80 mesh, acid washed and silanized, Applied Science). Columns were conditioned by heating from room temperature to 300 $^{\circ}$ C at 1 $^{\circ}$ /min. and maintained at 300 $^{\circ}$ C for 24 hours.

Preparative : Wilkens Aerograph A90P-3 instruments, each equipped with a thermal conductivity detector, were used for preparative g.l.c. Copper or stainless steel (20 ft.) columns $\frac{1}{4}$ or 1/8 in. in diameter were used. The columns were packed with 3% SE-30 on Gas Chrom P (100 - 120 mesh, acid washed and silanized) or 3% SE-52 on Chromasorb W (100 - 120 mesh, acid washed and silanized). (Wilkens Aerograph). The carrier gas was helium with flow rates of approximately 60 ml./min. at a pressure of 60 p.s.i. Injector and detector temperatures were 275 and 320 $^{\circ}$ C respectively. Collection of a requisite fraction was effected by trapping the eluate in a glass melting-point capillary (10cm x 1 m.m.). After collection both ends of the capillary were sealed with a small flame. The efficiency of collection was 60 - 70%. Sufficient material of each component for micro i.r. examination was isolated by repeated collections in the same capillary for corresponding peaks on the chromatogram. The capillaries were pre-cleaned by sonication in detergent solution, rinsing thoroughly with distilled water and acetone, and a final sonication in chloroform. Both ends of each capillary were flame-polished to prevent contamination from the silicone rubber septa.

Combined Gas Chromatography - Mass Spectrometry (G.C. - M.S.)

The fractions trapped by preparative g.l.c. were examined by G.C.- M.S. using the L.K.B. 9000 gas chromatograph - mass spectrometer. The columns used were 10 ft. x 3 m.m. i.d. glass columns packed with 1% S.E. 30 on Gas Chrom P (100 - 120 mesh, acid washed and silanized). The carrier gas, helium, had a flow rate of 30 ml./min. The mass spectrometer had a scanning period of approximately 4 seconds. An accelerating voltage of 3.5 kV was used in combination with an electron energy of 70eV. The multiplier voltage setting varied from 1.7 to 2.3eV and the separator temperature was 250^o.

Other procedures

At all stages, precautions were taken to minimise contamination and polythene gloves were worn at all manual operations.

All solvents were of "Analar" grade and were distilled through an 18 in. column packed with glass helices. Before distillation the solvents were heated under reflux (30 min.) and of 2l. of solvent only a middle cut of 1.8 l was distilled with partial reflux.

Glassware was cleaned ultrasonically (20 min.) in an ultrasonic tank (Dawe Instruments Ltd., type 1165/H60X; frequency 25.83 kcs; 150 watts) thoroughly rinsed with distilled water, stored in closed dust free jars, and rinsed with solvent before use. The detergent used for cleaning glassware was R.B.S.26 (Medical Pharmaceutical Developments Ltd.).

Solvents were evaporated in a rotary evaporator (Buchi) under water-pump vacuum or in a vacuum oven (Thomson and Mercer Ltd., Croydon) under water-pump vacuum when the quantity of solvent was small.

PROCEDURE

The sample of Green River shale used in this work was kindly donated by Dr. W.E. Robinson, U.S. Bureau of Mines, Laramie, Wyoming, U.S.A.

Preliminary Treatment

The outer surface of the rock was removed and discarded in order to minimise contamination and only fragments having freshly exposed surfaces were used. The fragments were broken up on a clean metal surface into smaller fragments 1 - 1½ inches in diameter with a hammer, the head of which was covered with several layers of aluminium foil. The resulting chips of rock were carefully washed 3 times (5 min. each time) by sonication in benzene/methanol (1:1) using a titanium probe (Dawe Instruments Ltd., Model S75).

The dried chips were then powdered in a cleaned rotary hammer mill (Glen Creston, Star Beates Mill). In order to again minimise contamination, the hammer mill was modified to accommodate lead gaskets rather than the standard rubber gaskets.

Final powdering was effected in a clean vibratory disc mill (Tema Machinery Ltd., Banbury), the milling operation lasting 15 minutes. The moveable parts of both mills in contact with the rock were cleaned by tank sonication in detergent (R.B.S.26, Medical Pharmaceutical Developments Ltd.), rinsed thoroughly with distilled water, washed with distilled acetone and finally with chloroform. It was found that, after pulverisation with the hammer mill, approximately 70% of the resulting powdered rock passed through a 200 mesh sieve. After the disc mill operation 100% of the powdered rock passed 200 mesh. The milling time for the disc mill was kept below 15 min. to prevent a substantial temperature rise ($\sim 60^{\circ}\text{C}$).

Extraction of Organic Matter

Ultrasonic extraction was used to liberate the organic matter from the powdered shale since it is known to be a rapid, effective and convenient method for removing soluble organic matter from sediments¹¹⁸. The powdered rock (100g) was placed in a glass centrifuge bottle (250ml.) with 100 ml. of solvent (3 : 1, benzene/methanol). The bottle was then adjusted in the

ultrasonic tank (Dawe Instruments Ltd., type 1165/H60X; frequency 25.83 kcs; coupled to a 300/150 watt Soniclean generator) such that the solvent was at the same level as the water in the tank. This was found to give the most efficient extraction. Sonication was allowed to proceed for 30 min. The resulting suspension was centrifuged at 2,500 r.p.m. for 30 min. and the clear solvent layer removed by pipette. The extraction was repeated seven times, fresh solvent being added on each occasion. The bulked solution (700 mls.) was evaporated under reduced pressure, (Buchi) to produce the total organic extract as a dark brown gum. (2.2056 g. : 2.2%).

Column Chromatography of the Total Organic Extract

An aliquot (2.15g) of the total organic extract, comprising the n-hexane soluble and suspended material was chromatographed on alumina (150 g Woelm Grade I neutral alumina, activated for one hour at 110°C and prewashed with n-hexane). A teflon stopcock was fitted to the glass column to enable an efficient seal to be formed without the use of contaminating grease. The column was eluted with n-hexane (850 ml.), 10 ml. fractions being collected by an automatic fraction collector (The Central Ignition Co., London, Cat. No. 150). Elution was continued with benzene (600 mls.), chloroform (150 mls.), ether (200 mls.), ethyl acetate (200 mls.) and finally methanol (250 mls.).

The hexane eluate was monitored by t.l.c. Of the 85 hexane fractions, nos. 11 to 20 were shown to contain most of the alkanes by comparison of the Rf. values to that of a standard hydrocarbon mixture. (n - C₂₂, n - C₂₄). The presence of alkenes was not apparent in this eluate, consequently all 85 fractions were bulked and the solvent evaporated to produce a total hexane eluate. (0.2228g., 0.22% by weight of shale). The i.r. spectrum (film) had absorption at 2920 (s, ν C - H), 1460 (m, δ CH₂CH₃), and 1378 cm⁻¹ (m, δ CH₃). (i.r.1).

The benzene eluate was similarly bulked and the solvent evaporated to produce 0.1015g of a straw coloured oil (0.10% by weight of the shale). The i.r. absorption (film) showed the presence of large amounts of unsaturation characterised by peaks at 3100 - 3020 (m, ν C - H), 1605 (w, ν C=C aromatic), 1510 (w, C=C aromatic), 810 (w), 730 (w), 720 (w), and 675 cm⁻¹ (m). (i.r.2).

The absorption suggested the presence of aromatics, trans, tri-substituted and possibly vinyl double bonds. The u.v. spectrum (202, 233 and 255 - 307 μ) confirmed the presence of aromatics and olefins, possibly conjugated to the aromatic rings. (u.v.2).

The chloroform and ether eluates were combined to give a total extract of 0.3703 g. (0.3% by weight of shale). The i.r. spectrum had absorption at 3400 - 3200 (w), 1690 (m), 1600 (w), 1040 - 1010 (m) and 720 cm^{-1} (m), suggesting the presence of hydroxyl and carbonyl functions. The u.v. spectrum, with intense absorption up to 400 μ , possessed maxima at 270, 300 and 397 μ which suggested the presence of ethers and esters.

The ethyl acetate and methanol eluates were similarly combined and produced a total of 0.2392g. (0.24% by weight of the total shale). The i.r. absorption showed the presence of hydroxyl ($\sim 3300 \text{ cm}^{-1}$, m) and carbonyl groups (1710 - 1650 cm^{-1} , s). (i.r.3., u.v.3).

Preparative t.l.c.

Preparative t.l.c. on silica impregnated with silver nitrate (10% by weight) enabled further fractionation of the hexane eluate. From an aliquot of 122 mg., fraction 1 (85 mg.) was obtained by t.l.c. The Rf. value was identical to that of a standard saturated hydrocarbon mixture (0.75). The remaining 37 mg. of the aliquot were extracted and re-plated on a similar plate but with a benzene/n-hexane solvent system (1.10). Two further fractions were extracted from this plate, fraction 2 (24 mg. Rf. 0.73 - 0.44) and fraction 3 (1 mg., Rf. 0.44 - origin). The gas chromatograms of fractions 1 and 2 (g.c. 3 & 4 respectively) show the presence of a complex mixture.

The infra-red spectrum of fraction 2 (film), (i.r.4) when compared to that of the total hexane eluate, showed that the concentration of unsaturated material had greatly increased. The i.r. spectrum showed absorption at 3100 - 3000 (m), 1610 (w \curvearrowright C=C aromatic), 1510 (w), 1500 (m \curvearrowright C=C aromatic), 975 (w), 880 (w), 850 (w), 815 (m), 675 cm^{-1} (m). The p.m.r. spectrum, recorded on a Perkin Elmer R10⁶⁰ Mg/c spectrometer with deuteriochloroform (CDCl_3) solvent and T.M.S. as internal standard, showed signals at 2.9 - 3.2 τ , 7.7, 8.75 and 9.15 τ - the interpretation of which appears above. The u.v. absorption confirmed the aromatic nature of this fraction (225 μ , benzenoid absorption; 275 μ , electron transfer band). (u.v.4).

Because of the small amount of material available (~ 1 mg.), fraction 3 was not investigated.

The benzene eluate was similarly fractionated by preparative t.l.c., an initial separation using n-hexane as developer affording 13.3 mg. of apparently non-polar (alkane) material (fraction A, Rf. 0.78, g.c.5.) The i.r. absorption (KCl disc) and u.v. absorption spectra of this fraction showed the presence of fully saturated compounds possessing un-substituted hydrocarbon chains (i.r. absorption, 730 and 720 cm^{-1} , m, - $(\text{CH}_2)_n$ - rock) (i.r.7). Further separation of the residue from the first chromatoplate was achieved by re-plating with benzene/n-hexane (1.10) as developer, to give fraction C (16 mgs. Rf. 0.0) and a less polar fraction (61 mgs.). The latter fraction was extracted and further separated using benzene/n-hexane (1.20) developer, yielding fractions D (6.4 mg., Rf. 0.2 - 0.37), E (8.6 mg., Rf. 0.37 - 0.6), and a third, more polar fraction (49 mg.). The latter fraction after re-plating with a benzene/n-hexane (1.10) solvent system afforded fractions F (20 mg.), G (23 mg.) and H (~ 1 mg.).

The i.r. absorption spectrum of fraction C (film) (i.r.8) had absorption at 3050 (w C-H), 1710 (w), 1600 (w, Δ C=C aromatic), 1510 (w, Δ C=C aromatic), 870 (w), 810 (m), 672 (w) cm^{-1} . The u.v. spectrum showed absorption at 220, 255, 260 and 290 μ . (u.v.5). The i.r. spectrum of fraction D (CCl_4 solution) suggested the presence of alkanes, a conclusion not in agreement with the Rf. value. The i.r. absorption recorded on a thin film showed a similar pattern except for absorption between 790 and 760 cm^{-1} , possibly due to trapped CCl_4 . The u.v. spectrum, with absorption at 210 μ confirmed the absence of aromatics, but suggested the presence of olefinic material. The i.r. spectrum of fraction E (CCl_4 solution) had absorption at 1730 (m, Δ C=O, ester), 1610 (w), 1290 (m), 1120 (w), 1075 (w), 1040 (w), 950 (w), and 862 (m) cm^{-1} . (i.r.9). The u.v. spectrum, with absorption at 210 and 236 μ , suggested the presence of conjugated olefins. (u.v.6). The u.v. spectrum of the combined fractions F, G & H showed intense absorption with peaks at 235, 260 and 378 μ . (u.v.7). The i.r. absorption of fraction F (film) occurred at 3060 (w, Δ C-H), 1610 (m), 1585 (m), 1160 (w), 1095 (m), 870 (w), 810 (m), 775 (m), 720 (w) cm^{-1} . The i.r. spectrum of fraction G (film) showed absorption at 3060 (w), 1700 (m) and 1600 cm^{-1} (w),

the remaining part being masked by contaminating silicone oil. Fraction H (film) showed i.r. absorption at 3070 - 3020 (w), 1610 (w), 1510 (w), 810 (m) and 675 (w). (i.r. 10, 11, & 12 respectively).

Preparative g.l.c.

Sixteen components of fraction 1 were isolated and collected by preparative g.l.c. employing a 20' x $\frac{1}{8}$ " i.d. stainless steel column packed with 3% S.E. 52 on Chromosorb W(100 - 120 mesh), acid washed and silanized. The column temperature was programmed from 100° - 300°C at 10°/m.m. with a carrier gas flow (helium) of 60 ml/min. The selected components are shown on chart g.c.3. Sufficient of each component was collected for micro i.r. spectra by repeated collections using the same capillary for corresponding peaks in the chromatogram. (~40 µg required).

The i.r. absorption spectra of the trapped fractions were recorded on carbon tetrachloride solutions (~40 µg/4µl) with a cell path of 0.5 m.m. (micro cell).

G.C.M.S. examination of the fractions was made using the L.K.B. 9000 gas chromatograph - mass spectrometer. A 10' x 3 m.m. i.d. glass column packed with 1% S.E. 30 on Gas Chrom P (100 - 120 mesh) acid washed and silanized. The carrier gas, helium, had a flow rate of 30 ml/min. The results appear in Table 1.

Nineteen components of fraction 2 (g.c.4) were isolated and collected by preparative g.l.c. under conditions identical to the above. Infra-red spectra were recorded as CCl₄ solutions with a cell path of 0.5 m.m. (micro cell).

An unsuccessful attempt to record the u.v. absorption spectra of individual components employed a u.v. micro-cell (R.I.I.C. cat. No. u.v. 01) of cell path 1 m.m., volume 20 µl. Selected components were subjected to G.C.M.S. examination and the results tabulated in Table 2.

Fraction A was examined by G.C.M.S. directly as the analytical g.l.c. showed an unusually simple mixture of only six components. (g.c.5). Mass spectral analysis allowed the assignment of the structures n - C₂₈ H₅₈, n - C₂₉ H₆₀, n - C₃₀ H₆₂, n - C₃₁ H₆₄, n - C₃₂ H₆₆, n - C₃₃ H₆₈ to the six components (Table 3)

OSMYLATION AND DIOL FORMATION

The osmylation method employed was a scaled down version of one applied to diterpenes⁹⁶. Consequently, the osmylation of a diterpene, iso-kaurene, was carried out in conjugation to that of the olefinic triterpenes, α -lupene, fernene and diploptene.

200 μ g of the olefin were dissolved in 7 μ l of anhydrous diethyl ether in a tapered 10 ml. B10 flask. 150 μ g of osmium tetroxide in 3 μ l. anhydrous ether were added. Excess ether was required to wash material from the sides of the flask. The reaction was allowed to proceed overnight after which a dark precipitate was seen to have formed. The ether was then evaporated off and the residue dissolved in benzene. Hydrogen sulphide was bubbled through the resulting solution for approximately 1 min. to decompose the osmyate ester formed. The solution was then centrifuged and the liquid phase (containing the diol) removed by pipette. Approximately 150 μ g of product was obtained, (estimated by g.l.c. peak areas).

The most simple method of identification of the product was found to be the G.C.M.S. examination of the T.M.Si. ether. The latter was formed by adding N, N-bis(trimethylsilyl)acetamide. It was found impossible to obtain a g.l.c. trace of the product using a 10' x 1/16", 3% S.E.30 column. However, a 6' x 3mm., 1% S.E.30 column enabled a gas chromatogram to be recorded, prior to G.C.M.S. analysis employing an identical column. A blank osmylation showed that no significant contaminating impurities had been included at any stage. Normalised mass spectra of the products are shown (m.s.14 - 20) and the results are summarised in Table 4.

QUANTITATIVE SUBLIMATION OF CHOLESTANE

A sublimation block was machined from a block of Dural alloy. An 80 watt cartridge heater, drawing power through a Variac magnetic transformer, was fitted as shown. The block was machined to allow a lead from a vacuum pump to connect directly (bore 0.25) with a cylindrical chamber (bore 0.06) into which was placed a short capillary tube (~ 30 m.m.) containing the sample. A pin-head thermometer was inserted in an aperture (bore $0.17 \pm \frac{1}{0}$) to one side of the sample tube. The top surface of the block was machined to accept an "O"-ring (bore 0.125), concentric with the capillary tube, on top of which was placed a glass microscope slide to act as a condensation surface.

A quantity of cholestane in a short capillary tube was placed in the sublimation block and the aperture covered with a microscope slide. The system was evacuated by an oil-diffusion pump to a pressure of 0.025 m.m. Hg and the pump then closed off. The rheostat controlling the cartridge heater was set to give a temperature of $\sim 200^{\circ}$ and after 35 minutes the temperature was 210°C . Retaining the vacuum, cooling was allowed for a further 15 minutes. The quantity sublimed was determined by weighing or by estimating the g.l.c. peak size. The melting point of the sublimed material was determined on a Kofler hot-stage incorporating a microscope (Leningrad United Optical-Mechanical Enterprises) of magnification $\times 56$.

The sublimation of an unsaturated triterpene, β -amyrene, was also carried out.

Sublimation Returns:-

(Cholestane) Initial Quantity	Sublimation Return
1.5 mgs.	38%
300 μg	66%
10 μg	70%*

*estimated by g.l.c. peak sizes (g.c.2)

PERKIN-ELMER F 11. DEVELOPMENTHeater for Effluent Column

To reduce peak "Tailing" at higher molecular weights, a heater was designed to prevent partial condensation of the high molecular weight material in the two inches of fixed column joining the end of the oven column to the base of the detector.

Firstly, the two inch column was insulated by a coating of Asbestos paper. Approximately two feet of 15% Vacrom wire (0.025 x 0.002, resistance 35 ohms/yard) was then wound over the insulation and the terminals connected to external leads by hard soldering with tinned copper. The power was supplied through a 12 volt variable transformer.

The g.l.c. traces (6 & 7) show the reduction of peak "tailing" brought about by incorporation of the heater. The possibility of the temperature being too high and causing cracking of compounds was investigated by W. Henderson of this group. It was found that the heater merely kept the temperature lag of the effluent column behind the programmed oven temperature to a minimum.

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Extraction and Separation of Green River Shale

Flow Diagram

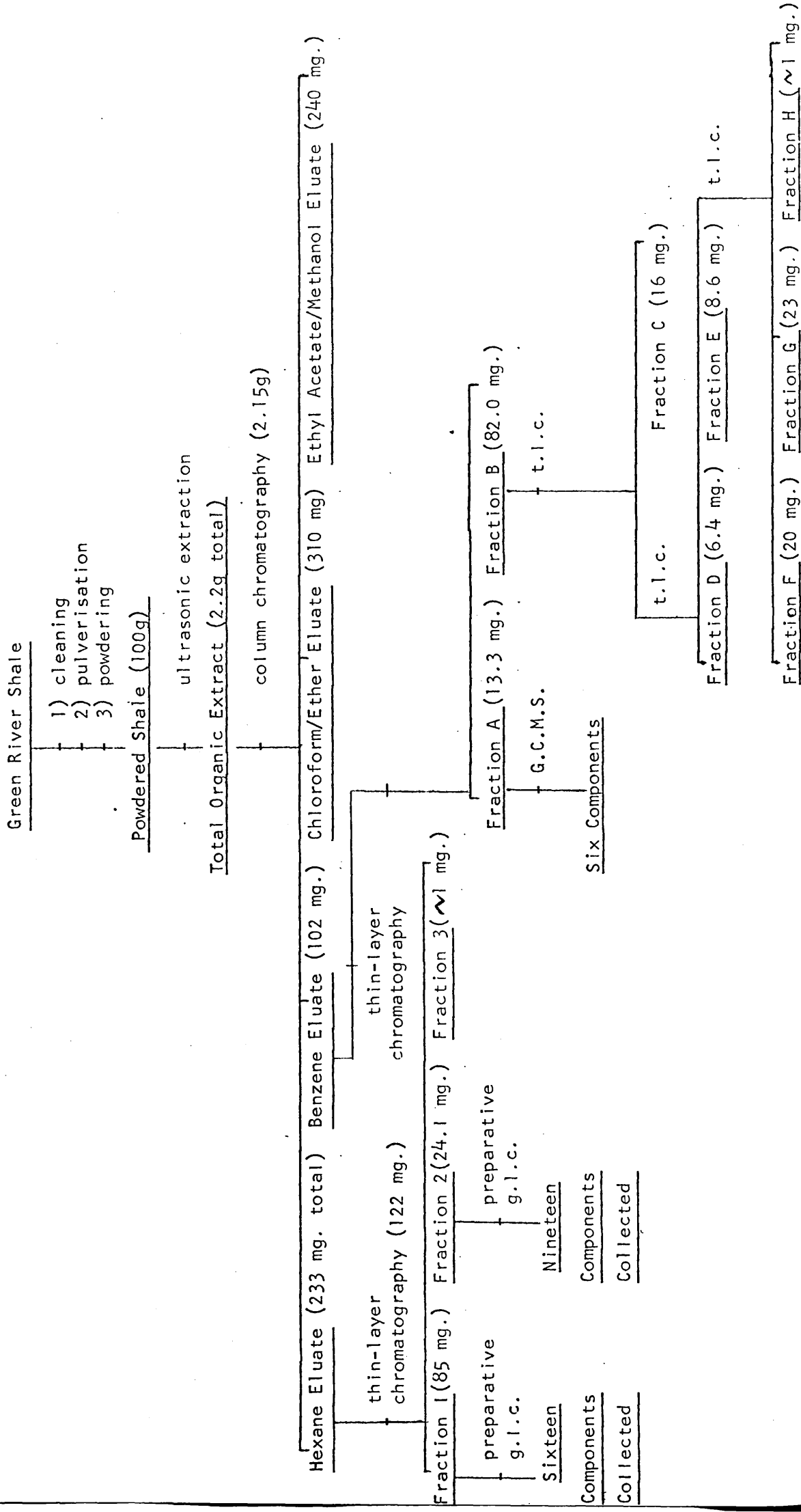


TABLE 1
COMPOUNDS ISOLATED FROM FRACTION 1.

Component	Molecular Ion (m/e)	Formula	Base Peak (m/e)	Comments
1.	372	C ₂₇ H ₄₈		a mixture containing "cholestane" 4 d.b.e.
2.	360	C ₂₆ H ₄₈	191	appears to be tetranor triterpene. tricyclic.
3.	380	C ₂₇ H ₅₆	57	a normal hydrocarbon.
4.	394	C ₂₈ H ₅₈	57	a normal hydrocarbon.
5.	366	C ₂₆ H ₅₄	57	a normal hydrocarbon.
8.	386	C ₂₈ H ₅₀		"ergostane"
10.	1)400 2)458	1)C ₂₉ H ₅₂ 2)C ₃₃	1) 2) 69	1) "sitostane" 2) contains a steroidal nucleus.
11.	412	C ₃₀ H ₅₂		"gammacerane" contaminated by a steroidal nuclear.
12.	412	C ₃₀ H ₅₂	191	a triterpene, possibly moretane.
13.	428	C ₃₁ H ₅₆	191	a tetracyclic triterpene.
15.	1)434 2)478	1)C ₃₁ H ₆₂ 2)C ₃₄ H ₇₀	1) 191 2) 157	
16.	558	C ₄₀ H ₇₈		"carotane"

TABLE 2

COMPOUNDS ISOLATED FROM FRACTION 2.

Component	Molecular Ion (m/e)	Formula	Base Peak (m/e)	Comments
1.	174	C ₁₃ H ₁₈	159	5 double bond equivalents : aromatic i.r. absorption
2.	202	C ₁₅ H ₂₂	119	5 double bond equivalents : -
3.	216	C ₁₆ H ₂₄	115	5 double bond equivalents : aromatic i.r. absorption
5A.	272	C ₂₀ H ₃₂	119	5 double bond equivalents : aromatic i.r. absorption
6.	286	C ₂₁ H ₃₄	69	5 double bond equivalents : aromatic i.r. absorption
7.	1) 300 2) 296	C ₂₂ H ₃₆ C ₂₂ H ₃₂	119 145	5 double bond equivalents : aromatic i.r. absorption
8.	1) 280	C ₂₁ H ₂₈	170 119	8 double bond equivalents : -
8A.	280	C ₂₁ H ₂₈	170	8 double bond equivalents : aromatic i.r. absorption
10.	1) 310 2) 354	C ₂₃ H ₃₄ C ₂₆ H ₄₂	157 157	1) 7 double bond equivalents : aromatic i.r. absorption 2) 6 double bond equivalents : aromatic i.r. absorption
17.	410	C ₃₀ H ₅₀	135	6 double bond equivalents : triterpene. non-aromatic trisub/trans double bond

TABLE 3

COMPOUNDS ISOLATED FROM FRACTION A.

Components	Molecular Ion (m/e)	Formula	Base Peak (m/e)	Comments
1.	394	C ₂₈ H ₅₈	57	normal hydrocarbon
2.	408	C ₂₉ H ₆₀	57	normal hydrocarbon
3.	422	C ₃₀ H ₆₂	57	normal hydrocarbon
4.	436	C ₃₁ H ₆₄	57	normal hydrocarbon
5.	450	C ₃₂ H ₆₆	57	normal hydrocarbon
6.	464	C ₃₃ H ₆₈	57	normal hydrocarbon

TABLE 4

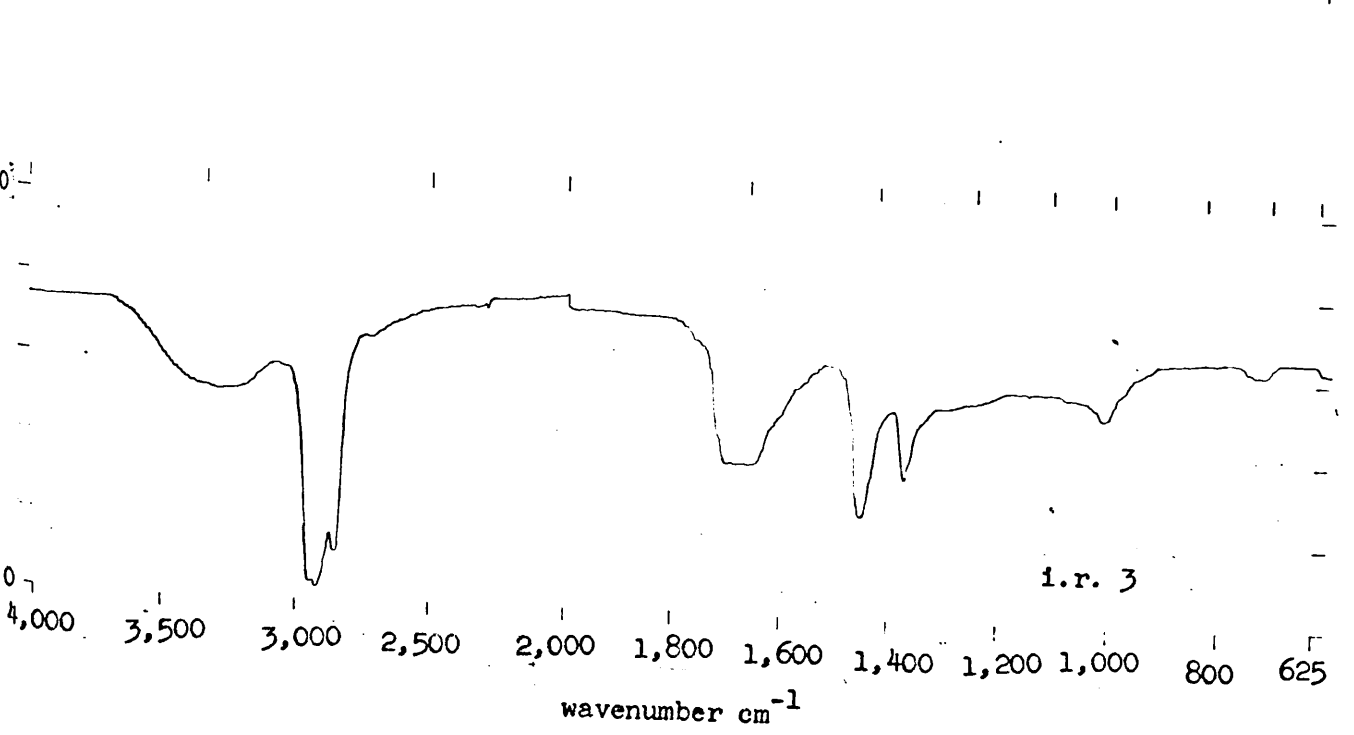
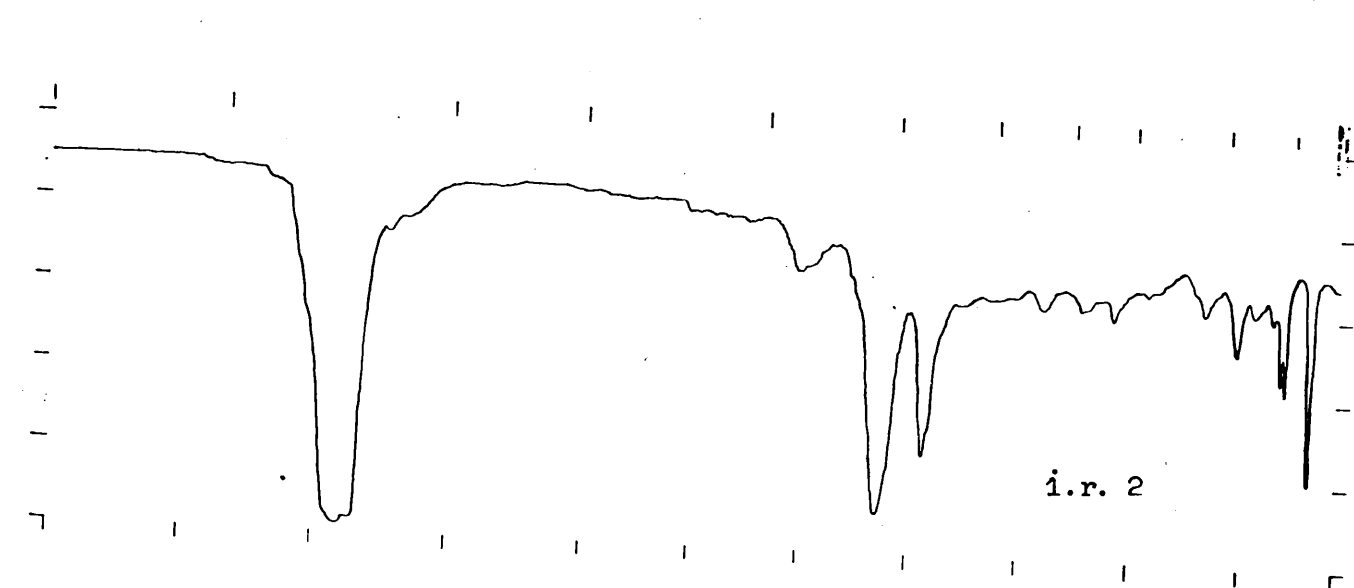
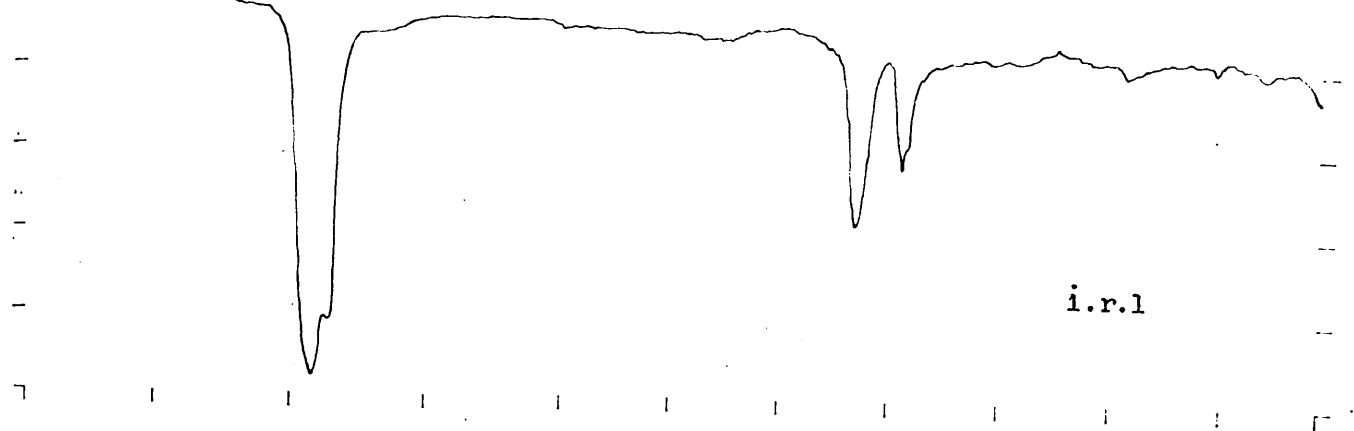
MAJOR OSMYLATION PRODUCTS.

Compound	Major Component		Other prominent components
	Molecular Ion (m/e)	Base Peak (m/e)	
diploptene	410	191	-
α -lupene	410	69	-
iso-karene T.M.Si.	378	73	450 : 360
fernene T.M.Si.	410	395	516
diploptene T.M.Si.	516	147	-
α -lupene T.M.Si.	516	191	-

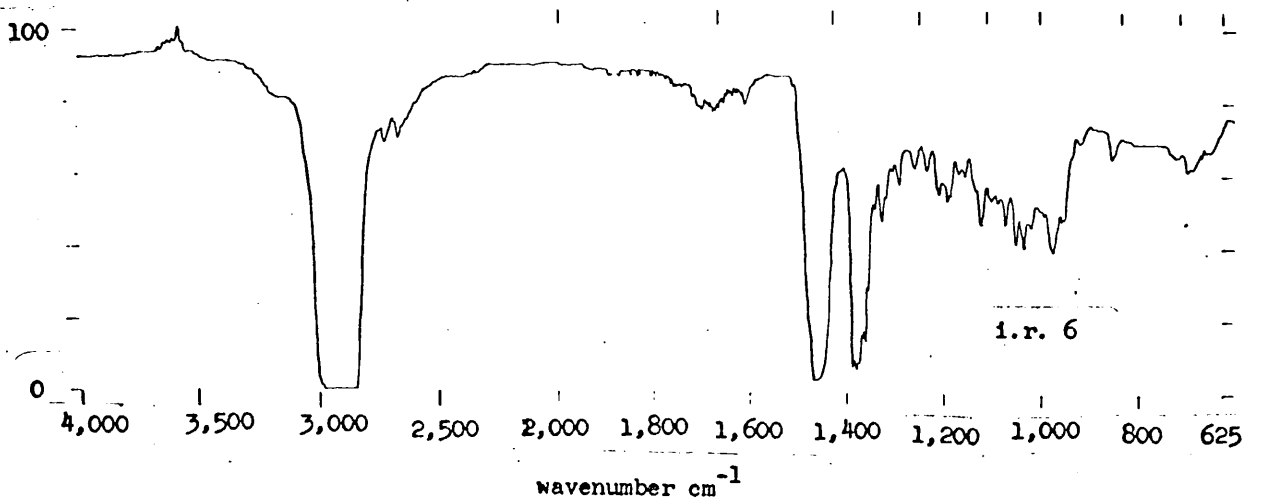
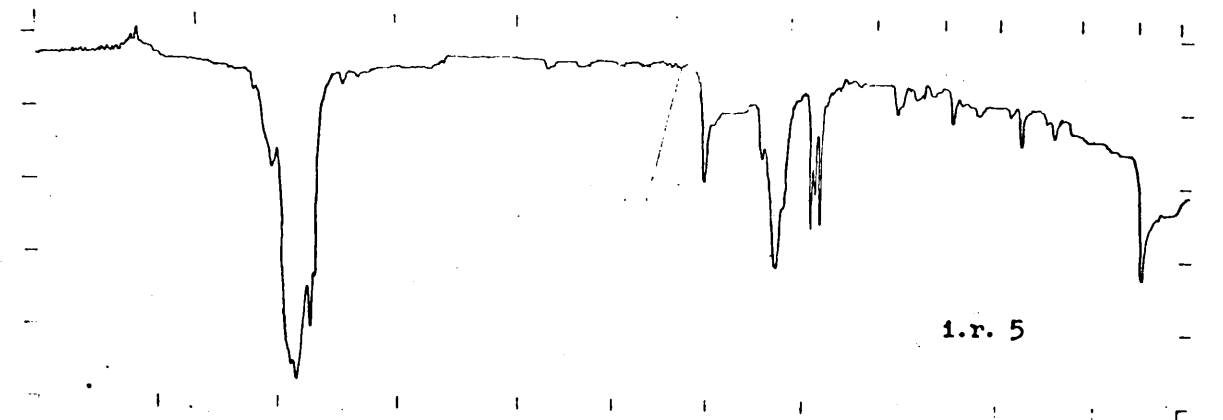
Legend for Infra-Red Spectra

- i.r.1. - Total hexane eluate, recorded as a thin film on a P.E.257 instrument; fast scan; normal slit.
- i.r.2. - Total benzene eluate, conditions as in i.r.1.
- i.r.3. - Ethylacetate/methanol eluate, " " " " .
- i.r.4. - Fraction 2, conditions as in i.r.1.
- i.r.5. - Fraction 2 Component iii; ca. 40 μ g in ca. 4 μ lCCl₄ solution; 0.5 m.m. cell path (beam condenser); conditions otherwise same as i.r.1.
- i.r.6. - Fraction 2 Component xvii; conditions as in i.r.5.
- i.r.7. - Fraction A, recorded as a KCl disc, conditions otherwise same as i.r.1.
- i.r.8. - Fraction C, conditions as in i.r.1.
- i.r.9. - Fraction E, " " " "
- i.r.10. - Fraction F, " " " "
- i.r.11. - Fraction G, " " " "
- i.r.12. - Fraction H, " " " "

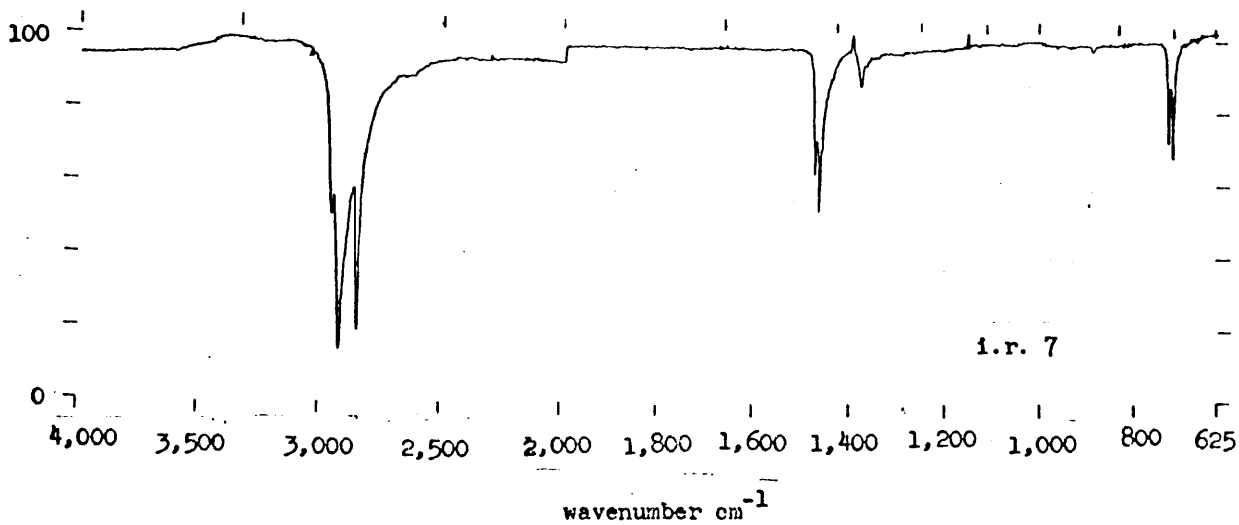
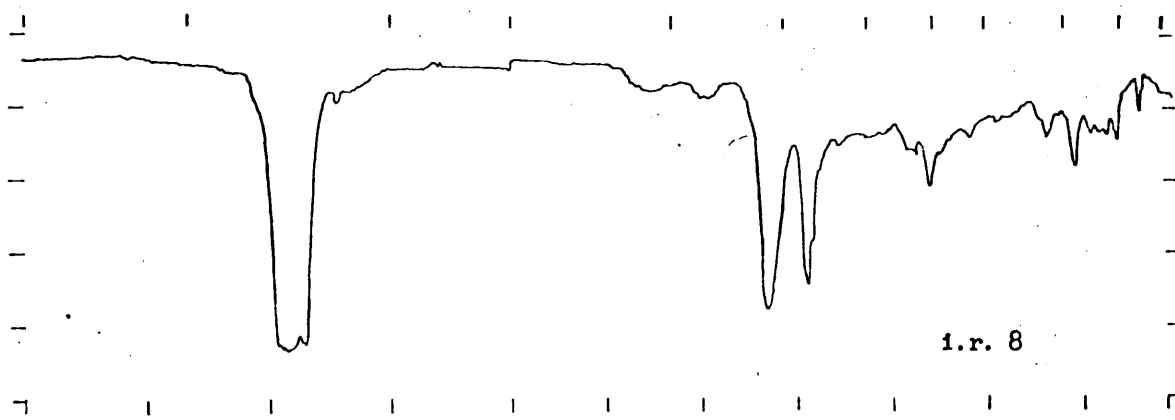
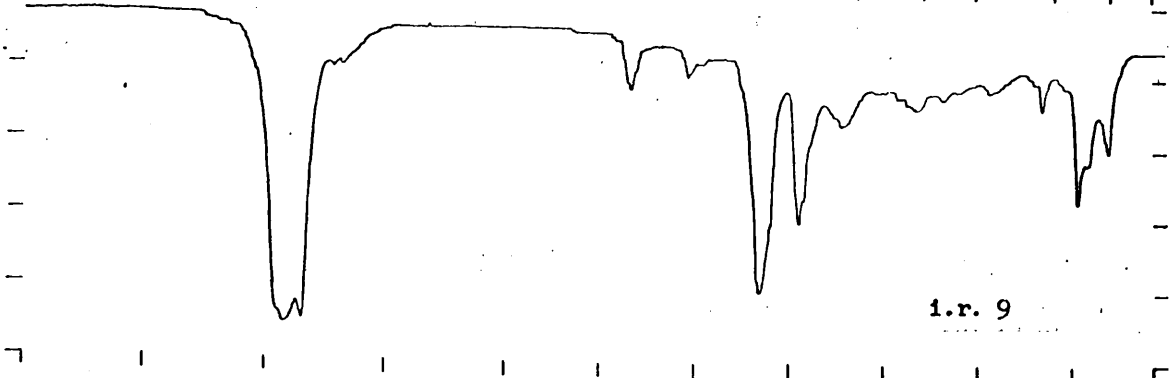
microns
2.5 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10 12 14 16

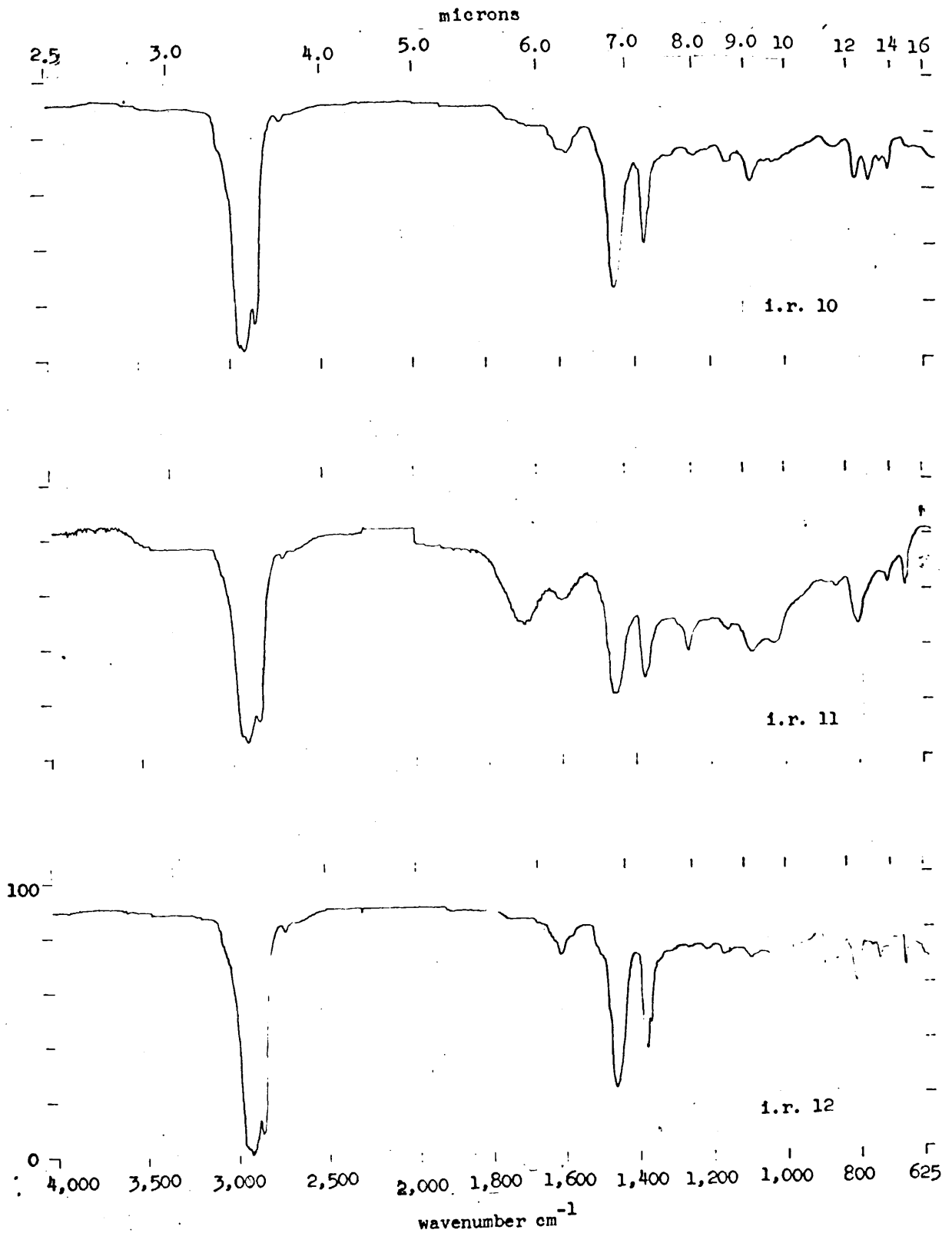


2.5 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10 12 14 16
Microns



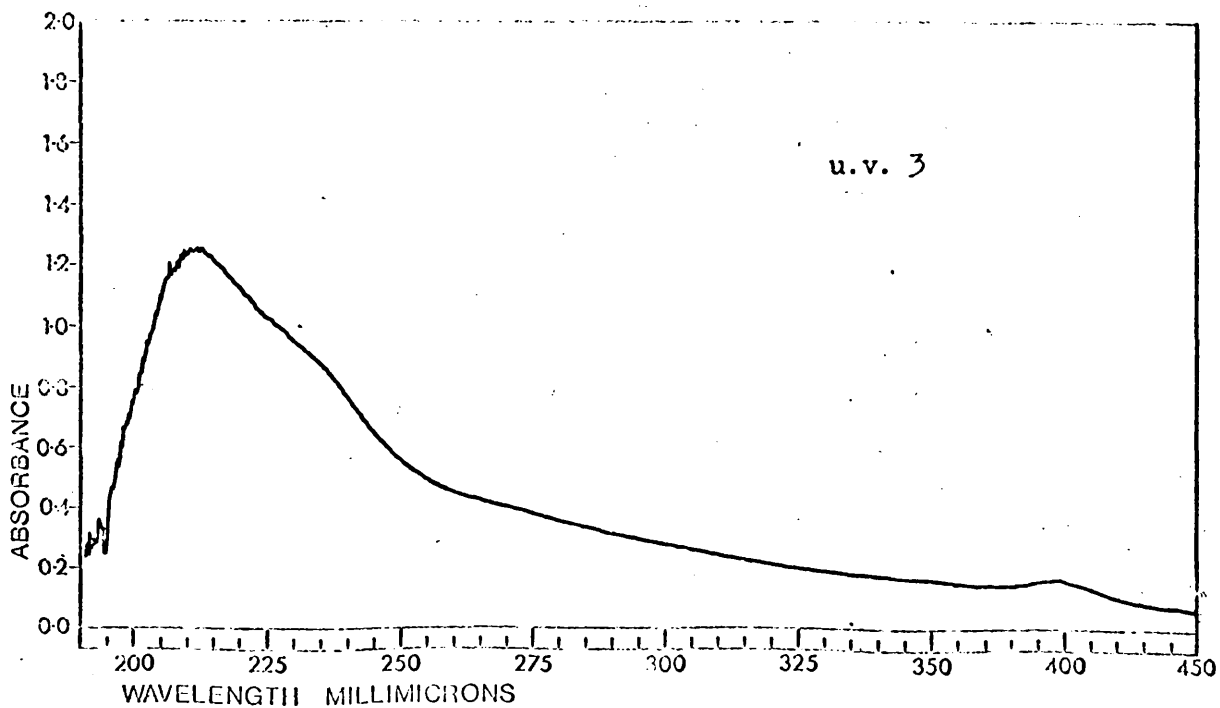
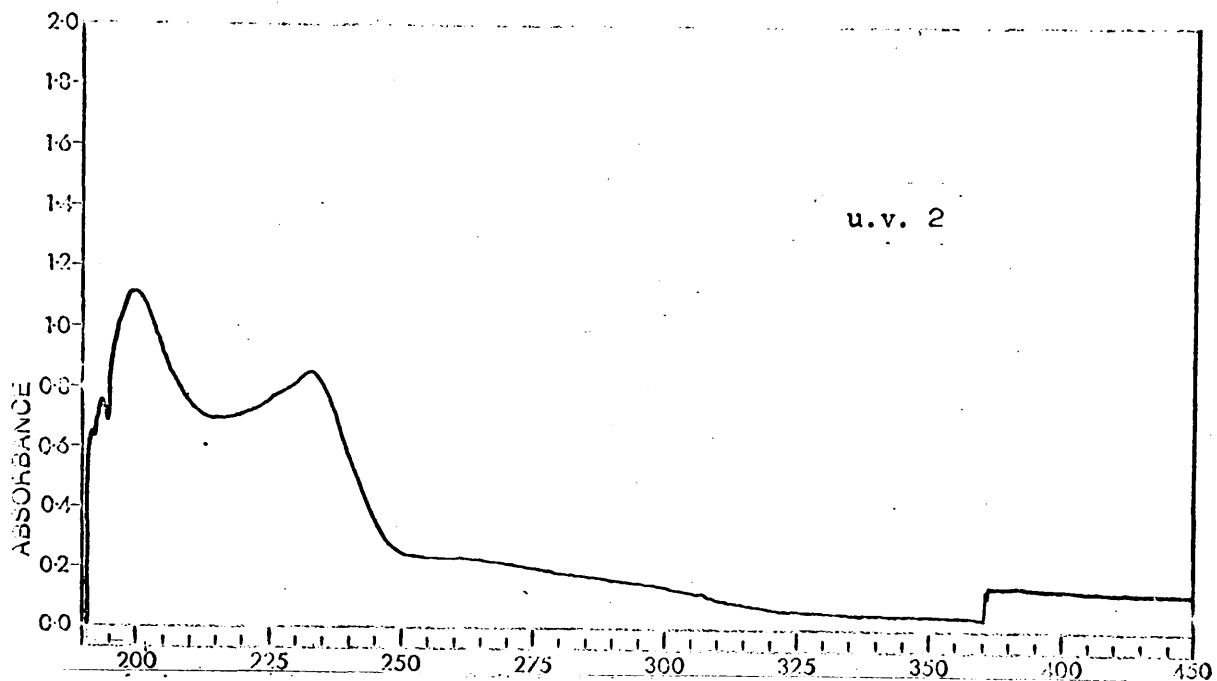
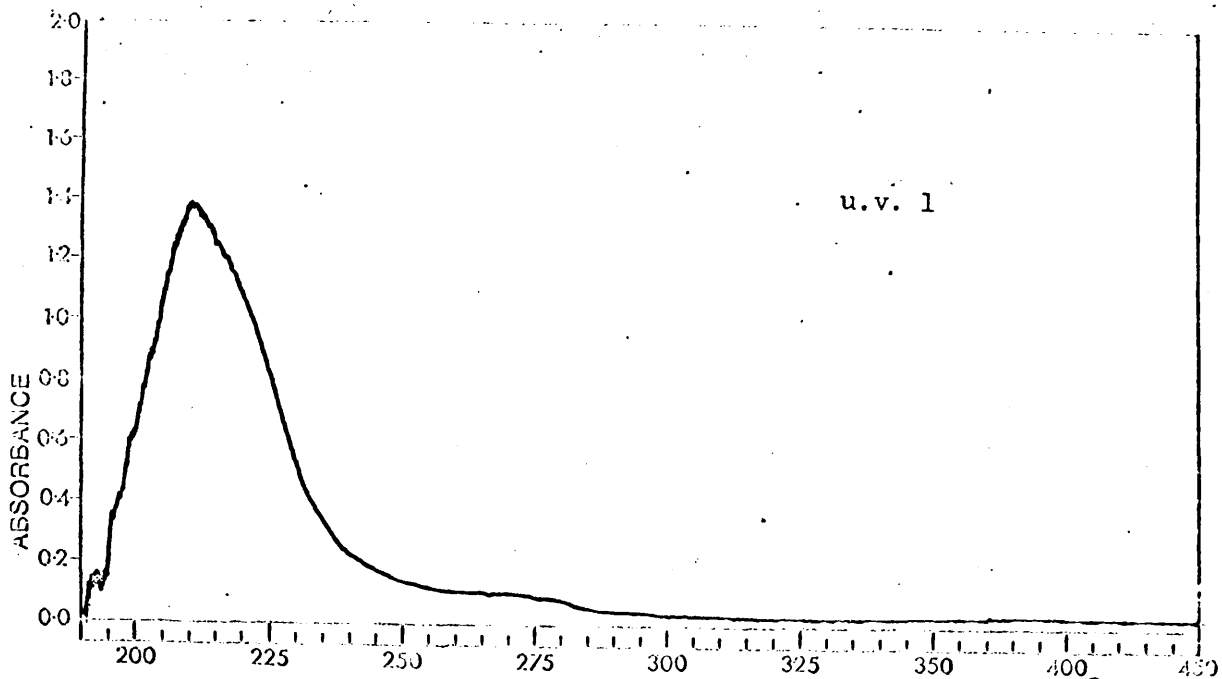
microns
2.5 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10 12 14 16

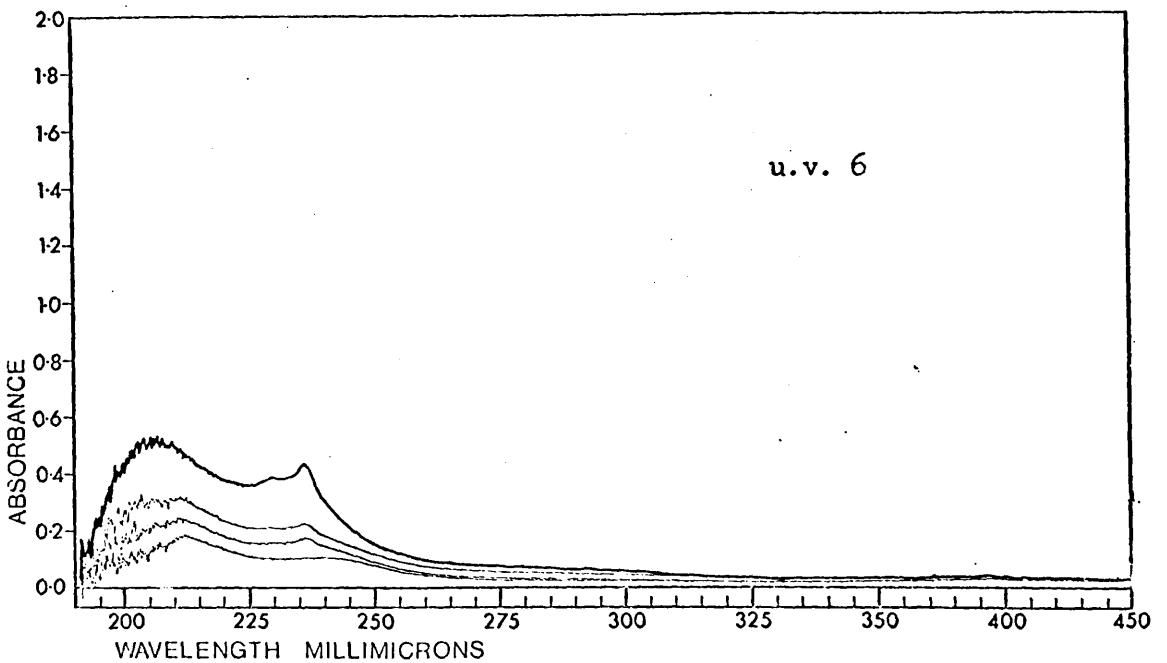
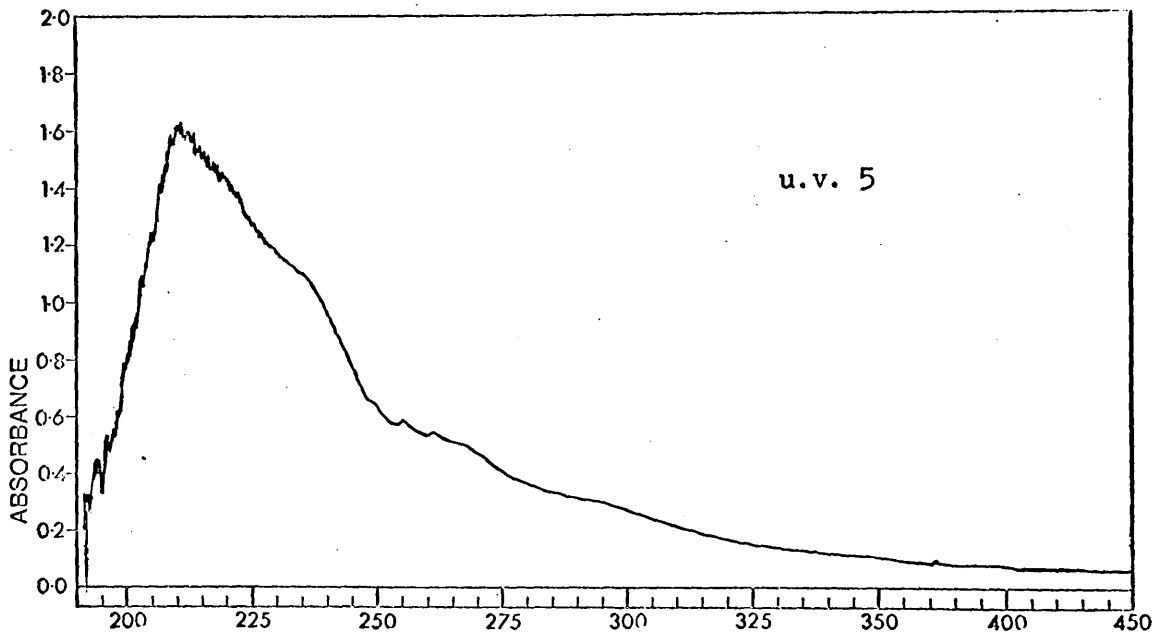


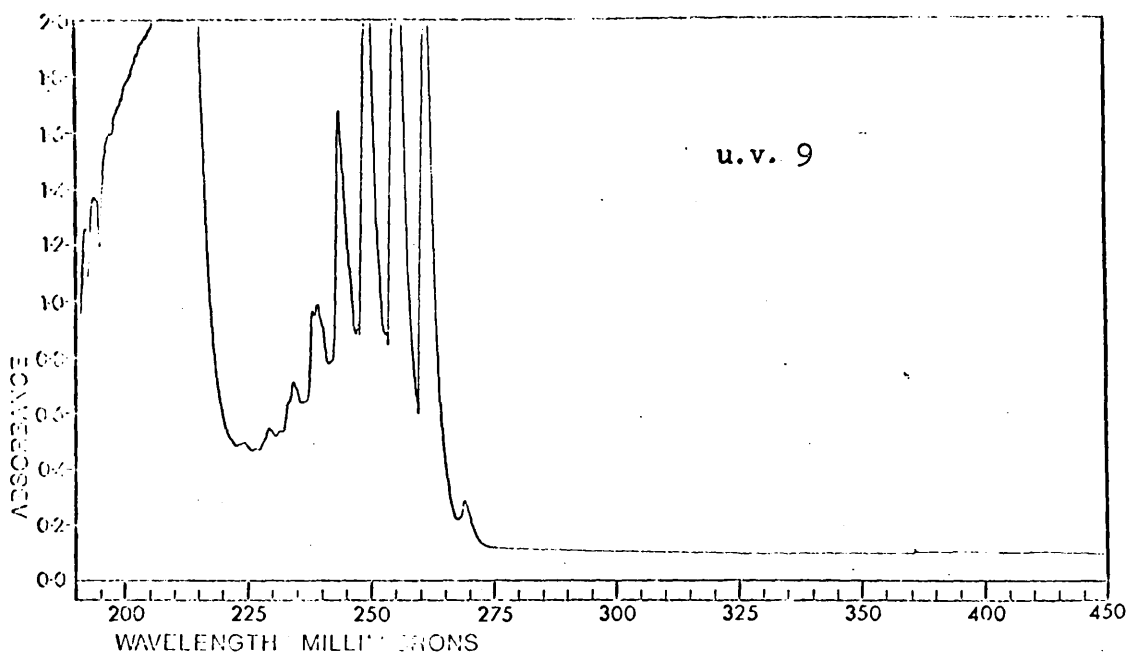
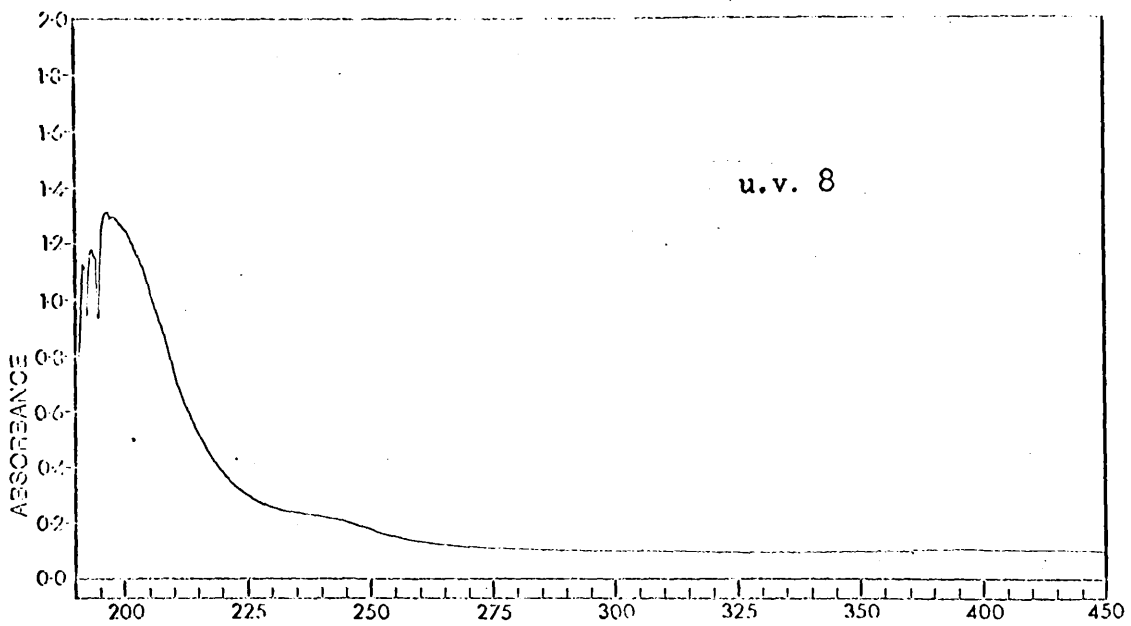
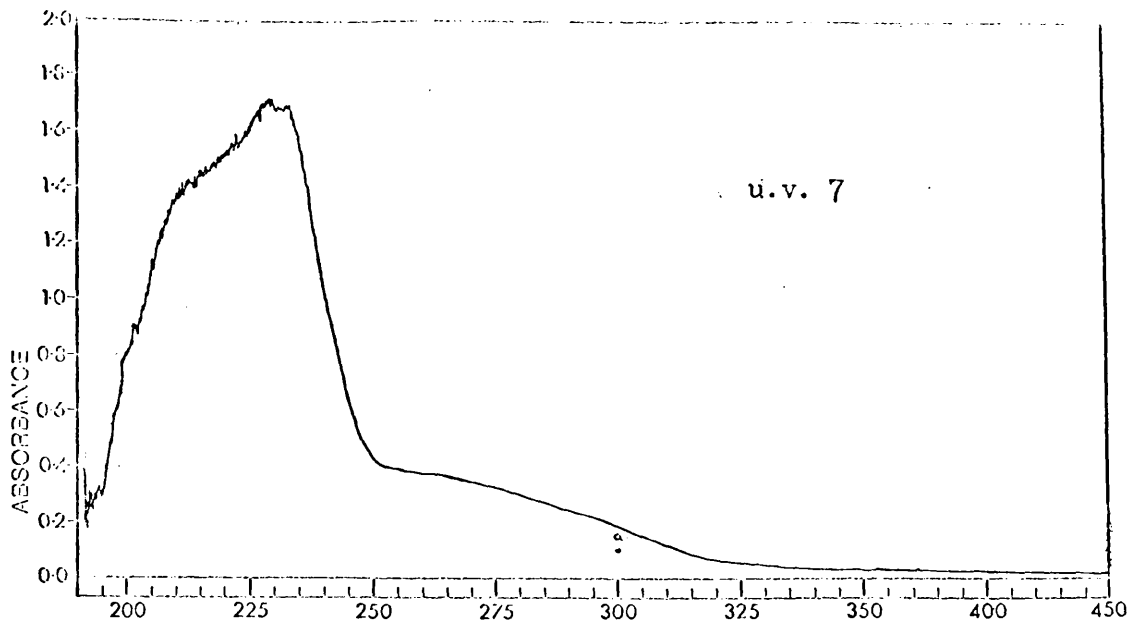


Legend for Ultra-Violet Spectra

- u.v.1. - Unicam SP800, slit width 0.002 m.m., fast scan, 10 m.m. cell path, ~ 0.5 mg/cell.
- u.v.2. - Conditions as for 1.
- u.v.3. - " " " " but 0.12 mg/cell.
- u.v.4. - " " " " but 1 m.m. cell path.
- u.v.5. - " " " " but 0.96 mg/cell.
- u.v.6. - " " " " but 8.6 mg/cell.
- u.v.7. - " " " " but 0.062 mg/cell.
- u.v.8. - " " " ".
- u.v.9. - " " " ".

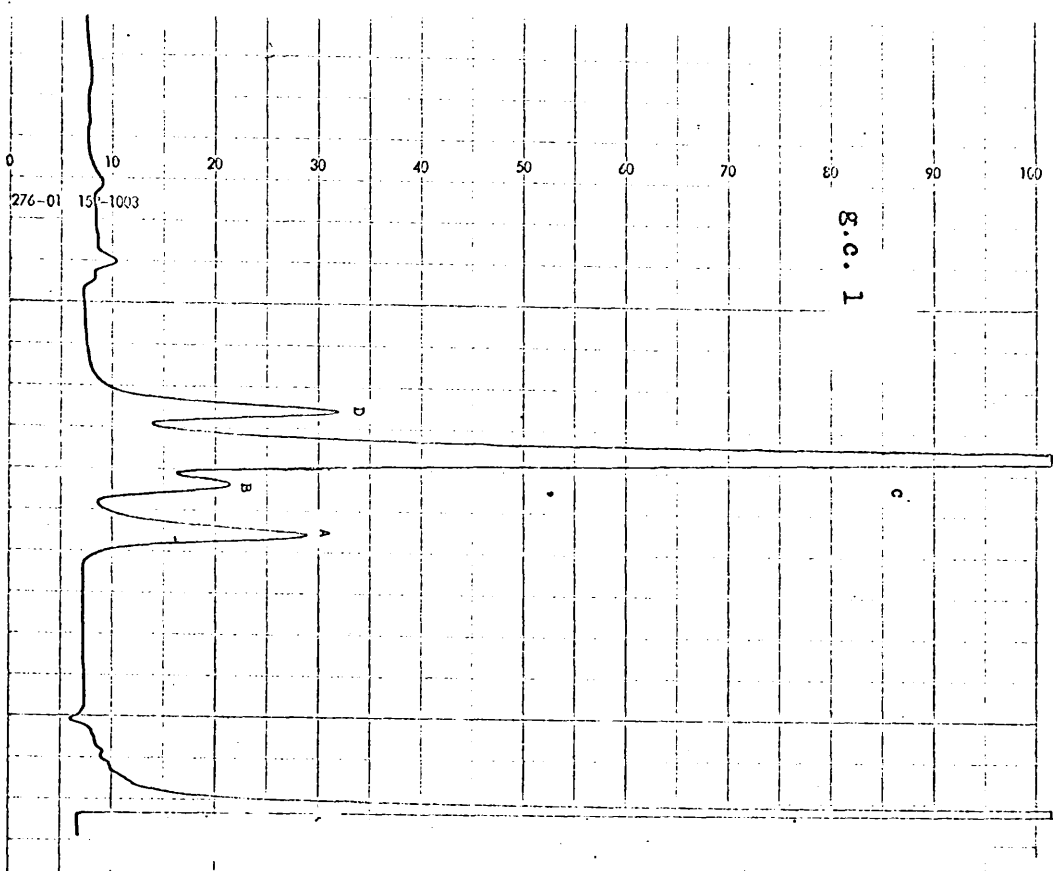




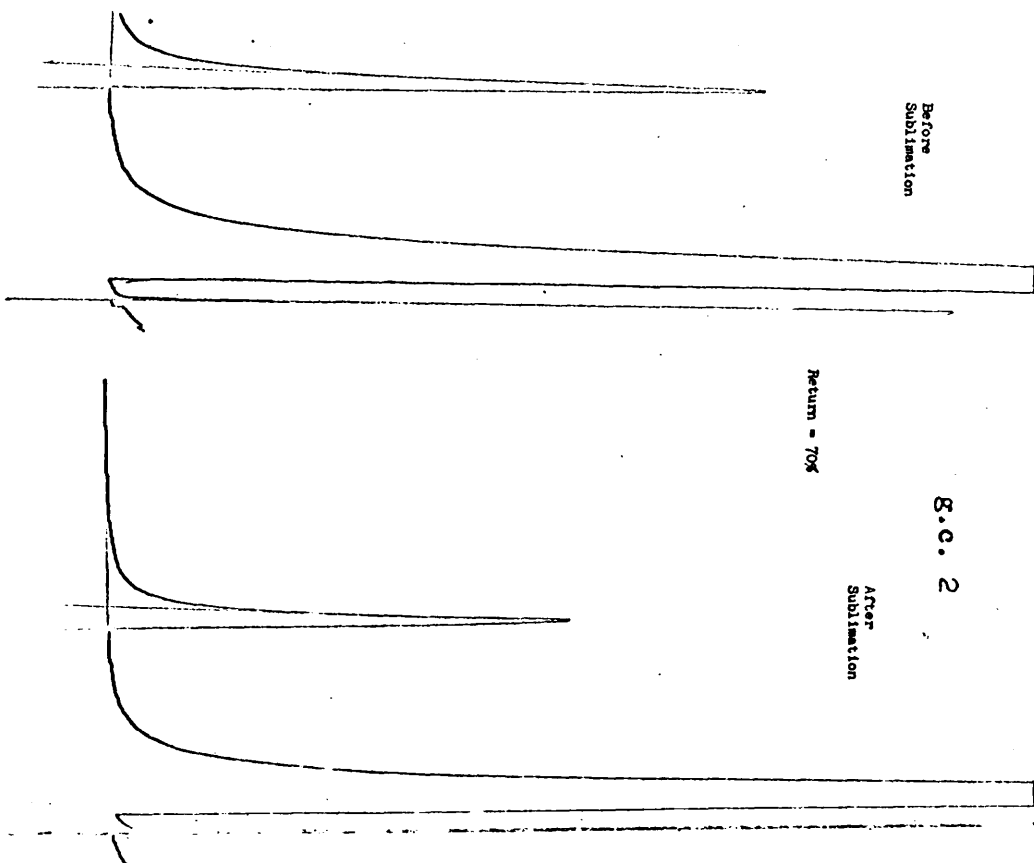


Legend for Gas-Chromatograms

- g.c.1. - Column 6' x $\frac{1}{4}$ " o.d. fitted to a P.E.F11 Dual Column; 1% S.E.30 on Gas-Chrom P, 100 - 120 mesh; N₂ carrier gas at 20 p.s.i.; temperature programmed from 100 - 280° at 50°/min; sample size 1 μ l of a solution in diethyl ether; attenuation 5 x 10²; chart speed medium (23.6"/hr.)
- A = Isophyllocladene, B = Phyllocladene, C = Isokaurene,
D = Kaurene.
- g.c.2. - Column 10' x 1/16" fitted to a P.E.F11; 3% S.E.30 on Gas-Chrom P, 100 - 120 mesh; N₂ carrier gas at 30 p.s.i.; temperature isothermal 250°; sample size 0.2 μ l of a solution in n-hexane; attenuation 20 x 1; chart speed 15"/hr.

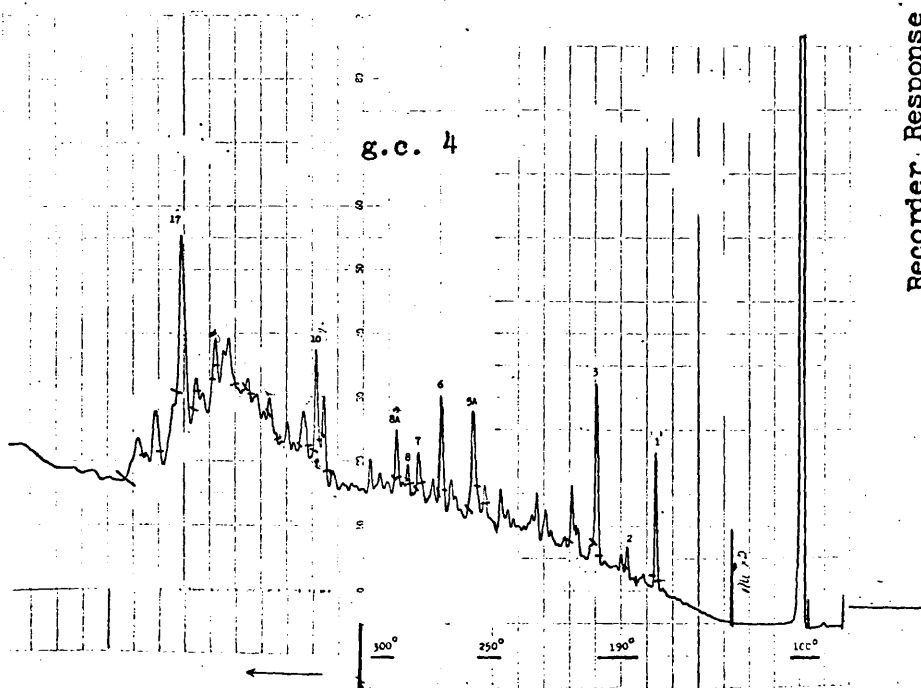
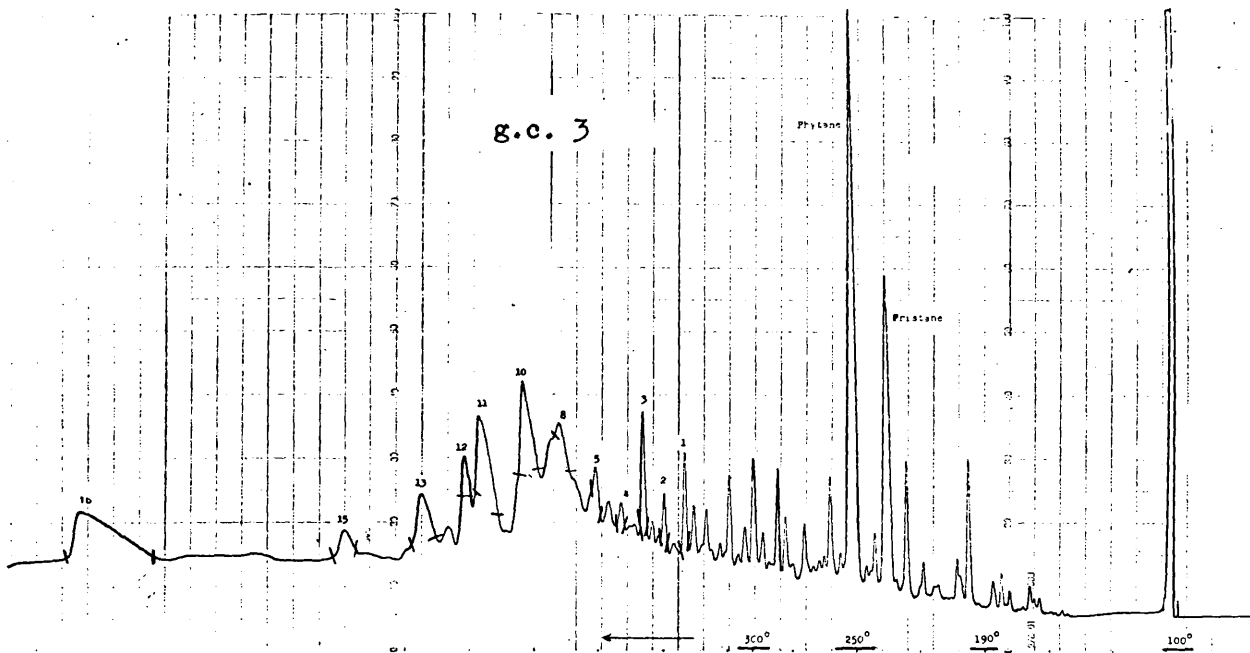


Recorder Response



Legend for Gas-Chromatograms

- g.c.3. - Column 20' x $\frac{1}{8}$ " fitted to Aerograph A 90 P3; 3% S.E.52 on Gas-Chrom P, 80 - 100 mesh; N₂ carrier gas at 60 ml/min; temperature programmed from 100 - 300° at 10°/min.; sample size 10 μ l of a solution in n-hexane; attenuation x 4; chart speed medium (23.6"/hr.)
- g.c.4. - Conditions as for g.c.3. except benzene used as solvent.

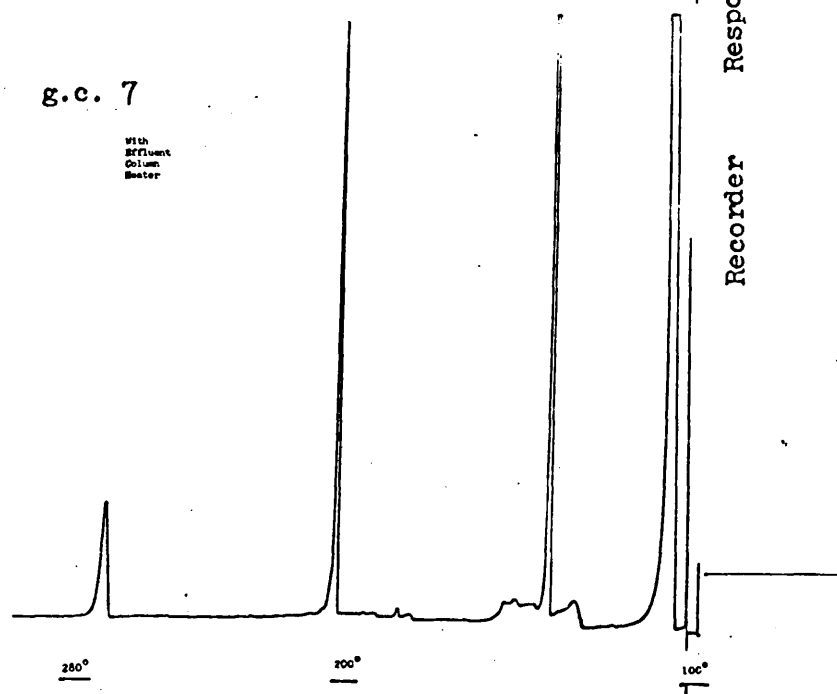
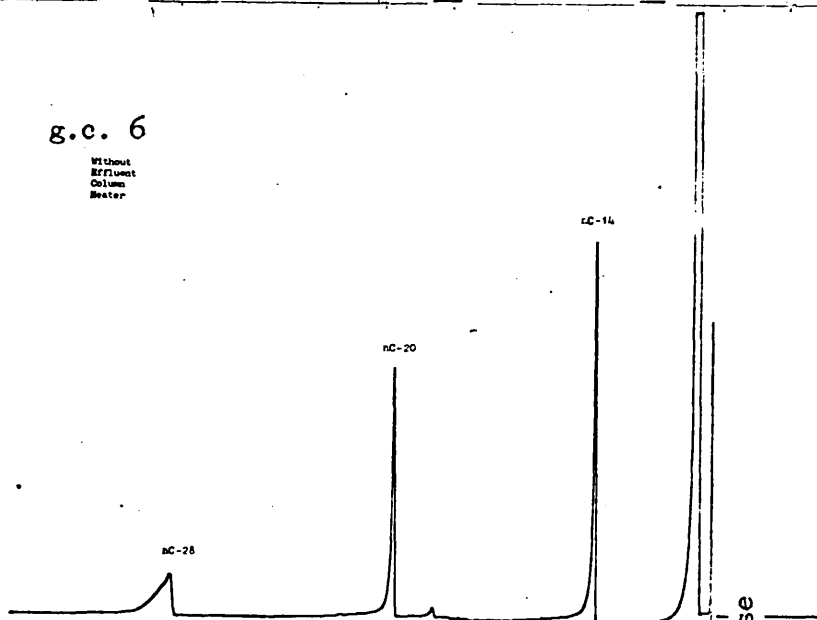
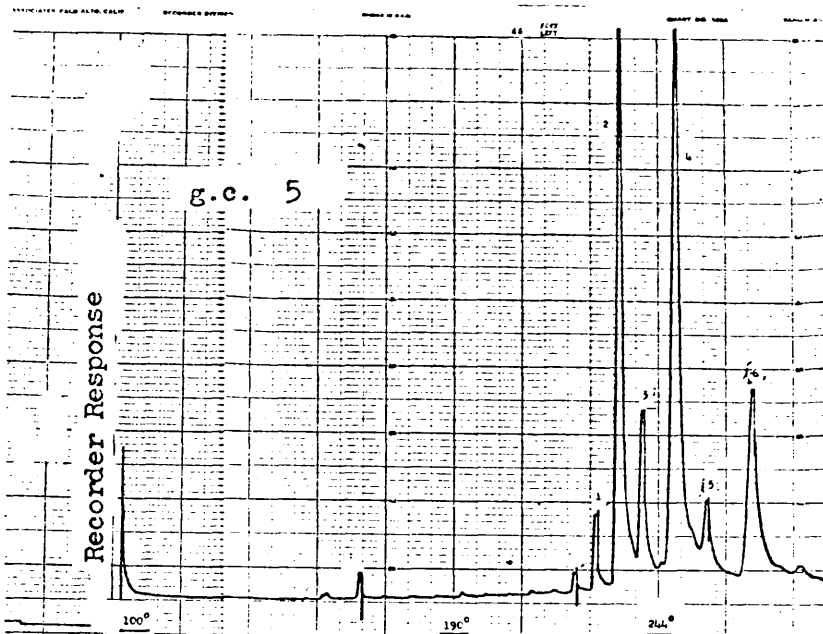


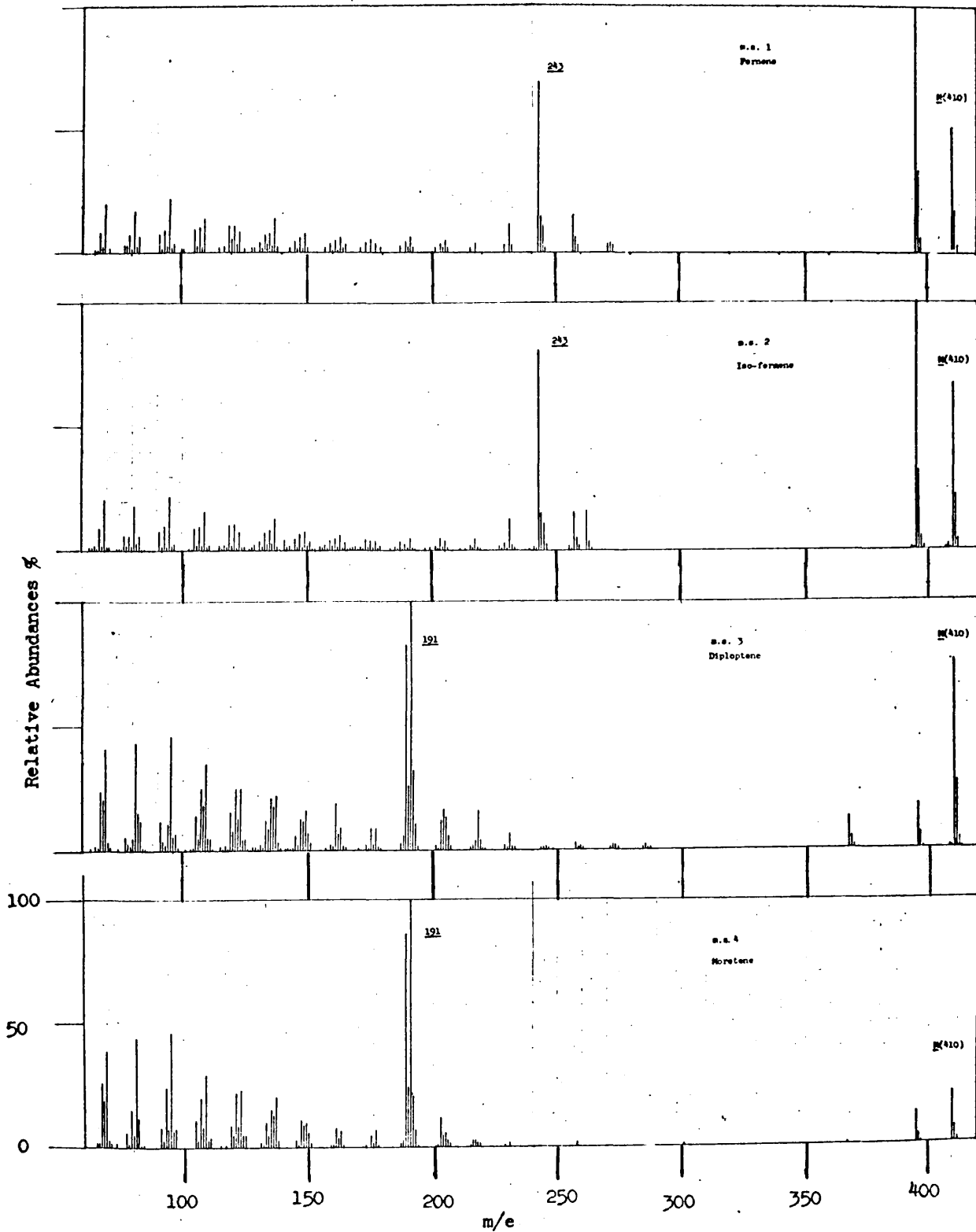
Recorder Response

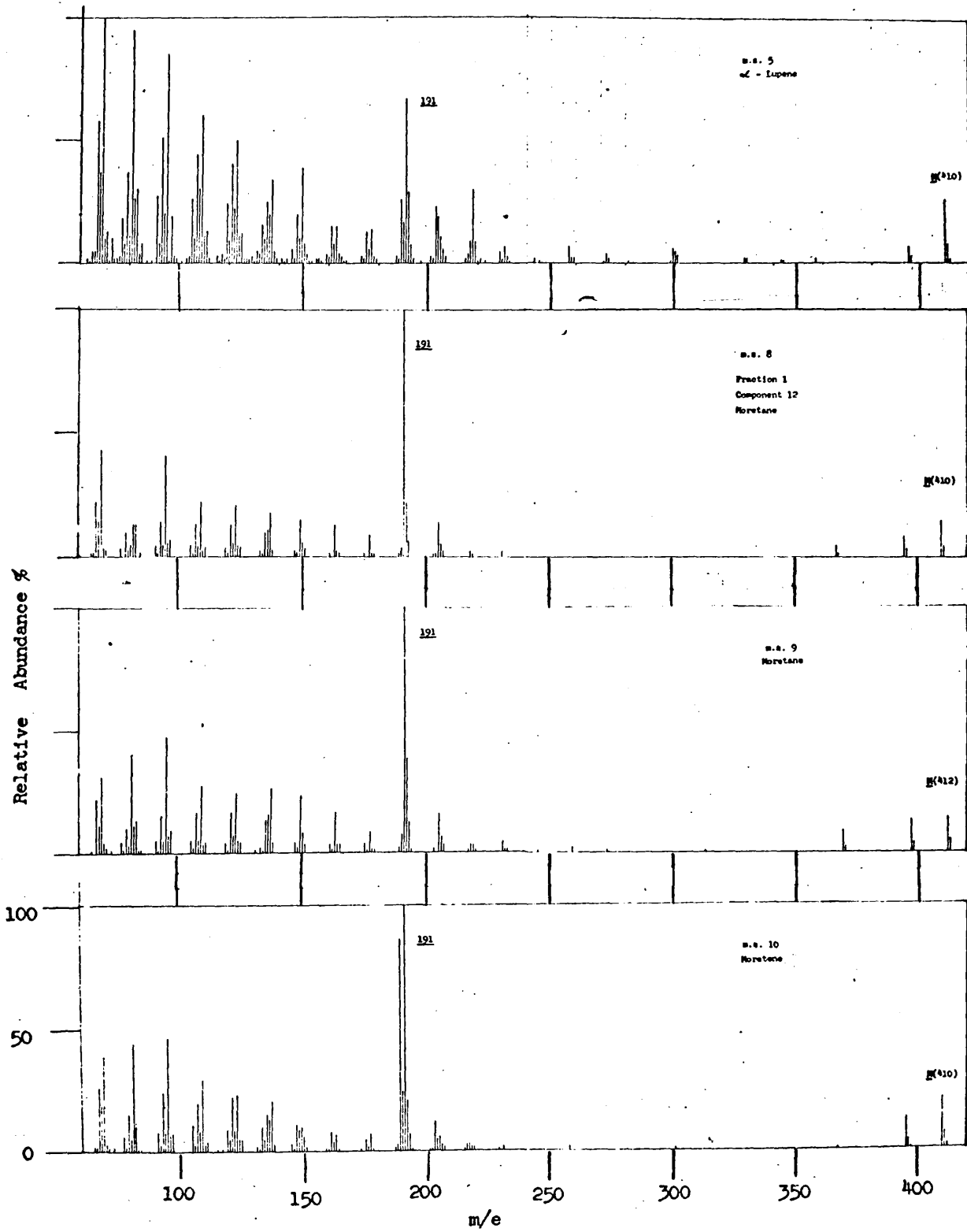
Legend for Gas-Chromatograms

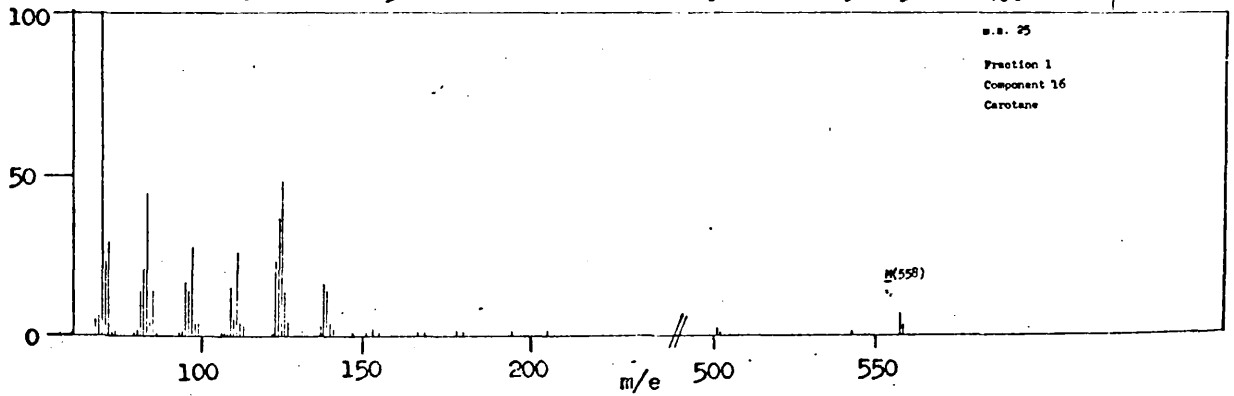
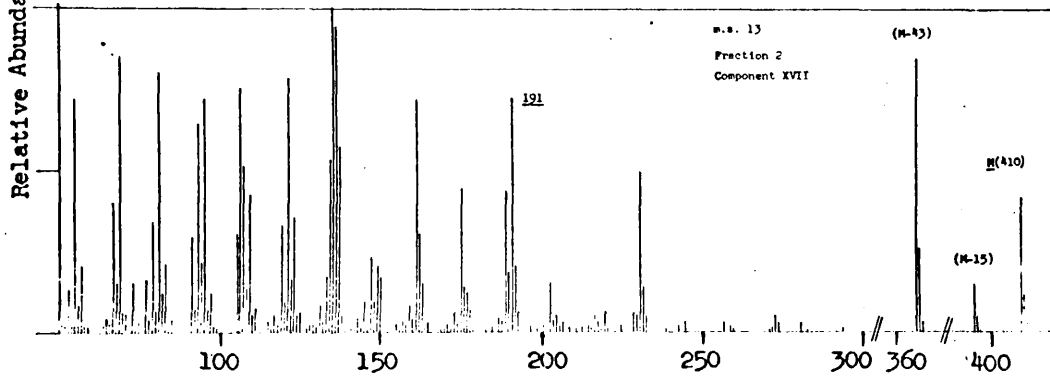
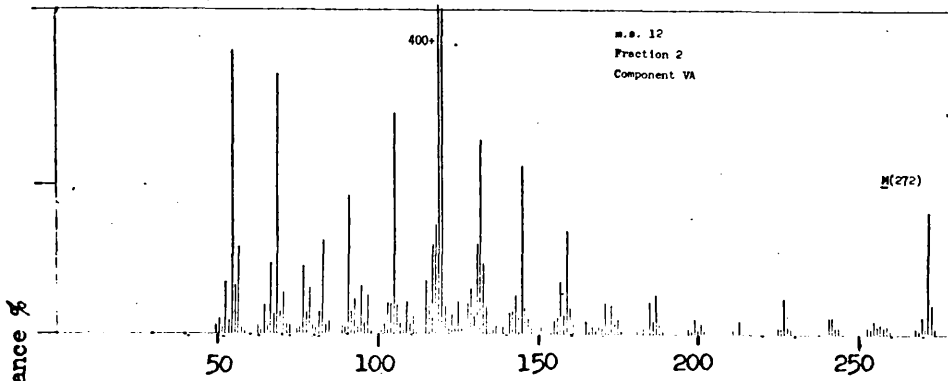
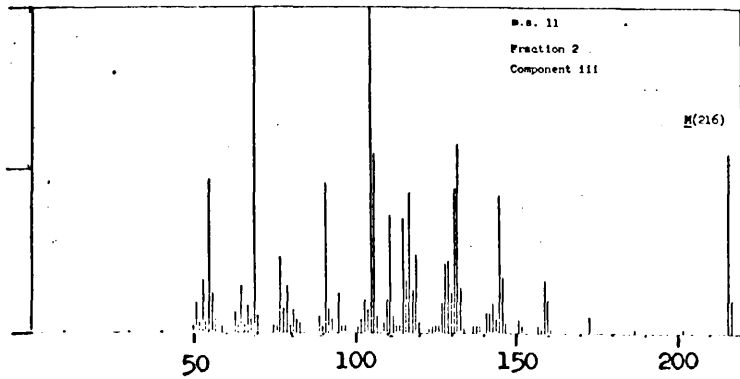
g.c.5. - Column 10' x 3 m.m.i.d. fitted to L.K.B. 9000; 3% D.V.1 on Gas-Chrom Q, 100 - 120 mesh; He carrier gas at 30 ml/min.; temperature programmed from 100 - 250° at 6°/min.; sample size 0.3 μl of a solution in n-hexane; sensitivity x 2000.

g.c.6 & 7 Column 10' x 1/16" fitted to a P.E.F11; 3% S.E.30 on Gas-Chrom P, 100 - 120 mesh; N₂ carrier gas at 30 p.s.i.; temperature programmed from 100 - 250° at 5°/min; sample size 0.2 μl of a solution in n-hexane; attenuation 50 x 1; chart speed 15"/hr.

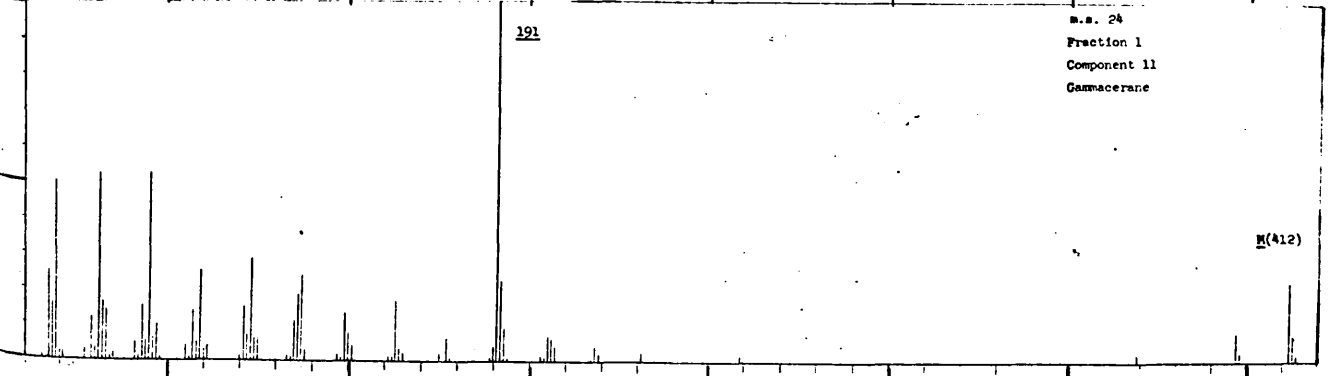
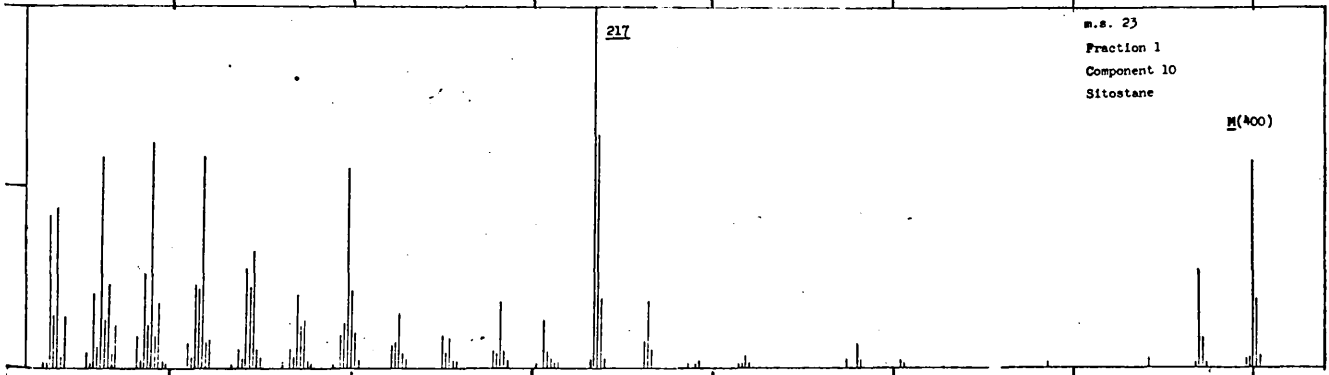
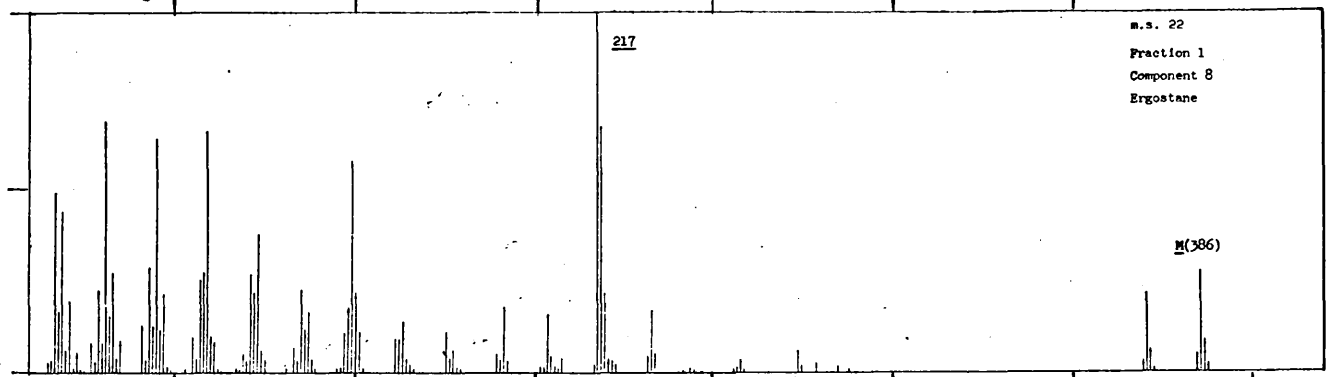
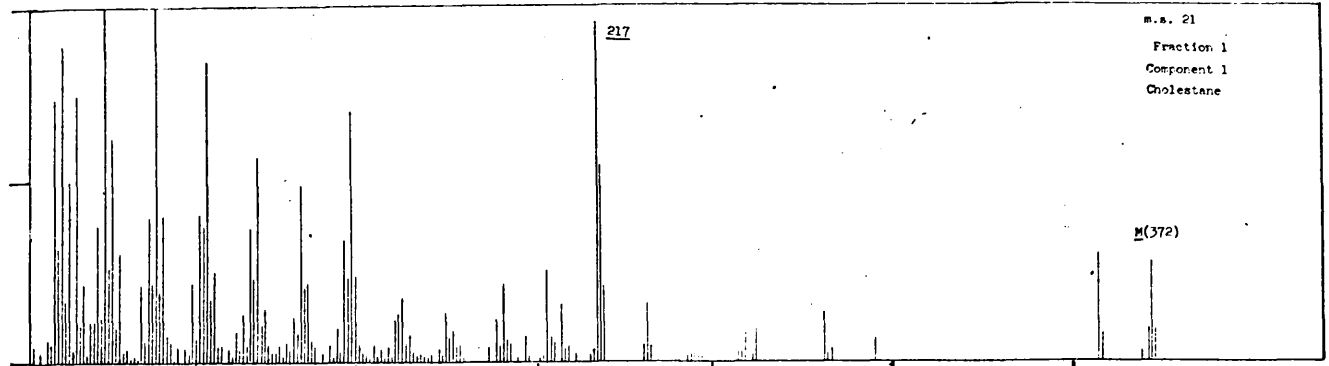








RELATIVE ABUNDANCE



100 150 200 250 300 350 400
m/e

