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INTRODUCTION.

The present study was begun at a singularly appropriate time (1958) with regard to the history of Gas Liquid Chromatography (G.L.C.). There were few commercial instruments available, all of which used the accurate but not very sensitive katharometer detectors. Thus it was that, when one of our projects required separation of a mixture of monoterpenes, we, like most industrial concerns at this time, were forced to build a preparative G.L.C. instrument and in so doing learned that there was more to the apparatus than a polished box.

Despite the inadequacy of the commercial apparatus, a great deal of work had been done, especially by the petroleum chemists who had effectively separated homologous series of the lower boiling hydrocarbons. In the fatty acid field the separation of a homologous series of fatty acid esters had been achieved and the search was on for new stationary phases to separate double bond isomers. It can be seen by the number of papers published the following year that the usefulness of G.L.C. to natural products had also been realised.

By means of the, then, new Pye Argon Chromatograph

with its very sensitive Lovelock Argon detector we were able to show that a very large range of compounds, including n-alkanes, triterpene hydrocarbons, C_{27} steroid hydrocarbons and ketones, e.g. methyl dehydroabietate, cholestanone, fluorene, docosane, hexatriacontane, friedel-3-ene, and 1,15-pentadecanolide, could be analysed at relatively moderate temperatures. At that time we suggested that the way was open to a considerable extension of the application of G.L.C., e.g. rapid screening of crude natural extracts, subsequently employed in Section II and by Horning (1961) for skin waxes. Re-investigation of diterpene and triterpene hydrocarbons, resin acids, waxes and essential oil constituents, as was suggested, has been pursued by Kirk (1961) for study of marijuana constituents, by Mazliak (1961) for the re-investigation of apple wax. The examination of reaction products (e.g. dehydrogenation) also mentioned, have since been studied by Solo (1961).

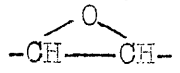
G.L.C. provides the linking thread in the thesis, five out of eight sections depending on G.L.C. as the chief means of identification. The methods of studying essential oils are now largely based on G.L.C. analysis

but at the beginning of our study of the essential oil of Aframomum mala, this application of G.L.C. had only started. Having shown as mentioned above that the n-alkanes C_{20}-C_{36} could be analysed by G.L.C., a study of alkanes in plant waxes was made and is reported in Sections II and III. Although several plant waxes had been studied by G.L.C. in the intervening period, the work in Sections II and III represent the first application of G.L.C. to many plant species in search of a taxonomic criterion.

The use of G.L.C. along with infra red spectroscopy and mass spectrography has been demonstrated in Section IV to confirm the assignments made to certain peaks in the wax alkanes. In Section VII, we attempted to show the high sensitivity of the G.L.C. detectors but were forced to admit that the fern prothalli are more effective in sensing very small quantities of volatile metabolites from fungi. The remainder of the thesis is concerned with the elucidation of the structures of several natural products. "Lindleyol", a diterpene diol, from Aeonium lindleyi (Sempervivoideae) is shown to be labdane-8α, 15-diol. The functional groups of "Sherungulone", a sesquiterpenoid, from Kaempferia roseae (Zingiberaceae)

are: $R\,C\,CH_2 = CH - CH = CH - CO -$

$CH_3\,C\,R_2$, $CH_3\,CH\,R_2$, $R_2C = C\,R_2$



From the study of the physical properties of two triterpene ethers from Arundo conspicua (Graminease), it is suggested that one belongs to a novel triterpene class and that the other is β-amyrin methyl ether.

G.L.C., to-day, is used almost entirely as an analytical tool. Only in the case of the steroids is G.L.C. making any contribution other than a purely analytical one. The workers in the steroid field feel that if they could show a relationship between retention time and structure, they would help considerably in the medical use of G.L.C. In this field progress is being made and it may be that the nature of a steroid may be determined by comparison of retention times of known steroids and increments for the different functional groups.

Since G.L.C. provides a large proportion of the thesis, a short review of the state of the technique follows.

The continued growth of gas liquid chromatography (G.L.C.) is so great that even ten years after its inception, it needs reviewing every six months.[1,2,3.] The theory of gas liquid chromatography was developed early in its history by Keulemans,[4.] Deemter[5.] and Kwantes[6.] and now most of the work is on specific applications of the technique. Several excellent books[7,8,9,10.] have been published on G.L.C. and it is proposed to deal here with the theory as briefly and simply as possible.

G.L.C. was first introduced in 1952 by James and Martin[11.] who demonstrated that the volatile fatty acids could be separated on a column of crushed firebrick coated with silicone oil-stearic acid. As the separated acids left the column, they were trapped in a solvent, collected in a series of small batches so that the time of emergence from the column could be recorded. The amount of acid in each sample was determined by titration. By standardising conditions, i.e. flow rate of gas, temperature of column, nature and amount of stationary phase and particle size of support, the emergence time of a compound can be taken as characteristic of that compound.

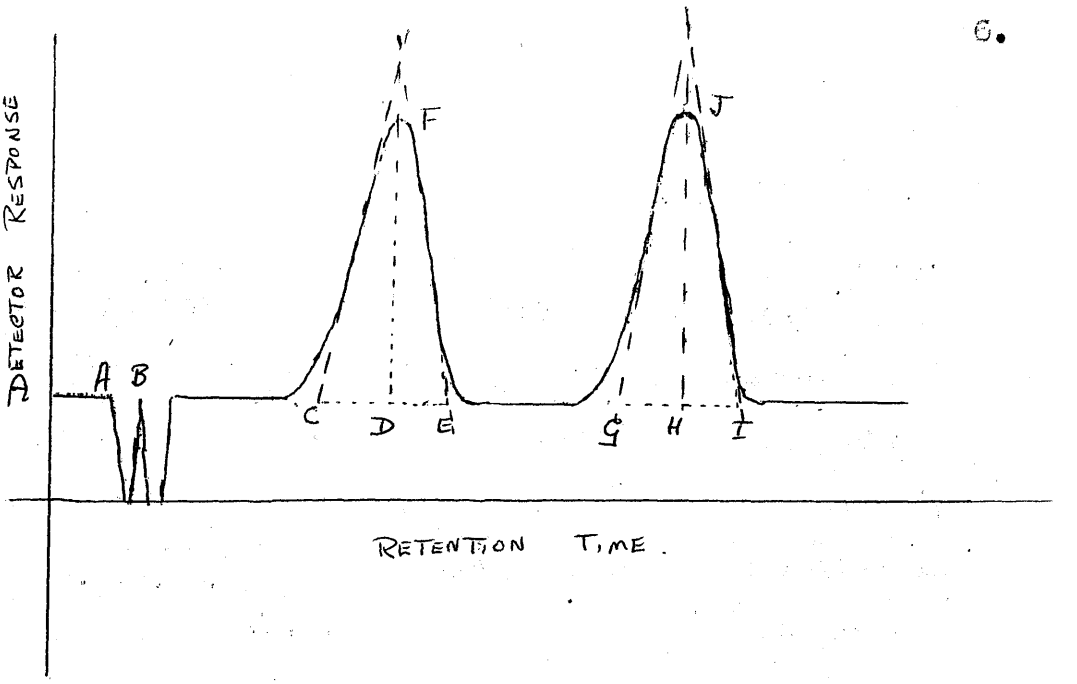


Fig. I.

The elution diagram (Fig. I) represents a typical chromatogram in which the "column resolution"[12.] is given by $\frac{2\ WJ}{CE + GI}$ **and the "column efficiency" by 16** $\left(\frac{BD}{CE}\right)^2$ **.**

Recent Changes in Apparatus.

Each part of the chromatographic instrument has undergone change or modification since the initial development of G.L.C. commercial apparatus in 1957.

The carrier gas now in most general use is argon, in place of the nitrogen or hydrogen formerly used. This retrograde step, since a gas of high "diffusivity"

such as argon leads to a lower number of theoretical plates, is necessitated by the introduction by Lovelock[13] of the β-ionisation detector. The new detectors are very sensitive and easily detect any impurity in the carrier gas. When the water vapour in argon rises above thirty parts per million, the sensitivity of the β-ionisation detector is greatly reduced[14]. though the hydrogen flame ionization detector, equally sensitive, is unaffected by water. Care is therefore taken to remove water vapour by using a drier of molecular sieve (4 Å).

Due to the increased sensitivity of detectors, the sample size has been reduced from 5-10 μ l. in katharometer detectors to 0.01-0.1 μ l. in β-ionisation or hydrogen flame ionisation detectors. Sample injection via a rubber septum on the column top has returned to fashion after a departure to columns requiring the gas flow to be interrupted before injection.

Peak tailing of high molecular weight compounds can be reduced by use of a flash volatilizer which is a small metal oven surrounding the top few inches of the empty column above the packing material. This oven is maintained 30-40°C. above the column temperature and

ensures that all of the sample is vapourised and reaches the column packing in a plug. When mixtures of wide boiling point range are to be analysed, temperature programming, in which the temperature is raised gradually throughout the analysis, is now being used in place of isothermal analysis.

The small sample volume has permitted the percentage stationary phase on the column packing to be reduced to 0.1 - 1.0%. Such a low proportion does not cover all the active sites of the support material which could cause decomposition of labile samples. The support and the glass column are therefore treated with trimethyl silyl chloride which neutralizes many of the active sites. The usual support for the stationary phase is Celite - a form of crushed kieselguhr but some attempts have been made to use micro glass beads.[15]. A new type of column - the Golay column - in which the stationary phase is coated along the walls of a capillary tube in a very fine film has produced remarkable increased resolution at least of the lower molecular weight hydrocarbons.[16]. Purnell[17]. has shown however that certain separations can be carried out equally effectively on packed columns. Golay columns[18].

require very small samples and the sample which is injected is usually split into two parts - one being diluted with argon before entering the column - the other is not analysed. Micro-detectors of very small cell volume must be used with this type of column.

Most of the analyses in this thesis have been carried out with a β-ionisation detector[13]. whose operation depends on the fact that argon is excited to a metastable state by collision with β- particles emitted by the radioactive Sr^{90} source. The excitation energy of argon, 11.6 electron volts, is higher than the ionisation potential of most organic molecules which in turn are ionised by collision with excited argon. The increased current produced between the electrodes is amplified and recorded. By increasing the applied voltage across the electrodes, the sensitivity can be increased. A modification of the β-ionisation detector is the electron capture unit in which compounds having a particular affinity for free electrons are detected in the presence of other components with little or no affinity for electrons. This detector has facilitated the analysis of chlorinated compounds of such importance in pesticides.

The mass spectrometer[19]. has been used as a detector for G.L.C. and functions adequately so long as the peak slope is maintained constant by means of temperature programming.

For analysis of mixtures, the accent is now on combinations of physical techniques and increasing use is made of the preparative aspect of G.L.C. In order to obtain a sample of the separated material only a small proportion is passed through the detector - the remainder through a side exit into a trap. Normal trapping methods of U-tubes and spirals cooled in ice, drikold/acetone or liquid air serve in most cases but some compounds - especially those analysed at high temperature - form aerosols when cooled rapidly. Recently electrostatic precipitators[20,21]. have been used to condense these fogs. Samples have been collected directly in an infra red gas cell by Chang[22].

The great beauty of G.L.C. has been the multiplicity of column packings available - some industrial analytical laboratories having a series of 30-40 columns ready for use. Highly specific columns have been developed to deal with small differences in polarity of the compounds, e.g. silver nitrate/glycol columns for ethylenic compounds[23].

Mixed packings of two stationary phases[24]. have been used for steroid separation, while to separate the four menthol stereoisomers[25]. two columns of different polarity were required.

Short pre-columns are used to bring about specific changes in the mixture to be analysed - use of Linde 5 Å sieve in subtractive G.L.C. to remove n-aliphatic hydrocarbons and permit passage of branched chain hydrocarbons[26]., the use of a calcium carbide precolumn to convert water to acetylene in analysis of aqueous solutions.[27].

Specific Applications.

It had long been realised that some groupings were too polar to allow easy G.L.C. so that many protecting procedures have been used - formation of methyl[28]. and trimethyl silyl[29]. ethers of sugars, of trifluoracetoxy steroids[30]., of silysated amino acids.[31]. With the increase in use of radioactive tracers in chemistry and biochemistry, G.L.C. has been used to separate tracers whose radioactivity can be recorded by a scintillation counter[32]. as they leave the column.

The separation of two optical isomers is one of the

most difficult imaginable, but Goldberg tackled this problem by using an optically active stationary phase. He had, however, to report failure.[33.] Corey[34.] claims to have succeeded while Denny[35.] showed that racemization of optically active halides took place on 40% polyethylene glycol. No relationship between retention times and structures has been found but among the many attempts are Ackman[36.] with fatty acids, Kovats[37.] with alicyclics, Horning[38.] with steroidal amines, Clayton[39.] with steroid methyl ethers and Lipsky[40.] with steroids on twenty different stationary phases.

The trend for organic chemists in G.L.C. has been to try to analyse larger and more complicated molecules with the resultant upward climb in column temperatures. Since our short communication[41.] reporting the analysis of some steroid and triterpene hydrocarbons, a great deal of work has been done in the steroid field.[42,43.]

McWilliams[2.] and Parriss and Holland[44.] have dealt with applications of G.L.C. to industry where the use of chromatographs in process control is becoming widespread.[45.] Scott[13.] has shown that cathode ray tubes in place of print out recorders can be used with short lengths of capillary column if only a visual check is

needed as in process control. Several simplified explanations of G.L.C. have been given by Pecsok,[46]. Keller[47]. and Lewin.[48].

G.L.C. has been used in conjunction with other physical techniques - thin layer chromatography (T.L.C.)[49]. for the study of methyl fatty esters; infra red spectroscopy[50]. for the study of octadecanoic acids; paper chromatography and alkaline isomerization[51]. for fatty acids of Chlorella pyrenoidosa.

Among the most unusual problems in gas chromatography is that of one manufacturer who is preparing an instrument small enough to fit into an American space rocket. It is hoped that when the apparatus lands on the moon, samples of the moon dust will be collected by the instrument, pyrolised at 150°, 325° 500° and 1000°C. successively and analysed. The record of the constituents data will then be transmitted back to earth and compared with that for terran dust. The sensitivity of the new detectors is very high but none can compare with the ability of Gypsy moth insects to sense two or three molecules of a sex attractant liberated by the female of the species. In the

synthesis of this compound, Jacobson[52]. used G.L.C. to separate the reaction mixture but instead of a detector they used a male moth which became agitated when the attractant left the column. Progress in G.L.C. has indeed been great but this "back to nature" attempt sounds like an admission of inability to match the living cell.

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SECTION I.

A STUDY OF THE ESSENTIAL OIL OF AFRAMOMUM MALA (THE MONOTERPENE FRACTION).

INTRODUCTION.

The isolation of the two "modifications" of an unusual hydrocarbon, $C_{16}H_{28}$, named α- and β-kayene by Worsley[1] was brought to our attention by Dr. Bray of Tropical Products[2] who very kindly supplied us with Aframomum mala (Zingiberaceae) seeds. Worsley isolated α- and β-kayene in highest proportion by fractional distillation of the essential oil of the seeds of Aframomum mala though he claimed the presence of the kayenes in the leaf and fruit peel essential oils. The essential oil of A.mala seeds was obtained in 3.4% yield by steam distillation at two atmospheres. His first fractionation by distillation at reduced pressure gave a mixture of cineole and 'kayene' which could only be further separated by the removal of the cineole as the resorcinol complex. The residual oil, 37% of the original oil, was further fractionated by distillation at reduced pressure over sodium in a current of hydrogen into two "modifications" α- and β-kayene whose physical properties were again well documented.

Worsley formulated kayene as a $C_{16}H_{28}$ hydrocarbon - vapour density determination of the molecular weight and C,H analysis - which is unlikely in a compound which distils in the same fraction as cineole and has a b.p. consistent with that of a monoterpene ($C_{10}H_{20}$). Worsley had no further suggestions to make on the structure of kayene but he considered that caryophyllene, the main constituent of the leaf essential oil, was changed to kayene in the peel and to cineole in the seeds. Kayene was said to contain a cyclopropane and/or cyclobutane ring which suggested its study would be very amenable to the more modern techniques of infra red and ultra violet spectroscopy and gas-liquid chromatography (G.L.C.). Our steam distillation of the seeds yielded an essential oil of similar but not identical physical properties to that of Worsley though in much lower proportion.

DISCUSSION.

Until the mid 1950's analysis of essential oils had been by classical methods of alumina chromatography[3], careful fractional distillation[4] and comparison of optical rotation, specific gravity and refractive index. Although these methods were still being used[5], at the

initiation of our study of Aframomum melo, Bernhard[6]. stated that the dramatic impact of G.L.C. would soon be felt by all workers in the essential oil field. He then showed that by varying the stationary phase, resolution of certain monoterpenes could be improved. His prophecy has been vindicated but reported progress in essential oil analysis by G.L.C. has been slower than in the fatty acid or hydrocarbon fields. Analysis of essential oils is fraught with difficulties due to the presence of a large number of components of wide boiling point range (150°-250°), of positional and geometrical isomers and of compounds which isomerize, autoxidize, polymerize or dehydrate readily. Successful attempts to overcome these difficulties have been made by Naves[7]. - a combination of fractional distillation, G.L.C. and I.R.; by Liberti and Cartoni[8]; also Stahl and Trennheuser[9]. - a combination of G.L.C. and silicic acid column and thin layer chromatography; by Brenner[10]. - removal of specific compounds by molecular sieves; von Rudloff[11]. - almost complete analysis by G.L.C. on special columns.

Most of the above methods have been developed and reported since the completion of our project and so we first tried unsuccessfully to separate the essential oil

of Aframomum mala by alumina chromatography and fractional distillation. We then analysed the essential oil by G.L.C. on several stationary phases, Apiezon L, silicone oil and tritolyl phosphate before the best separation was obtained on carbowax 600 (polyethylene glycol M.W.600). The assignment of the various peak identities was made by determination of the relative retention times of known compounds and comparison with those for the unknown peaks. Confirmation of these results was obtained by an enrichment procedure in which the known compounds were added one at a time to fresh portions of the A. mala essential oil and re-examined by G.L.C. As further confirmation, collections of the individual components by preparative scale G.L.C. and identification by formation of derivatives were carried out. A preparative scale G.L.C. unit was designed and constructed to effect this separation based on an original design of Dr. Blackman[13]. as no commercial apparatus was available at the time. By such means it was possible to identify all but one minor component of the monoterpene fraction of the seed oil of A.mala (Table I).

Klouwen and Heide[13]. have recently begun a systematic analysis of monoterpene hydrocarbons in which they claim that sabinene is decomposed by G.L.C. analysis on

silicone fluid stationary phase. We have accounted for the disappearance of the sabinene peak in run 1, Table I by assuming that this stationary phase does not resolve β-pinene and sabinene. It seems unlikely that complete decomposition occurs since our collection of sabinene from oil of savin was accomplished on a silicone fluid column.

In studies of essential oils which may contain twenty to twenty five major and minor constituents, very small quantities are now required - 5-10 mg. for comparative G.L.C. - and 2-3 ml. for preparative G.L.C. Analysis of many oils are therefore relatively easy and should facilitate studies of botanical variations, plant physiology, morphology and taxonomy. It is interesting, in this sense, to note the chemical similarity of related species and to speculate on a classification of the plant in the Natural Order by G.L.C. In the Zingiberaceae family, for example, a comparison, as below, can be made, though the essential oils of A. angustifolium and E. cardamomum were analysed by older conventional methods and that of A. mala is the result of our work by G.L.C.

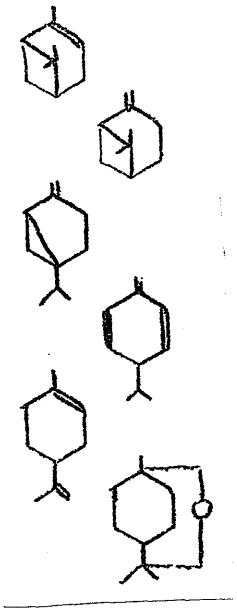
Aframomum mala	Aframomum[14] angustifolium	Elettaria[14] cardamomum
α-pinene	α-pinene	
β-pinene	β-pinene	
sabinene		sabinene
α-phellandrene		
limonene	limonene	limonene
cineole	cineole	cineole
	dipentene	
		terpineol

From the proposed biogenetic pathway (Fig.7) it can be seen that though geraniol is the precursor of both sabinene and α-pinene, there is a branching of the pathway. It would be interesting to see if by supplying labelled sabinene, the plant could metabolise it to the pinane skeleton.

CONCLUSIONS.

The fractionation which Worsley was able to achieve between α- and β-kayene was certainly not a separation between two sesquiterpenes (since the boiling points are much too low). It seems likely that Worsley partially separated β-pinene and sabinene. The constituents of the monoterpene fraction of the essential oil of Aframomum mala are:-

α-pinene	13%
β-pinene	21%
sabinene	14%
α-phellandrene	<1%
limonene	15%
1,8-cineole	39%
unknown	<1%



EXPERIMENTAL.

Isolation of the Essential Oil of Aframomum mala.

The three segments of the fruits of Aframomum mala (Zingiberaceae)[1]. which are similar but larger than the cardamom fruits contain dark-brown shiny ovoid seeds. Steam distillation of the seeds, macerated (but not ground) in water in an Atomix for two minutes, yielded an oil (1.5%, n_D^{23} 1.4668; Worsley[1]. reports 3.2%, n_D^{20} 1.4695). The oil was extracted with light petroleum. This extract was dried with sodium sulphate, filtered and the solvent removed under reduced pressure.

Infra red absorption bands of the essential oil at 3080, 1650, 890 cm.$^{-1}$ are due to an exomethylene group, at 3500 cm.$^{-1}$ due to a small amount of alcohol, and at 1210, 1160, 1085, 980 cm.$^{-1}$ due to an ether linkage, probably cineole from Worsley's analysis. The ultra violet absorption ($\lambda_{max.}$ 198 m u, ε =3250 assuming M.W. = 135 in alcohol) confirmed the presence of the ethylenic linkage.

In this extract it was impossible to find a component of the type suggested by Worsley, $C_{16}H_{28}$. A further extract was therefore prepared by steeping the seeds (100 g.) in light petrol (40-60°, 500 ml.) for two to three days after which the light petrol was filtered off and a further portion (500 ml.) of light petrol added. After a further two to three days the two light petrol portions were combined and dried over sodium sulphate. The extract (4 g.) remaining after the solvent had been removed under reduced pressure was distilled to give two fractions. The first fraction (20-40°, 8 mm.) which should have contained kayene (Worsley 35.5°/8 mm.) gave a G.L.C. analysis very similar to the steam distilled essential oil and the second fraction (40-100°/8 mm.) consisted of a mixture

of compounds whose boiling points were more consistent with kayene's formula $C_{16}H_{28}$. No one component in this second fraction was present to the extent of 37%.

Attempted Separation of the Steam Distilled Essential Oil.

The infra red absorption spectrum of each of the fractions, obtained by distillation at reduced pressure, was similar to that of the starting material. Alumina chromatography (Grade I), which has been used to separate the constituents of Puerto Rican Bay Oil[3], removed cineole and the alcohol but a G.L.C. analysis of the hydrocarbon fraction showed that the proportions of the individual components were unchanged.

Analytical Studies of the Steam Distilled Essential Oil.

Qualitative: A series of analyses by G.L.C. is shown in Table I. The relative retention times of known compounds were determined under the same column conditions as each of the runs shown, and confirmation made by enriching samples of the essential oil with these known compounds. It can be seen that whereas the components are eluted in boiling point order from a non-polar stationary phase (Apiezon L) a polar phase (polyethyleneglycol) reversed the elution order of peaks 5 and 6 (limonene and cineole).

Quantitative. It was found impossible to form the cineole-resorcinol complex[15] from the essential oil confirming the fact that cineole was present to less than 65%. To determine the quantity of cineole in the essential oil by the method of Cross, Gunn and Stevens[16] the optical densities of a series of carbon disulphide-cineole mixtures of known composition (in 25 mm. cells) were measured at the C-O stretching vibration 1085 $cm.^{-1}$. The optical density of the mixture of the essential oil and carbon disulphide was measured and from Figure 1 the weight percentage of cineole was calculated as 39%.

The first method of determining the percentage composition of a mixture by G.L.C. analysis is the 'marker method'[17] in which a series of mixtures of known composition of 1,8-cineole and p-cymene (the marker - a compound - not present in the mixture to be analysed, which has a retention time different from all the other peaks) was prepared and analysed under conditions as in run 6, Table I. The areas of the p-cymene - cineole peaks for each mixture were then measured by planimeter (average of six values for each peak) and plotted against the mole ratio as in Figure 2.

A known weight of p-cymene was added to the essential oil which was then analysed. From the area ratio of the cineole and p-cymene peaks the molar ratio could be read from Figure 2 giving the percentage cineole in the essential oil as 39%. The results of similar experiments for -pinene and limonene are shown in Table II.

In the 'internal normalization method'[17]. the areas of all the peaks in the essential oil (Figure 3) are measured by the planimeter (the average of six values for each peak). The percentage of each component is given as: $\frac{A_n}{\sum_{n=1}^{n=n} A_n}$ where A = area of peak n.

This internal normalization method assumes that the detector response is the same for each component irrespective of chemical type. From the marker method and the I.R. determination it is known that the percentage cineole is much higher than obtained by the internal normalization method suggesting that the detector response is very different for cineole compared with the hydrocarbons. By assuming that the area of the peak in the internal normalization method represents 39% of the total mixture it is possible to correct all the other areas which agree with the values found by the marker methods.

TABLE II.

Peak No.	Compound	Internal Normalization Area		Marker
		%	%*	%
1	α-pinene	15	13	13
2	β-pinene	25	21	
3	sabinene	17	14	
4	α-phellandrene	<1	<1	
5	limonene	18	15	12
6	1,8-cineole	25	39	39
7	unknown	<1	<1	

*Area percentage with the correction for cineole.

Separation of Constituents of the Essential Oil by Preparative G.L.C.

A large scale preparative G.L.C. column[18]. was constructed as in Figure 4. Separation was carried out at 155° on a 20% silicone oil column to give four fractions, collected in the traps of simple design cooled with liquid air at the points shown on Figure 5, which were then analysed on the analytical scale Pye Argon Chromatograph (P.A.C.), (Figure 6).

The first fraction ($n_D^{22.5}$ 1.4650, b.p. 154-155°/

760 mm., (α-pinene n_D^{23} 1.4658, b.p. 154°/760 mm.)[13,30]. had an I.R. absorption spectrum identical with α-pinene (a sample prepared by chromatography on the preparative scale G.L.C. from commercial α-pinene).

Preparation of the Nitrosochloride of α-pinene[21].

Ethyl nitrite, prepared by dropping 40% sodium nitrite (25 ml.) and ethanol (5 ml.) into 20% sulphuric acid (50 ml.) and ethanol (5 ml.) was passed into a stirred ice-cooled solution of concentrated hydrochloric acid (1.5 ml.) and α-pinene (0.75 ml.). After all the gas has been passed the stirring was continued for half an hour while a paste separated which solidified on trituration with cold ethanol (α-pinene nitrosochloride m.p. 112°). The material from the first fraction on treatment as above gave a solid (m.p. 112-114°, mixed m.p. 110-114°).

G.L.C. analysis of the second fraction (Figure 6) ($n_D^{21.5}$ 1.4700, b.p. 164-165°/760 mm.) showed the presence of two components though the I.R. absorption bands of the mixture at 3080, 1650 and 790 cm^{-1} were consistent with an exomethylene group. β-pinene ($n_D^{21.5}$ 1.4778, b.p. 164.5°/760 mm.) had the same relative retention time as that of the first component in the mixture.

It was known that sabinene (n_D 1.466 b.p. 165°/760 mm.) fractionated along with β-pinene and that it also contained an exomethylene group. Samples of the oil of savin were analysed on the preparative scale G.L.C. until a fraction containing 70% sabinene was collected.

Preparation of sabina ketone and nopinone.[22]

β-pinene (0.5 ml.) was ozonised in ethyl acetate (5.0 ml.) at -60° for one hour. The residue remaining after removal of ethyl acetate under reduced pressure, was covered with water and steam distilled to give an oil whose G.L.C. analysis (5% carbowax on celite (100-120 mesh) at 119° in P.A.C.) showed the presence of unchanged hydrocarbon and ketonic material (nopinone) (retention time = 6.2 mins.). The sabinene containing fraction was similarly ozonised to give a mixture of sabina ketone (retention time = 8.1 mins., conditions as above) and a minor component (retention time = 6.6 mins.). The infra red absorption at 1725 $cm.^{-1}$ (thin film) was due to a carbonyl conjugated with a cyclopropane ring (dihydro-β-umbellulone, 1723 $cm.^{-1}$).[23]

The second fraction from A.mala essential oil was also ozonised to give a mixture of ketones (retention time = 6.6 mins., and 8.3 mins.) whose I.R. absorption

at 1715 $cm.^{-1}$ only is caused by the large proportion of nopinone and small proportion of sabina ketone.

The third fraction ($n_D^{24.5}$ 1.4722, b.p. 174-176°) was considered to be limonene (n_D 1.4725, b.p. 176-176.5°) since their relative retention times were identical. (Nitrosochloride of fraction 3 (m.p. 107-109°), nitrosochloride of d-limonene (m.p. 101-103°).

The fourth fraction ($n_D^{21.5}$ 1.4625, b.p. 176°) had an I.R. spectrum which was identical with that of 1,8-cineole ($n_D^{21.5}$ 1.4562, b.p. 176°). A cineole-resorcinol complex (m.p. 78°, lit.m.p. 77°) was formed by mixing a saturated resorcinol solution with the contents of trap 4.

TABLE I.

Peak No.	Run 1.	Run 2.	Run 3.	Run 4.	Run 5.	Run 6.
1	1.00	1.00	1.00	1.00	1.00	1.00
2	1.26	1.78	1.38	1.46	1.43	1.87
3	-	-	1.23	1.30	-	2.08
4	-	2.37	-	1.78	2.00	3.12
5	1.68	2.90	1.98	2.30	2.33	3.56
6	1.68	3.40	1.80	2.18	-	4.43
7	-	-	2.00	2.38	-	6.42
α-pinene ret. time	28 mins.	10 mins.	6.1 mins.	4.7 mins.	8 mins.	2.3 mins.
Column	25% Sili.	20% T.T.P.	20% Ap.L.	5% Ap.L.	20% PEG 600	5% PEG 600
Temp. (°C.)	80	88	107	75	148	50
Efficiency T.P./ft.	400	1250	240	570	800	400

Footnote:

The peaks represent: No.1, α-pinene (b.p. 155°)[13]; No.2, β-pinene (b.p. 164°); No.3, sabinene (b.p. 164°); No.4, α-phellandrene (b.p. 171°); No.5, limonene (b.p. 176°); No.6, cineole (b.p. 176°); No.7, unknown.

Sili. represents Silicone fluid; T.T.P., tritolyl phosphate; Ap.L., Apiezon L; PEG 600, polyethylene glycol M.W. 600; PEG 1540, polyethylene glycol M.W. 1540; PEG 4000, polyethylene glycol M.W. 4000.

Efficiencies are quoted in theoretical plates per foot, T.P./ft.

Literature Retention Times.

Peak No.	Ref.No.11.	Ref.No.6.	Ref.No.13.	Ref.No.13.	Ref.No.15.
1	1.00	1.00	1.00	1.00	1.00
2	1.72	1.46	1.41	1.70	1.50
3	-	-	1.20	1.76	-
4	2.36	2.18	1.68	2.25	1.52
5	3.10	2.03	1.97	2.71	1.77
6	3.50	-			
7					

Column	20% PEG1540	40% PEG 600	20% Ap.L.	20% PEG 4000	20% Sili.
Temp. (°C.)	110	152	120	100	100

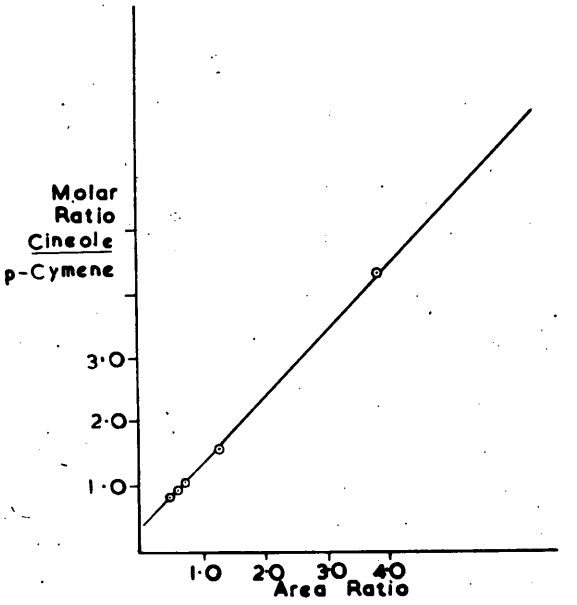


FIG. 2

% cineole by the " marker " method.

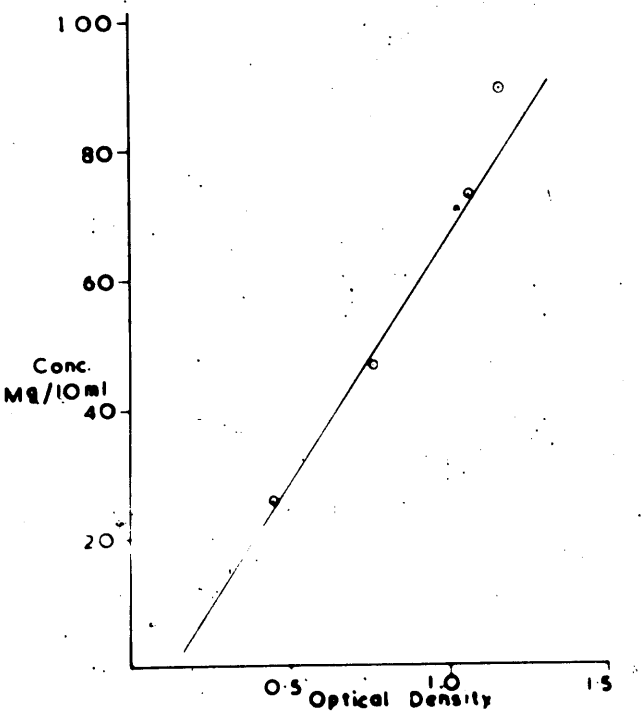


FIG. 1

Plot of optical density against concentration for solutions of cineole in carbon disulphide.

G.L.C. analysis as in Run 6.

FIG. 3

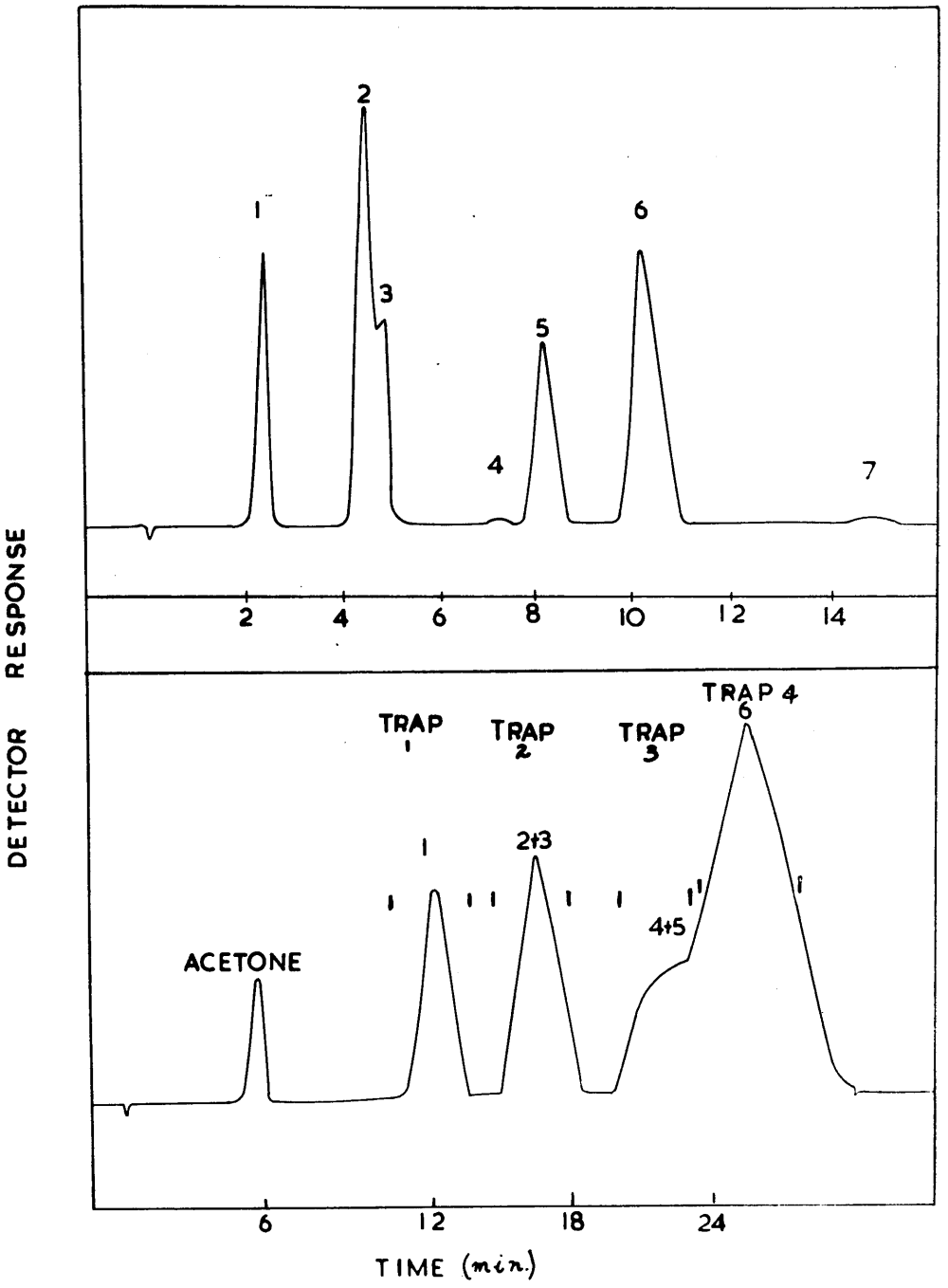
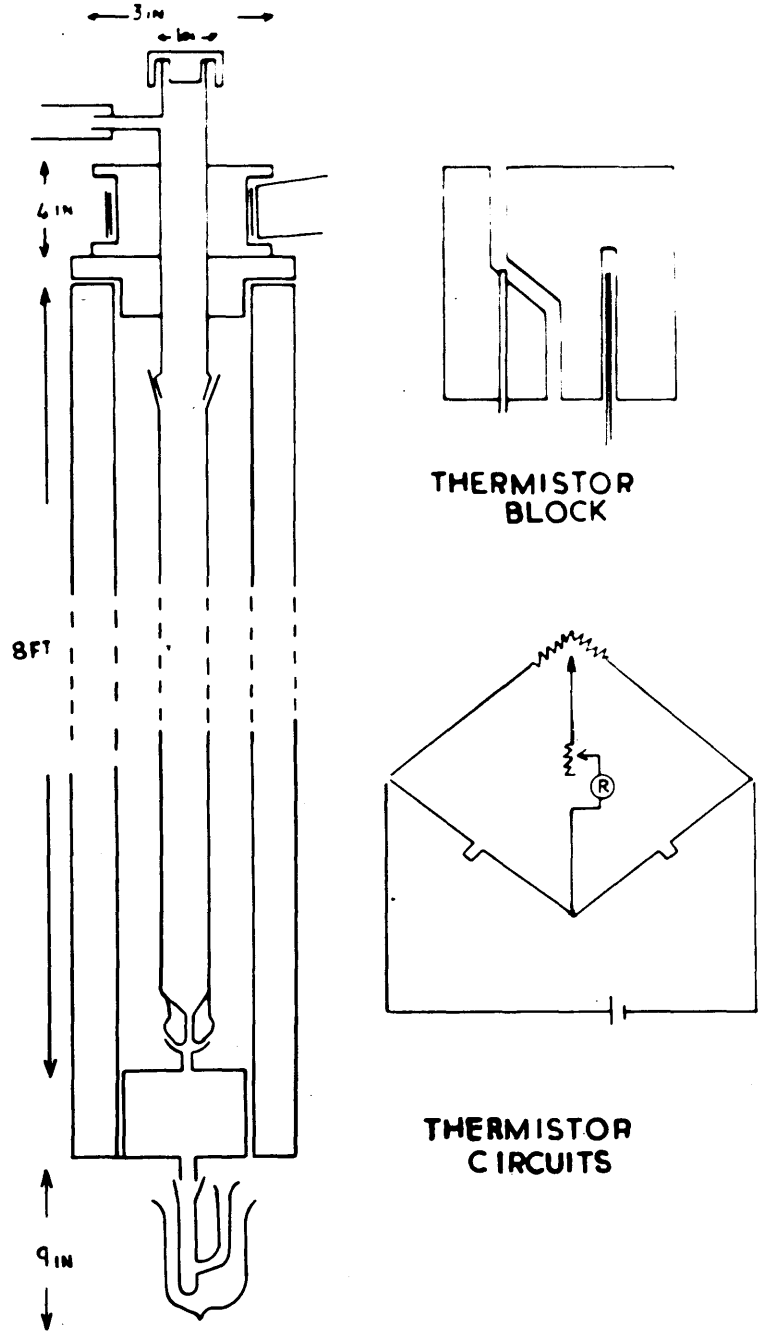


FIG. 5

Preparative G.L.C. on firebrick coated with 20% silicone oil at 155°. Traps were changed at points marked (. The acetone peak was due to the solvent which was used to clean the syringe with which the sample was injected.



F I G. 4

Drawing of the preparative scale G.L.C. apparatus showing injection port, preheater and an insert of the thermistor block and the thermistor circuits.

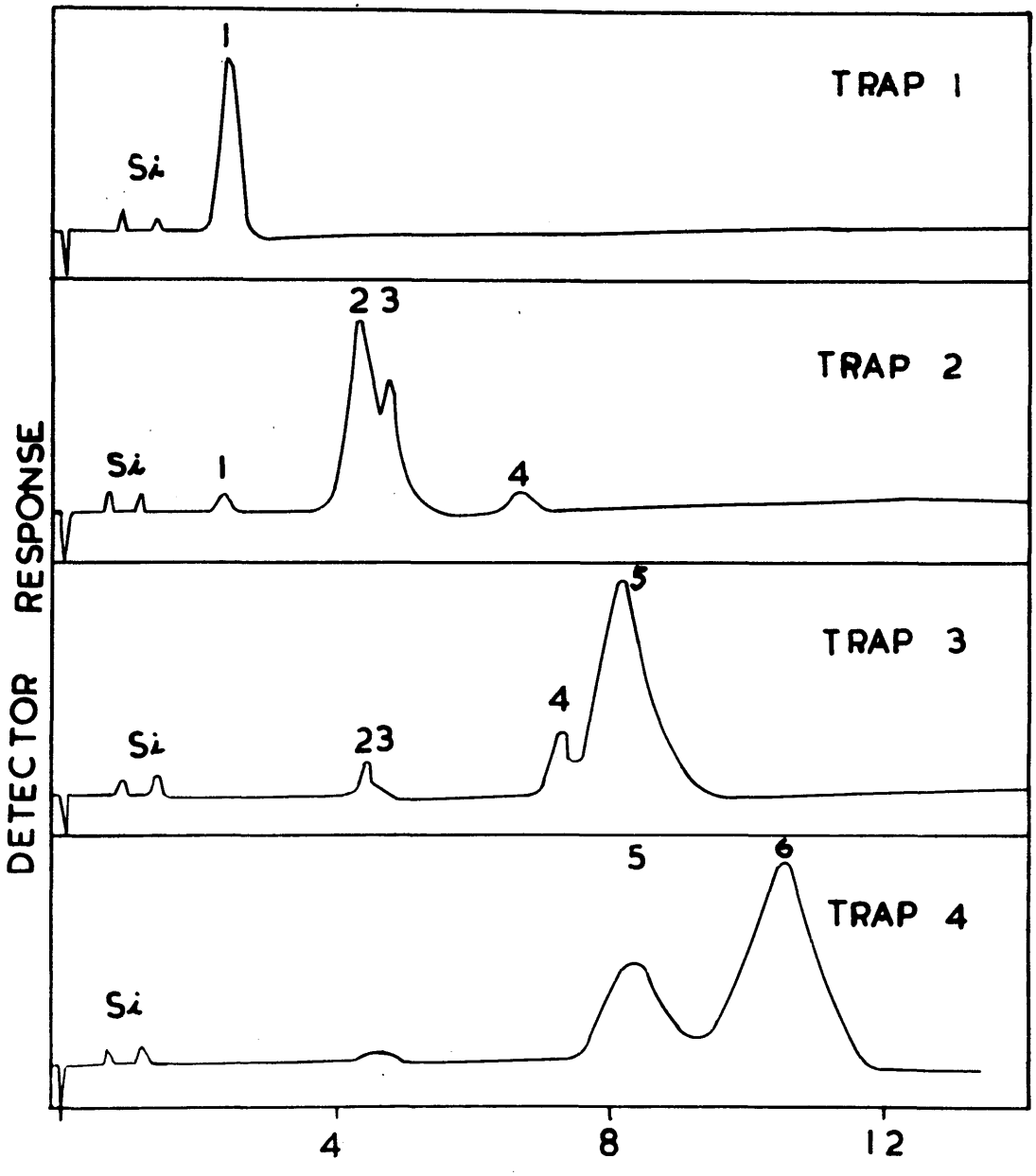
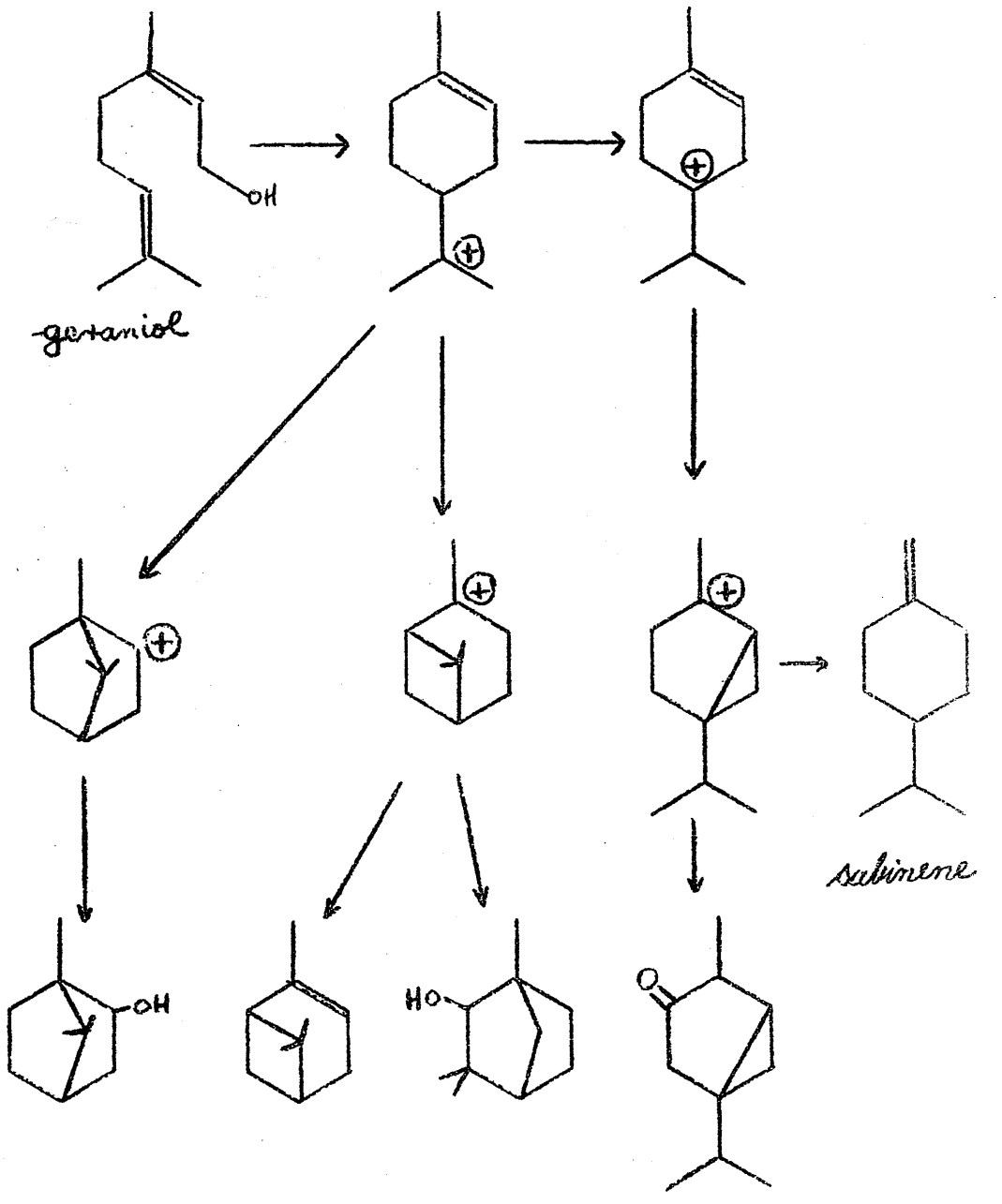


FIG. 6

Analyses of contents of traps showing slight impurities of silicone oil from the stationary phase (Si). Analyses carried out at 58°, 75 ml./min. on a 5% carbowax on celite column.



Proposed Biogenesis of Monoterpenes.

fig. 7.

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S E C T I O N II.

CONSTITUENTS OF PLANT WAXES.

INTRODUCTION.

The occurrence of a waxy coating on plant leaves is an almost universal phenomenon, but the detailed chemical investigation of the constituents of this protective covering has attracted less attention than might be expected. This is due in large part to the formidable problems of separation involved in the study of such a complex mixture of closely related long-chain aliphatic components. The most outstanding contribution has been that due to Chibnall and his collaborators[1]. in the 1930's using the classical method of fractional crystallisation, and employing as purity criteria precise melting points coupled with X-ray determination of crystal long-spacings. Their careful work demonstrated that many previously-isolated, "pure" components were in fact mixtures[1]. The main constituents of leaf waxes as determined from this and later work[2-15]. are shown in Table 1. This later work employing the combined techniques of gas-liquid chromatography and mass spectrometry, has complicated the original picture derived from the earlier work.

TABLE 1.

Major Constituents of Leaf Waxes.

Type	Range	Frequency
Alkanes	Normal: odd C_{21}-C_{37}	Common (especially C_{29} and C_{31})
	Normal: even C_{20}-C_{34}	Common minor constituents
	Branched: C_{27}-C_{33}	Infrequent
Alcohols (usually as esters)	Primary: even C_{22}-C_{32}	Common
	Primary: odd C_{25}-C_{31}	Infrequent
	Secondary: odd C_{21}-C_{33}	Common
	Diols and ketols	Rare
	Terpene alcohols	Infrequent
Aldehydes (as polymers)	Normal: C_{24}-C_{34}	Rare
Ketones	Di-n-alkyl ketones	Rare
Acids (usually as esters)	Normal: even C_{14}-C_{34}	Common
	Normal: odd C_{15}-C_{33}	?
	Ketoacids	Rare
	Dibasic acids	Rare
Esters	Between n-acids and primary and secondary alcohols	Common
	Estolides of hydroxy-acids.	Infrequent?

To take one example, the idea of the exclusive occurrence of odd carbon number n-alkanes has had to be modified in view of the detection of even-numbered n-alkanes[6,9]. and of branched-chain alkanes.[4,9] It is certain that more widespread application of the above techniques will result in considerable future modification of the information contained in Table 1.

As part of a thorough investigation of the chemical constituents of a group of New Zealand plants of medicinal value, still being carried out by members of the Experimental Pharmacology Section, we undertook to examine the wax fraction of these plants.

Originally investigations of the New Zealand plant waxes were performed on the total light petrol extracts, but interpretation of the G.L.C. chromatograms was exceedingly difficult (fig. 10a) since the total light petrol extracts probably contain several homologous series of compounds, e.g. n-alkanes, iso-alkanes, n-alcohols and esters. It was decided to study only the alkane components since these can be readily removed from the accompanying acids, alcohols, ketones and esters without fractionation of the alkanes themselves.

However, since this study was completed we have attempted to obtain a taxonomic fingerprint based on

all of the components (cf. fig 10a). To do this a small piece of plant leaf was placed in the preheater region of the G.L.C. column and all of the components of the wax which can be vaporized are detected producing a pattern of unidentified peaks. It is not yet known however if this pattern is consistent or characteristic. Should it prove feasible to obtain a characteristic pattern, we will have reached the stage which Kreger[16] strove to attain. Kreger scraped a small portion of the wax from a plant and tried to analyse its composition by X-ray crystallography. However, since the method is dependent on the spacings and intensities of lines in an X-ray photograph, it is limited to only the major constituents of waxes. Thus when Kreger applied his findings to the natural relationships of plants he was forced to suggest that the waxes had no taxonomic significance. This is not surprising when it is seen that he claimed that the wax of Copernicia cerifera (Carnauba wax) consisted of " almost pure n-C_{33} "(cf. No.30, fig.9B).

The taxonomic implications of this study of New Zealand plant waxes became apparent as the analyses of the alkane constituents were obtained. The

analyses were carried out over a range of C_{23}-C_{35} carbon numbers and it was possible to analyse all of the constituents (minor as well as major) quantitatively as well as qualitatively, improving considerably on Kreger's major constituent analyses. Such taxonomic possibilities had been hoped for by analogy with the fat and essential oil fields;[17] especially in the genus Hebe which is well known for the ease with which hybridisation occurs and Miss Moore, O.B.E., Botony Division, D.S.I.R., Wellington, New Zealand, carefully selected representative species of the subgroups in her reclassification of the genus.

At this stage it is worthwhile considering the biogenesis of the plant wax constituents.

Biogenesis: It should be noted that no work has been done on the biogenesis of the constituents of the surface leaf wax; the postulates of Chibnall and Wanless are made with reference to the total plant ~~wax~~ lipid constituents. With so little work directly concerned with the plant wax constituents, most of the conclusions and suggestions in this section are made by analogy with the work on glyceride fatty acid biogenesis. The two main biogenetic routesto the non-nitrogenous

plant products are firstly the acetate route[18]. to fatty acids, aromatics, terpenes and other branched compounds, and secondly the shikimic acid[19]. route to lignins and aromatics, e.g. coumarins. Acetic acid has long been accepted as playing a leading role in the biogenesis of plant products and one of the earliest theories concerning the formation of aromatic compounds by the plant, by Collie[20]. postulated a polyketone (fig. 1) derived from acetic acid units. Birch[21]. has very elegantly vindicated and extended this postulate with examples such as are shown in fig. 1.

Glyceride fatty acid synthesis was thought to be simply a reversal of β-oxidation since Clostridium kluyverii could degrade or synthesise acids in aerobic or anaerobic conditions respectively. For many years after Knoop's[22]. first suggestion of β-oxidation as the means of degrading fatty acids, it was felt that there was an "active ester" formed to accomplish this breakdown. This ester was shown to be the thio ester of the acid with Coenzyme A. β-Oxidation must be sparked by a member of the citric acid cycle[23]. and requires Mg^{2+}, PO_4^{3-} and A.T.P. (terminology as in fig. 2a). The probable mechanism of β-oxidation is

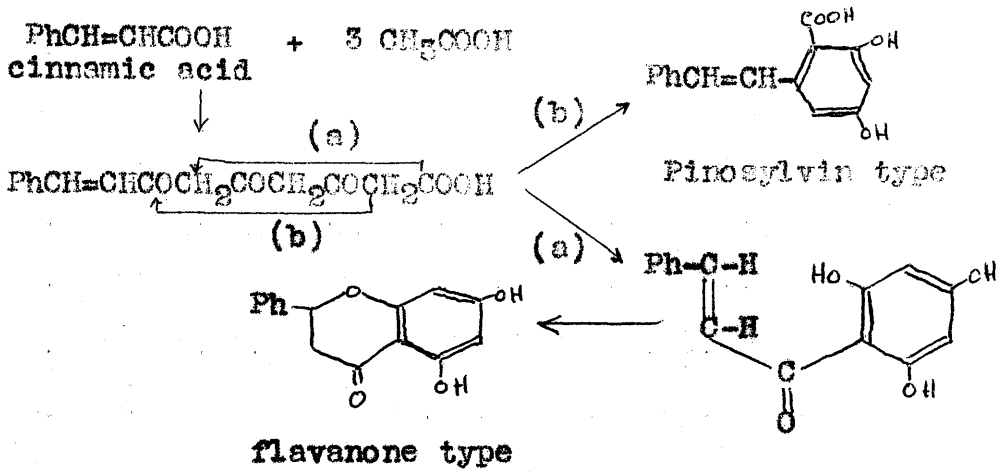


Fig.1. Birch's Postulates for in vivo Cyclisation of Polyketones.[21].

$$RCH_2CH_2COOH + ATP + CoA\text{-}SH \longrightarrow RCH_2CH_2COS\text{-}CoA + AMP + \text{pyrophosphate}$$

$$RCH_2CH_2COS\text{-}CoA + \text{acceptor} \xrightarrow{\text{enzyme}} RCH{=}CHCOS\text{-}CoA$$

$$RCH{=}CHCOS\text{-}CoA + H_2O \longrightarrow RCHCH_2COS\text{-}CoA \quad (OH)$$

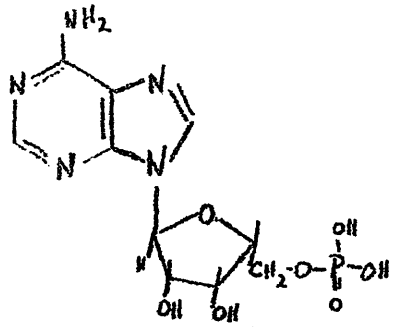
$$RCHCH_2COS\text{-}CoA + DPN \longrightarrow RCOCH_2COS\text{-}CoA + DPNH + H_2 \quad (OH)$$

$$RCOCH_2COS\text{-}CoA + CoA\text{-}SH \longrightarrow RCOS\text{-}CoA + CH_3COS\text{-}CoA$$

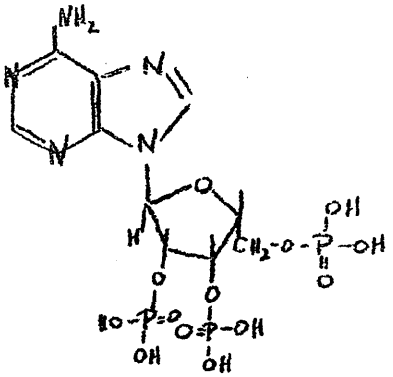
$$CH_3COS\text{-}CoA + \underset{OH}{\overset{CO_2H}{\overset{|}{C}}}{=}\underset{COOH}{\overset{H}{\overset{|}{C}}} \longrightarrow \underset{CH_2COOH}{\overset{CO_2H}{\overset{|}{C}}}\!\!-OH + CoA\text{-}SH$$

$$\underset{CH_2COOH}{\overset{CO_2H}{\overset{|}{C}}}\!\!-OH + AMP + Mg^{2+} + PO_4^{3-} \longrightarrow CO_2 + H_2O + ATP$$

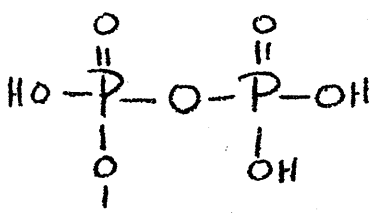
Fig.2. Glyceride Acid RCH_2CH_2COOH β-oxidised to $RCOOH$.[24].



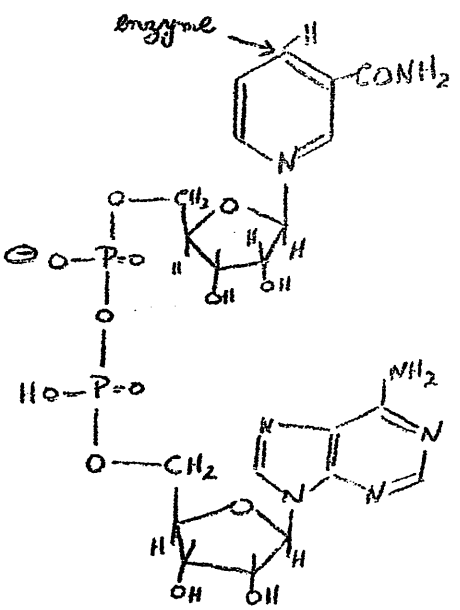
A.M.P. Adenosine monophosphate



A.T.P. Adenosine triphosphate



Pyrophosphate grouping



D.P.N. Diphosphopyridine nucleotide

fig. 2(a)

as in fig. 2 with citric acid fulfilling the second part of the coenzyme definition (see below). Clostridium kluyverii could only convert acetyl CoA to short chain glyceride fatty acids and it was later shown that there are two means of lengthening the acid chain.[24].

The first method depends on the use of animal or plant mitochondria to provide the enzyme system which can elongate the acid chain by one C_2 unit at a time.

Stearic, palmitic, a C_{20} acid, myristic and lauric acids are produced by the mitochondrial system whereas by the non-mitochondrial system, palmitic acid only is produced. These findings are in agreement with those found in the animal mitochondria where stearic acid can be formed from palmitic by addition of a C_2 unit but the reverse process cannot occur. Once stearic acid is started on a β-oxidation pathway, it is impossible to obtain intermediates before acetyl CoA is produced (fig. 3).

Footnote:

"A coenzyme is an entity which can form a complex with an enzyme to catalyse the reaction. It is essential that a second enzyme system is present to recycle the used coenzyme which must otherwise be present in stoicheiometric quantities."

In the non-mitochondrial system, bicarbonate ion is a requisite. The first intermediate has been shown to be malonyl CoA[25]. which then provides all the carbons of the chain except the two furthest from the carboxyl which acetyl CoA provides (Fig.4). Propionyl CoA[26]. can be substituted for acetyl CoA with the formation of a C_{17} acid.

The biogenesis of terpenes is also dependent upon the acetate mechanism but here the first intermediate is mevalonic acid[27]. whose formation is believed to be as in Fig.5. In Fig.6, the route to squalene is shown in which it is proposed that two molecules of Δ^3-isopentenyl pyrophosphate condense to give farnesyl pyrophosphate[28]. The next stage in the synthesis presents some difficulty in that it can be shown, by use of labelled farnesyl pyrophosphate, that squalene is asymmetrically labelled[29]. This may be caused by the isomerization of farnesyl to nerolidyl pyrophosphate. There is a recent suggestion that each terpene has its own site of formation in the plant. Labelled M.V.A. fed to germinating seeds of Pisum sativium[30]. was incorporated to the extent of 45% in β-amyrin and to only 2.2% in β-sitosterol, possibly because complete

cyclization of squalene yields β-amyrin but β-sitosterol requires partial cyclisation, external alkylation and reduction of double bonds.

Thirty years ago Chibnall[1]. had to explain fewer facts than those shown in Table I in attempting to explain wax biogenesis. It was then believed that only odd carbon-number alkanes and even carbon-number carboxylic acids were present in waxes. Chibnall accepted the then current view of fatty acid synthesis from sugars, e.g.

$$2\ C_6 + 2\ C_5 \longrightarrow C_{22}.$$
$$4\ C_6 \longrightarrow C_{24}.$$
$$C_6 + 4\ C_5 \longrightarrow C_{26}.$$

When he noted that some plants had compounds of similar size in different homologous series, e.g. apple cuticle wax with C_{29} alkane, a secondary C_{29} alcohol and brussel sprout wax with C_{29} alcohol, C_{29} ketone and C_{29} alkane, he realised that simple condensation of acids could not form the alcohols, ketones or alkanes. He preferred a β-oxidation scheme as in Fig.7. Such a scheme needed only slight modification by Wanless, King and Ritter[6]. to account for the even carbon-number alkanes and odd carbon-number acids (Fig. 8).

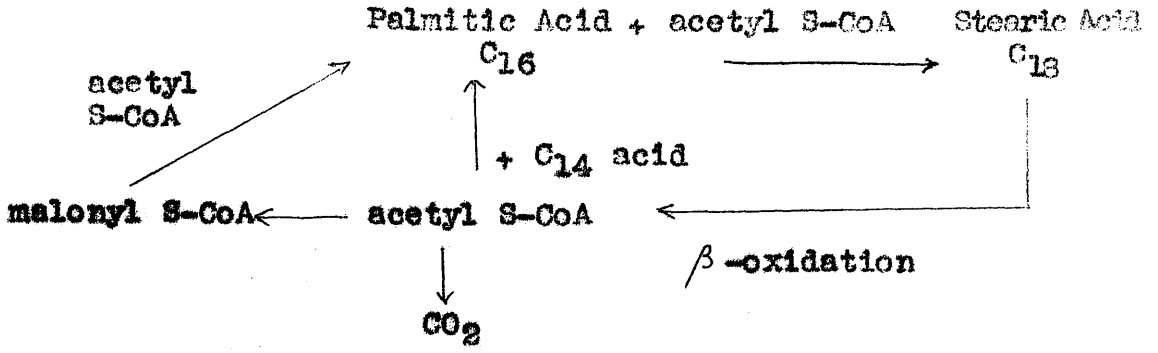


Fig. 3. Tricarboxylic Glyceride Acid cycle in live animals.[24].

$$CO_2 + CH_3COS\text{-}CoA + ATP \xrightarrow[(\text{bound biotin}) + Mn^{2+}]{\text{acetyl CoA carboxylase}} \underset{|}{CO_2H}\ CH_2COS\text{-}CoA$$

$$CH_3COS\text{-}CoA + 7\ \underset{|}{CO_2H}\ CH_2COS\text{-}CoA \longrightarrow CH_3(CH_2)_{14}COS\text{-}CoA + 14\ TPN^+ + 7\ CO_2 + 7\ CoA\text{-}SH + 7H_2O$$
$$+ 14\ TPNH + 14\ H^+$$

Fig. 4. Malonic Acid formation and overall reaction for the production of Stearic Acid[24].

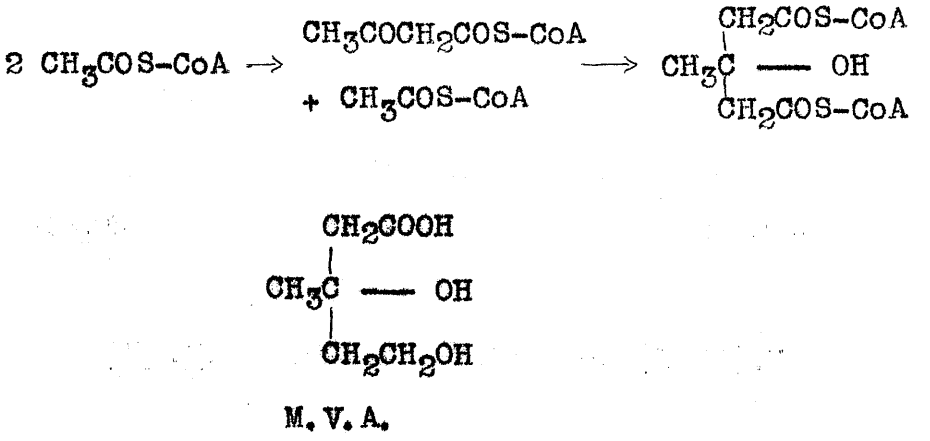


Fig.5. Proposed in vivo synthesis of Mevalonic Acid.[27].

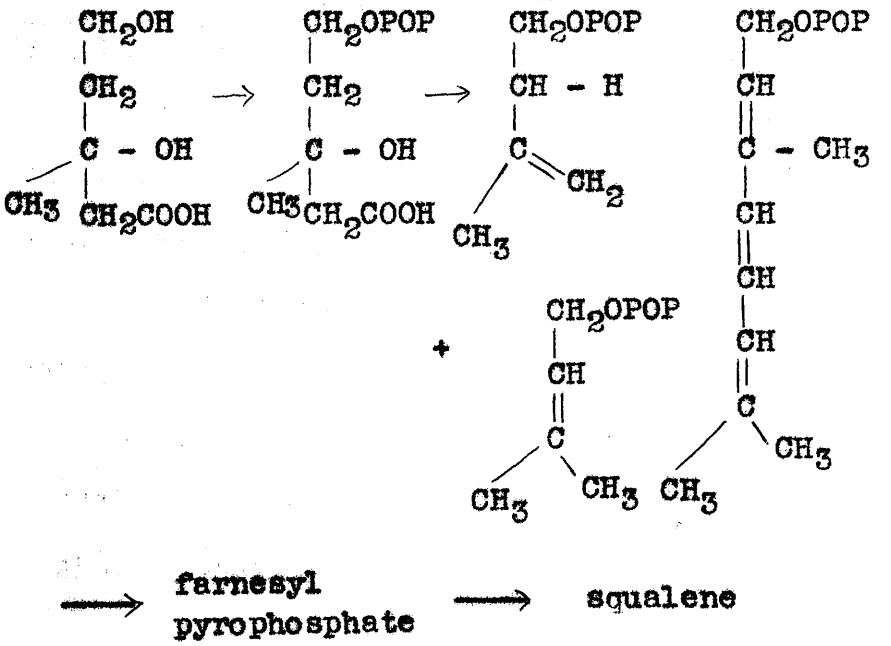


Fig.6. Proposed[29]. biogenesis of terpenes.

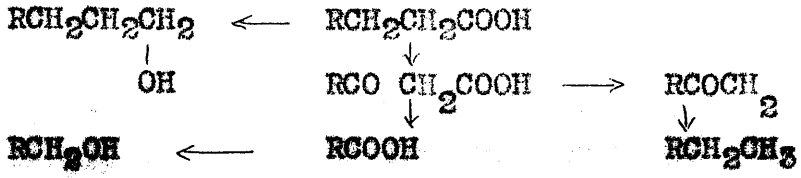


Fig.7. Chibnall Postulates (1934).[1]

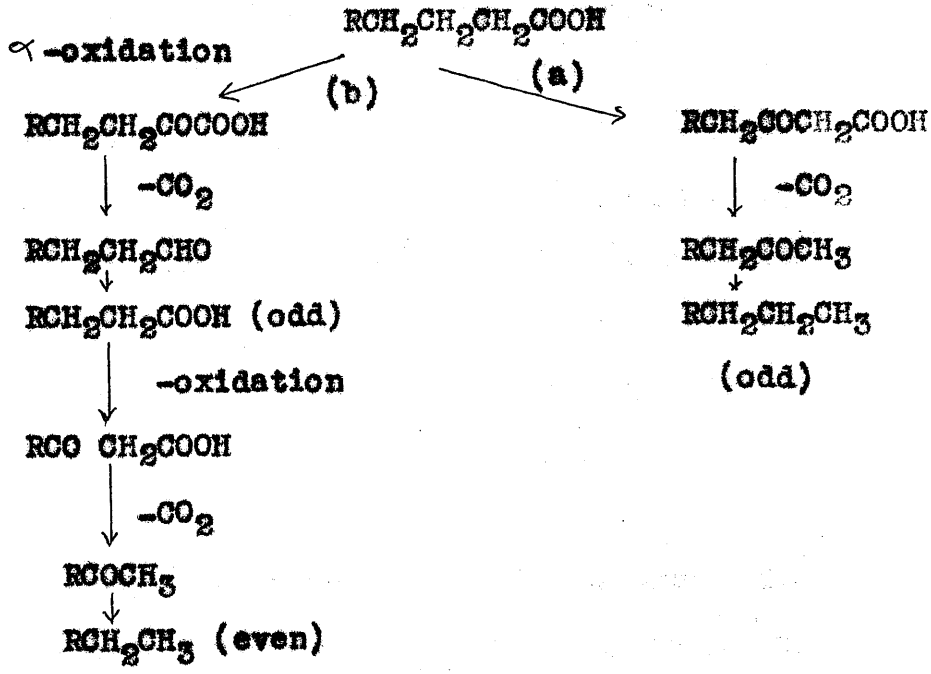


Fig.8. Wanless Postulates (1956).[6]

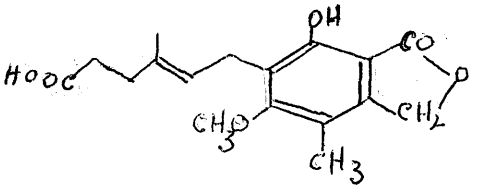
In terms of the acetate theory, it is known that stearoyl CoA will add acetyl CoA to give a C_{20} acid. It seems possible that this mitochondrial elongation by C_2 units as in the glyceride acids is continued until the wax acids of requisite chain lengths are built. However the stumbling block is that both schemes (figs. 7 and 8) require β-oxidation to get a smaller wax acid. In the C_{16} glyceride acid series, once β-oxidation is begun, the end product is acetyl CoA and not the shorter, by a C_2 unit, acid. Very little work has been done in comparing the acid, alcohol and alkane content of waxes but our few analyses suggest that where the major alkanes were C_{31} and C_{33}, the major acids were C_{28} and C_{30}. Further work is in progress to confirm this finding.

Odd carbon-number acids are now explainedin the in the glyceride series by using propionyl CoA[26]. at the chain initiation instead of acetyl CoA. It is reasonable to suppose that this is also true in the wax acids. Isobutyryl CoA,

isocaproyl CoA and iso valeryl CoA will initiate the chain of branched glyceride acids with adipose enzyme systems[26]. Once again this may be the means of producing iso- and ante-iso alkanes and acids in the waxes.

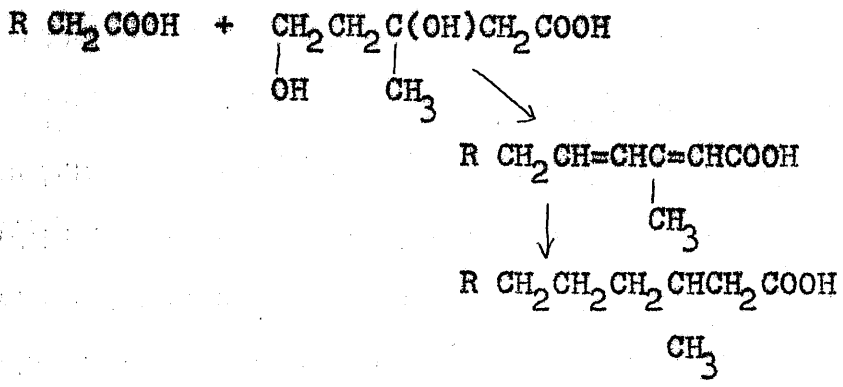
Long chain alkenes have only been found in paraffin waxes (petroleum distillate residues) but their presence in very small quantities in plant waxes would have been missed in our analyses and possibly in those of other workers. Their biogenesis could be explained in the same way as unsaturated compounds in the glyceride fatty acids via the saturated acid in aerobic conditions and via the alcohol and dehydration in anaerobic conditions[32].

"Crossing" of the biogenetic pathways of terpenes and fatty acids is suggested for the formation of mycophenolic acid where the side chain is due to mevalonic acid and the nucleus to acetate units[21].



MYCOPHENOLIC ACID

Such a "crossing" of pathways in the wax acids would result in a β-methyl branched, odd carbon-numbered acid e.g.



RESULTS AND DISCUSSION.

The plants studied are listed in Table II together with the pertinent experimental data. The alkane distribution patterns are represented diagrammatically in Fig. 9A. Fig 9B has been constructed for comparison from data in the literature, further details being given in Table III. The examples chosen for the construction of Fig. 9B are restricted to results which have been obtained, either by mass spectrographic[4,6,9]. or gas-liquid chromatographic analysis[4,5,10,13,14]. of unfractionated plant alkanes. Little reliance can be placed upon claims for the separation

and identification of plant alkanes by other methods, as shown by the outstanding collaborative work of Chibnall and Piper[1]; and the later mass spectrographic analysis of some of their original samples[9].

The New Zealand plants were collected by Dr. M. Martin-Smith, Department of Experimental Pharmacology, primarily for their pharmacological interest. The results indicated the potentialities of the method as a means of "fingerprinting" individual species and this aspect was subsequently examined in more detail in the study of the Sempervivoideae (Section III).

The results of the present studies may not be strictly comparable with those obtained with the Sempervivoideae since the plant material was pre-dried to facilitate transportation and the portions of plants employed were not confined to the leaves. Although it is probable that the alkane components are restricted to the protective surface coatings as evidenced by the fact that the alkane patterns of the leaf-surface

wax and of the total alkane extracts of the leaves of Arundo conspicua were virtually identical (see footnote Table II), there is no reason to suppose that the alkane distribution patterns are the same for different anatomical portions of the plant. In view of the marked difference between the alkane distribution in the rhizomes of Cordyline australis (9, Fig. 9A) and that in the leaves of Draco draconis (29, Fig. 9B), plants which are considered to be closely related botanically, the need for studies on the relative alkane distribution within the same plant is clearly indicated. In the present work comparison of the alkane distribution patterns of the different species within the same genus would appear to be valid inter se, as in all cases the comparisons are made using corresponding portions of the plants. In the case of the Gaultheria and Hebe species twigs and leaves were employed, and in the case of the two varieties of Phormium tenax, rhizomes were used.

It was found that the alkane analysis could be adequately performed on 40 g. of dried plant. A

crude fraction containing the total alkanes was obtained from the finely-ground plant material, which had been pre-dried at 80° for 24 hours in forced draught ovens by continuous extraction with boiling light petroleum (b.p. 40-60°) for 24 hours after which time exhaustive extraction had occurred. Infra-red analysis of the crude extract, which varied considerably in quantity (see Table II), indicated the presence of compounds containing hydroxyl, carbonyl and carboxyl functions. As simple chromatography over alumina proved unsatisfactory for the complete removal of these contaminants the esters were saponified and the carbonyl compounds were converted to their dinitrophenylhydrazone derivatives. After this pretreatment, alumina chromatography afforded the alkane fraction uncontaminated with oxygen-containing compounds (as evidenced by infra-red analysis) in all cases except that of Arundo-conspicua which showed a prominent band at 1120 cm.$^{-1}$ in the infra-red, due to ethers which are studied in Section V. The ethers were readily removed from the alkanes by

treatment of the mixture with concentrated sulphuric acid. The infra-red spectra and gas-liquid chromatograms of the light petroleum extracts of Arundo conspicua at the various stages of purification are shown in Fig. 10. A separate experiment established that the ethers were present in the true leaf-surface wax.

Complete analysis of alkanes possessing between 23 and 35 carbon atoms were achieved, but alkanes of higher and lower chain lengths which may have been present in small amounts were ignored. Eight of the plants studied, (nos. 2-4 and 7-11 in table II) appeared to contain branched alkanes as well as n-alkanes, although the former were present in very small amounts, usually representing less than 4% of the total fraction, except in the cases of nos. 8 and 9. Direct identification of the branched chain alkanes was not possible on the scale employed in the present work but further experiments are in progress. By analogy with the previously reported work and other studies, the odd-numbered, branched hydrocarbons are assumed to be iso-alkanes.

However, isomeric iso and anteiso-alkanes are reported to have almost identical retention times on Apiezon columns[6,11]. and it is possible that the even-numbered branched alkanes belong, in fact to the anteiso- series. Should this be so, the situation would be in the inverse of that reported for the branched hydrocarbons of wool wax[33]. A high content of branched alkanes is a relatively rare occurrence in leaf waxes. Very few of the examples in Fig. 9B show the presence of branched alkanes although trace quantities may have been overlooked by the original workers. Careful investigation of species 21-23 and species 29 in Fig. 9B, however, failed to show the presence of any iso-alkanes[34]. It is therefore all the more interesting that Nicotiana tabacum (19, Fig. 9B) has a high iso-alkane content and that certain Aeonium species, e.g. 13, Fig. 9B, show appreciable quantities of iso-alkanes[34].

In the gas liquid chromatograms of several of the plants (viz. those of nos. 1,3,6-10 in table II) small peaks were present which could not be assigned

to either n-alkanes or iso-alkanes. It is possible that these peaks are due to the presence of cycloalkanes or of compounds possessing double bonds or other functional groups which are present in such small amounts as to be not detectable in the infra red spectra. The intensity of these peaks, is however, so low that they in no way interfere with the interpretation of the major peaks.

In all cases n-alkanes with an odd number of carbon atoms form the major components of the paraffin fraction, which is in agreement with the results obtained by other workers[1,9]. Of the mixtures given in Figs. 9A and 9B the major constituent is either the C_{27}, the C_{29}, the C_{31} or the C_{33} n-alkane. It is of considerable interest that within the genus Hebe the major constituent is C_{29} in H. odora , C_{31} in H. parviflora and H. diosmaefolia, and C_{33} in H. stricta , thus giving an immediate chemotaxonomic distinction. In the case of the two Gaultheria species the differences in the alkane distribution patterns are not nearly so clear cut but they are still easily distinguishable.

The fact that the two varieties of Phormium tenax give virtually superimposable patterns may strengthen the utility of plant hydrocarbon analysis rather than weaken it, as distinction between species without differentiation between varieties would be useful in taxonomy.

In most cases the shortest and longest n-alkane chains differ by about ten carbon atoms with varying proportions of each homologue between these limits. In certain waxes such as that of Solanum tuberosum (18 Fig. 9B) and Pyrus communis (9), however, there appear to be only one or two individual alkanes present in appreciable quantities. An intriguing general point which emerges is that when the odd and even members are considered separately, in both cases the plot of percentage of constituent present against the number of carbon atoms is a simple distribution curve with a single maximum. This generalisation also seems to apply with the odd and even branched alkanes, though less data are available. Since the alkanes can be assumed to be end products of plant metabolism and not intermediaries.

this may be of significance in terms of the specificity of the enzyme systems which are involved in the elaboration of the alkanes from acetate units[7].

The variety of the hydrocarbon patterns is striking when Fig. 9, in which 12 families and 21 genera are represented, is considered as a whole and it augurs well for the future taxonomic application of plant hydrocarbon analysis. As yet no general patterns are apparent which might permit (a) monocotyledons (e.g. Fig.9, Gramineae species 12, 25-28 and Liliaceae species 7-9, 29 and 30) to be distinguished from dicotyledons (the remaining families illustrated in Figs. 9A and 9B), (b) assignment of a species to a given family, as there is marked variation in the hydrocarbon patterns from one constituent genus to another (e.g. within the Crassulaceae species 13-15, and the Gramineae and Liliaceae given in Fig. 9, although, incidentally, there is a marked resemblance between the two species Dracaena draco and Copernicia cerifera), (c) assignments of species to a particular genus, since again there is little constancy in the patterns

(e.g. the diversity within the Hebe species 1-4, the genus Aeonium[34]. and the genus Euphorbium species 21-24, but there is a similarity between the Lolium species, L. perenne and L. multiflora, species 27 and 28 respectively).

Footnote.

In a recent publication, Mazliak[35]. recorded an alkane distribution pattern for Carnauba wax (the wax of Copernicia cerifera). His pattern, however, is quite distinct from that in Fig. 9, see over. We have examined, at his request, the same sample as he used in his analysis and found that it was possible to improve considerably the resolution by use of the lower temperature 225° instead of 300° and in so doing permit easier area measurements. It seems possible that although both Mazliak's and Roberts and Robertson's samples were Commercial grade there had been some difference

in the extraction procedure and in the exact variety of Palm.

	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}
Mazliak's analysis.	8.3	13.3	16.0	16.0	12.3	7.9		
Our analysis of Mazliak's sample.	10.9	12.8	14.0	15.4	11.8	12.2		
Robertson's analysis (Fig. 9)	2.5	10.2	5.5	18.2	14.0	32.3	5.2	8.2

EXPERIMENTAL.

The following general procedure was adopted for the investigation of the alkane fractions. Light petroleum refers to the fraction b.p. 40-60°.

The finely ground plant material (40 g.) was extracted in a Soxhlet apparatus with light petroleum for 24 hours. After removal of the solvent under reduced pressure the extracts (usually of the order of 0.4 - 1.0 g. see table II) were refluxed with 2,4-dinitrophenylhydrazine (1.0 g.) and conc. hydrochloric acid (0.5 ml.) in ethanol (20 ml.) for two hours to convert carbonyl compounds into 2,4-dinitrophenylhydrazones. The solvent was then

removed under reduced pressure and the residue exhaustively extracted with light petroleum. The petrol-soluble material was reclaimed after removal of the solvent and refluxed for 2 hours in aqueous ethanol (1:2 - 20 ml.) containing sodium hydroxide (1.0 g.). The solution was then taken to dryness under reduced pressure and thoroughly extracted with light petroleum. The petrol extract was filtered through alumina (Brockmann grade I) and the hydrocarbon fraction completely eluted with further light petroleum and reclaimed.

Infra red spectra of the hydrocarbon fractions were recorded in order to establish the absence of compounds other than alkanes (Fig. 10). In the case of Arundo conspicua (Table II and Figs. 9 and 10 species 12) the fraction which remained after 2,4-dinitrophenylhydrazone formation, saponification, and alumina chromatography showed strong infra red absorption at 1120 $cm.^{-1}$, indicative of an ether grouping. The compounds responsible for this absorption were removed by treatment with hot concentrated sulphuric acid (140° C, 4 hours).

The light petroleum extract of the reaction mixture furnished the pure alkane fraction after alumina chromatography.

Gas liquid chromatography of the alkane fractions was generally accomplished by chromatographing 0.1 ul. of a solution of the fraction (2 mg. in 40 mg. of α-methylnaphthalene) on a column of Embacel (80-100 mesh) coated with Apiezon L (0.5%).

TABLE 1| DISTRIBUTION IN MOLE PERCENTAGE OF THE ALKANES*

No	Plant†	Family	Portions extracted	Total petrol extractives‡	Total alkane fraction§	C_{23} iso	C_{23} n	C_{24} iso	C_{24} n	C_{25} iso	C_{25} n	C_{26} iso	C_{26} n	C_{27} iso	C_{27} n	C_{28} iso	C_{28} n	C_{29} iso	C_{29} n	C_{30} iso	C_{30} n	C_{31} iso	C_{31} n	C_{32} iso	C_{32} n	C_{33} iso	C_{33} n	C_{34} iso	C_{34} n
1	*Hebe odora* (Hook f.) Ckn	Scrophulariaceae	Stems and leaves	3·9	4		+		1		2		2		10		3		52		2		25		2		3		
2	*Hebe parviflora* (Vahl.) var. *arborea* (Buchan.) L. B. Moore		,,	2·5	10						1		+	+	4		1	+	17		2	+	65		3		10		
3	*Hebe diosmifolia* (A. Cunn.) Ckn and Allan		,,	3·2	10										1		2	+	15		6	+	40		3	+	18	+	16
4	*Hebe Stricta* (Benth.) L. B. Moore		,,	1·7	4·5						+		1		2		2	+	10		4	+	24		1	1	37	1	1 16
5	*Gaultheria subcorymbosa* Col.	Ericaceae	,,	2·0	13·5						4		3		8		1		16		3		56		4		7		
6	*Gaultheria antipoda* Forst. f.		,,	1·6	11						1		1		8		3		26		3		52		2		7		
7	*Phormium tenax* J. R. and G. Forst var. S.S.	Liliaceae	Rhizomes	1·0	5·5				2	+	9	+	4	2	16	+	5	3	47	+	2	3	8		2				
8	*Phormium tenax* J. R. and G. Forst. var. Ngaro		,,	1·1	1					+	15	+	4	2	20	+	3	4	40	+	3	2	5						
9	*Cordyline australis* (Forst f.) Hook f.		,,	1·3	3·5						21		2	1	42		3	1	15		3	1	10						
10	*Pimelea prostrata* (J. R. and G. Forst.) Willd.	Thymelaeaceae	Stems and leaves	1·25	8						3		2		13		3	+	65		2	+	13						
11	*Acaena anserinifolia* (J. R. and G. Forst) Druce.	Rosaceae	,,	2·0	4						+		1		4		2	1	11	1	2	1	59		2	+	19		
12	*Arundo conspicua* Forst f.‖	Cortaderia	Leaves	2·5	4		+		+		4		2		9		5		60		5		12		2		3		

* The content of an individual alkane is expressed as a mole percentage of the total hydrocarbon content from C_{23}–C_{34} inclusive. The mole percentage is taken as being equivalent to the area percentage i.e. $100\ A_n / \sum_{23}^{34} A_n$, where A_n is the area of the peak corresponding to the hydrocarbon $C_nH_{(2n+2)}$, as measured by planimeter. The values are approximated to the nearest 1 per cent and peaks of relative area less than 1 per cent are indicated by +. The branched chain alkanes are designated *iso*-, but see text.

† The species were collected at the following locations: Porters Pass, Canterbury, 1; Rimutaka Saddle, Wellington, 2, 5; Ngaiotonga, North Auckland, 3; Blockhouse Bay, Auckland, 4; Akatarawa Valley, Wellington, 6, 11; Moutoa Development Area of the Department of Agriculture, Wellington, 7,9; Foxton Plantation of N.Z. Woolpack and Textiles Ltd., 8; Houghton Bay, Wellington, 10; Plimmerton, Wellington, 12.

Collection was made during the following months: July 7, 8, 9, 10, 11, 12; August 2, 3, 4, 5; September 1, 6.

Names used are, for dicotyledons as in Allan H. H. *Flora of New Zealand*, Vol. 1, 1961, Wellington, Government Printer; for monocotyledons as in Cheeseman, T. F., *Manual of the New Zealand Flora*, 2nd edition, Wellington, Government Printer.

Grateful acknowledgement is made to the following for aid in the collection of certain species: The director and staff, Botany Division, D.S.I.R., Christchurch, 1; Dr. R. C. Cooper, Auckland Museum, 4; Mrs. W. Reynolds, 3; Messrs. G. B. Miller, W. R. Boyce and W. E. Hale, 7, 8, 9; Miss Peggy Martin-Smith, 12.

‡ As per cent dry weight-values to the nearest 0·05 per cent.

§ Per cent total alkane hydrocarbons calculated on total weight of petrol extractives to the nearest 0·5 per cent.

‖ A separate sample of Species 12 has been examined: The leaves were collected from the same station in late December 1961 and the surface wax removed by brief immersion of the fresh leaves in chloroform. The alkane fraction was examined with the following results: C_{29}, +; C_{30}, +; C_{31}, 7%; C_{32}, 6%; C_{33}, 14%; C_{34}, 9%; C_{35}, 50%; C_{36}, 4%; C_{37}, 10%. This correlates quite well with the previous result.

Table III Literature Sources of the data depicted in Fig. 9B.

No.	Plant Species	Common Name	Portion extracted	Refs.
13.	Aeonium goochia W.B.		Leaf surface	34.
14.	Monanthes brauchycaula Lowe.		Leaf surface	34.
15.	Sedum anglicum Huds.		Leaf surface	34.
16.	Brassica nigra Koch.	Black mustard	Aerial parts	9.
17.	Bacopa Monnieri (L.) Pennell.		Aerial parts	12.
18.	Solanum tuberosum L.	Potato	Tuber cuticle	10.
19.	Nicotiana tabacum L.	Tobacco	Whole leaf	4.
20.	Phaseolus aureus Roxb.	String bean	Aerial parts	6.
21.	Euphorbia balsimifera Ait.		Leaf surface	34.
22.	Euphorbia aphylla Brouss.		Aerial parts	34.
23.	Euphorbia regis-Jubae W.B.		Aerial parts	34.
24.	Euphorbia cerifera Alcocer.	Candelilla	Aerial parts	13.
25.	Saccharum officinarum L.	Sugar cane	Stem surface	5.

Table III (contd.)

No.	Plant Species	Common Name	Portion extracted	Refs.
26.	*Leptochloa digitata* Domin.		Stem surface	5.
27.	*Lolium perenne* Aitch.	Perennial Rye Grass	Aerial parts	6.
28.	*Lolium multiflora* Lam.	Italian Rye Grass	Leaf surface	34.
29.	*Dracaena draco* L.	Dragon tree	Leaf surface	34.
30.	*Copernicia cerifera* Mart.	Carnauba palm	Leaf surface	14.

All of the alkane fractions were analysed by gas liquid chromatography except numbers 16, 20, and 27 where mass spectrometry was used.

The plant species belong to the following families, numbers 13, 14 and 15 to the Crassulaceae; number 16 to the Cruciferae; number 17 to the Scrophulariaceae; numbers 18 and 19 to the Solanaceae, number 20 to the Leguminosae; numbers 21, 22, 23 and 24 to the Euphorbiaceae; numbers 25, 26, 27 and 28 to the Gramineae; numbers 29 and 30 to the Liliaceae.

Fig. 9 Distribution in mole percentage of n- and branched chain alkanes C_{22} - C_{35} in the hydrocarbon fraction of the waxes from individual plant species. Alkane percentages less than 2 mole per cent have been omitted.

Hole 5

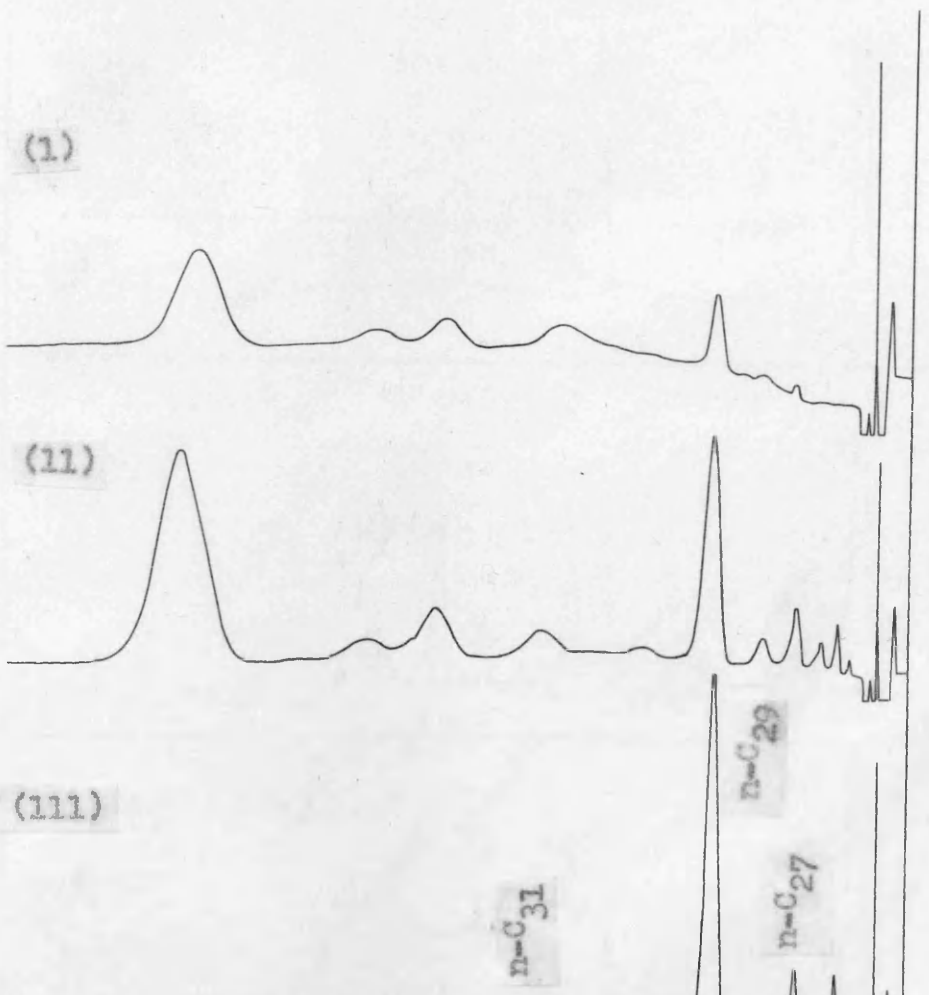
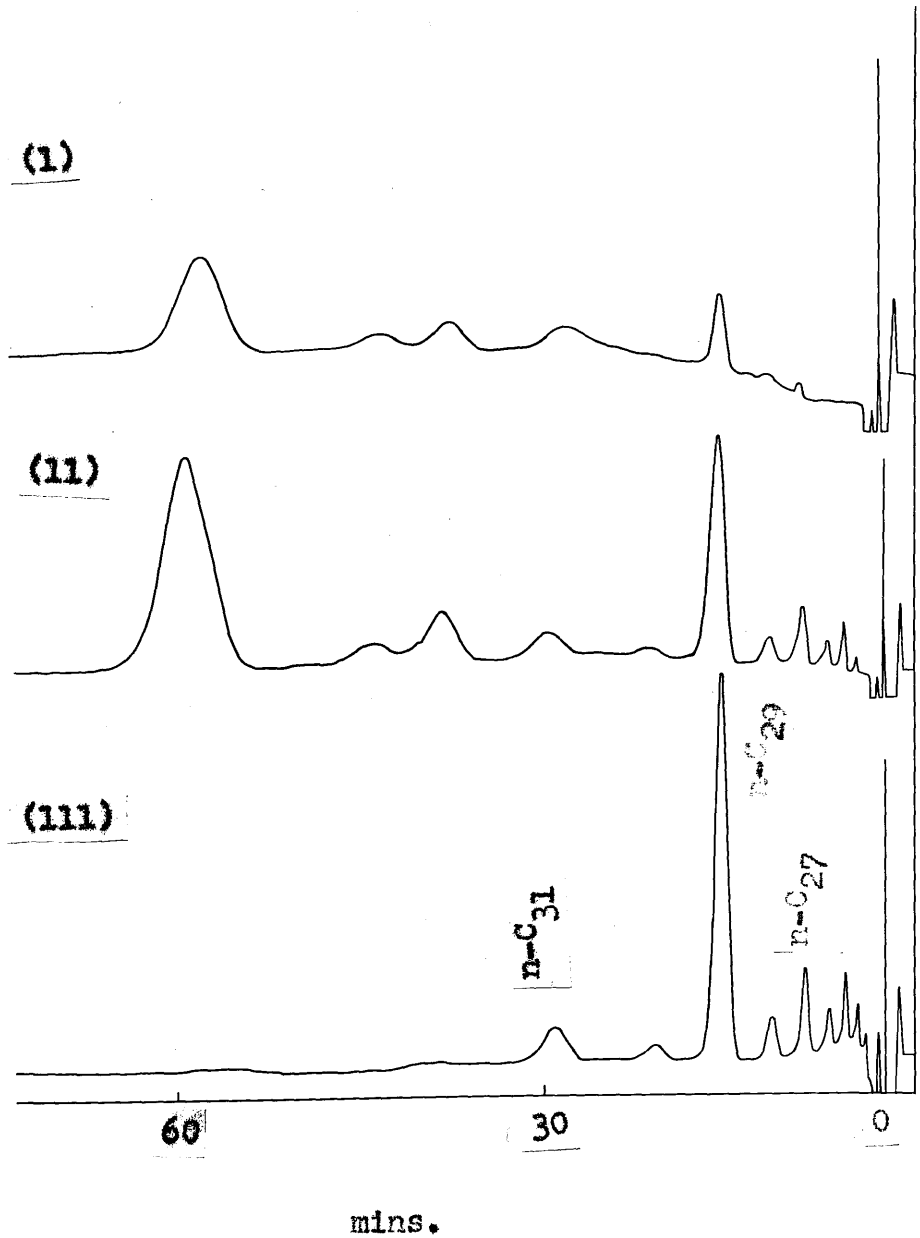
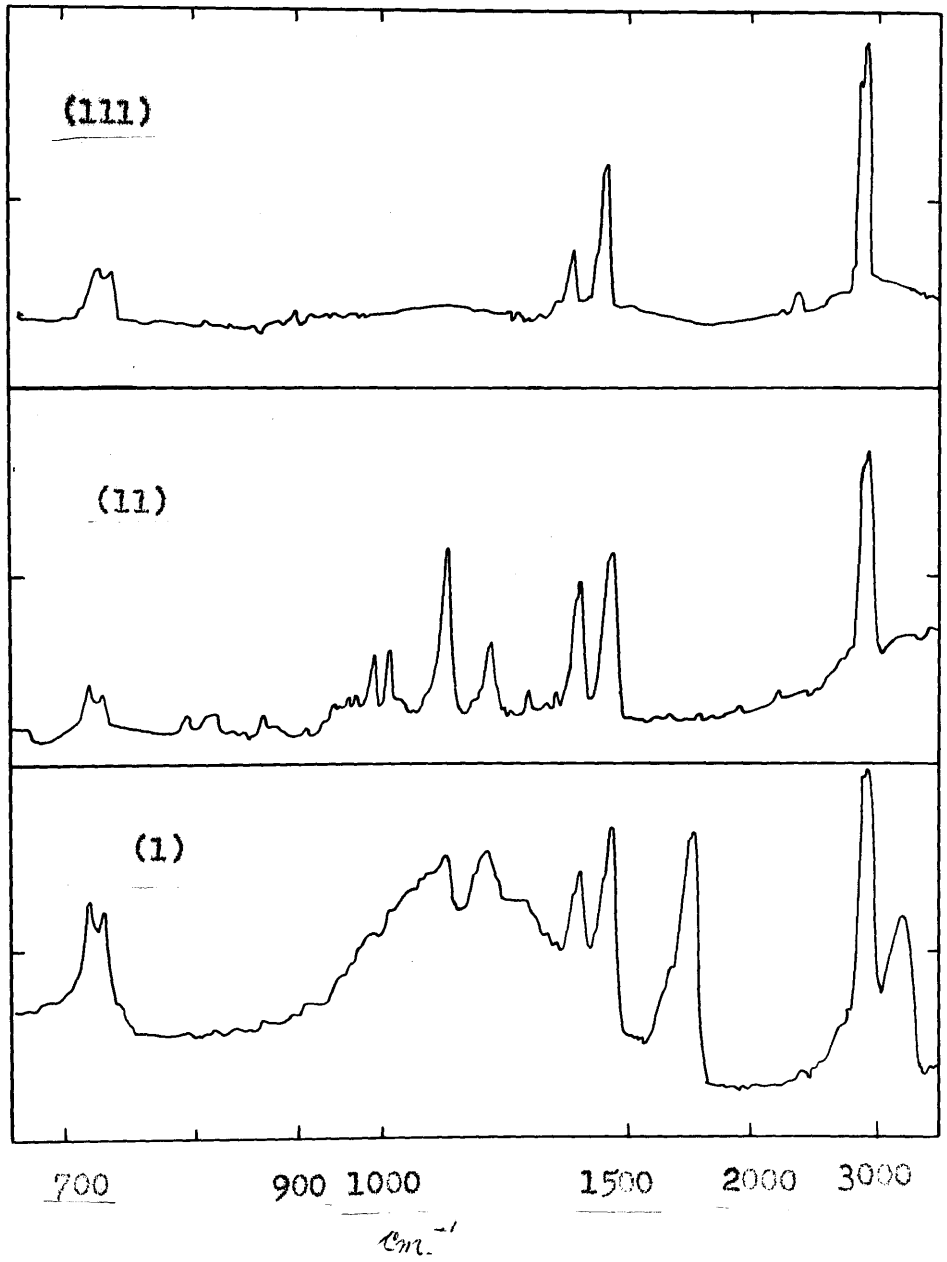


Fig. 10(a) Isolation of the hydrocarbon fraction from the plant wax of Arunda conspicua. (1) The crude plant wax as extracted from the dried plant. (11) The wax remaining after successive treatments with alcoholic potassium hydroxide and 2,4-dinitrophenylhydrazine reagent, followed by chromatography over alumina. (111) The wax remaining after treatment of fraction (11) with hot concentrated sulphuric acid. For these Gas Liquid Chromatograms, the load was ca 5u g. of solid wax, the column 130 cm. 0.4 cm. 0.5% Apiezon L on Embacel, 80-100 mesh at 225°, 45 ml./ min.


(1)
(11)
(111)
n-C31
n-C29
n-C27
60
30
0
mins.
SIGNAL



Infra red spectra . Solid films.

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SECTION III.

TAXONOMIC SURVEY OF ALKANES.

INTRODUCTION.

The use of chemical constituents of plants as an aid to their classification is now a familiar concept[1,2], the outstanding examples being the extensive work of Erdtman[3] on conifers and Bate-Smith[3] on monocotyledons. The employment[4] of leaf waxes as an advantageous taxonomic criterion is suggested by the considerations listed in Table I.

TABLE I.

(1) The universality of the occurrence of these waxy coatings.

(2) The already observed species variation in the wax composition.

(3) The reported lack[5] of seasonal variation in wax composition though the actual weight varies with both species[6] and season[6]. The unchanged wax composition may be due to the fact that the wax is extracellular as it is almost certainly an end product insulated from the regular essential metabolic functions of the plant.

(4) The simplicity of sampling.

(5) The present day availability of precise and rapid micro-analytical tools of G.L.C., mass spectrometry and infra red spectrometry.

The taxonomic possibility of alkanes seemed worthy of further investigations after our preliminary results in Section II, despite the forebodings that the differences between the waxes might not be sufficiently discriminating, too "conservative" as Erdtman[3]. has put it. Our results in Section II indicated that it was best to concentrate on one class of wax constituent (the alkanes which are present to a significant extent in most waxes). The alkane mixture is very amenable to exact and rapid analysis by G.L.C.[7,8,9,10]. and its very complexity serves as a positive advantage in providing a taxonomic "fingerprint".

In order to test the validity of these ideas a study of certain plants of the Canary Islands has been undertaken. This choice was made because of the availability of a compact grouping of closely related genera of the sub-family Sempervivoideae (Crassulaceae; Fig.1), which had already been extensively studied botanically. They are believed to be descended from a common ancestor which initially colonized the islands and to have been developed in isolation from the mainland. Because of the wide

climatic variation on these islands of diverse terrain, the variety of forms presents an excellent example of "adaptive radiation".[11] The members of this family are all xeromorphic and generally possess quite substantial waxy coatings. Several botanists, including Pitard and Proust,[12] Burchard[13] and particularly Praeger[14] have discussed the classification of these plants and Lems[11] devotes a recent lengthy paper to the evolutionary aspects of the group in which he states his belief that the Aeoniums present "a situation comparable in many ways to the finches of the Galapagos". His study concerns the plant forms of the Sempervivoideae and his conclusion is that "this group is composed of species of many forms, from shrub to biennial and annual herb; it is possible to derive all of them from a shrubby ancestor". The results of our own study are considered in relation to this approach.

RESULTS.

The plants studied are listed in Table 2. They include examples from all the constituent genera of the Sempervivoideae (Fig.1), with the exception of Sempervivum. For reasons discussed later seven species of the genus Euphorbia (Euphorbiaceae, 4,000 species) and two monocotyledons: Dracaena draco (Liliaceae, 3,700 species) and Lolium multiflora (Gramineae, 4,500 species) were included.

The waxes were extracted[15.] from both surfaces of the leaf samples by dipping the leaves either singly or in small bunches in three successive portions of cold chloroform. This treatment was found sufficient to remove all the surface wax without removing cytoplasmic constituents. The infra red spectrum of the residue remaining after removal of the solvent was then recorded (e.g. Fig. 2a).

The hydrocarbon fraction was isolated in 95% yield from the waxes by saponification followed by extraction with light petroleum and chromatography on basic alumina. (In two cases, Table 2, Nos. 33, 34, the saponification step was omitted with equally good results.) The infra red spectrum of the isolated fraction (e.g. Fig.2b)

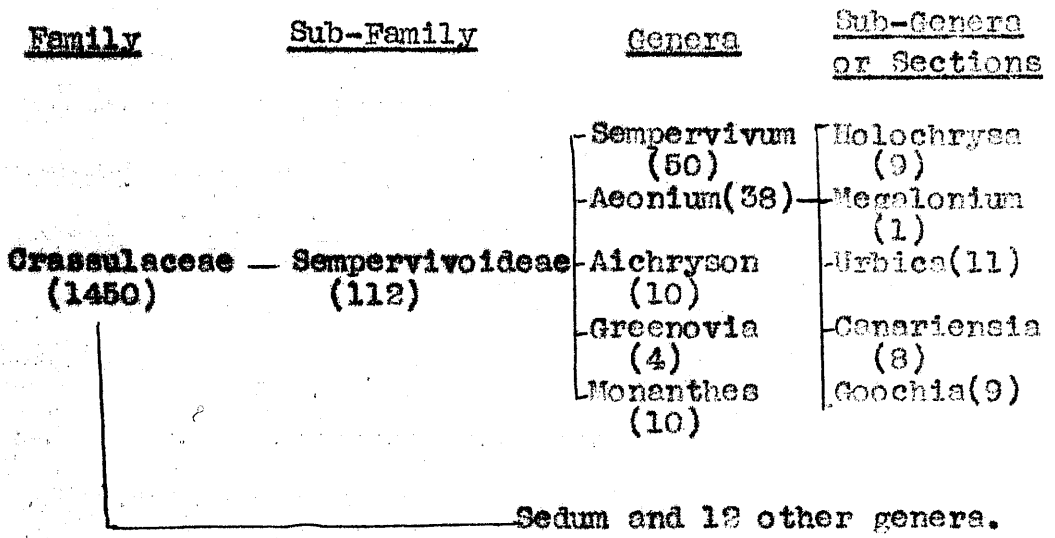


Fig.1. The Crassulaceae family showing the sub-division[11]. of the sub-family Sempervivoideae; number of species in parentheses.

showed no contaminants of different functional type, and repetition of the alkali treatment caused no change in the proportions of hydrocarbon present. Replicate extractions of leaves from the same batch gave hydrocarbon concentrates of almost identical composition (Table 2, Nos. 10-13).

The hydrocarbon fraction was analysed by gas chromatography (e.g. Fig.5) and the peaks of the n-alkanes assigned both by co-chromatography with known compounds and from the relationship of the retention times (Fig.4). The remaining peaks have all been assigned to iso-alkanes (2-methylalkanes)[9,16]. on the grounds of the relationship of the retention times (Fig.4) and the enhancement of the appropriate peak by addition of a genuine sample of 2-methyldotriacontane[17]. Although the presence of other types of saturated hydrocarbon cannot be rigorously excluded on the basis of the above techniques, it is certain that the components of the hydrocarbon mixtures belong overwhelmingly to the n- and iso-alkane series in the range C_{29} to C_{35} inclusive.

The quantitative results are given in Table 2; they were obtained by measurement of peak areas and are expressed as a percentage of the sum of the peak areas

of all compounds lying between C_{25} and C_{35}. The results were found to be reproducible to ± 5% by replicate analyses (Table 2, Nos. 44-46); and evaluation of synthetic mixtures showed that the areas were proportional to molar concentrations for each type of hydrocarbon.

DISCUSSION.

Some of the main findings of this study on the Sempervivoideae may be summarised as follows:-

(a) Alkanes of carbon number less than C_{25} and more than C_{35} are not present to any detectable extent;

(b) The content of odd carbon-number alkanes is greater than that of even carbon-number alkanes by a factor of more than ten. The distribution ratio of the odd and even series shows no parallelism;

(c) Some species contain quite high proportions of isoalkanes which occasionally even outweigh the n-alkane content. When present, an isoalkane constituent invariably accompanies its straight chain isomer, and for the major odd carbon number constituents (e.g. C_{31} and C_{33}) of any given leaf wax there are indications of a parallelism in the iso- to normal-hydrocarbon ratio.

This demonstration of the occurrence of branched chain structures makes it clear that this phenomenon should no longer be regarded as a biological "freak"[18]. although the only other reported occurrence of branched chain hydrocarbons is that found for tobacco leaf wax[19].

The principal requirement for a taxonomic criterion is that it be species specific. The present results indicate that the hydrocarbon composition of the leaf wax is not appreciably dependent on the position of the leaves with respect to the meristem (Table 2, 14 and 15), the age (20 and 21, 50 and 51) or the station (4 and 5, 13 and 17, 61 and 62) of the individual plant, provided that the species is the same. This was found to hold in spite of the differences in, for example, leaf size (e.g. 30 and 31, 38 and 40, 42 and 43) shown by those species which exhibit great seasonal variation in growth. Thus, within the limitations of this investigation and, in the absence of sufficient data for a thorough statistical treatment, it seems reasonable to conclude that the hydrocarbon pattern of the leaf wax of a species is a property characteristic of that species. Such fluctuations as there are (for example, with the A. urbicum examples 10-18) may be due to the occurrence of genetic and/or environmental factors, and do not invalidate the general conclusions.

In Fig.5 the hydrocarbon constituents of the leaf waxes studied are shown in histogram form, a single diagram for each authenticated species. The botanical classification is delineated, and within this the species are arranged such that, in general, the branched-chain isomer content increases from left to right and from top to bottom; the proportion of C_{31} to C_{33} seems to increase in a similar progression.

The most extensive survey was centred on the genus Aeonium (Fig.1) with the prime concern of unearthing any relationship between the hydrocarbon patterns and the botanical classification. Within the section Holochrysa the three species examined show closely similar hydrocarbon patterns and the same is true for the different hydrocarbon pattern obtained for the species in the section Urbica. The botanical subdivision of Urbica proposed by Lems[11] is not paralleled by these results. Six of the species (35, 28, 31, 29, 33, 36) of the section Goochia give mutually similar hydrocarbon patterns but the remaining three seem out of step in this respect, A. caespitosum (42), A. spathulatum (38) and A. cruentum (37). The species of the section Canariensia fall into two groupings.

The first three members (26, 22 and 24) show a close hydrocarbon pattern relationship allied to Holochrysa while the patterns of the next two (20 and 25) are much more akin to those of the section Urbica; one member of the Canariensia, the distinctive plate-like A. tabulaeforme(27), is quite anomalous in the reversal of its C_{31} to C_{33} n-alkane ratio.

In the less extensively examined genera Greenovia, Aichryson and Monanthes the hydrocarbon patterns are internally consistent with the exception of one of the last, M. amydros(58). Lems has suggested that the genus Greenovia is evolutionarily related to the Canariensia section of the genus Aeonium but the hydrocarbon patterns of the two are quite different and on this latter basis one might rather propose a relationship between the genera Greenovia and Monanthes and the sections Urbica and Megalonium of the genus Aeonium. On the other hand, the branched hydrocarbon content of the Aichryson species examined does give some support to Lems' contention that this genus is related to the Goochia section of the genus Aeonium.

Although such comparisons of hydrocarbon patterns may serve to establish relationships, it would seem

that the differences between related genera may be insufficiently discriminating to act as "fingerprints", e.g. in the cases of the genera Monanthes and Greenovia studied in this work.

To show the results obtained in a completely different plant family a few readily available species of the genus Euphorbia have been included. This genus (750 species) is not closely knit botanically and the observed hydrocarbon distributions are of at least two types. In comparison with the Sempervivoideae, where the C_{31} and C_{33} hydrocarbon content generally approaches about 90% of the whole, the Euphorbiae examined are much more variable in this respect and include a higher proportion of C_{27} and C_{29} hydrocarbons.

The monocotyledons Lolium multiflora (Italian rye grass) and Dracaena draco (Canary dragon tree) possess hydrocarbon patterns strikingly different from those of the Sempervivoideae representatives.

CONCLUSIONS.

Even the present limited investigation on a restricted area has shown at least some glimmerings of a taxonomic relevance in the leaf-wax hydrocarbon pattern. The power

of gas chromatography for work of this kind is rapidly increasing with development[20] of temperature-programmed, high-efficiency, high-speed columns and of automatic tabulation of quantities and retention times of the separated constituents. Such refined techniques should render surveys of the type reported here readily feasible on a very large scale, not only for the hydrocarbons, but for the oxygenated constituents[20,21]. Such investigations would not only be of taxonomic interest but could be used in the study of the possible relationships existing between plant waxes and such matters as plant metabolism[22], water balance[23], plant diseases[15,24], weather damage[15, 23,24], atmospheric pollution damage[25], efficacy of crop-spraying[15,26], the carcinogenetic effect of tobacco smoke[19], and the origin of petroleum[27]. Other waxy coatings such as those possessed by micro-organisms[28], insects[6], and animals[16] should certainly be amenable to similar study.

EXPERIMENTAL.

Collection and Identification of Plant Material.

The plants listed in Table 2, with the exception of the Sedum and the grass, were collected during the period September 1960 to April 1961 in the Western group of the Canary Islands - Tenerife, Gran Canaria, Gomera, La Palma and Hierro. A few species were collected from several stations and at different seasons. All were identified by Dr. S. Sventenius of the Jardin del Aclimatacion at Orotava, Tenerife. In order to prevent contamination of the surface waxes, contact between the leaves of different species was carefully avoided and stations were chosen which were not adjacent to road traffic or subject to urban atmospheric pollution.

Extraction of Surface Waxes[15].

In all cases, except certain Euphorbia species, only leaf waxes were extracted. Although the quantity and appearance of the leaf wax is known[15,24] to differ on the abaxial and adaxial surfaces, the present work concerns the total wax from both surfaces. The leaves were generally examined as soon as possible after collection of the entire plant which, in most cases, survived even prolonged storage.

A representative sample (100 g.) of leaves (picked from several individual plants of the particular species, collected at the same station) was extracted by dipping small bunches or individual leaves for 30 sec. in each of three successive small volumes (50 ml., 25 ml., and 25 ml.) of chloroform. In some cases complete buds, unopened or partially opened, of small leaves were extracted. The combined extract was filtered to remove suspended matter and the filtrate and washings evaporated to dryness under reduced pressure. The residue was weighed and its infra red spectrum recorded as a solid film (Perkin-Elmer Model 13 or 137; cf. Fig. 2a).

Tests with further volumes of chloroform and also with light petroleum demonstrated that this rapid and simple procedure adequately dissolved the surface wax without removing any of the cytoplasmic constituents. After this treatment the fleshy-leaved species frequently exuded in a dramatic manner considerable quantities of water.[23].

Isolation of the Hydrocarbon Content.

The wax (50-250 mg.) was heated for 2 hr. with ethanolic potassium hydroxide (1 g. in 30 ml.); the

ethanol was removed under reduced pressure and the residue extracted consecutively with three 120 ml. portions of 60-80° light petroleum. After concentration to small bulk, the petroleum extract was passed down a column of basic alumina (10 cm. by 1 cm. Brockmann, Grade II) followed by elution with light petroleum. The solvent was removed from the first 50 ml. of eluate under reduced pressure; the residue was weighed and its infra red spectrum recorded (solid film). The residue represented about 95% of the total hydrocarbon content of the wax and contained no contaminants of different functional type (Fig. 2b). Repetition of the alkali treatment on this product caused no change in the hydrocarbon proportions. An alternative simpler procedure in which the hydrolysis step was omitted, was employed in two cases (Table 2; 32 and 34). The residue from the chloroform extract was chromatographed directly[29,30]. as above. The first 50 ml. of the eluate furnished a hydrocarbon fraction identical in composition with the product obtained by the above alkali treatment.

Gas-Liquid Chromatography of the Hydrocarbon Extracts.

A Pye "Argon Gas Chromatograph" (^{90}Sr detector) was used with a 120 x 0.5 cm. column of Celite (80-100 mesh[31] coated with 0.5% of Apiezon L grease deposited from 60-80° light petroleum. Generally, the hydrocarbon mixture (~2 mg.) in warm α-methylnaphthalene (~50 mg.) was applied to the heated column (~230°) in 0.1 ul loads. Several columns were used as prolonged operation at this temperature resulted in denudation of stationary phase. Typical chromatograms are illustrated in Fig.3. The peaks were assigned on the basis of addition of genuine n-alkanes which resulted in intensification of the appropriate peaks. Further, a linear relationship was obtained between the log of the retention time (R_t) and the assigned n-alkane carbon number (Fig.4). Nearly all the remaining peaks showed a parallel straight line relationship (Fig.4) and were consequently attributed to isoalkanes (2-methyl-alkanes)[9,16]. Confirmation of this latter conclusion was derived from the enhancement of the appropriate peak by addition of a genuine sample[17] of 2-methyl-dotriacontane and from the infra red characteristics of the hydrocarbon mixture itself. The absence of

unsaturated hydrocarbons and oxygen-containing components was further checked in one case (41) by the virtual constancy of the gas chromatogram pattern even after several hours' treatment of the hydrocarbon mixture (40) with hot concentrated sulphuric acid.[22]

The content of individual hydrocarbons in the mixtures is expressed in Table 2 as an area percentage derived from the area of the relevant peak and the total area of all the peaks between C_{25} and C_{35} inclusive. Alkanes below and above these limits have been ignored as control experiments with weighed mixtures show them to be present in only trace amounts. Areas were determined by planimeter (six determinations) for chromatograms with all peaks on scale and with the major constituents registering almost full scale. Accuracy was checked by repeat analyses (e.g. 44-46) and the percentages in Table 2 seem to be reproducible to ± 5%, a variation due to detector-amplifier and planimetering errors. While not negligible this deviation is definitely too small to invalidate the general conclusions. Evaluation of synthetic mixtures

of n- and isoalkanes confirmed the detector response to be proportional to the molar concentration and was virtually the same for the two types of hydrocarbon.

"Column efficiencies"[32]. were of the order of 1500-2500 theoretical plates for the hydrocarbons in the C_{30} region (retention times ca 35 min.). In Fig.3, the "peak resolutions"[32]. were, for example, 1.3 for the n- and iso-C_{33}-alkane peaks and 2.9 for the n-alkane C_{31} and C_{32} peaks.

Table 2.

Species	No.*	Leaf Wax: Wet (wt.%)†	Leaf Wax: Dry (wt.%)‡	Leaf Wax: H C (°)§	Distribution in mole percentage of the hydrocarbons in the hydrocarbon fraction of the leaf wax: C_{28} iso	C_{28} n	C_{27} iso	C_{27} n	C_{26} iso	C_{26} n	C_{29} iso	C_{29} n	C_{30} iso	C_{30} n	C_{31} iso	C_{31} n	C_{32} iso	C_{32} n	C_{33} iso	C_{33} n	C_{34} iso	C_{34} n	C_{35} iso	C_{35} n
Aeonium																								
Section Holochrysa																								
A. manriqueorum	1	0·95	5·5	5		•		•		1	•	•	2	2	1	1	10	21	1	4	15	38		1	1	2
A. holochrysum	2	0·15	1·5	65		•		•		•		•	1	1	•	•	4	18	1	4	12	48	•	2	6	3
A. rubrolineatum	3	0·15	1	60				•		•		•		•		•	2	17	•	3	14	54		2	3	4
Section Urbica																								
A. percarneum	4	0·2		35		•		•	•	•		•		•		•	1	13		2	1	74		2		6
A. percarneum	5	0·3	4	15		•		•	•	•		•		•		•		9		3	4	79		2		4
A. percarneum	6	0·1	1	55		•		•	•	•		•		•		•	1	8		2	4	80		1		4
A. haworthii	7	0·1	1	20		•		•		•		1		•		•		21		3	3	68		•		2
A. decorum	8	0·1		15		•		•		•		1					1	25		5	5	59	1	1		2
A. castello-paive	9	0·1		60		•		•		•		1		•			1	30		3	1	59		1		5
A. urbicum	10	0·25	1·5	25		•		•		•		1		•			1	20		3	3	68		2		3
A. urbicum	11	0·1		55		•		•		•		1		1			1	21		4	2	63		•	1	6
A. urbicum	12	0·1		60		•		•		•		•		•			•	15		2	4	69		3		6
A. urbicum	13	0·2		50		•		•		•		•		•			•	16		3	6	70		1		3
A. urbicum	14	0·2		35		•		•	•	1		•		•			1	22		2	1	68		1		2
A. urbicum	15	0·1	1	65		•		•	•	•		•		•			1	15		4	4	72		1		2
A. urbicum	16	0·1		55						•		•					•	12		3	1	69		2	•	13
A. urbicum	17	0·05				•		•		1		•	•	1		•	•	12		3	2	74		1	•	5
A. urbicum	18	0·1		25		•		•		•		•	•	1		•	1	21		3	1	64		2		5
Section Megalonium																								
A. nobile	19	0·2	2·5							•		•		1		•	•	12	•	3	1	76		2		5
Section Canariensia																								
A. cuneatum	20	0·1	0·5	35		•		•		•	•	•		1		•	•	27		3	2	63		•		4
A. cuneatum	21	0·05	0·5	35		•		•		1	•	•	•	1		•	•	29		2	2	60		•		4
A. canariense	22	0·05	0·5	55		•		•		•	•	•	•	1	•	•	9	21	1	4	23	36		•	1	2
A. canariense	23					1		•	•	2		•	1	3			11	10	1	1	39	25			•	4
A. palmense	24	0·25	2·5	30		•		•	•	2		•	1	3	•	•	9	14	1	3	26	35	•	1	2	2
A. subplanum	25	0·05	1	45		1		•		1		•	•	1		•	3	36		3	4	45		1	1	3
A. virgineum	26	0·05	0·5	40		1		1		2	2	2	1	1	•	•	7	14	1	2	23	33			3	4
A. tabulaeforme	27	0·05		45						•		•		2		1		79		2		17				
Section Goochia																								
A. goochiae	28	0·7	9	10		1		•		3			8	7		2	16	14	3	2	17	21			3	4
A. viscatum	29	1·1		5		2		•	2	8			16	12			13	18			12	16			•	•
A. lindleyi	30	1·2		1		•		•		1		•	3	8	•	•	11	22	1	2	18	27			2	2
A. lindleyi	31			5	•	2		•	•	2	•	•	12	9	1	1	16	17	1	1	15	19				2
A. lindleyi	32				•	2		•		2		1	11	8	1	1	16	17	1	2	17	19				•
A. saundersii-Bolle	33			15		•		•	•	1	•	1	13	5	1	1	20	16	1	3	20	16	•	•	2	1
A. saundersii-Bolle	34					•		•	•	1	•	1	13	5	1	1	22	16	1	2	20	14		•	2	1
A. sedifolium	35	0·9	12	5		1				1			1	2		•	10	17	1	3	25	34			2	2
A. smithii	36	0·1	1	20						•			1	1	•	•	30	17	1	3	22	19	•	•	3	1
A. cruentum	37	0·4	4·5	25				•		•		•		3		1	2	53		3	4	29				3
A. spathulatum	38	3·8	7·5	2				•		•		•		2		1	7	56		4	5	24				
A. spathulatum	39					•	•	•		•		•	•	2		•	15	54		2	8	18				
A. spathulatum	40			3		•		•		•		•	•	3		1	15	59		2	5	14				
A. spathulatum	41					•		•		•			•	3		1	13	51	•	2	7	21				
A. caespitosum	42	0·25		25									1	1			4	13	1	3	16	57		1	1	2
A. caespitosum	43	0·25	3	20						•		•		•			3	13		2	16	58		•	2	3
Hydrocarbon mixture	44					1				1			1	2			10	13	1	2	35	35				
Repeat run	45					1				2			1	2			10	12	1	2	35	34				
Repeat run	46					•				2			1	3			11	12	1	3	34	33				
Aichryson																								
Ai. dichotomum	47	0·2	2	55		•		•		•		•	1	1		1	2	12	4	2	4	62	2	1	•	6
Ai. punctatum	48	0·05	0·6	35										1		•	1	17		4	3	62		1	3	6
Ai. unidentified	49	0·05		35						1			2	2		4	7	13	7	2	14	41		2	2	3
Greenovia																								
G. aurea	50	0·1						•		•				•			•	9		1	1	80		1		7
G. aurea	51	0·05	1	35		•		•		•				•		•	•	8		1	1	76		2		12
G. diplocycla	52	0·05	1	45		•		•		•				•			•	4		1	2	78		2		9
G. unidentified	53	0·05	1	20				•		1		•	2	2		•	2	8		1	3	72		1		7
G. unidentified	54	0·3		15		•		•		•				•				4		1	3	75		1	2	14
G. unidentified	55		1	50														7		3	2	77		1		10
G. unidentified	56	0·05	1	20		•		•	•	1		•		1			•	5		1	1	82		1	1	8
G. unidentified	57	0·05		30		•		•		•		•		•			•	5		2	2	81		2		8

TABLE 2 *continued.*

SPECIES	No.*	Leaf Wax			Distribution in mole percentage of the hydrocarbons in the hydrocarbon fraction of the leaf wax																			
		Wet (wt.%)†	Dry (wt.%)‡	+H/C (%)§	C_{25}		C_{27}		C_{29}		C_{31}		C_{33}		C_{35}		C_{37}		C_{39}		C_{41}		C_{43}	
					iso	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*
MONANTHES																								
M. amydros	58			45					1		•	•	1		3		52		2	1	43			
M. laxiflora	59	3·1		65			•		•		•		•		•		2		1		94		1	1
M. polyphylla	60	0·4	4	30		•	•		•		•	•	•		•		12		2	2	84		•	1
M. muralis	61			15					•				•				9		1		87		1	2
M. muralis	62			25		•	•		1		•	•	1				15		2	1	77			4
M. anagensis	63	2·0	12	35					•				•				4		1		89		1	3
M. brauchycaula	64	0·1		70		•	•	•	•			•	1		•		10		1	•	84		1	2
M. pallens	65	0·25		25		•			1			1	1		1		13		2	1	80		1	3
SEDUM																								
S. anglicum	66								1				8		1		80		1		9			
DRACAENA																								
D. draco	67			1		4		5		12		7		22		6		31		1		4		
LOLIUM																								
L. multiflora	68			10		4		1		7		1		42		1		40				3		
EUPHORBIA																								
E. peplus	69	0·2	1	3		3		2	1	9	•	1	1	17		1		45		1		18		
E. balsimifera	70	1·2		5		20		1		51		1		24										
E. atropurpurea	71	0·4		10		1		•	•	5		•	•	13	1	1	•	70		1		8		
E. regis-jubae	72	0·2	1	2		1		•		3		•		11		1		67		1		15		
E. aphylla	73	0·8		2		14		2		15		3		23		3		33		•		2		
E. bourgaeanea	74					•		•		17		1		25		1		56						

* The species were collected at the following stations: (i) on the island of *Tenerife* at Buenavista, 7, 17, 18, 59, 65 and 74; La Laguna, 10, 11, 12, 13 and 67; Las Mercedes, 14 and 15; the Forestry Gardens, La Laguna, 20 and 21; San Juan de la Rambla, 27; Santa Ursula, 16; Bajamar, 30, 31, 32; Canadas, 36; Aguamansa, 38, 39, 40, 41; Punta de Hidalgo, 48; La Esperanza, 50 and 51; Playa de Martianez, 60 and 64; Punta de Anaga, 63; Taco, 72; and at undetermined locations, 2, 22, 23, 47, 69, 70 and 71: (ii) on the island of *Gran Canaria* at Tafira, 1; Santa Brigida, 5 and 6; Firgas, 26; Lagunetes, 4, 42 and 43; San Mateo, 54 and 55; and at an undetermined location, 49: (iii) on the island of *Gomera* at Chejelipe, 8, 33, 34, 57 and 58; Agulo, 53; Tunel, 56; and at undetermined locations, 3, 9, 25, 29, 52 and 73: (iv) on the island of *La Palma* at Tazacorte, 35; Mazo, 37 and 62; and at undetermined locations, 19, 24 and 28: (v) on the island of *Hierro* at Sabinosa, 61: and (vi) at *Portencross, Scotland*, 66 and 68.

The species were collected during the following months: September 1960, 1, 2, 3, 4, 5, 9, 10, 11, 12, 13, 20, 22, 25, 26, 27, 30, 42, 47, 48, 49, 54, 55, 70 and 71; October, 38, 52, 53, 56, 57 and 63; November, 6, 14, 15 and 43; December, 7, 8, 17, 18, 19, 24, 28, 29, 35, 37, 59, 65, 74; January 1961, 21, 36, 60, 62, 64, 69 and 73; February, 16, 50, 51 and 72; March, 39, 40 and 41; April, 23, 31, 32, 33, 34, 58, 61 and 67; May, 66 and 68.

Specific points concerning the origins of the wax extracts are as follows:

Species numbers: 10–13 are repeat extractions of fresh leaves from the same batch of individuals of that species; 15, immature leaves; 16 and 55, dead leaves in a naturally desiccated condition; 17 and 18 differ in that they were collected at different altitudes in the same district; 32 and 34, hydrocarbon fractions isolated solely by column chromatography; 39, the plant was in flower; 41 is the hydrocarbon fraction from sp. 40 after treatment with concentrated H_2SO_4 for 3 hrs at 120°C; 44–46 are replicate analyses of the same hydrocarbon mixture; 51, young individuals; 53 and 54, possible *G. diplocycla* and *G. aurea*, respectively but identification awaits appearance of flowers; 69 and 73, stalk wax in addition to leaf wax—all other species numbers were derived from leaf surface waxes only.

† *Wet wt.* (%) = wt. of wax extract/wt. of undried leaves. Values are approximated to nearest 0·05 per cent.

‡ *Dry wt.* (%) = wt. of wax extract/wt. of leaves after air drying to constant weight. Values are approximated to nearest 0·5 per cent. Data are incomplete due to difficulties in drying many species.

§ *H/C* (%) = wt. hydrocarbon fraction/wt. of wax extract. Values greater than 5 per cent are approximated to nearest 5 per cent.

‖ *The content* of an individual hydrocarbon is expressed as a mole percentage of the total hydrocarbon content from C_{25}–C_{35} inclusive. The mole percentage is taken as being equivalent to the area percentage, i.e. $A_n / \sum_{n=25}^{n=35} A_n$, where A_n is the area of the peak corresponding to the hydrocarbon C_nH_{n+1} in the gas-liquid chromatograms (cf. Fig. 3). The values are approximated to the nearest 1 per cent and peaks of relative area <1 per cent are indicated by •. The species 67–74 were measured over the range C_{25}–C_{35} as there is an appreciable content of these hydrocarbons C_{37} and C_{39} thus: 67 has *n*-C_{37} 1 per cent, *n*-C_{39} 2 per cent; 68 has traces of *n*-C_{37} and $C_{n\text{-}39}$; 70 has *n*-C_{37} 2 per cent, *n*-C_{39} 1 per cent; 73 has *n*-C_{39} 5 per cent.

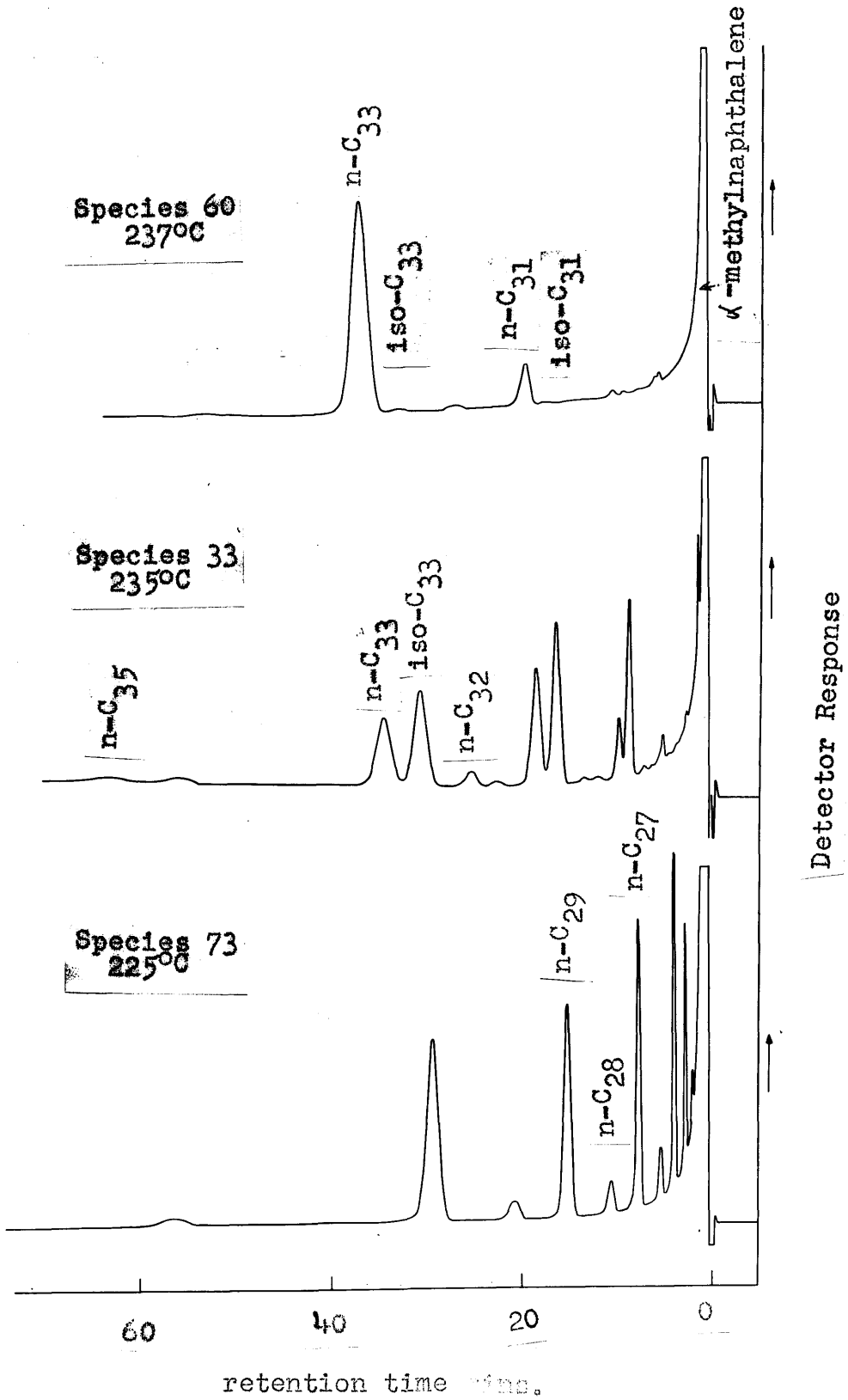

Species 60
237°C
n-C33
iso-C33
n-C31
iso-C31
α-methylnaphthalene
Species 33
235°C
n-C35
n-C33
iso-C33
n-C32
Species 73
225°C
n-C29
n-C28
n-C27
Detector Response
60
40
20
0
retention time (min.)

Fig. 3

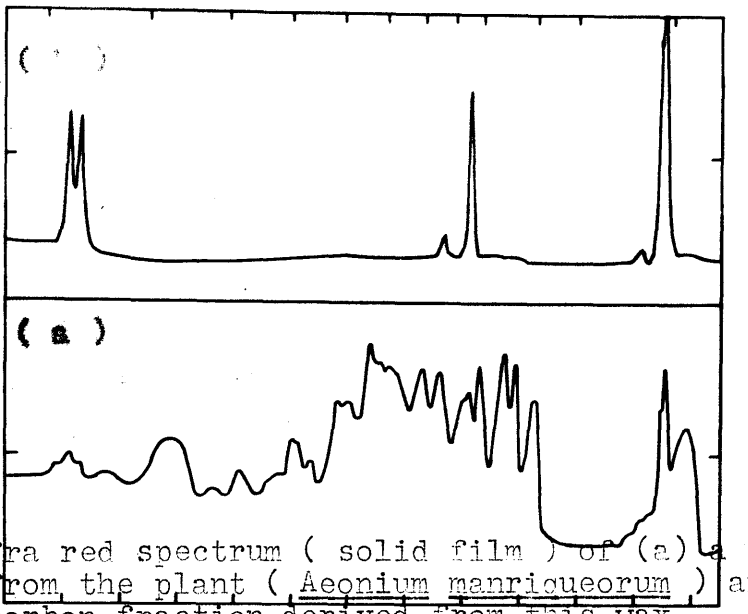
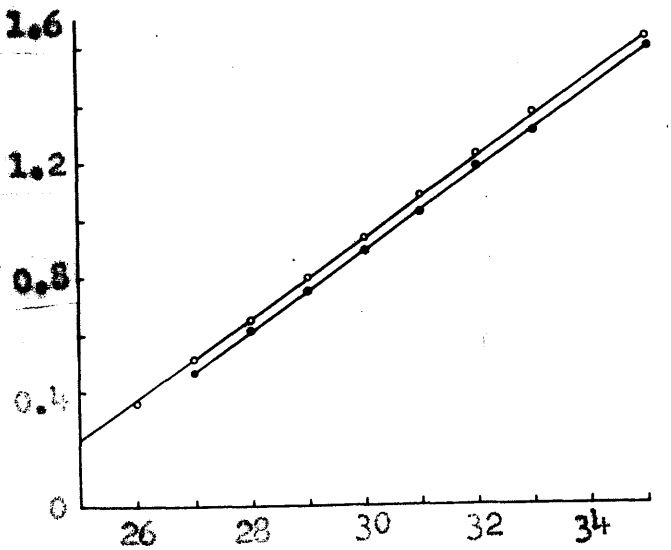
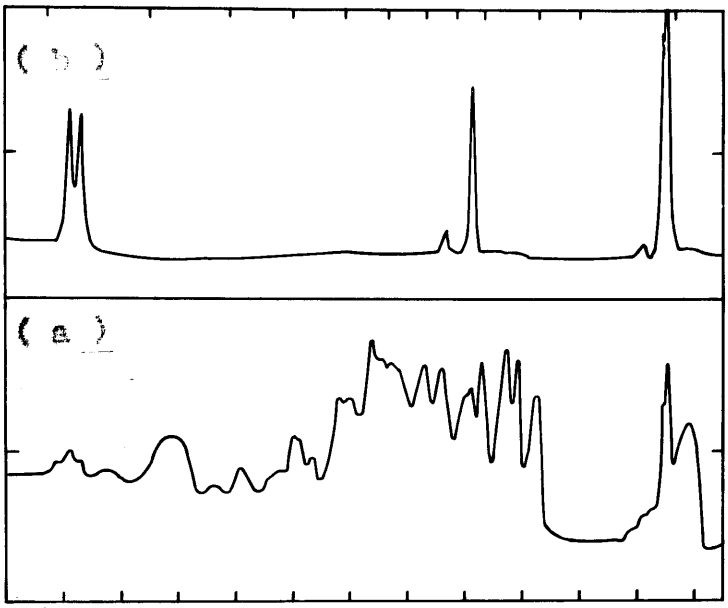
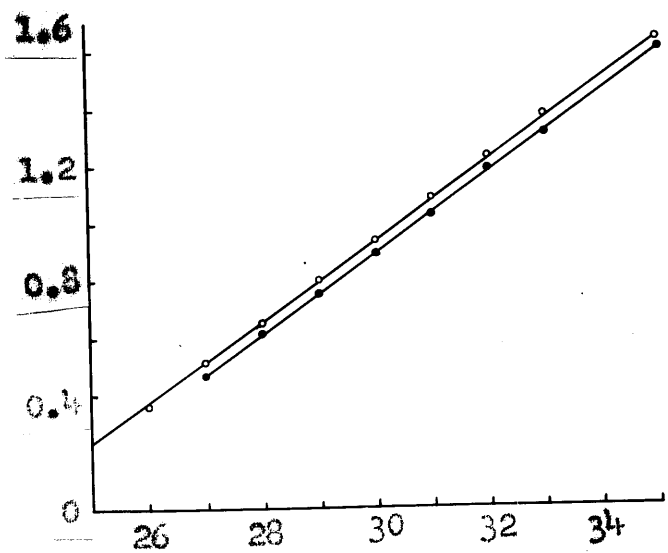


Fig. 2 The infra red spectrum (solid film) of (a) a leaf wax as extracted from the plant (Aeonium manriqueorum) and of (b) the hydrocarbon fraction derived from this wax.

Fig. 4 Plot of the log of the retention time against carbon number for the peaks assigned to n- and iso- alkanes. Data from the Gas Liquid Chromatogram (Fig.3) of the hydrocarbon fraction of the leaf wax of Aeonium saundersii-Bolle.
= n. alkanes, = iso- alkanes.




(b)
(a)


1.6
1.2
0.8
0.4
0
26
28
30
32
34

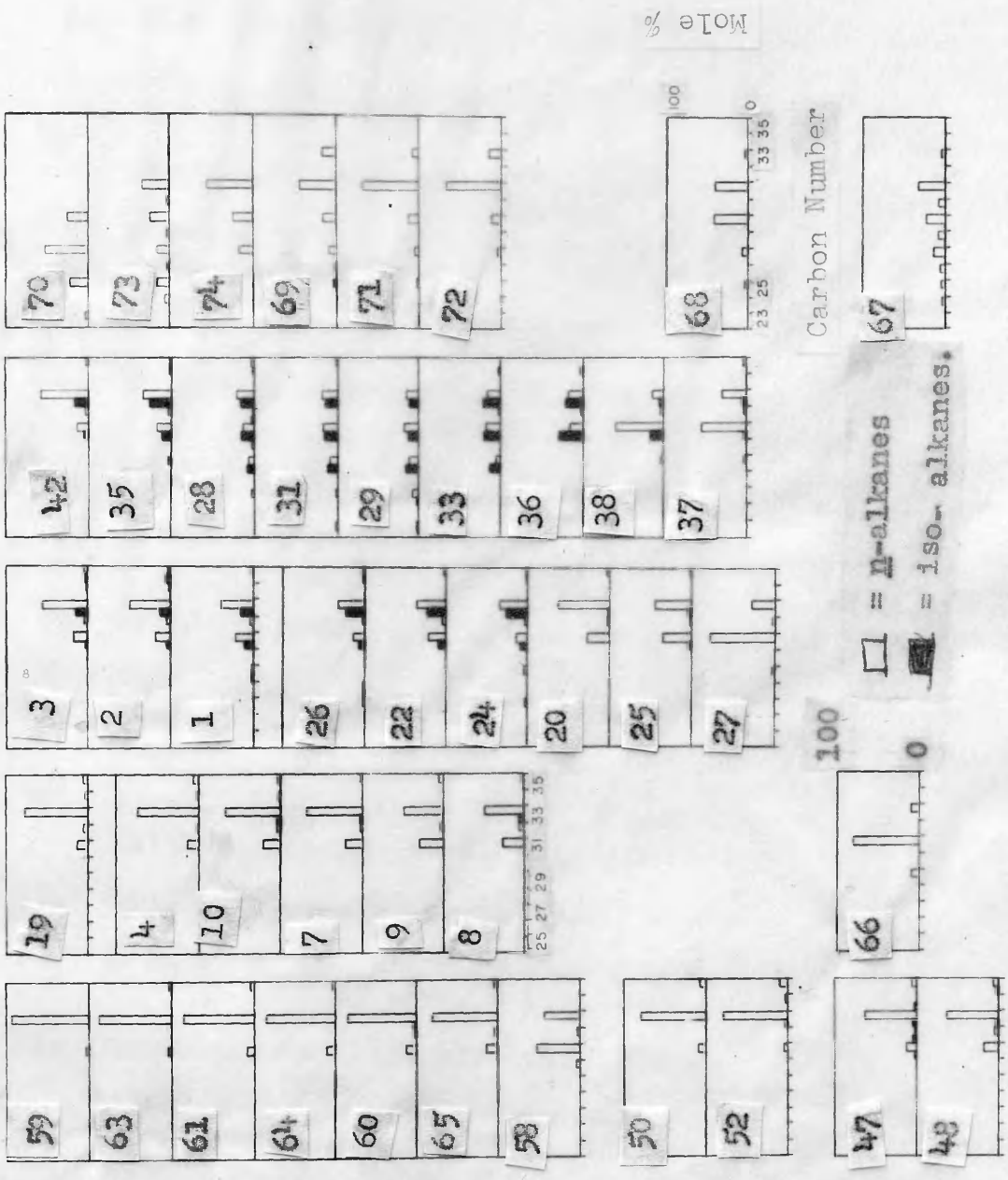
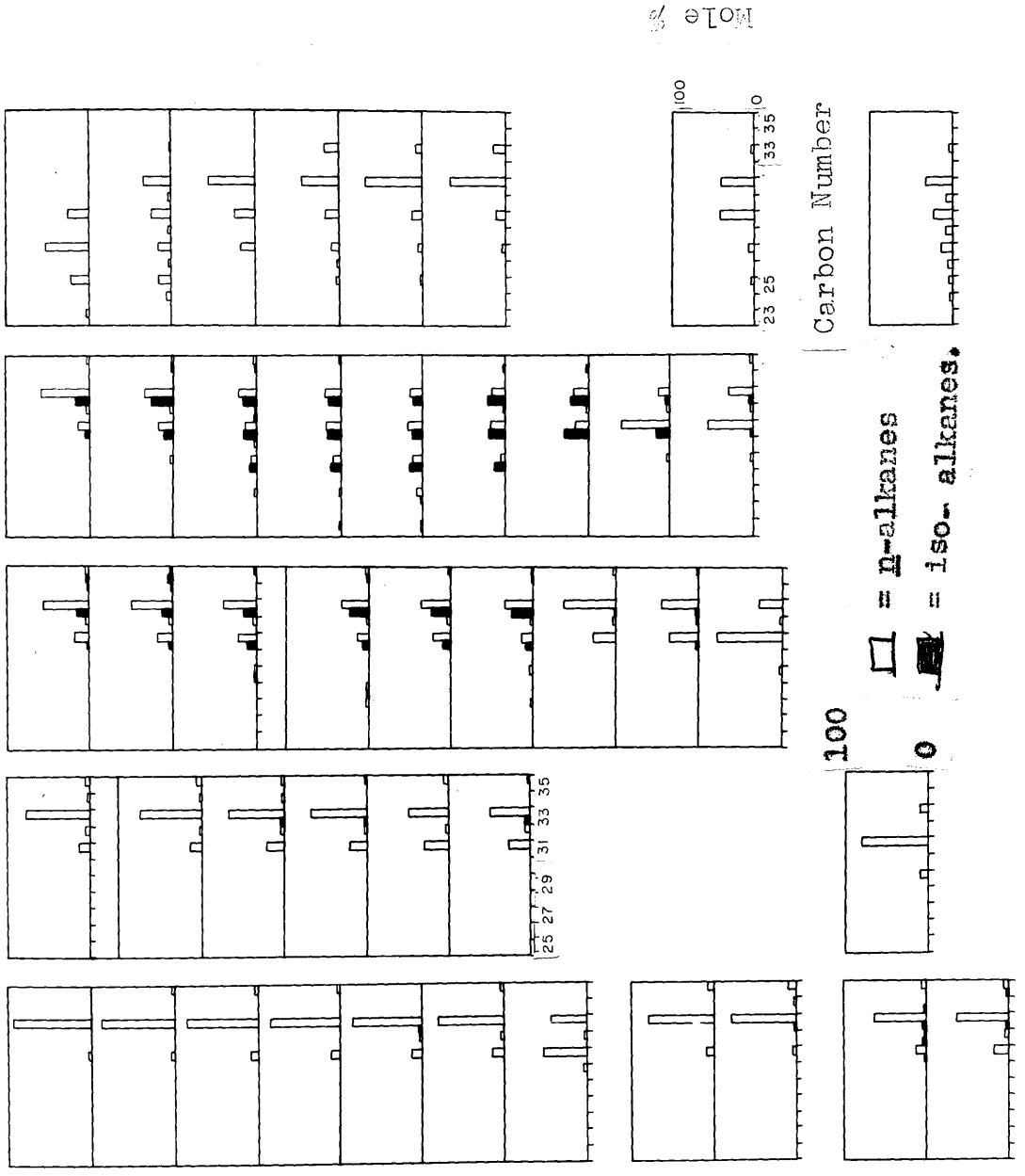


Fig. 5 Distribution in mole percentage of n- and iso- alkanes C_{23} - C_{35} in the hydrocarbon fraction of the surface waxes from the leaves of individual species of the genera Monanthes, Greenovia, Aichryson, Aeonium, Sedum, Euphorbia, Lolium and Dracaena. (In two species Euphorbia aphylla and E. peplus the wax originates from stalk and leaves). Alkanes present as less than 2 mole per cent have been omitted.



5 Distribution in mole percentage of n- and iso- alkanes C_{23} - C_{35} in the hydrocarbon fraction of the surface waxes from the leaves of individual species of the genera Monanthes, Greenovia, Aichryson, Aeonium, Sedum, Euphorbia, Lolium and Dracaena. (In two species Euphorbia aphylla and E. poplus the wax originates from stalk and leaves). Alkanes present as less than 2 mole per cent have been omitted.

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S E C T I O N IV.

ELUCIDATION OF ALKANE STRUCTURES BY G.L.C. AND SPECTROSCOPIC METHODS.

INTRODUCTION.

In Sections II and III it was assumed that the constituents of the alkane fraction of leaf waxes were n and iso-alkanes. This assumption was based on analogy with the plant alkane studies of Murray[1]. and of Carruthers[2] and their collaborators. Further, in our case, the peaks assigned to n-alkanes fell on a line when their logarithm retention times were plotted against carbon number and the iso-alkanes on a second parallel line. However it was realised that there were other compounds which might fit such G.L.C. data e.g. unsaturated compounds, alkanes with branched chains and alkanes with alicyclic rings attached, and it was decided to investigate these other possibilities by infra red spectroscopic, gas liquid chromatographic and mass spectrometric study of a few wax fractions and of standard alkanes. We were fortunate in obtaining pure standard hydrocarbons from Dr. A.C. Chibnall, F.R.S., Dr. Wibaut of the University of Amsterdam,

Holland and Dr. Dixon of the American Petroleum Institute, Pennsylvania State University, to whom we are greatly indebted.

RESULTS AND DISCUSSION.

The assumption, which was made in Sections II and III, and mentioned above, was that the components of the alkane fraction were all either iso or n-alkanes. The only evidence for this was the relative retention time of the n and iso-alkanes. From Tables I A and B it can be seen that 5-methylpentadecane and 5-butyldodecane have retention times which would distinguish them from either n or iso-alkanes. However 2-ethyltetracosane has the same retention time relative to n- C_{26} as 2-methylpentadecane relative to n- C_{16}, and 2-methylpentadecane cannot be separated from 3-methylpentadecane. n-Heptacosane, 1-cyclohexyleicosane and 1-cyclopentylheneicosane cannot be resolved and 2-cyclohexyleicosane could be mistaken for iso- C_{27}. It is reasonable to suppose that these findings concerning specific hydrocarbons are true for all hydrocarbons in the C_{22} - C_{36} range.

It would thus seem that it is not possible to make positive identification of the alkane constituents of waxes by G.L.C. alone. In other types of G.L.C. analyses it is possible to use a second more polar stationary phase and to compare the relative retention times of standards on the two columns. However with these high molecular weight compounds, the alternative stationary phase (silicone gum) was not capable of separating 1-cyclopentylheneicosane and 1-cyclohexyleicosane.

The infra red spectra of the standard alkanes in the range 1300 - 1500 $cm.^{-1}$ are helpful in distinguishing between different types. The data from the region 2850 - 2960 $cm.^{-1}$ also gives information concerning the methyl and methylene groups in standard hydrocarbons (Table III) but it is more difficult to interpret than that in the 1300 - 1500 $cm.^{-1}$ region so it was decided to concentrate on this latter range. In the range 1300 - 1400 $cm.^{-1}$, the n- alkanes absorb at 1380 $cm.^{-1}$, due to the symmetric C-H deformation of the methyl

group and at 1370, 1355, and 1340 cm.$^{-1}$ in broad diffuse peaks, probably due to skeletal vibrations, which increase in intensity with increasing molecular weight. Introduction of a cyclohexane or cyclopentane ring to the hydrocarbon chain (Table II) confirms that the 1380 cm.$^{-1}$ peak is due to methyl deformation since its intensity is reduced when the alicyclic ring is in the 1- position compared with the intensity when it is in the 2- position. Introduction of an alkyl side chain increases the methyl absorption (1380 cm.$^{-1}$) except when the side chain is methyl in the 2- position where, the gem dimethyl group so formed, has very intense characteristic absorption at 1386 and 1368 cm.$^{-1}$ (Fig. 1, nos. (11) and (111)). Thus for standard hydrocarbons it is possible to distinguish between 2-methylpentadecane and 3-methylpentadecane or 3-ethyltetracosane i.e. in general terms infra red absorption will distinguish between the iso-alkane and an alkane with any other branched chain. It is not however possible to distinguish between 3-ethyltetracosane and 3-methyl-pentadecane though with these two specific

examples, the effect of increased molecular weight on the skeletal vibrations is noticeable (Table II). This means that the other branched alkanes cannot be distinguished by infra red.

It was impossible to resolve n- C_{27} from 1-cyclohexyleicosane and 1-cyclopentyleicosane by G.L.C. As mentioned above the infra red absorption (1300 - 1400 $cm.^{-1}$) of 1- cyclo compounds is such that the intensity of the 1380 $cm.^{-1}$ band is reduced with the result that these compounds cannot be distinguished by infra red spectroscopy. However, 1-cyclohexyleicosane absorbs at 1445 $cm.^{-1}$ due to unperturbed methylenes in an alicyclic ring much more intensely than 1-cyclopentylheneicosane, thus infra red would distinguish between n- C_{27} , 1-cyclohexyl-eicosane and 1-cyclopentyleicosane. Table I A also showed that <u>iso</u>- C_{27} and 2-cyclohexyleicosane had the same retention time but infra red absorption in the 1300 - 1500 $cm.^{-1}$ region clearly distinguishes between <u>iso</u>- alkanes and 2-cyclohexyl derivatives (Fig. 1 (11) and Fig. 2 (11) and (111)).

That there are iso- alkanes in the wax hydrocarbon fraction is best confirmed by considering Aeonium Smithii whose alkane fraction has 57% branched hydrocarbon and exhibits the very characteristic gem dimethyl absorption Fig. 1 (V1). The only difference between the absorptions of Aeonium Smithii and 2-methyldotriacontane is in the 1379 $cm.^{-1}$ peak which is more intense in the natural wax due to the 43% n-hydrocarbons present. The I.R. absorption of the wax hydrocarbons of Monanthes laxiflora is very similar to the absorption of n-dotriacontane confirming the G.L.C. analysis.

In the 1400 - 1500 $cm.^{-1}$ region, there is absorption due to methylene scissoring vibrations, 1467 $cm.^{-1}$ and to methyl asymmetric deformation, 1458 $cm.^{-1}$. The diagrams in Fig. 2 can be seen to be very similar and there is no way of distinguishing between Aeonium Smithii and Monanthes laxiflora . R. N. Jones[3] claims that the asymmetry on the lower frequency side of the 1467 $cm.^{-1}$ peak is more marked at higher molecular

weight and due to coupling between the scissoring vibrations of suitably orientated vicinal methylene groups in randomly coiled chains or from coupling of a methylene scissoring vibration with the first overtone of the rocking vibration at 722 - 719 cm.$^{-1}$. Our results confirm that the asymmetry is greater at higher molecular weight but in the hexadecane series the calculated absorption coefficient is much lower than we have found.

By use of the formulae derived by R. N. Jones[3]. (Table III) it is possible to calculate an apparent molecular extinction coefficient for the n-hydrocarbons. The calculated values (Table II) agree with the values found for the n-hydrocarbons except in the case of dotriacontane where the calculated is much higher than the observed value. This finding is in agreement with the fact that G.L.C. analysis shows it to contain a high proportion of n- C_{28} . The observed extinction coefficient for Monanthes laxiflora of the 1379 cm.$^{-1}$ peak, 53, is in good agreement with the calculated value, 53, assuming the Molecular Weight

for n- C_{33} which G.L.C. suggests. The intensity of the absorption, 176, of the peak at 1379 $cm.^{-1}$ in squalane is much higher than that value calculated, 136, by assuming that it is due to four methyl groups each with a weight of the single methyl group in 2-methyldotriacontane. This suggests that these methyl groups in the middle of the chain have greater absorption than those at the end.

Thus far we have shown that at least in the wax of Aeonium Smithii, the components which fall on the second parallel line in the plot of logarithm retention time against carbon number are iso- alkanes. Both Murray [1.] and Whitham [4] had shown that n- alkanes could be removed from a mixture by absorbing them on to Linde 5A^{o} sieve. They had applied this to G.L.C. analysis and removed n- alkanes from a mixture to permit the analysis of the iso- alkanes. We attempted unsuccessfully to use a short column of Linde sieve to analyse the iso- alkanes of Aeonium lindleyi but since our attempt Whitham [5] has published results showing that although iso-C_{22}

is not absorbed by Linde Sieve, 2-methyldotriacontane is absorbed which may explain why we could not detect any components from Aeonium lindleyi alkanes using subtractive G.L.C. O'Connor[6] has shown recently that n- alkanes can be removed from paraffin wax by refluxing it in iso- pentane with pellets of Linde Sieve. This may be the easiest method of tackling the separation of iso- alkanes in any future work.

A study of the nuclear magnetic resonance spectra of the hydrocarbon fractions of some waxes and of some standard hydrocarbons was not very instructive since the methyl peaks, τ = 9.0, 9.04 and 9.10 were much smaller than the methylene peak, τ = 8.73 making quantitative area measurement difficult.

The experiment described in Section III in which the hydrocarbon fraction of Aeonium urbicum was unchanged after treatment with concentrated sulphuric acid suggested that only a small proportion of alkenes could be present (if at all). Small amounts of alkenes would be difficult to

detect (e.g. ordinary C and H analysis would be useless to determine 1% of alkene material, infra red absorption in the 1600 - 1700 $cm.^{-1}$ would be of too low an intensity to be detected, in the ultra violet spectrum, $\log \varepsilon = 1$ or 2 at λ_{max} 195-205mμ would be the intensity of a 1% alkene mixture and this is much too small to detect. It was not possible to determine the alkene content of the wax fractions (if present) but means of tackling the problem include oxidation of a large quantity of the hydrocarbon fraction with potassium permanganate and identification of the oxygenated portion as well as G.L.C. analysis of the unreacted alkanes or hydrogenation of the wax fraction, followed by G.L.C.

As conclusive evidence for the assignment of the G.L.C. peaks in the wax alkane fractions to iso- and n- alkanes, separation of individual peaks was accomplished by preparative G.L.C. followed by mass spectrographic analysis. The results of these analyses can be seen in Fig. 3 in which the breakdown pattern of the peak assigned to the

iso-C_{33} hydrocarbon from A. lindleyi is very similar to that of synthetic 2-methyldotriacontane. The diagram for 2-methyldotriacontane shows that the parent peak (the only peak of even mass number) is 20 - 30% the abundance of the (parent - $C_3H_7^+$) ion, removal of the $C_3H_7^+$ ion (the gem dimethyl group) leaving a normal hydrocarbon chain of three carbon atoms fewer than the parent. The breakdown pattern is then like that of an n- alkane (compare with the review of Ryhage[8]), a series of ions each one CH_2 unit less than the preceeding one, increasing in abundance as in n-C_{30} (synthetic). It is suspected that the slight deviation from the steadily increasing abundance peaks in the natural iso-C_{33} is due to varying vapour pressure in the mass spectrometer (which may also explain the sudden drop in abundance at C_7) .

The breakdown pattern of the peak assigned to n-C_{33} in Monanthes laxiflora is typical of an n- alkane (with again the slight variation in abundance due to pressure changes). Murray [1].

had stated that the peaks associated with the n- alkanes of even carbon number are not iso- alkanes but ante-iso alkanes (3-methylpentadecane). We attempted to isolate the component which elutes from the G.L.C. column before n-C_{32} but G.L.C. analysis showed that the fraction trapped contained a mixture with the peak in which we were interested present to only 30%. Mass spectrographic analysis of this fraction gave a breakdown pattern reminiscent of an iso- alkane but in fact each of the peaks for C_{33}, C_{32}, C_{31}, and C_{30}, appeared at an even mass number showing that they were parent peaks and not ions from iso- C_{33}.

One other assumption which was made in Section III was that the detector response was the same for branched chain alkanes as for n- alkanes. In order to check the detector response, analyses of solutions of known concentration of 3-methylpentadecane and n-hexadecane in chloroform were carried out by G.L.C. and the area ratio of the peaks measured. The area of the branched hydrocarbon peak was found

to be larger than would be expected. The calibration factor varies for each type of branched hydrocarbon -- almost 1.0 for the iso- alkane and as low as 0.75 for 5-methylpentadecane (Table IV).

In the hexadecane series, mole ratio and weight ratio are interchangeable but in comparisons of n-triacontane and 2-methyldotriacontane the area ratio can be seen to be more nearly equal to the mole ratio.

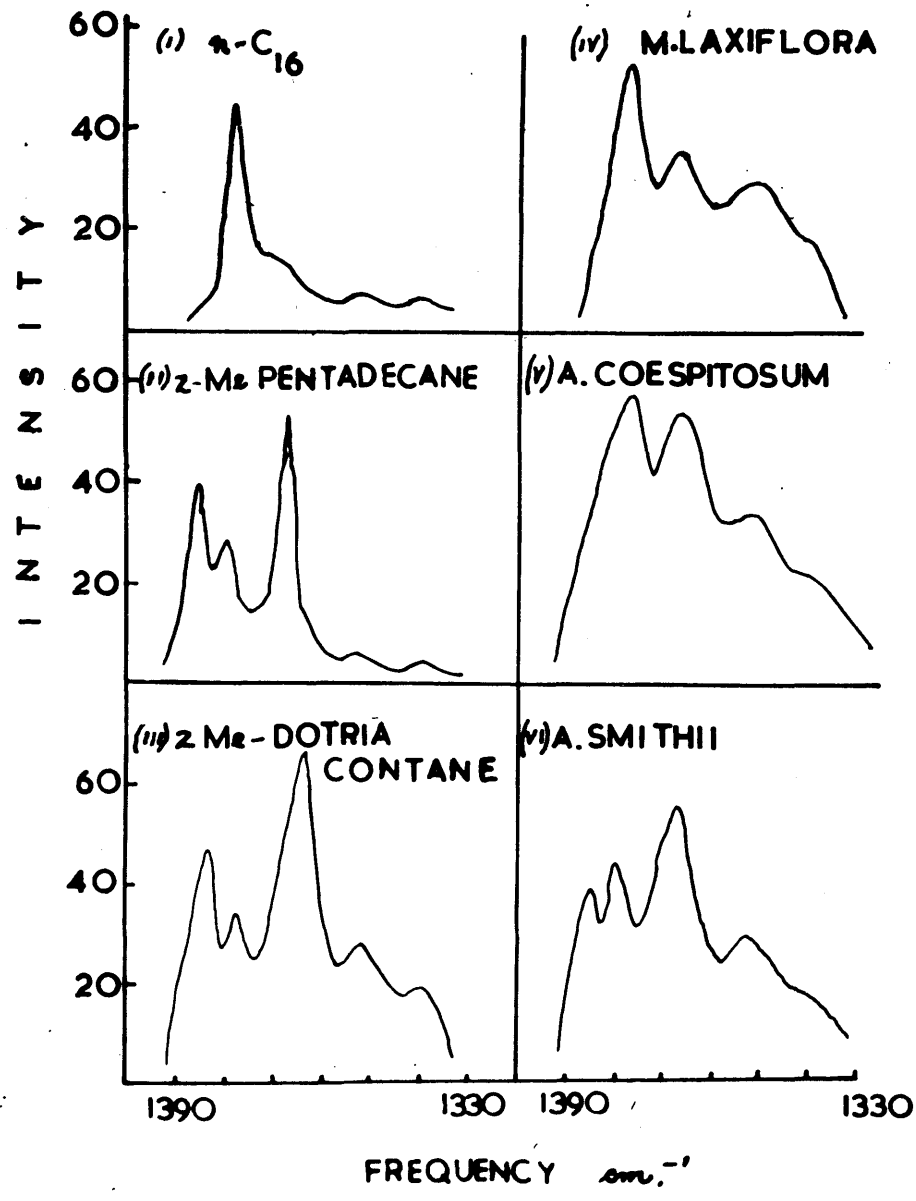
In Murray's analyses of the wax hydrocarbons, he determined the proportion of the alkanes unmeasured by G.L.C. by weighing his sample probe before and after analysis. Comparison of the peak heights and sample weight with peak heights and sample weight of mixtures of known hydrocarbons showed that of the alkane was analysed. We were unable to use this means of determining the unmeasured proportion of the wax fraction but an alternative was found. Solutions of mixtures of hydrocarbon fraction of Monanthes laxiflora and n-nonacosane of known concentrations in chloroform were analysed by G.L.C. at 229°

and the peak areas measured. From our analyses in Section III, Monanthes laxiflora is 98% pure n-tritriacontane, it follows then that the area ratio of the n-tritriacontane peak to the n-nonacosane peak should be equal to the mole ratio. The measurements of these two ratios show that the area ratio differs from the mole ratio by 5-9% suggesting the presence of limited quantities of components in the Monanthes laxiflora wax of molecular weight higher than C_{35} or lower than C_{23}.

CONCLUSIONS.

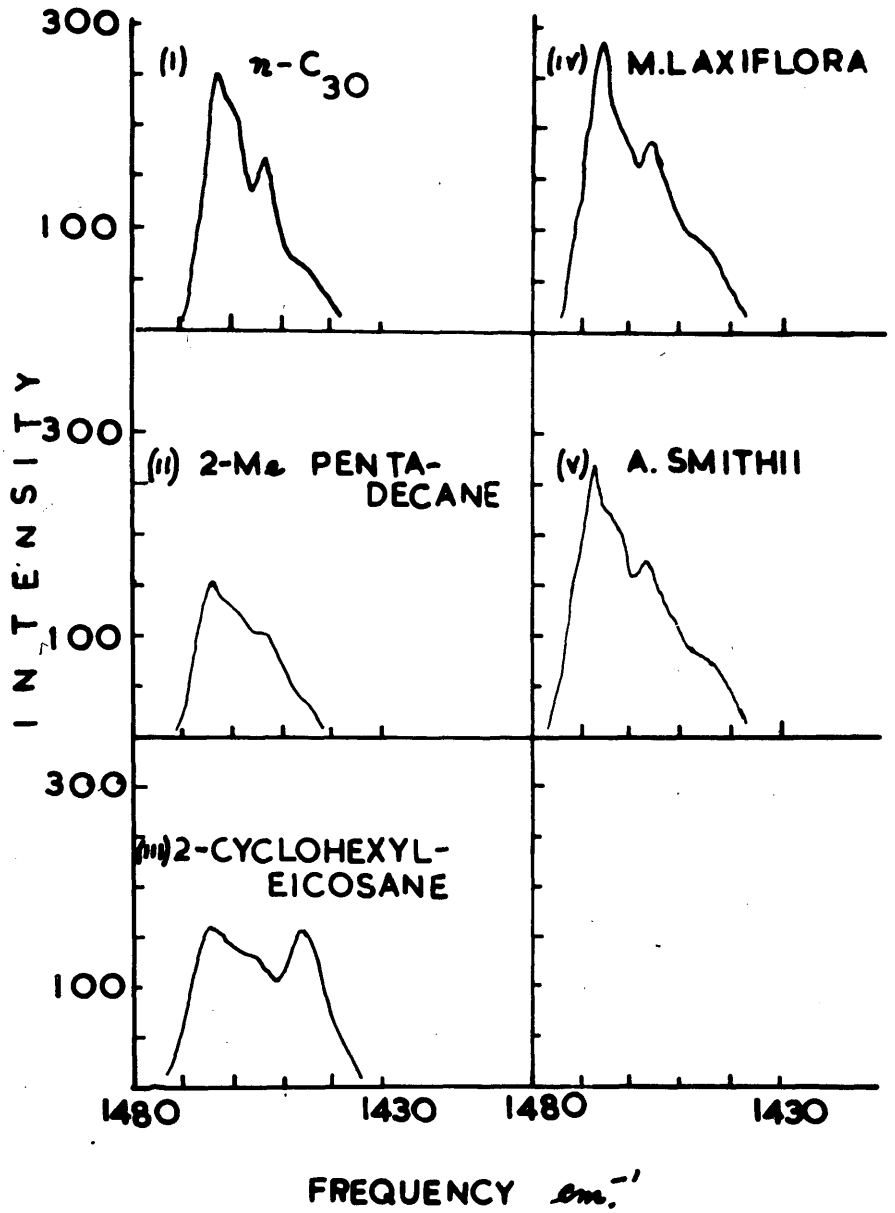
In Aeonium Smithii and Aeonium lindleyi, we have shown that the components which were assigned to iso- alkanes are iso- alkanes. However, it is clear, from the number of different alkanes with similar retention times on G.L.C. analysis that positive identification of components in the wax hydrocarbon fraction can only be made after separation, collection and mass spectrographic analysis.

F I G. I



Infra red spectra over the region 1300 - 1400.

F I G. 2



Infra red spectra over the region 1400 - 1500 cm.$^{-1}$.

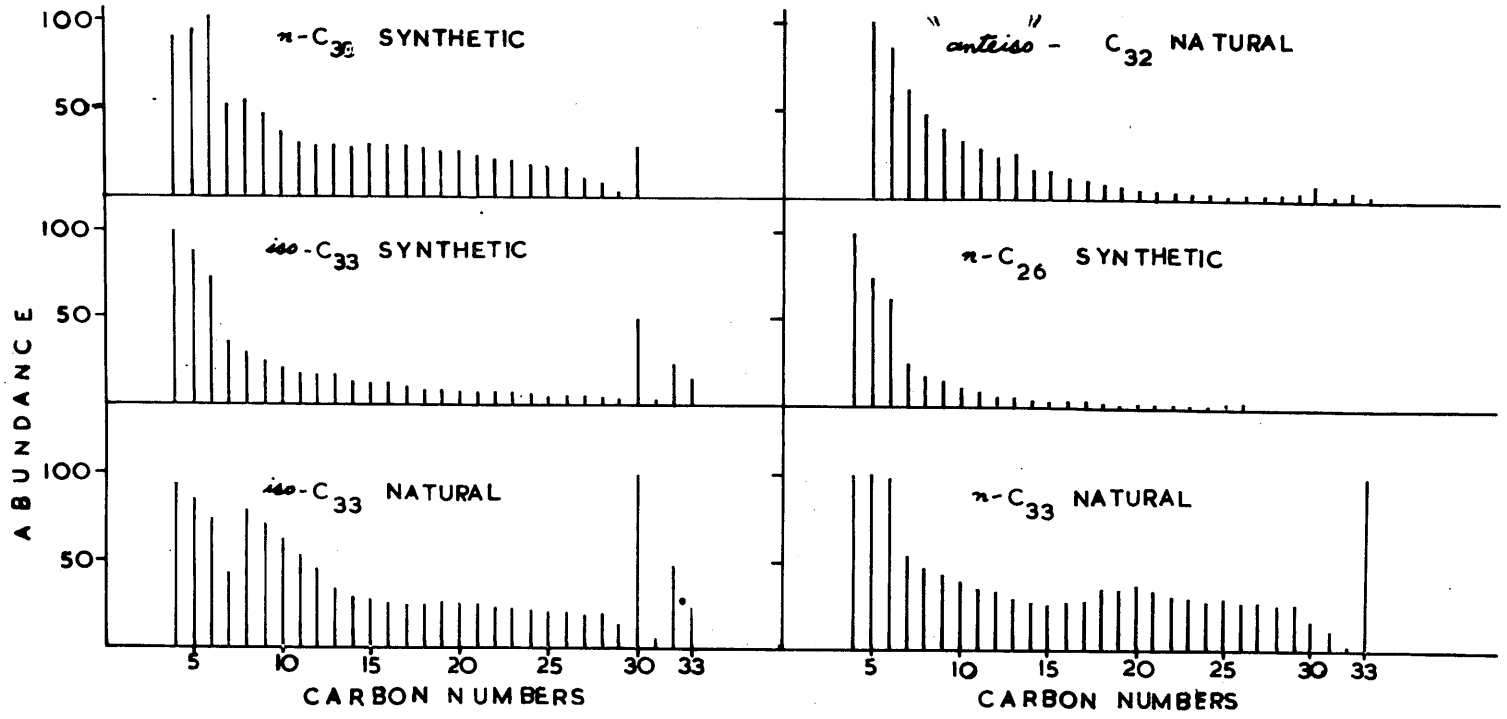


FIG. 3

mass spectra with only the major peaks shown.

Table I A. Gas chromatographic data for C_{16} alkanes.

Compound	b.p.	Relative retention time	Relative retention time
5-butyldodecane	156°	0.56	0.52
5-methylpentadecane	163°	0.72	0.74
3-methylpentadecane	166°	0.80	0.83
2-methylpentadecane	165°	0.82	
n-hexadecane	170°	1.00	1.00
		Run I	Run 2

Run I was accomplished at 154°, 60 ml./min. on a 5% Apiezon column, and Run 2 at 124°, 50 ml./min. on a 0.5% Apiezon column.

Table I B Gas chromatographic data for C_{25}, C_{26}, C_{27}, C_{28}, alkanes.

Compound	Relative retention time	Relative retention time
n-pentacosane	0.64	0.66
2-ethyltetracosane		0.82
n-hexacosane	1.00	1.00
2-cyclohexyleicosane		1.35
1-cyclopentylheneicosane	1.54	
n-heptacosane	1.54	1.52
1-cyclohexyleicosane		1.52
n-octacosane	2.34	2.37
	Run 3	Run 4

Runs 3 and 4 were accomplished on a 0.5% Apiezon column with a flow rate of 60 ml./min. and temperatures of 180° and 184° respectively.

Table II. Intensities of the principal bands in the infra red spectra of alkanes in the region 1330 - 1400 cm.$^{-1}$

	1386cm.$^{-1}$	1380		1368	1354	1341
	ϵ	ϵ	ϵ_{cal}	ϵ	ϵ	ϵ
n-hexadecane		43	46	14	8	7
2-methylpentadecane	43	27	45	53	7	6
3-methylpentadecane		60	45		9	5
5-methylpentadecane		69	45		8	8
5-butyldodecane		68	45		8	9
n-triacontane		51	52	37	28	21
n-dotriacontane		40	53	28	17	10
2-methyldotriacontane	46	34	53	66	28	19
squalane	122	176	47	130		
A. Smithii	37	43		56	28	18
A. coespitosum		41		54	33	23
M. laxiflora		53	53	36	30	20
n-octacosane		47	51	31	25	17
3-ethyltetracosane		69	49	33	24	13
1-cyclohexyleicosane		32	49	28	25	
2-cyclohexyleicosane		63	47	32	22	
1-cyclopentylheneicosane		29	49	26	22	16
7 n-propyltridecane		65	45	23		12
n-hexacosane		47	56	30		17

Footnote: ϵ_{cal} is obtained from formulae in Table III. The modes to which these peaks are assigned are given in Table III.

Table II. Intensities of the principal bands in the infra red spectra of alkanes in the region 1430 - 1480 $cm.^{-1}$.

	1468$cm.^{-1}$		1453$cm.^{-1}$		1443$cm.^{-1}$
	ε	ε_{cal}	ε	ε_{cal}	ε
n-hexadecane	150	147	105	107	46
2-methylpentadecane	164	131	95	97	34
3-methylpentadecane	143	131	116	97	39
5-methylpentadecane	153	131	116	97	44
5-butyldodecane	161	131	127	97	57
n-triacontane	252	259	172	181	72
n-dotriacontane	248	299	163	191	59
2-methyldotriacontane	299	291	180	191	79
squalane	305	155			
A. Smithii	268		171		84
A. coespitosum	245		162		58
M. laxiflora	286		187		91
n-octacosane	235	243	159	170	78
3-ethyltetracosane	210	211	155	149	100
1-cyclohexyleicosane	167	195	130	139	159
2-cyclohexyleicosane	160	171	127	121	157
1-cyclopentylheneicosane	177	195	137	139	66
7 n-propyltridecane	134	131	123	97	35
n-hexacosane	229	227	157	159	74

Table III. Relationship between band intensity and chain length for n- alkanes.

Symbol	Vibrational mode	Absorption mode $cm.^{-1}$	Intensity
Methylene vibrations			
ν	Asym. C-H stretch	2927	77n - 18
ν	Sym. C-H stretch	2855-2853	46n - 64
δ	Scissor	1467	8.0n + 35
γ	Wag	1307-1304	1.15n + 1.5
Methyl vibrations			
ν	Asym. C-H stretch	2959-2954	8.0n + 258
ν	Symm. C-H stretch	2872-2869	8.3n + 110
δ	Asym. C-H deformation	1458-1457	5.2n + 35
γ	Sym. C-H deformation	1379	0.4n + 41

Intensity can be calculated by these formulae given by Jones[3] for n- alkanes where n is the number of methylenes in the chain $5 < n < 37$.

Table IV. Detector Response.
Comparison of branched and normal hydrocarbons.

Mixture components	Runs	Area ratio	Wt. ratio	Mole ratio	Calibration factor.
n-hexadecane and 3-methylpentadecane	1	2.58	2.29	2.29	0.89
	2	2.26	1.81	1.81	0.80
n-hexadecane and 5-methylpentadecane	1	2.92	2.29	2.29	0.79
	2	2.74	2.05	2.05	0.74
n-hexadecane and 5-butyldodecane	1	0.76	0.67	0.67	0.88
	2	1.14	1.01	1.01	0.88
	3	2.61	2.13	2.13	0.82
n-hexadecane and 2-methylpentadecane	1	0.34	0.33	0.33	0.95
	2	0.49	0.46	0.46	0.94
	3	0.48	0.47	0.47	0.98
	4	1.18	1.13	1.13	0.95
	5	1.65	1.50	1.50	0.91
n-triacontane and 2-methyl-dotriacontane	1	0.42	0.46	0.43	1.01
	2	0.94	1.05	0.97	1.03
	3	1.17	1.33	1.22	1.04
n-hexacosane and n-nonacosane	1	2.36	2.18	2.42	1.03
	2	1.29	1.33	1.48	1.14

The area ratio is the ratio of the areas of the peaks for the two components of the mixture (analysed by G.L.C. under conditions as in Table I).
The weight ratio is the ratio of the weights of the two components in the mixture.
The mole ratio is the ratio of weights multiplied by the reciprocal ratio of the molecular weights of the components. The calibration factor is the factor by which the area ratio must be multiplied to obtain the mole ratio.

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S E C T I O N V.

STUDY OF THE TRITERPENE ETHERS FROM ARUNDO CONSPICUA.

INTRODUCTION.

During the investigation of the alkane constituents of Arundo conspicua (Gramineae), Section II, it was observed that the crude petroleum ether extracts showed a prominent peak in the infra red at 1104 $cm.^{-1}$ which was absent in the corresponding extracts of the other New Zealand plants. Moreover, the fraction remaining after complete removal of all alcoholic, carboxylic and carbonyl components from the total petroleum ether-soluble material still exhibited this strong infra red absorption, whilst G.L.C. analysis showed the presence of two additional components, clearly not belonging to the alkane series as indicated by their positions on a plot of the logarithm of retention time against carbon number. Absorption at ca 1100 $cm.^{-1}$ in the infra red is characteristic of the ether grouping as is solubility in concentrated sulphuric acid, a property shown by the two non-alkane components which was used to advantage in removing them from the

alkanes (Section II). It was therefore assumed that ethers, lacking any hydroxyl or carbonyl functions were present in Arundo conspicua and in view of the rarity of such compounds in nature (see Karrer[1].) attention was directed towards the isolation and characterisation of these two components. On the large scale, isolation of the two compounds in the pure form was accomplished by a combination of alumina chromatography and preparative G.L.C.

The total petroleum ether extractives from the finely ground dried leaves of Arundo conspicua were chromatographed on alumina (Brockmann) and the initial fatty fractions rejected. The crystalline material which eluted in subsequent fractions was then subjected to further chromatography over alumina (Woelm), when the material eluting after the initial small quantities of alkanes was found to consist of a single component (G.L.C. and thin layer chromatography). This compound was designated Ether A. Later fractions from the second alumina column were found to contain Ether A together with progressively increasing proportions of a second component which was designated Ether B. Small quantities of Ether B were obtained in pure form from one such fraction by means of preparative G.L.C.

DISCUSSION.

ETHER B.

Although available in such small quantities, Ether B was deduced to be the methyl ether of β-amyrin. Initially the observation that Ether B and taraxerol methyl ether (prepared from an authentic sample of taraxerol kindly supplied by Dr. C.J.W. Brooks) showed identical retention times on our G.L.C. columns, led to the supposition that Ether B might be taraxerol methyl ether. Our supposition was strengthened by the fact that the methyl ether of taraxerol had previously been reported[2,3] as a constituent of the seed oil of Echinochloa crusgallis (which like Arundo conspicua belongs to the family Gramineae) and given the trivial name sawamilletin.[2] A related compound miliacin[4] which was concluded to be the methyl ether of germanicol was isolated from Panicum miliaceum (Gramineae) (Table I).

However, the infra red spectra of taraxerol methyl ether and Ether B in carbon tetrachloride solution showed significant differences especially in the 1500 - 1300 $cm.^{-1}$ region thus clearly demonstrating the non-identity of the two compounds. Moreover, the mass

spectrographic analysis whilst indicating a certain similarity in cracking pattern and showing both compounds to have a molecular weight, 440, also showed significant differences. Thus taraxerol methyl ether showed an intense peak at 318 m/e. due to rings A, B and C with carbons 15 and 16 and a methyl at C_{13}, and a peak at 305 m/e. due to this ion minus the methyl group at C_8. Taraxerol methyl ether also gave an intense peak at 219 m/e assignable to the ion $C_{15}H_{24}^+$ which is considered to be due to rings D and E together with C_{12} arising by rupture of the 11-12 and 8-14 bonds.

Ether B, on the other hand, showed a strong peak at m/e 218 and another peak at m/e 203 with 20% the intensity of the 218 peak and these were characteristic of the oleana-12-ene or ursa-12-ene systems strongly suggesting that Ether B could be either β- or α-amyrin methyl ether.

Indeed its melting point (248-250°) was very close to that of the methyl ether of β-amyrin which was reported[6] as 247-248°, thus suggesting that it was this compound rather than α-amyrin methyl ether which has a melting point 221-223°[6].

Further evidence for the β-amyrin rather than the α-amyrin derivative was provided from the infra red spectrum of Ether B (Fig. 1) in carbon tetrachloride when it was found that the ratio of the intensity of the angular methyl absorption at 1374 $cm.^{-1}$ (ε = 75) to the intensity of the gem dimethyl absorption at 1364 $cm.^{-1}$ (ε = 132) gave a value of 0.57. This very low value suggested that Ether B had fewer methyl groups relative to angular methyl groups than α-amyrene for which the ratio is 1.88.[7] However, taraxerol methyl ether has a value of 1.55 and it may be that the different environments of the methyls in Ether B and taraxerol methyl ether invalidate this comparison. The peak at 1364 $cm.^{-1}$ was taken for the gem dimethyl absorption as angular methyl absorption interferes with the peak cs. 1380 $cm.^{-1}$ [8].

Ether B also showed infra red absorption in carbon tetrachloride solution at 1645 $cm.^{-1}$ and 810 $cm.^{-1}$ consistent with the presence of a trisubstituted double bond (already placed in the 12-13 position from the mass spectrographic evidence) but no absorption could be detected at 3040-3020 $cm.^{-1}$, (β-amyrin itself shows no absorption at 3040-3020 $cm.^{-1}$). Absorption at 1440 $cm.^{-1}$

in Ether B, however, was indicative of an allylic methylene group.

In the ultra violet Ether B showed no absorption above 220 mμ. Absorption at 203 mμ. of ε = 5,900 compared with the absorption of β-amyrin of ε = 8,900 and of taraxerol of ε = 6,100. The ratio of $\varepsilon_{210}/\varepsilon_{220}$ was 4.4 for Ether B and it is known[9]. that for trisubstituted double bonds this ratio is 3-6 (taraxerol had a ratio of 6.3).

Authentic β-amyrin methyl ether was therefore prepared by the method of Morice and Simpson.[10]. β-amyrin methyl ether and Ether B proved to have almost identical infra red absorptions in the 1500-1300 $cm.^{-1}$ region.

Ether A.

Ether A ($[\alpha]_D$ = -9°; c = 1.69 in $CHCl_3$), was found to exhibit dimorphism, the two forms having melting points 235-237° and 271-273°. That the two forms were indeed dimorphs and not isomers was shown by their interconversion on crystallization, the lack of a mixed melting point depression and by their identical infra red spectra (in CCl_4 solution) and G.L.C. retention times. Mass spectrographic analysis

indicated a molecular weight of 458±8 and elemental analysis was consistent with the molecular formula $C_{31}H_{50\pm2}O$. These values, taken in conjunction with the N.M.R. spectrum (Scheme I) and the occurrence of β-amyrin methyl ether in the same plant provided fairly conclusive evidence that Ether A is also the methyl ether of a monohydroxytriterpene.

Unfortunately a direct methoxyl determination by the Zeisel method proved to be impossible owing to the insolubility of Ether A in the hydriodic acid reagent, but the peak at $\tau = 6.58$ in the nuclear magnetic resonance spectrum in deuterochloroform showing the presence of three protons is strong evidence for the presence of the methoxyl group. At the same time, the N.M.R. spectrum made it quite clear that the methoxyl group was not derived from a primary alcohol as there was no absorption from CH_3O-CH_2-. Further evidence for the presence of a methoxyl group was afforded by the mass spectrum. In common with the mass spectra of Ether B and taraxerol methyl ether, prominent peaks occured at Parent - 15, $\overline{\text{Parent - 15}}$-14 and $\overline{\text{Parent - 15}}$-32. The first peak obviously represented elimination of an angular methyl group (allylically activated) or one

member of a gem dimethyl group but the Parent - 15-14 and the Parent - 15-32 peaks arose from the methoxyl function as they were absent in the mass spectra so far reported[5]. of all triterpenes without this function. In the case of Ether A the peak at Parent - 15 was much stronger than the Parent peak itself whereas in the case of Ether B and taraxerol methyl ether the reverse was true indicating a much higher probability for the formation of the ion Parent minus methyl group in Ether A than in the case of Ether B or taraxerol methyl ether.

In addition to the strong absorption at 1104 $cm.^{-1}$ in the infra red ascribable to the ether function, Ether A in CCl_4 solution showed peaks at 3028 $cm.^{-1}$, 1639 $cm.^{-1}$ and 810 $cm.^{-1}$, which absorptions would not be inconsistent with the presence of a trisubstituted double bond. The peak at 3028 $cm.^{-1}$ could conceivably arise from a cyclopropane methylene group which usually absorbs at ca. 3040 $cm.^{-1}$ but there was no peak in the N.M.R. spectrum corresponding to a cyclopropane methylene at τ = 9.4. The ultra violet spectrum showed no absorption above 220mμ and has ε = 7,450 at 203mμ, which was comparable with the absorption of multiflorenol[11]. (3β -hydroxy-D:C-friedo-oleana-7-ene) which

occurs at 205mμ with ε = 4,500 and of bauerenol[12]. (3β-hydroxy-D:C-friedo-urs-7-ene) at 205 mμ with ε = 4,100. Convention for naming triterpenes adopted in this thesis was that suggested by Allard and Ourisson[13].(fig. 2). The ratio of $\varepsilon_{210}/\varepsilon_{220}$ for Ether A was 6.0 which was in good agreement with Bladon's ratio[9]. of 3 to 6 for trisubstituted double bonds. Further evidence for the presence of at least one double bond in Ether A was provided by the weak yellow colour produced when tetranitromethane was added to a chloroform solution of Ether A. Several attempted hydrogenations failed to yield any compound other than unreacted starting material (infra red spectra), and this can be taken as further evidence of the trisubstituted or tetrasubstituted nature of the double bond in view of the known resistance to hydrogenation of double bonds of this type in the triterpene series[14]. The absence of a peak at ca. 910 $cm.^{-1}$ in the infra red ruled out the presence of a vinyl methylene group. The N.M.R. of the Ether A showed no recognisable olefinic proton in the region τ = 3 to τ = 5, but should the double bond be such that coupling between this proton

and an adjacent CH_2 group occurred, then grave difficulty would be expected in distinguishing the resultant multiplet from the background. At the same time it is to be noted that the single proton $R_2CH-OCH_3$ of the ether likewise can not be distinguished in the N.M.R. spectrum if indeed it is present. Should the molecular weight of Ether A be 438, the presence of two double bonds in the pentacyclic system would be necessary. As all triterpenoids are elaborated in Nature in such a way as to possess six double bond equivalents (Scheme II),[15]. the second double bond in Ether A (if present) must be introduced after the basic skeleton is complete.

With concentrated sulphuric acid, Ether A gave a red fluorescent colour similar to that produced in the Salkowski modification of the Liebermann Burchard test with stenols[16]. and this might indicate cleavage of the methyl ether to give an unsaturated triterpene alcohol.

However, attempted cleavage of Ether A with hydrochloric acid in chloroform served only to yield unchanged starting material as shown by infra red analysis whilst the action of concentrated sulphuric acid generated carbonyl functions as shown by infra red analysis. The absence of absorption at ca.1100 $cm.^{-1}$ indicated

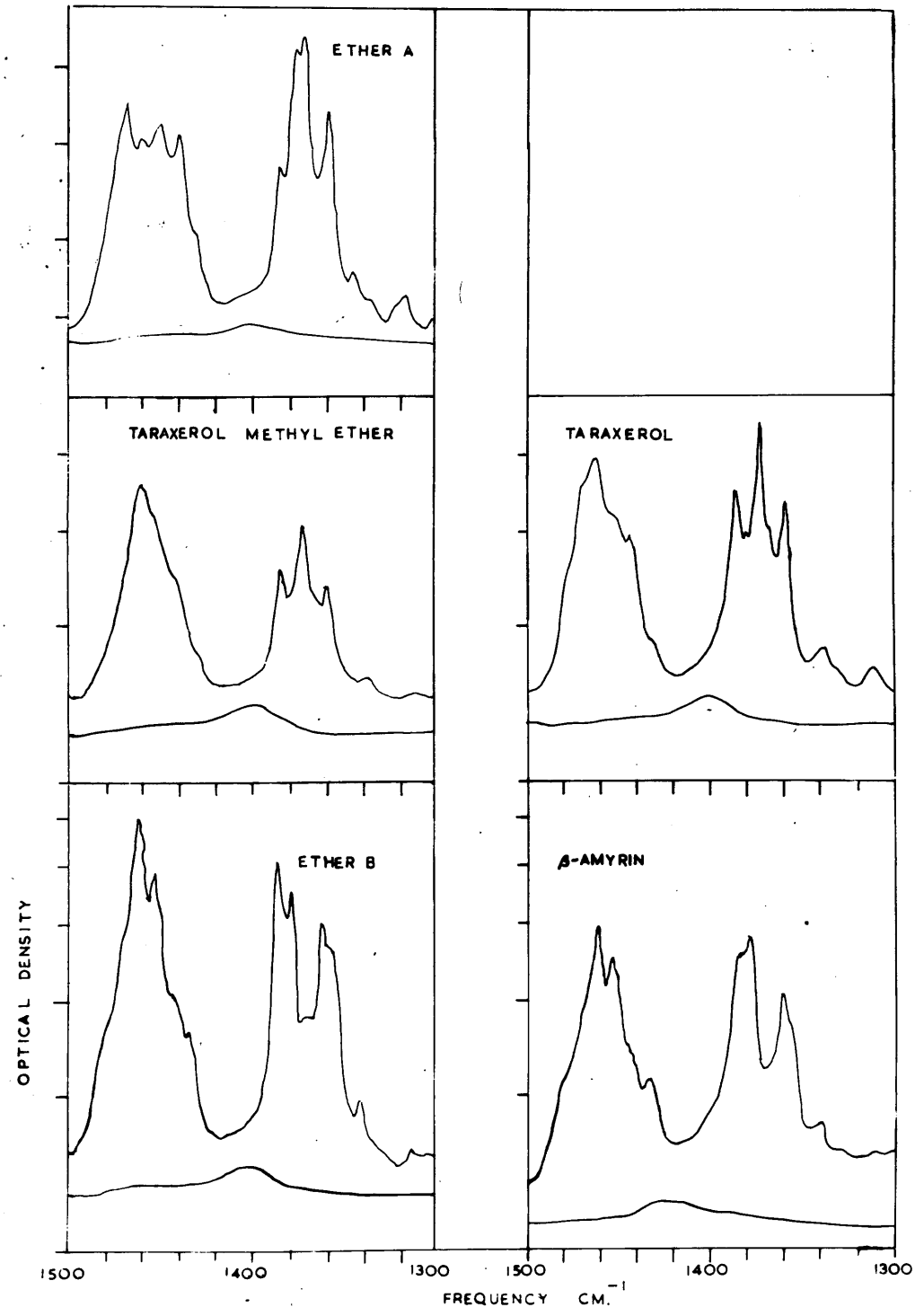
that the ether function was involved. Unfortunately careful chromatographic work up was not carried out on the $HCl/CHCl_3$ treated product and so a small proportion of rearranged material may have been missed and so no unambiguous information concerning the lability of the double bond was obtained.

Further evidence bearing on the triterpene skeleton of Ether A was afforded by a study of the $C-CH_3$ stretching absorptions in CCl_4 solution in the infra red. Peaks at 1381 and 1364 $cm.^{-1}$ (ε = 197 and 158) were ascribed to gem dimethyl groups attached to six membered rings.[17.] Peaks at 1389 $cm.^{-1}$ (ε = 93) and 1376 $cm.^{-1}$ (ε = 230) could be ascribed to angular methyl groups. Considering the gem dimethyl absorption at 1364 $cm.^{-1}$ and the angular methyl absorption at 1376 $cm.^{-1}$ it is seen that the value of

$$\frac{\text{intensity of angular methyl absorption}}{\text{intensity of gem dimethyl absorption}} = \frac{230}{158} = 1.45$$

In view of the fact that the value for taraxerol methyl ether was 1.55 and that for α-amyrene is 1.88[7.], it would seem safe to conclude that Ether A possesses two pairs of gem dimethyls.

FIG. 1



Infra red spectra of the ethers and alcohols in carbon tetrachloride solutions over the range 1300 - 1500 cm.$^{-1}$.

Evidence that Ether A is a pentacyclic triterpene was afforded by the absence of peaks in the mass spectrum at 326 or 327 (parent minus 111 or 112) or at 312 or 313 (parent minus 125 or 126) which would be expected to arise from elimination of the side chain at C-17 in the trimethyl steroid type - such a side chain having eight carbon atoms (e.g. in lanosterol, euphol, tirucallol, butyrospermol, cycloarterol) or nine carbon atoms (e.g. in euphorbol, cycloloudenol) since Reed and De Mayo[18]. have shown such side chain elimination is usually easy. Weak peaks attributable to such side chain elimination were detectable in the mass spectra of a variety of steroidal ketones which were recently reported[19].

Similarly the absence of a peak at P - 41 (loss of isopropenyl group) suggested that Ether A did not belong to the lupane series. In view of all this evidence (infra red and mass spectral) it would seem probable that Ether A belongs to the oleanane group.

A study of the mass spectrum cracking pattern of Ether A revealed that the most prominent peaks between m/e 200 and m/e 363 were at m/e 241 and m/e 273. It was immediately appreciated that these peaks differ by 32 mass units, a fact which was readily explicable by

the loss of methanol from the ion of higher mass number. Hence it can be reasonably concluded that the species of molecular weight 273 retains the methoxyl group as loss of $C_2H_6 + H_2$ (mass number 32) is highly unlikely as is the loss of $2CH_4$ or $2CH_3 + H_2$. In the latter case it would be expected that a strong ion resulting from loss of CH_3 alone would also be present but there is no strong peak at m/e 258.

Recently the results of an extensive study of the mass spectrographic cracking patterns of a number of triterpenes has been published.[5] These clearly indicate that Ether A is not oleana-12-ene or ursa-12-ene derivative, since the most characteristic ions of compounds belonging to these two groups occur at m/e = 218 and m/e 203 due to the species V and VI and such peaks are entirely absent from the mass spectrum of Ether A. The species V and VI are considered to arise from the reverse Diels Alder reaction shown in VII and elimination of the angular methyl group attached to C - 17. Moreover it was also shown that oleana-13(18)-enes give double allylic cleavage at the 11-12 bond and the 8-14 bond, the molecule splitting into two virtually equal halves with no ions between m/e 205 and the parent peak for oleana-13(18)-enes bearing

the usual substituents. The existence of strong peaks at 241 and 273 in the mass spectrum of Ether A indicated that Ether A was not of this type (the highest peak possible for 3-methoxyoleana-13(18)-ene would be 236 arising from rings A and B and Carbon 11). Similarly taraxerol methyl ether gave characteristic peaks at m/e 204, 318 and 305 which were absent in Ether A indicating that Ether A was not of the D-friedo-oleana-14-ene type. The cracking patterns of oleana-18-enes showed less predictable cleavages but peaks at 177, 189, 203, 204 and 218 assignable to fragments containing the D and E rings and portions of ring C should be present in the mass spectrum of Ether A if it were of germanicol type by analogy with the mass spectra of oleana-18-ene and germanicol acetate. The absence of these peaks in the mass spectrum of Ether A indicated therefore that Ether A is not germanicol methyl ether, a conclusion supported by the absence of a vinylic proton singlet in the N.M.R. spectrum and the difference in its m.p. (271-273°) from that of 282° reported for germanicol methyl ether.

Moreover, such cleavages as have been found in the germanicol series would not afford a methoxyl containing fragment of as high a mass number as 273.

Thus mass spectrographic analysis indicated that Ether A was not an oleana-12-ene, an ursa-12-ene, an oleana-13(18)-ene, a D-friedo-oleana-13-ene or an oleana-18-ene, which together represent the more common triterpene types and so it must be concluded to be of a more novel type. The most likely type of structure (bearing in mind the simultaneous presence of β-amyrin methyl ether in the same plant and the probability of a close chemical relationship between the compound and Ether A) would seem to be that of a D:C friedo-oleana-7-ene (VIII), a D:B friedo-oleana-9(11)-ene (IX) or a D:B friedo-oleana-1(10)-ene (X). None of these structures would give a methoxyl containing ion of mass number 273 by reverse Diels Alder reactions but in view of the type of cleavages observed in the germanicol series at non-allylic positions these structures cannot be ruled out on these grounds alone.

It is of interest to note that the doubly unsaturated compound XI, which involves a hitherto unknown type of E-friedo re-arrangement would give an ion of mass number 273 on reverse Diels-Alder reaction, as shown, followed by elimination of a methyl group. The formation of XI

from germanicol (itself formed biogenetically from the same intermediate as is β-amyrin)(Scheme II), can be envisaged as shown in XII, suggesting that XI is yet another possible structure for Ether A.

CONCLUSIONS.

Ether B has been shown to be β-amyrin methyl ether.

Mass spectral analysis gave a molecular weight 438 for Ether A. (However, reliance cannot be made to 2 units at this end of the spectrum and the other data make it apparent that the true molecular weight is, in all probability, really 440.) Some of the possible structures for Ether A are VIII, IX, X, XI from mass spectral data.

Examination of the relationship of the melting points and M_D's of the known pairs of triterpene alcohols and their corresponding methyl ethers (Table I) indicates that, in the oleanane series (β-amyrin and germanicol), the D friedo-oleanane series (taraxerol) and the ursane series (α-amyrin), the melting point of the methyl ether is not only above 220° but also higher than that of the alcohol, whilst the ΔM_D is approx. +50. This suggests that the pentacyclic triterpene alcohol from which Ether A has been formed has a m.p. 180-220° and a negative optical rotation.

Table II gives some of the alcohols which have physical properties (m.p., $[\alpha]_D$) in this range. It is possible to eliminate many of these alcohols as possible parents for Ether A, e.g. spinasterol has a side chain (mass spectral data rule it out); taraxasterol, 3β-hydroxyurs-13(18)-ene and bauerenol belong to the ursane series (the presence of 2 pairs of gem dimethyls in Ether A rule them out).

3α-hydroxyoleana-18-ene cannot be the parent alcohol (mass spectral analysis of oleana-18-enes as above) but it is interesting to note that 3α isomers of some of the other Friedo-oleananes might possibly fit.

Of the alcohols given in Table II, multiflorenol (VIII) is the most likely structure for the parent alcohol of Ether A.

As an initial step in further work directed towards the elucidation of the structure of Ether A it would seem essential to establish with certainty whether Ether A undergoes re-arrangement with HCl/$CHCl_3$ as friedo derivatives are known to revert to a mixture of the oleana-12-ene and the oleana-13(18)-ene.

Another obvious approach is to investigate the product of the action of SeO_2.

EXPERIMENTAL.

Light petroleum refers to the fraction b.p.40-60°. Infra red spectra were measured on the Unicam SP 100; ultra violet on the Hilger-Watts.

Extraction and Separation of A. conspicua ethers.

For this large scale separation and extraction mentioned below we are indebted to Miss Irene Wilson. The finely ground aerial parts of Arundo conspicua were extracted with refluxing light petrol for 24 hours in a Soxhlet apparatus. After removal of this first portion of solvent, a further portion was added and the extraction continued for a further 24 hours. The two extracts were combined and the solvent removed under reduced pressure. This extract was absorbed from light petrol onto alumina (Brockmann, Grade V) and the first fractions eluted with light petrol rejected as they contained only fatty material. Subsequent fractions eluted with light petrol gave a white solid on removing the solvent. These white needles were chromatographed on basic alumina (Woelm, Grade I) and the initial eluants (m.p. = 80°) rejected. Several of the later fractions (m.p. = 220-230°) were combined. These combined fractions were recrystallised from ethyl acetate to

give Ether A, $[\alpha]_D$ -9°, (C = 1.69 in $CHCl_3$), (Found C, 85.37, 83.6; H, 11.93, 12.35, $C_{31}H_{52}O$ requires C, 84.48; H, 11.89, $C_{31}H_{50}O$ requires C, 84.87; H, 12.73). Ether A can exist in two forms, m.p. 235-237° and 271-273°, which are identical in G.L.C. retention time (45 mins. on 0.5% Apiezon L at 235°) and in thin layer R_f value.

Alternative Extraction.

For this extraction carried out in New Zealand we are indebted to Miss Peggy Martin-Smith. The leaves of Arundo conspicua, collected at Plimmerton, North Island, New Zealand, during December, 1961, were briefly immersed in cold chloroform. The chloroform extract again showed ether absorption in the I.R., and the G.L.C. showed that there was a smaller proportion of the ethers in the leaf wax extract than in the total aerial parts extract.

Further Separation of Fraction (m.p. 215-230°).

G.L.C. of the fraction (m.p. 215-230°) on an Apiezon L column (0.5%) on Embacel (80-100 mesh) at 235° showed that it contained a high proportion of another ether. Isolation of this ether, Arundo conspicua ether B was accomplished by preparative G.L.C.

on a silicone gum column (1%) on Embacel (60-80 mesh) trapping in spiral traps in an ice bath. The trapped material was then sublimed at 180°/0.02 mm. to remove silicone gum eluted from the column, to give pure Ether B.

Preparation of β-amyrin methyl ether.

β-amyrin (60 mg.) and potassium (100 mg.) were stirred in dry benzene (5 ml.) in a current of nitrogen at room temperature for three hours. Methyl iodide (1 ml.) in benzene (2 ml.) was added every 2 hours with refluxing for 12 hours. Methyl alcohol was added to decompose the unreacted potassium and water to extract the potassium salts. The infra red spectrum of the chloroform extract of this aqueous solution showed that it contained no alcohol and that there was an ether present. Sublimation of the chloroform extracted material gave a solid whose G.L.C. retention time was identical with that of Ether B.

Attempted Hydrogenation of Ether A (m.p. 234-235°).

The ether A (10 mg.) was added to pre-reduced platinum in an ethyl acetate-acetic acid mixture (3 - 1, 15 ml.) and allowed to absorb hydrogen. After

eight hours, the platinum was filtered off and the solution taken to dryness under reduced pressure. The infra red spectrum of the resulting material showed no significant difference from the starting material.

Treatment of Ether A with Concentrated Sulphuric Acid.

The Ether A (10 mg.), dissolved in chloroform (20 ml.), was washed with concentrated sulphuric acid (2 x 5 ml.). The sulphuric acid layer was added to water (100 ml.) and almost neutralised with potassium hydroxide, then extracted with diethyl ether. The infra red spectrum of the solid remaining after the solution had been dried with magnesium sulphate and the solvent removed under reduced pressure, showed the presence of acid and also of an enone (1710 $cm.^{-1}$).

Treatment of Ether A with Acid.

To Ether A (10 mg.) dissolved in chloroform was added concentrated hydrochloric acid (3 drops). After standing for half an hour, the solution was washed with water to remove the acid and then dried with magnesium sulphate. The infra red spectrum of the material recovered from the chloroform solution showed no significant difference in absorption.

Preparation of Taraxerol Methyl Ether.

Potassium (50 mg.) and taraxerol (27 mg.) were stirred in dry benzene (5 ml.) in a current of nitrogen at room temperature for three hours. Methyl iodide (1 ml.) in benzene (2.5 ml.) was added and the mixture refluxed for three hours. After working up this reaction mixture, it was found that only a small proportion of the alcohol had been converted to the ether. The reaction product was therefore refluxed for twelve hours in dry benzene with potassium (25 mg.) and fresh portions of methyl iodide every two hours. Methyl alcohol was added to decompose the unreacted potassium and water to extract the potassium salts. Benzene extraction of this aqueous mixture gave material whose I.R. showed no hydroxyl absorption. This material was chromatographed on alumina (Grade V) and the light petrol eluate sublimed. As the temperature was raised in sublimation, a liquid fraction (125-130°/0.02 mm.) was removed. On re-sublimation of the residue, a solid was obtained (180-190°/0.02 mm.) whose m.p. range 270-275° was wide despite the fact that G.L.C. analysis showed that this fraction was almost pure.

TABLE I.

TRITERPENE ALCOHOL.

Compound	m.p.	$[\alpha]_D$	M_D	Ref.
3β-hydroxyoleana-12-ene (β-amyrin)	197-8°	+ 89°	+379	6
3β-hydroxyursa-12-ene (α-amyrin)	186-7°	+ 83°	+354	6
3β-hydroxy-D-friedo-oleana-14-ene (taraxerol)	269°	+5±2°	+ 22	1
3β-hydroxyoleana-18-ene (germanicol)	176°	+ 6°	+ 36	1
3β-hydroxyoleana-13(18)-ene	213°	- 52°	-222	4

TRITERPENE METHYL ETHER.

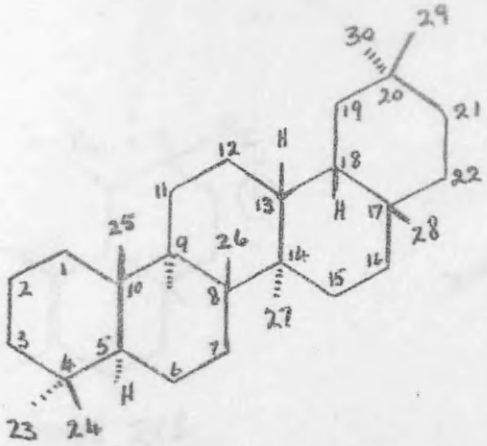
					ΔM_D
3β-methoxyoleana-12-ene	247-8°	+98°	+431	-	+52°
3β-methoxyursa-12-ene	221.2°	+93°	+407	-	+53°
3β-methoxy-D-friedo-oleana-14-ene(sawamilletin)	278°	+ 8°	+351	2	+13°
3β-methoxyoleana-18-ene	282°	+ 3°	+ 36		±0°
3β-methoxyoleana-13(18)-ene	189°	-24°	-105	4	+117°

TABLE II.

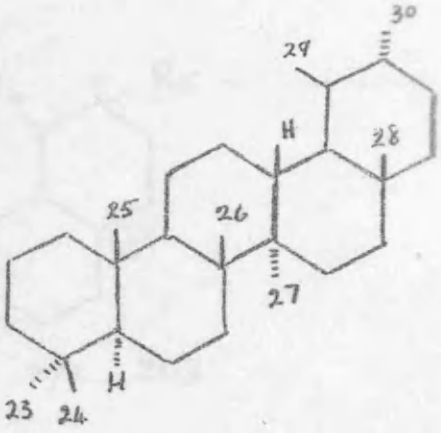
ALCOHOL	m.p.	$[\alpha]_D$	M_D	Ref.
3β-hydroxyursa-20-ene (taraxasterol)	186°	+83°	+354	1
α-spinasterol	172-5°	- 4°	- 17	1
3α-hydroxyoleana-18-ene	221°	-34°	-145	-
3β-hydroxy-D:C-friedo-olean-7-ene (multiflorenol)	188-9°	-28°	-119	12
3β-hydroxyursa-13(18)-ene	201-4°	-36°	-153	-
3β-hydroxy-D:C-friedo-ursa-7-ene(bauerenol)	207-8°	-30°	-130	12

Peak No.	τ value.	Assignment.
1	9.25	Methyls
2	9.22	
3	9.16	
4	9.12	
5	9.04	
6	8.88	
7	8.70	Methylenes
8	8.53	
9	8.47	
10	8.39	
11	8.16	
12	6.68	OCH_3

Scheme I. N.M.R. Spectrum.



I oleanane



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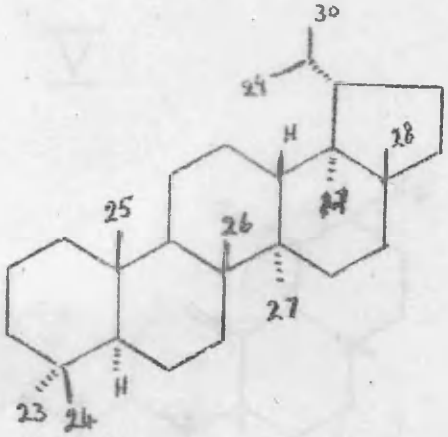


III lupane

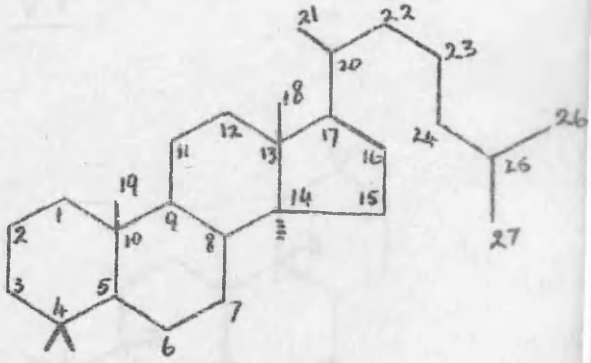
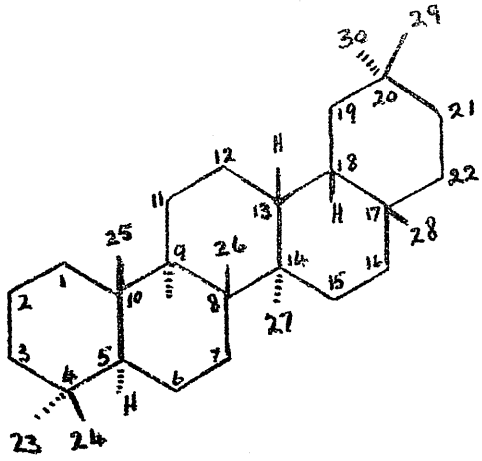
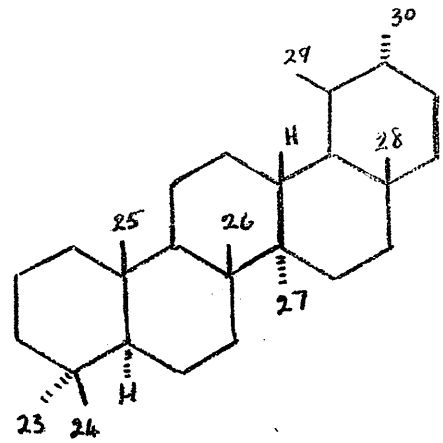


Fig.2 : The above structures are regarded as the basic hydrocarbons and the derivatives of them formed by migration of methyl groups are named friedo-lupane. Where the methyl group on C_{14} migrates to C_{13} the derivative is a D-friedo compound: where the methyl group on C_{14} migrates to C_{13} and the methyl group on C_8 migrates to C_{14} the derivative is a D:C-friedo compound: where migration of the methyl groups on C_{14} and C_8 is followed by migration of the methyl group on C_{10} to C_9 the derivative is named as a D:B-friedo compound: and where migation of the methyl groups on C_{14}, C_8, and C_{10} is followed by the migation of the axial group on C_4 to C_5 the derivative is named as a D:A-friedo compound.



I oleanane



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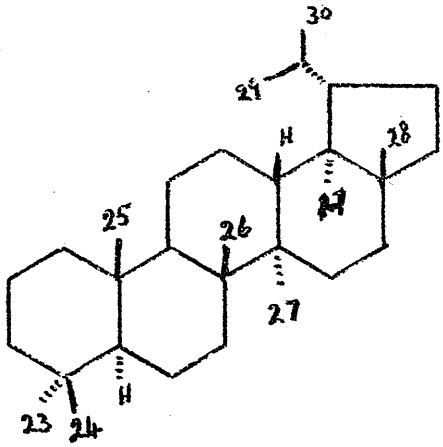
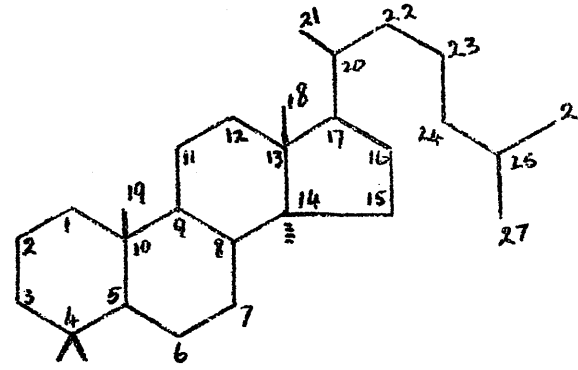
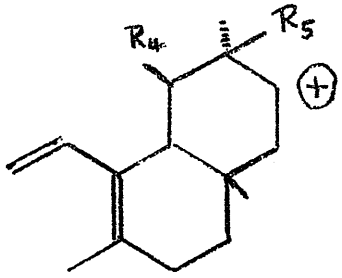


III lupane



IV lanostane

fig. 2.

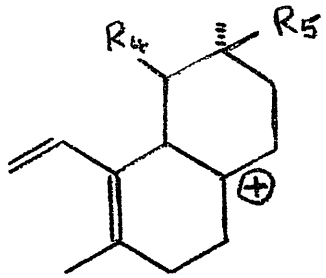


m/e 218

$R_4 = H$ & $R_5 = CH_3$

or $R_4 = CH_3$ & $R_5 = H$

V

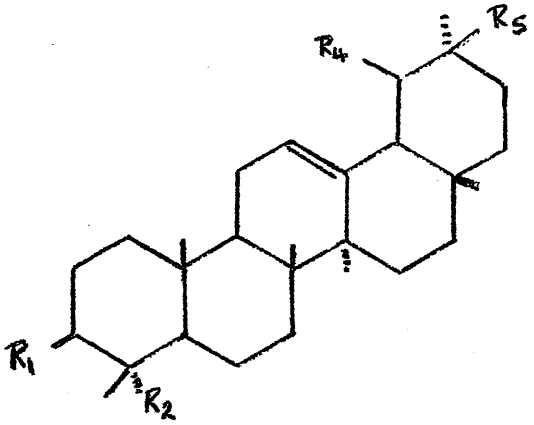


m/e 203

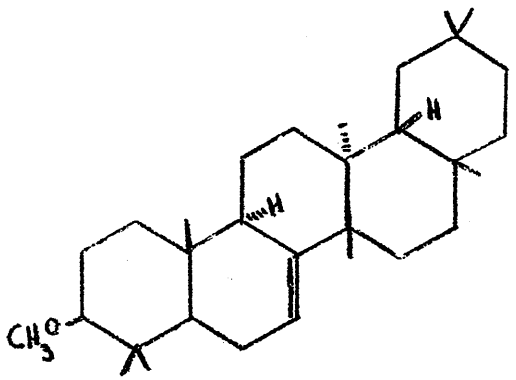
$R_4 = H$ & $R_5 = CH_3$

or $R_4 = CH_3$ & $R_5 = H$

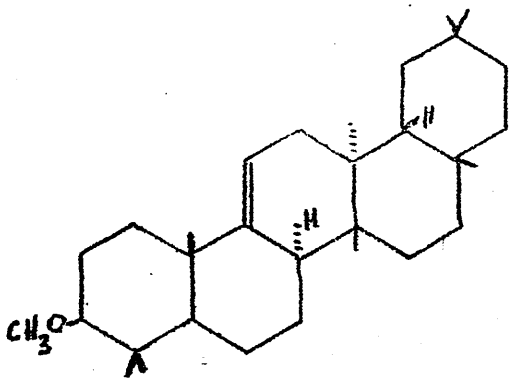
VI



VII



VIII



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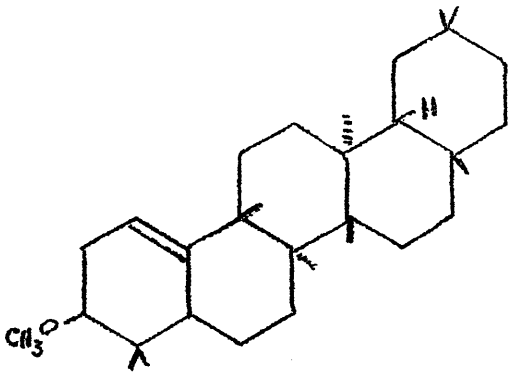
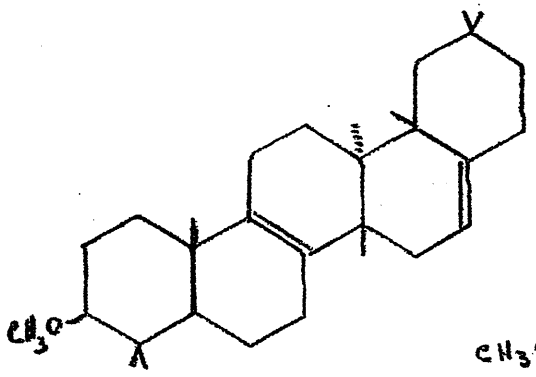
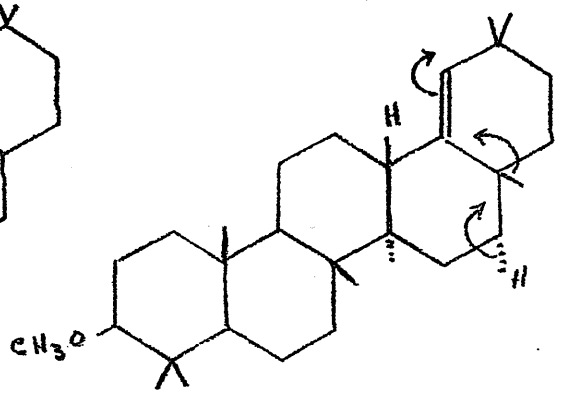


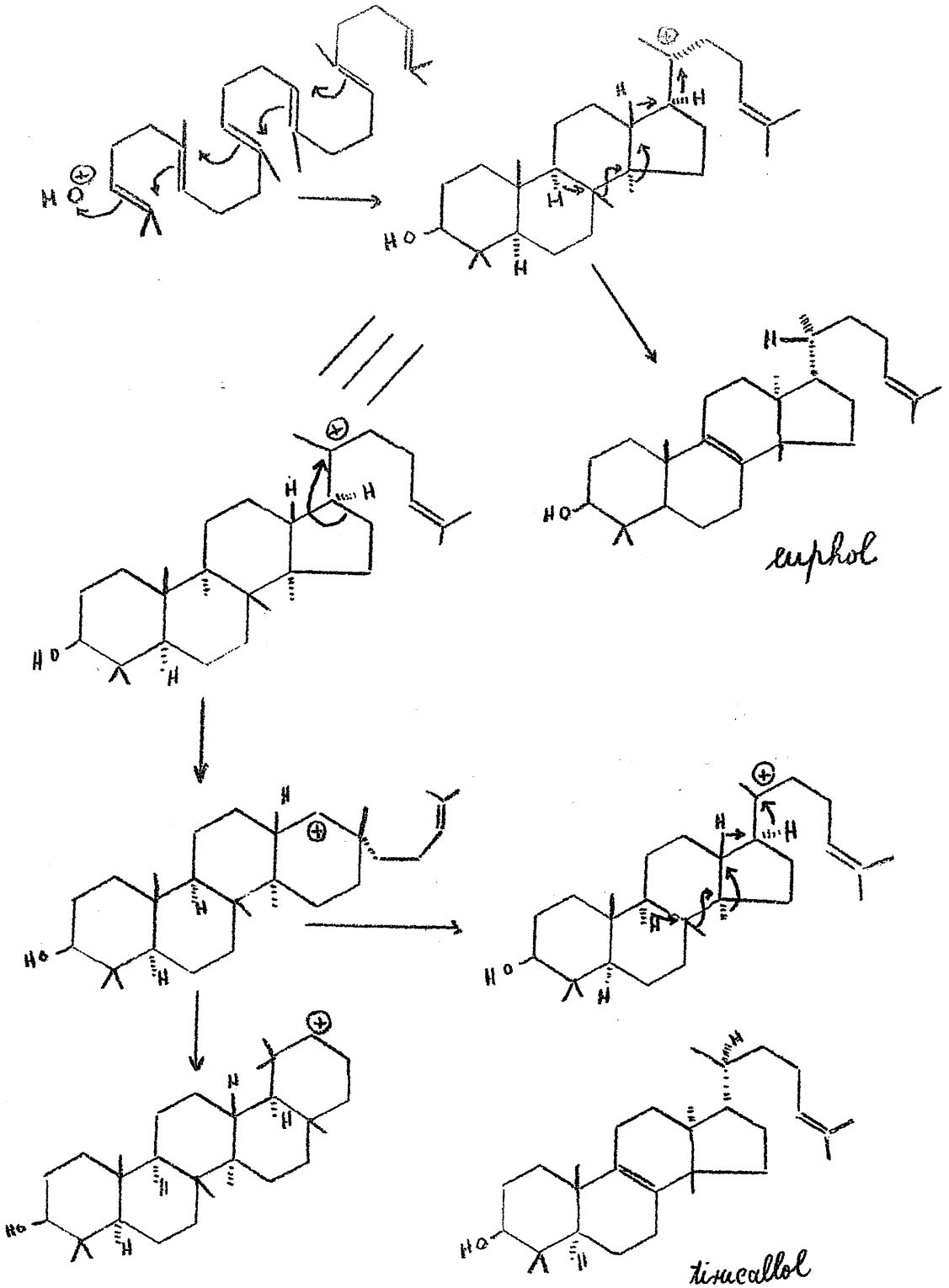
X



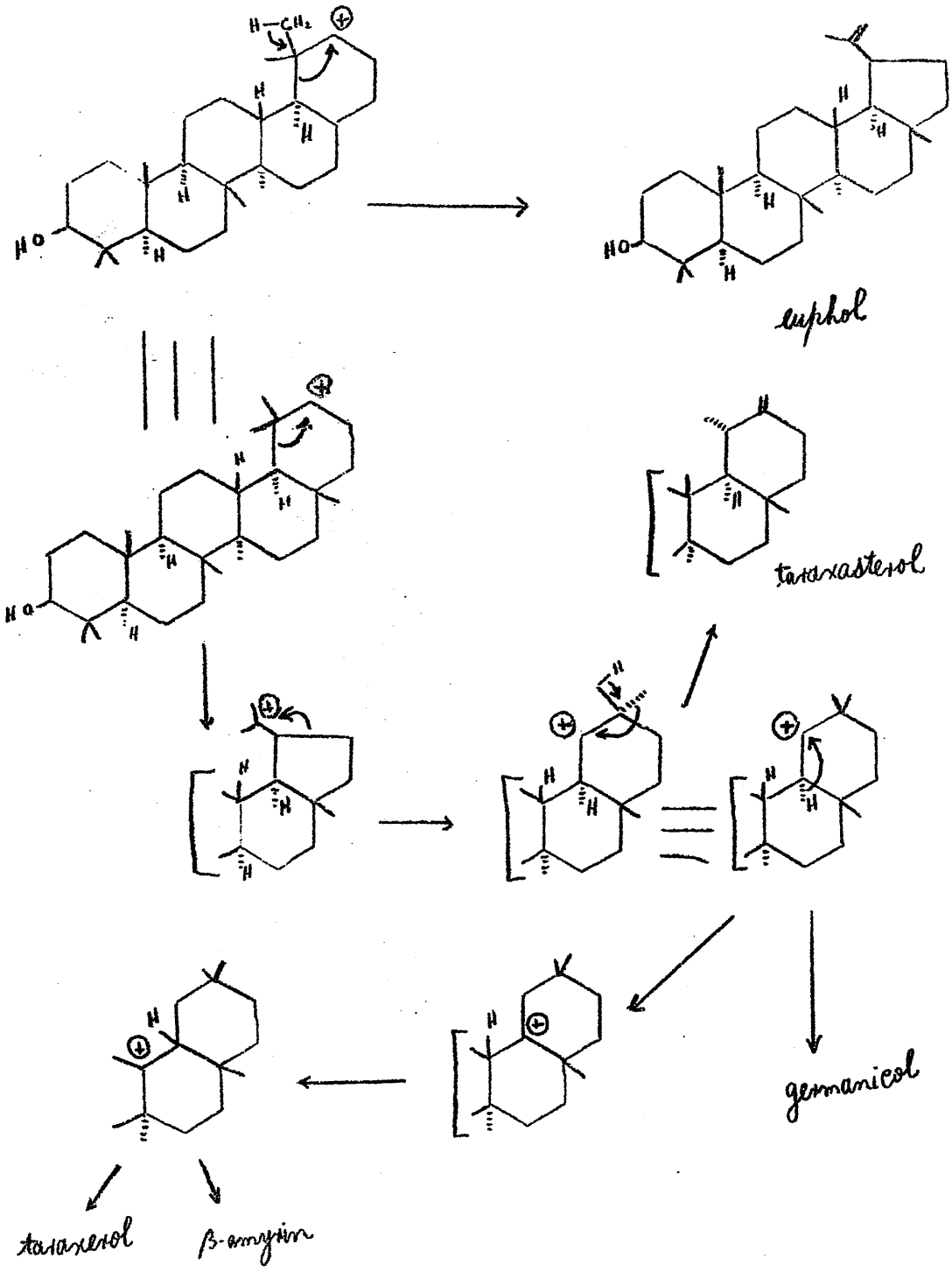
XI



XII



Scheme II Proposed Biogenesis of Triterpenes.



Scheme II (continued)

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SECTION VI.

THE ELUCIDATION OF THE STRUCTURE OF LINDLEYOL.

INTRODUCTION AND DISCUSSION.

In the course of an extensive study of the leaf waxes of plants of the subfamily Sempervivoideae (Crassulaceae)[1]. it was observed that a gummy exudate coated the leaves of Aeonium lindleyi (W. and B.), and to a lesser extent some others in the Goochia group, and that extraction of this gummy exudate resulted in a much higher proportion of chloroform soluble material than the majority of Aeonium species examined. This gum, obtained by washing the leaves with ethanol, was dissolved in ether and separated into acidic and neutral fractions by extraction with sodium hydroxide solution. The neutral portion on chromatography afforded a compound m.p. 82-84° for which analytical figures and molecular weight determinations indicated a formula $C_{20}H_{38}O_2$. The infra red spectrum showed only the presence of hydroxyl groups primary and tertiary 3634, 3604 and 1030 $cm.^{-1}$ and a gem dimethyl (1380, 1370 $cm.^{-1}$). The nuclear magnetic resonance spectrum indicated that it was a primary-tertiary diol

(τ = 6.32 triplet) (for this information we are obliged to Dr. A. Melera), and this was confirmed by the fact that lindleyol gave only a mono-acetate on mild acetylation. From these facts it followed that lindleyol was bicyclic and probably a diterpene - the first fully saturated diterpene found. The non-crystalline lindleyol mono-acetate on dehydration with phosphoryl chloride in pyridine gave a homogeneous product, the infra red spectrum of which indicated the presence of a vinylidene group (bands at 890 $cm.^{-1}$ and 1645 $cm.^{-1}$). This compound was characterised by conversion with osmium tetroxide in pyridine into a triol which proved identical in melting point and rotation with that obtained from methyl labdanolate[2], with the result that the correct name for lindleyol is labdane-8α,15-diol.

Though lindleyol (IV) (Scheme I) appears to be the first saturated diol isolated, sclareol[3] (II) and torulosol[4] (III), diols of increasing unsaturation, are known. Biogenetically (Fig. 5), labdanolic[2] (X), daniellic[5] (IX), eperuic[6] (VII), and catavic[7] (VIII) acids must be related to communic acid[8] (V), though why oxidation should take place at so many varying centres is not known.

It seems possible that among the many compounds with the labdane skeleton in Spanish gum labdanum there will be labdane-8 α,15-diol since the enzyme system required for its formation should differ only at the last step from that of labdanolic acid. It seems likely from their gummy appearance that the leaves of Aeonium spathulatum and Aeonium goochiae also contain labdane-8 α,15-diol.

The region, 3000-3700 $cm.^{-1}$, where most hydroxyl stretching frequencies occur, is now amenable to precise study using commercially available, high resolution, grating infra red spectrometers.[9] Measurements are conveniently carried out at high dilution (0.01 - 0.001 M) in long-path length cells (0.5 - 5 cm.). It is frequently assumed that intermolecular hydrogen bonding is absent at such low concentrations except in special cases such as the carboxylic acids[10] where rather stable dimeric species are known. In this and other work, however, several types of compounds have been encountered where inter-molecular association, presumably dimeric, is unusually persistent.

We have examined several compounds of labdane type and it is clear from our measurements (Table 2 and Figure 1)

that intramolecular hydrogen bonding involving "closure" of a ten-membered ring does occur to a detectable extent in very dilute solutions in carbon tetrachloride. Such a structure is sterically unexceptional and models indicate many possible favourable conformations of such a "ring" involving minimal bond oppositions and transannular interactions; one of these (XX, Fig.2) is shown for methyl labdanolate.

Similar measurements show the presence of an analogous intramolecularly hydrogen bonded structure in the solution of labdane-8α,15-diol monoacetate (XV). The hydroxyl absorption pattern for each compound is concentration independent up to the highest concentration tested (0.01M). It does not seem feasible to assign the absorption near 3550 $cm.^{-1}$ unambiguously either to -OH.....O=C-O or to OH.....O-C=O.[11,13] The carbonyl absorption band is a little wider than normal (c.f. labd.-8(20)en-15-ol acetate (XVI Table I) and methyl stearate $\nu_{max}(CCl_4)$ 1743 $cm.^{-1}$, $\Delta\nu^{a}_{\frac{1}{2}}$, 16 $cm.^{-1}$; ε_a 500), presumably as a result of this partial bonding. There is evidence in the incompletely resolved absorption near 3610 $cm.^{-1}$ for more than one conformation of the non-bonded hydroxyl grouping (c.f. t-butanol, (CCl_4) 3617 $cm.^{-1}$, $\Delta\nu^{a}_{\frac{1}{2}}$ 17, ε_a= 60).

Figs. 3 and 4 show the same conformation of labdane-8 α,15-diol drawn from shadows cast by a Dreidling model with a pin-hole source of light.

Labdane-8 α,15-diol (IV) is an interesting case. Even at the relatively low concentration of 0.0078 M. there is a prominent, concentration-dependent, broad absorption band at 3370 $cm.^{-1}$, which, in view of other studies, could be ascribed to (OH) of a fully-bonded dimeric species involving an 8-membered ring of four hydrogen bonds as depicted in (XIX). Progressive dilution to 0.00078 M. reveals a second band at 3515 $cm.^{-1}$ which almost certainly represents an intra-hydrogen bonded -OH....OH monomeric structure. The bulk of the attached alicyclic ring system undoubtedly facilitates both the inter- and intra-molecular hydrogen bonding by restricting the number of available conformations; thus heptane-1,7-diol shows no absorption band in this region other than that due to free hydroxyls. The asymmetry of such free hydroxyl bands has been discussed in conformational terms by Oki and Iwamura.[12]

The above results emphasize the importance of carrying out such hydrogen-bonding studies under precisely controlled and calibrated conditions with a

range of dilutions if misleading conclusions are to be avoided. Methyl labdanolate is a case in point; Bigley, Rogers and Barltrop[13]. have reported that this compound does not show intramolecular hydrogen bonding in carbon disulphide solution, and they therefore allocate a configuration at C_{13} in methyl labdanolate on the basis that the molecular rotation difference method will be valid in the absence of such bonding. In view of the results in this work this assignment, and those made for related compounds such as methyl eperuate and methyl cativate, are again open to question.

Meakins[14]. has shown that the -OH stretching absorption of monohydric alcohols can vary from shoulders on the side of the main peak - propan-2-ol, 3626 $cm.^{-1}$, $\Delta\nu^{a}_{\frac{1}{2}}$ 17, and ε = 50; 3611 $cm.^{-1}$, $\Delta\nu^{a}_{\frac{1}{2}}$ 14, ε = 8; to two distinct peaks in 5α-cholestan-5α-ol 3629 $cm.^{-1}$, $\Delta\nu^{a}_{\frac{1}{2}}$ 20, ε = 28; 3611 $cm.^{-1}$, $\Delta\nu^{a}_{\frac{1}{2}}$ 18, ε = 26. He suggests that these facts are best explained by "conformational heterogeneity".

EXPERIMENTAL.

Rotations refer to solutions in chloroform at room temperature. M.p.s were determined on a Kofler block and are uncorrected. The alumina used for chromato-

graphy had activity III (Brockmon). Light petroleum refers to the fraction with b.p. 40-60°. Infra red spectra were measured on the Infracord PE 137 for thin films and on the Unicam SP 130 for solutions and KCl discs.

Isolation of labdane-8 α,15-diol.

The fleshy leaves of Aeonium lindleyi (10 kg.) were treated with ethanol (5 l.) for several days with occasional shaking. After filtration, evaporation afforded a brown gum (10.4 g.) which was taken up in ether and washed with N sodium hydroxide solution (3 x 50 ml.) and water (3 x 50 ml.). The ether solution was dried and evaporated and the residual gum (8.7 g.) was absorbed from light petroleum onto alumina (200 g.). Elution with ether-methanol (3 : 2, 300 ml.) afforded a pale yellow gum (7.3 g.) which partially solidified. This solid from light petroleum gave labdane-8 ,15-diol (5.6 g.) as large colourless prisms. Pumping at reduced pressure for 24 hrs. gave crystals (free of solvent) m.p. 83-84°, $[\alpha]_D$ = -10°(C, 1.7). (Found C, 77.0, H, 12.19. Calculated for $C_{20}H_{38}O_2$, C, 77.35, H, 12.35%). Molecular weight by mass spectroscopy 310, calculated for $C_{20}H_{38}O_2$ = 310.

Mass Spectrum:

The following are the major peaks in the mass spectrum:-

310 (s)		
295 (m)	Parent -	15 (CH_3)
292 (s)	Parent -	18 (OH)
277 (s)	Parent -	33 (CH_3 + OH)
239 (m)	Parent -	71
238 (m)	Parent -	72
195 (m)	Parent -	115
191 (m)	Parent -	119
177 (m)	Parent -	133
157 (m)	Parent -	153
137 (m)	Parent -	173

The molecular weight 310 together with the CH analysis suggest that $C_{20}H_{38}O_2$ is the formula for lindleyol. From the breakdown pattern, it can be seen that one methyl and one OH are easily removed - probably the tertiary methyl.

The nuclear magnetic resonance spectrum disclosed the presence of five methyl groups (8.84 for R R $C(CH_3)OH$, and 9.03, 9.12 and 9.2), sixteen skeletal hydrogens

(principal peaks at 8.27, 8.38, 8.45, 8.65), two hydroxyl protons (7.94) and the two α hydrogens of the grouping RCH_2CH_2OH (6.21, 6.32 and 6.42).

Significant I.R. absorption bands at 3450, 1380, 1375 and 1030 $cm.^{-1}$ (KCl disc) which were identical with those of an authentic sample of labdane-8α,15-diol. The U.V. spectrum of labdane-8α,15-diol in hexane showed no end absorption. Hydrogenation of labdane-8α,15-diol in ethyl acetate over Pd-10% showed no absorption.

Gas liquid Chromatography of labdane-8α,15-diol on an Embacel (80-100 mesh) column coated with 1% Silicone Elastomer 30 at 220°, flow rate 30 ml./min. gave a single broad peak r_t = 12 mins. G.L.C. examination of the residual oil from which labdane-8α,15-diol crystallised, whose I.R. was very similar to that of the 8α,15-diol, showed a small peak r_t = 18 mins. as well as the major peak due to the 8α,15-diol. The large prisms of the diol turn to a fine powder on standing for several days and in order to obtain an analytical sample it is necessary to pump them for 24 hrs. at 25°C. It would seem that the large crystal is due to a clathrate compound formed with one of the solvent molecules (l. petrol) since heptane and other

solvents are not suitable for recrystallizations. From the above extraction procedure it can be seen that the diol is present to the extent of approximately 0.06% based on the weight of green plant. It is however present to the extent of 50-60% of the alcohol extract.

Alternative methods of extraction.

(1) The green leaves (50 gm., 5 gm. when dry) were extracted with chloroform (3 x 50 ml.) to give a gum (0.9 g.) i.e. 18% of dry weight is due to the gum. Ether, chloroform and ethanol all serve as a solvent to extract the diol but ethanol is preferred as it removes less of the wax leaf coating.

(2) Fleshy leaves (7.5kg.) collected at Bajamar, Tenerife in November, 1960, were steeped in batches for 4 mins. in ethanol (4 l.), then washed with a further portion of ethanol (2 l.). The ethanol extracts were filtered and evaporated to 200 ml. under partial vacuum and the small deposit of waxes removed. The extracts were taken up in ether and washed with N sodium hydroxide (3 x 100 ml.) and water (3 x 100 ml.), and the ether evaporated off to give a gum (16 g.) (2.2% of green weight).

Labdane-8 α,15-diol monoacetate (XV).

Labdane-8 α,15-diol (500 mg.) in pyridine (70 ml.) was allowed to stand for 24 hrs. at 20° with acetic anhydride (1.5 ml.). The mixture was poured into water and extracted with ether to give an oil (482 mg.) which was absorbed from benzene on silica gel (15 g.). Labdane-8 α,15-diol-15 monoacetate (376 mg.) was eluted from silica in benzene-ether (1:1) as a gum $[\alpha]_D$ -4.1°(C, 1.02) (lit. $[\alpha]_D$ -3.7° (C, 0.98). A dilute solution in carbon tetrachloride shows infra red absorption bands at 3605, 3565, 1745 and 1238 $cm.^{-1}$, the relative proportion of the first two is unchanged on further dilution suggesting only intra-molecular hydrogen bonding (see Table 2 and text).

Labd-8(20)en-15-ol acetate (XVI).

Labdane-8 α,15-diol monoacetate (0.52 g.) in dry pyridine (20 ml.) was treated with phosphoryl oxychloride (0.9 g.). After 45 min. at reflux temperature it was cooled to 0° and added drop wise with stirring to a mixture of ice and water (100 ml.). Ether extraction afforded a product which was absorbed from light petroleum on alumina (20 g.). Elution with benzene afforded labd-8(20)en-15-ol acetate (0.44 g.) as

a colourless syrup $[\alpha]_D$ + 44° (C, 0.98). (Found : C, 78.63, H, 11.59. $C_{22}H_{38}O_2$ requires C, 78.98; C, 78.63, H, 11.59. $C_{22}H_{38}O_2$ requires C, 78.98090, 1738, 1645, 1240 and 890 $cm.^{-1}$

Labdan-15-ol-15 acetate (XVIII).

Labd-8(20)en-15 ol-15 acetate was hydrogenated in ethyl acetate over 10% palladium on charcoal. After filtering off the catalyst, removing the solvent, the saturated acetate was obtained as a colourless syrup $[\alpha]_D$ + 38°(C, 0.84). (Found : C, 78.57, H, 11.48, calculated for $C_{22}H_{40}O_3$, C, 78.51, H, 11.98%.)

Labd-8(20)en-15-ol(IXX).

This compound, obtained by alkaline hydrolysis of the corresponding acetate, was a colourless syrup $[\alpha]_D$ + 40°(C, 1.1). Infra red absorption bands at 3620, 3090, 1645 and 890 $cm.^{-1}$. Lit. $[\alpha]_D$ + 40°(C, 0.98).

Labdane-8 α,15:20-triol (XVII).

The above labd-8(20)-en-15-acetate (0.9 g.) in pyridine-chloroform (1:1) (80 ml.) was treated with osmium tetroxide (1.0g.) and kept at 20° for six days. Removal of the solvents under reduced pressure gave a

black residue which was heated under reflux for 5 hrs. with benzene (20 ml.), methanol (20 ml.), potassium hydroxide (4 g.) and mannitol (4 g.) in ethanol (20 ml.) and water (10 ml.). After dilution with water, the ether extract was washed with dilute hydrochloric acid, sodium bicarbonate solution and water. Evaporation gave a gum (0.757 g.) which on chromatography on alumina gave labdane-8 α,15,20-triol (0.12 g.) m.p. 126-127°. (After four recrystallisations from light petroleum benzene.) $[\alpha]_D$ - 21° (C, 0.86) 126-127°. (Found C, 73.41, H, 11.53, calculated for $C_{20}H_{38}O_3$, C, 73.55, H, 11.75%) Lit. values (Halsall) for this compound are: m.p. 124-125.5°; $[\alpha]_D$ -17° (C, 1.01).

TABLE 1.

Stretching Absorptions of Carbonyl Groupings.

(CCl_4)

Molarity	Cell cm.	ν(C=O) $cm.^{-1}$	$\Delta\nu_{\frac{1}{2}}^{a}$ $cm.^{-1}$	ε_a
Methyl labdanolate				
0.00152	0.5	1741	18	480
Labd-8(20)-en-15-ol-15-acetate (XV)				
0.0023	0.5	1742	17	470
Labdan-15-ol-15-acetate (XVI)				
0.00154	0.5	1743	15	690

"Frequency" values are accurate to $\pm$ 1 $cm.^{-1}$ for sharply defined peaks. ε_a values are quoted to the nearest 5 units.

TABLE 2.

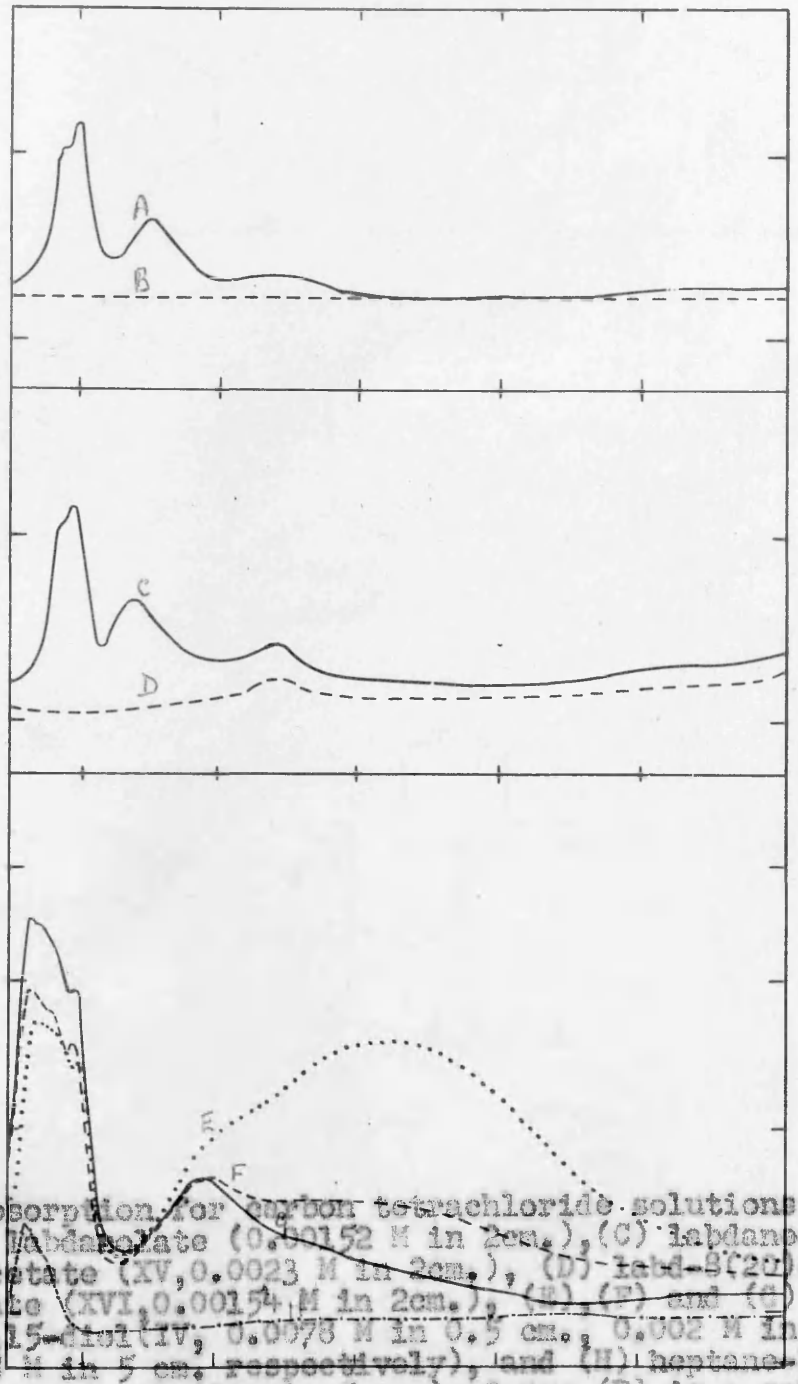
Stretching Absorptions of Hydroxyl Groupings.

(CCl_4)

Molarity	Cell cm.	ν(OH) $cm.^{-1}$	$\Delta\nu_{\frac{1}{2}}$ $cm.^{-1}$	ε_a	Assignment
Methyl labdanolate*					
0.00152	2	3613 sh. } 3605 }	36	30	'free'
		3550	47	15	'intra',OH...O
Labdane-8α,15-diol monoacetate* (XV)					
0.0023	2	3612 sh. } 3605 }	30	20	'free'
		3563	56	10	'intra',OH...O
Labdane-8α,15-diol (IV)					
0.00078	5	3636 } 3607 sh. }	48	65	'free'
		3515	90	15	'intra',OH...OH
0.0078	0.5	3637 } 3605 sh. }	52	50	'free'
		3500 sh.	-	30	'intra',OH...OH
		3370	270	42	'inter',OH...OH
Heptane-1,7-diol Saturated Solution	5	3637+	25	-	'free'

The hydroxyl absorptions for methyl labdanolate and labdanediol monoacetate were concentration independent up to 0.01 M. "Frequency" values are accurate to ±1 $cm.^{-1}$ for sharply defined peaks. ε_a values are quoted to the nearest 5 units.

*These compounds have a weak (ε_a 5) concentration independent band at ca.3460 $cm.^{-1}$ which is ascribed to the (C=O) first overtone. + Unsymmetrical band.



Hydroxyl absorption for carbon tetrachloride solutions of (A) methyl labdanolate (0.00152 M in 2cm.),(C) labdane-8 ,15-diol monoacetate (XV,0.0023 M in 2cm.), (D) labd-8(20)en-15-ol acetate (XVI,0.00154 M in 2cm.), (E), (F) and (G) labdane-8 ,15-diol(IV, 0.0078 M in 0.5 cm., 0.002 M in 2 cm., and 0.00078 M in 5 cm. respectively), and (H) heptane-1,7-diol (saturated solution in 5 cm.). Curve (B) is a solvent background (2 cm. cells).

Fig. 1.

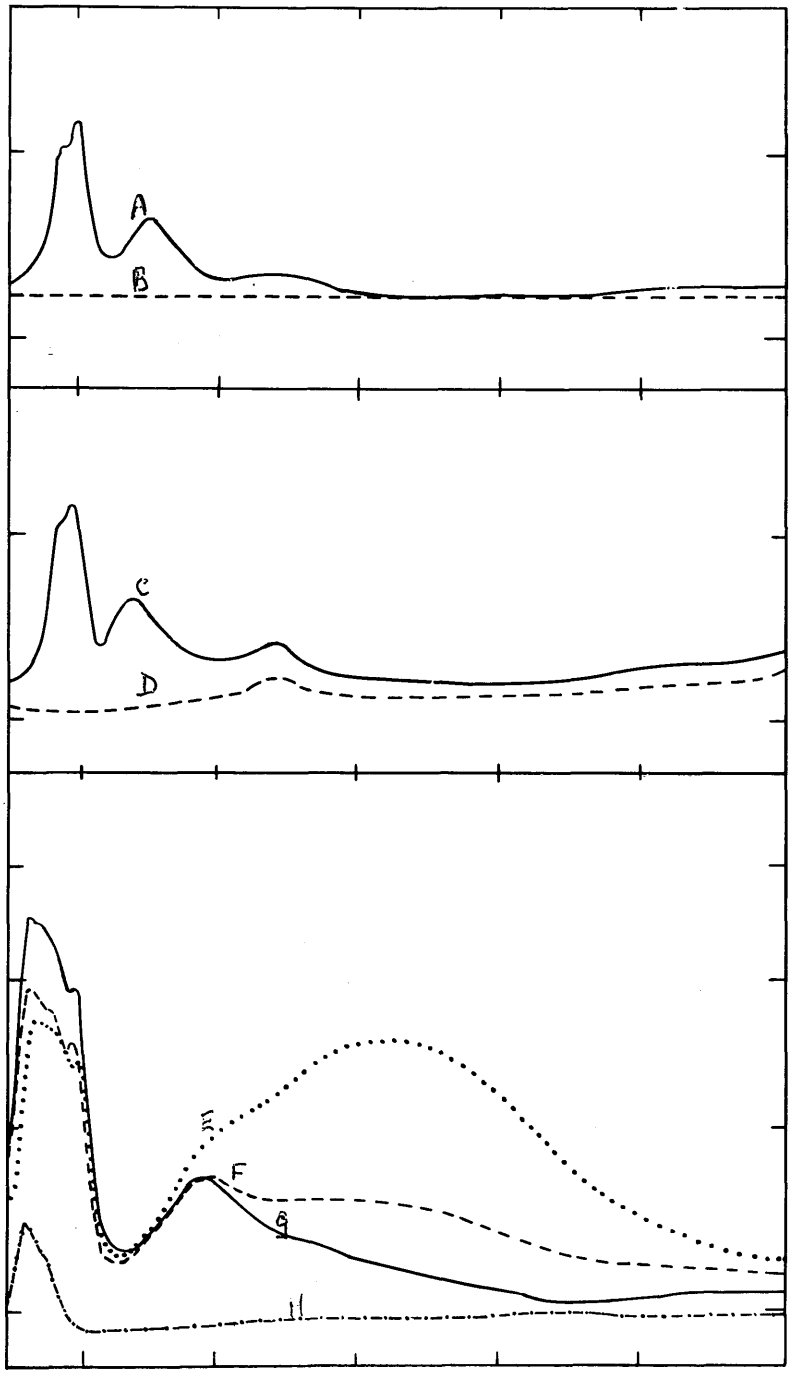
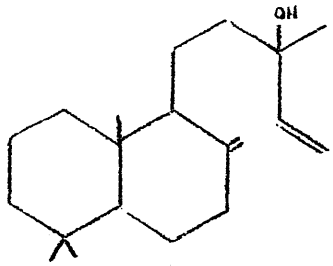
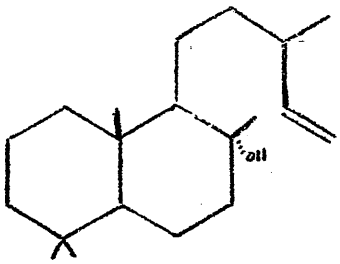


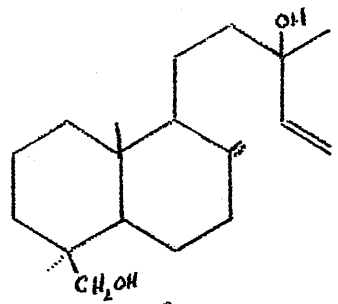
Fig. 1.



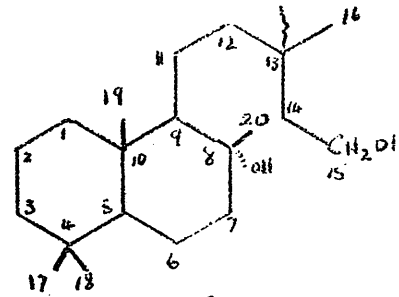
I manool



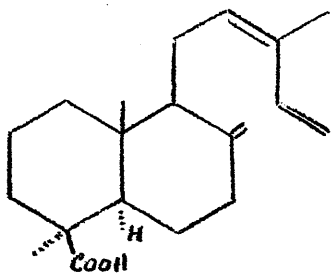
II sclareol



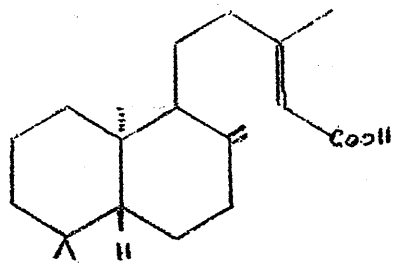
III torulosol



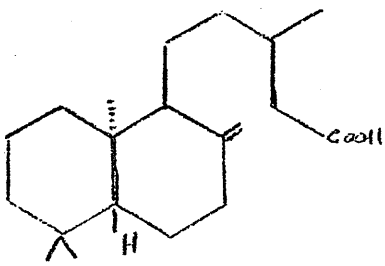
IV lindleyol.



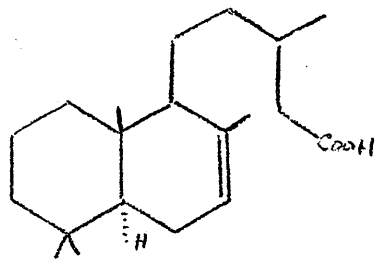
V communic acid



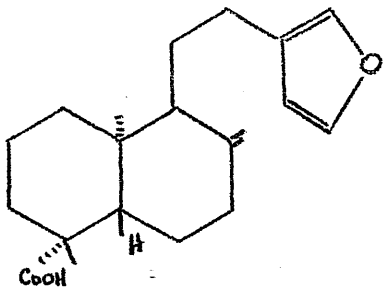
VI copalic acid



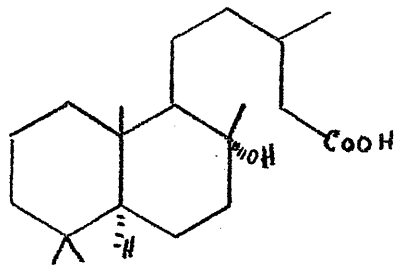
VII eperuic acid



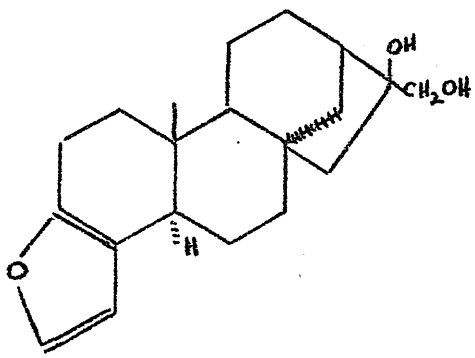
VIII cativic acid



IX daniellic acid



X labdanolic acid.



XI Cafestol

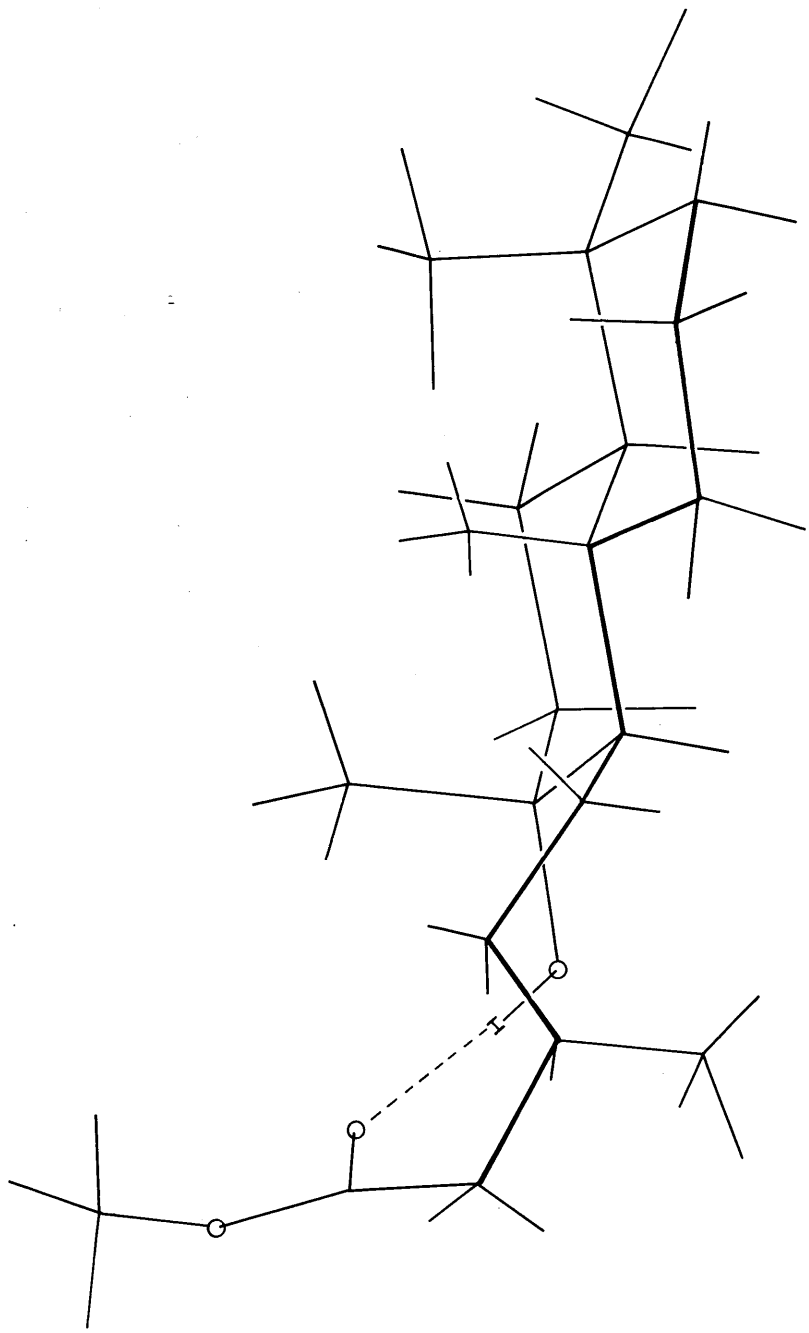


Fig. 3 One conformation of labdane-8α,15-diol drawn from the shadow cast by a Dreidling model with a pinhole source of light.

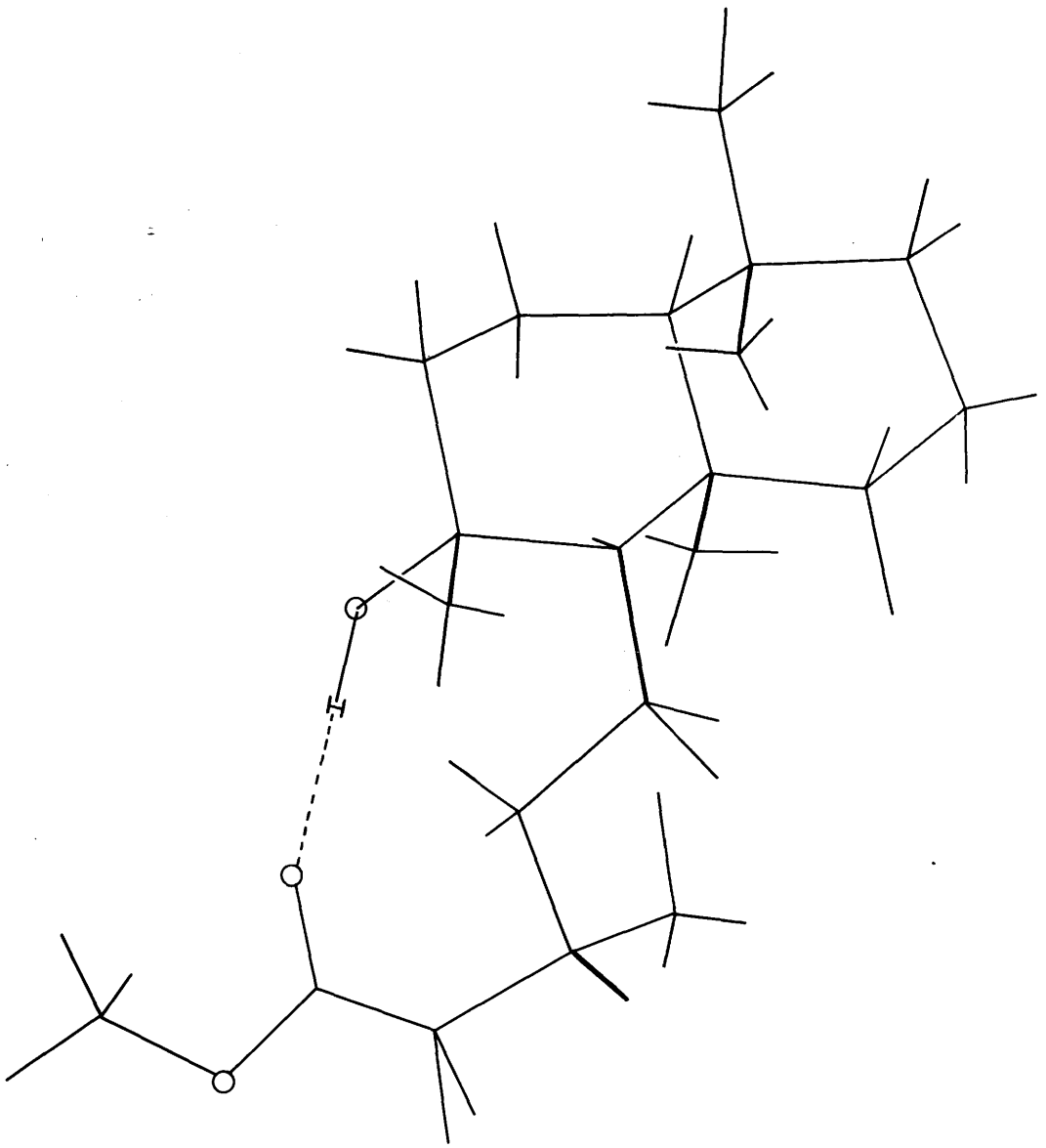


Fig. 4 Same conformation of labdane-8α,15-diol as in Fig. 3 but drawn from the shadow cast by the source of light in a different position relative to the model.

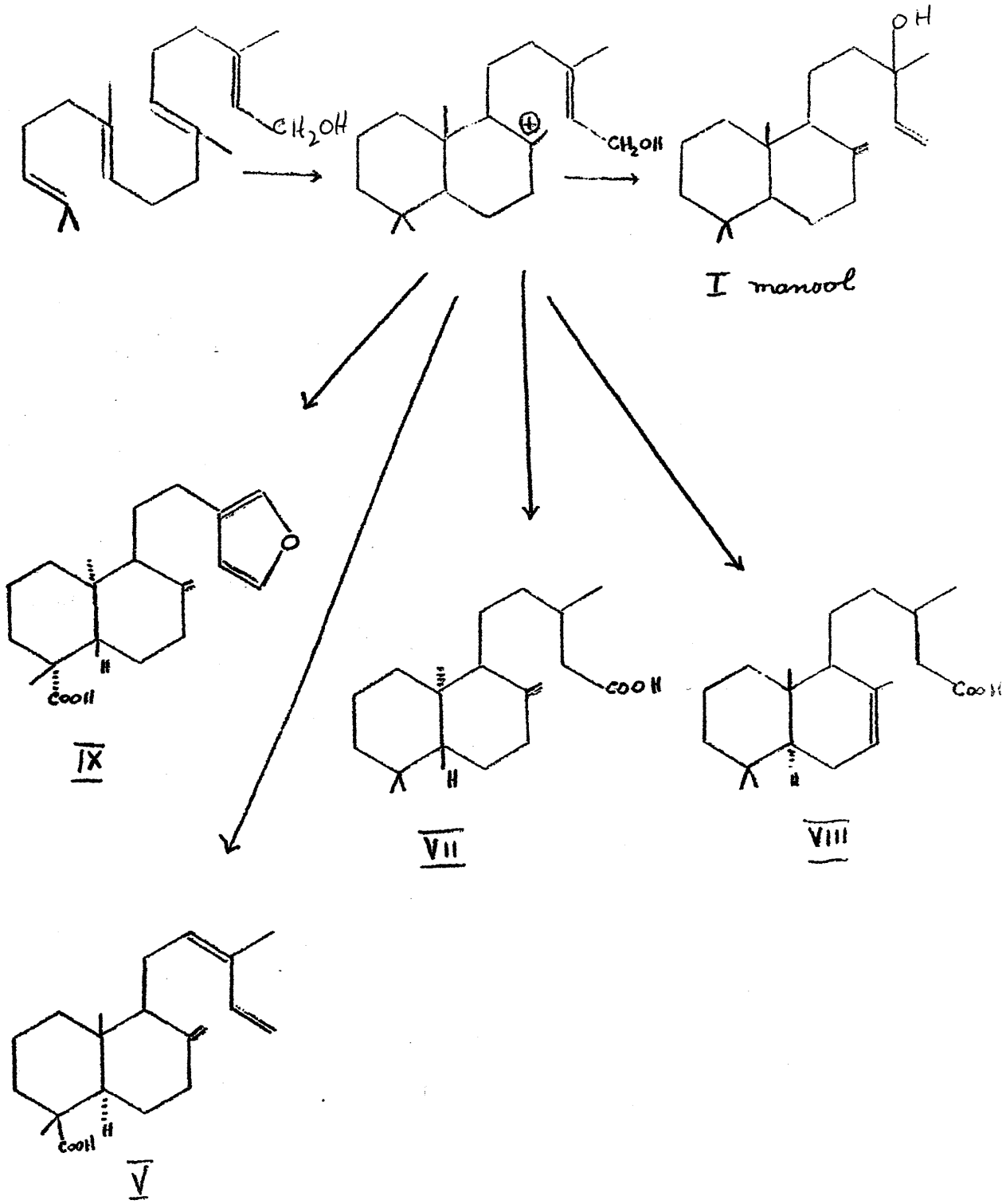
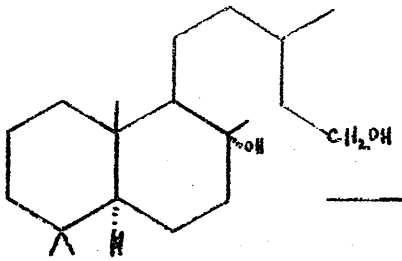
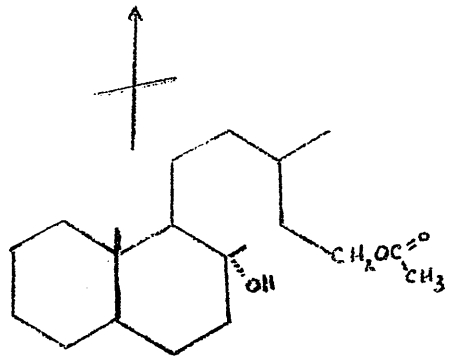


fig. 5. Suspected Biogenesis of Labdane Diterpenes.

Diacetate

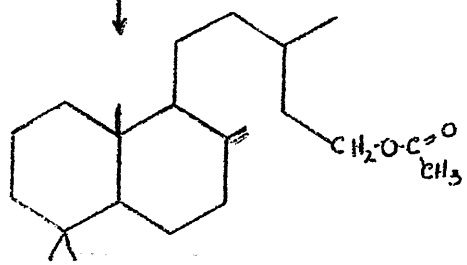


IV



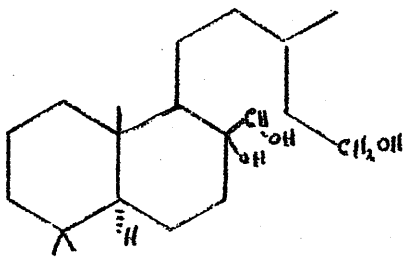
XV

$POCl_3$/pyridine



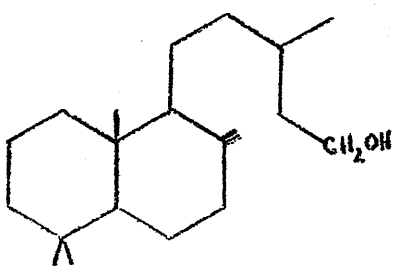
XVI

O_5O_4 / pyridine

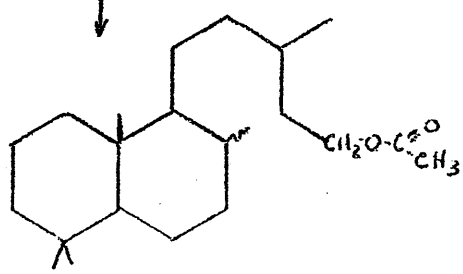


XVII

H_2/Pd./charcoal



IXX



XVIII

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S E C T I O N VII.

VOLATILE FUNGAL METABOLITES.

INTRODUCTION.

The effects of ethylene on higher plants, e.g. its influence on rate of ripening of fruits and vegetables, its effect on rate of growth of leaf surfaces (epinasty), have been well studied.[1] The effects of volatile metabolites from fungi on microbial life and on higher plants have been much less investigated. Fungal metabolites can have three effects:-

(a) they can influence the growth of the fungus from which they emanated or the germination of the spores of that fungus, e.g. the unknown self inhibitor in germination of rust uredospores,[2] the self inhibitor of uredospores of Puccinia graminis claimed to be trimethylethylene[3];

(b) the volatile from one fungus can affect the growth of another, e.g.

(i) the unknown gas from Mucor spinosus stimulates growth of Phytophthora citrophthora[4],

(ii) the volatile from Mucor plumbeus which stimulates asexual reproduction in Pilobolus kleinii is claimed to be ammonia[5];

(c) the volatiles from fungi can affect the growth of green plants, e.g. Dr. Hutchinson of the Botany Department of this University has shown the effects of volatiles from many different fungi on higher plants (using fern prothalli as test organism) (fig.1).

Hutchinson and McTeague had grown Agaricus campestris in the bottom plate of a Petri dish, with A. campestris spores in the top plate and, by placing a cellophane membrane between top and bottom plates, had shown that the compounds, which stimulated spore germination, given off by A. campestris were volatile. By passing air over a culture of A. campestris and collecting the effluent material at -60° they had trapped the volatile metabolite and shown that it was 2,4-dimethylpent-1-ene.[7]

When Dr. Hutchinson invited us to collaborate with him in these studies, it was decided to carry out a number of preliminery experiments with A. campestris as test organism to ensure that our analytical tool (G.L.C.) was sufficiently sensitive. A. campestris was grown in a Petri dish, as above, with the spores replaced by a culture of fern prothalli in the upper plate. After ten to fifteen days incubation, samples of the air from

the Petri dish were injected into a G.L.C. column but no components, other than water vapour, could be detected. Later, when A. campestris was replaced by other fungi (Table I), no components could be detected by this direct air sampling. A trapping system was also used similar to that in Hutchinson, McTeague and Reed's original work but with the phosphorus pentoxide drying tubes replaced by milder dessicating agents (fig. 2). An attempt was made to isolate the volatile from A. campestris but G.L.C. analysis of the trapped material gave inconclusive results.

These preliminary experiments amply demonstrated the difficulties of these analyses. In particular, it seemed likely that we would have difficulties due to:-

(a) the very low concentration of volatiles;

(b) the very high concentration of other components, e.g. water vapour and carbon dioxide concentrations which are certain to be increased by the processes of respiration and evaporation.

The ideal form of analysis of the volatile fungal metabolites would be to make a complete analysis of all the possible constituents of the culture gas.

However, such a complete analysis was beyond our capabilities with G.L.C. and it was decided to concentrate

on volatiles, whose boiling point range was 0 - 80°, which might have an effect on prothallial growth. To confirm that such compounds might have an effect, a preliminary experiment was carried out. The hydrocarbons, 4-methylpent-1-ene, 4-methylpent-2-ene, 2,2-dimethylbutane and 2,4,4-trimethylpent-2-ene were investigated by placing vapour samples of them in bottles containing prothalli. The choice of hydrocarbons was determined by the desire to consider the effect of a saturated and unsaturateds with a mono-, di-, and trisubstituted double bond. After ten to fifteen days incubation it was noted that 2,2-dimethylbutane, 2,4,4-trimethylpent-2-ene showed a significant inhibitory effect on prothallial growth and 4-methylpent-2-ene an almost significant effect. The results of this small experiment can only be taken as a pointer to the course that future work might take; they could not be taken as conclusive proof of the presence of these hydrocarbons among the fungal volatiles.

DISCUSSION.

As mentioned earlier, there is an increase in the water and carbon dioxide level of the air in which fungi or fern prothalli grow. It was realised that

as the prothalli were photosynthetic the increase in carbon dioxide level might be itself the cause of the stimulatory effects noted above. The experiments required to confirm this theory are very tedious, however, and it was therefore decided to investigate any other volatile metabolite. The fungus Schizosaccharomyces octosporus was chosen as a suitable test organism since it had an inhibitory effect on prothallial growth which is more difficult to explain in the carbon dioxide scheme. Sterile air was blown over a culture of Schizo. octo. and the volatiles collected in a trap at -60° (fig.2). Samples of the trapped material were analysed by G.L.C. and the presence of ethanol consistently detected amongst a number of other components which varied from sample to sample. This was a very interesting finding since it was easy to show that 5% ethanol/water solutions inhibited the prothallial growth. Cultures of prothalli were grown with solutions of ethanol and water (10%, 5%, 1%, 0.1%) in the bottom plate and after ten to fifteen days incubation, inhibition of growth was noted in the cultures above 10% and 5% ethanol but not those above the 1% or 0.1% ethanol

solutions. G.L.C. analysis of samples from these incubated cultures showed the presence of ethanol down to 0.1%.

However, G.L.C. analysis of direct air samples from cultures containing S. octosporus did not show any peak for ethanol. This suggested that though ethanol inhibited prothallial growth, it was not present in sufficient concentration from S. octosporus to be the sole cause of inhibition.

Although ethanol had been detected in the volatiles from S. octosporus it had not been possible to detect consistently any other component. It was suspected that the long lengths of water and carbon dioxide removing agents were adsorbing the very small quantities of volatiles and a change was made to removing the water by means of a refrigerating column. In order to obtain a more concentrated sample the air supply was changed from blowing fresh sterile air to blowing a continuously recycled sample of air. With these two modifications, material was again trapped from S. octosporus but it again gave no conclusive results.

Another modification was made to the trapping procedure in which the continuously recycled air from

S. octosporus was passed through the refrigeration coil and then through a short length of G.L.C. column packing. The G.L.C. column-packing absorbed the volatiles which were then flushed onto a G.L.C. analytical column by argon. Mackay used this method of trapping very successfully for the volatiles of cheese and coffee[8].

Using this method, it was possible to detect a component from S. octosporus consistently but as yet no identification has been made.

CONCLUSIONS.

Our problem has been, throughout this study, one of obtaining a sample of the volatiles sufficiently rich in the fungal metabolites to permit identification. The results so far have been disappointing but there is great hope that, with this latest means of trapping (use of G.L.C. packing), the continuously recycled air and the use of a Swodoba column[9]. (which permits analysis of aqueous solutions), success will be achieved.

Despite these trapping difficulties, we have shown that Schizosaccharomyces octosporus, Saccharomyces cerevisiae, Tilachlidium, and aspergillus niger give off small quantities of volatiles though we have not

been able to identify them. We have also shown that ethanol is not the volatile from Schizosaccharomyces octosporus which plays the important part in inhibiting prothallial growth.

The results of this work far from being of purely academic interest should help in the explanation of many well known biological interactions, e.g. in soil mycology, mycorhizal associations, etc.

EXPERIMENTAL.

First Sampling Method.

Sterile air was blown over a culture of Agaricus campestris, the common mushroom, and then passed through a trap K cooled at -60° (fig. 2). The material in this trap was vacuum transferred to a smaller trap from which vapour samples (1-2 ml.) could easily be removed for G.L.C. analysis. The samples were injected onto a squalane (16.6%) column on celite (80-100 mesh) in a copper spiral (4 ft. x ¼") at 0° and 30° but no peaks could be seen. The results of analyses of standard hydrocarbons of the type suspected are shown in Table II. Hexane (10 mg.) was placed in the system (fig. 2) and the air flow started. After

half an hour the material in trap K was vacuum transferred to sampling trap L from which gas samples were removed and analysed by G.L.C. as above. A peak due to hexane was obtained consistently.

Using the above procedure with Schizosaccharomyces octosporus, G.L.C. analysis showed the presence of one component consistently among a variable mixture of other components. Comparison of the retention times of this component on 20% carbowax and 16.6% squalane identified it as ethanol.

Second Sampling Method (Direct Air Sampling).

In the biological test for activity, Schizosaccharomyces octosporus was grown in the bottom plate of a Petri Dish and separated by a cellophane membrane from the test organism - fern prothalli - grown on medium in the top plate. After ten to fifteen days incubation, the fern prothallial growth was inhibited relative to the controls. Samples of the vapour above the Schizosaccharomyces octosporus were then analysed on a carbowax (20%) column at 60° but no peaks could be seen.

The vapours from standard mixtures of ethanol/water were analysed on a carbowax column (20%) at 60°

and the peak heights measured - average of six samples for each mixture. The ethanol peak could still be measured from the vapour of a 0.01% ethanol/water mixture. Fern prothalli were grown on the medium in the upper plate of Petri dishes as before but with ethanol/water mixtures (0.1, 1.0, 5.0 and 10.0% ethanol) in the bottom plate. After ten to fifteen days incubation, G.L.C. analysis showed that the amount of ethanol in the vapour had fallen - 5% ethanol/water vapour sample gave a peak corresponding to 0.5% ethanol/water mixture, while 1% ethanol had fallen to 0.05%. It was found that a minimum of 5.0% ethanol/water mixture was required to inhibit prothallial growth.

Third Sampling Method (Trapping in Stationary Phase).

A large stirred culture of Schizosaccharomyces octosporus was grown in a closed system with a continuously cycled volume of air (5 gallons). After ten days the air was passed through a cooling coil at -10° to remove water then over a drying agent Calcium Oxide and into a short length of G.L.C. packing material (20% carbowax on celite). Portions of this packing material were then placed at the top of an

analytical column (5% carbowax on the P.A.C.) at 60°. The gas flow was restarted and the chromatogram showed the presence of water vapour and a sharp peak (retention time = 8 mins.) which has not been identified as yet but is definitely not ethanol or methanol.

Cultures of fern prothalli were incubated in litre bottles and the vapours of the hydrocarbons listed below injected.

	Vol.of vap.	Average growth.
4-methylpent-1-ene	10 ml.	146
4-methylpent-2-ene	"	97.7
2,2-dimethylbutane	"	51.0
2,4,4,-trimethylpent-2-ene	"	78.3
Control		135
Least significant difference		41.7

TABLE I.

Summary of Results.

Fungal Species	Biological Effect	Method of trapping &/or sampling	Results.
1	Inhibits prothallial growth	1. Direct air sampling. 2. Trapping at -60°.	Negative 2 peaks, first ethanol, second b.p.=70°
2	Inhibits prothallial growth	Direct air sampling of 6 Petri dishes.	Ethanol in only one Petri dish.
3	Stimulates spore formation. No significant effect on prothalli.	Trapping at -60°.	Inconclusive.
4	Stimulates prothallial growth.	Trapping at -60°.	2 minor peaks C_6 or C_7 range.
5	Stimulates prothallial growth.	Trapping at -60°.	Inconclusive.
6	Stimulates prothallial growth.	Direct air sampling.	Negative.
7	Stimulates prothallial growth.	Direct air sampling.	Negative.

Footnote: The fungal species are:

1, Schizosaccharomyces octosporus, 2, Saccharomyces cerevisiae, 3, Agaricus campestris, 4, Thlaschidium spp., 5, Aspergillus niger, 6, Verticillium albo-atrum and 7, Colletotrichum lindemuthianum.

TABLE II.

G.L.C. analysis of Standard Hydrocarbons.

Hydrocarbon	Retention Time (in mins.)
4-methylpent-2-ene (cis and trans)	22.8
2,3-dimethylpentane	52.4
2-methylpent-1-ene	37.4
2,3-dimethylbutane	26.2
Mixture of:	
2,2-dimethylbutane	18.4
4-methylpent-1-ene	23.2
4-methylpent-2-ene (cis and trans) 2,3-dimethylbutane	25.2
2-methylpent-1-ene	37.2

Footnote: All analyses were carried out on a 16.6% squalane column at 30°.

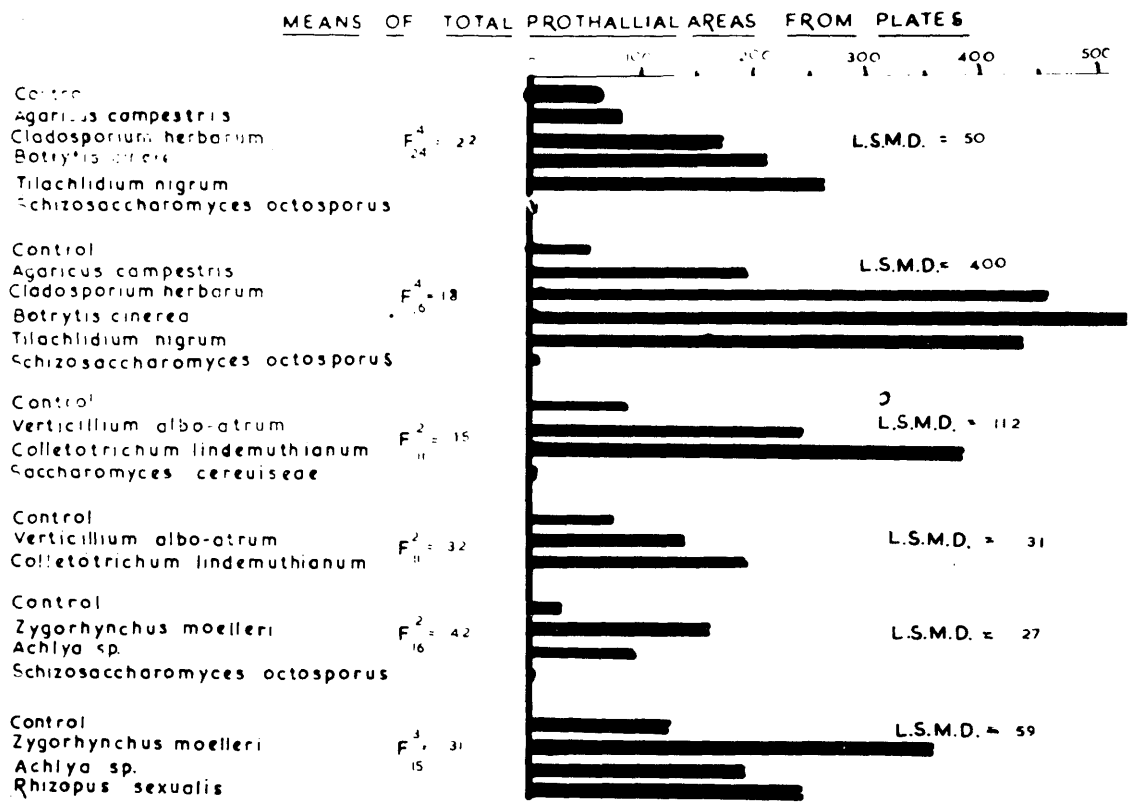
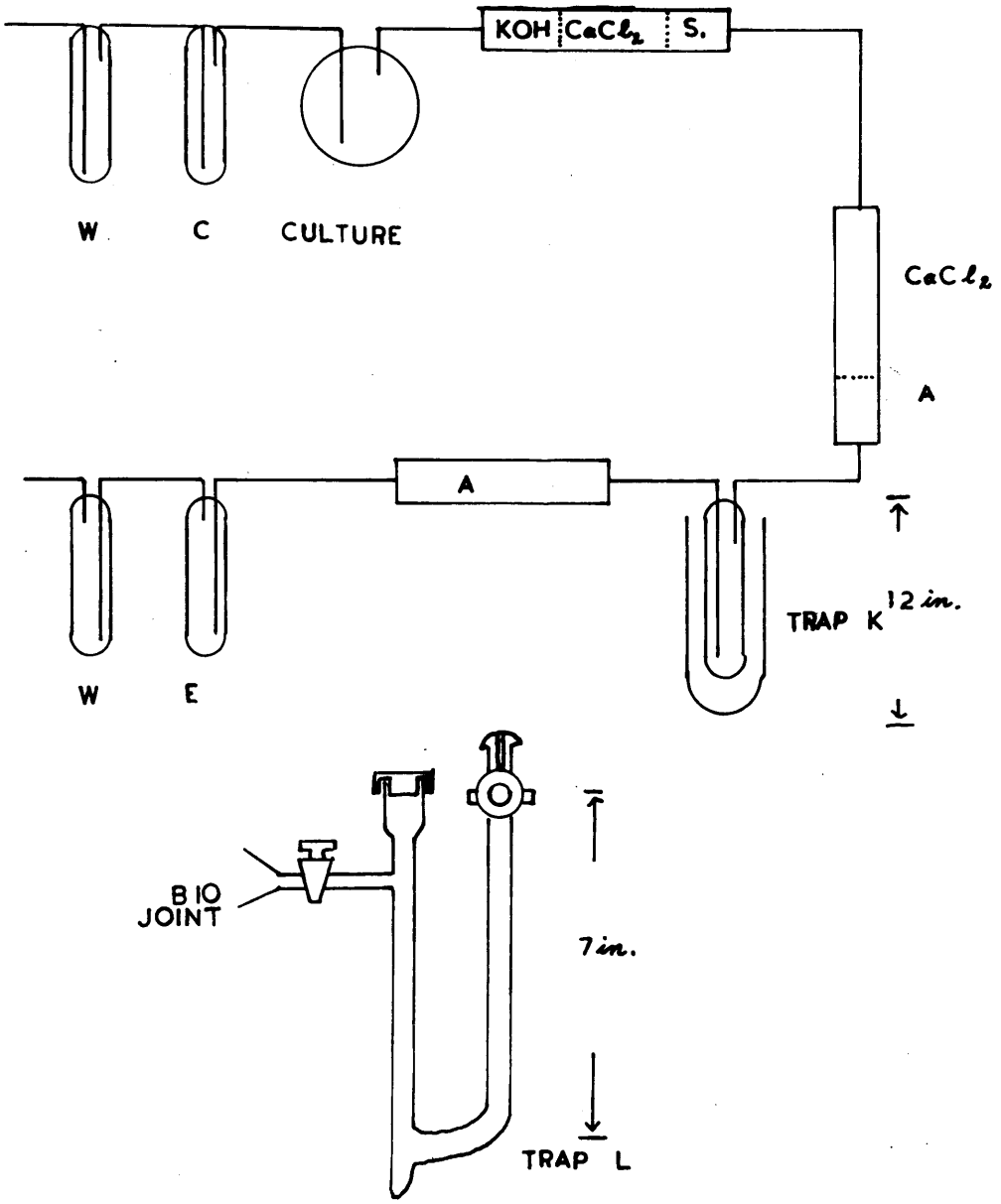


Fig. 1 The effects of the volatiles from two fungi are measured by comparing the prothallial areas, after incubation in the upper plate of the petri dish with fungi in the bottom, with the areas of the control plates of prothalli alone. Only those values which differ from the control by the least significant mean difference (L.S.M.D.) are taken as significant.

F I G . 2



A - Drying tube with anhydrone.
S - Sofnolite, carbon dioxide removing agent.
C - Wash bottle with sterile cotton wool to prevent contaminants entering the culture.
E - Empty Wash bottle.
W - Wash bottle with water, first to provide moisture for the culture, the second to show that air is flowing.

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S E C T I O N VIII.

PARTIAL STRUCTURE OF "SHERUNGULONE".

INTRODUCTION.

An interesting compound, with the unusual empirical formula $C_{24}H_{28}O_4$, was reported in 1915 by Goulding and Roberts.[1] It had been isolated from the essential oil of the tubers of Kaempferia ethelae (Zingiberaceae), a plant known to the natives of the Transvaal as "Sherungulu". Goulding and Roberts were unable to suggest a structural formula but they showed the presence of a ketone (formation of an oxime), a hydroxyl group (although it did not form an acetate it formed a "benzoyl derivative", m.p. 260° with decomposition), and unsaturation (uptake of three times its weight of bromine). This highly unsaturated ketone seemed worthy of re-investigation and Tropical Products kindly supplied tubers of a Kaempferia species. Unfortunately, at the time of collection in Portuguese East Africa, no flowers were available and the species was believed to be Kaempferia roseae.

The quantity of crystalline ketone obtained from the essential oil of Kaempferia roseae did not permit the more customary degradative approach, so it was decided to attempt a structural determination mainly by physical techniques.

RESULTS AND DISCUSSION.

Steam distillation of the tubers of Kaempferia rosoae yielded an essential oil containing α-pinene, p-cymene and 1,8-cineole among six other monoterpene constituents (G.L.C. analysis). A colourless solid, which could be recrystallized from ethanol, separated from the essential oil. Mass spectrographic analysis of this recrystallized material (m.p. 105-6°, $[\alpha]_D^{19}$ +272°; Goulding and Roberts had reported m.p. 102°, $[\alpha]_D$ +192°) indicates a molecular weight of 230 and elemental analysis is consistent with $C_{15}H_{18}O_2$. The infra red and ultra violet absorption data (Table I and Fig. 4) suggest an $\alpha\beta$-unsaturated ketone (-CH=CH-CO-), and infra red absorption at 1440 $cm.^{-1}$ and 1419 $cm.^{-1}$ is not inconsistent with a methylene adjacent to the double bond and a methylene adjacent to the carbonyl. It was decided to name this ketone "Sherungulone" since it was first isolated from "Sherungulu" tubers.

Hydrogenation of Sherungulone produces a hexahydro-derivative ($\lambda_{max.}$ 273 mμ, ε =55) whose mass spectrum shows a molecular weight of 236 ($C_{15}H_{24}O_2$ requires 236). The infra red spectrum of hexahydro Sherungulone (Table I) with strong absorption at 1712 $cm.^{-1}$ indicates that the carbonyl group is not in a five membered ring

though the data in Table IV show that it may be in a six or seven membered ring. The absorption bands of Sherungulone between 1000-1200 cm^{-1} and the absence of -OH stretching absorption suggest that the second oxygen function is present as an epoxide or ether link. This fact is partly confirmed by the presence of a peak (P-33) in the mass spectrum (Table II, fig. 2) which is probably due to successive elimination of a methyl group (angular or gem dimethyl) and a molecule of water. A study of the infra red absorption of Sherungulone (Table I, fig. 1) suggests that there are no gem dimethyl groups so that the peak (P-15) in the mass spectrum is probably due to an angular methyl group. The presence of three double bonds (uptake of three moles of hydrogen), one carbonyl and one ether link (infra red spectrum) in Sherungulone is suspected so that the ketone must have either one ring and one unhydrogenated double bond or two rings ($C_{15}H_{18}O_2$).

Interpretation of both infra red and ultra violet spectra is made more difficult by the fact that adjacent groups may affect the chromophore, e.g. the allylic hydroxyl in T (Table IV) is claimed to be the cause of the low λ_{max}. The elucidations below are based on

the assumption that there are no neighbouring group interactions. The ultra violet absorption of Sherungulone (Fig. 4) is unusual in that the maximum shifts only 2mμ on changing from hexane to ethanol. In ethanol, but not in hexane, addition of base produces a new absorption band at 206mμ (ε = 19,150) and a shoulder at 222mμ (ε = 14,500). Assuming the chromophore (222 mμ) to be -CH=CH-CO-, this new absorption (206mμ) may be due to a chromophore present in the molecule but not seen because of the absorption of the $\gamma\beta$-unsaturated ketone, e.g. -CH=CH-C$\overset{CH_2}{\diagup\diagdown}$HC-CO (212 mμ)[2]; or it may be due to a chromophore formed by addition of base.

Although the cyclopropane ring is possible, the N.M.R. spectrum (Fig. 3) does not indicate a methylene group of this type and its presence in Sherungulone would require an eleven membered system for the second ring. A saturated ketone in an eleven membered ring would absorb at 1700 $cm.^{-1}$ [3]. and a carbonyl conjugated with a cyclopropane ring probably at 1685 $cm.^{-1}$ (compare with dihydro-β-umbellulone[4].) whilst hexahydro Sherungulone absorbs at 1712 $cm.^{-1}$ The absence of absorption in the infra red of hexahydro Sherungulone at 3040 $cm.^{-1}$ is taken as conclusive evidence against the cyclopropane ring.

The chromophore $CH_3-CH=CH-CO-CH_2-(CH_2)_{1 \text{ or } 2}$ (B)

was based tentatively on the infra red absorption at 1419 $cm.^{-1}$ (Table I) which we assigned to CH_2CO (Jones[5]. gave a range 1426 to 1415 $cm.^{-1}$).

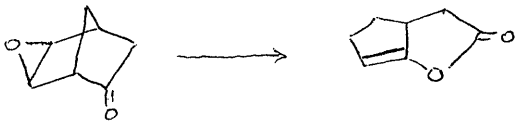
Hexahydro Sherungulone, which almost certainly contains a CH_2CO (hydrogenation of the $\alpha\beta$ - unsaturated ketone) absorbs at 1429 $cm.^{-1}$ which we assigned to CH_2CO following Herz[6]. who assigned the 1430 $cm.^{-1}$ band to CH_2COCH_2. Herz, however, assigned the band at 1420 $cm.^{-1}$ to CH_2COCR_3. It is impossible to assign the 1419 $cm.^{-1}$ band in Sherungulone to CH_2COCR_3 since the group adjacent to the carbonyl must be CH=CH-CO. It would appear therefore that the infra red absorption by no means rigorously defines the group adjacent to the carbonyl.

It was hoped that the nuclear magnetic resonance spectrum (Table III, fig. 3) of Sherungulone would determine the group adjacent to the carbonyl. The peak at τ = 8.98 due to one angular methyl group confirms the evidence of infra red and mass spectrographic analyses whilst the doublet at 8.05, 8.09 is due to one vinylic methyl group. The peaks in the region τ = 3 - 5 can be

attributed to three protons RCH=CH-R and $R_2C=CHR$ and the splitting of the peaks in this region and at 8.05, 8.09 is such that the grouping

$$RCH_2 - CH - CH - CH - CO -$$

is strongly suggested. This leaves nine protons of which two or three are at τ = 8.81, 8.69 due to either CH_3CH or $R_3C - CH_2 - CR_3$ and five or six at τ = 7 to 7.6 due to CH_2CO and $-HC\overset{O}{\diagdown\!\diagup}CH-$ and other methylenes. One of the reactions of Sherungulone helps to confirm the presence of an epoxide. After standing Sherungulone in carbon tetrachloride for six hours, the solution is found to contain lactonic material. Epoxyketones are known to give unsaturated lactones[7]. and this may be the reaction which Sherungulone undergoes, e.g.



The N.M.R. spectrum of Sherungulone would suggest that both an epoxide and CH_2CO are present.

The N.M.R. spectrum of the hexahydro derivative helps to confirm some of the assignments made for Sherungulone. It is difficult to determine the number

of methyl groups but it is possible that the peaks at τ = 9.12, 9.03 and 9.08, 8.97 are due to two CH_3CH groups and the peak at τ = 8.85 to one angular methyl. The absence of peaks in the region τ = 3 to 5 confirms the grouping RC CH_3 = CH - CH = CH - in Sherungulone.

That the grouping R C CH_3 = CH - CH = CH - is adjacent to the carbonyl in Sherungulone is confirmed by the presence of peaks τ = 7.84, 7.90, 7.97 due to CH_2CO in the hexahydro derivative. The peaks τ = 6—7 do not confirm the epoxide since they are normally due to - CH$\overset{O}{\diagdown\!\diagup}$$(CH_2)_n$CH - . This suggests that some rearrangement has occurred at the same time as hydrogenation which might also account for the unusual methyl absorptions (1300 - 1500 $cm.^{-1}$) in the hexahydro derivative.

A number of reactions was attempted as in Table V. Sodium borohydride reduction of Sherungulone yielded a red solution containing a mixture of compounds containing hydroxyl carbonyl and double bond groups. To study this red colouration, which was thought to be produced by an azulene, a larger quantity of Sherungulone was reduced with borohydride. The

infra red absorption of the product showed the presence of lactonic material and absorption in the ultra violet ($\lambda_{max.}$ 240, $\lambda_{shoulder}$ 275, $\lambda_{max.}$ 435, $\lambda_{shoulder}$ 550 mμ) suggested that it was not an azulene. This was partly confirmed by the fading of the red colour on standing in air for six to eight days.

Reduction of the hexahydro derivative with lithium aluminium hydride gives a secondary hydroxyl group which is not intramolecularly bonded confirming that the -OH is in a position in which it cannot bond to the second oxygen function.

CONCLUSIONS.

It would seem, therefore, that the data available at present is insufficient to allow the structure to be deduced unambiguously. It is possible to give the functional groups in Sherungulone. It would appear to contain

R C CH_3 = CH - CH = CH - CO - but the trisubstituted double bond must be twisted out of the plane of the α/β-unsaturated ketone since neither infra red nor ultra violet data will fit a normal

$\alpha\beta\gamma\delta$ unsaturated ketone. The group adjacent to the carbonyl is probably CH_2CO. Sherungulone also contains an angular methyl group CH_3CR_3, a CH_3CHR_2, and a tetrasubstituted double bond ($R_2C = CR_2$) and an epoxide, $-HC\overset{O}{<}CH-$, leaving one proton to be accounted for. It is also clear that the hexahydro derivative is unusual and one may postulate that isomerisation has occurred at the same time as hydrogenation.

Sherungulone seems to be a new sesquiterpene and merits further work which might follow the following lines:-

- a study of the ozonolysis products of Sherungulone,
- a study of the lactonic material and the red colouration,
- selective hydrogenation with a less active catalyst to obtain the di- and tetrahydro derivatives,
- hydrogenation and dehydrogenation with platinum to give the ring system.

Lastly, as an entirely different approach, the X-ray pattern of a derivative of Sherungulone might be studied. Such a derivative would require a heavy atom and the bromophenyl hydrazone might be useful. A simple bromine addition compound would not be useful since it would ruin the stereochemistry of the double bonds.

EXPERIMENTAL.

Light petroleum refers to petroleum ether (b.p. 40-60°C.). M.p.'s are uncorrected. Ultra violet spectra are measured on the Hilger-Watts Uvispec Spectrometer or the Perkin Elmer "Ultracord", infra red analyses on the Unicam SP 100. For the nuclear magnetic resonance spectra we are indebted to Dr. A. Porte of this Department.

Steam distillation of the tubers of Kaempferia roseae (which had been macerated but not ground in an Atomix) yielded an essential oil. During the course of the distillation a white solid was seen forming in the condenser. After extraction of the total steam distillate with ether, the ether solution was dried over magnesium sulphate. Removal of ether, under reduced pressure, gave an essential oil from which separated a colourless solid.

Recrystallization of the solid from ethanol gave a crystalline solid, m.p. 105-106°, $[\alpha]_D$ +272°. (Found C, 78.34, H, 7.63; $C_{15}H_{18}O_2$ requires C, 78.23 and H, 7.88). G.L.C. analysis of the solid on a 5% Silicone column (S.E.30) at 167° gave a single peak retention time = 14 mins. and on a 1% QF column at 135°, retention time = 8 mins.

The molecular weight, 230, was given by mass spectrographic analysis ($C_{15}H_{18}O_2$ requires 230.2).

Catalytic reduction of Kaempferia ketone.

Palladium charcoal (10%, 60.2 mg.) in ethyl acetate (10 ml.) was treated with hydrogen until uptake of gas ceased and the ketone (51.95 mg.) was added. Uptake (15.54 ml.) of hydrogen corresponded to three moles. After removal of the catalyst and the solvent, the hydrogenated enone was sublimed at 80°/0.05 mm. pressure to remove any contaminants (catalyst, silicone grease).

G.L.C. of the hydrogenated enone on 1% QF (a fluorinated alkyl silicone gum) at 135° showed that the hydrogenated enone was 85% pure (retention time = 10 mins.).

Hexahydro Sherungulone had infra red and ultra violet absorptions as in Table I and Fig.4. The molecular weight by mass spectroscopy was 236 ($C_{15}H_{24}O_2$ requires 236) (Table II and Fig.2). Figure 2, the histogram representation of the mass spectrum, does not show any of the peaks below 55 mass units.

Sodium Borohydride Reduction.

To Kaempferia ketone (4.2 mg.) in dioxan - water (1 ml. - 0.1 ml.) was added sodium borohydride (3.1 mg.)

and the mixture allowed to stand at room temperature for thirty minutes. Water was added and the solution extracted with ether (3 x 5 ml.). The infra red spectrum of the ether soluble material showed hydroxyl absorption (3,500 $cm.^{-1}$) carbonyl absorption and broad absorption peaks at lower frequencies due to borate ester. To hydrolyse this borate ester, it was dissolved in methanol and concentrated hydrochloric acid (2 drops) added. During removal of the methanol, white fumes were produced and the solution became pink. The infra red absorption of the substance remaining showed a hydroxyl group at 3500 $cm.^{-1}$, carbonyl at 1710 $cm.^{-1}$ and ethylenic group 1610 $cm.^{-1}$

Lithium Aluminium Hydride Reduction.

The hexahydro ketone (3.4 mg.) was stirred in ether (10 ml.) and lithium aluminium hydride (20-50 mg.) added. After the vigorous reaction had ceased the mixture was heated for two hours, then ethyl acetate added to decompose the remaining lithium aluminium hydride. A white gelatinous precipitate, formed on addition of water to this ethereal solution, was filtered off and washed with ether. The combined ether extracts were washed with water (three times),

dried over magnesium sulphate and the ether removed under vacuum. The infra red spectrum of the resulting material (in carbon tetrachloride) showed sharp hydroxyl absorption at 3638 $cm.^{-1}$ (concentration independent) and broad absorption at 3348 $cm.^{-1}$ (concentration dependent) indicative of intermolecular bonding but no intramolecular bonding.

TABLE I.

Infra Red Spectra.

Sherungulone.				Hexahydrosherungulone.			
ν	$\Delta\nu_{\frac{1}{2}}$	ε	Possible assignments	ν	$\Delta\nu_{\frac{1}{2}}$	ε	Possible assignments
3026		35	γ = C-H				
2968		106		2965			
				2954			asymγ(C-H)CH_3
2930		80	asymγ(C-H)CH_3	2926			asymγ(C-H)CH_2
2842		61		2920			
				2873			symmγ(C-H)CH_3
1682	11	718	Conj.C=O	2853			symmγ(C-H)CH_2
1647	11	110	C=C endo	1713	17	448	C = O
1617		25		1484		23	
				1461		83	
1458		95	CH_2 skeletal	1457		110	CH_2 skeletal
1440		69	CH_2adj.to C=C	1446		48	
1419		67	CH_2 adj.to C=O	1429	10	85	CH_2 adj.to CO
1391		89	}	1377	8	96	asg. CH_3
			} methyl	1365	9	41	
1385		46	} absorption	1331		44	
			}	1320		58	
1375		127	}				
1115		125		1119*			
1090		146	(C-O)	1100*			
1085		125	ether	1084*			
994		33					
986		27		987*			
949		27		963*			
920		40		937*			
906		25		917*			
877		53	γ (CH=)	890*			

All values for CCl_4 soln. except * which are for liquid film.

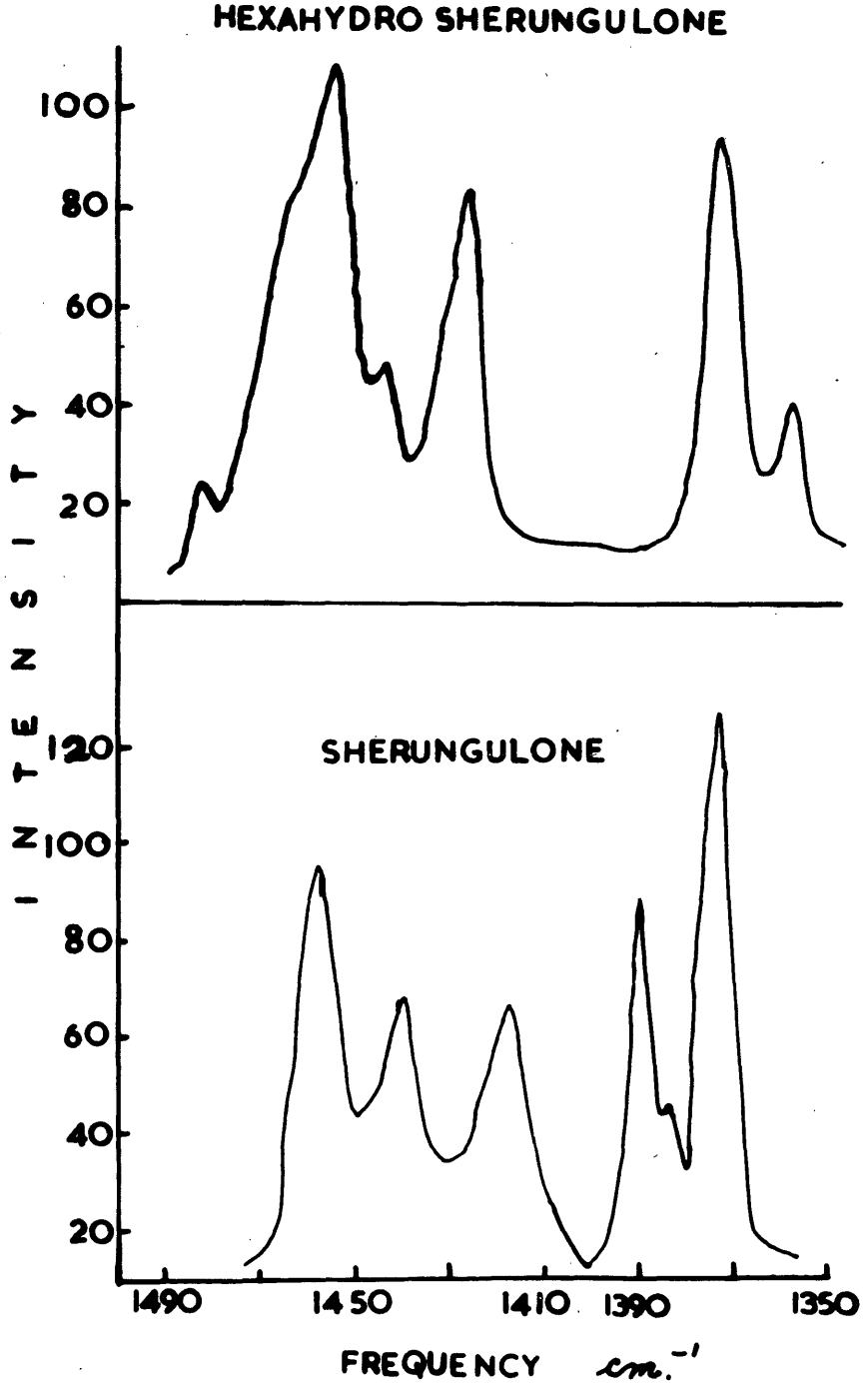


FIG. 1

Infra red spectra over the 1360 - 1530 cm.$^{-1}$ region.

TABLE II.

Mass Spectra.

Sherungulone.			Hexahydrosherungulone.		
		Groups removed			Groups removed
230	P		236	P	
215	P-15	CH_3	221	P-15	CH_3
197	P-33	CH_3, H_2O	205	P-31	CH_3O,
187	P-43	C_3H_7, C_2H_3O, (CH_3, CO)	203	P-33	CH_3, H_2O
159	P-71	C_5H_{11}, C_4H_7O $C_3H_3O_2$	177	P-59	(CH_3, C_3H_7); (CH_3, C_2H_3O)
145	P-85	C_5H_9O, $C_4H_5O_2$	138	P-98	C_7H_{14}, $C_6H_{10}O$
119	P-111	C_8H_{15}; $C_7H_{11}O$	99	P-137	
108	P-122		97	P-139	
95	P-135		83	P-153	
91	P-139		81	P-155	
83	P-147		69	P-167	
69	P-161		55	P-181	
55	P-175				

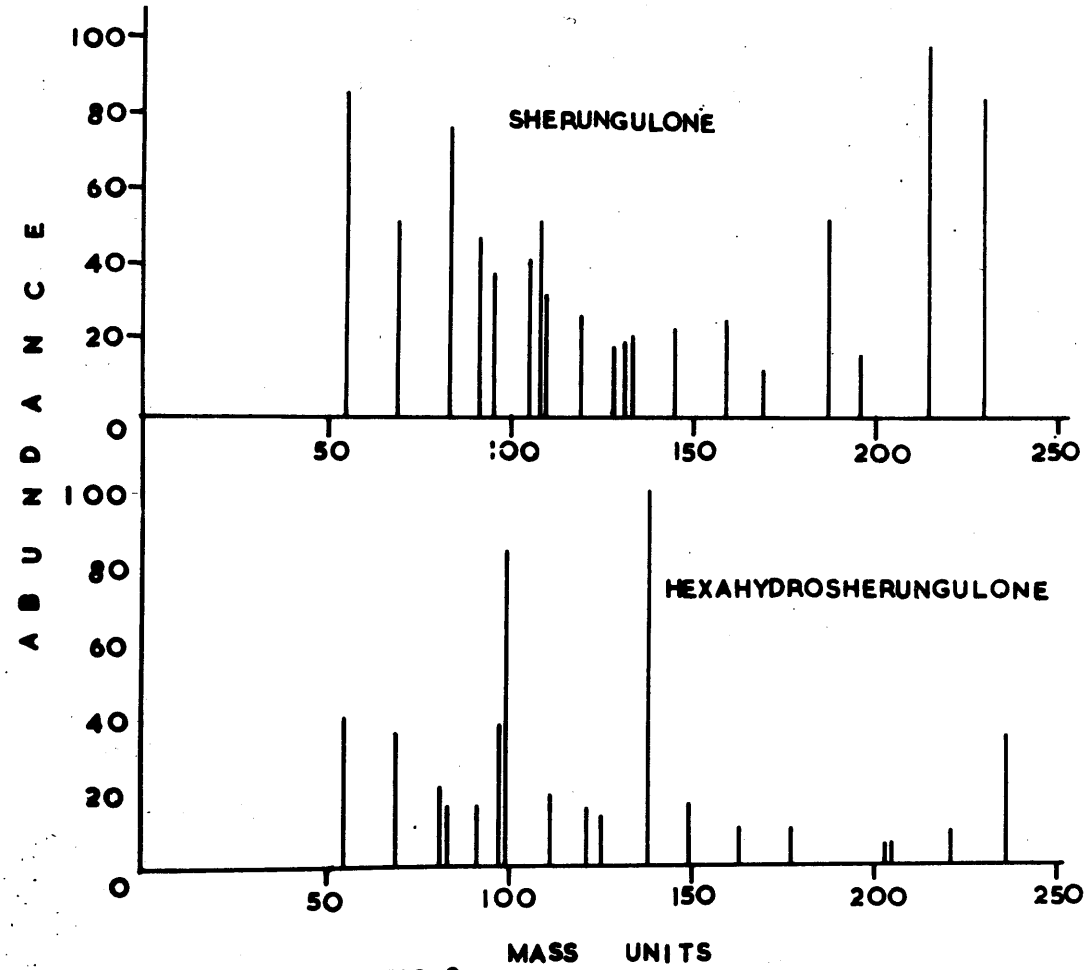


FIG 2

Histogram representation of the major peaks in the mass spectra. The peaks below 50 mass units are not included.

TABLE III.

N.M.R. Spectra.

Sherungulone.		Hexahydrosherungulone.	
—	Assignment	—	Assignment.
9.14		9.12)	2 CH_3's split;
8.98	1 ang CH_3	9.08)	possibly
		9.03)	CH_3CH
8.81)	CH_3CH	8.97)	
8.69)	or		
)	$R_3CH_2-R_3$	8.85	1 ang. CH_3
8.25			
		8.79)	
8.09)	1 CH_3	8.66)	$-CH_2$
8.05)	|	8.62)	
	C = C	8.53)	
7.79		8.39)	
7.67		8.32)	
		8.21)	
7.40)	CH_2CO and	8.18)	
7.34)		8.07	
		7.97	
7.17		7.90	
		7.84	
4.24)			
4.20)		7.64)	$-CH_2-CO$
	3 protons	7.54)	
4.10)			
4.04)	H H	6.90)	
	| |	6.78)	
	C = C split by	6.74)	CH
3.47)		6.61)	H-C-O-
3.42)	$-CH = CCH_3-$	6.40)	CH
		6.26)	
3.30)		6.10)	
3.27)		6.07)	
		6.04)	
3.04			

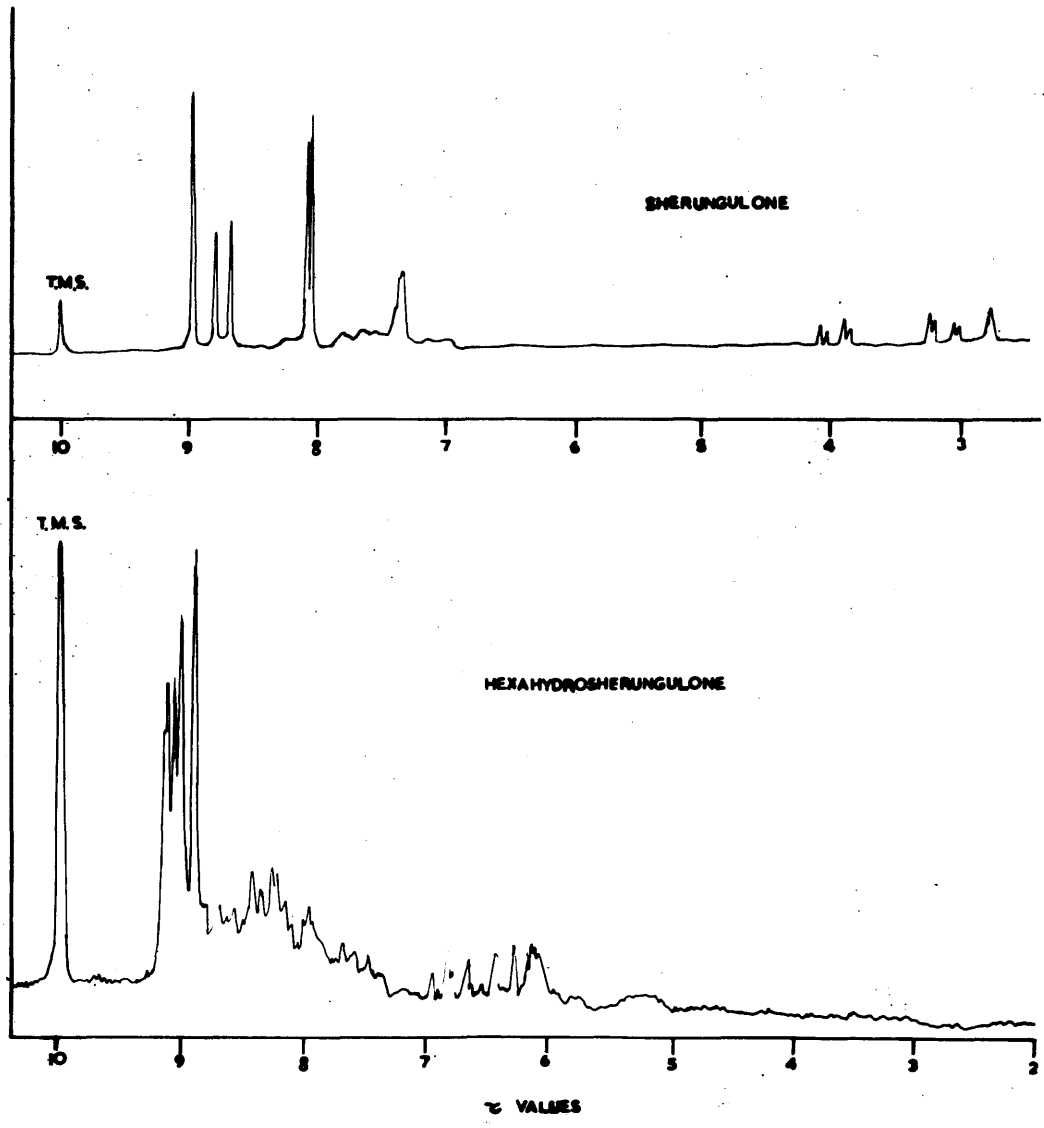


FIG. 3

Nuclear magnetic resonance spectra with tetramethylsilane (T.M.S.) as marker.

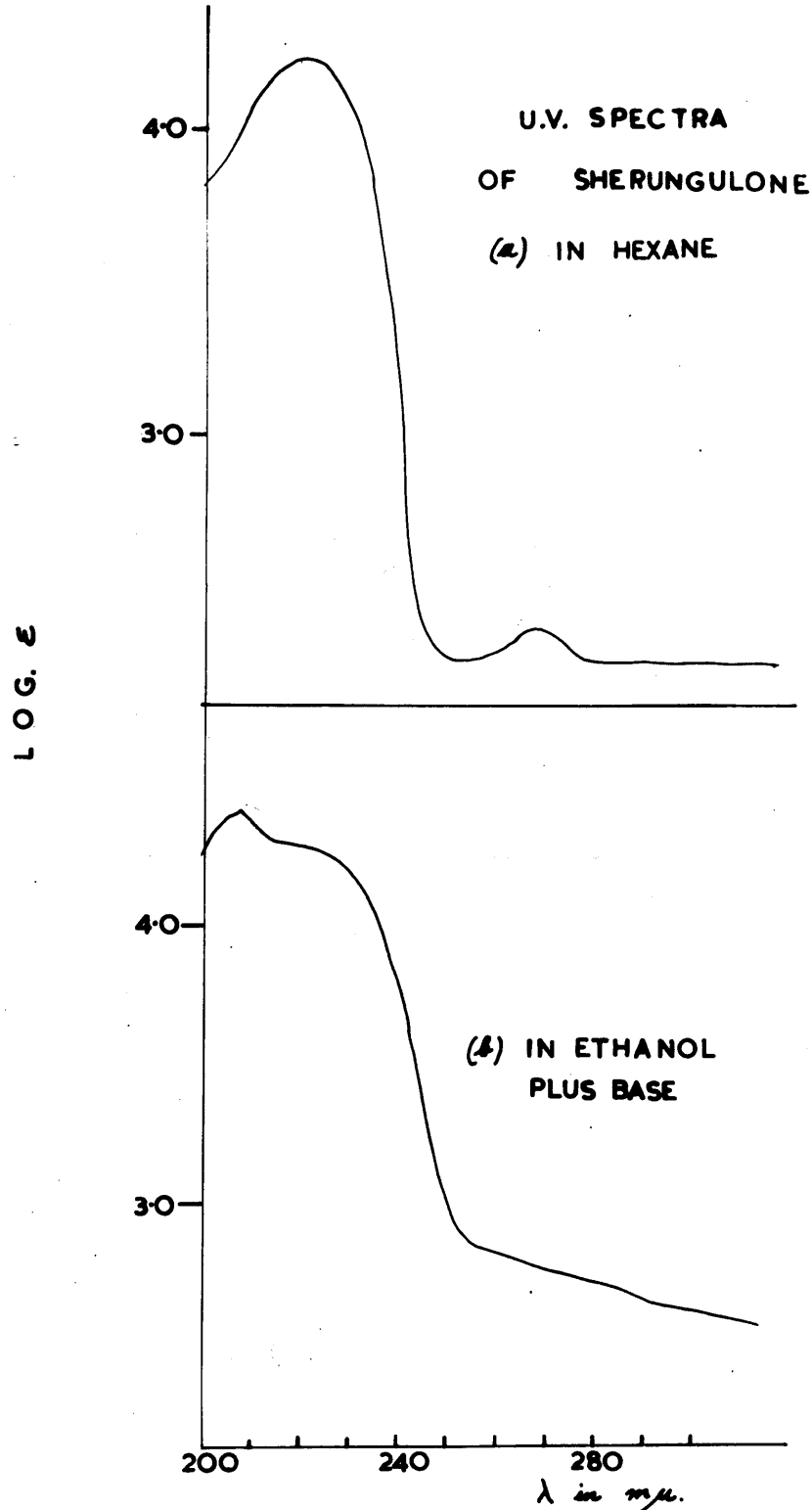

U.V. SPECTRA
OF SHERUNGULONE
(a) IN HEXANE
4·0
3·0
LOG. ε
4·0
3·0
(b) IN ETHANOL
PLUS BASE
200
240
280
λ in mμ.

FIG. 4

TABLE IV.

Literature Values.

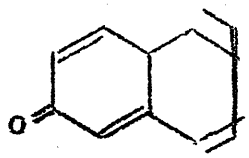
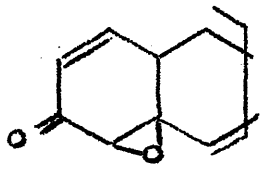
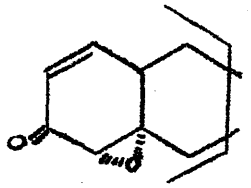
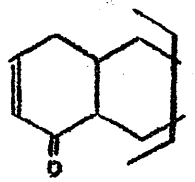
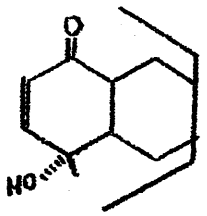
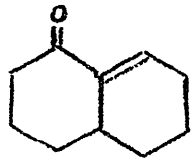
		mu	$cm.^{-1}$	Ref.
P		243,		8
Q		232,	1690, 1610	9
R		227,(10,470)	1677, 1610	9
S		225,(8,350);	1680	
T		216,	1680	10,11
U		243,		12

TABLE IV. (Cont'd)

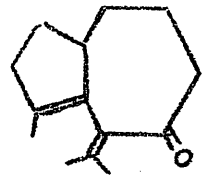
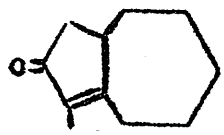
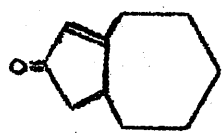
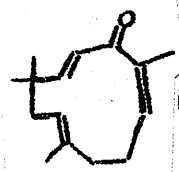
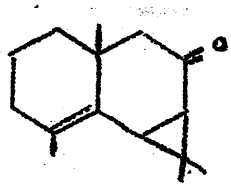
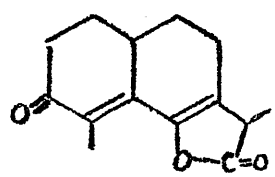
		mμ	$cm.^{-1}$	Ref.
V		245,(8,200);	1680, 1591	13
W		239,	1682	14
X		219	1695	15
Y		(shoulder)253,(8,000) 248,(8,480)	1662 1650(shoulder)	5
Z		212,		2
Z(a)		270,(21,000)		8
Hexahydrosherungulone		273,	1712	

TABLE IV. (Cont'd)

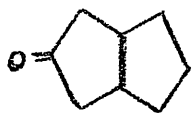
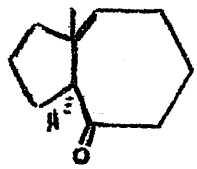
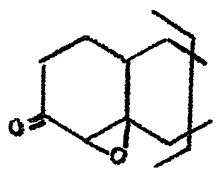
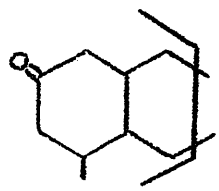
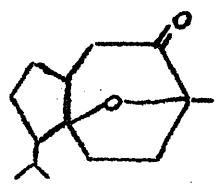
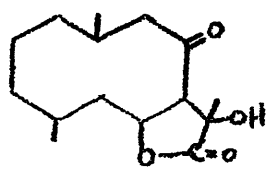
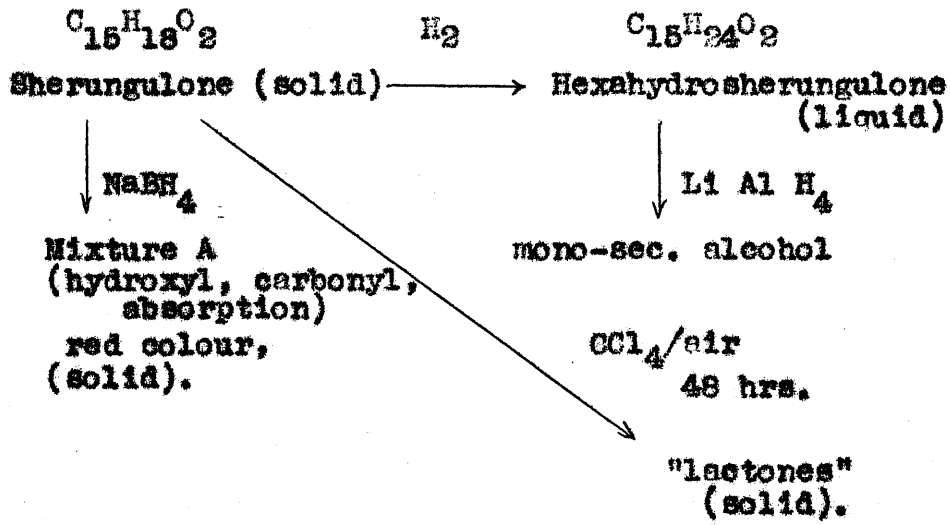
		mu	$cm.^{-1}$	Ref.
Z(b)		-	1742	16
Z(c)		-	1692	9
Z(d)		210(3,090)	1700	9, 8
Z(e)		-	1715	6
Z(f)		-	1719	16
Z(g)		-	1700	17
Z(h)		-	1700	3

TABLE V.


$C_{15}H_{18}O_2$
Sherungulone (solid)
H_2
$C_{15}H_{24}O_2$
Hexahydrosherungulone (liquid)
$NaBH_4$
Mixture A (hydroxyl, carbonyl, absorption) red colour, (solid).
Li Al H_4
mono-sec. alcohol
CCl_4/air 48 hrs.
"lactones" (solid).

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