V O L A T I L E C H E M I C A L S

O F T H E A N T

M Y R M I C A R U B R A

by

LESTER J. WADHAMS

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C O N T E N T S

INTRODUCTION

INTRODUCTION

As early as the 18th century it was recognised by Bonnet[1] that some ants use odour trails as a means of recruitment to food. He showed that by drawing a finger over the ground in front of returning ants he was able to impede their travel and only after a period of some minutes did they cross over the place and, apparently by chance regain the trail. This disturbance is not due to the smell of fingers, for the same result can be obtained by wiping the ground in front of the ants with cotton wool. Bonnet concluded that the power of the trail consisted of an odour deposited by the ants themselves and that odour was removed by the finger or cotton wool. Bethe[2] suggested that this odour trail was laid down automatically by the feet of the ants. Forrel[3] showed that the ability of the ants to follow the odour trail depended on their antennae. He also noticed that the antennae were well adapted, like a pair of callipers, for the estimation of shape and he suggested that the ants possessed a composite sense of shape and odour whereby they perceived the trail as an odour form.

However, interruption experiments only show that the ants will not cross clear ground and does not show what was on the ground or how the ants use it. Later investigators were able to show that the tracks of worker ants recruited to the food source are nearly the same as the track of the scout back to the nest. That the scouts do not lead the recruits to the food can be shown by capturing the scout as soon as recruits start to

leave the nest. The path of the recruits to the food still approximates to the path taken by the returning scout[4,5]. Returning foragers of Tapinoma nigerrimum press the tips of their gasters to the ground and smear the secretions of glands on the ground[6]. The smears made in a similar manner by Lasius fuliginosus can be visualised by dusting with lycopodium powder[7]. Goetsch[8] was able to establish artificial trails by drawing the tip of the gaster of a freshly killed worker along the ground. These early experiments showed that ant trails are due to chemicals deposited on the ground by the ants themselves.

In the past twenty years there has been considerable interest in organic compounds that are used to carry messages between individuals in nature. Regnier[9] has recently proposed the name semiochemical for these chemical signal vehicles that carry information between organisms. These semiochemicals are clearly different from hormones and nucleic acids, since the latter two are used for communication within an organism. Semiochemical communication can occur between members of the same species or between different species. Those compounds that carry signals between members of the same species, intraspecific communication, have been called pheromones[10,11]. Pheromones are of two types, releasers and primers. The two are easily differentiated on the basis of the behaviour of the receiver. The response of an organism on reception of a primer pheromone is delayed. Primer pheromones often induce physiological responses in an organism which ultimately manifest themselves in a behavioural response sometime after the initial pheromone perception. The control of gyne production by worker

ants in response to a queen producing "queen substance" is an example of the action of a primer pheromone. On the other hand, when a releaser pheromone is detected by an organism an immediate behavioural response occurs. An example would be a sex attractant produced by a female insect which upon reception by a mature male releases sexual behaviour.

The rapid exploitation of newly discovered food sources is important for social insects and is assured by the evolution of efficient recruiting systems. In bees, the returning forager that has found a good food source, communicates the quality, distance and direction of the food from the hive by means of a dance performed on the interior of the hive[12]. Fine navigational directions around the food source are achieved by the forager marking the spot with the contents of the Nasanoff gland[13].

The terrestrial social insects such as termites[14,15] and ants have also evolved recruiting systems based on chemical signals. In both cases recruitment to food sources is achieved by an odour trail laid from food source to the nest by the returning forager.

It is probable that odour trail recruitment evolved through a stage where one ant led another directly to the food source. This type of behaviour still exists in some species of Camponotus[5,16]. When Monomorium pharaonis workers are evenly spaced out along a trail there is no preference shown by ants arriving at a branch relative to the fork taken by the ant immediately preceeding them. However, workers which are following very closely behind another worker are likely to follow it into

the branch that it chooses[17]. This seems to be a remnant of the original tandem running behaviour where one ant led another directly to the food, though in most species there has been an evolution in behaviour so that the distance between ants on the trail can be quite large.

The mechanism of trail laying and the glandular origin of the trail pheromone varies considerably (Fig.1). In the Myrmicinae, where the sting is well developed, trails can be made from the venom gland itself, as for example in the Attini[18] and in Tetramorium guineense[19]. In other members of this subfamily the Dufour gland is implicated. Wilson[20] has shown that foragers of the imported fire ant, Solenopsis saevissima, lay trails by extruding their stings and depositing the material from the Dufour gland on the ground. Hangartner[21] later showed that the related species, Solenopsis geminata, is able to communicate food quality by the type of trail a forager lays. This is done by controlling the sting pressure on the ground and presumably the amount of pheromone deposited. In the Dolichoderinae, which have no sting, Pavan's gland is implicated[22]. The Formicinae, which are also stingless, produce trail substance from their hindgut. Thus in Lasius fuliginosus the trail consists of extruded droplets of hindgut material smeared out by the tip of the gaster[23,24]. Acanthomyops interjectus tends to withdraw its gaster immediately after the deposition of each droplet of hindgut material[25]. The trail of Acanthomyops interjectus therefore, consists of odour spots rather than a continuous streak, which means that it provides less uninterrupted directional information than that of Lasius fuliginosus.

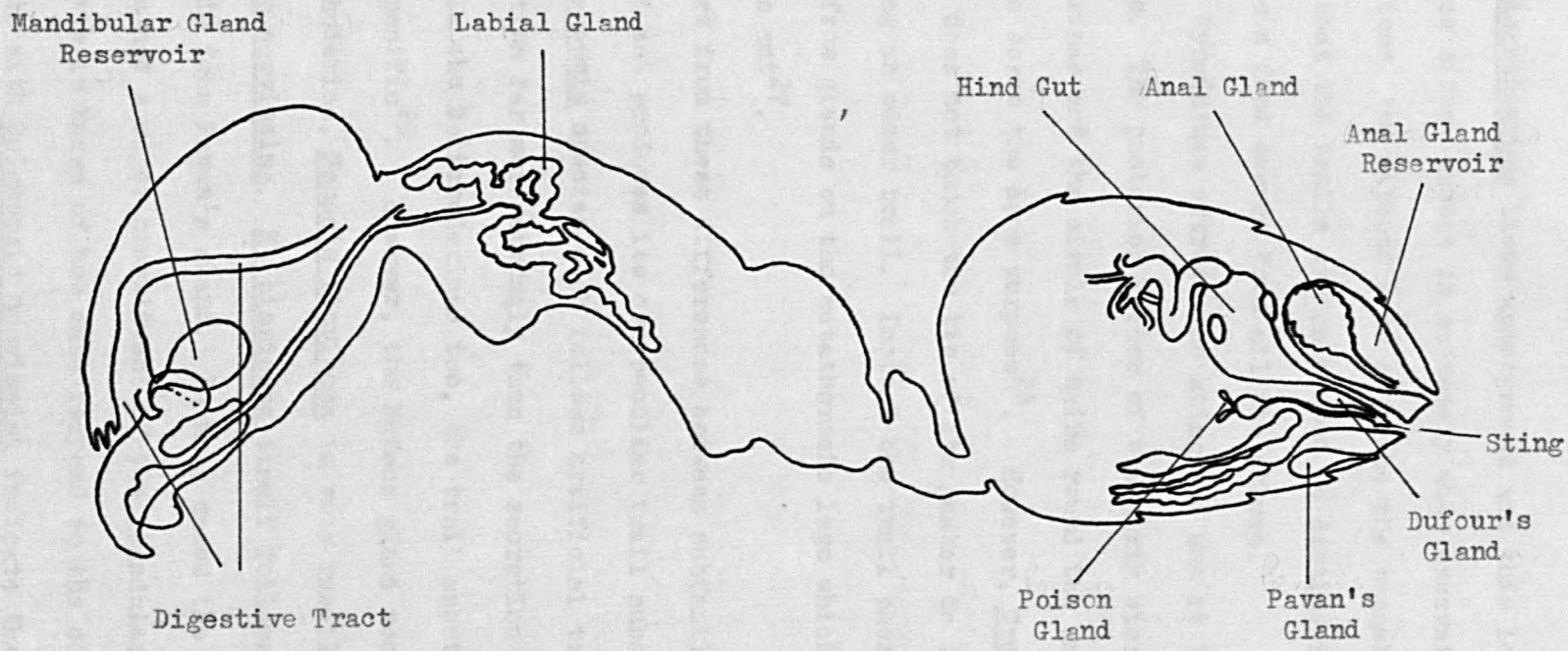


Fig.1 Part of the exocrine system of a worker ant

However, Acanthomyops lives underground and this loss is of little consequence since, except in extremely wide excavations or ramifications, the direction is given by the tunnel and it is probable that the trails are only used to distinguish the tunnels leading to a food source from all the others.

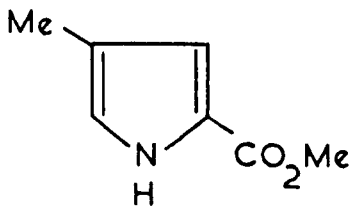
The Myrmicinae extrude the sting and use it like a pen to lay trails. The posterior border of the sixth sternite in the Dolichoderinae and the circle of hairs round the anus in the Formicinae serve the same purpose[26]. However, Crematogaster ashmaedia does not touch the tip of its gaster to the ground when laying an odour trail. Instead the trail pheromone is released from glands on the metathoracic legs which are trailed behind the ant[27].

Apart from these differences between subfamilies each species of ant produces its own peculiar trail substance. Of three Solenopsis species each follows artificial trails of its own secretion far more strongly than the secretion of the other two[28]. In the Dolichoderinae too, the trail substances are species specific[29]. However, the Dufour gland secretion from the dolichoderine, Monacis bispinosa is very readily followed by Solenopsis saevissima. M. bispinosa itself follows artificial trails made from Pavan's gland. In many cases the scent the ant follows is not a major constituent of the glandular secretion[30].

Little is known of how ants respond to the odour trail. Experiments with Solenopsis saevissima indicate that the trail exists in the air rather than on the ground[31]. The trail left by the returning forager evaporates and diffuses outwards in the air. The ant is only able to detect the pheromone when its concentration

is above a certain threshold value. Thus the trail is a sausage shaped portion of space, the dimensions of which are dependent on the amount of pheromone released by the ant, on its rate of diffusion and its threshold concentration. It is probable that once the ant has entered the scented sausage shape, it remains there by turning back whenever it passes through the threshold concentration at the skin on the sausage. This would account for the characteristic weaving behaviour shown by M. rubra workers when they follow a trail.

At the present time only one ant trail pheromone has been identified. The trail of the town ant, Atta texana, consists of at least two components, one of which is volatile and the other nonvolatile[32]. A methylene chloride extract of homogenised worker poison sacks was used to lay an artificial odour trail on a cardboard sheet. This still showed activity after two hours and even after four months at 0.4 mm Hg the artificial trail still elicited trail following behaviour. The volatile component has been identified as methyl 4-methylpyrrole-2-carboxylate (I). The detection threshold is 0.08 pg/cm and it is claimed only 0.33 mg of this material would be sufficient to lay a trail round the world[33].



I

Dufour's gland in myrmicine ants is a uniform, tube-like sack running onto the base of the sting beside the duct from the poison vesicle. The morphology of the sting apparatus in

this subfamily has been investigated in Solenopsis saevissima[34] and Pogonomyrmex badius[35]. In both these species there are special muscles that can close the poison vesicle so that the contents of the Dufour gland can be emitted separately. In Myrmecia gulosa it has been shown that the contents of the poison vesicle can be emitted separately from the Dufour gland[36]. Similarly in the pseudomyrmecine, Pseudomyrmex pallidus, examination of the poison apparatus after the proteinaceous venom had been ejected, showed the venom sacks to be collapsed, but in every case the Dufour gland was distended[37].

In the stingless ants of the subfamily Formicinae, the Dufour gland and poison vesicle have only short ducts and open close to each other. No muscles have been described which would allow the contents of each gland to be emitted separately and it is not known whether the Dufour gland secretion can be ejected separately or if it can only be ejected together with the contents of the poison vesicle.

The Dufour gland secretion can serve as a releaser of alarm behaviour, a trail pheromone or both. Wilson has demonstrated that in Solenopsis saevissima the Dufour gland secretion contains a trail pheromone[20]. In Pogonomyrmex badius, on the other hand, the material from the Dufour gland serves as a releaser of alarm behaviour[38,39]. Maschwitz has reported that species of Tetramorium, Messor, Pheidole and Myrmica possess abdominal alarm substances produced in the poison apparatus[40]. These species also produce alarm pheromones from their mandibular glands. A multiple securing of the alarm function can also be found in Formica (F. polyctena, F. cinerea and F. fusca). Mandibular gland secretions as well as Dufour

gland and venom gland contents alarm the workers. In general the alarm substances of the social Hymenoptera are associated with the organs and glands of defence.

Unlike trail pheromones, alarm substances are not species specific. Thus in Myrmica the venom of M. laevonodis, M. sulcinodis and M. rubida stimulates alarm in M. ruginodis and vice versa[40]. Similarly the mandibular gland alarm substances in Myrmica may be cross effective. The mandibular glands of M. brevinodis contain 3-octanone and 3-octanol as major components, as well as smaller amounts of 6-methyl-3-octanone and 3-nonanone[41]. All four of these compounds are capable of releasing alarm behaviour in this species. Among eight myrmecine species the ratios of 3-octanone and 3-octanol vary from 1.2:1 to 12:1 which would indicate that each species may have its own private blend of alarm pheromones. Thus it is possible that a species possesses high olfactory sensitivity to its own alarm pheromone because the releaser actually consists of a mixture of compounds to which the insect responds most efficiently.

Little is known of the chemical composition of the Dufour gland secretion. In the formicine, Acanthomyops claviger, Regnier and Wilson[42] have reported that the Dufour gland contains the normal hydrocarbons n-undecane, n-tridecane and n-pentadecane, as well as the ketones 2-tridecanone and 2-pentadecanone. In accordance with Maschwitz's[40] observations they were able to show that n-undecane acts as a releaser of alarm behaviour. They also demonstrated that the contents of both Dufour's gland and the venom gland are emitted together and suggested that n-undecane, the principle component of the Dufour gland, acts as a spreading agent for formic acid.

Cavill and Williams[36] reported that gas chromatography of the Dufour gland contents of the myrmecine, Myrmecia gulosa, gave twelve peaks, the major component being cis-8-heptadecene, together with smaller amounts of the normal hydrocarbons tetra-, penta-, hexa- and heptadecane. The same authors also reported on preliminary investigations of Dufour gland secretions in one ponerine, Amblyopone australis, one myrmecine, Podomyrma gratiosa and two formicine ants, Camponotus species, which also showed the presence of aliphatic hydrocarbons. However, in another myrmecine, Aphaenogaster longiceps, the Dufour gland contains only one component - an isomer of the sesquiterpene α-farnesene[43]. This material was identical with a sample of α-farnesene isolated from the natural coating of Granny Smith apples. Subsequent investigations showed this to be trans,trans- α-farnesene[44].

An isomer of α-farnesene has also been reported in the Dufour glands of the slave keeping ants, Formica sanguinea and Polyergus rufescens, in which it was present as a major component, as well as in one of their slaves Formica fusca[45]. The same farnesene isomer is also present in another formicine, Camponotus ligniperda[46]. The corresponding acetate, farnesyl acetate, has also been reported in this species. The same authors also examined the Dufour gland secretions of several other formicine ants. The main components of the Dufour gland secretion in the nine formicine species comprise mainly acetates, alkanones and hydrocarbons. The major components of the secretion of Lasius flavus and L. carniolicus are 2-tridecanone and 2-pentadecanone. Of the normal hydrocarbons only n-undecane was consistently present in the Dufour gland and in Camponotus ligniperda it was again shown that this compound elicited strong

alarm behaviour. This is in accordance with the observations of Maschwitz[40] and Regnier and Wilson[42], though surprisingly, n-tridecane which is a major component of the Dufour gland secretion in this ant, showed no alarm pheromone activity.

DISCUSSION

DISCUSSION

It has been known for a long time that the social insects use chemicals as a means of communication. However, only recently has it been possible to isolate and identify these chemical signal vehicles. In the past twenty years considerable interest has been shown in these pheromones and many have now been isolated, identified and synthesised.

When this work was initiated no ant trail pheromone had been identified. The project began as an investigation into the chemical nature of a trail substance, using the ant Myrmica rubra since this species was readily available in Britain and colonies could be maintained in the laboratory. Eidmann[4] had already showed that Myrmica rubra used odour trails as a means of recruitment to food.

Although this ant is common in England, difficulty was experienced in obtaining a ready supply and it was not until July 1970 that an abundant supply of M. rubra was found close to the University.

Initial attempts to extract the active material from homogenised whole ants gave an extract that showed no trail pheromone activity on bioassay. Either the active components were not extracted or, more likely, their activity was masked by other chemicals. In the subfamily Myrmicinae the trail pheromone is produced in the poison apparatus and the odour trail is laid by the ant extruding its sting and depositing the secretion from the glands on the ground in the form of a streak[20,21]. A pentane extract of homogenised Dufour glands and poison vesicles gave a strong positive response on bioassay, with one ant following the circle for 17½ minutes.

However, investigation of this extract by gas chromatography,

both preparative and analytical, led us to believe that the trail pheromone was a minor component of the glandular secretion. At this stage the supply of ants was strictly limited and it was felt that they were insufficient to continue work on the trail pheromone. Instead, it was decided to concentrate on the volatile components of the Dufour gland and poison vesicle and to return to the trail pheromone when the supply of ants permitted.

The greater part of the identification and separation of the volatile components of the glandular secretion was achieved using the techniques of gas chromatography and combined gas chromatography-mass sepctrometry.

Three main methods of extraction of the volatile materials from the poison apparatus were employed. Initial investigations were carried out on a pentane extract of homogenised glands. However, this method suffered from several disadvantages. The extraction was time consuming and on gas chromatography it was thought that the solvent peak obscurred several of the glandular components. Temperature programmed gas chromatography of this extract was not possible since at the high sensitivities employed there was excessive baseline drift.

In order to overcome these difficulties the solid injection method was developed. The technique consists of sealing individual glands in glass vials and injecting them directly onto the column by means of a solid sample injector. Using this technique we were able not only to remove the interference from the solvent but also to analyse even single glands at a much lower sensitivity. The technique was also most useful for combined gas chromatography-mass spectrometry and enabled mass spectrometric data of the glandular

components to be obtained using relatively small samples.

However, larger samples were required for preparative gas chromatography. In the third method the volatile materials were extracted from whole ants, or gasters and head separately, by distillation under reduced pressure. In order to separate the organic material from the large volume of water obtained during the distillation, the organic volatiles were trapped in activated charcoal and subsequently recovered by eluting the charcoal with carbon disulphide.

Several different columns were used in the investigation, the choice being dictated by the requirement of the experiment. Initial investigations were carried out using a 5% SE 30 column which is non-polar and separates predominately by molecular weight. It was found to be most useful for preliminary estimations of the volatile materials and also provided some information on the molecular weight range of the compounds being studied. Further separation of the glandular components was achieved using a more polar 10% polyethylene glycol adipate column which provided a good separation of alkanes and alkenes. The characteristics of this column are such that the alkanes are eluted before the alkenes. Comparison data for co-chromatography and log plots were obtained using a 15% Apiezon L column which again provided a good separation of the hydrocarbon components of the secretion. The characteristics of this column are the opposite of the polyethylene glycol adipate column in that the alkenes are eluted before the corresponding alkanes.

Combined gas chromatography-mass spectrometry was carried out using the analytical 10% polyethylene glycol adipate column since it achieved the best separation of the glandular components.

Gas chromatography of the homogenised pentane extract of two hundred Dufour glands and poison vesicles on the 10% polyethylene glycol adipate column showed twenty components (Fig.2), four of which constitute over 85% of the total volatile material in the glands (Table 1). However, gas chromatography of this extract on the 5% SE 30 column showed only three major peaks corresponding to compounds differing in chain length by two carbon atoms. To ensure that no volatile material was lost during the concentration of the pentane extract or components obscured by the solvent peak a single poison apparatus (Fig.1) was analysed on the 10% polyethylene glycol adipate column using the solid injection technique. The gas chromatographic trace showed the same twenty components (Fig.3) and in the same relative proportions to those obtained from the pentane extract of homogenised gland, together with several further components that had been obscured by the solvent peak. However, these components represented a very small fraction of the whole and were also present in the chromatographs obtained from the solid injection of mandibular glands. It is probable that one of these components is water, introduced during the disection of the glands, and that another is acetone. Under the conditions for combined gas chromatography-mass spectrometry the three major components in this area were not resolved, probably due to the large volume of water present in the samples. However mass spectra of the "solvent peak" showed strong ions at m/e 18 and m/e 58 corresponding to water and acetone. Gas chromatography of a mixture of acetone and water gave two peaks of retention time 0.4 mins and 0.2 mins respectively. Under the same conditions the retention times of the first two components of the glandular secretion were also 0.2 mins and 0.4 mins.

Figure 2

Gas chromatograph of the volatile material from a pentane extract of homogenised glands

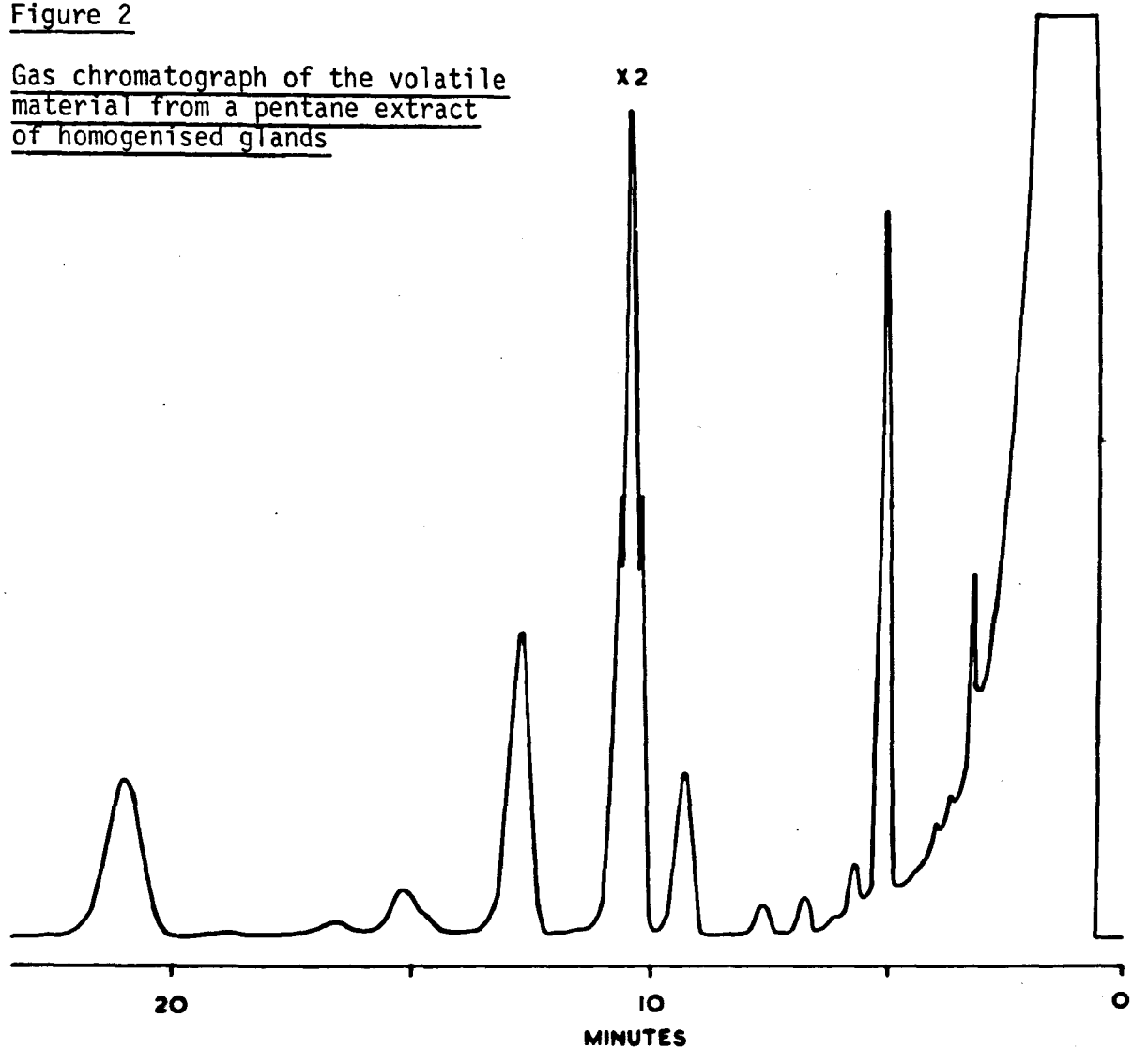


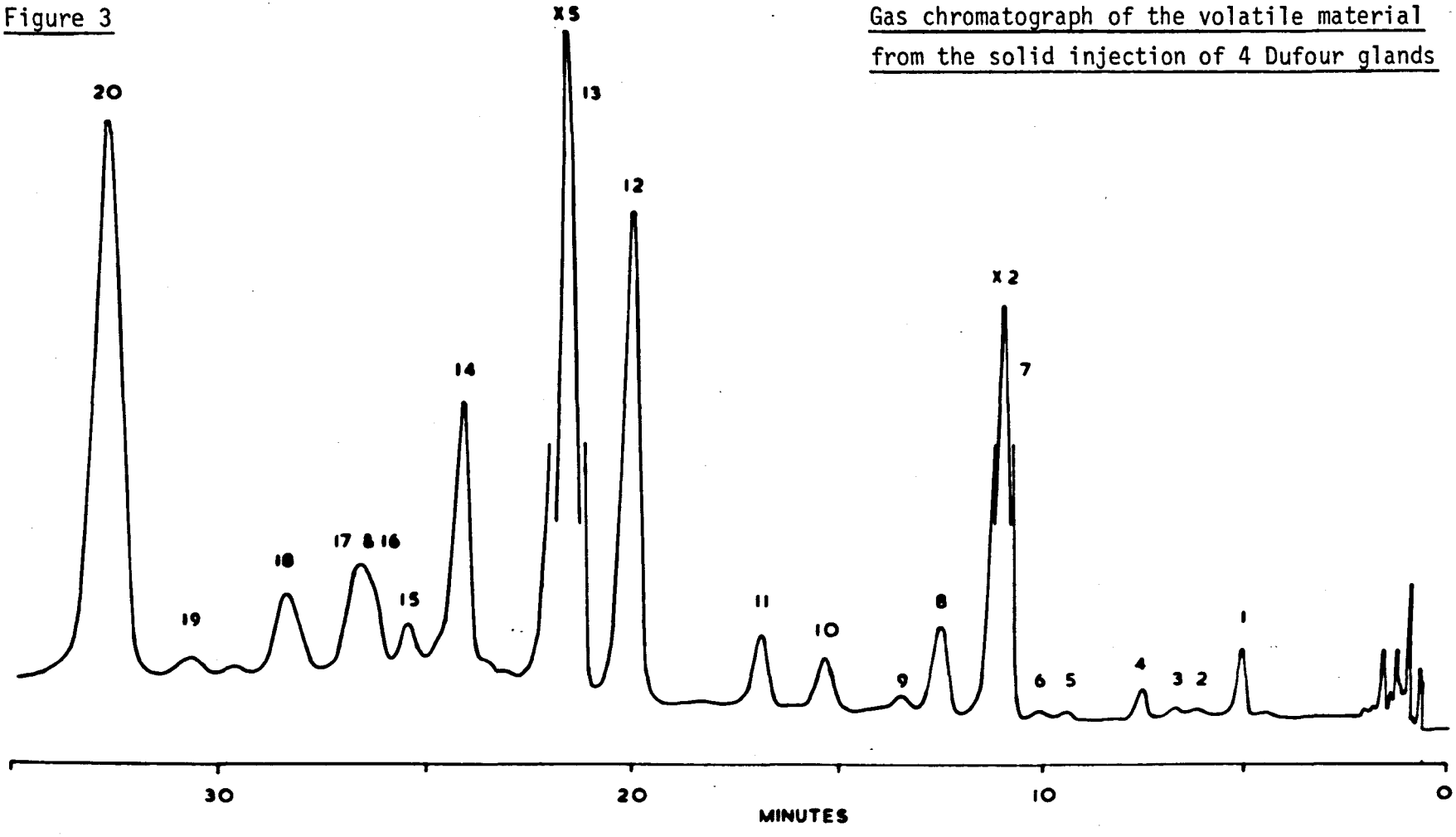
TABLE 1

Composition of Volatile Constituents of Dufour's Gland

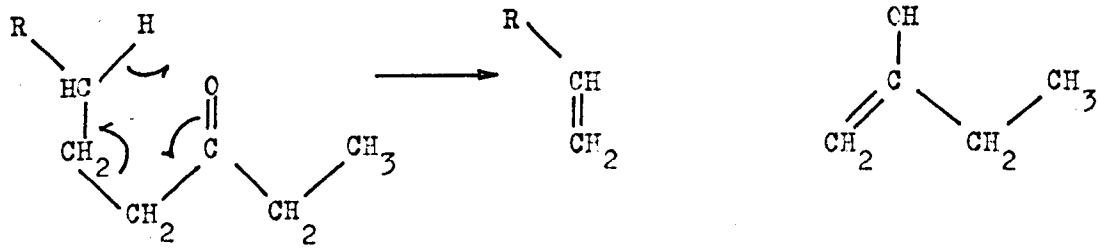
Peak No.	Compound	% Age
1	Tridecane	0.6
2		Trace
3		Trace
4	Tetradecane	0.3
5		0.2
6		Trace
7	Pentadecane	14.2
8	Pentadecene	2.1
9		Trace
10	Hexadecane	1.0
11	Hexadecene	1.0
12	Heptadecane	7.2
13	Cis-8-Heptadecene	53.3
14	Farnesene Heptadecadiene	4.5
15	Octadecane	Trace
16	Octadecene	0.8
17	Homofarnesene	0.8
18		1.6
19	Nonadecane	Trace
20	Cis-9-Nonadecene Bishomofarnesene	12.2

Figure 3

Gas chromatograph of the volatile material from the solid injection of 4 Dufour glands



Gas chromatography of the material obtained from the distillation of whole ants gave a gas chromatographic trace, which, except for the presence of two major peaks of short retention time, was similar to that obtained from the homogenised pentane extract and the solid injection of whole glands (Fig.4). These two additional components originate in the mandibular glands (Fig.5). Twenty worker heads were analysed, using the solid injection technique, by combined gas chromatography-mass spectrometry. The mass spectrum of peak A, (Fig.6) identified as 3-octanone, showed a molecular ion at m/e 128 and a fairly strong ion at m/e 72 corresponding to fragment (II) which is characteristic of a McLafferty rearrangement and indicating the presence of an ethyl ketone



II

Additional fragments at m/e 99 and m/e 57 arise by fragmentation on either side of the carbonyl group, whilst the peak at m/e 71 could arise by fragmentation at the carbonyl group with retention of charge by the larger alkyl fragment.

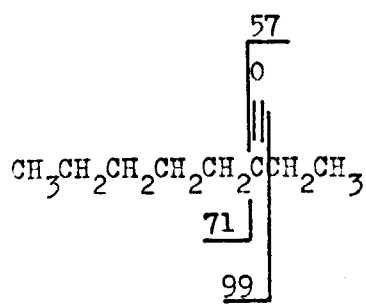


Figure 4

Gas chromatograph of the volatile material isolated by the distillation of whole ants

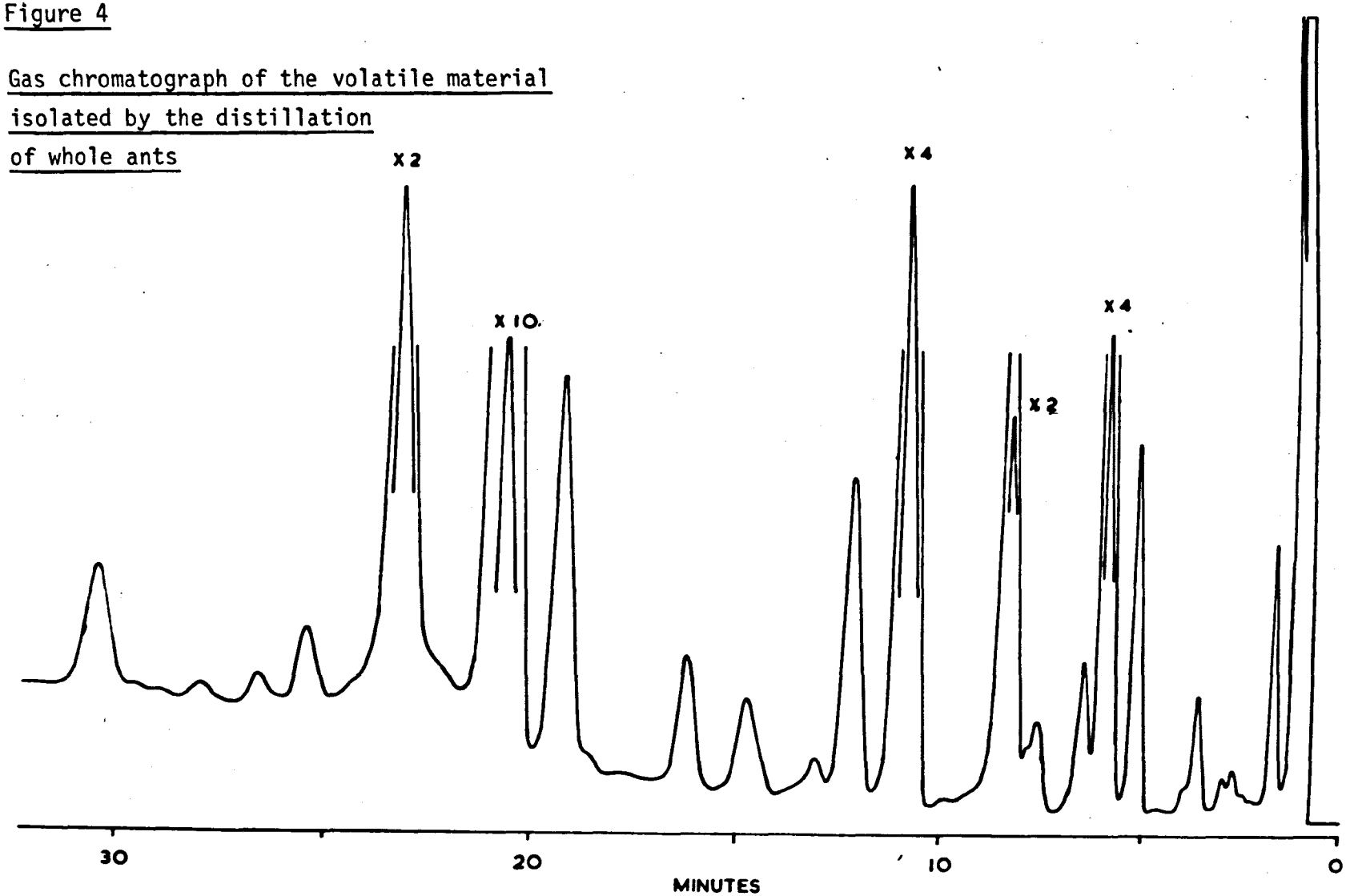


Figure 5

Gas chromatograph of the volatile material from the solid injection of a single mandibular gland

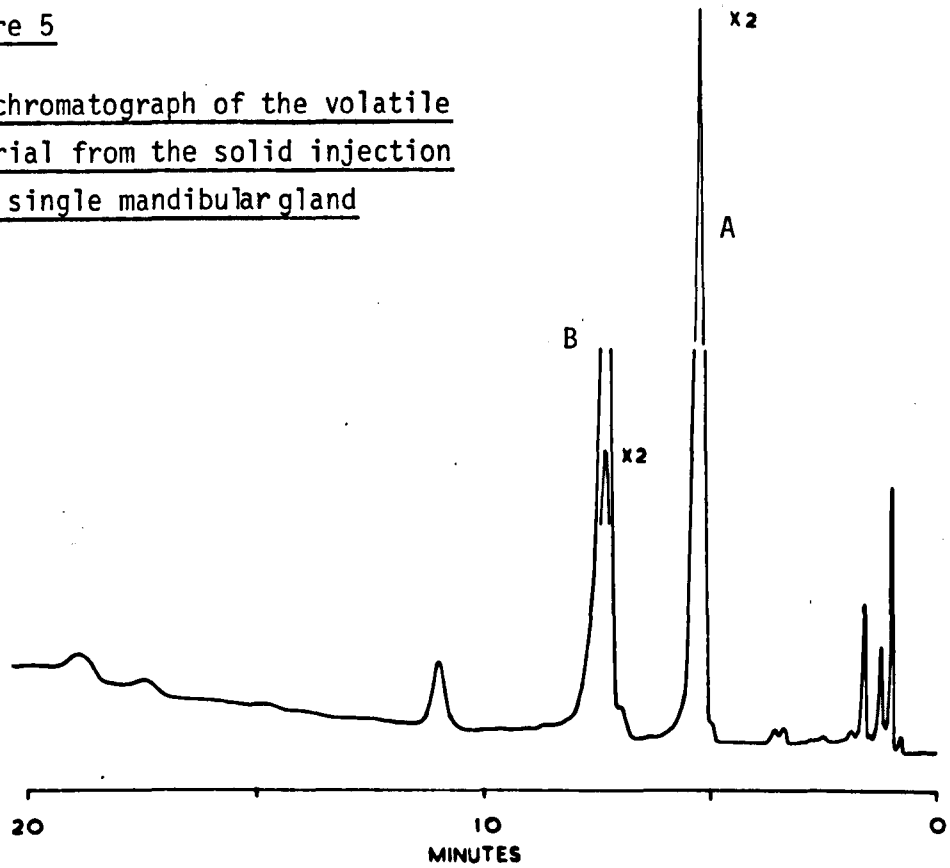


Figure 6

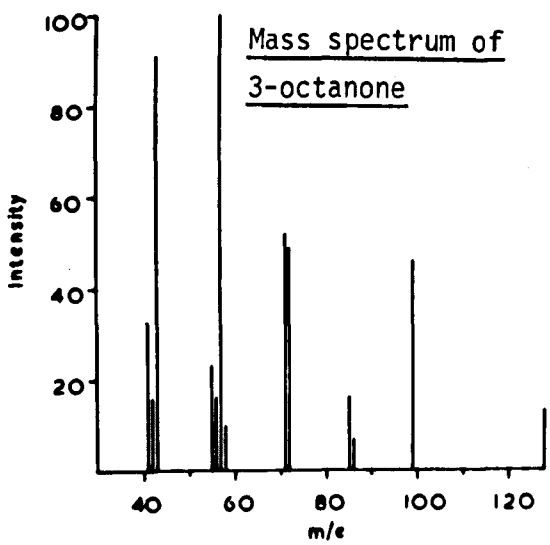
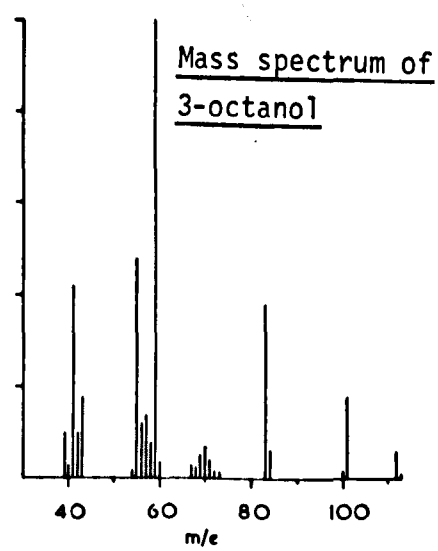


Figure 7



An authentic sample of 3-octanone, run under the same conditions, gave an identical mass spectrum. Co-chromatography of the authentic sample together with the material from the distillation of heads, on both the 10% polyethylene glycol adipate column and the 15% Apiezon L column, gave no new peaks but showed enhancement of peak A.

Peak B was identified as 3-octanol. The fragment of highest mass obtained in the mass spectrum (Fig.7) of this component was at m/e 112, but the gas chromatographic retention time and the slight tailing of the peak indicate a polar compound, possibly an alcohol. If this compound were the alcohol corresponding to the ketone A, then the peak at m/e 112 would correspond to M - 18 associated with the elimination of water from higher alcohols. Peaks at m/e 101 and m/e 59 could then arise by α-cleavage on either side of the carbon atom carrying the hydroxyl group. The mass spectrum of the 3-octanol isolated from the mandibular glands of M. rubra workers and queens was identical with the spectrum published by Crewe and Blum[41] who identified 3-octanone and 3-octanol in the mandibular glands of M. laevinodis (=rubra)

One hundred Dufour glands and poison vesicles were analysed by combined gas chromatography-mass spectrometry. Peaks 1,4,7 and 12 were identified as the normal alkanes tri-, tetra-, penta- and heptadecane. Their mass spectra (Figs.8,9,10,11) showed parent ion at m/e 184, 198, 212 and 240 respectively and the cracking patterns of straight chain alkanes characterised by groups of peaks 14 mass units apart, corresponding to the loss of methylene, whose relative intensity increased regularly with decreasing m/e ratio to the base peak at m/e 57. Authentic samples of n-pentadecane and n-heptadecane run under the same conditions gave identical mass spectra to those

Figure 8 Mass spectrum of n-tridecane

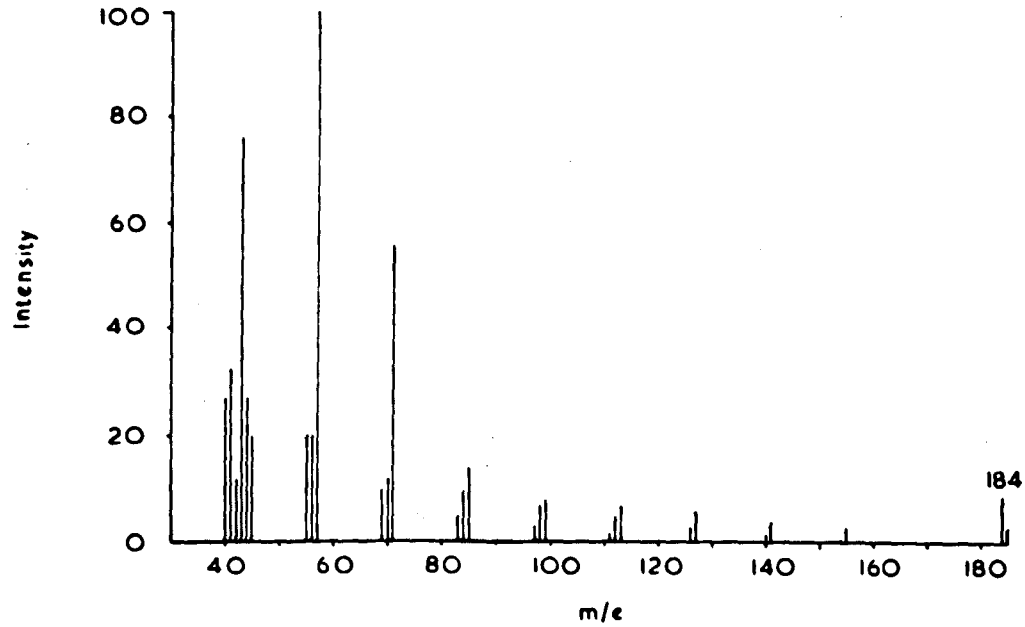


Figure 9 Mass spectrum of n-tetradecane

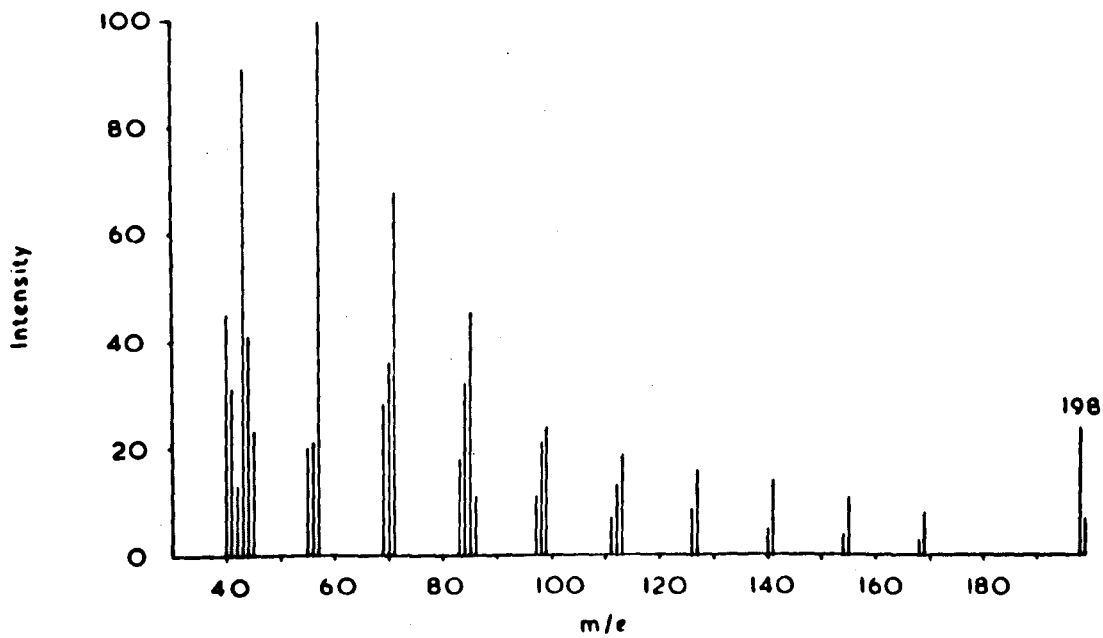


Figure 10 Mass spectrum of n-pentadecane

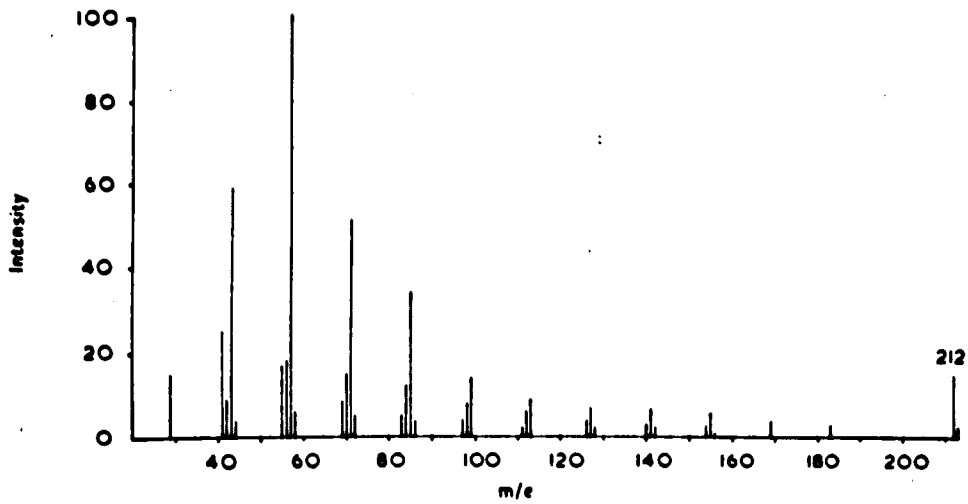
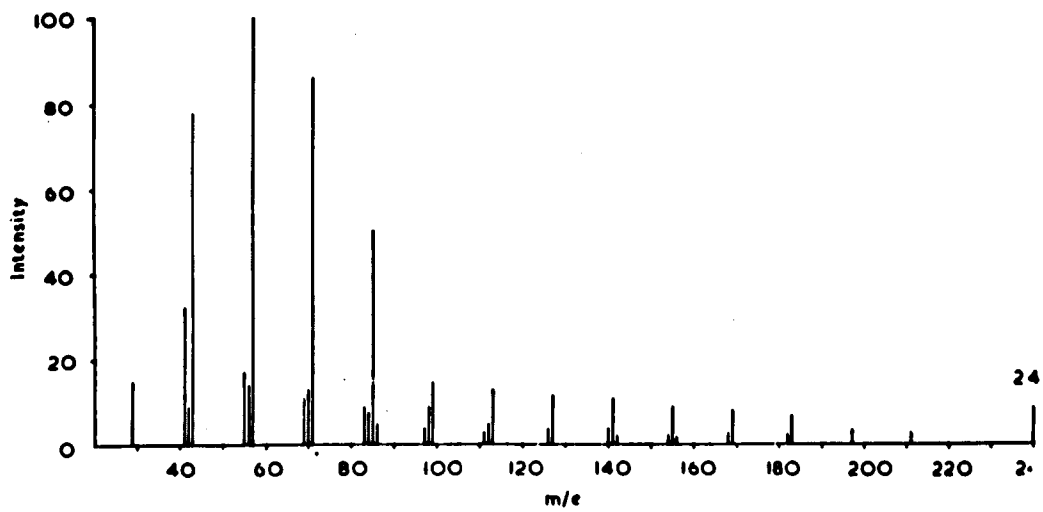


Figure 11 Mass spectrum of n-heptadecane

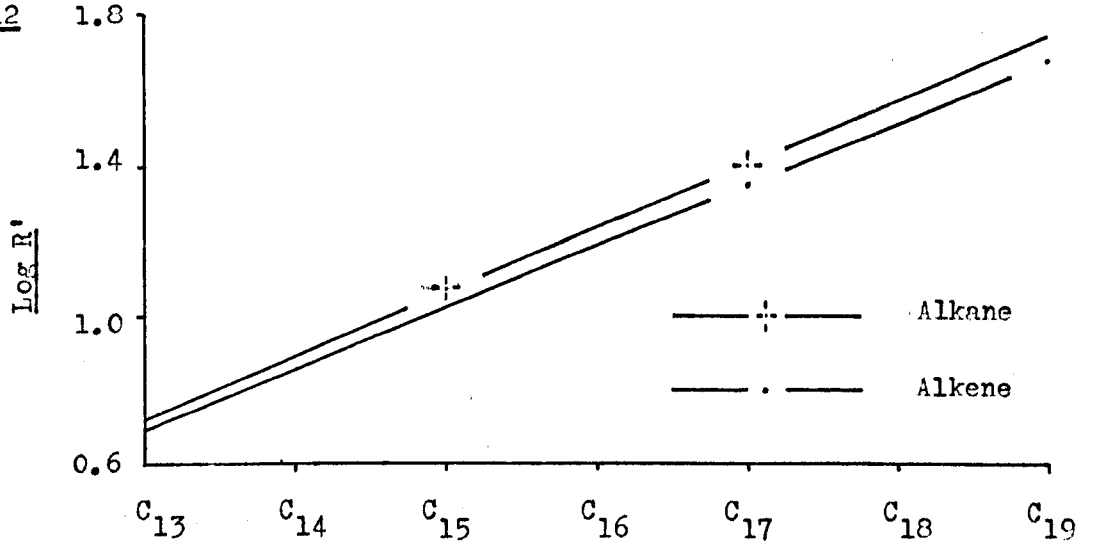


obtained from peaks 7 and 12. Co-chromatography of these authentic samples, on both the polyethylene glycol adipate and Apiezon L columns, together with the gland extract gave no new peaks but showed enhancement of peaks 7 and 12. A plot of the log of the retention time of the components against carbon chain length, on polyethylene glycol adipate and Apiezon L columns, using pentadecane and heptadecane as internal standards, showed peaks 10, 15 and 19 to be hexa-, octa- and nonadecane respectively (Figs.12,13).

As a further check on the assignment of the hydrocarbon components, three Dufour glands and poison vesicles were injected together with 1 µl of concentrated sulphuric acid, using the solid injection technique. Under these conditions the sulphuric acid will react with all compounds except alkanes and aromatic hydrocarbons. Since the reaction goes to completion the gas chromatographic trace should show the absence of all components except alkanes. Accordingly, the trace obtained on the polyethylene glycol adipate column showed the elimination of all except peaks 1, 4, 7, 10, 12, 15 and 19, corresponding to the alkanes tri-, tetra-, penta-, hexa, hepta-, octa- and nonadecane. No impairment of column efficiency by this treatment was noted and subsequent injection of samples were not affected by the concentrated sulphuric acid, though the glass wool plug on top of the column packing was replaced as a precautionary measure.

Similarly, the injection of glands together with 1 µl of bromine in carbon disulphide (1:1, v:v) again resulted in the elimination from the gas chromatographic trace of all peaks except those mentioned, together with the production of several new peaks presumably derived from the bromination products of low molecular

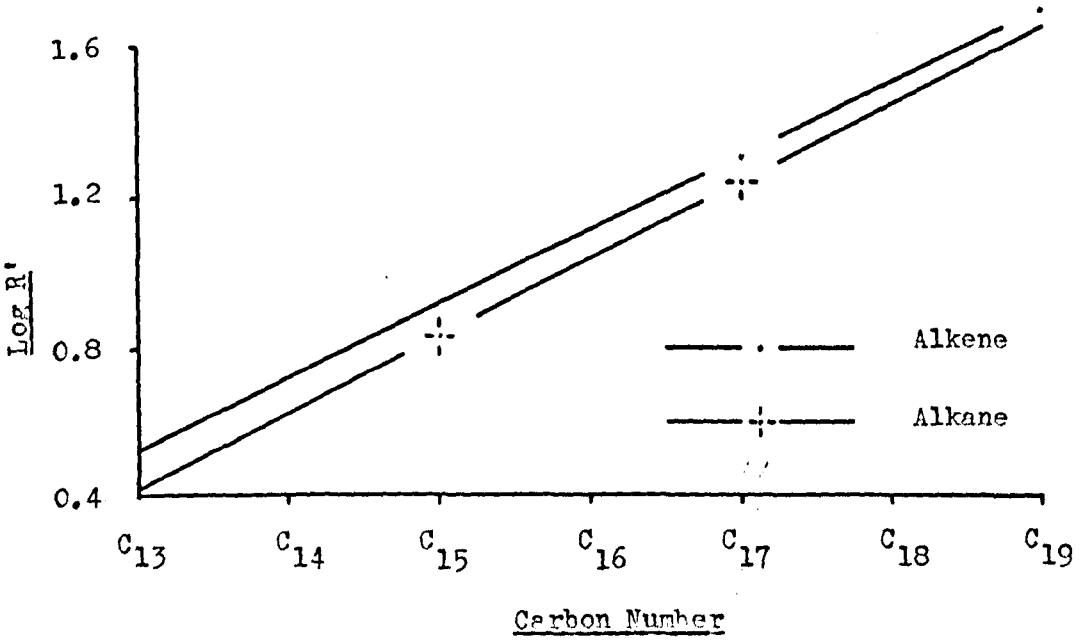
Fig.12



Column: - 20% Apiezon L.

Compound	Expected R' from Log Plot		R'
Tridecene	5.00	mins	5.00
Tridecane	5.25	"	5.25
Tetradecene	7.2	"	
Tetradecane	7.8	"	7.9
Pentadecene	10.5	"	10.6
* Pentadecane		Standard	
Hexadecene	15.3	"	15.6
Hexadecane	17.0	"	17.0
* Heptadecene		Standard	
* Heptadecane		Standard	
Octadecene	33.1	"	33.3
Octadecane	36.8	"	36.7
* Nonadecene		Standard	
Nonadecane	54.0	"	54.2

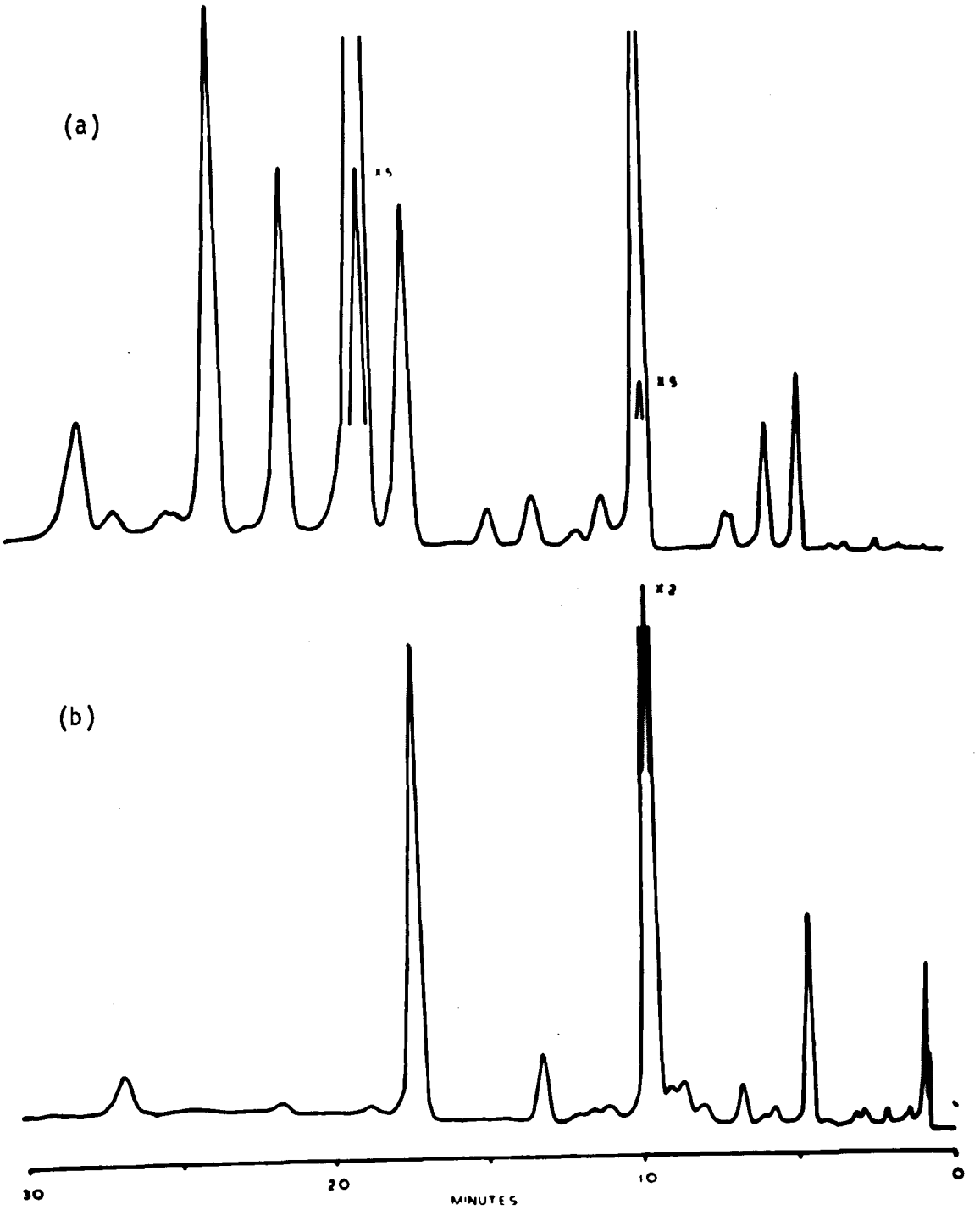
Fig. 13



Column:- 10% PEGA

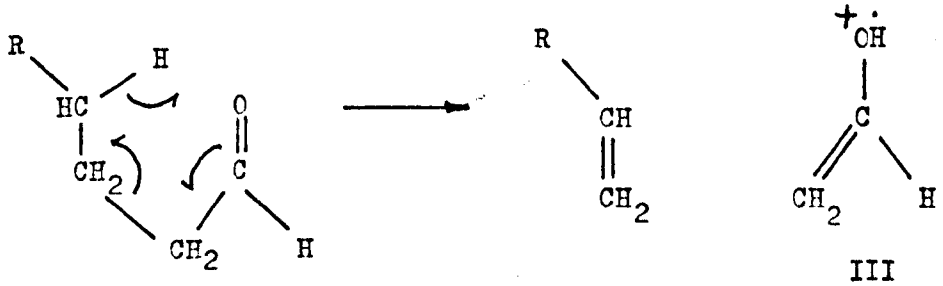
Compound	Expected R' from Log Plot	R'
Tridecane	2.6 mins	2.6 mins
Tridecene	3.4 "	
Tetradecane	4.3 "	4.3 "
Tetradecene	5.3 "	
* Pentadecane	Standard	
Pentadecene	8.3 "	8.3 "
Hexadecane	11.1 "	11.1 "
Hexadecene	13.2 "	13.2 "
* Heptadecane	Standard	
* Heptadecene	Standard	
Octadecane	29.5 "	29.7 "
Octadecene	32.1 "	32.6 "
Nonadecane	44.7 "	45.0 "
* Nonadecene	Standard	

Figure 14 Gas chromatograph of the Dufour gland secretion before (a) and after (b) reaction with bromine



weight components (Fig.14). Identical results were obtained on the material from the distillation of gasters using the syringe reaction technique described by Hoff and Feit[47,48].

The major component of the secretion, peak 13, was identified as cis-8-heptadecene. The mass spectrum of this component (Fig.15) showed a parent ion at m/e 238 and a typical alkene cracking pattern characterised by groups of peaks 14 mass units apart whose relative abundance increased fairly regularly with decreasing m/e ratio in a series two mass units lower than that shown by the alkanes. Assignment of the double bond position in alkenes from mass spectrometric data alone is difficult due to rearrangement processes occuring in the molecule. However, a sample of heptadecene, obtained by preparative gas chromatography on the material from the distillation of gasters, was treated with ozone. Gas chromatography of this ozonised material showed the elimination of the heptadecene peak and the production of two new peaks of shorter retention time and approximately equal area (Fig.16). These two new components were identified as octanal and nonanal. Their mass spectra (Fig.17) showed parent ions at m/e 128 and m/e 142 respectively and fairly strong ions at m/e 44 corresponding to the fragment (III) which is characteristic of the McLafferty rearrangement expected from an aldehyde.



III

Co-chromatography on both polyethylene glycol adipate and

Figure 15

Mass spectrum of heptadecene

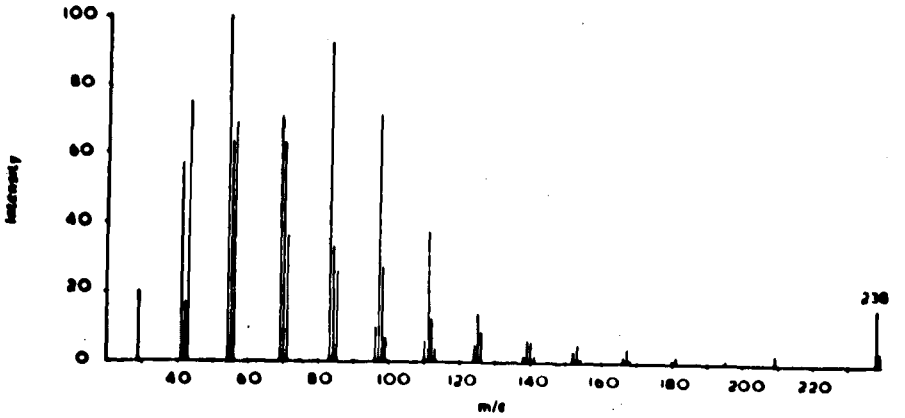
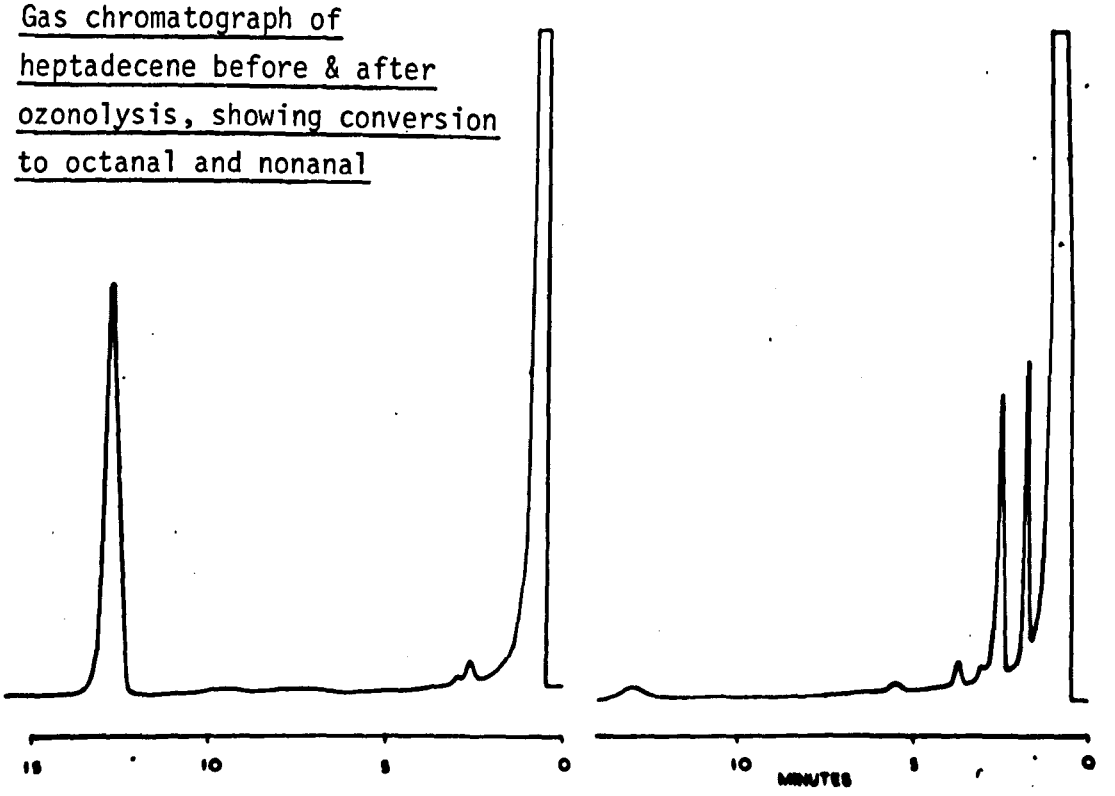


Figure 16

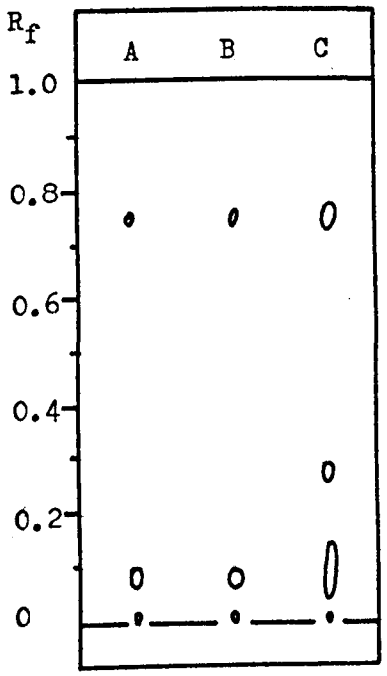
Gas chromatograph of heptadecene before & after ozonolysis, showing conversion to octanal and nonanal



Apiezon L columns of the ozonised material together with an authentic sample of octanal gave no new peaks but showed enhancement of octanal peak (Fig.18).

Assignment of the stereochemistry of the double bond was achieved using samples of cis and trans-8-heptadecene provided by Coggins[49] and Johnson[50]. The mass spectra of the two isomers, run under the same conditions as the gland extract, were inconclusive. The base peak of cis-8-heptadecene and the 8-heptadecene isolated from ants is at m/e 55, whilst the trans isomer showed a base peak at m/e 69. However, there were significant differences in the intensities of several peaks, notably m/e 83, between the synthetic cis isomer and the ant heptadecene. Thin layer chromatography of the cis- and trans-isomers on t.l.c. plates impregnated with silver nitrate together with the material from the distillation of gasters, showed the major component of the gland extract to have the same Rf value (0.087) as the synthetic cis-isomer (Fig.19).

Fig.19



A Cis-8-heptadecene R_f 0.087

B Ant heptadecene R_f 0.087

C Trans-8-heptadecene R_f 0.28

(Sample contaminated with

8-heptadecyne R_f 0.1)

Figure 17 Mass spectrum of (a) octanal and (b) nonanal

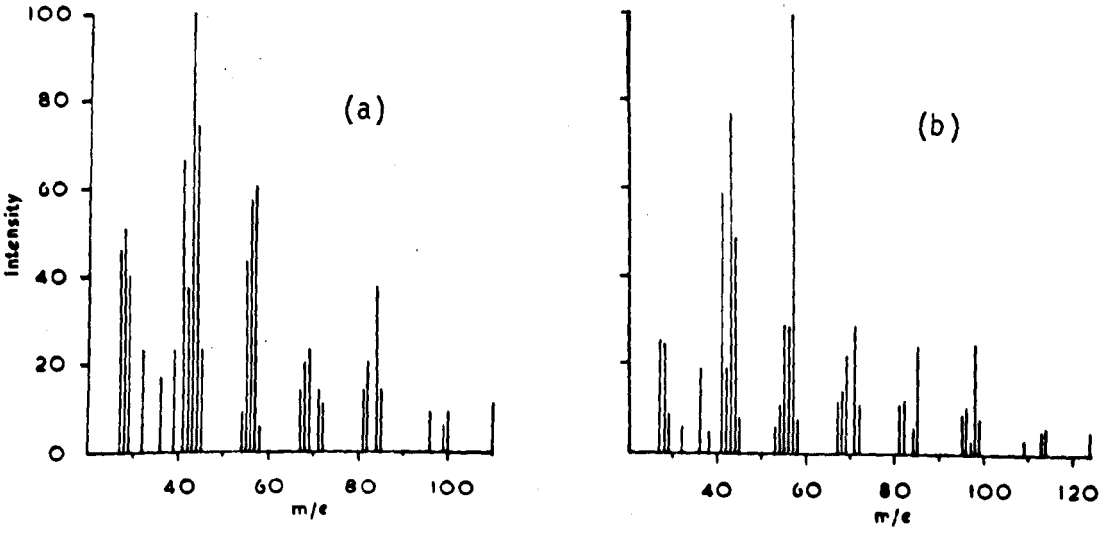
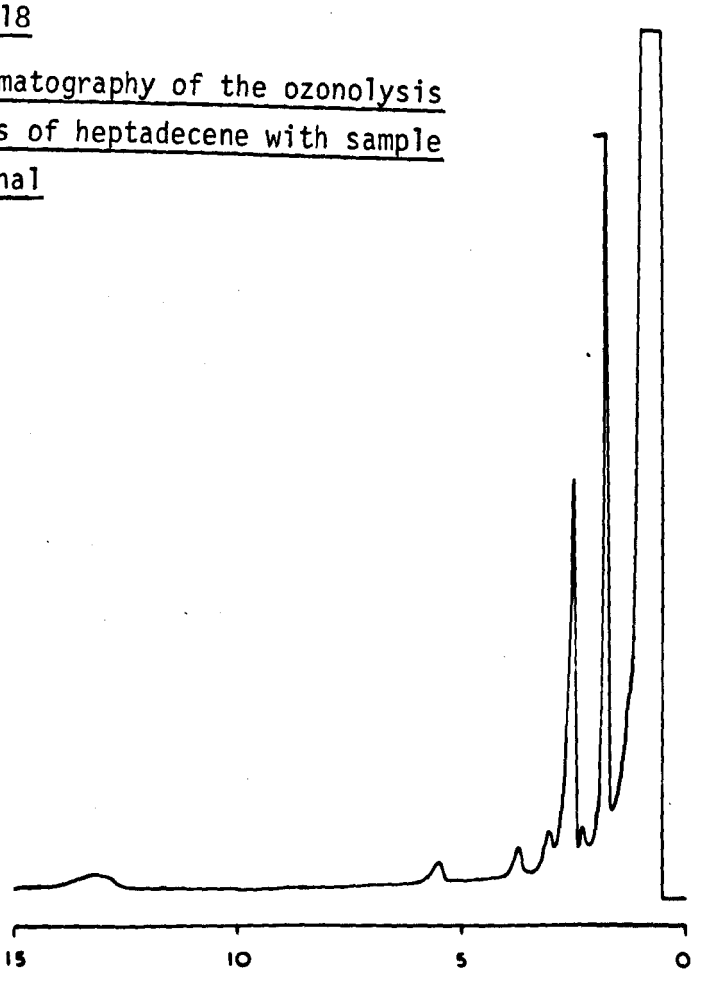


Figure 18

Co-chromatography of the ozonolysis products of heptadecene with sample of octanal



The mass spectrum of peak 20 showed a parent ion at m/e 266 and a typical alkene cracking pattern, together with a small peak at m/e 93 derived from a minor constituent of the peak (Fig.20). The major component of this peak was identified as cis-9-nonadecene. Ozonolysis of a sample of nonadecene, obtained by preparative gas chromatography of the material from the distillation of gasters, showed elimination of the nonadecene peak and the formation of two new components of shorter retention time and approximately equal area (Fig.21). Unfortunately, insufficient material was available to obtain a mass spectrum of these components. However, co-chromatography on both polyethylene glycol adipate and Apiezon L columns of an authentic sample of octanal together with the ozonised material gave three peaks (Fig.21). A plot of the log of the retention times of these three peaks against carbon chain length showed the ozonolysis products to be nonanal and decanal. Thus the double bond in nonadecene can be assigned to the 9-position.

Thin layer chromatography of authentic samples of cis and trans-8-heptadecene on t.l.c. plates impregnated with silver nitrate together with the material from the distillation of gasters showed only one spot with an Rf identical to that of cis-8-heptedecene and no spot corresponding to the trans-isomer. The Rf values of alkenes run on silver nitrate impregnated t.l.c. plates is dictated primarily by the stereochemistry of the double bond. Since there is no component in the glandular secretion showing a trans double bond we have assigned the nonadecene, which represent approximately 10% of the secretion, the structure cis-9-nonadecene.

A plot of the log of the retention times against carbon chain length for the remaining components in the gland extract, on

Figure 20 Mass spectrum of nonadecene

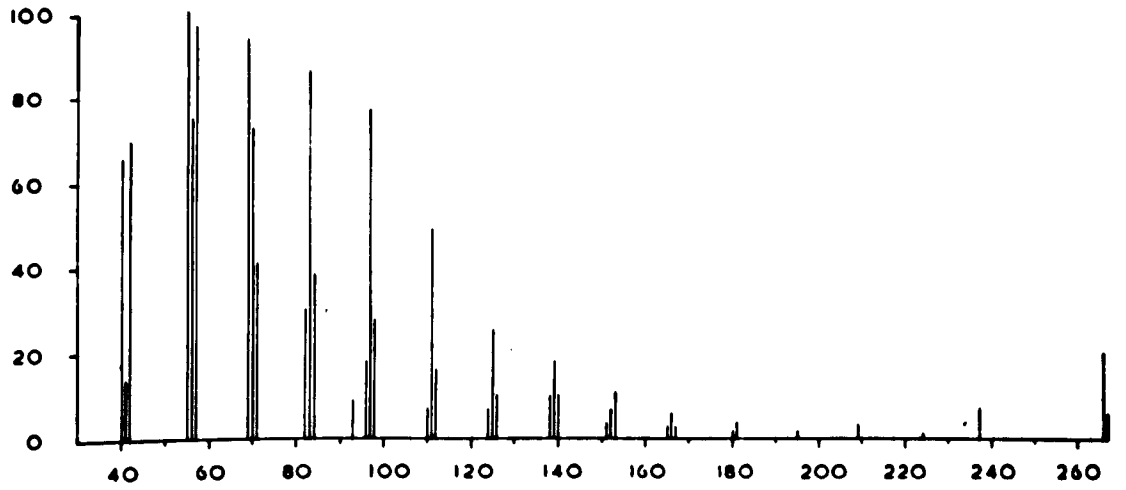
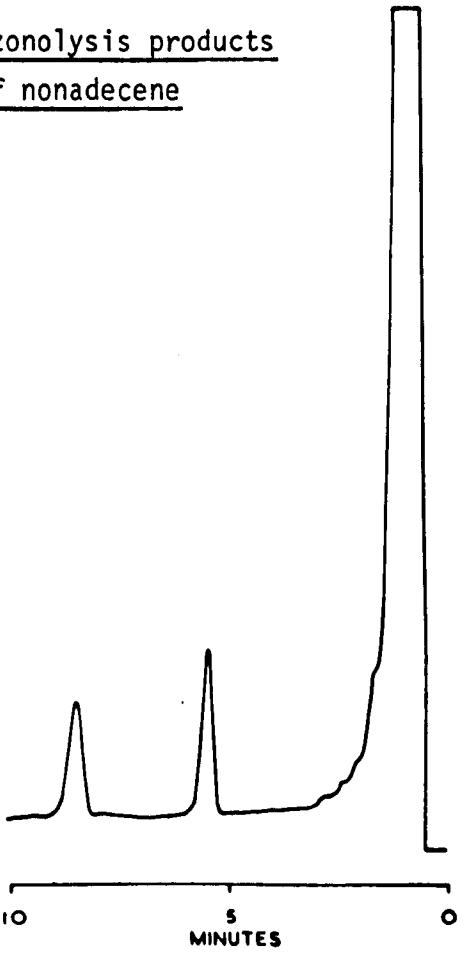


Figure 21

Co-chromatography of the ozonolysis products with a sample of octanal

Ozonolysis products of nonadecene



polyethylene glycol adipate and Apiezon L columns, showed the minor components corresponding to peaks 8, 11 and 16 to be penta-, hexa- and octadecene respectively (Figs.12 & 13). Assignment of peak 11 was confirmed by means of its mass spectrum (Fig.23) which showed a molecular ion at m/e 224 and a characteristic alkene cracking pattern. The components so far discussed, which represent over 90% of the volatile material in the poison apparatus, are straight chain aliphatic hydrocarbons.

The C13 to C17 straight chain alkanes and alkenes presumably arise by metabolic decarboxylation of the common fatty acids. The position and stereochemistry of the double bond in cis-8-heptadecene corresponds to that of oleic acid and the proportions of n-pentadecane, n-heptadecane and cis-8-heptadecene correspond to those of palmitic, stearic and oleic acid respectively in common lipids. Cis-8-heptadecene has been reported as the major constituent of the Dufour gland secretion in the myrmecine, Myrmecia gulosa[36] and a C17 alkene has been found in several formicine ants[46]. The same authors have also reported the presence of a C19 alkene in two other formicines, Lasius alienus and Camponotus ligniperda. Presumably the cis-9-nonadecene isolated from M. rubra is derived from the less common 11-eicosenoic acid. The total fatty acid composition of a sample of 1.5 gms of workers was determined to see if this acid was present in significant amounts (Table 2). Although small quantities of acids higher than C18 were present, and there is an approximate relationship between the proportions of C16 and C18 acids and C15 and C17 hydrocarbons present in the gland extract, this relationship does not hold for 11-eicosenoic acid and cis-9-nonadecene.

The mass spectrum of peak 14 showed two molecular ions at

T A B L E 2

Fatty Acid Composition of Whole Bodies of M. rubra Workers

Acid	Total (%)
Myristic	5.2
Palmitic	17.2
Palmitoleic	4.1
Stearic	7.7
Oleic	52.5
Linoleic	10.4
Eicosanoic Eicosenoic	Approx. 2

Figure 23 Mass spectrum of hexadecene

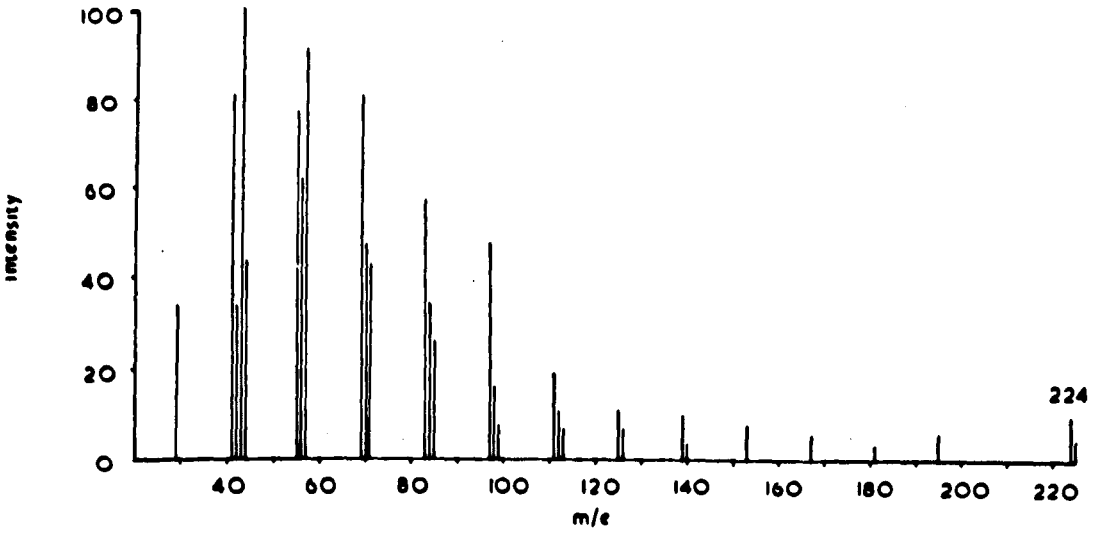
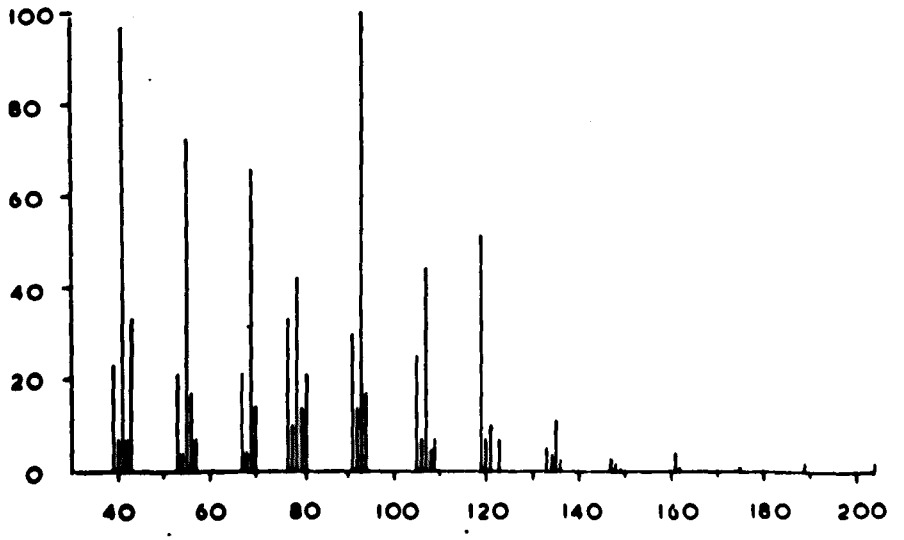


Figure 24 Mass spectrum of cis, trans-α-farnesene

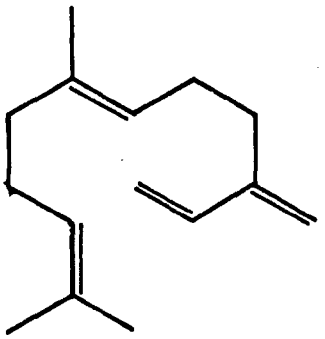


m/e 204 and m/e 236. The gas chromatographic retention time of this peak indicated that the later component was a C17 diene. It is probable that the alkanes and alkenes present in the glandular secretion arise by metabolic decarboxylation of the corresponding fatty acid. If this is the case, then the heptadecadiene would be derived from linoleic acid, which represents 10% of the total fatty acid content of M. rubra workers (Table 2). On this assumption we have tentatively assigned the C17 diene constituent of peak 14 the structure cis, cis-8,11-heptadecadiene.

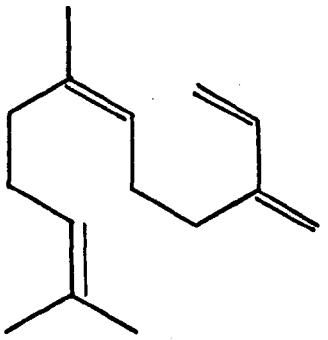
A pure spectrum of the other component of peak 14 was obtained using the 5% diethylene glycol succinate column. The compound was identified as cis,trans-α-farnesene. The mass spectrum (Fig.24) showed a molecular ion at m/e 204 and a base peak at m/e 93. Strong ions were also present at m/e 69, 105, 107 and 119, which are typical of a sesquiterpene[51]. Monocyclic or bicyclic sesquiterpenes gave base peaks at m/e 93 or higher mass numbers, as well as fairly intense molecular ions: of the order of 10-20% of the base peak, whereas acyclic sesquiterpenes show only weak molecular ions and the base peak is usually at m/e 69 or m/e 93[51,52]. Although this spectrum did show a base peak at m/e 93, the relative intensity of the molecular ion is only 2% and we have assigned this compound the structure of an acyclic sesquiterpene. The mass spectrum of this compound is identical with one published by Bergström and Löfqvist[45] and identified by them as that of an isomer of the sesquiterpene α-farnesene.

In order to determine which farnesene is present in M. rubra the various isomers (Fig.25) were prepared by the dehydration of nerolidol[44]. Gas chromatography of the crude mixture gave only

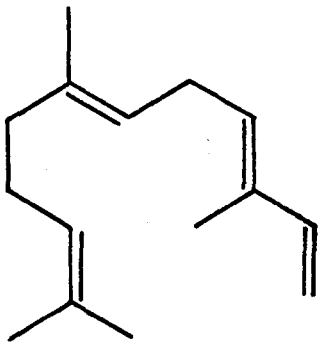
Fig. 25



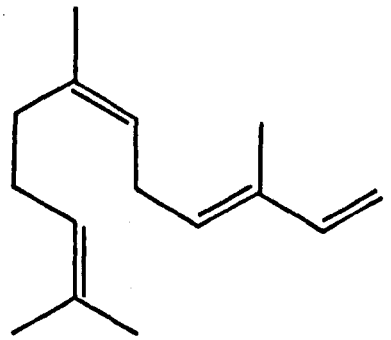
Trans-β-farnesene



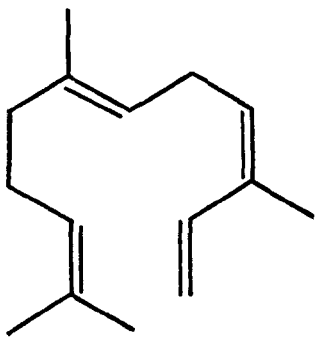
Cis-β-farnesene



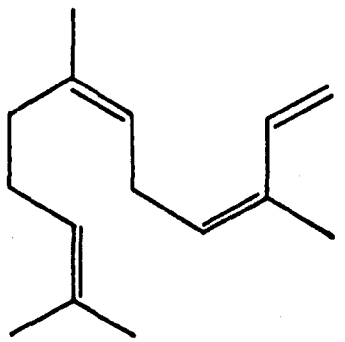
Trans,trans-α-farnesene



Trans,cis-α-farnesene



Cis,trans-α-farnesene



Cis,cis-α-farnesene

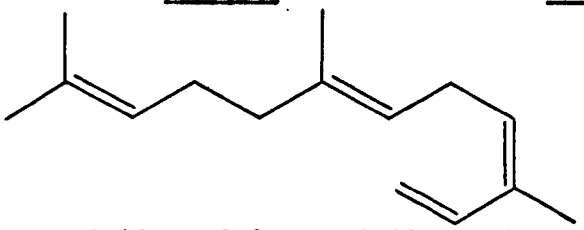
five components.

Peak No.	Retention Time
1	15.0 mins.
2	17.3 "
3	20.8 "
4	23.3 "
5	25.8 "

According to Anet[44], the order of elution of the farnesenes is cis β; trans β; cis,cis α; cis,trans α and trans,cis α together, and finally trans,trans-α-farnesene. Peak 4 of the crude farnesene extract is broader than its retention time would dictate and it is probable that it is a composite peak containing both the cis,trans α and trans,cis α isomers. Attempts to separate these two isomers were unsuccessful. The gas chromatographic retention time of the farnesene component of the glandular secretion, run under the same conditions, was 23.6 mins. Thus, on retention time evidence this compound is either cis,trans-α-farnesene or trans,cis-α-farnesene.

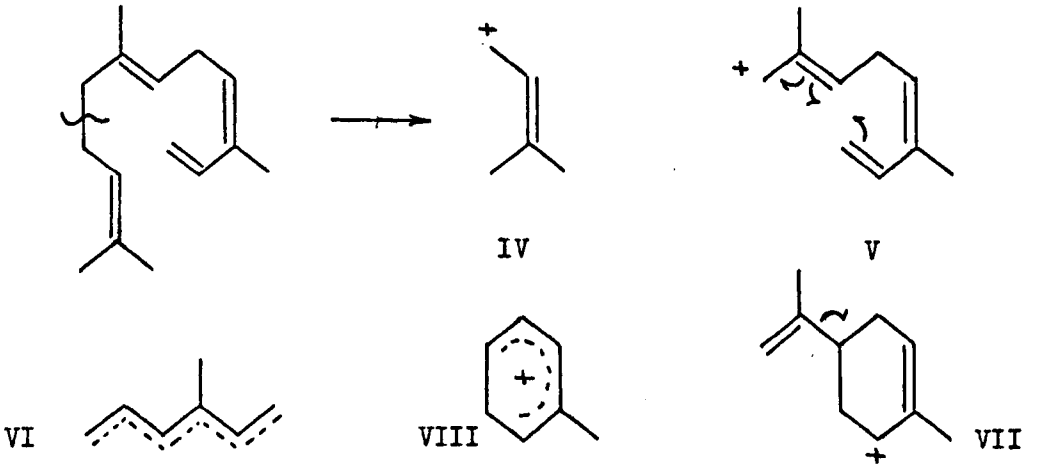
The mass spectra of the two β isomers, peaks 1 and 2, showed base peaks at m/e 69, whereas a sample of pure trans,trans-α-farnesene, obtained from the natural coating of Granny Smith apples, and found to be identical with component 5, gave, as did components 3 & 4 of the crude farnesene mixture, mass spectra with base peaks at m/e 93. These three were very similar to the spectrum of the farnesene present in the glandular secretion. Although all α-farnesenes give essentially the same mass spectra there are however, significant differences in the intensities of some of the

peaks. From the data supplied by Anet[44], it can be seen that the ratio of m/e 135 to m/e 133 is dependant on the configuration about the C6 double bond. If the configuration at this position is trans, then the ratio of m/e 135 to m/e 133 is approximately 2:1, whereas if the configuration is cis then the ratio is 1:2. In the case of trans,trans-α-farnesene the ratio if 7:4, whereas in cis,cis-α-farnesene the ratio is 4:9. The mass spectrum of the farnesene isolated from M. rubra gave a ratio of m/e 135 to m/e 133 of 11:5 and thus this compound must have a trans configuration at the C6 double bond. Gas chromatographic retention times showed that this component was either cis,trans-α-farnesene or trans,cis-α-farnesene, both components being eluted together under the conditions of the experiment. By a process of elimination the isomer present in the glandular secretion of M. rubra was found to be cis,trans-α-farnesene.

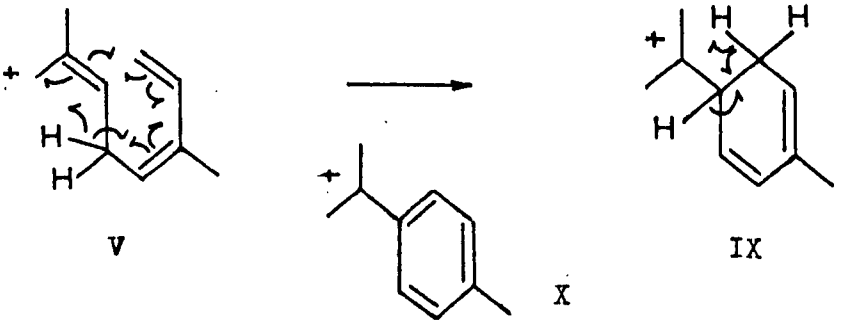


The interpretation of fragmentation patterns of sesquiterpenes must be treated cautiously. Allylic cleavage at the 8-9 bond is however, highly favoured giving rise to peaks at m/e 69 (IV) and m/e 135 (V) and the further degradation of this fragment (V) can then give rise to the rest of the major peaks in the spectrum. It may break down simply by loss of consecutive methylenes along the pathway m/e 135 - 121 - 107 to give finally the base peak at m/e 93 (VI). Alternatively, (V) may cyclise in one of two ways depending on the configuration at the C6 double bond. If the configuration is trans, then the favoured cyclisation gives a limonene type ion (VII) with the charge on the cyclohexene ring. Loss of the side

chain again gives the base peak at m/e 93 (VIII). There is a metastable ion at m/e 64.1 (Calc. 64.07) for this transition.



If, however, the configuration at the C6 double bond is <u>cis</u>, then the favoured cyclisation proceeds along different lines involving a proton transfer between C5 and C8 to give (IX), which can then either lose the side chain to give (VIII), or it can aromatise, by loss of hydrogen, to give the <u>p</u>-cymene type ion (X). This can then break down further along the pathway m/e 133 - 119 - 105.



A <u>cis</u> configuration at the C6 double bond favours the proton transfer with subsequent aromatisation to give the fragment of m/e 133 (X) since the stereochemistry of the ion (V) is such that the C5 and C8 positions are in close proximity. If, however, the configuration at the C6 double bond is <u>trans</u>, then there must be rotation about this bond for the 5 and 8 positions to be set up

correctly for the proton transfer. For this reason a trans configuration at the C6 double bond results in the predominance of m/e 135 over m/e 133, whilst the situation is reversed if the configuration at the C6 double bond is cis.

Two other components in the glandular secretion also showed sesquiterpene type mass spectra. The mass spectrum of peaks 16 and 17, which were usually eluted together under the conditions of combined gas chromatography-mass spectrometry, showed two molecular ions at m/e 252 and m/e 218 (Fig.26). The former component (Peak 16) had already been assigned as octadecene. A pure spectrum of the component of molecular weight 218 was again obtained using the 5% diethylene glycol succinate column. The compound was identified as an homofarnesene ($C_{16}H_{26}$). The mass spectrum (Fig.27) was like that of an acyclic sesquiterpene and it is probable that this compound is an homologue of farnesene with an additional $-CH_2$. The strong ion at m/e 189 (P - 29) indicated the presence of an ethyl side chain and it would seem that one of the methyl groups at C3, C7 and C11 in farnesene is replaced by an ethyl group. The possibility of the ethyl group being at C11 could be discounted. Allylic cleavage at the 8-9 bond would again be highly favoured. If the ethyl group were at C11 one would expect a decrease in the intensity of the peak at m/e 69, relative to that of the farnesene spectrum, and the appearance of a fairly strong ion at m/e 83. Thus, although there was a peak at m/e 83 there was no corresponding decrease in the intensity of the peak at m/e 69. If, however, the ethyl group were at C3 or C7 the spectrum could be interpreted using the same fragmentation pattern postulated for farnesene. Allylic cleavage would now, however, give an ion at m/e 149 instead of the m/e 135 ion found in farnesene.

Figure 26

Mass spectrum of a mixture of octadecene and homofarnesene

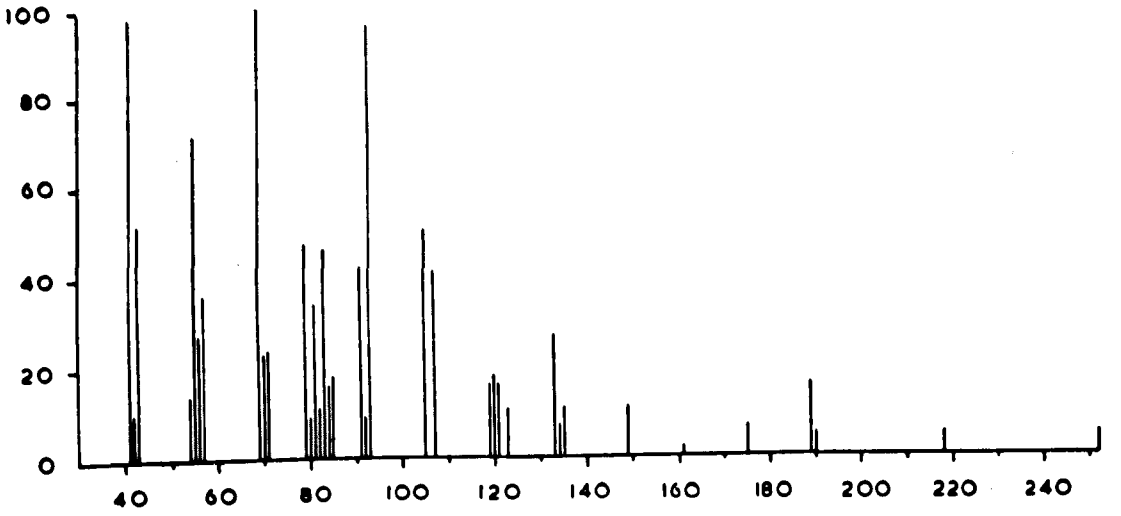
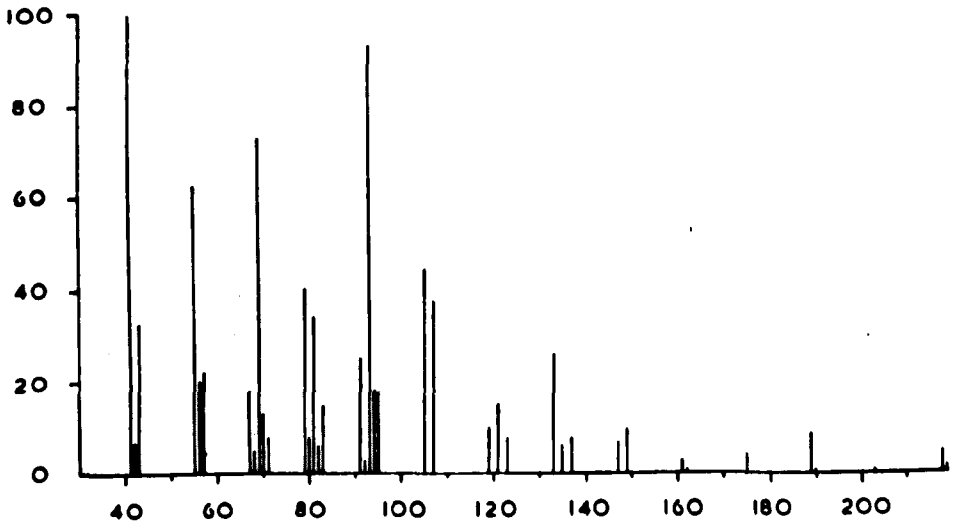
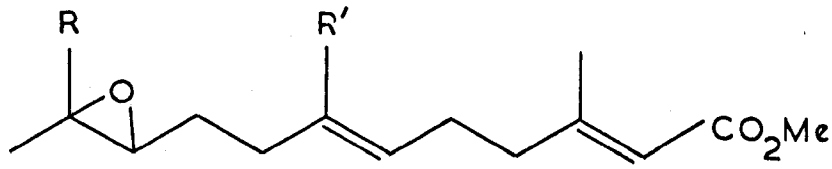


Figure 27

Mass spectrum of homofarnesene



The breakdown of the m/e 149 fragment should give rise to peaks 14 mass units higher than those found in the farnesene spectrum. This is the case and the ion m/e 133 in the homofarnesene spectrum corresponds to the m/e 119 ion in the farnesene spectrum. However, it is not possible to decide between the C3 and C7 positions for the ethyl group, though consideration of related compounds such as those isolated from Cecropia[53] (XII) and (XIII) would indicate an ethyl group at the C7 position.



XI $R = R' = CH_3$ XII $R = CH_3$ $R' = C_2H_5$

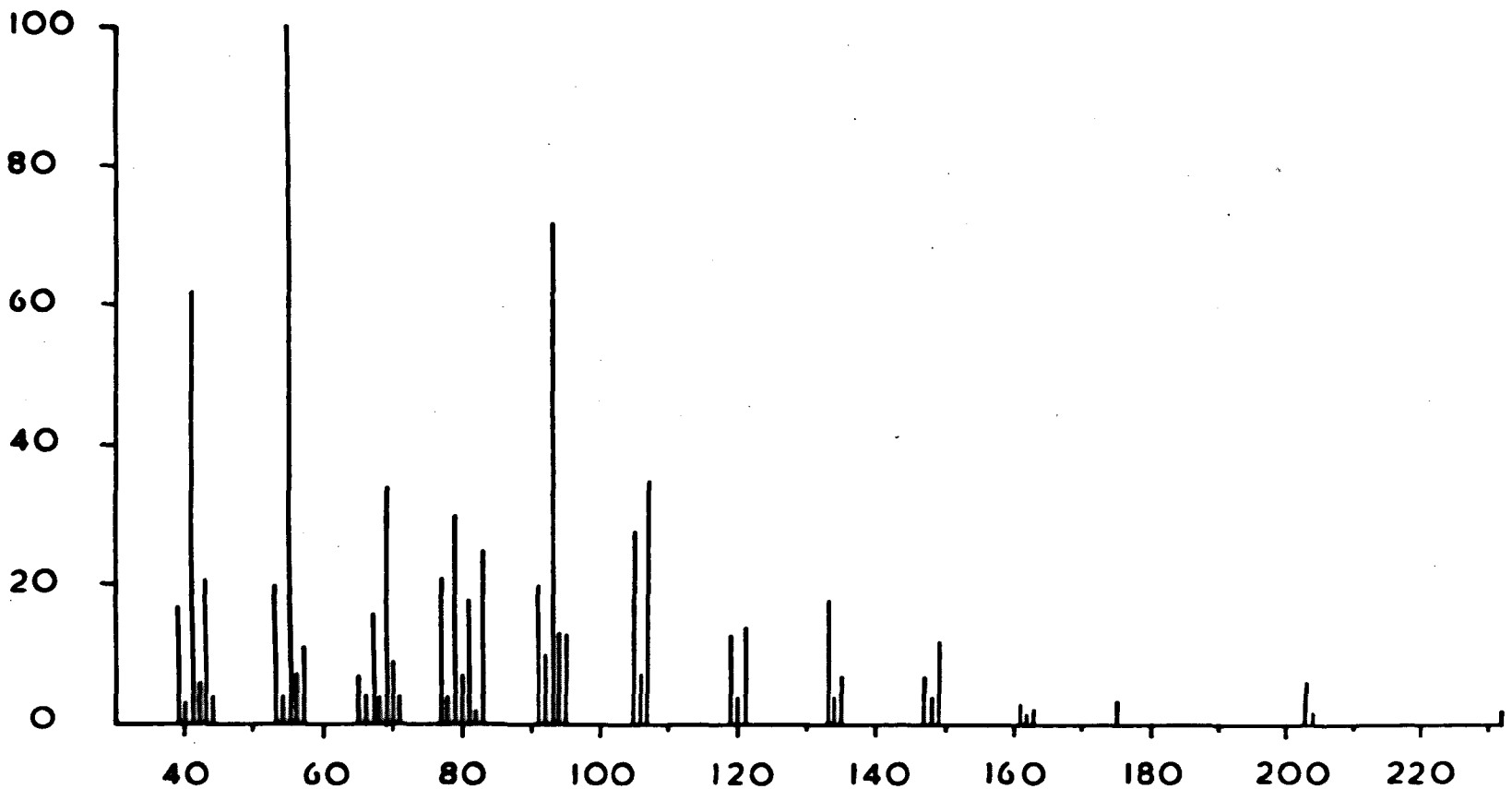
XIII $R = C_2H_5$ $R' = CH_3$ XIV $R = R' = C_2H_5$

In farnesene the configuration about the C6 double bond determined the ratio of m/e 135 to m/e 133. Since it is probable that the fragmentation of the homofarnesene is governed by similar considerations an examination of the ratio of m/e 149 to m/e 147 should indicate the configuration about this bond. The ratio is 5:3 and thus it would seem that the homofarnesene too has a trans configuration at the C6 double bond.

Peak 20 contained a small amount of a similar compound which, when separated on the 5% diethylene glycol succinate column, gave a mass spectrum with a molecular ion at m/e 232 (Fig.28). We have identified this compound as a bishomofarnesene. The mass spectrum

Figure 28

Mass spectrum of bishomofarnesene



showed a strong ion at m/e 203 (P - 29) again indicating the presence of an ethyl side chain and the similarity of the three spectra (Figs.24,27,28) suggested that this compound was a further homologue of farnesene with two additional $-CH_2$ groups ($C_{17}H_{28}$). This time it was possible to assign one of the ethyl groups to the C11 position. The mass spectrum showed the expected decrease in the intensity of the ion at m/e 69, relative to that in farnesene and homofarnesene, together with the appearance of a fairly strong ion at m/e 83, formed by the highly favoured allylic cleavage at the 8-9 bond. However, as with the homofarnesene, it was not possible to distinguish between the C3 and C7 positions, though again it is probable that the second ethyl group is at C7. The ratio of m/e 149 to m/e 147 is 12:7 showing that the bishomofarnesene too has a trans configuration at the C6 double bond.

An isomer of α-farnesene has been reported in the Dufour gland secretion of the slave keeping ants Formica sanguinea and Polyergus rufescens, as well as in one of their slaves, Formica fusca[45]. Trans,trans-α-farnesene has been found to be the only component of the Dufour gland secretion of the myrmecine, Aphaenogaster longiceps[43,44]. The Dufour gland secretion of the formicine, Camponotus ligniperda contains an isomer of farnesene and the corresponding acetate, farnesyl acetate[46]. Thus although the presence of farnesene in the Dufour gland secretion is not unusual this is the first report of an homofarnesene or bishomofarnesene.

The similarity of these three related sesquiterpenes to the juvenile hormones (XI), (XII), (XIII) and (XIV) isolated from Cecropia[53] is worth noting. Further, all these compounds have a trans configuration at the C6 double bond. Farnesol and farnesal also show some juvenile hormone activity and recent work has shown that they are only active as juvenile hormones when there is a trans configuration at the C6 double bond[54]. These similarities make it tempting to postulate that the three sesquiterpenes isolated from M. rubra could be derived either as degradation products of compounds similar to the Cecropia juvenile hormones perhaps also possessing juvenile hormone activity or, alternatively, they could be derived from common precursors to such a group of compounds, perhaps farnesyl pyrophosphate.

In an attempt to discover whether the Dufour gland or poison gland was responsible for the peaks shown on the gas chromatographic trace, samples of individual Dufour glands and poison vesicles were examined by the solid injection method. Contamination of the poison vesicle sample with the contents of the Dufour gland was unavoidable since it was necessary to rupture the latter during the disection. It was thus essential to be able to obtain quantitative results on the composition of the glands. This was achieved by calibrating the gas chromatograph with serial dilutions of n-heptadecane and running these at the appropriate attenuation. Under the conditions of the experiment it was found that 1.7×10^{-8} g. of n-heptadecane gave a peak area of 1 sq. cm. at an attenuation of 1×10^{3}. Using the same conditions it was found that the Dufour gland of a mature worker contained $0.5 - 1.0 \times 10^{-7}$ g. n-heptadecane, whereas a large poison vesicle contained only 1.5×10^{-9} g. n-heptadecane, and moreover the

same peaks were present in the gas chromatographic trace and in the same proportions as in the Dufour gland. It may, therefore, be safely concluded that the small amount of volatile material in the poison vesicle arose only from contamination with the Dufour gland secretion and that the poison vesicle itself contains no volatile material.

Attempts have been made to determine whether the Dufour gland contents were discharged with the proteinaceous venom[55] from the poison vesicle. However, although Jentsch[55] was able to collect venom from Myrmica rubra workers by subjecting them to electric shocks we have had no success with this method. Neither have we been able to collect venom by inducing workers to sting through a membrane[36]. However, disection of ants that had been allowed to attack and kill a fly showed the venom sacks to be collapsed, whereas the Dufour gland was still distended with fluid. This is in accordance with observations on Myrmecia gulosa[36] and Pseudomyrmex pallidus[37].

The volatile constituents of the Dufour gland showed considerable quantitative variation in the components of the different samples. This was most noticeable at the colony level, particularly between colonies taken from different parts of the country. However, there were also significant differences shown between individual members of the same colony. It was thought that these quantitative variations between colonies could have resulted from dietary differences, but even with nests maintained on identical diets under laboratory conditions for several months, considerable quantitative variations still existed. It was found, however, that over this period there was a gradual decrease in the aggressive response shown when an ant from another colony was introduced into the nest. In one instance,

with two nests that had been maintained under artificial conditions for four months, the intruder was not attacked at all and was even allowed to feed without interference. Thus it would seem that the colony or nest odour is not derived from the volatile constituents of the Dufour gland secretion.

The Dufour gland secretion in Myrmica rubra has a dual function. Maschwitz[40] reported that M. rubra possesses an abdominal alarm pheromone, produced in the poison apparatus, as well as an alarm substance produced in the mandibular glands. Gas chromatography of individual Dufour glands and poison vesicles has shown that the only volatile materials present in the poison apparatus are derived from the Dufour gland. Thus alarm is one function of the Dufour gland secretion.

In Myrmecinae, artificial trails can be laid with the venom gland itself, as in Tetramorium guineense[19] or, in other members of the subfamily, with the contents of the Dufour gland. Solenopsis saevissima lays trails by extruding its sting and depositing the material from the Dufour gland on the ground[20]. Eidmann[4] has shown that M. rubra also lays trails. Returning foragers have been seen to touch the tips of their gasters to the ground and it is probable that the mechanism and glandular origin of the trail pheromone in M. rubra is the same as in S. saevissima. Thus another function of the Dufour gland secretion in M. rubra is that it contains the trail pheromone.

Initial investigations of the trail pheromone in M. rubra were carried out on a pentane extract of one hundred homogenised whole ants. This extract, however, elicited no trail following behaviour, either because the pheromone was not extracted or, more probably, because of

masking by other compounds. However, a pentane extract of two hundred homogenised Dufour glands and poison vesicles showed strong trail following activity.

The conditions for the bioassay were standardised as far as possible. A known volume of the sample was applied to the porous tile place on a rotating stage turned at a constant speed by an electric motor. The ants used for each test were taken from the nest soon after they had been fed. Only individuals that had been feeding or were in the immediate area of the food were used. Care was taken not to alarm the workers when placing them on the porous tile. If roughly handled they became alarmed, running around at high speed with open mandibles and waving their antennae, and paid no attention to the artificial trail even though the same extract may have proved active in a previous test.

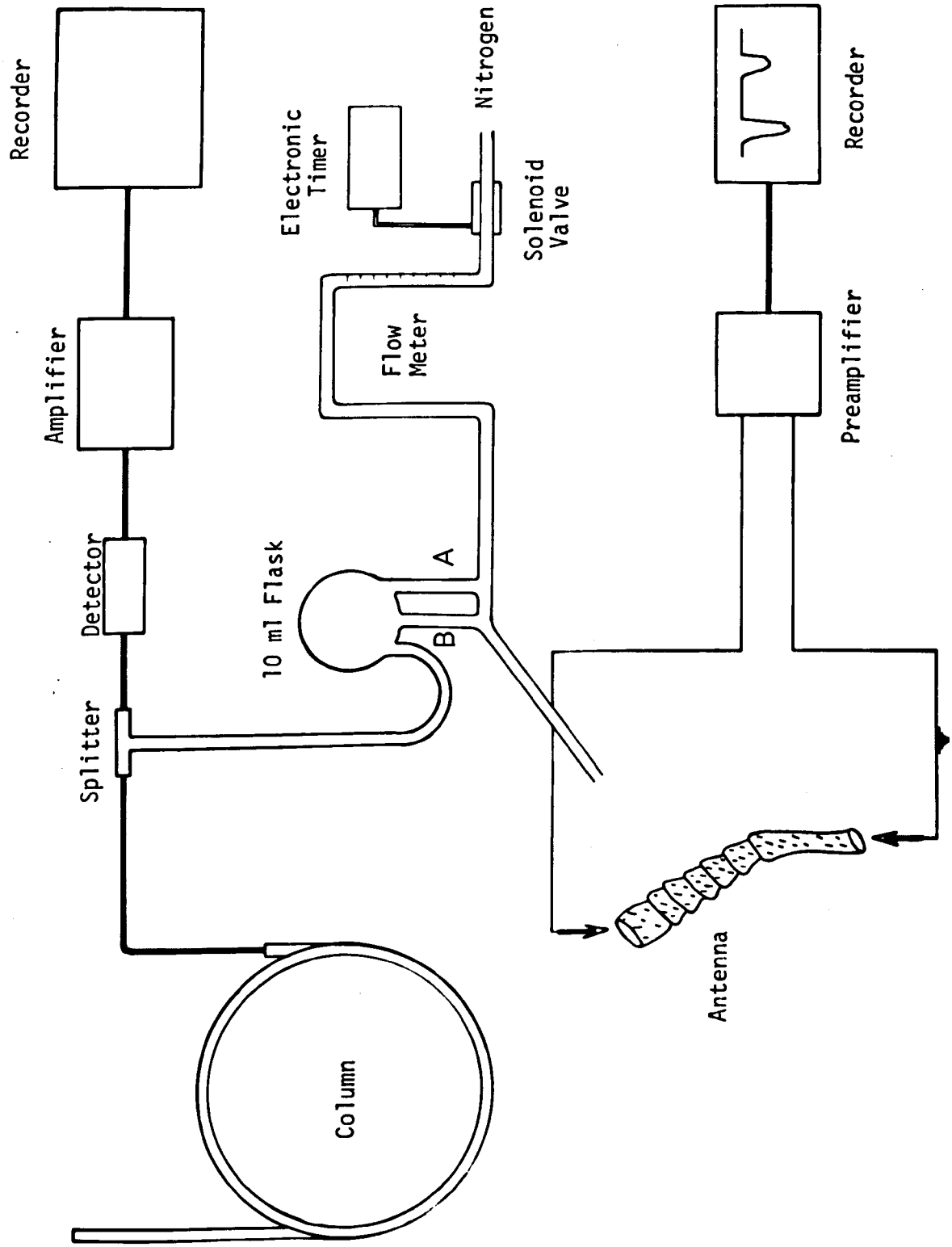
In order to isolate and identify which of the twenty components of the Dufour gland secretion contained the trail pheromone, the material from the distillation of gasters was fractionated by preparative gas chromatography. Sixty µl of this extract was chromatographed (6 x 10 µl samples) on the analytical 10% polyethylene glycol adipate column. Of the twelve fractions collected, three showed trail pheromone activity on bioassay. The active fractions corresponded to peaks 7, 8, 9, 10 and 11. To avoid the possibility of the contamination of the fractions by differences in the timing of trap changes a single 200 µl sample of the extract was chromatographed. This time, the peaks showing trail pheromone activity in the previous fractionation were collected separately, only peaks 8 and 9 were collected in one trap. Bioassay of these fractions again showed trail pheromone activity to be present in peaks 7 - 11 inclusive.

The occurence of five active components in the Dufour gland secretion seemed unlikely. One constituent of the trail pheromone must be present in peak 7 since all earlier fractions proved inactive. It was, however, improbable that this was the only active constituent in the glandular secretion. If this were the case, the activity in the three succeeding fractions could arise only by contamination and one would then expect a gradual decrease in the strength of the trail following behaviour in each subsequent fraction. Thus, the fraction from peak 11 should elicit weaker trail following than the fraction corresponding to peaks 8 and 9. This was not so. In fact, the fraction from peak 10 gave the strongest response on bioassay. Thus it seemed probable that there were at least two active components in the secretion.

At this stage it was decided to discontinue the attempt to isolate the trail pheromone by preparative gas chromatography since the probability of the activity from one fraction being carried over to contaminate the next meant that we were unable to determine how many active components were present in the Dufour gland secretion. Instead, it was decided to try and identify the peaks corresponding to the active components by means of an electroantennogram. The technique consists of linking a gas chromatograph with the insects antenna prepared for the recording of electroantennograms. The electroantennograms response is essentially produced by many olfactory receptor potentials recorded by an electrode implanted into the antenna. Simultaneous recordings of the gas chromatograph detector and insect responses enable the correlation of retention time with electroantennogram responses (Fig.29, Appendix).

However, it must be remembered that not all potentials

Figure 29 The electroantennogram/gas chromatograph link



recorded from the electroantennogram are necessarily in response to a behaviourally active substance. Inhibitors too can produce relatively large electroantennogram responses. An example of this effect is the case of two species of Gelichiidae which are morphologically similar but are reproductively isolated by employing different sex attractants. Bryotropha similis is attracted to cis-9-tetra-decenyl acetate and Bryotropha sp. (unnamed) to trans-9-tetradecenyl acetate. Males do not respond to the attractant of the other species and are actually inhibited from being attracted to their pheromone by the presence of the geometrical isomer. Thus a mixture of cis and trans-9-tetradecenyl acetate is not attractive to males of either species. However, electroantennograms show that the male antennae of both species respond to cis and trans isomers. In this case the electroantennogram recordings alone would lead to the conclusion that the species are cross attractive when in fact they are inhibitory to one another[56]. Thus, electroantennogram data must be interpreted in conjunction with the results from a reliable bioassay.

Initially, electroantennograms were recorded on excised antennae with glass electrodes inserted into both cut ends. However, this technique suffered from several disadvantages; difficulty was experienced in making a good electrical contact between electrodes and antennae, and also the size of the antennal response decreased with time until about twenty minutes after excision it approached zero. This was presumably due to the gradual death of the antenna. In order to prolong the life of the antenna it was necessary to use whole ants. However, the fine glass electrodes were not sufficiently robust to pierce the cuticle. Instead tungsten electrodes were employed, initially in an attempt to make a hole in the cuticle into which the

glass electrodes could be inserted. Unfortunately, with the micomanipulators available it proved impossible to reinsert the glass electrodes into the hole made by the tungsten needles and it was decided to use the latter as the electrodes . It was still found necessary to excise the distal segment(s) of the antenna, since even with the tungsten electrodes it was not possible to pierce the antennal cuticle. The second electrode was inserted into the antennal socket where it joins the head.

The magnitude of the antennal response varied from 1.5 to 3.0 mv for a positive response. The voltage obtained seems to be dependant on the segment at which the distal cut is made. The maximum response being obtained by removing only the first distal segment and the response becoming weaker as the antennal length becomes shorter. Roelofs and Comeau[56] suggested that this is due to a lowering of the internal resistance which allows more injury current to flow through internal blood channels, but it could also be due to a decrease in the number of sensilla available as receptor sites. Scanning electron micrographs show that the distribution of the sensilla on the antenna is highest on the three distal segments (Fig.30). Thus the more segments removed, the fewer sensilla remain and the lower the potential generated.

Electroantennograms obtained using excised antennae or whole ants, with the Dufour gland secretion, were in agreement. Responses, on the polyethylene glycol adipate column, indicated three areas of antennal activity corresponding to peaks 1, 7 and 10, though in one instance a positive response was recorded for the low molecular weight components (Fig.31). Taken in conjunction with the results of the bioassay of fractions obtained from the preparative gas chromatography

Figure 30

Scanning electron micrograph of the 8 distal segments of a worker antenna showing the distribution of the sensillae (x 1600)

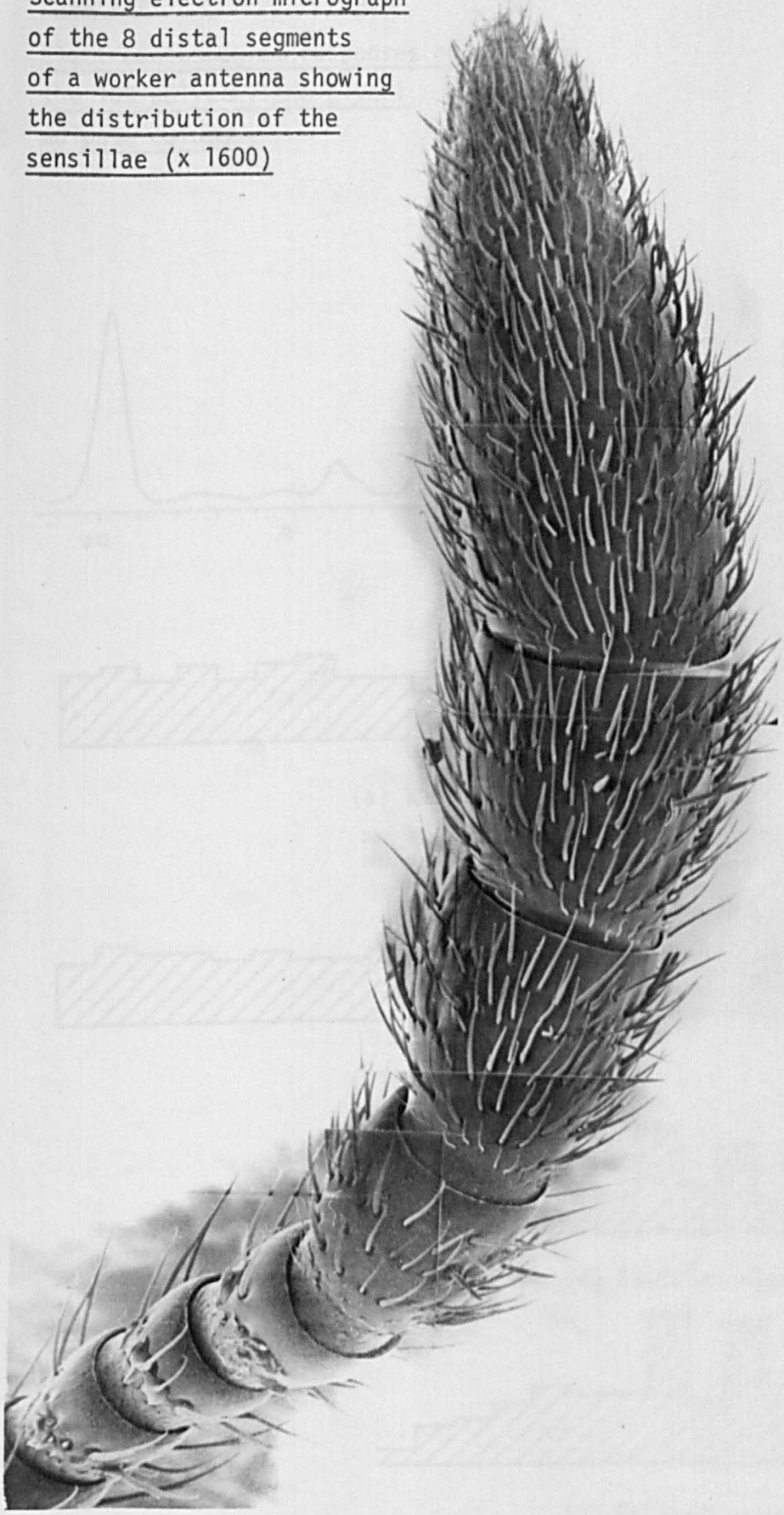
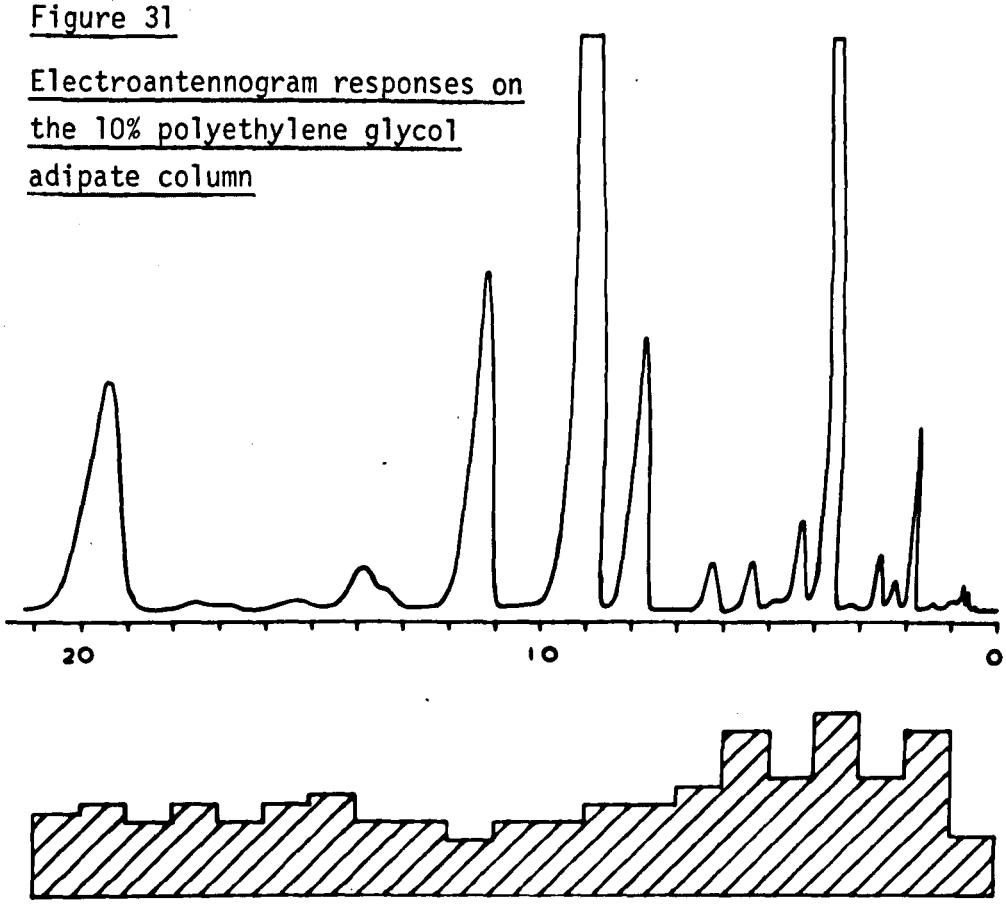
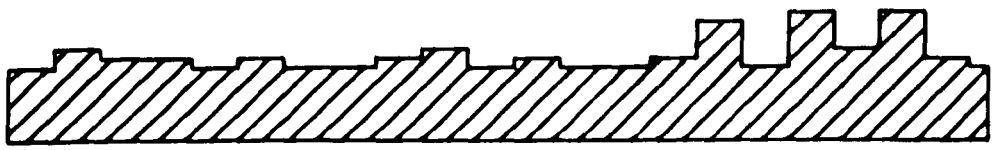


Figure 31

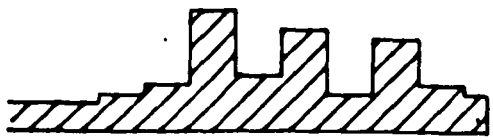
Electroantennogram responses on the 10% polyethylene glycol adipate column



(a) Whole ants



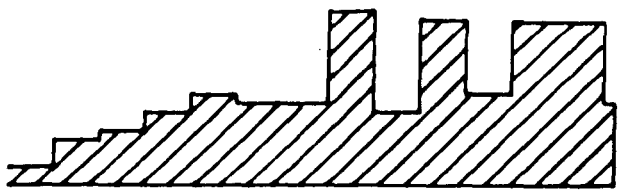
(b) Whole ants



(c) Excised antennae

Scale Response

1 mV



(d) Excised antennae

of the material from the distillation of gasters, the electro-antennograms results indicate that there were two active components in the trail pheromone of the ant Myrmica rubra. The bioassay showed trail following activity in the fractions corresponding to peaks 7, 8, 9, 10 and 11, whilst the electroantennogram results indicated activity to be present in components of peaks 7 and 10. The activity shown on the bioassay of the fractions corresponding to peaks 8 and 9 and peak 11 could easily have arisen from contamination.

Peaks 7 and 10 have already been identified as the normal hydrocarbons n-pentadecane and n-hexadecane, though neither of these components was active as a trail pheromone. A characteristic of the polyethylene glycol adipate column is that alkanes are eluted before all other compounds of a similar molecular weight. Thus, if these active components are not alkanes they must be polar compounds, probably with a molecular weight below 200. The elution of these components in the n-pentadecane and n-hexadecane peaks is interesting and it is tempting to postulate that the trail pheromone components are related compounds differing only by one Carbon atom. However, there is no evidence, other than retention times, to substantiate this postulate.

Peak 1, on the polyethylene glycol adipate column, also gave consistently positive electroantennogram responses, though no trail following activity was present in the preparative gas chromatograph fractions. Maschwitz[40] reported that M. rubra possessed an abdominal alarm substance, produced in the poison apparatus. Since it was found that the poison vesicle contained no volatile material it was probable that the alarm pheromone too was produced in the Dufour gland.

This component, peak 1, has been identified as n-tridecane. In Acanthomyops claviger, Regnier and Wilson[42] have shown that the $C_{10} - C_{13}$ alkanes were optimally efficient in respect to the alarm function. The behavioural threshold concentrations, approximately 10^{12} molecules/cm^3, of these alkanes was such that they were more effective than substances of lower molecular weight and at the same time their vapour pressure was still high enough to generate active spaces of centimeter distances from the release of microgram quantities over a very short period of time. They showed that n-undecane not only acted as a spreading agent for formic acid but also served as an alarm pheromone.

When a nest of M. rubra was presented with a small amount of n-tridecane in a capillary tube, mild alarm behaviour was released over the whole of the nest within a few seconds of the introduction of the capillary. The ants ran around in a weak state of excitement, some with open mandibles and raised antennae. The degree of alarm released was only slightly less than that obtained from crushing a whole poison apparatus in the nest.

The possible occurence of both trail and alarm pheromones in the same glandular secretion was surprising since one would expect the alarm signal to override the information carried by the trail pheromone. Thus, any ant finding the trail should become alarmed and not follow it. This is not the case and we suggest that n-tridecane does not act as a true alarm pheromone.

The odour trail is thought to be a narrow corridor of scent[31] and the probability of an ant finding the trail by chance is not great. If, however, the glandular secretion contained a volatile compound that diffused outwards rapidly, then ants at some distance

could be sensitised to begin searching for the trail.

Bioassay of n-tridecane released only mild excitement in the nest and not the full alarm response generated by either alarmed workers or crushed mandibular glands. This is in accordance with the postulate that n-tridecane acts not as an alarm perhomone but as an "interest generator" for the trail pheromone.

In order to try and obtain further information on the chemical nature of the active components of the trail pheromone, the glandular material was fractionated by preparative gas chromatography using the 15% Apiezon L column. Bioassay showed trail following activity to be present in two of the early fractions (Fig.32). The first of these two fractions was strongly active and it is probable that the weak activity shown by the succeeding fraction arose only by contamination.

Unfortunately, attempts to determine the retention times of these active components by means of electroantennograms, using the Apiezon L column, were not successful and gave anomolous antennal responses. This could have been caused by the high oven temperature used.(225°C). Not only did this result in excessive column bleed, which had a strong odour, but the temperature of the effluent passed over the antenna was above 30°C and this could have damaged the antenna. Certainly, the life of the preparations was shorter than when using the polyethylene glycol adipate column.

The gas chromatographic trace from the Apiezon L column showed two minor components to be present in the first active fraction. From the data obtained on the polyethylene glycol adipate column it was thought that there were two active components in the trail pheromone and that they were both polar compounds. Their occurence in the n-pentadecane and n-hexadecane peaks led us to suggest that

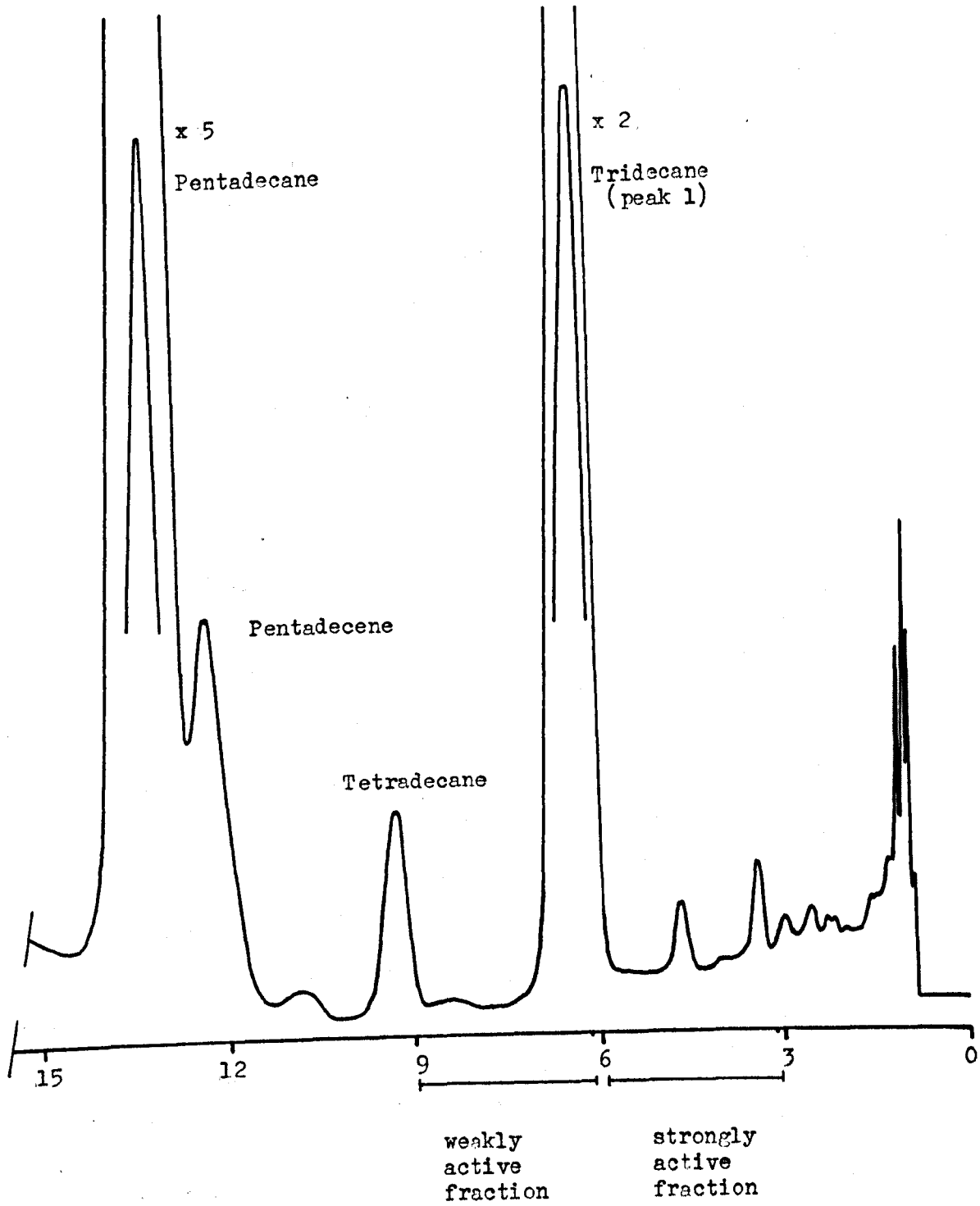


Fig.32 Part of the gas chromatograph of the volatile components of the Dufour gland run on the 15% Apiezon L column.

Preparative gas chromatography gave two fractions that showed positive trail pheromone activity on bioassay.

they were related compounds differing by one carbon atom. The bioassay results obtained on the Apiezon L column tend to confirm this. Peak 1, on the Apiezon L column, has been identified as n-tridecane and since this column is less polar than polyethylene glycol adipate, the shift to shorter retention time of the active components of the trail pheromone suggested that they were polar compounds. Furthermore, their occurence in the C_{11} and C_{12} region again suggested that they were related compounds differing by only one carbon atom.

There would seem to be considerable differences in the chemical composition of the Dufour gland secretion between the Myrmecinae and Formicinae. In Formicinae, the major components in the gland are the C_{11} and C_{13} alkanes. Other components comprise mainly alcohols, acetates and ketones, which may or may not be present as major components, and to a lesser extent, straight chain alkenes[46]. Although the composition of the Dufour gland secretion is less well documented for Myrmecinae the components so far reported have mainly been alkenes and alkanes and the major components of the secretion are of longer chain length than those found in the formicines. Thus in both Myrmecia gulosa[36] and Myrmica rubra the major component of the Dufour gland secretion is cis-8-heptadecene.

It is probable that these differences in the chemical composition of the Dufour gland secretion reflect differences in the function of the gland in the two subfamilies. In Formicinae, the Dufour gland secretion serves as a releaser of alarm behaviour. A major component in most of the formicines studied is n-undecane and it has been shown that this compound elicits strong alarm behaviour in workers of Acanthomyops claviger[42] and Camponotus ligniperda[46].

Similarly, 2-tridecanone, present in the Dufour gland, releases alarm behaviour in A. claviger. This compound is also present as a major component of the Dufour gland secretion of Lasius alienus and L. carniolicus[46]. Furthermore, Regnier and Wilson[42], have shown that substances in the C_{10} - C_{13} range are optimally efficient with respect to the alarm function and it is interesting to note that the major components of the Dufour gland secretion of formicine ants fall within these limits. Thus, in Formicinae, the Dufour gland secretion is optimally suited to the alarm function.

Otto[57] and Osman and Kloft[58] have shown that the principal venom toxicant in the Formicinae is formic acid. The contents of the Dufour gland are ejected together with the contents of the poison vesicle and Regnier and Wilson[42] have shown that n-undecane acts as a spreading agent for formic acid in A. claviger. Thus, the Dufour gland secretion in this ant actually serves as both a defensive substance and as an alarm pheromone.

In Myrmecinae, on the other hand, the Dufour gland secretion contains the trail pheromone. However, the function of the aliphatic hydrocarbons in the gland is not understood. It has been suggested that the secretion acts as a sting lubricant[59], though there is no evidence that the Dufour gland contents are ejected with the proteinaceous venom: rather the reverse. Thus, we consider this suggestion unlikely and suggest that the function of the hydrocarbons is to regulate the volatilisation of the trail pheromone.

Beroza and Bierl[60] have shown that the activity of the gypsy moth sex attractant disparlure, can be prolonged by means of what they term "keepers" which are used to regulate the volatilisation of this attractant. Keepers are of two types. The "nonvolatile" type is

virtually nonvolatile compared to the attractant; whilst the "equivolatile" type has a similar volatility to that of the attractant. With either type of keeper, the vapour pressure of the attractant will parallel its mole fraction in the mixture. With the nonvolatile keeper the concentration of the attractant and hence its volatility, will be greatest initially and then decrease gradually over a period of time. Thus the attraction will likewise be greatest initially and then decrease. With an equivolatile keeper the attractant is volatilised at a similar rate to the keeper until the mixture is depleted. Attraction will therefore remain constant. The duration and effectiveness of disparlure could be regulated to some extent by the attractant-keeper mixture used. We suggest that the aliphatic hydrocarbons in the Dufour gland of M. rubra act as an equivolatile type keeper to regulate the volatilisation of the trail pheromone components.

The threshold concentrations of pheromones are low. There is also, in some cases, a maximum concentration above which the pheromone is either no longer active or produces a different response. Thus, high concentrations of the trail pheromone methyl 4-methylpyrrole-2-carboxylate are actually repellant to Atta texana workers[33]. Insects must therefore be able to regulate, within fairly narrow limits, the concentration of pheromone used.

Results indicate that the trail pheromones in M. rubra are volatile polar compounds. If they were used alone, the concentration of the pheromone in the air directly above the trail would be high, perhaps enough to cause it to be repellant, and in order to remain within the limits of detection, workers would have to follow a parallel course to the original trail but at some distance from it.

If, however, the hydrocarbons in the Dufour gland acted as an equivolatile-type keeper, then the vapour pressure, and hence the concentration of the pheromone in the air above the trail could be regulated so as to fall within the active range of the pheromone. The trail substance would be volatalised at a constant rate - since its mole fraction in the mixture would remain constant - and the attraction of the trail itself would also remain fairly constant.

Further work is necessary to identify the volatile components of the trail pheromone. When there are isolated vapour pressure and behavioural studies, on synthetic mixtures of the glandular constituents, can be used to check the idea that the Dufour glands aliphatic hydrocarbons act as an equivolatile-type keeper for the trail pheromone.

EXPERIMENTAL

EXPERIMENTAL

SECTION 1

(a) Collection of Ants

The ants were collected from two main areas. The first sample, some 9,000 individuals, was taken from the Isle of Purbeck, Dorset in September 1969. The second and third samples, some 30,000 individuals in all, were collected from Chesterton, Staffordshire between July and October 1970 and again over the same period in 1971.

The nests were dug up, the soil placed on a sheet and the ants collected with an aspirator. In the laboratory the ants were separated from any remaining soil and checked under the microscope to ensure that they were the correct species. The only species likely to be confused with Myrmica rubra is M. scabrinoidis, of which there seems to be large numbers in the Chesterton area, particularly in the coal mine shale heaps.

(b) Maintenance of Colonies

After identification, the colonies were transferred to artificial nests. Each nest consists of a 300 ml conical flask filled with 1 cm of moistened calcium sulphate (Fig.33). Arranged radially round the bottom of the flask are eight short lengths of tubing, five of which are connected to 10 x 80 mm test tubes covered so as to provide darkened accommodation for the queens and brood. Two of the other tubes serve as exits from the nest,

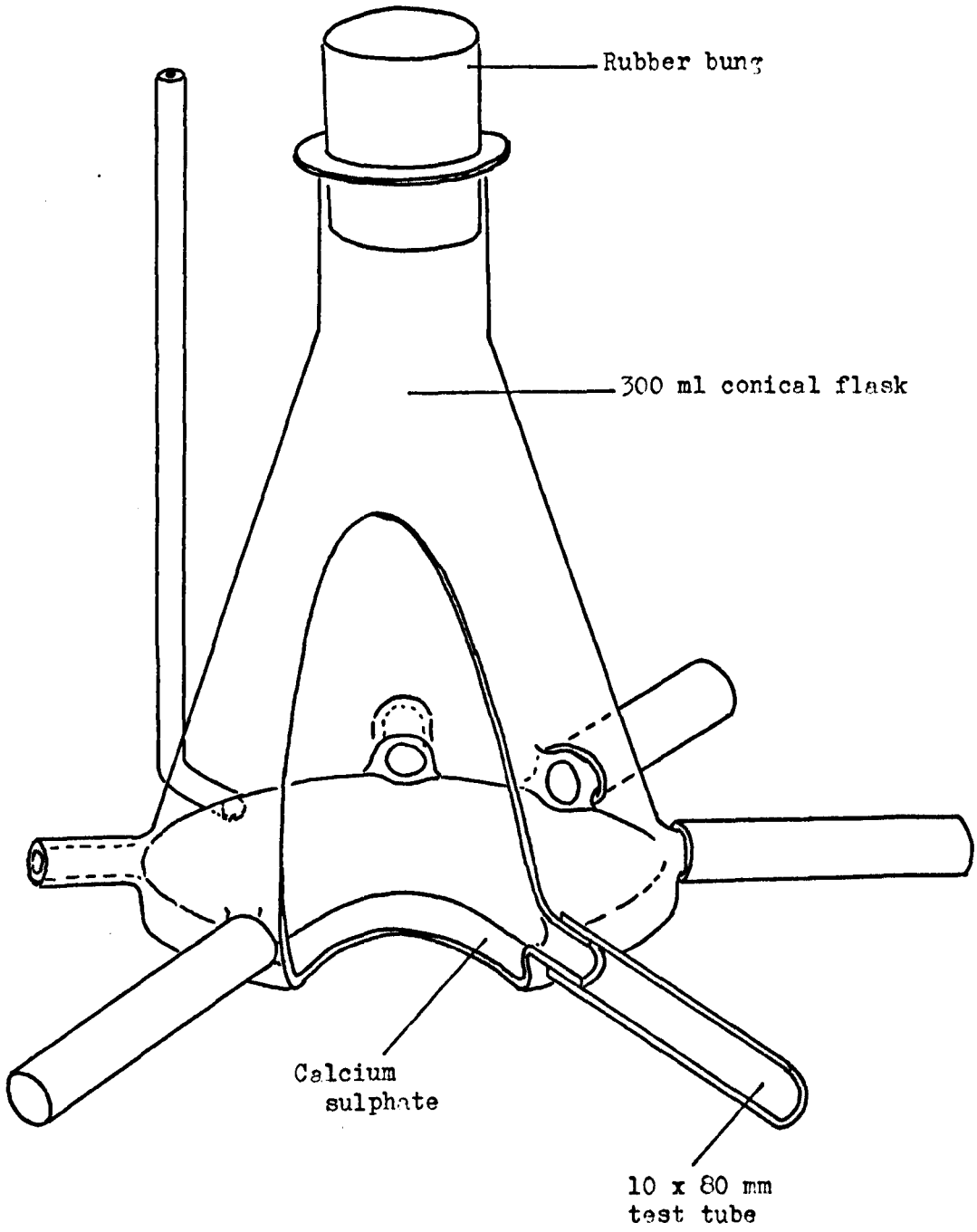


Fig.33 Artificial nest

whilst the third, which opens below the level of the calcium sulphate, provides a means of keeping the nest moist. The top of the nest is closed with a rubber bung.

After the ants were placed in the nest the exits were closed with cotton wool plugs and these left in position for 24 hours both to allow the ants to familiarise themselves with their environment and to ensure that the nest acquired the necessary colony odour. Each nest, containing one to four thousand ants, was placed in a 6 x 12 inch plastic washing up bowl, the sides of which were treated with polytetrafluoroethylene (ICI Fluon) to ensure that no ants escaped from their feeding area. The food consisted of 20 - 40% sugar solution, administered in specimen tubes fitted with cotton wool wicks, and live insects, which were either flies, mealworms or locusts depending on availability.

In this way stable colonies of Myrmica rubra were maintained from one year to the next.

(c) Gas Chromatography

The identification and isolation of the volatile constituents of the glands was carried out using a Pye Series 104 gas chromatograph fitted with dual hydrogen flame ionisation detectors.

Initial investigations of the glandular secretions were carried out on a 5 ft. x 0.25 inch stainless steel column of 5% SE 30 on 100/120 mesh Chromosorb M at a temperature of 140°C with a nitrogen flowrate of 50ml/min.

Further separation of the components was achieved using a 9 ft. x 0.25 inch glass column of 10% polyethylene glycol adipate on 100/120 mesh acid washed Gas Chrom M run isothermally at 135°C

and also programmed from 105°C to 160°C at 4°C/min. In both cases the nitrogen flowrate at 135°C was 50ml/min.

Comparison data for log plots and co-chromatography was obtained using a 9 ft. x 0.25 inch glass column of 15% Apiezon L on 100/120 mesh acid washed Gas Chrom M at a temperature of 225°C and with a nitrogen flowrate of 60ml/min.

The separation and identification of methyl esters of the fatty acids was carried out on a 9 ft. x 0.25 inch glass column of 5% diethylene glycol succinate on 100/120 mesh Chromosorb G at a temperature of 170°C and a nitrogen flowrate of 40 ml/min.

(d) Mass Spectroscopy

Mass spectra of the volatile constituents of the glandular secretions were obtained using combined gas chromatography-mass spectroscopy. The glandular material was introduced onto the gas chromatograph using the solid injection technique described in section 2(b).

Mass spectrometric data were obtained on an Hitachi Perkin-Elmer RMU 6 mass spectrometer (excitation energy 80 ev, accelerator voltage 1.8 kv and ion source temperature 200°C) linked through a Watson-Biemann separator by an all glass system to a modified Pye Series 104 gas chromatograph with a hydrogen flame ionisation detector.

One hundred and twenty Dufour glands and poison vesicles were analysed using the solid injection method [section 2(b)] However, with the large number of glands involved it was not possible to use the capillary method for introducing the glands into the soda glass vial. Instead a 20 x 2 mm length of soda

glass tube was employed. By careful manipulation with a pair of tweezers the end of this tube was formed into a shovel shape. Excised glands were placed on the tip of the shovel and gently pushed up into the tube. At all times during the disection the tube containing the glands was cooled on a slab of solid carbon dioxide. When all the glands had been placed in the tube it was introduced into the usual 2.5mm soda glass vial which was then carefully sealed in a flame. The contents of the vial were injected onto the gas chromatograph in the usual manner [section 2(b)].

Mass spectra of the alkane and alkene components (Figs.8,9,10, 11,15,20,23) of Dufour's gland were obtained using the 10% polyethylene glycol adipate column. The helium flowrate was 10 ml/min and the temperature was programmed from 125°C to 170°C at 4°C/min. Mass spectra of the terpene components (Figs.24,27,28) in the secretion were obtained from a further sample of one hundred and twenty glands using a 5% diethylene glycol succinate column programmed from 110°C to 170°C at 4°C/min with a helium flowrate of 10 ml/min.

Mass spectra of the volatile components of the mandibular glands (Figs.6,7) were obtained using the 10% polyethylene glycol adipate column programmed from 105°C to 170°C at 4°C/min with a helium flowrate of 10 ml/min.

It was found that the solid injection of whole worker heads gave the same gas chromatographic trace as that given by the injection of excised mandibular glands alone. Mass spectra of the two major components of the mandibular gland secretion were obtained on a sample of twenty whole worker heads analysed using the solid injection technique.

SECTION 2

Extraction of volatile components

Three main methods of extraction of the volatile material from the poison apparatus were employed.

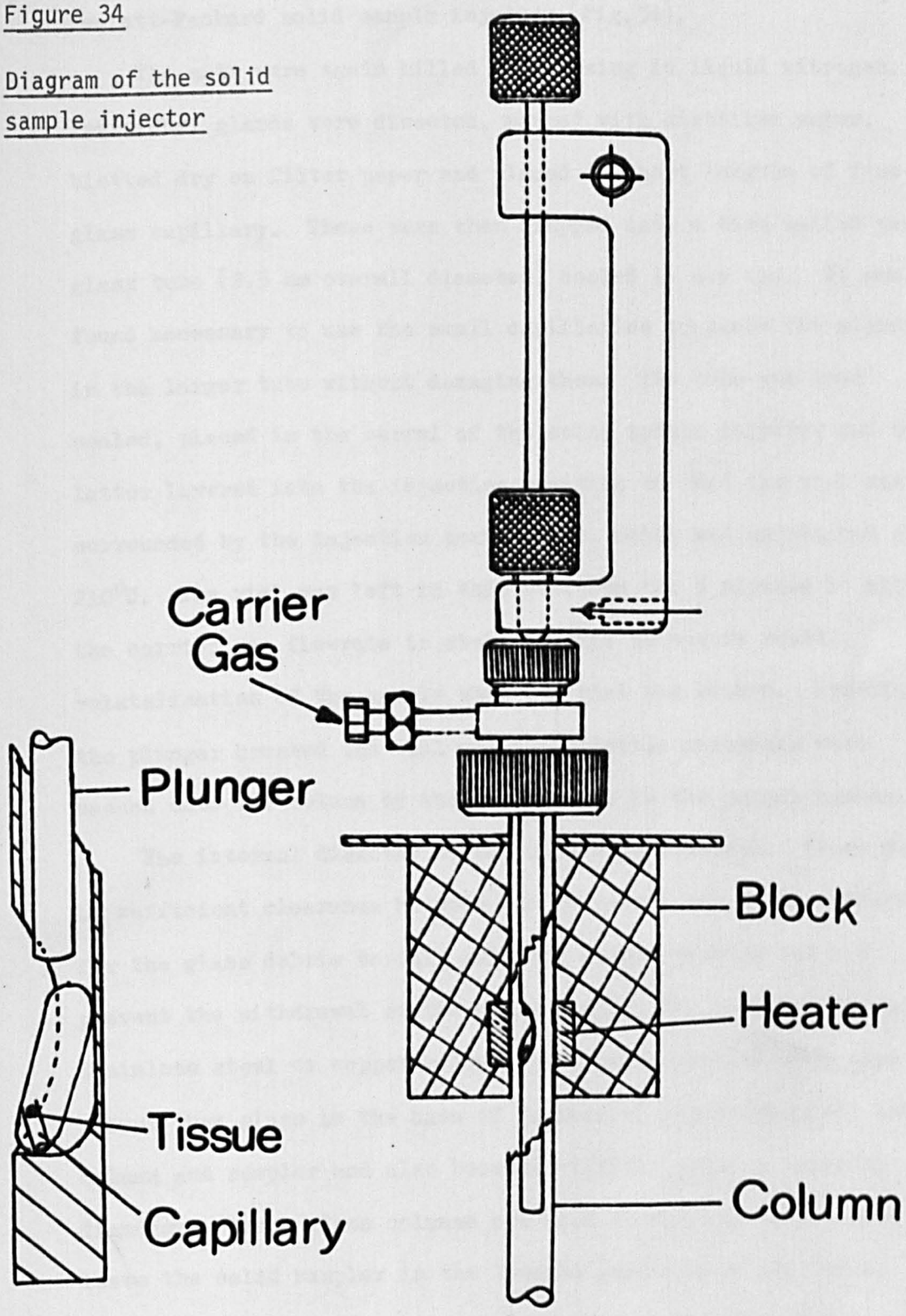
(a) In the first, some two hundred Dufour glands and poison vesicles were excised from Myrmica rubra workers. A gentle pull on the sting was usually sufficient to remove the glands and it was found that killing the ants by freezing them in liquid nitrogen resulted in the sting being extruded and greatly facilitated removal of the poison apparatus. The glands were removed with fine tweezers under a disecting microscope, washed in distilled water, blotted dry on filter paper and transferred to pentane cooled in liquid nitrogen. The glands were homogenised in a tissue grinder and the extract concentrated to 100 µl under a stream of nitrogen at room temperature.

Gas chromatography of this extract on a 5% SE 30 column showed only three major components. However, gas chromatography on the 10% polyethylene glycol adipate column gave twenty components, four of which constitute over 85% of the volatile material in the poison apparatus. (Fig.1).

(b) To ensure that no volatile components were lost during the concentration of the pentane extract, individual glands were analysed using the solid injection method. Essentially, the technique consists of sealing the glands in glass vials and inserting them into the heated zone of the injection block of a Pye Series 104 gas chromatograph modified to accommodate a

Figure 34

Diagram of the solid sample injector



Hewlett-Packard solid sample injector (Fig.34).

The ants were again killed by freezing in liquid nitrogen. Individual glands were disected, washed with distilled water, blotted dry on filter paper and placed in short lengths of fine glass capillary. These were then dropped into a thin walled soda glass tube (2.5 mm overall diameter) cooled in dry ice. It was found necessary to use the small capillaries to place the glands in the larger tube without damaging them. The tube was then sealed, placed in the barrel of the solid sample injector and the latter lowered into the injection position so that the vial was surrounded by the injection port heater, which was maintained at 210°C. The vial was left in this position for 5 minutes to allow the carrier gas flowrate to stabilise and to ensure rapid volatalisation of the sample when the vial was broken. Lowering the plunger crushed the vial and the volatile materials were washed onto the column by the carrier gas in the normal manner.

The internal diameter of the column is critical. There must be sufficient clearance between the walls and the solid sampler for the glass debris to fall onto the column packing and not prevent the withdrawal of the solid sampler for repeated injections. Stainless steel or copper columns were preferred as being more robust than glass in the case of jamming of glass fragments between column and sampler and also because of their greater internal diameter. Where glass columns are used it has been found best to leave the solid sampler in the lowered position at all times. The carrier gas flow must then be interrupted before the addition of each sample.

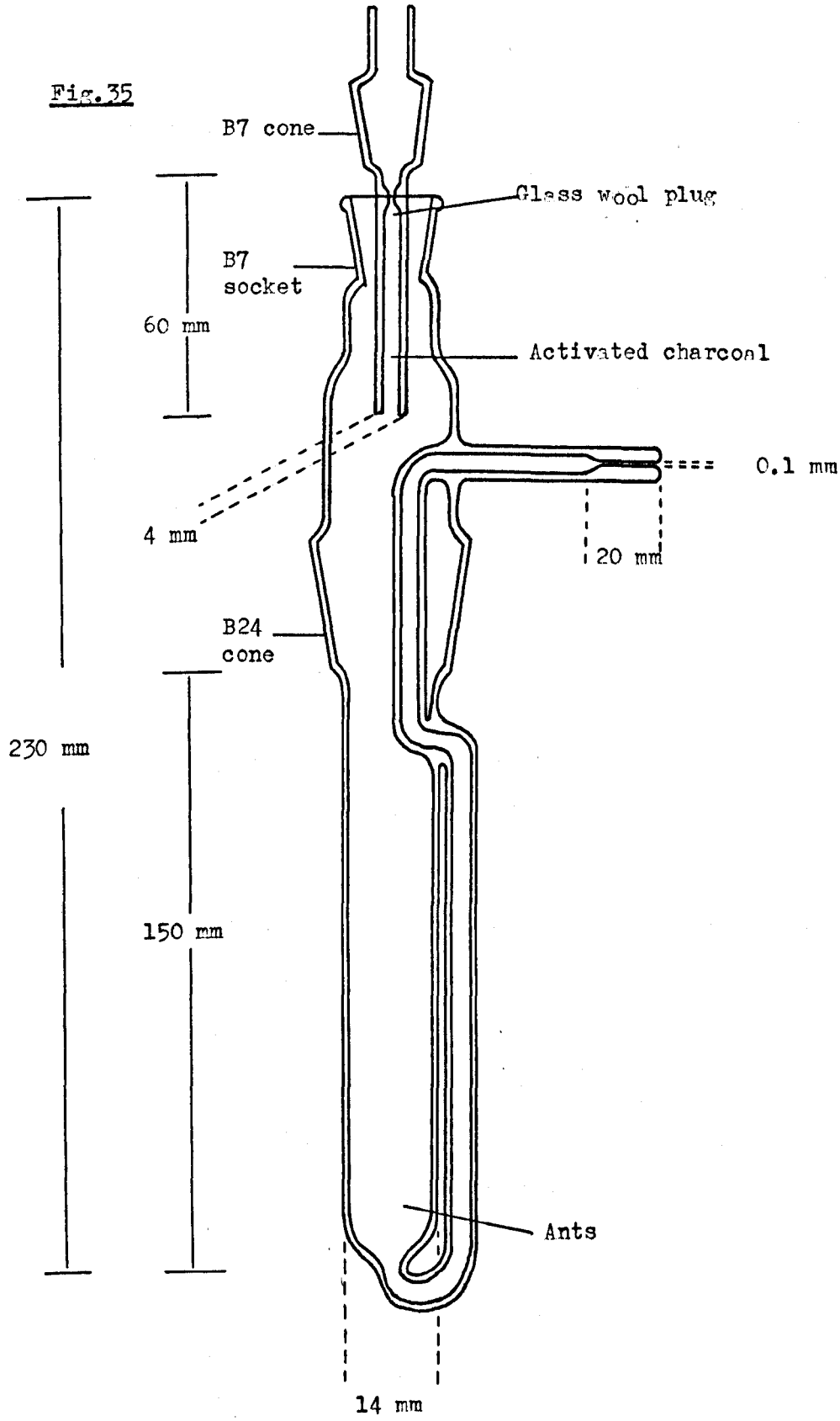
There was no evidence of thermal degradation or reaction of the

glandular material analysed in this manner. Gas chromatography of a single poison apparatus, using the solid injection technique, gave the same twenty peaks (Fig.3) and in the same proportions as those shown in the trace from the pentane extract of two hundred glands, together with several further components that had been obscured by the solvent. However, these components represented a very small fraction of the whole gland extract.

(c) In the third method the volatile materials were extracted from whole ants, or gasters and heads separately, by distillation under reduced pressure. The organic material was trapped in activated charcoal and recovered at the end of the distillation by eluting the charcoal with carbon disulphide.

The apparatus (Fig.35) consists of a 14 x 230 mm glass tube with a B 24 cone 150 mm from the bottom and a B 7 socket at the top into which fits a 4 x 60 mm tube containing 50 mgs of 30 - 60 mesh charcoal which was activated by heating for eight hours at 300°C under a stream of nitrogen. The other end of this tube is connected via a liquid nitrogen trap to an oil pump. One to two thousand ants, killed by freezing in liquid nitrogen, were placed in the distillation tube, the charcoal trap inserted and the apparatus placed in one arm of a three necked 5 litre flask containing refluxing Diglyme (b.p. 164°C). Distillation under nitrogen, introduced via the fixed bleed, was continued for four to five hours at 164°C and a pressure greater than 0.1 mm Hg. The volatile organic material was collected in the charcoal trap whilst the large volume of water obtained passed through this and was condensed in the liquid nitrogen trap which also ensured that

Fig.35


B7 cone
Glass wool plug
B7
socket
60 mm
Activated charcoal
0.1 mm
4 mm
20 mm
B24
cone
230 mm
150 mm
Ants
14 mm

there was not back diffusion of pump oils into the system. At the end of the distillation the charcoal was removed from the trap, the volatile components eluted with 4 mls of carbon disulphide and the extract concentrated under a stream of nitrogen at room temperature.

Except for the presence of two additional peaks of short retention time, the material isolated in this manner gave the same gas chromatographic trace (Fig.4) as that obtained from the solid injection of whole glands. These two additional components originate from the mandibular glands (Fig.5) and in later extractions heads and gasters were distilled separately.

(d) Reaction Gas Chromatography

Reaction of the Dufour gland secretion with bromine and concentrated sulphuric acid was achieved using a modification of the solid injection technique described in section 2 (b).

Approximately 1 µl of reactant in another short length of capillary was placed in the soda glass tube together with the glands. The vial was then sealed and the contents injected onto the gas chromatograph in the usual manner.

Reaction of the glandular components with concentrated sulphuric acid resulted in the elimination from the gas chromatograph trace of all peaks except 1,4,10,12,15 and 19. Similarly injection of three Dufour glands together with 1 µl of bromine in carbon disulphid (1:1, v/v) again resulted in the elimination of all peaks except those mentioned (Fig.14), together with the production of several new components presumably derived from bromination products of low molecular weight components.

(e) Preparative Gas Chromatography

Preparative gas chromatography of the material from the distillation of whole ants was carried out on a Pye Series 104 gas chromatograph with the 10% polyethylene glycol adipate column and fitted with an all glass splitting system. The splitting ratio was 20:1. Fractions were collected in separate traps cooled in liquid nitrogen. The material was recovered by washing the traps with 4 mls of carbon disulphide and the sample then concentrated to 100 µl under a stream of nitrogen at room temperature.

In the case of material for ozonolysis the solvent used was methylene chloride.

(f) Ozonolysis

Ozonolysis of the material obtained from preparative gas chromatography was carried out at room temperature using a British Oxygen Company mark 2 ozoniser. The sample, in 0.5 mls methylene chloride, was placed in a glass tube 60 x 4 mm with a side arm vent 20 mm from the top. Ozone was bubbled through the solution at 10 ml/min until a starch iodide paper held at the outlet showed that there was excess ozone present. The solution was then purged with nitrogen and a crystal of triphenyl phosphine added to decompose the ozonides. Gas chromatography and mass spectrometry were carried out immediately as the sample deteriorated rapidly on standing.

Gas chromatography of an ozonised sample of heptadecene showed elimination of the heptadecene peak and the production of two new peaks of shorter retention time and approximately equal area (Fig.16). The mass spectra of these components showed parent ions at m/e 128 and m/e 142 respectively (Fig.17).

Co-chromatography on both Apiezon L and polyethylene glycol adipate columns with an authentic sample of octanal gave no new peaks but showed enhancement of the first of the two peaks.

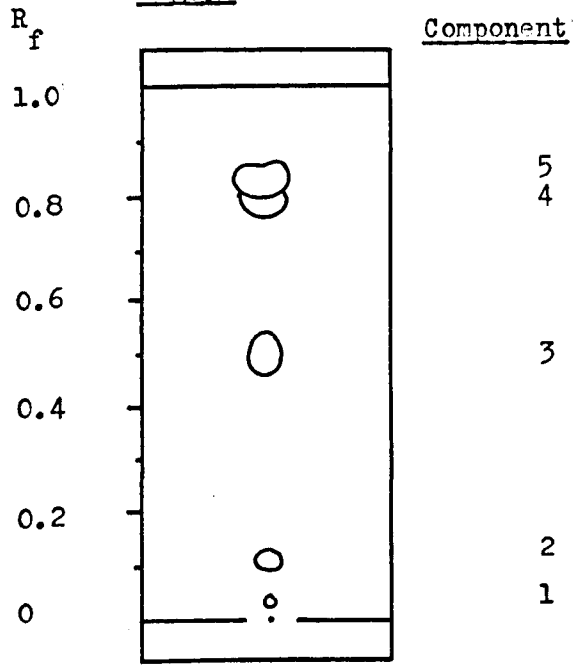
Similarly, gas chromatography of an ozonised sample of nonadecene showed elimination of the nonadecene peak and the production of two new peaks of shorter retention time and approximately equal area (Fig.21). Co-chromatography of the ozonised material with a sample of octanal on both Apiezon L and polyethylene glycol adipate columns gave three peaks (Fig.22). A plot of the log of the retention times of these three components against carbon chain length gave a straight line.

(g) Thin Layer Chromatography

Thin layer chromatography was carried out on 5 x 20 cm glass plates coated with silica gel PF 254 (Merck). The solvent, light petroleum (b.p. 40-60°C) and ether (9:1), was allowed to run 15 cms up the plate. Components were visualised with either iodine, concentrated sulphuric acid or antimony pentachloride in carbon tetrachloride (2:8, v/v).

Thin layer chromatography of a pentane extract of 30 glands showed 5 components (Fig.36) all of which were visualised with iodine, concentrated sulphuric acid and antimony pentachloride. Similar results were obtained with the material from the distillation of gasters.

Fig.36



Gas chromatography of components 3 and 5 was achieved using a modification of the solid injection technique described in section 2 (b). A constriction was pulled in the soda glass tube and this plugged with glass wool. One end of the tube was attached with plasticine to a vacuum line and the silica sucked off the t.l.c. plate into the soda glass tube. This was then sealed and the contents injected onto the gas chromatograph in the usual manner. The gas chromatographic trace showed all twenty peaks to be present in both t.l.c. components.

Assignment of the stereochemistry of the double bond in 8-heptadecene and 9-nonadecene was achieved using silver nitrate impregnated t.l.c. plates. These were prepared by immersing standard 5 x 20 cm glass plates coated with silica gel PF 254 (Merck) face downwards in a solution of 10% silver nitrate in acetonitrile for 30 minutes. The plates were then removed from the bath, dried in an oven at 120°C for 1 hour and stored in the dark. The solvent, petroleum ether (b.p. 40-60°C), was again allowed to run 15 cms up

the plate and the components were visualised with concentrated sulphuric acid.

Thin layer chromatography of samples of cis and trans-8-heptadecene, provided by Coggins[49] and Johnson[50], together with the material from the distillation of gasters showed the major component of the Dufour gland extract to have the same Rf value (0.087) as the synthetic cis isomer (Fig.19). No component was present in the extract that would correspond to a trans configuration.

(h) Fatty Acid Determination

Analysis of the total fatty acids of worker ants was carried out by a conventional method. Whole bodies (1.5 g) were allowed to stand in 50 ml of 0.6M potassium hydroxide in methanol overnight, then refluxed for 1 hour, allowed to cool and acidified with dilute sulphuric acid. The free acids were extracted with ether, washed twice with water and dried over anhydrous potassium sulphate. The solvent was removed and the sample esterified by heating with methanol containing boron trifluoride (15 ml) for 6 minutes. The solution was cooled, water and ether added and the ether phase dried over molecular sieves.

The methyl esters were chromatographed on the 9ft. column of 5% diethylene glycol succinate at 170°C.

SECTION 3

(a) Bioassay

Samples were tested for trail pheromone activity using the method described by Blum. A circular trail nine centimeters in diameter was laid on a porous tile and 6 ants placed in the centre. A positive response was recorded when one ant completed a full circle without deviating from the trail.

To ensure reproducibility and even distribution of the sample, the tile was placed on a rotating stage turned at constant speed by an electric motor (Fig.37). Ten microlitres of the sample, in carbon disulphide, was sucked up into a capillary mounted on an arm above the stage and the trail laid by bringing the tip of the capillary into contact with the rotating tile. At the end of each test the tiles were heated at 120°C for 1 hour to ensure removal of all volatile material. All solvents used were pretested for trail pheromone activity.

A pentane extract of one hundred homogenised whole ants in 0.5 ml of pentane showed no trail pheromone activity. However, an extract of two hundred Dufour glands and poison vesicles in 1 ml of pentane gave a strong positive response.

Bioassays of fractions obtained from the preparative gas chromatography of the material from the distillation of gasters showed pheromone activity to be spread over peaks 7 - 11 (Fig.3).

(b) Electroantennogram

Electroantennograms were obtained initially on excised antennae using a modification of the method described by Roelofs *et al*[56].

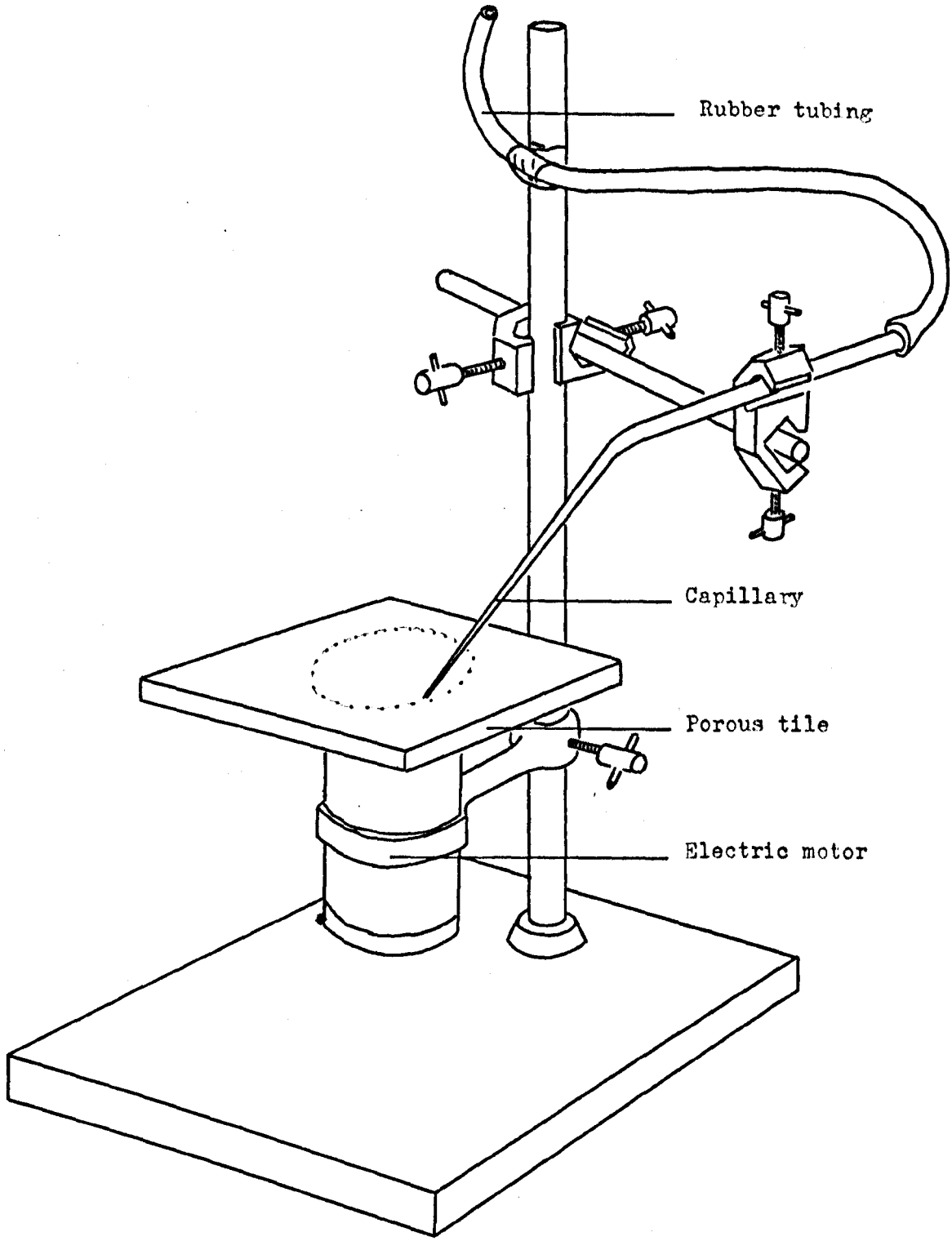


Fig.37 Artificial trail applicator

Essentially the technique consists of linking a gas chromatograph with the insects antennal sense organs prepared for the recording of electroantennograms. The volatile constituents of the poison apparatus were separated on the gas chromatographic column and the effluent split between a flame ionisation detector and the antenna (Fig.29 - Appendix). Simultaneous recordings of the gas chromatograph detector and insect responses enable the correlation of retention time with electroantennogram responses.

The gas chromatograph was linked to the antenna using the system described by Yeadon et al[61]. A Pye Series 104 gas chromatograph was fitted with an all glass splitting system. The split ratio was such that 80% of the column effluent went to the dectector and the rest passed into a 10 ml, three necked flask located in the chromatograph oven. The effluent accumulating in this flask was flushed out at intervals by a stream of nitrogen passed in through A and out through B (Fig.29). A second nitrogen stream was used to cool the gasses below 30°C before they reached the antenna. The nitrogen stream was monitored by a flowmeter and the timing sequence controlled by an electronic timer in series with a solenoid valve. The nitrogen flowrate was 5 litre/min and the column effluent was allowed to accumulate for 55 secs and then flushed over the antenna for 5 secs.

The excised antenna was mounted on the stage of a disecting microscope with a lump of wax and the two distal segments removed with a pair of scissors. Two glass microelectrodes, 2 microns in diameter were prepared using a microelectrode puller. These were filled with locust ringer solution - NaCl 7.6 gms., KCl 0.75 gms., $CaCl_2$ 0.22 gms., $MgCl_2$ 0.19 gms., $NaHCO_3$ 0.37 gms., NaH_2PO_4 0.48 gms.

made up in 1 litre of distilled water. The electrodes, mounted on a pair of Prior micromanipulators, were inserted into the two cut ends of the antenna. Short lengths of chloridised silver wire connected the electrodes with the input of a Fenlow AD 55 d.c. amplifier mounted on one of the micromanipulators. The preamplifier signals were amplified 10 times and fed into a Tectronix storage oscilloscope. The responses were either measured directly from the oscilloscope trace or fed into a Servoscribe chart recorder. The whole apparatus was located inside a grounded Faraday cage to screen out extraneous electronic signals.

However, this technique suffered from several disadvantages. The main being the death of the antenna after only twenty minutes. In order to prolong the life of the antenna it was found necessary to use whole ants. Unfortunately, the glass electrodes used in the original technique were not sufficiently robust to pierce the cuticle of the ant. Instead, fine tungsten electrodes were used.

The ant was pinned to a cork placed on the microscope stage with short lengths of wire used rather like staples. One electrode was inserted into the cut distal end of the antenna and the other electrode was inserted in the antennal socket where it joins the head. In this way antennal life was prolonged and one preparation was sufficient to monitor several runs on the gas chromatograph.

Whole glands were injected onto the gas chromatograph using the solid injection method described in section 2 (B). Four glands were used in each experiment. In order to ensure that the solid injection technique did not result in any degradation or reaction of the active components in the glandular secretion, electroantennograms were also recorded on an extract from the distillation of gasters.

This extract gave a positive response on bioassay and the electroantennograms responses were in agreement with those obtained from the solid injection of whole glands. (Fig.31).

REFERENCES

REFERENCES

1. C. Bonnet, Oeuvres d'Histoire Naturelle et de Philosophie Neuchatel. 1779 - 1783, I, 523.
2. A. Bethe, Arch. gesamt. Physiol. 1898, 70, 15.
3. A. Forel, "The Senses of Insects", Methuen, London, 1908.
4. H. Eidmann, Rev. Zool Russe, 1927, 7, 39.
5. R.W.G. Hingston, "Problems of Instinct and Intelligence", Arnold, London, 1928.
6. F. Santschi, Rev. Zool. Afr., 1923, 11, 111.
7. J.D. Carthy, Behaviour, 1951, 3, 304.
8. W. Goetsch, Z. Morphol. Ökol. Tierre, 1934, 28, 319.
9. F.E. Regnier, Biol. of Reproduction, 1971, 4, 309.
10. P. Karlson and M. Lüscher, Nature, 1959, 179, 55.
11. P. Karlson and A. Butenandt, Ann. Rev. Entomol., 1959, 4, 39.
12. K. von Frisch, "The Dancing Bees", Methuen, London, 1927.
13. R. Boch and D.A. Shearer, Nature, 1962, 194, 704.
14. A.M. Stuart, Nature, 1961, 189, 419.
15. B.P. Moore, Nature, 1966, 211, 746.
16. E.O. Wilson, Psyche, Camb., 1959, 66, 29.
17. J.H. Sudd, Nature, 1960, 183, 1588.
18. J.H. Sudd, "An Introduction to the Bheaviour of Ants", Arnold, London, 1967.
19. M.S. Blum and G.N. Ross, J. Insect Physiol., 1965, 11, 857.
20. E.O. Wilson, Science, 1959, 129, 643.
21. W. Hangartner, Z. Vergl. Physiol., 1969, 62, 111.

22. E.O. Wilson, Ann. Rev. Entomol., 1963, 8, 345.

23. J.D. Carthy, Nature, 1950, 166, 154.

24. W. Hangartner and S. Bernstein, Experientia, 1964, 20, 392.

25. W. Hangartner, J. Insect Physiol., 1969, 15, 1.

26. E.O. Wilson, Ann. Rev. Entomol., 1963, 8, 345.

27. D. Fletcher and J. Brand, J. Insect Physiol., 1968, 14, 783.

28. E.O. Wilson, Anim. Behaviour, 1962, 10, 134.

29. E.O. Wilson and M. Pavan, Psyche, Camb., 1959, 66, 70.

30. M.S. Blum and G.N. Ross, J. Insect Physiol., 1965, 11, 857.

31. E.O. Wilson and W.H. Bossert, Recent Progr. in Hormone Research, 1963, 19, 673.

32. J.C. Moser and R.M. Silverstein, Nature, 1967, 215, 206.

33. J.H. Tumlinson, R.M. Silverstein, J.C. Moser, R.G. Brownlee and J.M. Ruth, Nature, 1971, 234, 348.

34. P.S. Callahan, M.S. Blum and J.R. Walker, Ann. ent. Soc. Am., 1959, 52, 573.

35. R.H. Hermann and M.S. Blum, Ann. ent. Soc. Am., 1967, 60, 661.

36. G.W.K. Cavill and P.J. Williams, J. Insect Physiol., 1967, 13, 1097.

37. M.S. Blum and P.S. Callahan, Psyche, Camb., 1963, 70, 69.

38. E.O. Wilson, Psyche, Camb., 1958, 65, 41.

39. D.J. McGurk, J. Frost, E.J. Eisenbraun, K. Vick, W.A. Drew and J. Young, J. Insect Physiol., 1966, 12, 1435.

40. U.W. Maschwitz, Nature, 1964, 204, 324.

41. R.M. Crewe and M.S. Blum, J. Insect Physiol., 1970, 16, 141.

42. F.E. Regnier and E.O. Wilson, J. Insect Physiol., 1968, 14, 955.

43. G.W.K. Cavill, P.J. Williams and F.B. Whitfield, Tetrahedron

Lett., 1967, 23, 2201.

44. E.F.L.J. Anet, Aust. J. Chem., 1970, 23, 2201.

45. G. Bergström and J. Löfqvist, J. Insect Physiol., 1968, 14, 955.

46. G. Bergström and J. Löfqvist, Proc. 2nd. Int. IUPAC Congr. of Pesticide Chem., III, 1971, 195.

47. J. Hoff and E. Feit, Anal. Chem., 1963, 35, 1298.

48. J. Hoff and E. Feit, Anal. Chem., 1964, 36, 1002.

49. P. Coggins, Undergraduate Research Project, Keele Univ., 1972.

50. Q. Johnson, Undergraduate Research Project, Keele Univ., 1972.

51. R.C. Hill, R.I. Reed and M.T. Robert-Lopes, J. Chem. Soc., 1968, 93.

52. Y. Hirose, Shitsuryo Bunseki, 1967, 15, 162.

53. A.S. Meyer, E. Hanzmann, H. Schneiderman, L.I. Gilbert and M. Boyette, Archiv. Biochem. Biophys., 1970, 137, 190.

54. R. Yamamoto and M. Jacobson, Nature, 1962, 196, 908.

55. J. Jentsch, Proc. int. Congre. social insects, 1969, 6, 69.

56. M. Beroza and B.A. Bierl, Proc. 2nd. Int. IUPAC Congr. of Pesticide Chem., III, 1971 115.

57. D. Otto, Zool. Anz., 1960, 164, 42.

58. M.F.A. Osman and W. Kloft, Insectes Soc., 1961, 8, 383.

59. F. Bernard, Traité de Zoologie, 1951, 10, 713.

60. W.L. Roelofs and A. Comeau, J. Insect Physiol., 1971, 17, 1969.

61. J.E. Moorhouse, R. Yeadon, P.S. Beevor and B.F. Nesbitt, Nature, 1969, 223, 1174.

A P P E N D I X

A P P E N D I X

Photograph No.1

The electroantennogram/gas chromatograph set up.

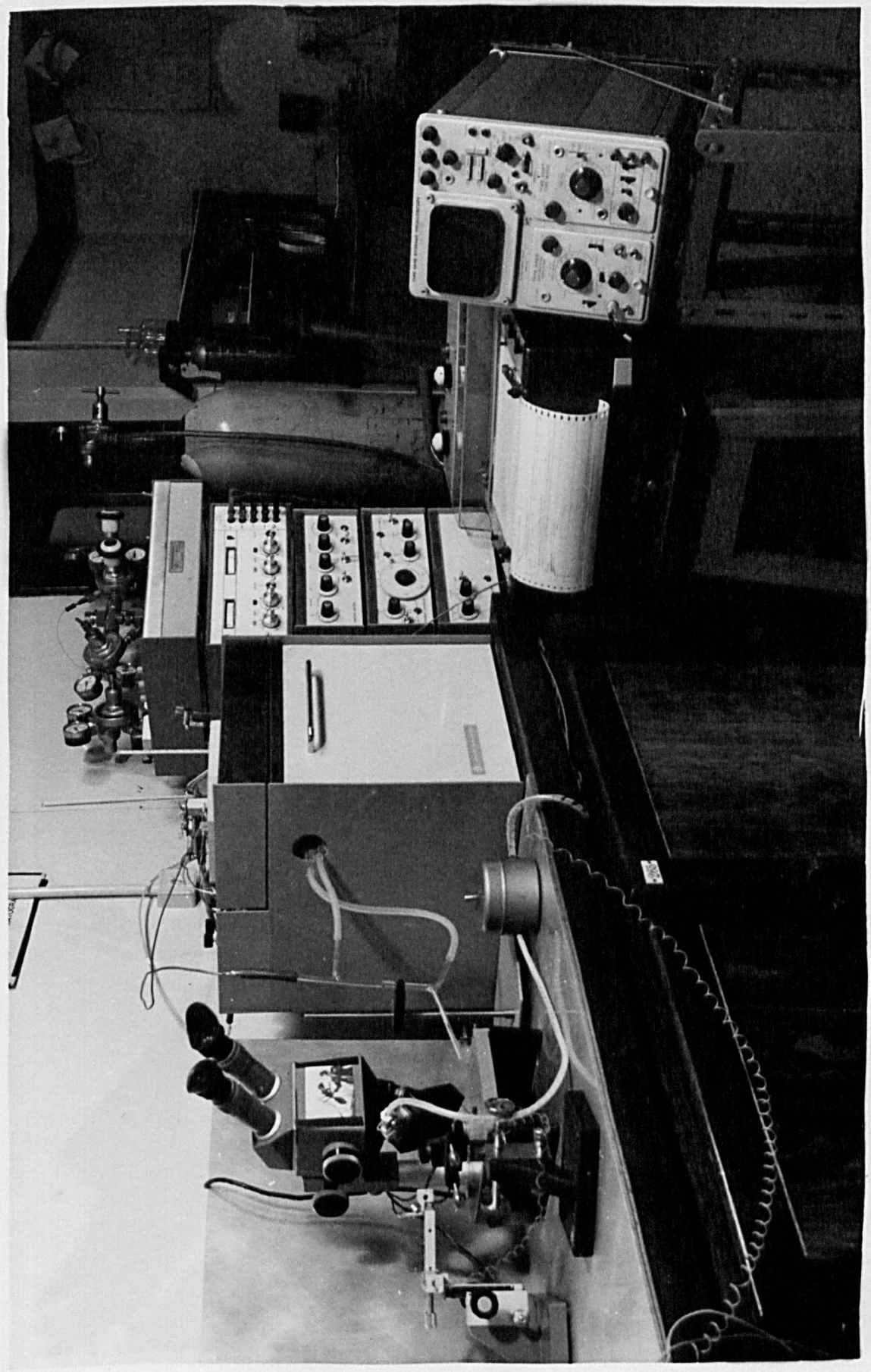
Photograph No.2

The electroantennogram apparatus showing the amplifier and electrodes mounted on the micromanipulators.
The effluent from the gas chromatograph is passed over the antenna by the glass tube on the right.

Photograph No.3

Scanning electron micrograph of the distal segment of the antenna showing the two different types of sensilla (x 8,800).

Photograph No. 1



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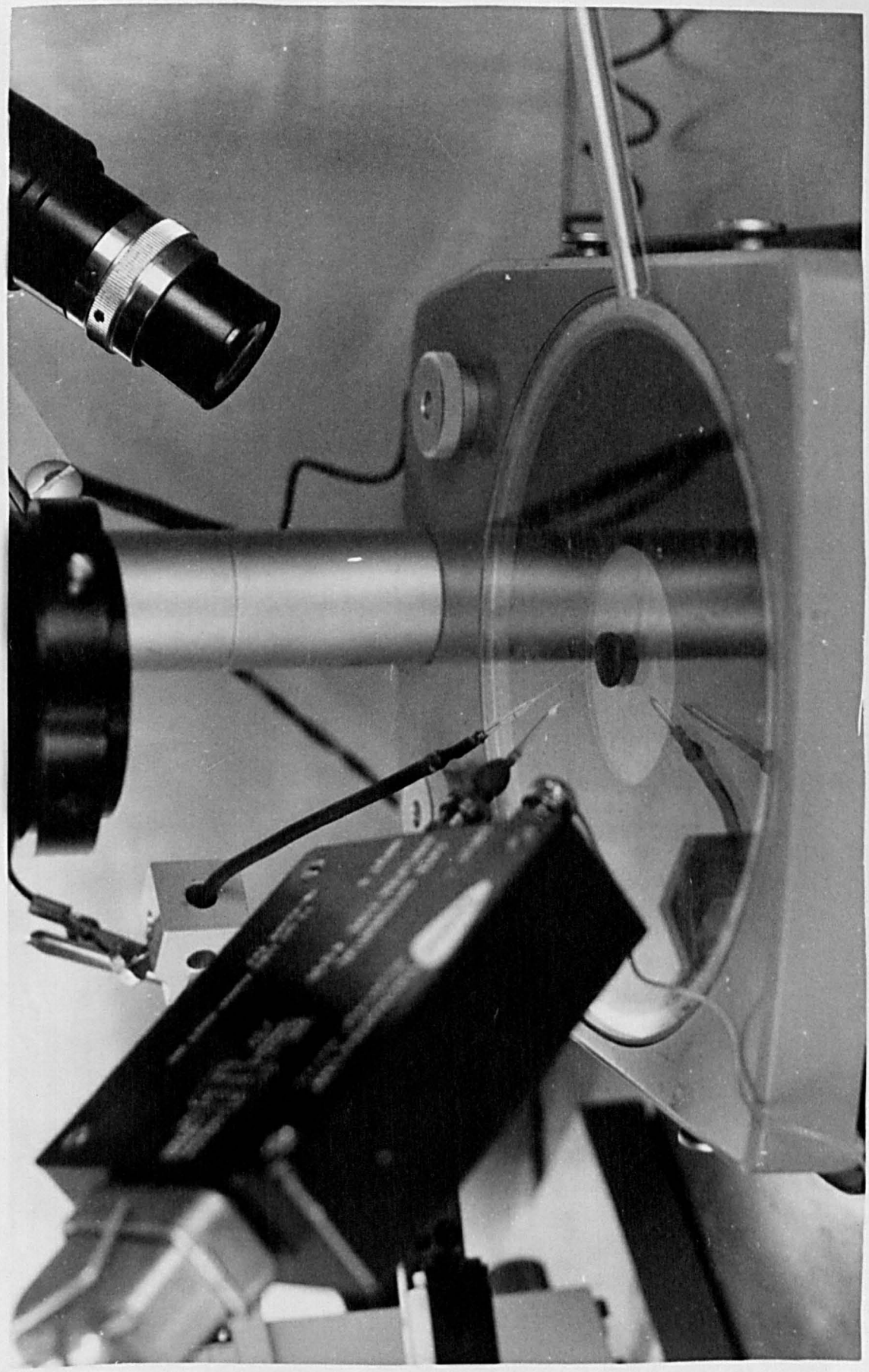

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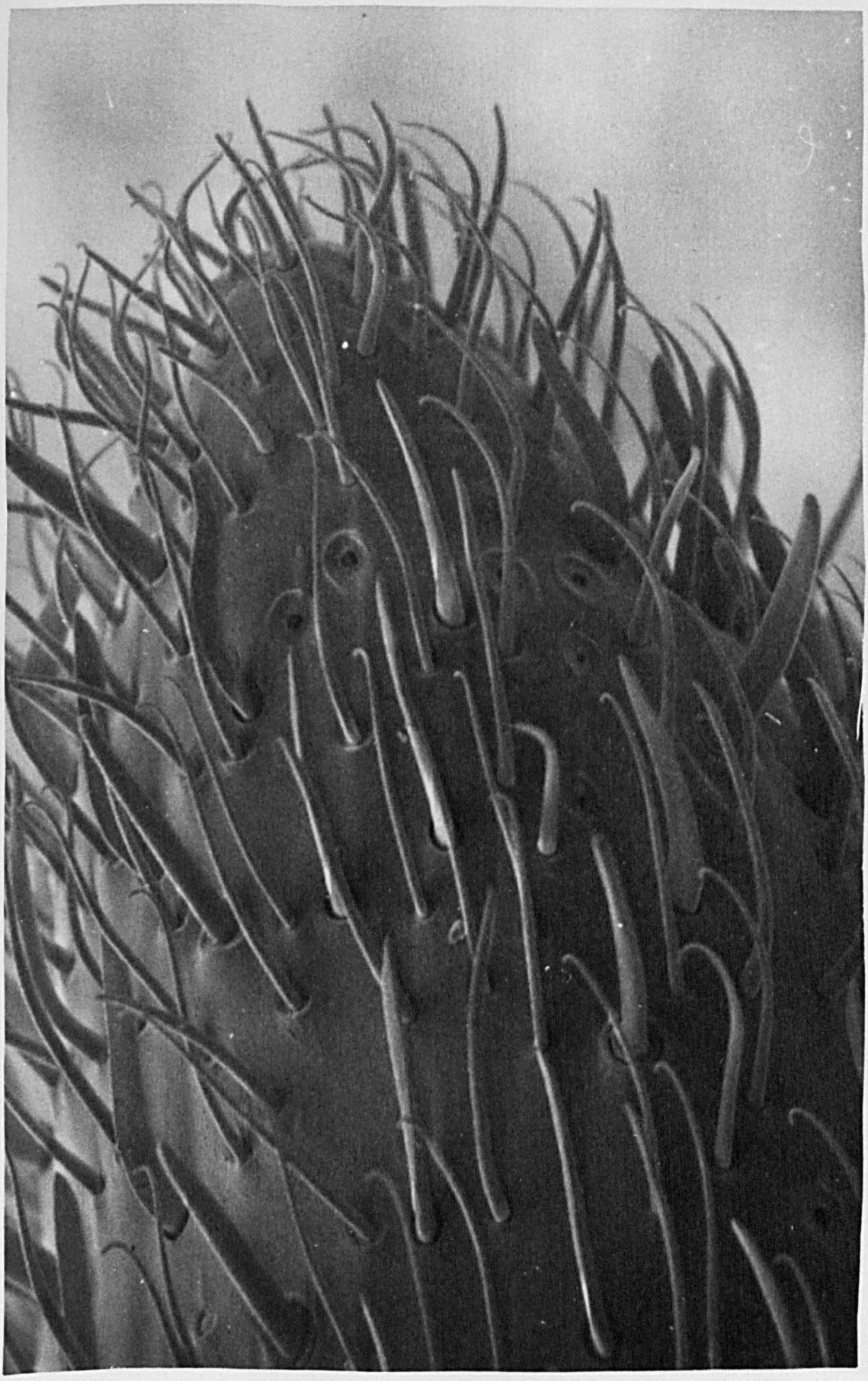

SUMMARY

The volatile constituents of the Dufour gland secretion in *Myrmica rubra* have been examined by combined gas chromatography-mass spectrometry. The principal components are *cis*-8-heptadecene, n-pentadecane, n-heptadecane and *cis*-9-nonadecene. The other constituents comprise mainly other alkanes and alkenes, together with three related sesquiterpenes. These are *cis,trans*-α-farnesene, homofarnesene and bishomofarnesene.

A technique has been developed for the direct on-column injection of individual glands. Essentially, this consists of sealing the glands in a thin walled glass vial and inserting them into the heated zone of the injection port of a Pye Series 104 gas chromatograph, modified to accommodate a Hewlett-Packard solid sample injector. When the vial is crushed the volatile material is carried onto the column by the carrier gas in the usual manner.

The glandular origin and chemical nature of the trail pheromone in *M. rubra* has been investigated by means of an electroantennogram. The pheromones are minor components of the Dufour gland secretion. There are two active constituents, both of which are polar and it is thought that they are related compounds differing only by one carbon atom. It is suggested that the function of the aliphatic hydrocarbons in the Dufour gland secretion is to regulate the volatilisation of the trail pheromone.