VOLATILE CHEMICALS OF THE ANTS

MYRMICA RUBRA L. AND *M. SCABRINODIS* NYL.

by

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ABSTRACT

The most volatile constituents of the sting apparatus of the ants Myrmica rubra L. and M. scabrinodis Nyl. have been examined by reaction gas chromatography and found to contain nanogramme quantities of simple alcohols, aldehydes and ketones.

A technique has been developed to permit the extraction of glandular liquid directly from the component glands of the sting apparatus. Essentially, for this technique, the appropriate gland was dissected from the ant, pierced with a fine glass tube and its contents withdrawn by capillary action. The filled portion of capillary was then sealed into a small glass ampoule and introduced into the gas chromatograph using an existing solid sampling technique. The technique was also used to calculate the volume and water content of the glandular liquid.

In both species, Dufour's gland contains between 100 ng and 150 ng of oxygenated volatiles dissolved in anhydrous hydrocarbons. The principal components are ethanal, propanone, methylpropanal, butenone and butanone with smaller quantities of methanol, ethanol, butanol and propanal.

The poison vesicle of both species also contains methanol, ethanal, ethanol, propanal, propanone, methylpropanal and butanone, together with 2- or 3-methylbutanal (probably the latter), butane, traces of methane, ethane and propane and three unidentified components. No butenone or butanol is present in this gland.

The concentration of the volatiles in the poison vesicle, where they are in aqueous solution, is some five times less

than in Dufour's gland, but is still about twenty times greater than in the haemolymph.

It is suggested that these oxygenated compounds are deposited by older workers on their foraging area and are used as a recruitment pheromone to a freshly laid trail in the case of the poison vesicle secretion. In the Dufour's gland secretion the same compounds are volatilised relatively slowly from their hydrocarbon solution: they attract other workers and induce them to move in a rapid, sinuous and exploratory manner, thus serving as a recruitment and foraging pheromone for new territories.

Ethological tests performed in another laboratory on M. rubra have shown that three effects recognised earlier for the Dufour's gland can be attributed to four of its components. Ethanal synergised by ethanol produces an attractive effect on foraging workers. Propanone induces an increased linear speed, and changes in sinuousity of movement are induced by ethanol synergised by butanone. Ethanol, butanone or mixtures of all four compounds induce the deposition of Dufour's gland secretion on the foraging area.

The electrophysiological response of ants to these four compounds was assessed by recording their electroantennogram.

The poison vesicle contents of each species will induce trail following in either species, but none of the volatiles which have been tested was found to elicit trail following behaviour.

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'Go to the ant, thou sluggard;
consider her ways, and be wise:'

Proverbs vi. 6

INTRODUCTION

INTRODUCTION

The survival of any animal species, above a certain level of complexity, is dependent upon communication between the members of that species. In solitary animals this communication may be confined to the sexual attraction of a mate, but for more gregarious animals an efficient system of communication is vital for the co-ordination of social behaviour.

It was originally thought that animals only employed two senses in communication: consequently their behaviour was interpreted solely in visual and auditory terms. Whilst accounting for much of the behaviour of the higher vertebrates, these modes of communication could not account for the long-ranging sexual attraction observed in invertebrates, particularly among insects and notably for some lepidoptera.

As early as the 18th century, naturalists like Réne de Réaumur[1] observed that virgin females of the silk moth Bombyx mori could lure males of that species from distances of one kilometre. Clearly, visual and auditory communication could not be effective at this range, so it was suggested that a third sense, that of smell, might be involved.

In 1879 Jean Henri Fabre[2] demonstrated clearly that males of the Oak Bombyx moth were attracted by the 'odourous effluvia' of the female, by picking up a female moth and sealing her under a glass bell jar. He observed that male moths paid no attention to her, but were attracted to the oak branch on which she had been sitting a few minutes earlier and which she had 'impregnated with her emanations'. He wrote, 'This quintessance easily impregnates every object on which the female rests for

any length of time; and thenceforth the actual object becomes as potent a centre of attraction as the mother herself, until the emanations are dispelled'. He concluded, 'It is the smell therefore that guides the moths, that gives them information at a distance'.

Until comparatively recently little was known of the chemical nature of these scents used in communication, because of the technical difficulties involved in their extraction and analysis. In the case of Bombyx mori, Adolf Butenandt[3] and his colleagues worked for almost 10 years to extract a quarter of a million virgin female glands in order to obtain 10 milligrammes of the pure sexual attractant 'Bombykol' (I), identified as (E,Z)-10,12-hexadecadien-1-ol.

(I)

In the last 25 years technological advances in scientific instruments, making possible the separation and identification of tiny samples, have led to an enormous increase in the number of chemicals identified in a whole range of insect species.

Pheromones

Karlson[4] suggested that these volatile chemicals which are used for chemical communication between members of the same species should be termed Pheromones. These pheromones are different from hormones in that they originate in exocrine glands and are released from the body of the animal: hormones are produced by endocrine glands and are secreted internally to regulate the organism's own physiology.

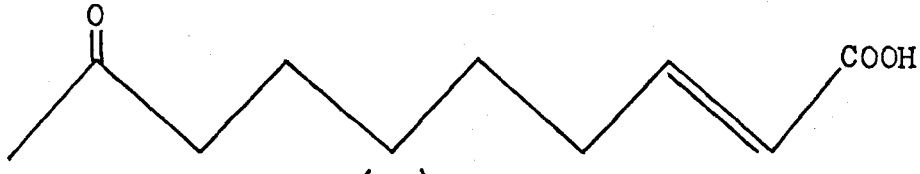
A number of other terms have been suggested for different

categories of exocrine secretions used in chemical communication, although these have not been as widely adopted as the term 'pheromone'.

Law and Regnier[5] suggested the name Semiochemicals as a general term for volatile chemicals used in communication either within a species (intraspecific) or between different species (interspecific). The terms allomone and kairomone[6] have also been used for interspecific semiochemicals.

Although the term pheromone was originally suggested for sex attractants such as 'Bombykol', it is now applied to the range of chemicals used to control both development and organisation in social insect life. It has been suggested[7] that the social insects make extensive use of this pheromonal communication because of their need for a system of communication which is both operable and efficient in the dark interiors of their nests.

There are two distinct categories of pheromone which are classified according to their mode of action. Those which initiate a chain of physiological changes affecting the development of the recipient are termed Primer pheromones.[8] One of the few primers identified in social insects is (E)-9-oxo-2-decenoic acid (II), which forms the 'queen substance' of the honey bee Apis mellifera.[9,10] This compound inhibits both queen rearing and ovarian development in worker bees.



(II)

No primer pheromone has been identified in ants, although an acidic extract of fertile queen heads has been reported to

depress larval growth in the ant Myrmica rubra.[11]

The other category of pheromone, Releaser pheromones,[8] release behavioural activity in a classical stimulus-response manner. The most extensively studied of these releasers in social insects are those concerned with alarm communication and colony defense. This group of releasers, often loosely referred to as 'alarm pheromones', are widely distributed in the social insects and are concentrated in exocrine glands in the insect body.

The earliest report of the extraction and analysis of this type of releaser was by Wray[12] in 1670, who identified formic acid in a distillate of formicine ants. The full behavioural significance of this compound was not recognised at the time, but it is now known to be a universal generator of alarm in many species of Formica[13] and Camponotus.[14]

The more complex 6-methyl-5-hepten-2-one was identified much later in 1953 by Cavill and Ford[15] in a distillate of the dolichoderine ant Iridomyrmex detectus. This compound has been shown to be a releaser of alarm in this species and in other members of the same subfamily.[16]

The identification of the 'alarm substances' in these cases was possible by classical means, since they form some 1% of the total body weight of the insect. Releaser pheromones which serve different functions, however, may be more potent and in consequence synthesised and stored in very small quantities by the insect.

Workers of the fire ant Solenopsis richteri for example, can detect 10 fg cm^{-3} in air of an unsaturated hydrocarbon extracted from their gasters.[17] This sensitivity of insects to some of their releaser pheromones reaches its limit in the lepidoptera, where it is claimed that a silk moth can detect

a single molecule of its attractant (compound I).[18]

The purification and analysis of these small quantities of volatile compounds was not really practicable with conventional techniques and it was not until the 1960's, with the development of gas chromatography,[19] that exocrine products present in only microgramme quantities could readily be separated and their structures determined.

Pheromones in ants

The majority of these compounds subsequently analysed were from ants, where over 140 different volatile chemicals have been identified in some 30 genera. These compounds are largely aliphatic hydrocarbons and aliphatic carbonyl compounds (some terpenoid), often with smaller quantities of the corresponding alcohols, but include a considerable number of chemicals only known as natural products because of their discovery in ants.

The various exocrine glands in which these chemicals may be stored or produced are illustrated in Fig.1.[20]

Often a large number of compounds may be found in a single gland. In Camponotus ligniperda[21], for example, the Dufour's gland contains more than 50 compounds, of which 41 have been identified. However the strong alarm behaviour released by this secretion was shown by bioassay to be released by the C_9 to C_{12} alkanes, the other components eliciting a weak searching behaviour.

The rôle of the range of compounds produced by this gland in other species has not been fully elucidated. Often large numbers of compounds are apparently redundant or contribute to the informational content of the secretion by the regulation or enhancement of the odour of the pheromonally active

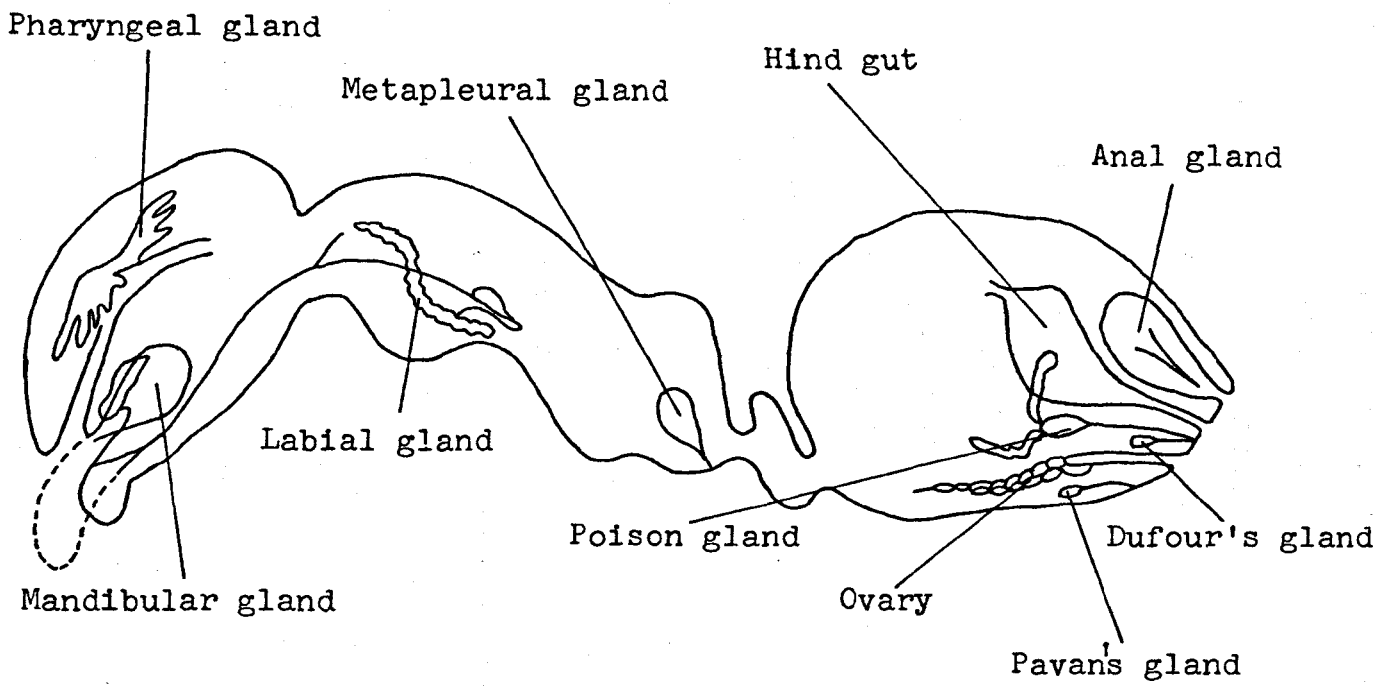


Fig.1 The exocrine system of a worker ant (Iridomyrmex pruinosus)

(After Pavan and Ronchetti[20])

substances.

In one of the species in the present study, Myrmica rubra, the major Dufour's gland components have been identified as hydrocarbons[22] and are summarised in Table 1. These components appeared to be inactive pheromonally and it was suggested that they may have a similar volatility to minor pheromonal components in the mixture. The vapour pressure of the pheromone would be reduced in such a system, and its activity correspondingly prolonged[23]. In this case the major hydrocarbon components are acting as equivolatile 'keepers' for the pheromonally active minor components. In this species the Dufour's gland secretion is not a potent releaser of alarm activity and it has been reported to be laid down on its foraging area as a series of attractive spots[24].

In Myrmica rubra and Myrmica scabrinodis the true alarm pheromones are produced by the mandibular glands. The alarm pheromones in the genus Myrmica have been identified as 3-alkanones[25] (Table 2), whose activity in Myrmica rubra have been reported to be synergistically enhanced by the presence of smaller quantities of the corresponding alcohols[26].

The class of releaser pheromone used for alarm and defense have been extensively reviewed[16,27,28].

Although the majority of releasers reported fall into this category of alarm pheromones, releasers are also used for sexual attraction and to direct and organise foraging.

Sex attractant pheromones are used during the 'nuptual flight' and generally originate in the mandibular glands of males; however few of these have been studied[28].

Pheromones in foraging

The releasers in the present study play a part in foraging.

TABLE 1

Alarm pheromones in the genus Myrmica

Compounds present	M. rubra	M. scabrinodis
3-octanone	+	+
3-octanol	+	+
6-methyl-3-octanone	Trace	+
6-methyl-3-octanol	-	+
3-nonanone	Trace	Trace

(from Crewe and Blum[25])

TABLE 2

Dufour gland contents of Myrmica rubra L.

Compounds present	%age composition
tridecane	0.6
tetradecane	0.2
pentadecane	14.2
pentadecene	2.1
hexadecane	1.0
hexadecene	1.0
heptadecane	7.2
(Z)-8-heptadecene	53.3
α-farnesene heptadecadiene	4.5
octadecane	Trace
octadecene	0.8
homofarnesene	0.8
nonadecane	Trace
(Z)-9-nonadecene bishomofarnesene	12.2

(from Morgan and Wadhams[22])

The selective advantage to social insects of an efficient means for communicating the direction and quality of food sources has led in many species to the evolution of complex pheromonal systems for the co-ordination of foraging.

One aspect of this foraging behaviour is the recruitment of workers to newly discovered food sources. Lindauer and Kerr[29] have shown that many species of stingless bee can rapidly recruit large numbers of workers to a food find by laying an odour trail from the food source to the nest. This pheromone originates in the mandibular glands and is laid as a series of droplets.

In the terrestrial hymenoptera the laying of odour trails is also an important means of recruitment. The ants and termites[30] lay these odour trails normally by dragging their gasters and releasing chemicals along the ground. In this way a trail from the nest to the food source can be established. Successive workers reinforce the trail until the food source is exhausted or overcrowded[16], when workers returning to the nest cease to lay a trail. The existing volatile pheromone then disperses within a few minutes to a concentration at which workers can no longer detect it. This system thus makes for great efficiency during foraging.

The glandular origin and mechanism of trail laying varies considerably in the various subfamilies and has been reviewed[16].

In the Ponerinae, Dorylinae and Formicinae the hindgut is the source and the trail pheromone is emitted from the anus. Thus the formicine Lasius fuliginosus lays its trails by smearing hindgut material along the ground with its gaster[31].

In the Dolichoderinae, material originating in Pavan's gland is emitted from between the IV and V urosternal outlets and is smeared along the ground to form a trail[32].

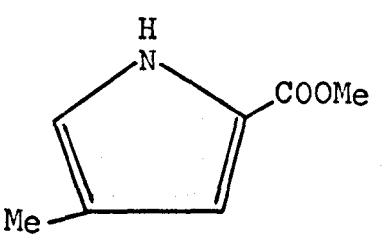
In the Myrmicinae the source of the trail pheromone is more varied. In most members of this subfamily the source is either the poison gland or Dufour's gland, and the trail substance is emitted from the sting, which is extruded and dragged along the ground like a pen. However in Crematogaster peringueyi[33] and C. ashmeadi[34], workers return to the nest after discovering a new food source without touching the ground with their gasters. The trail pheromone in this genus was found to originate in the distal segments of the meta-thoracic legs and is probably emitted from the terminal tarsal segment.

In the genus Myrmica the trail pheromone originates in the poison gland and is emitted through the sting[35].

At the present time only three trail pheromones have been identified in ants: two in the Myrmicinae and one in the Formicinae. In these cases the compounds identified are responsible for the principal pheromonal activity of the secretions, although other components which elicit weaker trail following activity remain unidentified.

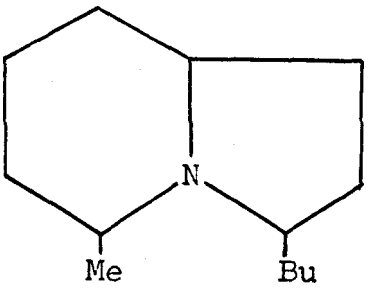
The first of these trail substances to be identified was methyl 4-methylpyrrole-2-carboxylate (III), which was isolated from the Texas leaf-cutting ant Atta texana[36] and later also found in the related species A. cephalotes[37]. The poison reservoirs of these species contain at least two components, one volatile and the other involatile, which contribute to a composite trail pheromone. Synthetic III was found to elicit strong trail following behaviour on bioassay, the threshold concentration being 0.08 pg cm^{-3}.

(III)



In another myrmicine, Monomorium pharaonis[38], an extract in methylene chloride of 6000 workers was separated by gas chromatography and found to contain three broad fractions which elicited trail following behaviour. One of these compounds was identified as 5-methyl-3-butyl-octahydroindolizine (IV)

(IV)



Two other compounds with molecular formulas of $C_{13}H_{27}N$ and $C_{15}H_{29}N$ were partially identified.

In the formicine Lasius fuliginosus[39], the trail pheromone consists of a major acidic fraction and a minor non-acidic fraction. Both fractions were extracted from the rectal fluid with ether, although only the acidic components were identified. They consisted of six fatty acids:- hexanoic, heptanoic, octanoic, nonanoic, decanoic and dodecanoic acids.

The trail pheromones have been isolated from a further four species of the genus Solenopsis, but only partially identified[17]. In this genus the trail pheromones are located in Dufour's gland[40]. Solenopsis richteri and S. invicta were found to contain species specific trail pheromones, whilst S. geminata and S. xyloni appeared to have a common trail pheromone. The molecular formula of the main component in S. richteri was found to be $C_{16}H_{26}$. This species also contained small amounts of a lower molecular weight compound, possibly $C_{15}H_{24}$, whilst this compound formed the major component of the trail pheromone in S. invicta. In S. geminata and S. xyloni the trail pheromone may be a $C_{17}H_{28}$ compound.

Blum and Brand[28] have suggested that many pheromones are

actually multicomponent systems, which function synergistically. Variation in the blend of components in these cases could then help explain the species specificity of many pheromones. The studies on trail pheromones to date are at least in agreement with this suggestion.

Other foraging activities may also be explained by such a system. Wilson suggests[41] that the odour trail exists not on the ground, but as a corridor of scent rather like a long sausage. In most cases studied the pheromones constituting this trail possess an attractive component which directs foragers to the trail.

Blum and Brand[28] conclude from their studies that for many different ant species, a newly laid trail produces a strong recruitment response. They have found that Solenopsis invicta workers can be drawn off well-established food trails by a fresh trail, laid by a single worker, to a new food source. It would appear that the trail pheromone here is a mixture of compounds differing in volatility; the most volatile fraction eliciting a short-acting attractive response.

The more volatile fraction of these secretions has not been analysed for any species, as they are either lost on extraction of the less volatile components or their presence is obscured by the occurence of a large solvent peak on gas chromatographic analysis.

Studies on Myrmica rubra

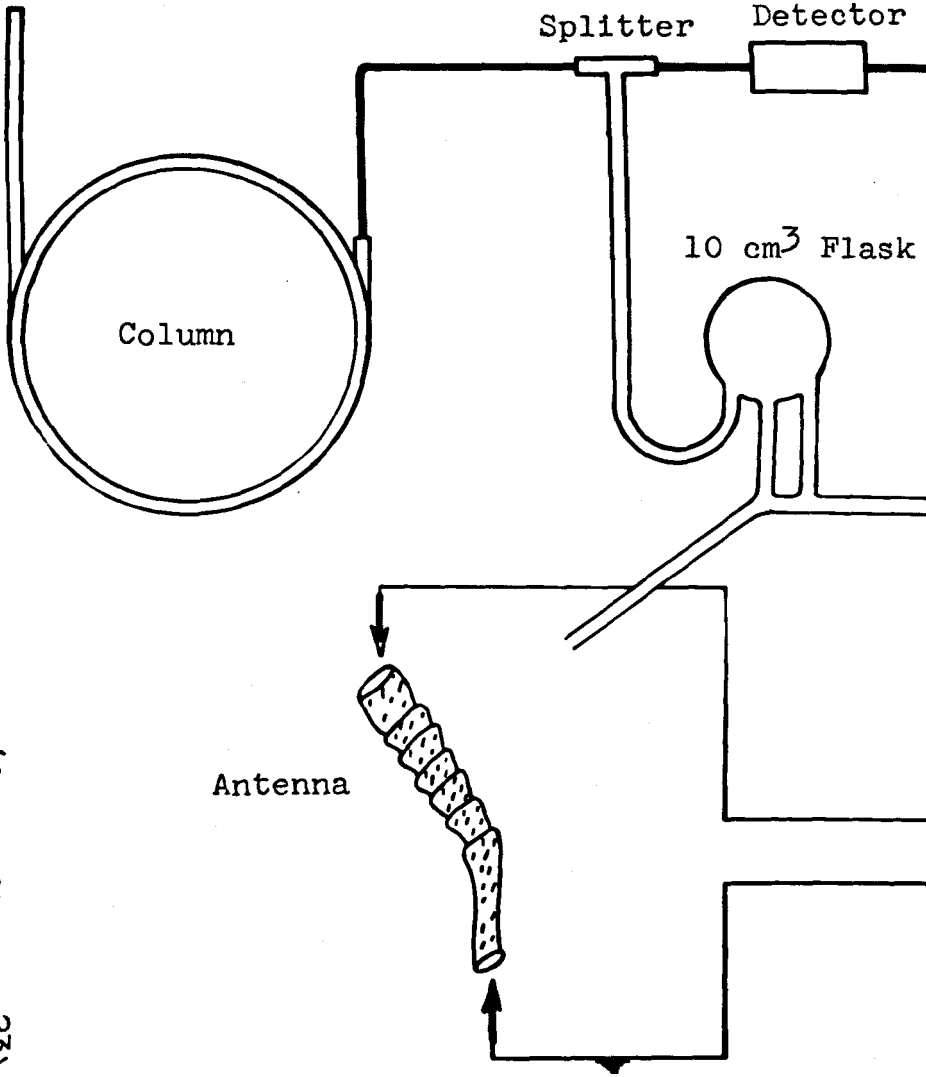
In the work presented here the presence of these volatiles has been observed in the ant Myrmica rubra by use of a solid sampling technique[42], enabling the analysis of samples without the addition of solvents. In this case the perception by the ants of various volatile fractions of their whole sting

apparatus (Fig.5), was assessed by the recording of their electroantennogram. This technique enables electrical impulses, generated in the insect antenna, to be measured by the insertion of recording electrodes into the antenna.

Schneider first used the technique of electroantennography in the late 1950's[43], when he recorded an electrophysiological response from the silk moth Bombyx mori by passing a stream of air containing the sex attractant (I), over an isolated antenna of the insect mounted between two electrodes.

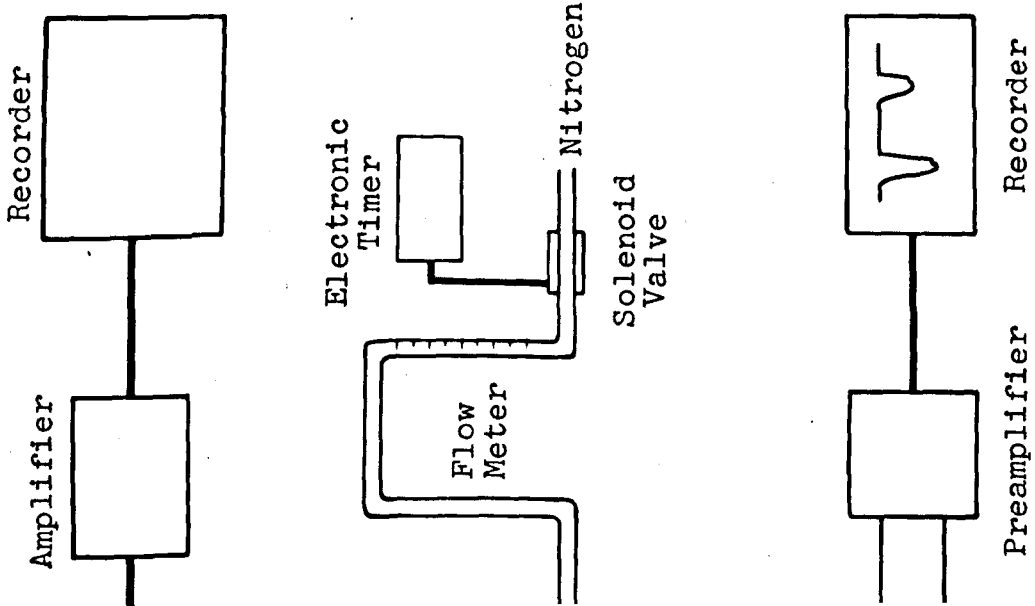
Roelofs and Comeau used Schneider's technique to identify the sex lures of lepidoptera[44]. The major functional group of the active component of the sex lure was determined by subjecting a crude extract to simple microchemical reactions. After reaction, any loss of activity from the crude extract was assessed by recording the electroantennogram. The various components of the sex lure were separated by gas chromatography and the column effluent trapped at one minute intervals in glass tubes. The contents of each tube were re-eluted with air over the male moth antenna. In this way biologically active compounds in the multicomponent system may be located and partially identified.

In the study on Myrmica rubra[23] the volatile constituents of the sting apparatus were separated by gas chromatography and the column effluent split between the flame ionisation detector and the antenna. The gas chromatogram and the electro-antennogram were recorded simultaneously to enable the correlation of retention time with insect response. The gas chromatograph was linked to the insect antenna using the system described by Moorhouse and co-workers[45]. The column effluent was split through an all-glass system designed to allow 80% of the column effluent through the flame ionisation detector;



(After Wadhams[23])

Fig.2 The electroantennogram/gas chromatograph system

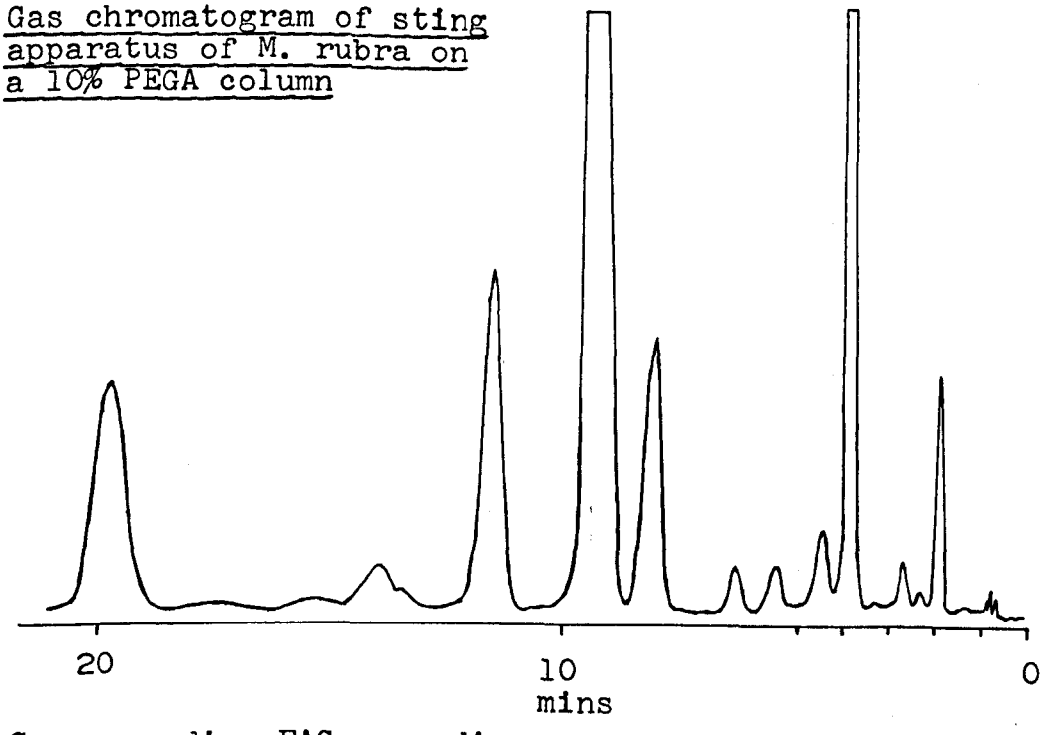


the remaining 20% passed into a 10 cm^3 three necked flask located in the chromatograph oven (Fig.2). The effluent was allowed to accumulate in the flask for 55 seconds and then flushed over the antenna with nitrogen for 5 seconds. An electroantennogram recording, therefore, indicates that an active component has eluted from the column in the previous minute.

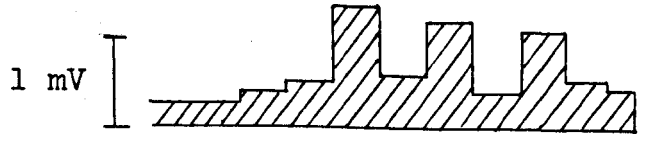
The results obtained using this system suggested that three fractions were active (Fig.3). The first of these active fractions is of interest in the present study as the electroantennogram response corresponds to the highly volatile fraction of the sting apparatus. This fraction was not identified but it was suggested that two of the components may be water and acetone.

Fig.3

Gas chromatogram of sting apparatus of M. rubra on a 10% PEGA column



Corresponding EAG recording



The less volatile major components of the sting apparatus were identified[22], but none of them elicited trail following behaviour on bioassay. The possibility therefore remained that this very volatile fraction contained or contributed to the trail pheromone of this species.

The object of the present study was to determine the glandular origin of the most volatile fraction in the sting apparatus of the ant Myrmica rubra, to isolate and identify the components and to assess their biological rôle, looking in particular for trail following activity. Similar studies were to be undertaken with M. scabrinodis in order to study the qualitative and quantitative variation in the composition of these volatiles in a closely related species.

DISCUSSION

DISCUSSION

The original objective of this study was to isolate and identify the trail pheromone of the ant Myrmica rubra L. The interest in ant trail substances stems from a desire to develop highly specific and non-toxic methods of control for those ant species which are serious pests. Thus the first trail pheromones identified were in Atta texana[36] and in Monomorium pharaonis[38], the former being a serious pest in the tropics, particularly on Citrus plantations, and the latter being a vector of disease in European hospitals.

Ants of the genus Myrmica are not serious pests, but have been chosen for the present study since they present a readily available source of insect material for use in the development of microchemical techniques: these techniques could subsequently be applied to other species of economic importance. From the entomological viewpoint the species M. rubra are ideal for chemical studies as their foraging behaviour is relatively well documented, whereas the chemical releasers of this behaviour have not been identified.

Colonies of both M. rubra and M. scabrinodis may be found throughout the year, although M. rubra are more numerous in the Spring and Summer and M. scabrinodis in the Autumn.

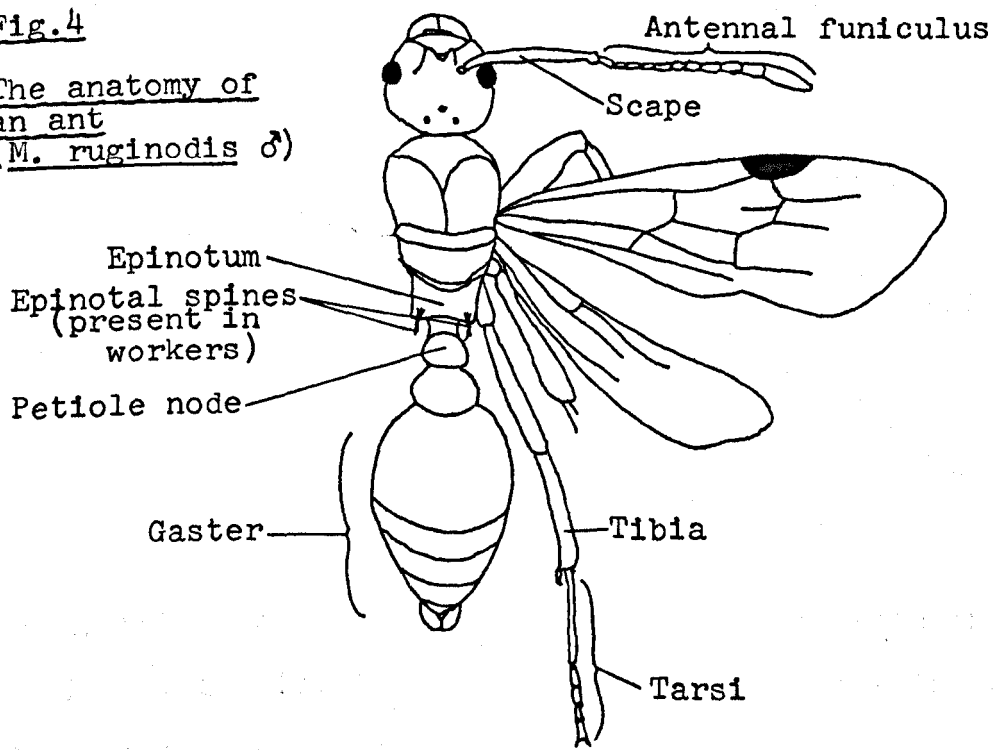
Nests occur in open ground and under hedgerows, but are most conveniently located and collected from under flat, well-established stones on short turfed pasture and on heathland. The large black ant Formica fusca and the yellow hill ant Lasius flavus also occur in these habitats but are readily distinguishable by their pigmentation from Myrmica red ants.

In the field M. rubra or M. ruginodis may be distinguished from M. scabrinodis or M. sabuleti as the former have antennal scapes with gently curved bases, whereas the latter two have sharply curved bases to their scapes[46].

Microscopic examination reveals that in M. rubra the petiole node is peaked or rounded in profile and the epinotal spines are shorter than the distance between their tips (Fig.4) In M. ruginodis the petiole node is flattened dorsally and bluntly angled behind; and the epinotal spines are about as

Fig.4

The anatomy of an ant (M. ruginodis ♂)



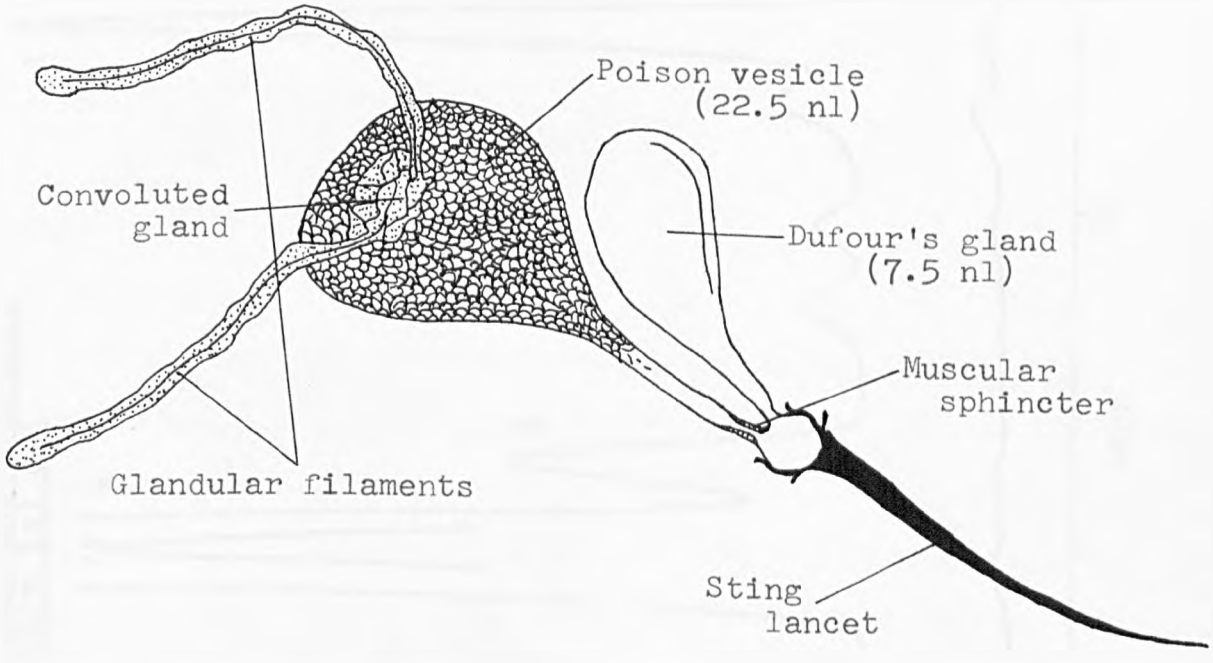
long as the width between their tips.

In M. scabrinodis the base of the antennal scape is simply angled or with a slight extension on the bend, whereas M. sabuleti has a distinct extension from the bend on the scape and a ridge running from this bend towards the funiculus. (Plate 1)

Dissection of workers of the species M. rubra and M. scabrinodis revealed that the structure of the sting apparatus is similar in both species, consisting of a large

rounded poison vesicle and an accessary gland called Dufour's gland, of approximately one third of the volume of the poison vesicle (Fig.5). Apart from the difference in size, the glands differ in overall appearance allowing them to be readily distinguished under the microscope. The poison vesicle has a textured surface and two glandular filaments. The convoluted

Fig.5 The sting apparatus of M. rubra



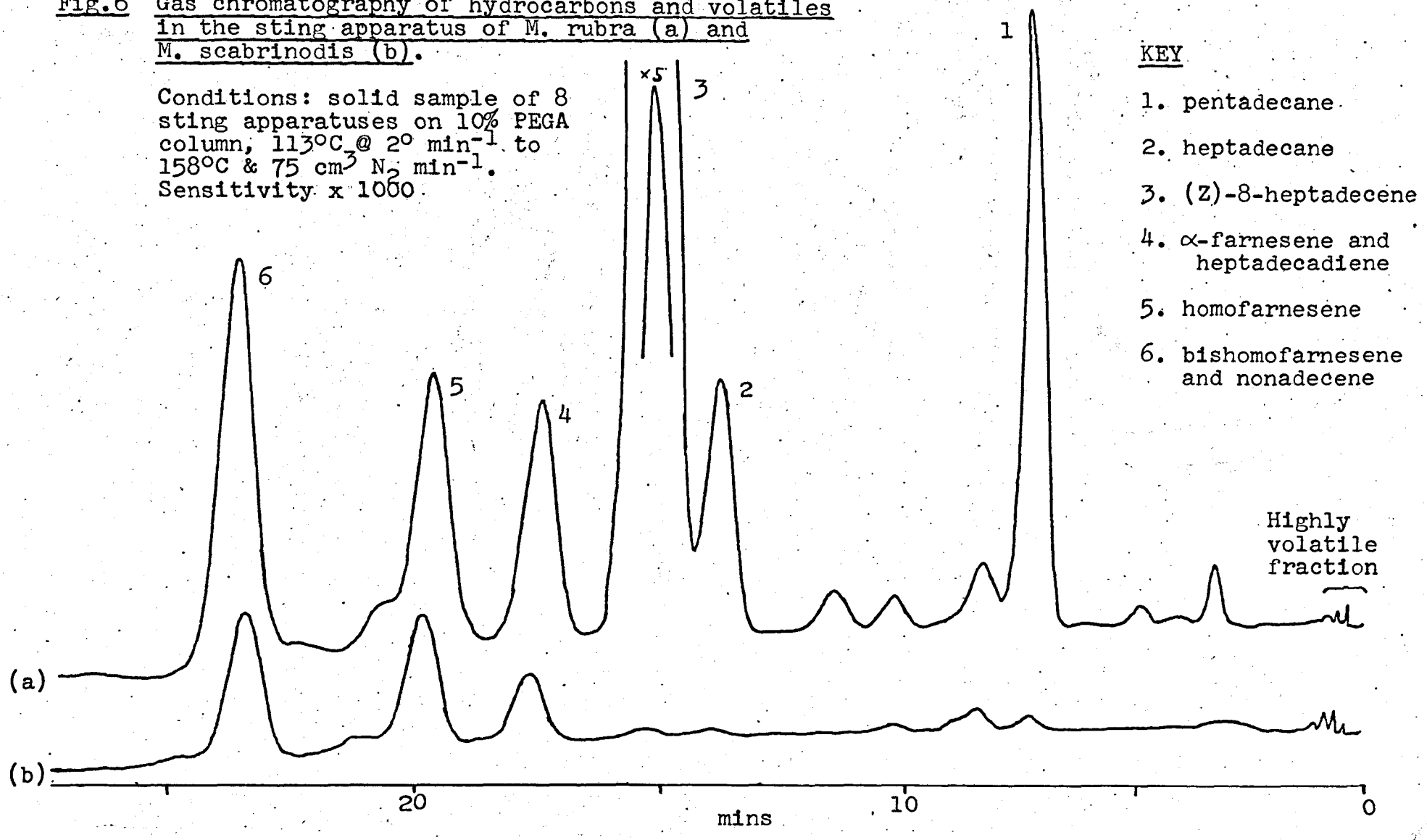
gland, believed to be the site of venom synthesis in other species[47], is visible as an opaque patch on the inner surface of the poison vesicle.

Dufour's gland is a clear pear-shaped sac whose base opens, together with the duct from the poison vesicle, at the base of the lancet of the sting.

Analysis of the volatile contents of the whole sting apparatus on a 10% PEGA column, using the solid sampling technique, revealed distinct profiles for the two Myrmica species, which subsequently aided the confirmation of identity (Fig.6). M. rubra contains some twenty components (Table 1), whereas M. scabrinodis contains only three major components

Fig.6 Gas chromatography of hydrocarbons and volatiles in the sting apparatus of M. rubra (a) and M. scabrinodis (b).

Conditions: solid sample of 8 sting apparatuses on 10% PEGA column, 113°C @ 2° min^{-1} to 158°C & 75 cm^3 N_2 min^{-1}. Sensitivity x 1000.



corresponding in retention times to components present in M. rubra. The absence of other alkanes and alkenes in M. scabrinodis makes it tempting to suggest that these three major components may be the sesquiterpenes farnesene, homofarnesene and bishomofarnesene previously identified by Wadhams[23] in M. rubra. No attempt was made, however, to analyse these components.

Both species contain a number of very volatile compounds forming less than two percent of the total contents of the glands.

STUDIES ON M. RUBRA

Previous work

Early studies were undertaken on the species M. rubra as a number of observations had indicated that the very volatile components were pheromonally active in this species. Wadhams results on the electroantennogram[23] indicated that workers could at least detect some or all of these components: furthermore, Cammaerts-Tricot showed[48] in three experiments that the Dufour's gland contains a highly volatile fraction which elicits characteristic behavioural movements from workers.

In the first of these experiments a freshly isolated Dufour's gland was found to lose its activity after two or three minutes. Secondly, when the distillate from 500 ants was passed into an ant colony, reactions similar to those induced by the gland were observed when the temperature of the distillate was near 35°C. Furthermore the behaviour decreased and did not reappear when the temperature rose to 40°C or more. Finally, 10 Dufour's glands were placed in a small closed bottle with its top lined with a filter paper. After 10 minutes the filter paper had a short-acting attraction for worker ants,

whereas the glands themselves were inactive[48].

Extraction of volatiles

Numerous unsuccessful attempts were made to extract these volatiles; firstly ant gasters were vacuum distilled into chilled traps, but no material was collected. Distillation into traps of Poropak Q or Tenax GC surrounded with water-cooled jackets enabled the volatiles to be trapped out, but they could not easily be recovered from the absorbant. If a small amount of absorbant, loaded with volatiles, was sealed in a glass vial and introduced into the gas chromatograph with the solid sampling device, the volatiles could be rapidly desorbed by heating and crushing the vial. To desorb large enough quantities for analysis, however, a number of types of pre-column furnace were built, but these all failed to desorb the volatiles rapidly, resulting in the elution of very broad peaks. It was not economically feasible to build a suitable furnace, due to the high cost of the valves required, so alternative extraction procedures were investigated.

Three different extraction procedures were found to be successful. Initially, 600 workers were degassed by ampoule distillation to yield 0.5 cm^3 of a dilute aqueous solution of the volatiles.

This technique was developed by Bergström[49] for the extraction of volatiles from a single gaster of formicine ants. Briefly, the technique consists of sealing the ant material under vacuum in an ampoule. The material is degassed by heating and the volatiles collected in the chilled end of the vial.

To ensure that all the volatiles in the ampoule distillate originated in the sting apparatus, the freshly dissected glands were analysed whole via the solid sampling technique. A

comparison of the results from these two different extractions revealed that the major component in the ampoule distillate did not originate in the sting apparatus. It was apparent, however, from both extractions that each worker only contained a few nanogrammes of this volatile fraction in its sting apparatus.

The analysis of these volatiles by gas chromatography -mass spectrometry (GC-MS) was not feasible, partly because the low molecular weight compounds are readily lost through the sinter of the Watson-Biemann separator fitted to the mass spectrometer available.

Reaction gas chromatography

These restrictions led us to investigate suitable micro-chemical reactions for the analysis of the volatiles. The original techniques were developed by Hoff and Feit[50], but a large number of reactions have since been published. These fall into two basic categories called pre-column and post-column reactions. Pre-column reactions are typically conversions such as oxidations or reductions, so that the effect of the reaction may be observed by a change in the chromatographic profile after passage of the reaction products through the column. This type of reaction is of limited use in a post-column as the products would appear at the same retention times as the corresponding starting materials. Consequently post-column reactions are normally abstractions such as complex formation for carbonyl compounds or esterification for alcohols. In this case compounds containing the appropriate functional group are completely removed from the chromatographic profile.

The use of pre- and post-column reactions in gas chromato-graphy for the identification of functional groups has been

reviewed[51].

The application of pre-column reactions to the analysis of the volatiles in the sting apparatus of M. rubra presented the problem that involatile material, present in the tissue surrounding the glands, might be rendered volatile by contact with the reagents. Triglycerides, for example, would be hydrolysed by both acidic and basic reagents to yield free fatty acids and glycerol, the acids being more volatile than the original lipid. To avoid this complication the technique of capillary extraction was developed.

Capillary extraction technique

In brief, the appropriate glandular sac was carefully removed from the ant, laid on the glass stage of a binocular microscope and its liquid glandular contents withdrawn by the capillary action of a fine glass tube. This technique has three main advantages over other extraction techniques in the analysis of highly volatile compounds: firstly, only 1% of the surface of the extracted liquid is exposed to the air, so that the evaporative loss of the volatiles is minimal. This enables the percentage composition of the glandular liquid to be determined accurately.

Secondly, it can clearly be seen under the microscope when all the liquid has been extracted from the gland, permitting both the volume and the absolute composition of the gland to be calculated.

Finally, by this technique, material can be extracted separately from the Dufour's gland and the poison vesicle without cross-contamination or contamination with cell wall tissue. These extracts, free of contamination, can then be analysed with the aid of pre-column reactions.

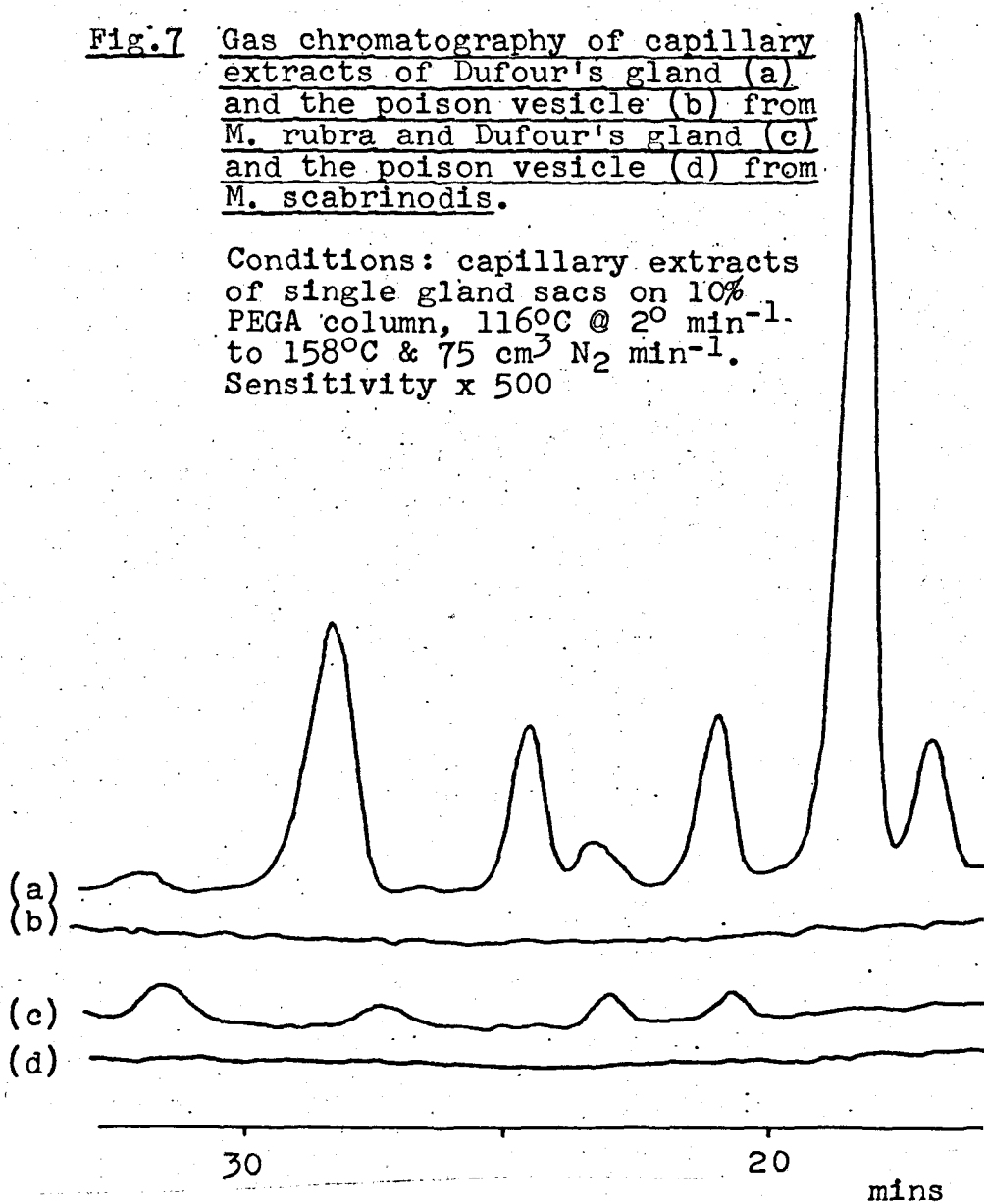
Use of this capillary extraction technique confirmed Wadham's findings[23] that in M. rubra all the hydrocarbons were located in Dufour's gland and none were in the poison vesicle (Fig.7). In the related species M. scabrinodis the high molecular weight volatile components of the sting apparatus were again located in Dufour's gland, with the poison vesicle containing only the very volatile components (Fig.7).

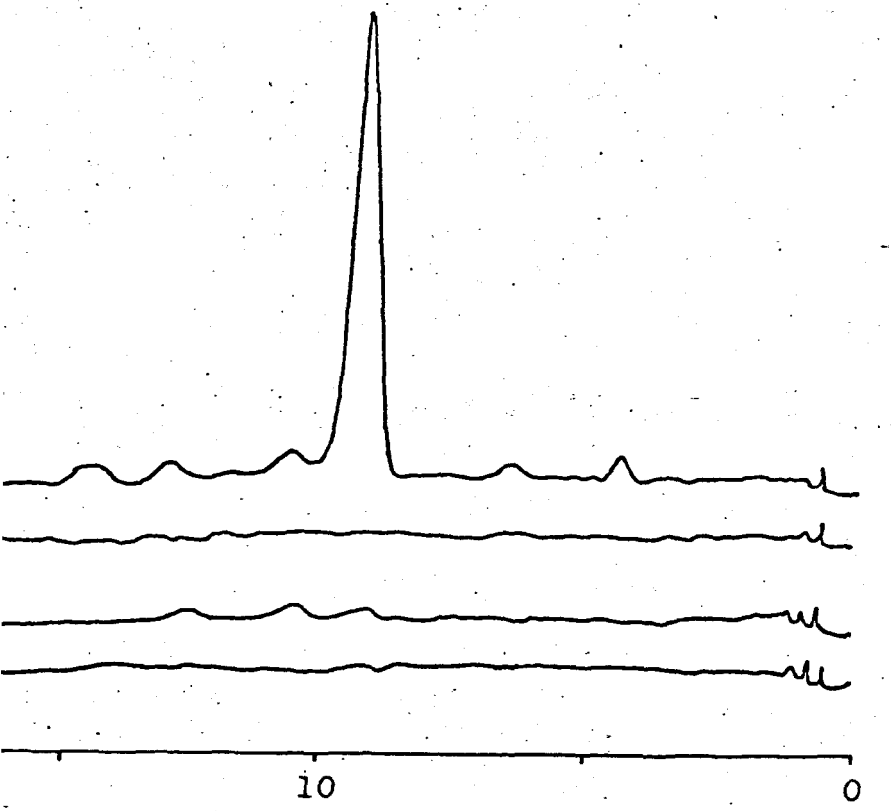
The separation of the highly volatile fraction, present in both glands, was very poor on conventionally coated phases such as SE-30 or PEGA (Figs.6 & 7). This problem has been overcome by other workers by the use of porous polymer phases such as Poropak Q. In one case[52] volatile microbial metabolites of low molecular weight were analysed by the injection of various culture supernatants directly onto the column. Involatile matter such as lactose and amino acids in the sample, remained on the top of the column, avoiding the need for pre-treatment of samples. Using this technique simple oxygenated compounds were rapidly and quantitatively analysed. Poropak Q has also been used for the determination of volatiles in the head space over saliva samples[53]. Simple oxygenated and sulphur-containing compounds were well resolved on this phase and the chromatogram was found to be a useful tool in the investigation of disorders in the oral cavity.

Porous polymers in general are stable up to 250°C and are used uncoated to give low detector noise even at high sensitivities. Poropak Q itself is a cross-linked polystyrene-divinyl benzene polymer and is slightly polar, separating principally according to molecular weight and shape. It is one of the most retentive of the polymer phases and can even be used at low temperature for the separation of permanent gasses. All these properties make it an ideal phase for the separation of the

Fig. 7 Gas chromatography of capillary extracts of Dufour's gland (a) and the poison vesicle (b) from M. rubra and Dufour's gland (c) and the poison vesicle (d) from M. scabrinodis.

Conditions: capillary extracts of single gland sacs on 10% PEGA column, 116°C @ 2° min^{-1}. to 158°C & 75 cm^3 N_2 min^{-1}. Sensitivity x 500

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very volatile components present in Dufour's gland. One big advantage was that the major hydrocarbon components present in this gland were retained on the top of the column, as reported for the involatile microbial metabolites[52], so that it was not necessary to temperature programme off the high molecular weight components before proceeding with further analyses. The only disadvantage in this technique is that the top few centimetres of the column become yellow with accumulated hydrocarbons. This contamination, however, was removed by repacking the discoloured portion of the column top after each three or four weeks of continued use.

The identification of low molecular weight compounds on this phase and on the related phase Chromosorb 102 is aided by the limited number of compounds with a specific retention time.

Analysis of the ampoule distillate of 600 ants

It was recognised that the components extracted from whole ants by the ampoule distillation technique did not necessarily reflect the true composition of the combined glands of the sting apparatus. Nevertheless the aqueous extract was chromatographed on the Poropak Q phase and found to have six components (Fig.8). Five of these components were also to be found in the chromatogram of whole glands introduced into the chromatograph by the solid sampling technique. The major component of the ampoule distillate was found to correspond in retention time to a minor component in the glands themselves, but the other four components were present in similar proportions. In order to gain insight into the type of compounds with which we were dealing we chose to analyse the ampoule distillate first. The use of the ampoule distillate allowed quantitative assessment of the amount of each component which had reacted after

treating the sample with various reagents. Such a comparison is not easy with whole gland samples, since tissue adhering to the sample introduces variable quantities of impurities from one sample to the next.

The presence of simple amines and carboxylic acids in the ampoule distillate was sought, but both of these classes of compound were found to give a very poor flame response in the detector of the chromatograph: it could not be said with certainty, therefore, that these compounds were not present in the ampoule distillate. These classes of compound, however, do give a good response in a thermal conductivity detector (TCD) and use of one of these detectors showed no peak other than water; nevertheless, this result merely indicated that amines and carboxylic acids were not present in microgramme quantities or more per insect, since the TCD has a relatively low sensitivity.

The next compounds considered as possible candidates for the components observed in the ampoule distillate were simple alcohols, aldehydes and ketones. The retention times of five of the six components present in the ampoule distillate were found to correspond well with authentic samples of certain of these compounds (Table 3). The possible candidates for each component were selected with the aid of published retention data on the Chromosorb and Poropak phases used.

The functional groups present in each component were confirmed by reaction gas chromatography.

The identity of the alcohols was confirmed by using a boric acid pre-column similar to that described by Darbre and Islam[54]. The originally described loading of 20% boric acid on Diatomite S was found to remove 47.6% methanol and 82.6% ethanol at 100°C. However a 50% loading on Poropak Q completely

removed authentic aqueous samples of both alcohols, with the appearance of a peak after six minutes, presumed to be the methyl ester. If this component is the ester it would suggest the formation of methyl metaborate as opposed to the orthoborate, since the retention time indicates a molecular weight of around 60 for a moderately polar compound. This suggestion is consistent with the known dehydration of orthoboric acid to form metaboric acid at 100°C.

Passage of the distillate through the boric acid pre-column resulted in the drastic reduction of the methanol peak and the complete removal of the ethanol peak (Table 4).

After continued use of the boric acid pre-column, the analytical column gave irreproducible results: methanol was partially absorbed from the injected samples and a peak of area corresponding roughly to that removed from the methanol component, appeared after six minutes. This suggested that the column had become contaminated with metaboric acid.

TABLE 3

Retention times of volatiles in ampoule distillate of M. rubra

Conditions: Poropak Q @ 170°C & 40 cm^3 N_2 min^{-1}

Chromosorb 102 @ 120°C & 40 cm^3 N_2 min^{-1}

Component identity	Retention times on Poropak Q (mins)		Retention times on Chromosorb 102 (mins)	
	Authentic sample	Component in distillate	Authentic sample	Component in distillate
methanol	1.6	1.6	1.6	1.6
ethanal	2.0	2.0	1.8	1.8
ethanol	2.7	2.7	2.9	3.0
unknown	-	3.4	-	12.5 ?
propanone	4.1	4.1	4.5	4.7
butanone	8.6	8.5	11.0	11.2

Fig.8 Gas chromatography of ampoule distillate from M. rubra

Conditions: Poropak Q @ 176°C & 40 cm^3 N_2 min^{-1}

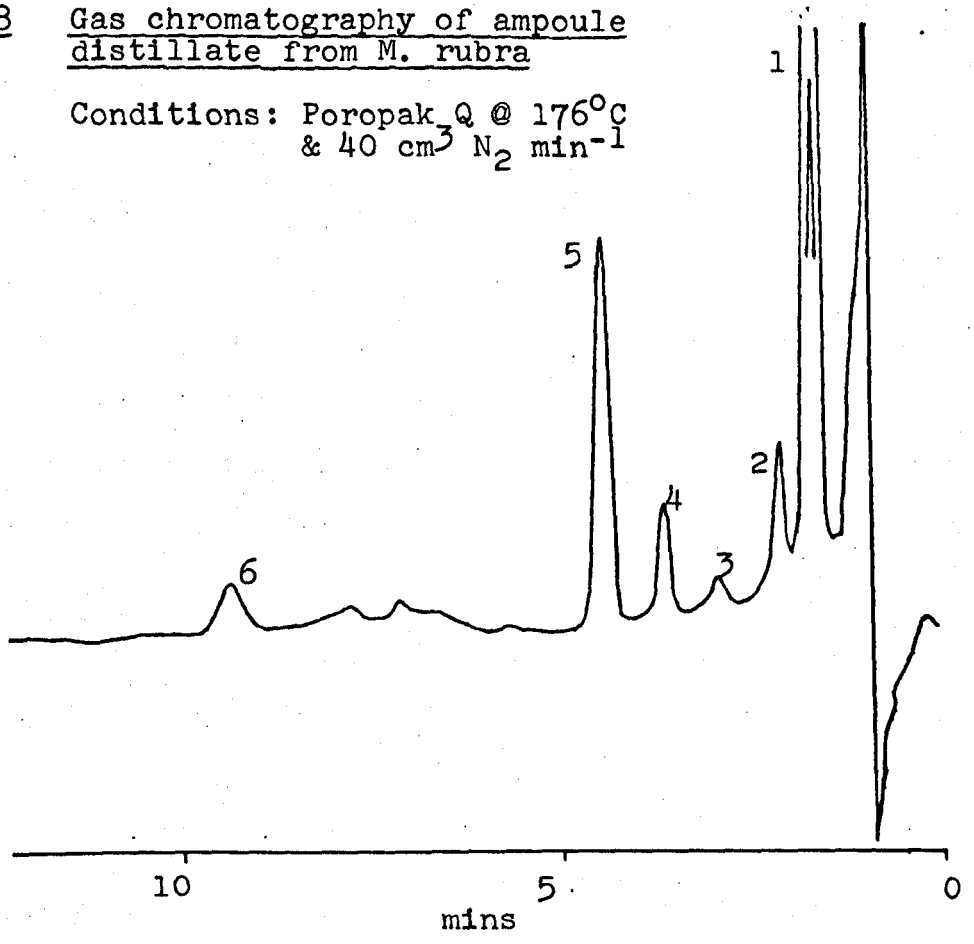


TABLE 4

Analysis of ampoule distillate from M. rubra

Component identity	%age composition of distillate			
	Before treatment	After passage through boric acid pre-column	After borohydride reduction for 1.5 mins	After borohydride reduction for 5 mins
1 methanol	60.9	6.3	67.8	64.3
2 ethanal	11.9	7.0	1.4	0.1
3 ethanol	0.6	0	6.0	7.5
4 unknown	4.9	4.8	5.1	5.2
5 propanone	17.0	15.7	9.6	0
6 butanone	4.7	4.3	2.3	0

The aldehydes and ketones in the distillate were reduced to their corresponding alcohols in a syringe reaction with borohydride reagent. The reagent has previously been used in ethanolic solution[55], but freshly prepared aqueous reagent was found to be stable if a small amount of base was added.

After five minutes reaction with the reagent, authentic standards of ethanal, propanone and butanone were all completely removed with the enhancement of the ethanol peak and the production of two new peaks corresponding in retention times to 2-propanol and 2-butanol.

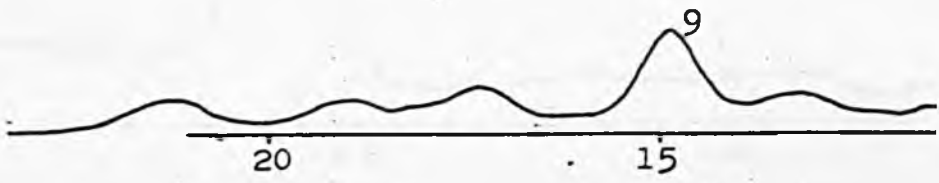
The percentage composition of the distillate after partial and complete reduction with borohydride reagent is given in Table 4.

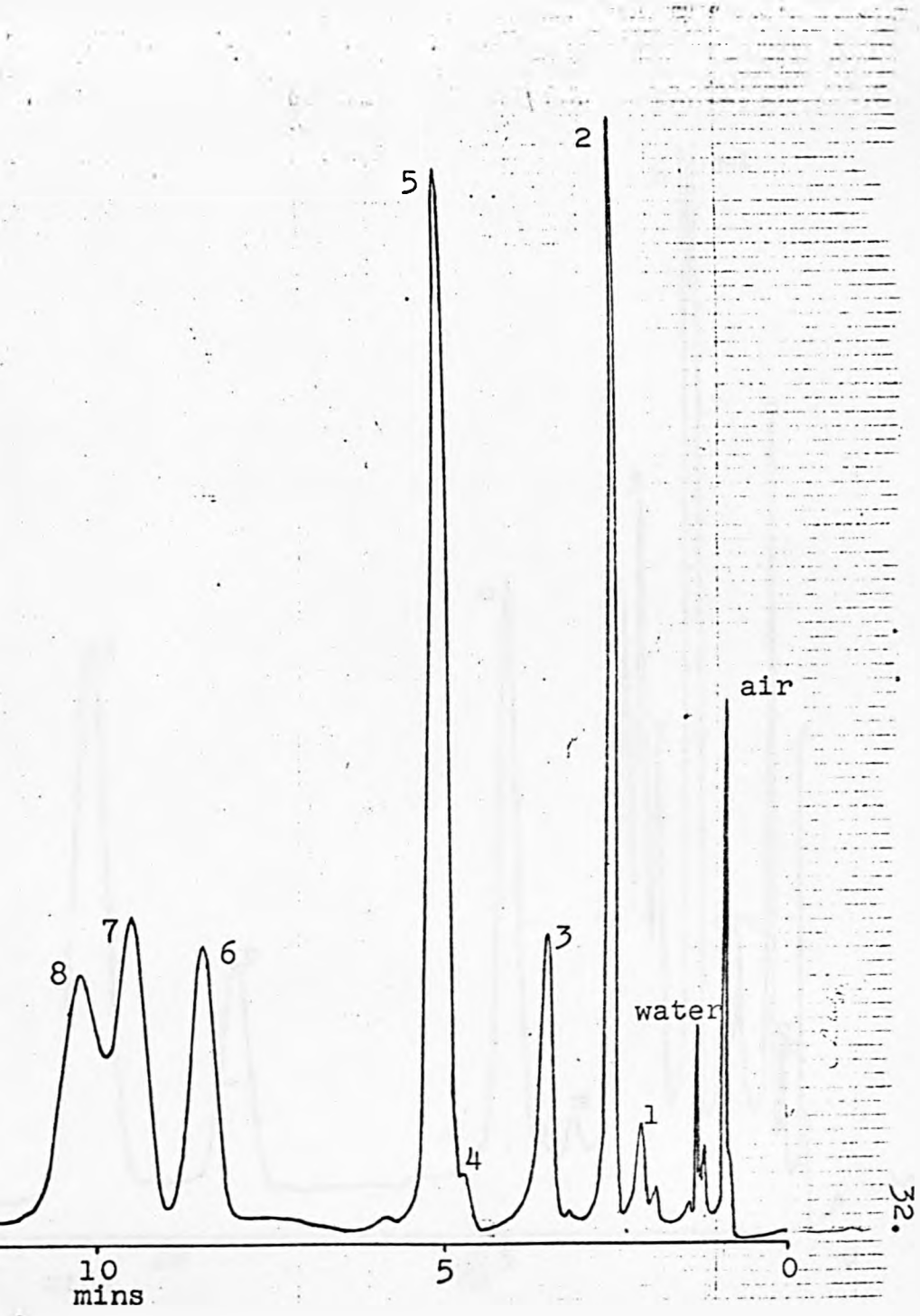
Glands analysed via the solid sampling technique revealed that methanol and the unknown component in the ampoule distillate were not present in the sting apparatus. The methanol component could possibly have arisen from the pyrolysis of ant tissue, similar to the pyrolysis of lignin in wood. A number of minor components not present in the distillate were now found in the sting apparatus.

The development of the capillary extraction technique allowed samples of liquid to be removed from the glands without contamination from the neighbouring tissue. These capillary extracts were extremely reproducible qualitatively from one ant to another, allowing successive samples to be compared before and after reaction. Extracts from either gland taken from ants within the same nest were almost as reproducible as if they had been drawn successively from a single large reservoir in each case. In this respect the capillary extracts resembled the ampoule distillate, but unlike the material extracted by the latter technique, capillary extracts contained

Fig.9 Gas chromatography of volatiles in Dufour's gland of M. rubra

Conditions: capillary extract of 9 Dufour's glands on Poropak Q @ 187°C & 45 cm^3 N_2 min^{-1}
Sensitivity x 200

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7
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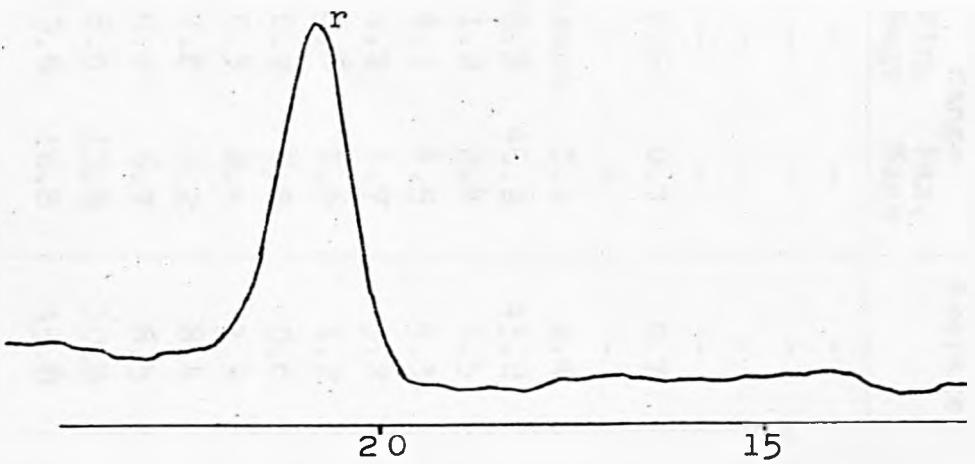
Fig.10 Gas chromatography of volatiles in poison vesicle of M. rubra

Conditions: capillary extract of 3 poison vesicles on Poropak Q @ 167°C & 50 cm^3 He min^{-1}
Sensitivity x 20



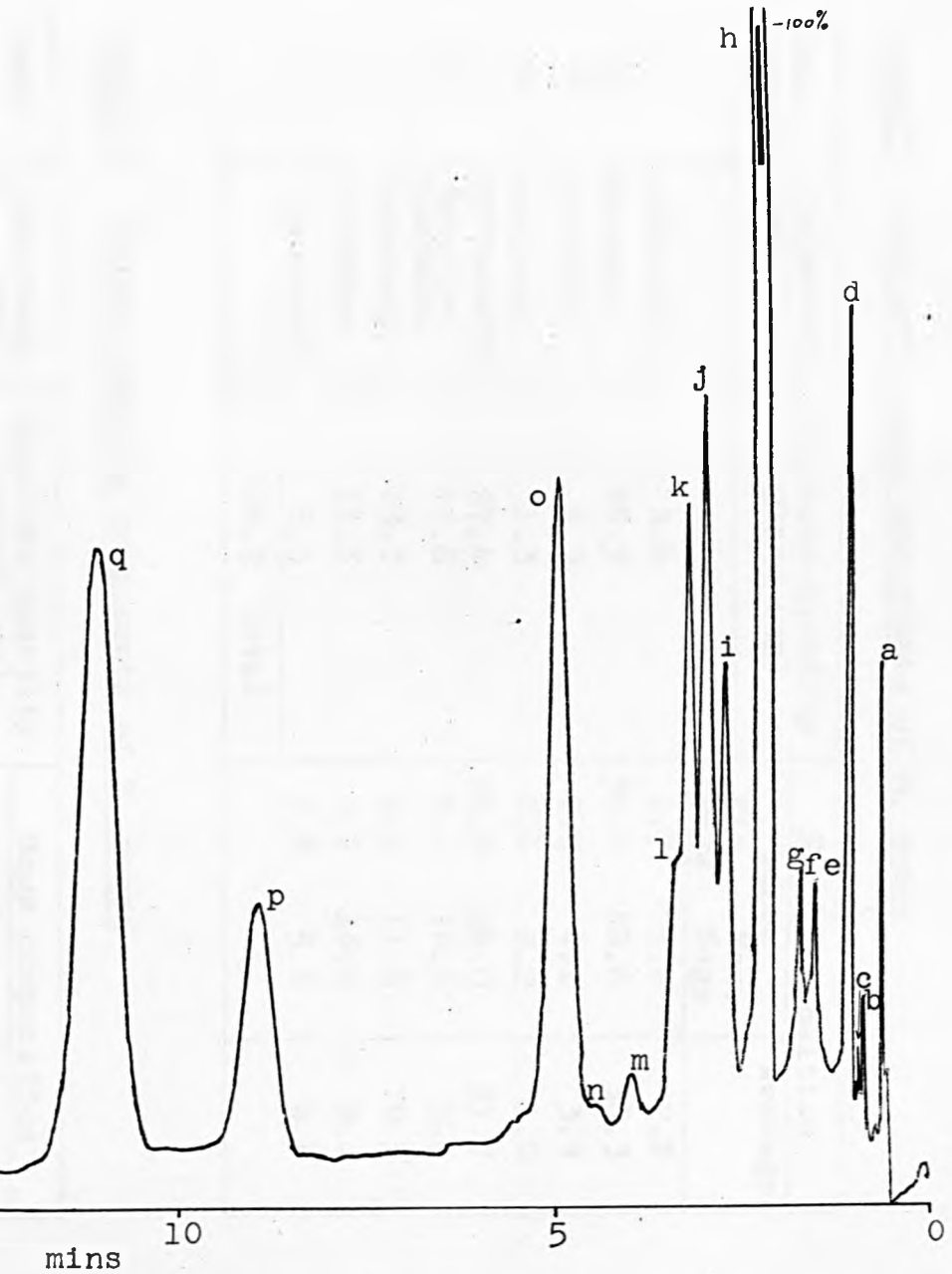

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TABLE 5 Dufour's gland components of M. rubra

Peak number	Component identity	Absolute quantity per gland (ng)	%age composition		
			Range Min. %age	Max. %age	Average
1	methanol	2.8	1.7	3.2	2.2
2	ethanal	48.3	30.0	48.6	38.3
3	ethanol	4.3	2.9	4.1	3.4
4	propanal	1.3	0.4	2.2	1.0
5	propanone	27.4	20.6	24.0	21.7
6	methyl-propanal	11.8	8.1	10.8	9.4
7	butenone	13.3	9.3	11.6	10.5
8	butanone	11.5	7.7	10.8	9.1
9	1-butanol	5.5	3.8	5.6	4.4
		126.2 Total			

TABLE 6 Poison vesicle components of M. rubra

Peak letter	Component identity	Absolute quantity per gland (ng)	%age composition		
			Range Min. %age	Max. %age	Average
a	air	-	-	-	-
b	methane	trace	-	-	-
c	ethane	trace	-	-	-
d	water	*	-	-	-
e	unknown	0.5	0.6	0.7	0.7
f	propane	trace	-	-	-
g	methanol	1.7	trace	3.1	2.4
h	ethanal	31.2	39.3	45.2	43.2
i	unknown	1.1	1.2	2.4	1.5
j	unknown	3.1	4.1	4.5	4.3
k	butane	2.3	1.2	4.7	3.2
l	ethanol	0.9	0.6	1.6	1.2
m	unknown	0.4	0.2	0.9	0.6
n	propanal	1.0	0.3	2.4	1.4
o	propanone	6.1	7.8	10.2	8.4
p	methyl-propanal	4.7	5.9	6.7	6.5
q	butanone	7.8	8.5	13.6	10.8
r	methyl-butanal	11.4	13.6	16.8	15.8
		72.2 Total			

* not estimated

no contaminants originating in other parts of the ants.

Samples from Dufour's gland and the poison vesicle were analysed separately on Poropak Q by the capillary extraction technique in order to determine the glandular origin of the volatiles.

Chromatograms of the separate secretions revealed that they have different compositions, despite certain similarities (Figs.9 & 10). The composition of the Dufour's gland was sought first since the behaviour of M. rubra workers perceiving the secretion of this gland has been well documented[26], but the volatile components responsible have not been identified.

Analysis of the volatiles in capillary extracts of Dufour's gland (M. rubra)

The nine components observed in the capillary extracts of Dufour's gland (Fig.9), were assigned a tentative identification by comparing their Kováts' indices with those of authentic compounds on a matrix of indices for the phase (Fig.11).

The identification was aided by reaction of the extract with solid sodium borohydride reagent. The conditions required for complete reduction of aldehydes and ketones to their corresponding alcohols with this reagent, were determined with aqueous samples of authentic compounds. The concentration of the standard samples used was chosen to give peak heights comparable with those observed after gas chromatography of the contents of four Dufour's glands. It was subsequently discovered that the Dufour's gland contents are essentially anhydrous, but the reduction may still occur in aqueous solution due to the presence of moisture on the inner walls of the vial.

Components 2,4,5,6,7 and 8 of the glandular extracts were all removed by this treatment with the appearance of peaks

Fig.11 Matrix of Kovats' indices on Poropak Q

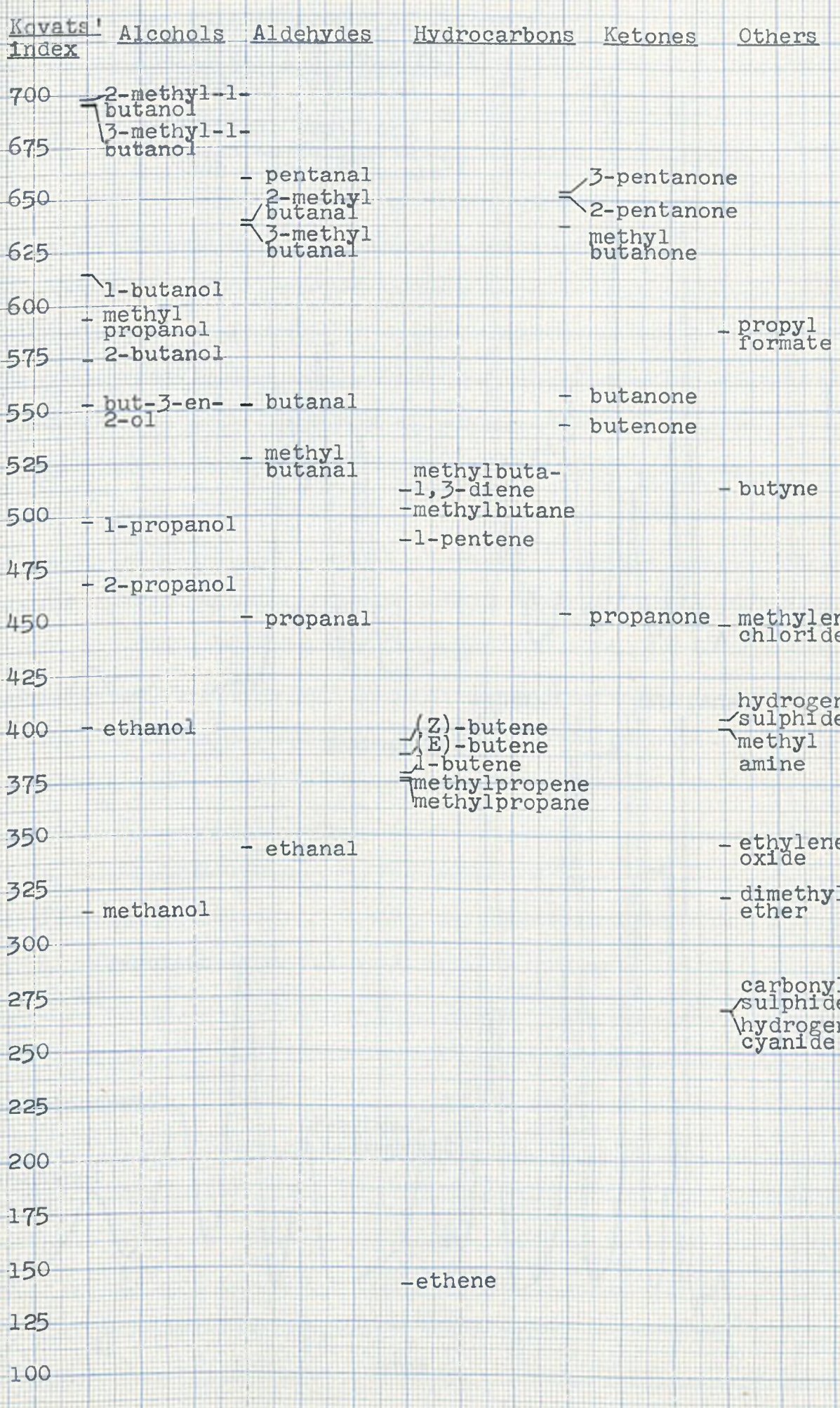
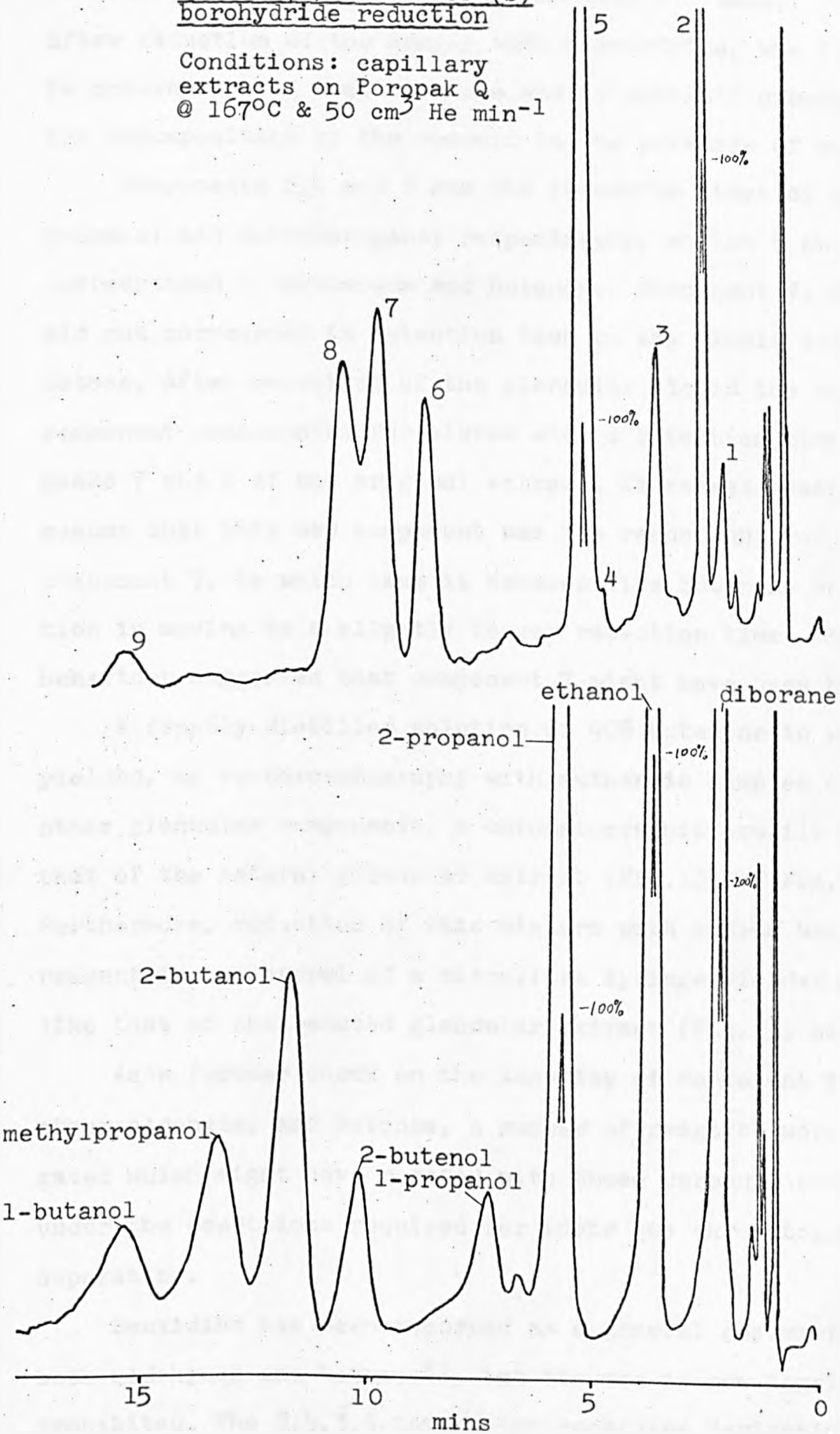


Fig.12 Gas chromatography of Dufour's gland contents of M. rubra before (a) and after (b) borohydride reduction

Conditions: capillary extracts on Porapak Q @ 167°C & 50 cm^3 He min^{-1}



having the retention times of the corresponding alcohols (Fig.12). A large peak of retention time 2.1 mins., observed after reduction of the sample with borohydride, was found to be present in the reagent alone and is possibly diborane from the decomposition of the reagent in the presence of moisture.

Components 2,4 and 6 had the retention times of ethanal, propanal and methylpropanal respectively, whilst 5 and 8 corresponded to propanone and butanone. Component 7, however, did not correspond in retention time to any simple aldehyde or ketone. After reduction of the glandular liquid the only component unaccounted for eluted with a retention time between peaks 7 and 8 of the original extract. It seemed reasonable to assume that this new component was the reduction product of component 7, in which case it behaved like butanone on reduction in moving to a slightly longer retention time. This behaviour suggested that component 7 might have been butenone.

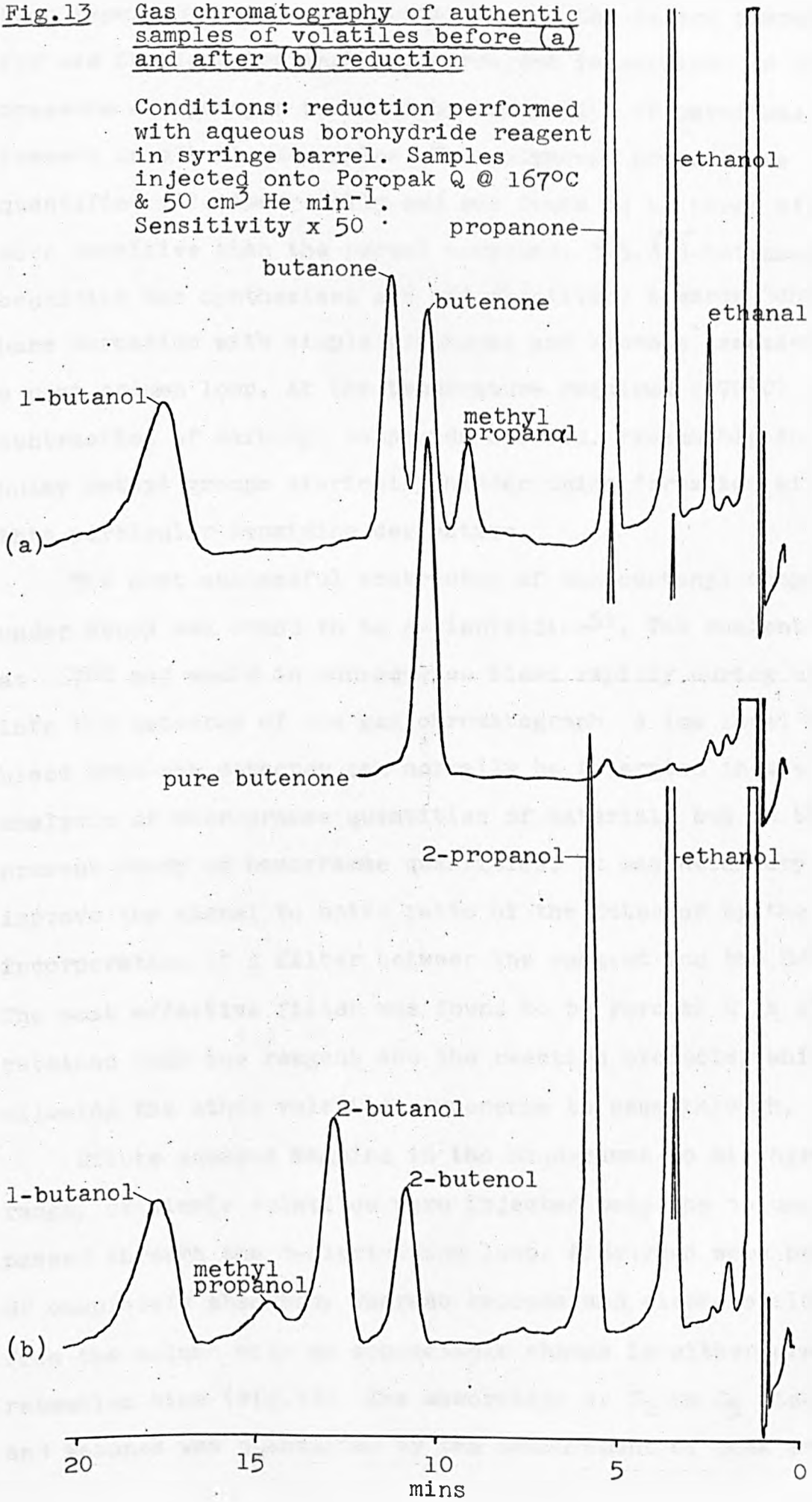
A freshly distilled solution of 90% butenone in water yielded, on co-chromatography with authentic samples of the other glandular components, a chromatographic profile like that of the natural glandular extract (Fig.13; cf.Fig.9). Furthermore, reduction of this mixture with sodium borohydride reagent in the barrel of a microlitre syringe yielded a profile like that of the reduced glandular extract (Fig.13; cf.Fig.12).

As a further check on the identity of component 7 and the other aldehydes and ketones, a number of reagents were investigated which might have reacted with these carbonyl compounds under the conditions required for their gas chromatographic separation.

Benzidine has been reported as a general abstractor for both aldehydes and ketones[51], but its use is now legally prohibited. The 3,5,3′,5′-tetramethylbenzidine derivative has

Fig.13 Gas chromatography of authentic samples of volatiles before (a) and after (b) reduction

Conditions: reduction performed with aqueous borohydride reagent in syringe barrel. Samples injected onto Poropak Q @ 167°C & 50 cm^3 He min^{-1}. Sensitivity x 50



been reported as a safer substitute for the parent compound for use in blood testing[56]. The reagent is oxidised in the presence of hydrogen peroxide by the action of peroxidase present in all blood samples. The polymeric product was quantified colorimetrically and was found to be three times more sensitive than the parent compound. 3,5,3',5'-tetramethyl-benzidine was synthesised and its reactivity towards Schiff base formation with simple aldehydes and ketones assessed in a post-column loop. At the temperature required (170°C) no subtraction of carbonyl compounds occured. Presumably the bulky methyl groups sterically hinder imine formation with this particular benzidine derivative.

The most successful abstractor of the carbonyl compounds under study was found to be o-dianisidine[51]. The reagent melts at 167°C and would in consequence bleed rapidly during use into the detector of the gas chromatograph. A low level of bleed into the detector can normally be tolerated in the analysis of microgramme quantities of material, but in the present study on nanogramme quantities, it was necessary to improve the signal to noise ratio of the detector by the incorporation of a filter between the reagent and the detector. The most effective filter was found to be Poropak Q as it retained both the reagent and the reaction products, whilst allowing the other volatile components to pass through.

Dilute aqueous samples, in the nanogramme to microgramme range, of simple volatiles were injected onto the column and passed through the o-dianisidine loop. Aldehydes were partially or completely absorbed, whereas ketones and alcohols eluted from the column with no appreciable change in either area or retention time (Fig.14). The absorption of C_2 to C_5 aldehydes and ketones was quantified by the measurement of peak areas

with and without the subtraction loop. Straight chain aldehydes were abstracted more completely than their 2-methyl branched isomers and the absorption was found to be greater for the higher homologues (Table 7).

TABLE 7

%age absorption of aldehydes and ketones by o-dianisidine

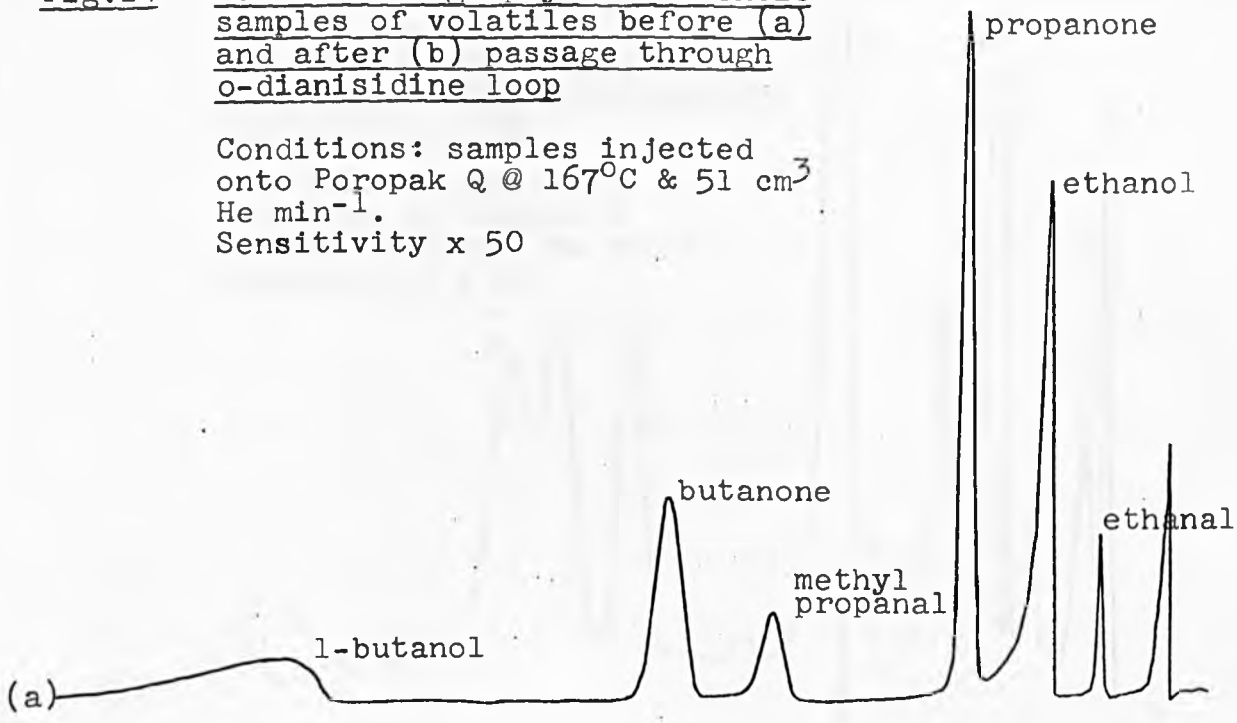
Aldehydes	%age absorption	Ketones	%age absorption
ethanal	83	propanone	0
propanal	88	butanone	0
methyl propanal	76	methyl butanone	0
butanal	100	2-pentanone	0
2-methyl butanal	80	3-pentanone	0
3-methyl butanal	100		
pentanal	100		

The passage of capillary extracts of Dufour's gland through the o-dianisidine loop in the gas chromatograph, resulted in the removal of peaks 2 and 4 and the partial removal of peak 6. Peak 7 however remained, supporting its identification as a ketone (Fig.15).

A number of attempts were made to reduce the double bond of the butenone in the extract without reducing the carbonyl compounds present to their alcohols. It was obviously not possible to separate and trap out component 7 from the mixture with the small quantity of material present, so the reagents chosen would have to be highly specific in reducing the butenone to butanone. None of the reagents investigated met this requirement. Raney nickel and hydrogen reduced the carbonyl compounds present to their alcohols and the addition of halogens readily yielded the substitution products of the carbonyl compounds.

Fig.14 Gas chromatography of authentic samples of volatiles before (a) and after (b) passage through o-dianisidine loop

Conditions: samples injected onto Poropak Q @ 167°C & 51 cm^3 He min^{-1}.
Sensitivity x 50



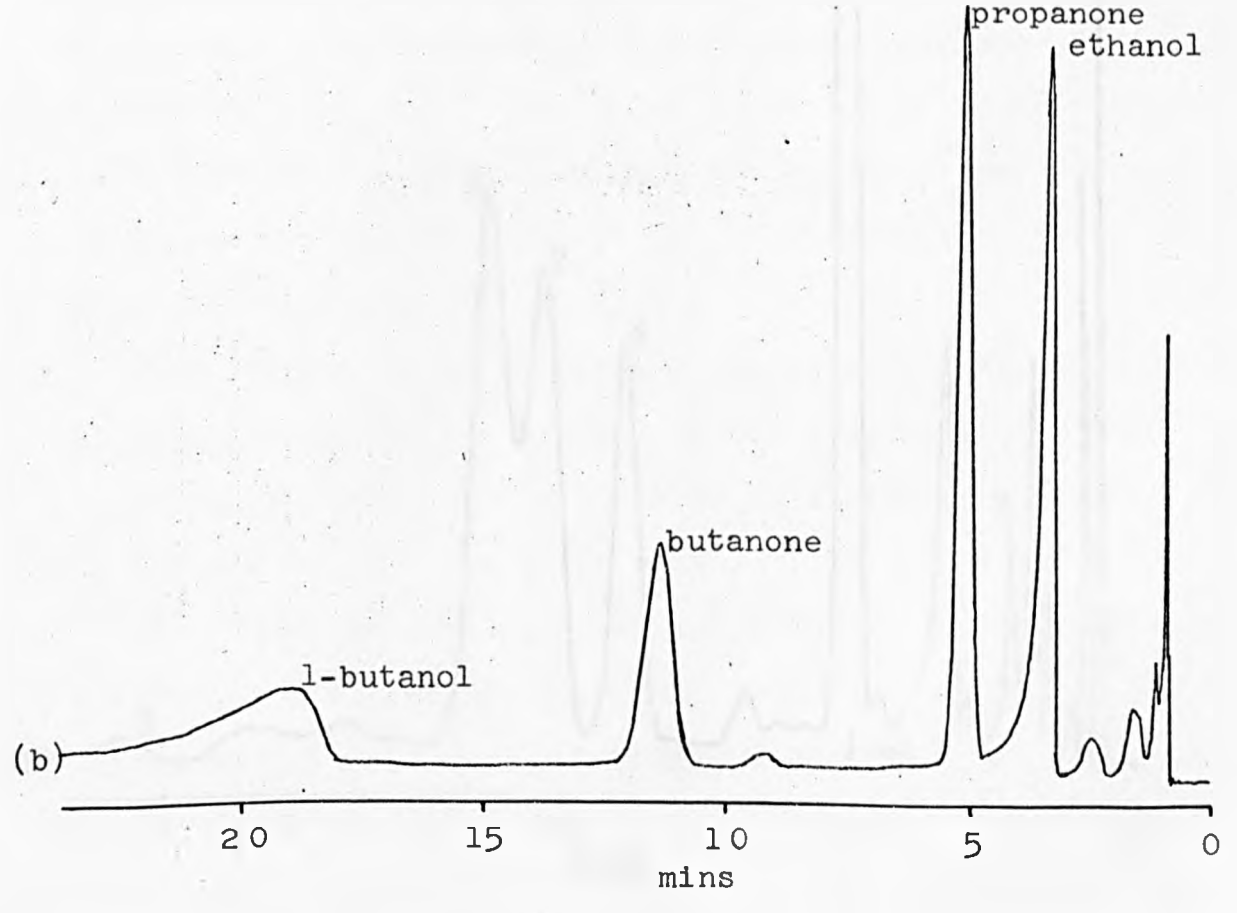
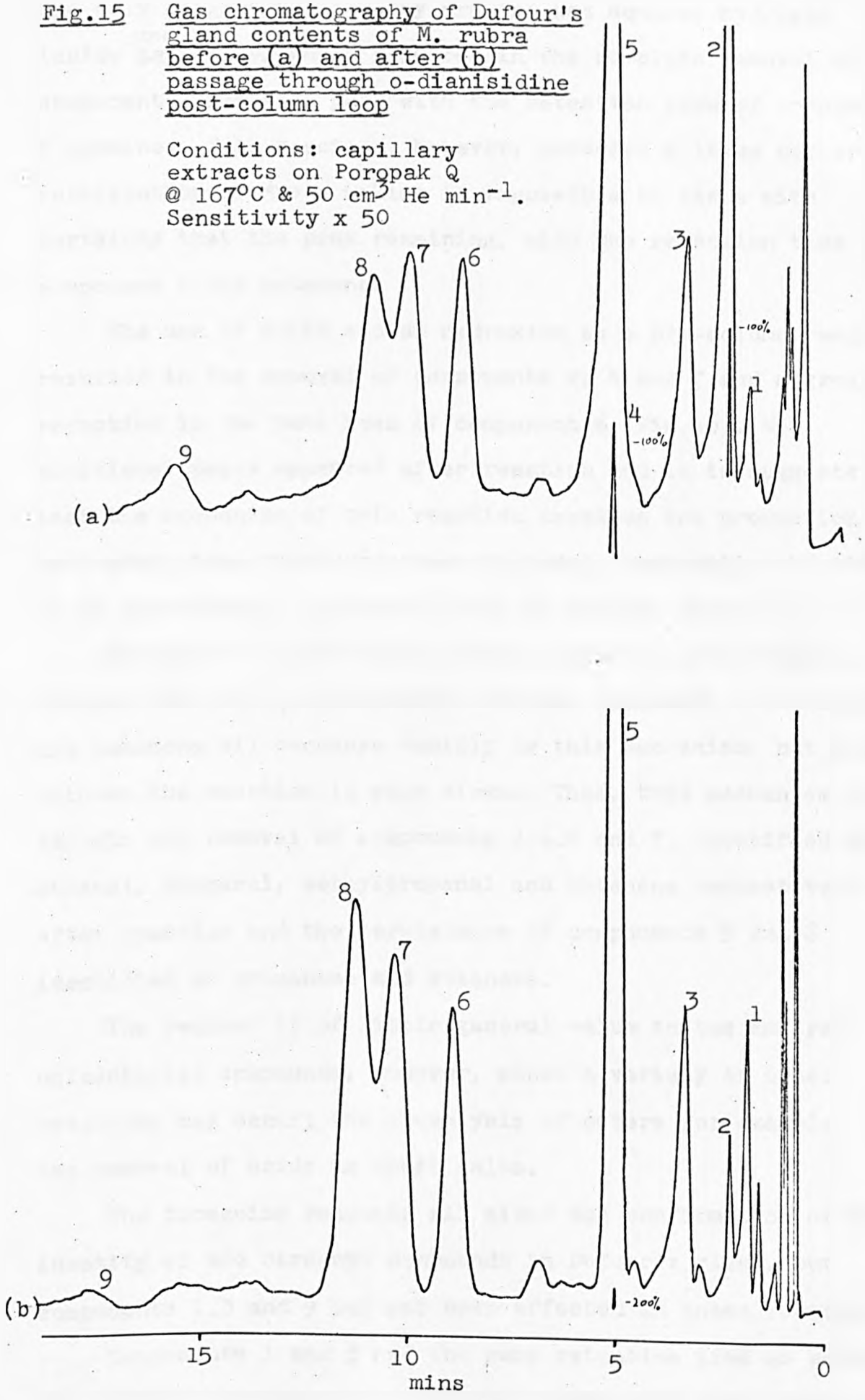


Fig.15 Gas chromatography of Dufour's gland contents of M. rubra before (a) and after (b) passage through o-dianisidine post-column loop

Conditions: capillary extracts on Porapak Q @ 167°C & 50 cm^3 He min^{-1}. Sensitivity x 50



The only reagent to show any promise was aqueous hydrogen iodide and iodine, which resulted in the complete removal of component 7 whilst a peak with the retention time of component 8 remained. This reaction, however, produced a large number of substitution products making it impossible to state with certainty that the peak remaining, with the retention time of component 8 was butanone.

The use of solid sodium hydroxide as a pre-column reagent resulted in the removal of components 2, 4 and 7 and a great reduction in the peak area of component 6 (Fig.16). No additional peaks appeared after reaction and it is suggested that the mechanism of this reaction involves the production of an enolate ion, which undergoes an aldol condensation by attack on an electrophylic carbonyl group of another molecule.

In Dufour's gland there exists a mixture of aldehydes, ketones and the α,β-unsaturated ketone, butenone. The aldehydes and butenone all condense rapidly by this mechanism, but with ketones the reaction is much slower. Thus, this mechanism would explain the removal of components 2,4,6 and 7, identified as ethanal, propanal, methylpropanal and butenone respectively after reaction and the persistance of components 5 and 8 identified as propanone and butanone.

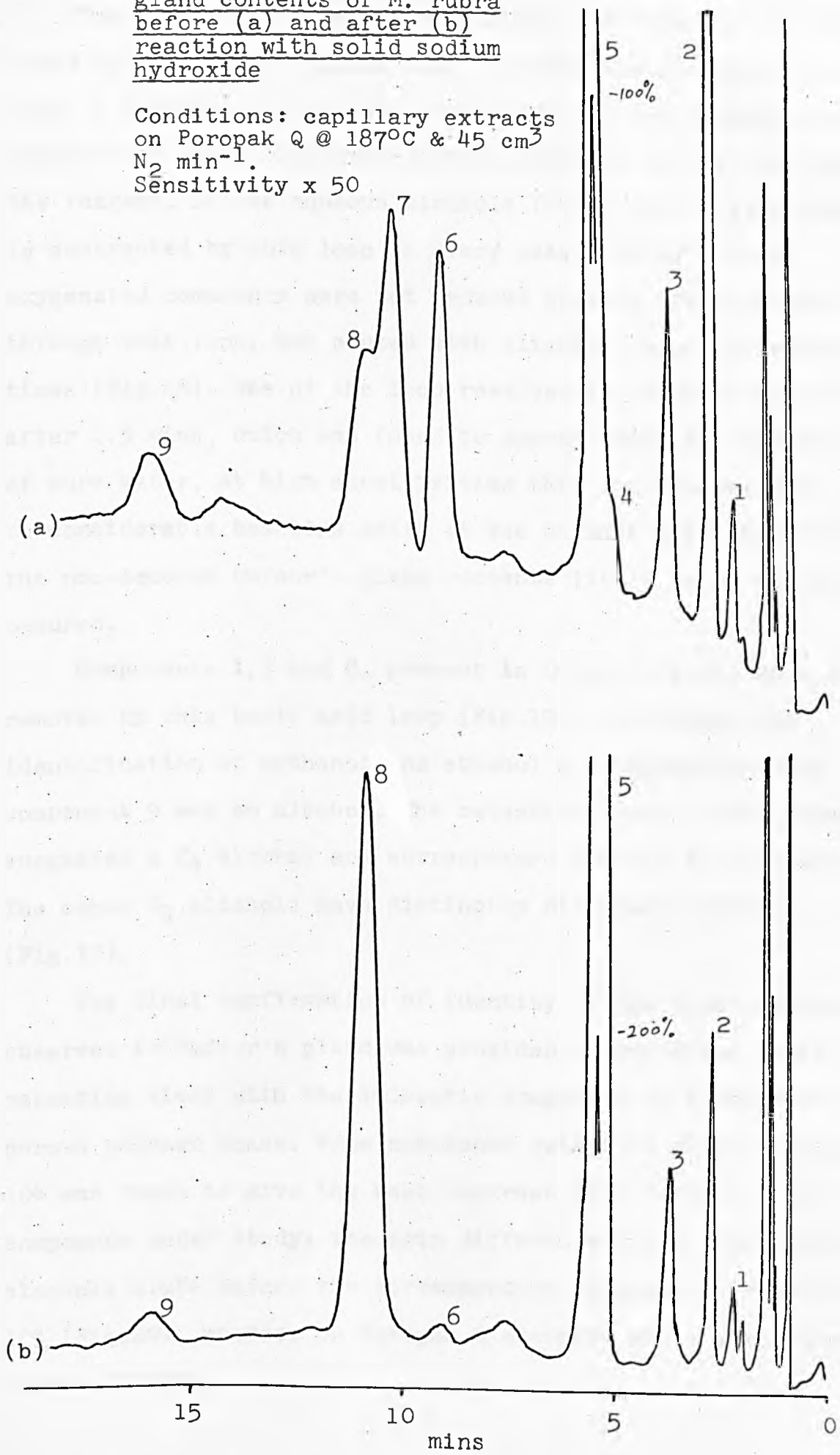
The reagent is of little general value in the analysis of unidentified compounds, however, since a variety of other reactions may occur; the hydrolysis of esters for example, or the removal of acids as their salts.

The foregoing reagents all aided the confirmation of the identity of the carbonyl compounds in Dufour's gland, but components 1,3 and 9 had not been affected in these reactions.

Components 1 and 3 had the same retention time as methanol and ethanol respectively, which had previously been identified

Fig.16 Gas chromatography of Dufour's gland contents of M. rubra before (a) and after (b) reaction with solid sodium hydroxide

Conditions: capillary extracts on Poropak Q @ 187°C & 45 cm^3 N_2 min^{-1}.
Sensitivity x 50



in the ampoule distillate.

The identity of these alcohols was confirmed by the use of boric acid in a post-column loop. As with the o-dianisidine loop, a Poropak Q filter was placed between the reagent and the detector to reduce background noise produced by the bleeding of the reagent. Dilute aqueous alcohols from C_1 to C_4 were completely subtracted by this loop in every case (Fig.17). Other oxygenated compounds were not reduced in area after passage through this loop, but eluted with slightly longer retention times (Fig.18). Use of the loop resulted in baseline disturbance after 1.5 mins, which was found to appear after the injection of pure water. At high sensitivities this disturbance gave rise to considerable baseline drift on the chromatogram, but with the non-aqueous Dufour's gland contents little or no disturbance occured.

Components 1,3 and 9, present in Dufour's gland were all removed by this boric acid loop (Fig.19), confirming the identification of methanol and ethanol and indicating that component 9 was an alcohol. The retention time of this component suggested a C_4 alcohol and corresponded exactly to 1-butanol. The other C_4 alcohols have distinctly different retention times (Fig.17).

The final confirmation of identity of the nine components observed in Dufour's gland was provided by comparing their retention times with the authentic compounds on a contrasting porous polymer phase. From published retention data Chromosorb 104 was found to give the best contrast with Poropak Q for the compounds under study; the main difference being that secondary alcohols elute before the corresponding ketones on Chromosorb 104 (Fig.20), whereas on Poropak Q alcohols elute after their parent ketones.

Fig.17 Gas chromatography of authentic alcohols before (a) and after (b) passage through boric acid loop

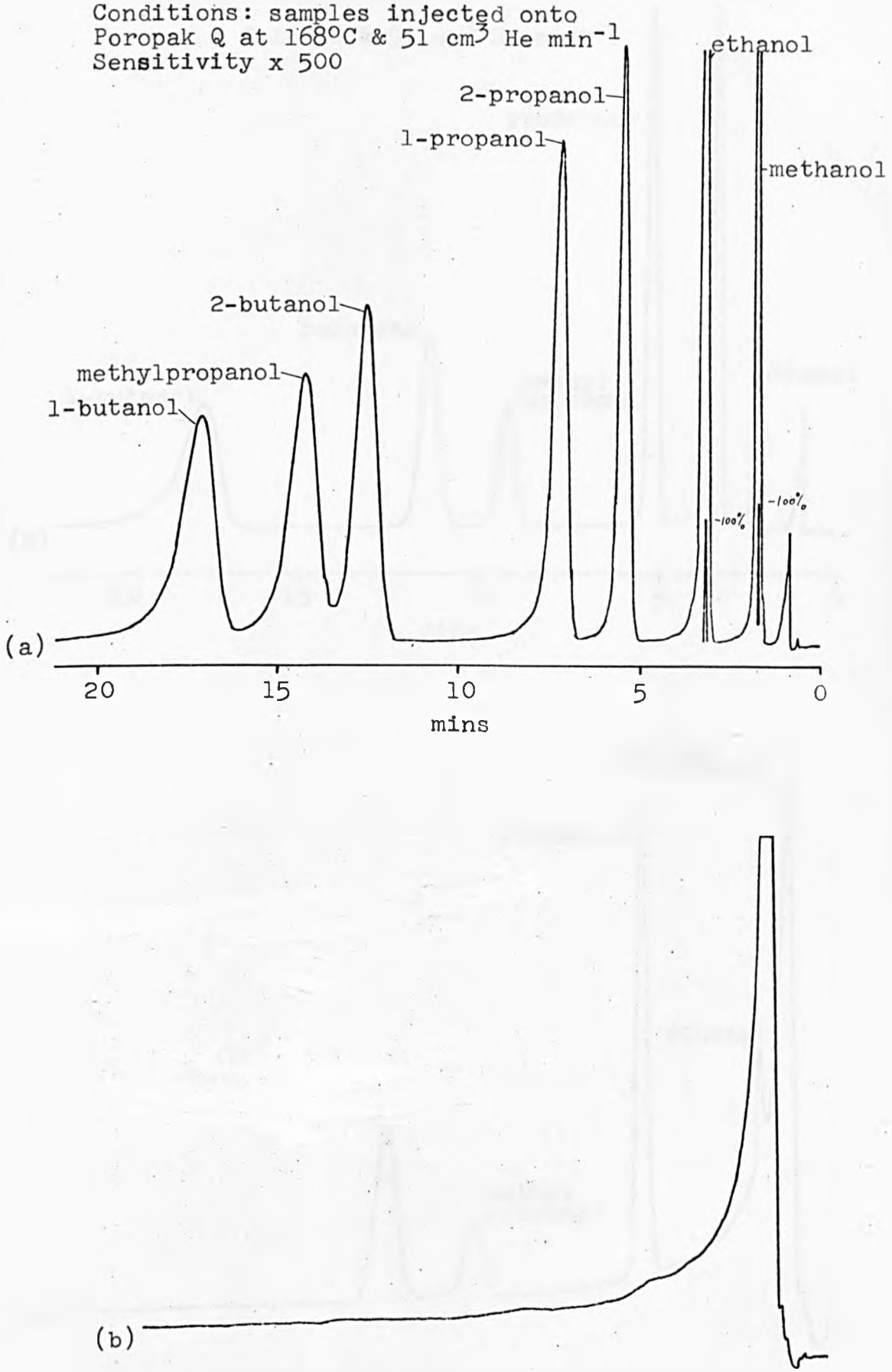
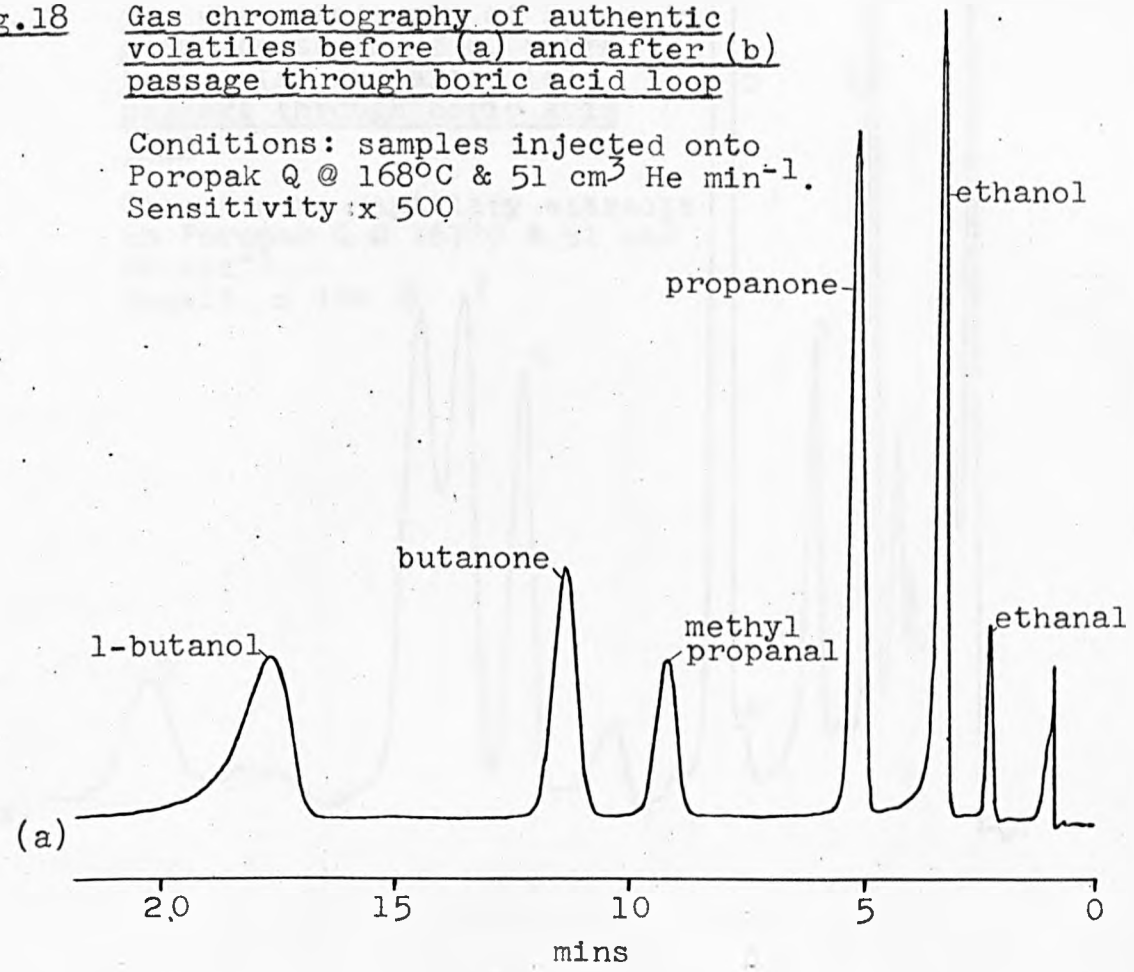


Fig.18 Gas chromatography of authentic volatiles before (a) and after (b) passage through boric acid loop

Conditions: samples injected onto Poropak Q @ 168°C & 51 cm^3 He min^{-1}. Sensitivity x 500



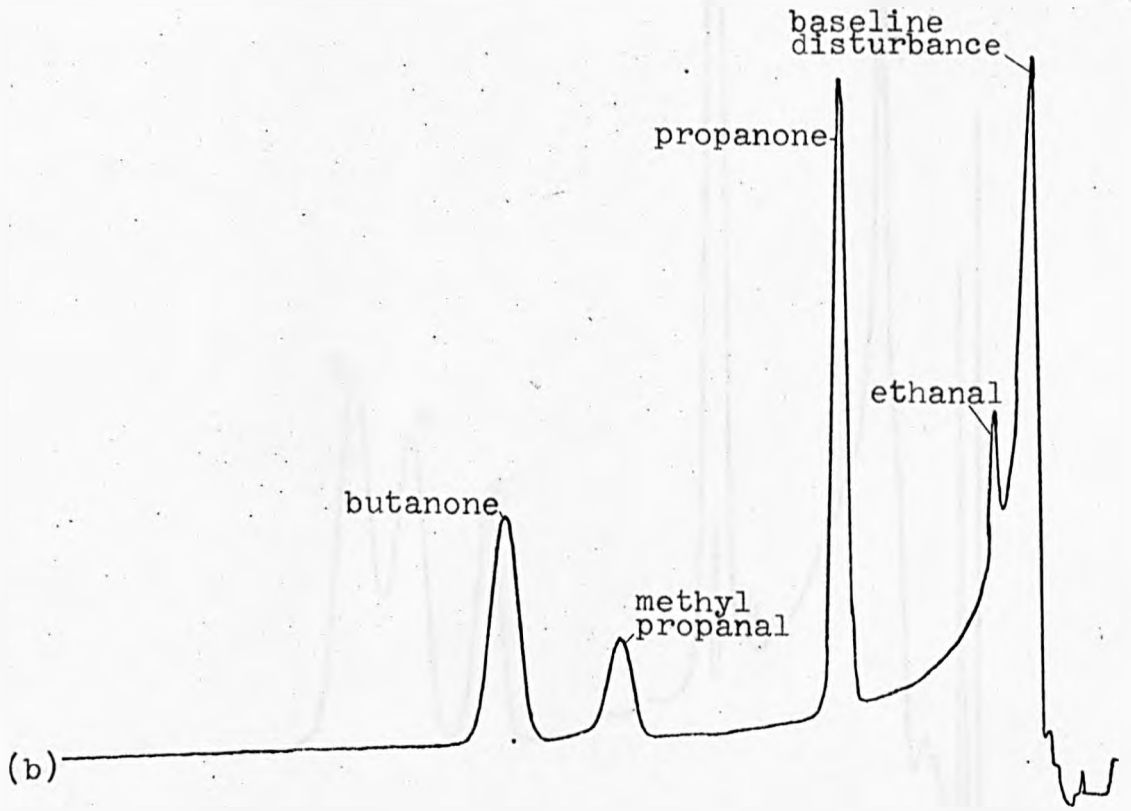
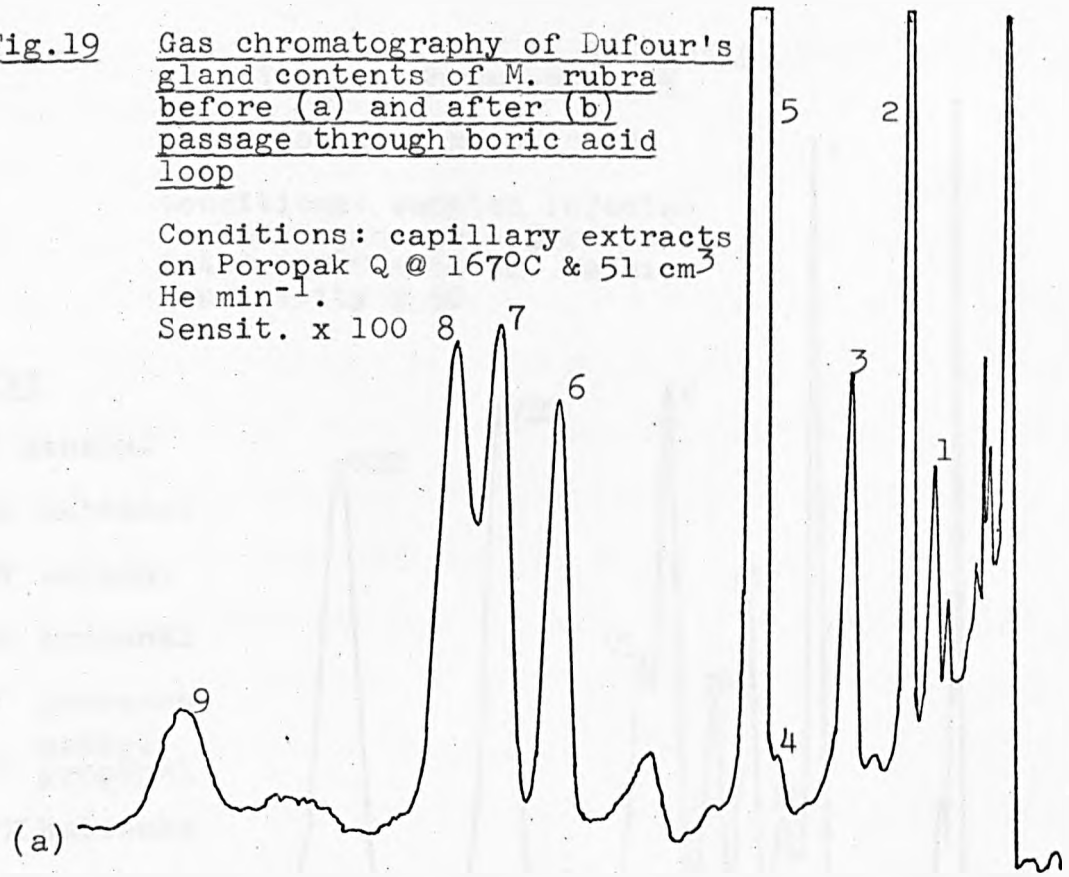


Fig.19 Gas chromatography of Dufour's gland contents of M. rubra before (a) and after (b) passage through boric acid loop

Conditions: capillary extracts on Poropak Q @ 167°C & 51 cm^3 He min^{-1}.
Sensit. x 100



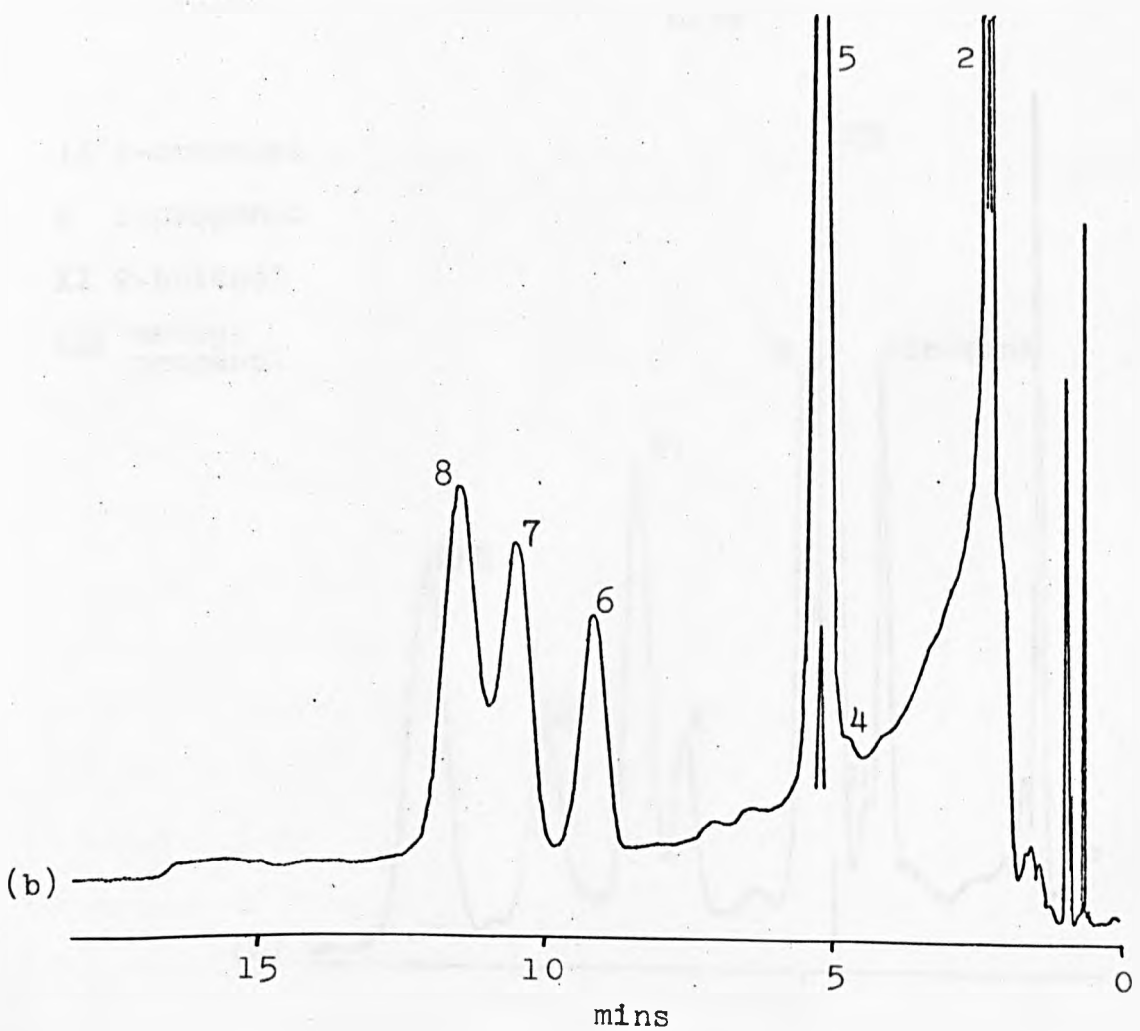
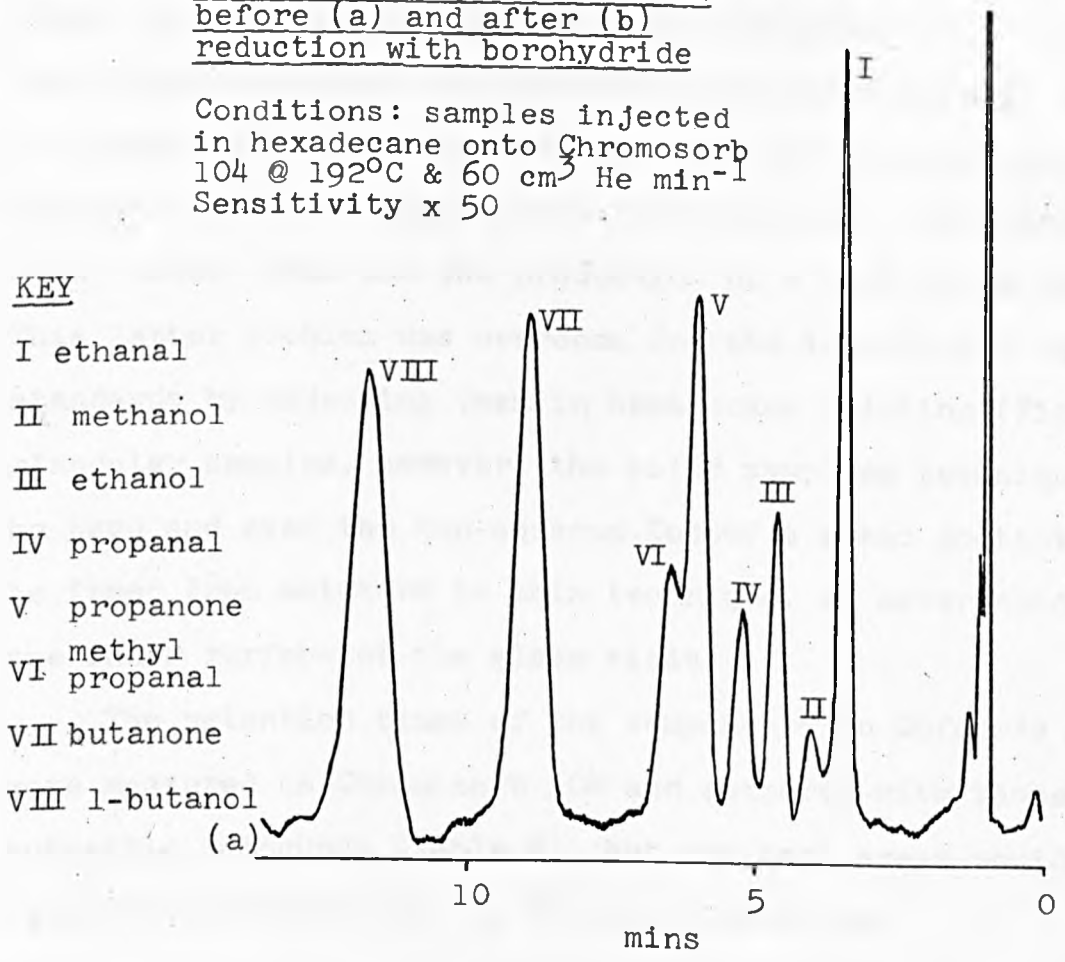
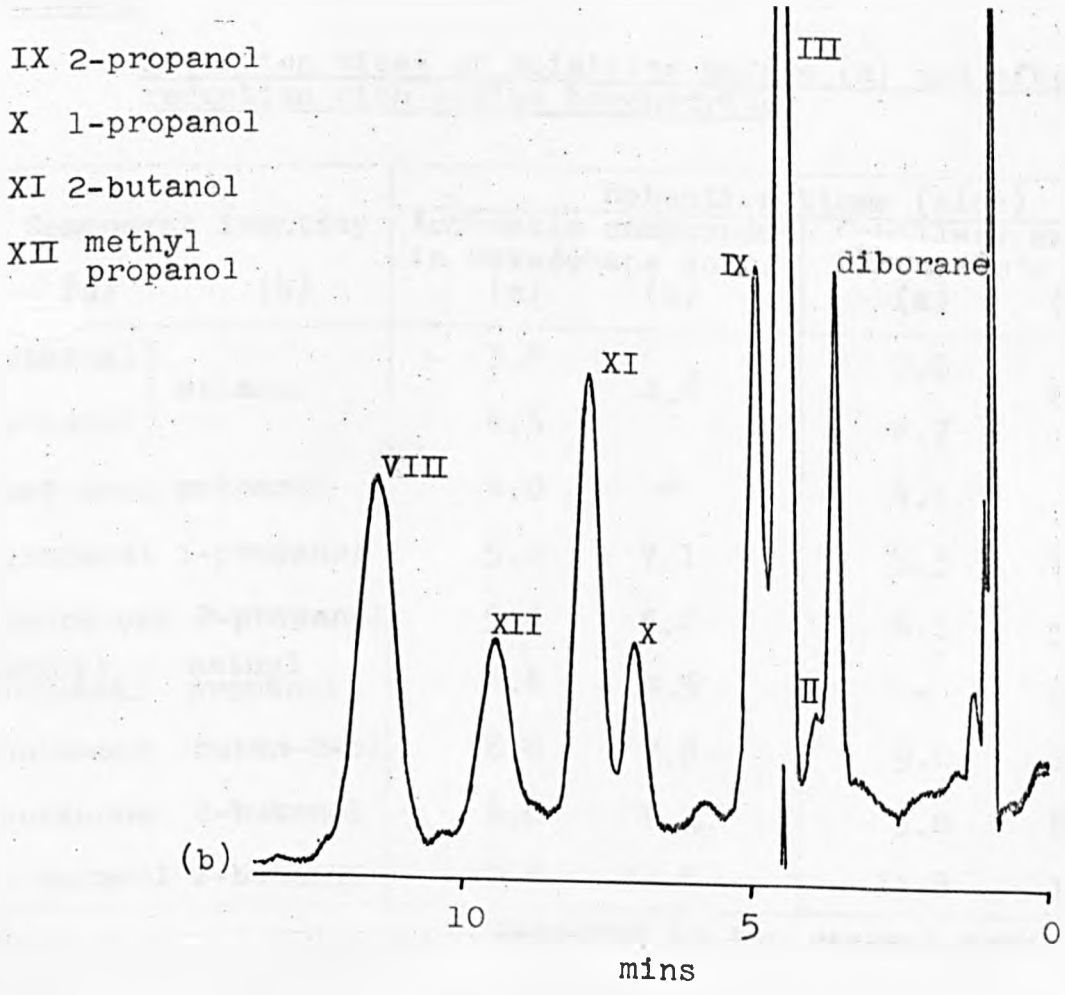


Fig.20 Gas chromatography of authentic volatiles on Chromosorb 104 before (a) and after (b) reduction with borohydride

Conditions: samples injected in hexadecane onto Chromosorb 104 @ 192°C & 60 cm^3 He min^{-1} Sensitivity x 50





The behaviour of this Chromosorb 104 phase, however, was found to make it most unsuitable for continual use. During use the phase discolours and contracts continually. Small samples of ethanal in particular were absorbed by the phase and the injection even of large samples in water led to the elution of a 'water' peak and the production of a very noisy baseline. This latter problem was overcome for the injection of authentic standards by injecting them in hexadecane solution (Fig.20). For glandular samples, however, the solid sampling technique had to be used and even the non-aqueous Dufour's gland contents cannot be freed from moisture in this technique, as water condenses on the inner surface of the glass vials.

The retention times of the components in Dufour's gland were measured on Chromosorb 104 and compared with those of authentic compounds (Table 8), but the peak areas could not be measured accurately due to the noisy baseline.

TABLE 8

Retention times of volatiles before (a) and after (b) reduction with sodium borohydride

Component identity (a)	(b)	Retention times (mins) Authentic compounds in hexadecane soln. (a)	(b)	Capillary extracts of Dufour's gland (a)	(b)
ethanal	ethanol	3.4	4.6	3.5	4.7
ethanol	ethanol	4.6		4.7	
methanol	methanol	4.0	*	4.1	*
propanal	1-propanol	5.2	7.1	5.3	7.2
propanone	2-propanol	5.9	5.0	6.1	5.1
methyl propanal	methyl propanol	6.4	9.5	-	9.7
butenone	buten-2-ol	8.8	7.8	9.0	8.0
butanone	2-butanol	8.8	7.9	9.0	8.0
1-butanol	1-butanol	11.6	11.6	11.8	11.9

* Obscured by the reagent peak

The results on Chromosorb 104 confirmed the identity of components 1,2,3,4,5 and 9. Components 7 and 8 were not completely resolved under these conditions, nor were components 5 and 6. The chromatogram of the corresponding sample after reduction with sodium borohydride still showed a double peak for the reduction products of components 7 and 8, but the reduction products of components 5 and 6, 2-propanol and methylpropanol respectively, were well resolved.

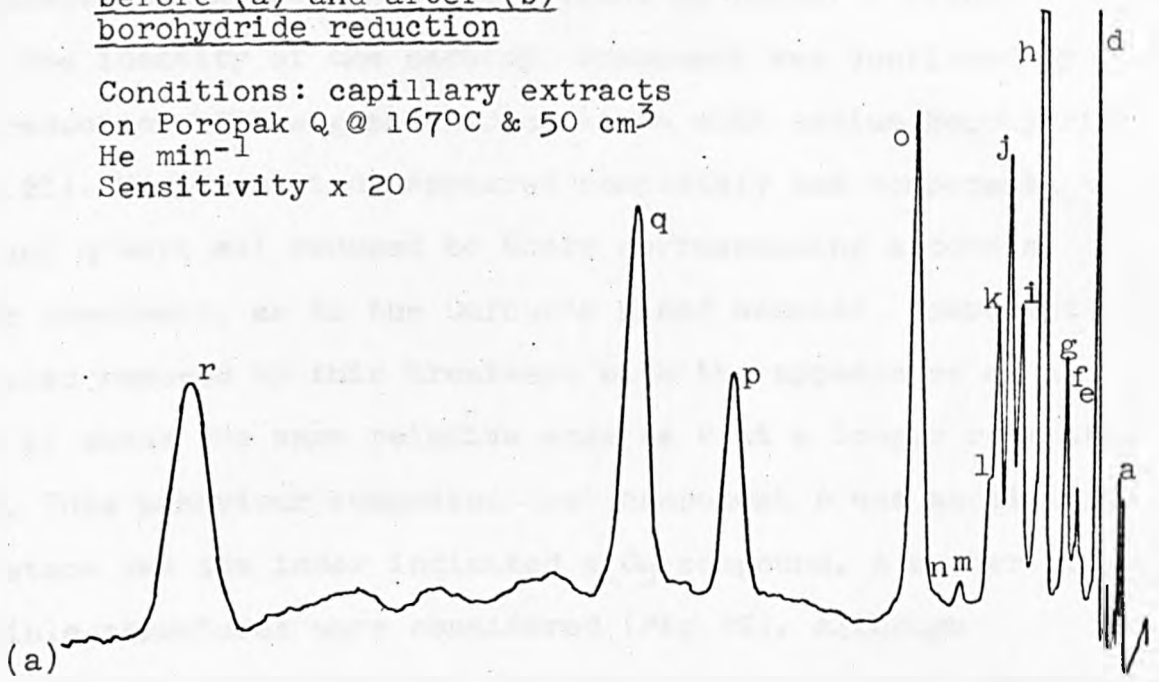
Analysis of the volatiles in capillary extracts of poison vesicles (M. rubra)

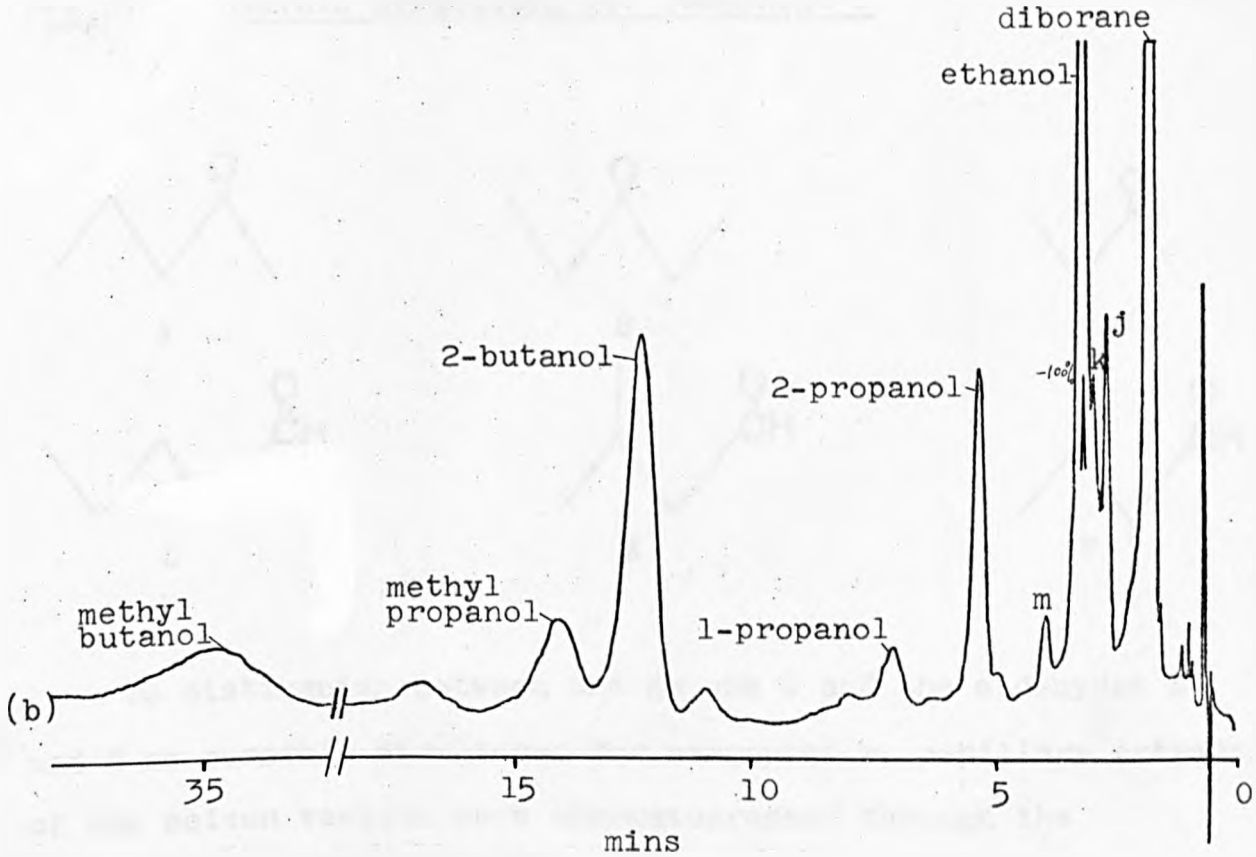
The removal of the poison vesicle contents by the capillary extraction technique proved to be more difficult than for Dufour's gland, as the proteinaceous venom coagulates on contact with the air. This difficulty was alleviated by the use of fine capillaries (50 μm) drawn to give very thin walls. These can pierce the poison vesicle cleanly, making only a fine hole and thus reducing the contact of the gland contents with the air.

The gas chromatogram of the volatile contents of this gland when contrasted with the Dufour's gland revealed that despite its greater volume it contains quantitatively less material. The absolute quantity of each component is discussed in a later section. Qualitatively the poison vesicle contains more material than Dufour's gland, having 18 components (Fig.10). Two of these components, a and d, were found to correspond in retention times to air and water respectively and the components g,h,l,n,o,p and q corresponded to the methanol, ethanal, ethanol, propanal, propanone, methylpropanal and butanone respectively found in Dufour's gland and chromatographed under identical conditions. No peaks corresponding to butenone or 1-butanol were present in the chromatogram of the poison vesicle, but one of the major

Fig.21 Gas chromatography of poison vesicle contents of M. rubra before (a) and after (b) borohydride reduction

Conditions: capillary extracts on Poropak Q @ 167°C & 50 cm^3 $He\ min^{-1}$
Sensitivity x 20

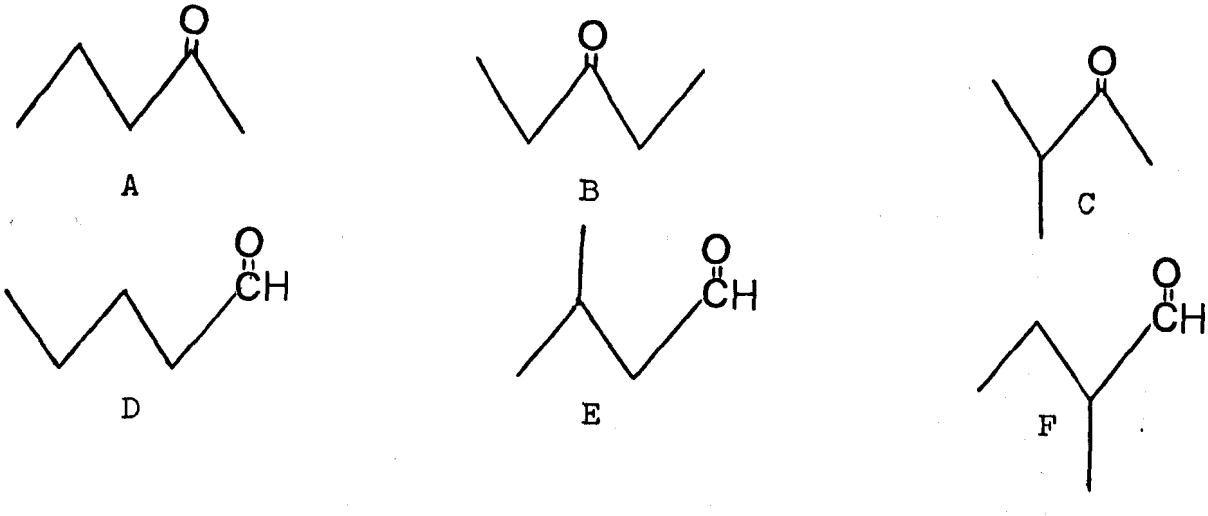




volatile components of this gland, component r and the minor components i,j,k and m are not present in Dufour's gland.

The identity of the carbonyl compounds was confirmed by the reduction of the glandular contents with sodium borohydride (Fig.21). Component i disappeared completely and components h,n, o,p and q were all reduced to their corresponding alcohols, after treatment, as in the Dufour's gland samples. Component r was also removed by this treatment with the appearance of a peak of about the same relative area as r at a longer retention time. This behaviour suggested that component r was an aldehyde or ketone and its index indicated a C_5 compound. A number of possible structures were considered (Fig.22), although 2,2-dimethylpropanal was not included as being highly improbable on biogenetic grounds. Authentic samples of the straight chain compounds A,B and D were all found to have higher retention indices than component r, ruling out these as possibilities.

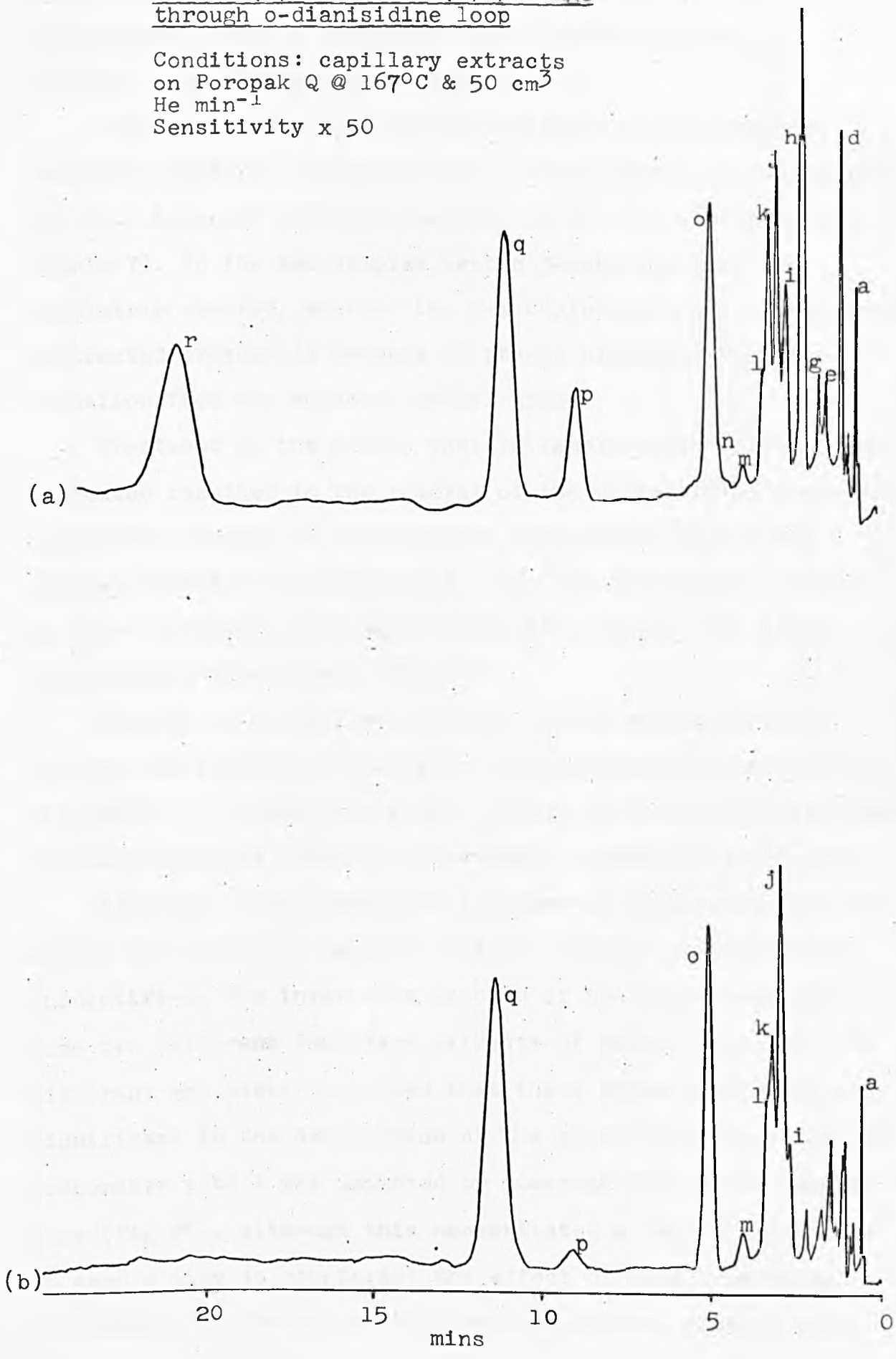
Fig.22 Possible structures for component r



To distinguish between the ketone C and the aldehydes E and F as possible structures for component r, capillary extracts of the poison vesicle were chromatographed through the o-dianisidine loop (Fig.23).

Fig.23 Gas chromatography of poison vesicle contents of M. rubra before (a) and after (b) passage through o-dianisidine loop

Conditions: capillary extracts on Poropak Q @ 167°C & 50 cm^3 $He\ min^{-1}$
Sensitivity x 50



Component r was completely removed by this treatment indicating an aldehyde structure (E or F). The removal of components h,n and p confirmed their identity as ethanal, propanal and methylpropanal respectively.

Component r was tentatively assigned the identity of 3-methylbutanal by comparison of its abstraction by o-dianisidine with those of authentic samples of 2- and 3-methylbutanal (Table 7). In the two samples tested 3-methylbutanal was completely removed, whereas the 2-methylbutanal was incompletely abstracted presumably because of steric hindrance to imine formation from the adjacent methyl group.

Treatment of the poison vesicle samples with solid sodium hydroxide resulted in the removal of the unidentified component i, and the removal of the aldehyde components h,n and the partial removal of p. Component r was not discernably reduced by this treatment, presumably since it undergoes the aldol condensation more slowly (Fig.24).

Passage of a capillary extract of the poison vesicle through the boric acid loop after chromatography, resulted in the removal of components g and l which is consistent with their identification as methanol and ethanol respectively (Fig.25).

After all these reactions a number of components, particularly those eluting rapidly from the column, still remained unidentified. The invariable pattern of the minor components in some ten different capillary extracts of poison vesicles from different ant nests suggested that these trace components were significant in the composition of the gland. The resolution of components a to l was improved by lowering the column temperature (Fig.26), although this necessitated a two-fold increase in sample size to counteract the effect of peak broadening. Calibration of the column with methane,ethane, propane and

Fig.24 Gas chromatography of poison vesicle contents of M. rubra before (a) and after (b) treatment with solid sodium hydroxide

Conditions: capillary extracts on Poropak Q @ 167°C & 60 cm^3 He min^{-1}
Sensitivity x 50

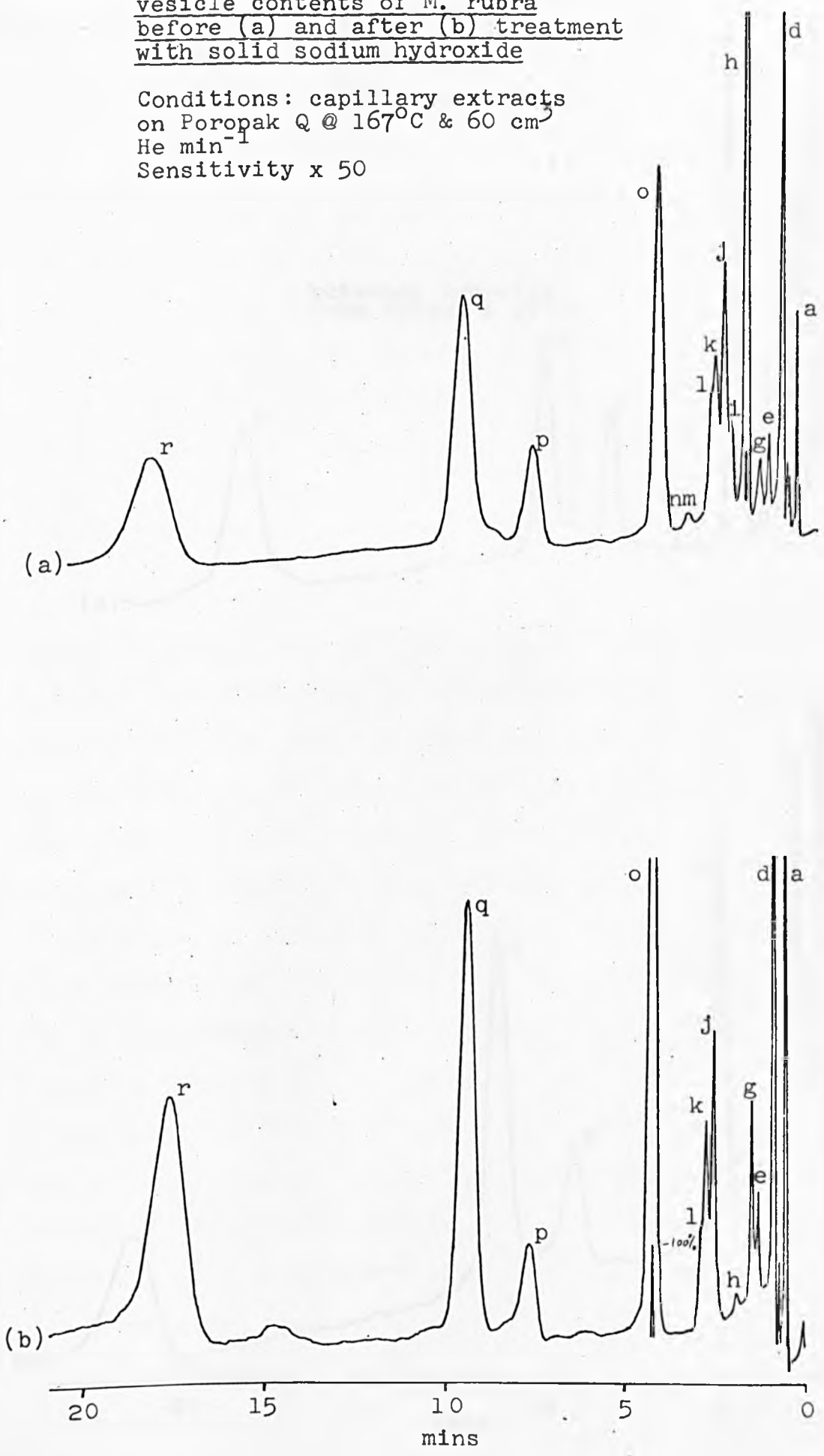
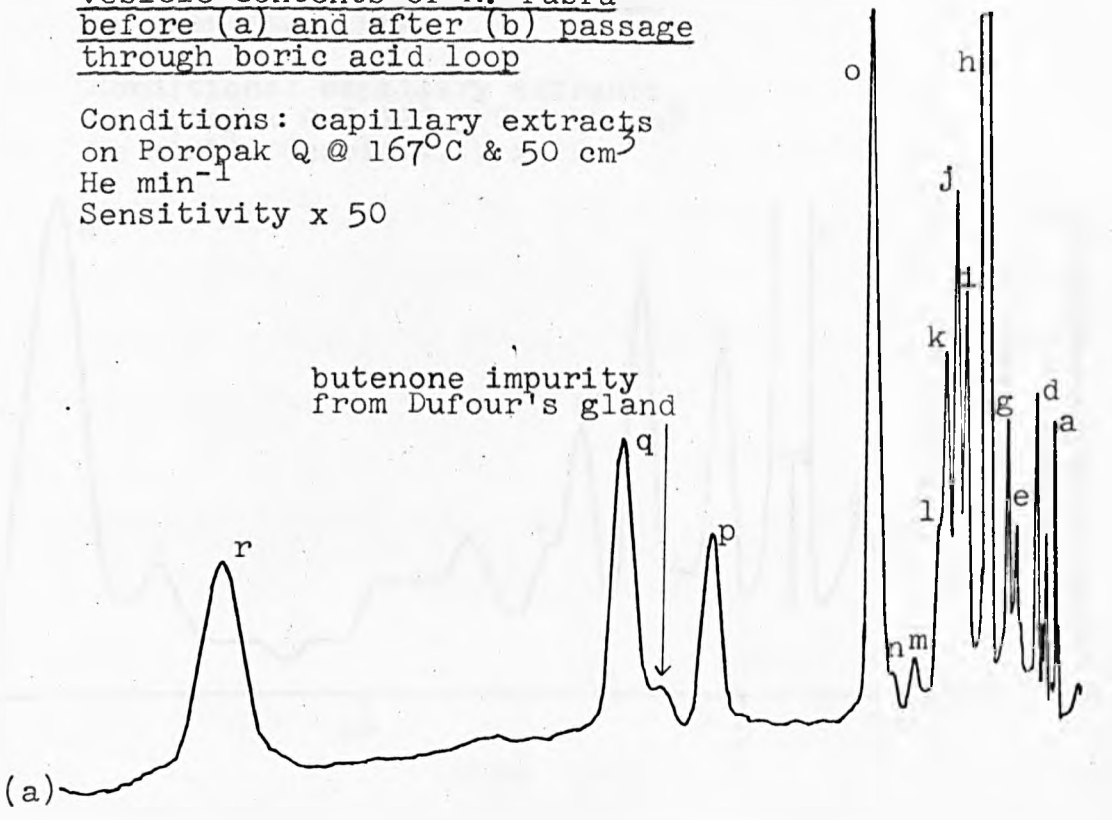


Fig.25 Gas chromatography of poison vesicle contents of M. rubra before (a) and after (b) passage through boric acid loop

Conditions: capillary extracts on Poropak Q @ 167°C & 50 cm^3 $He\ min^{-1}$
Sensitivity x 50



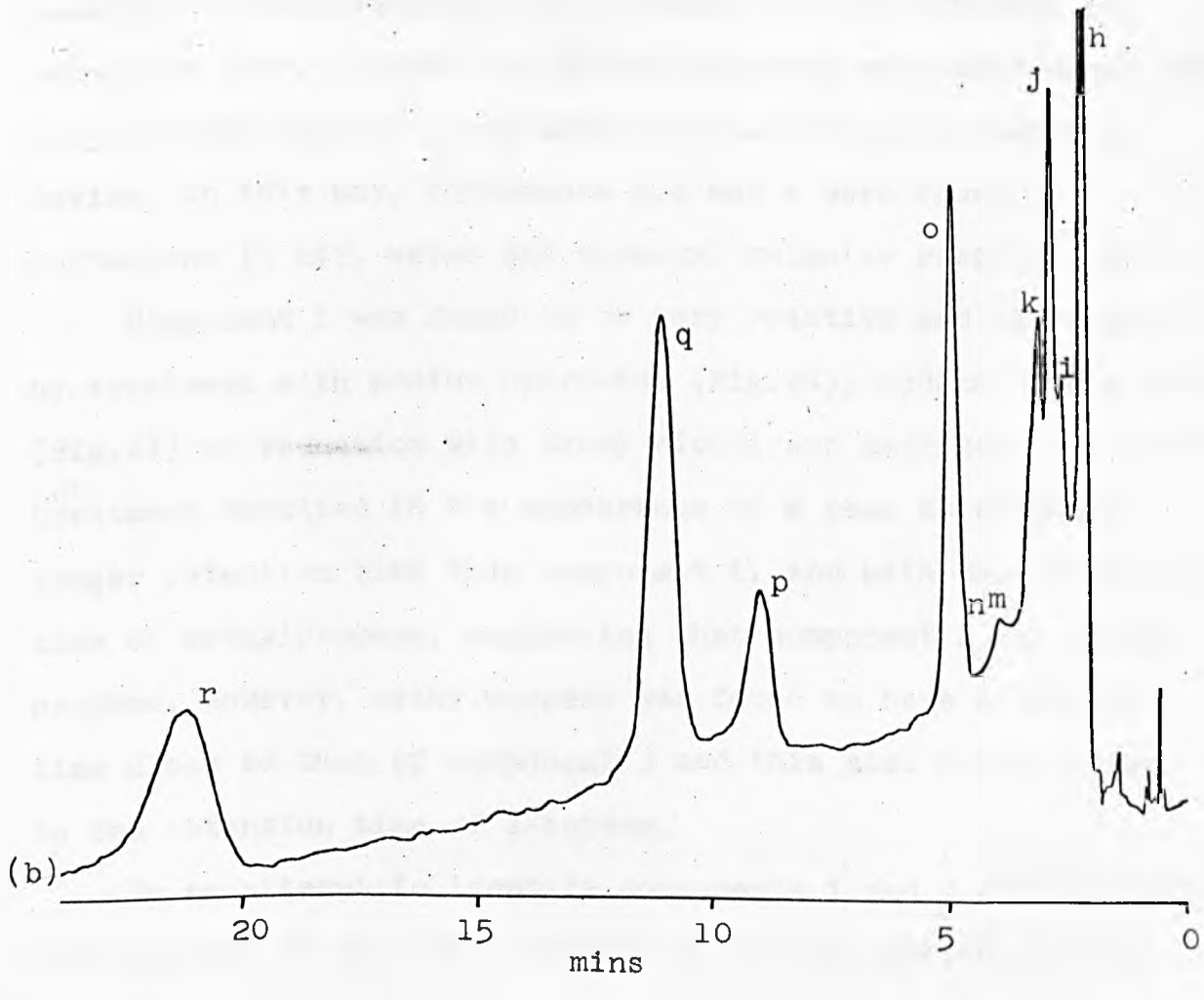
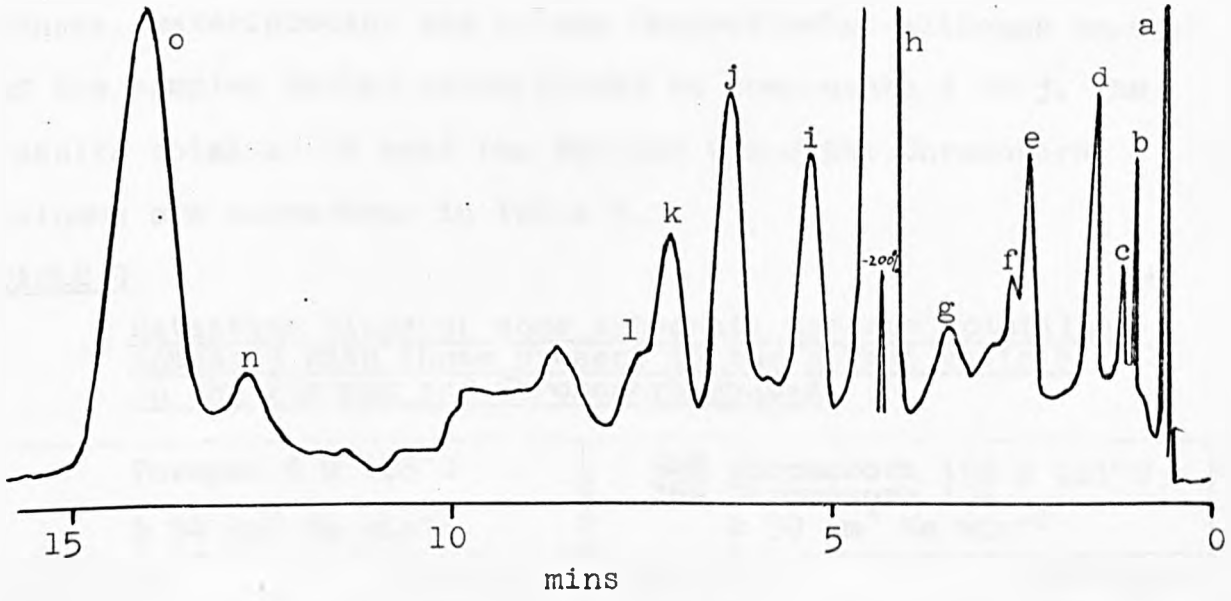


Fig.26 Gas chromatography of 8 poison vesicles of M. rubra, showing gaseous volatiles

Conditions: capillary extracts on Poropak Q @ 127.5°C & 54 cm^3 He min^{-1}. Sensitivity x 10



butane revealed that trace components b,c and f and the minor component k respectively, corresponded to these alkanes in retention time. A number of authentic gases were introduced into the chromatograph in glass ampoules via the solid sampling device. In this way, components a,d and e were found to correspond to air, water and carbonyl sulphide respectively.

Component i was found to be very reactive and was removed by treatment with sodium hydroxide (Fig.24), sodium borohydride (Fig.21) or reduction with Raney nickel and hydrogen. The latter treatment resulted in the appearance of a peak at slightly longer retention time than component i, and with the retention time of methylpropane, suggesting that component i was methylpropene. However, methylpropene was found to have a retention time close to that of component j and this also corresponded to the retention time of 1-butene.

In an attempt to identify components i and j and to confirm the identity of the other gaseous volatiles, poison vesicle

extracts were chromatographed on a contrasting phase. To reduce the problems encountered with Chromosorb 104, this phase was combined with Chromosorb 102 (36:64 $^{w}/_{w}$ respectively). Components a,b,c,d,f and k still corresponded to air, methane, ethane, water,propane and butane respectively, although none of the samples tested corresponded to components i or j. The results obtained on both the Poropak Q and the Chromosorb columns are summarised in Table 9.

TABLE 9

Retention times of some authentic gaseous volatiles compared with those present in the poison vesicle on the Poropak and Chromosorb phases

Poropak Q @ 128°C & 54 cm^3 He min^{-1}			64% Chromosorb 102 @ 101°C 36% Chromosorb 104 & 50 cm^3 He min^{-1}		
Retention time of components in ant	Compound	Retention time of authentic compounds	Retention time of components in ant	Compound	Retention time of authentic compounds
a 0.6	air	0.6	0.7	air	0.7
b 0.9	methane	1.0	0.8	methane	0.8
c 1.1	ethane	1.2	1.3	ethane	1.3
d 1.4	water	1.4		ethene	1.5
e 2.3	carbonyl sulphide	2.3	1.4	water	1.4
f 2.6	propane	2.6	2.9	propane	2.8
g 3.2	methanol	3.3		methyl propene	7.2
h 4.2	ethanal	4.3		methyl propane	7.4
	ethylene oxide	4.5		1-butene	7.4
i 5.2	-		7.5	butane	7.6
	methyl propane	5.8	7.9	-	
	methyl propene	6.0	8.5	-	
j 6.2	1-butene	6.1		1,3-buta-diene	8.8
	(E)-2-butene	6.7			
k 7.0	butane	7.0			
	(Z)-2-butene	7.1			

Absolute quantities of volatiles per gland (M. rubra)

During the chemical analysis of the volatiles in Dufour's gland and the poison vesicle, authentic samples of various compounds had been injected into the gas chromatograph in dilute aqueous solution. The resulting peak areas revealed that the same weight of different compounds did not give the same detector response.

In gas chromatography the percentage composition of mixtures analysed is frequently calculated from the peak areas of the components, making the assumption that the number of ions produced by components in a flame ionisation detector is directly proportional to the molecular weight of each component. Whilst this relationship may hold well for relatively high molecular weight compounds in the same homologous series, it clearly cannot be applied to the small oxygenated molecules in the present study.

Authentic samples of the components identified were chromatographed, and a flame response factor calculated for each compound. In the case of ethanal this factor proved to be high, reflecting the fact that this compound ionises poorly in the detector flame. The unidentified components i and j in the poison vesicle were arbitrarily assigned the response factor of ethanol to allow an estimation of the total volatile contents of each gland (Tables 5 & 6).

Determination of the glandular volumes and water content

In the capillary extraction of glandular samples for analysis it could clearly be seen under the microscope that in many cases essentially all the glandular liquid had been removed. This suggested that it might be possible to estimate the glandular volumes by calculation of the volume of liquid removed from the gland.

As the Dufour's gland contents proved the easiest to remove by capillary extraction, the volume of this gland was measured first. Two different methods were used: in the most direct method the inside diameter of the tube was measured and assuming it to be uniformly circular the volume was calculated from the equation $\pi(c/2)^2(a)$, where (c) is the diameter of the tube and (a) is the length of the glandular liquid contained in the tube. In the second technique the 'water equivalent volume' was calculated. In this technique a Katharometer detector was calibrated by injection of water in dry ethanol using the Poropak Q column. A calibration graph was compiled of water volume injected versus peak height on the chromatogram. The peak heights were corrected for the residual water content of the 'dry' ethanol (Fig.27).

A uniform capillary was filled with the glandular liquid and its length measured to give (a) in Table 10. The immediately adjacent capillary was then filled with distilled water to a length (b). Analysis of sample (b) by gas chromatography gave a water peak height of (e). This value was corrected for the average water content of an empty vial to give (g). Using the calibration graph, the volume of water for each sample was calculated as (h). The volume of liquid removed from Dufour's gland must then have had a volume of (h) x (a)/(b) in each case.

The results obtained by these two methods were found to be in reasonable agreement for five Dufour's gland samples from differently sized workers. The average volume is around 7.5 nl for this gland (Table 10). This average volume is identical with that reported by Cammaerts-Tricôt[57] for the volume of an older forager's Dufour's gland of the species M. rubra. The estimate in this case was made by microscopic examination of the dissected gland on a graticule.

We have been able to estimate the volume of hydrocarbons

Fig.27 Calibration graph of water volume injected versus water peak height on the chromatogram

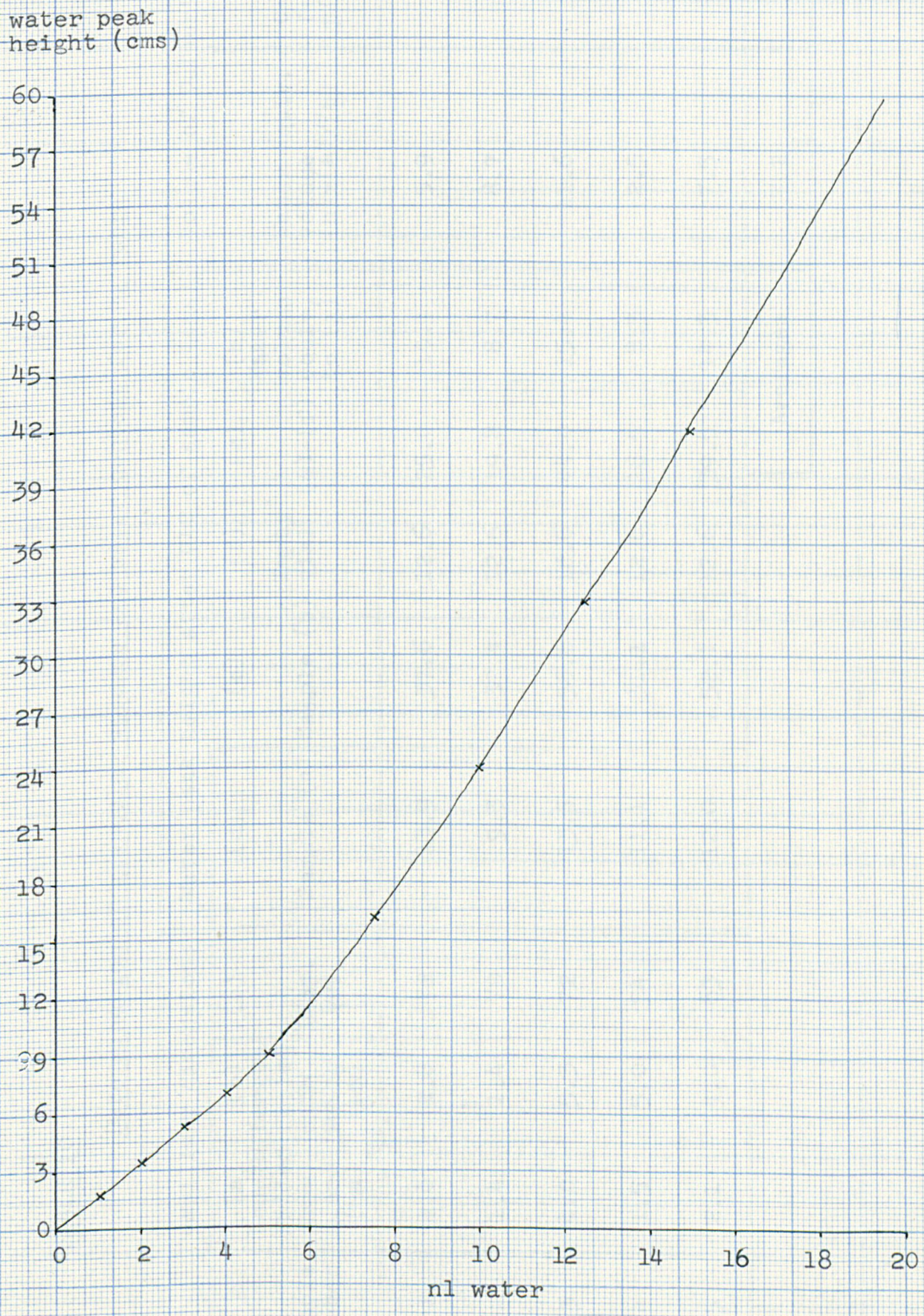


TABLE 10

Determination of glandular volumes and water content for M. rubra

	Capillary dimensions			Water peak height after GLC of sample (cms)					Volume of Dufour's gland (nl)	
Sample number	(a) Length filled from gland (mm)	(b) Length filled with water (mm)	(c) Diam. (μm)	(d) Sample (a)	(e) Sample (b)	(f) Empty vial	(g) (e)-(f)	(h) Water volume equiv. to (g)	Water equiv. volume (h)x(a)/(b)	Calculated from $\pi(c/2)^2(a)$
1	1.6	2.8	72	13.9	37.6	12.6	25.0	10.4	5.9	6.5
2	1.4	1.4	70	9.3	32.4	12.6	19.8	8.7	8.7	5.4
3	4.0	5.3	72	13.5	56.6	12.6	44.0	15.4	11.6	16.3
4	1.5	5.0	54	11.1	31.1	12.6	18.5	8.3	2.5	3.4
5	2.1	4.0	63	12.0	52.1	12.6	39.5	14.3	7.5	6.6
							Averages		7.2	7.6

in Dufour's gland using the figures quoted by Wadhams[23]. The total hydrocarbon content was found to be some 1.4 µl per Dufour's gland. Assuming a density of 0.8 for the total mixture contained in the gland, the volume was calculated as:

$$1.4 \times 10^{-6} \div 0.8 = 2 \times 10^{-6} \text{ ml or } 2 \text{ nl}$$

It was originally thought that the difference between this value and that found for the total glandular volume could be accounted for by the presence of water. However, gas chromatography of the samples of liquid taken from Dufour's gland gave peak heights (column (d) of Table 10) which revealed that this gland contains little or no water when compared with the water content of an empty vial (column (f)).

The volume of the poison vesicle was only measured in one experiment, because of the difficulty of extracting all of the liquid from two successive glands in the same ant. On this one occasion the length of liquid extracted from the poison vesicle was 4.5 mm, whereas that from the corresponding Dufour's gland was only 1.5 mm. This result is at least consistent with observation under the microscope, where the poison vesicle is about three times the volume of Dufour's gland in mature workers. In young and very old workers, however, the poison vesicle may be of about the same volume as Dufour's gland. No serious attempt was made to estimate the water content of the poison vesicle as Jentsch[58] reported that it contains an aqueous solution of proteins.

Electroantennogram studies on M. rubra

The electrophysiological response elicited from worker ants to the volatiles found in the ampoule distillate was assessed by recording their electroantennograms (EAG's). It did not prove practical to test the other volatiles found later by the

capillary extraction technique, since the EAG had to be dismantled and moved after the initial studies and would have certainly have taken many weeks to put back into operation.

EAG recordings are made by inserting electrodes into the insect antenna and represent mainly a summation of the receptor potentials of excited sense cells[59], but the results must be interpreted in conjunction with those from a reliable bioassay to ensure that the insect actually responds to odours which it senses.

In the study of multicomponent secretions EAG has commonly been used to determine which fraction of the secretion can be detected by the insect, thus avoiding the need to analyse all the components present.

The small number of compounds present in the sting apparatus made it feasible to identify the components first and to record the corresponding EAG responses from the ant by using authentic samples of the components identified. This approach avoids the difficulty of handling the small quantities of material present in the natural gland secretion.

The EAG responses to the volatiles identified in the ampoule distillate revealed that M. rubra could at least detect methanol, ethanol, ethanal, propanone and butanone (Fig.28). The compounds were presented to the preparation in different orders each time to avoid saturation effects on the antennal chemoreceptors. The responses varied from one specimen to another in the range 0.1-1.0 mV even for the same compound. This variation of response can be partially explained by the position of the microelectrode tip relative to the nerve. The recordings are made from the haemolymph in the region of the nerve and arise from the eddy currents generated in the haemolymph when an impulse passes down the nerve. These eddy currents have been studied in mammalian

Fig.28

Electroantennograph responses to volatiles from M. rubra L.

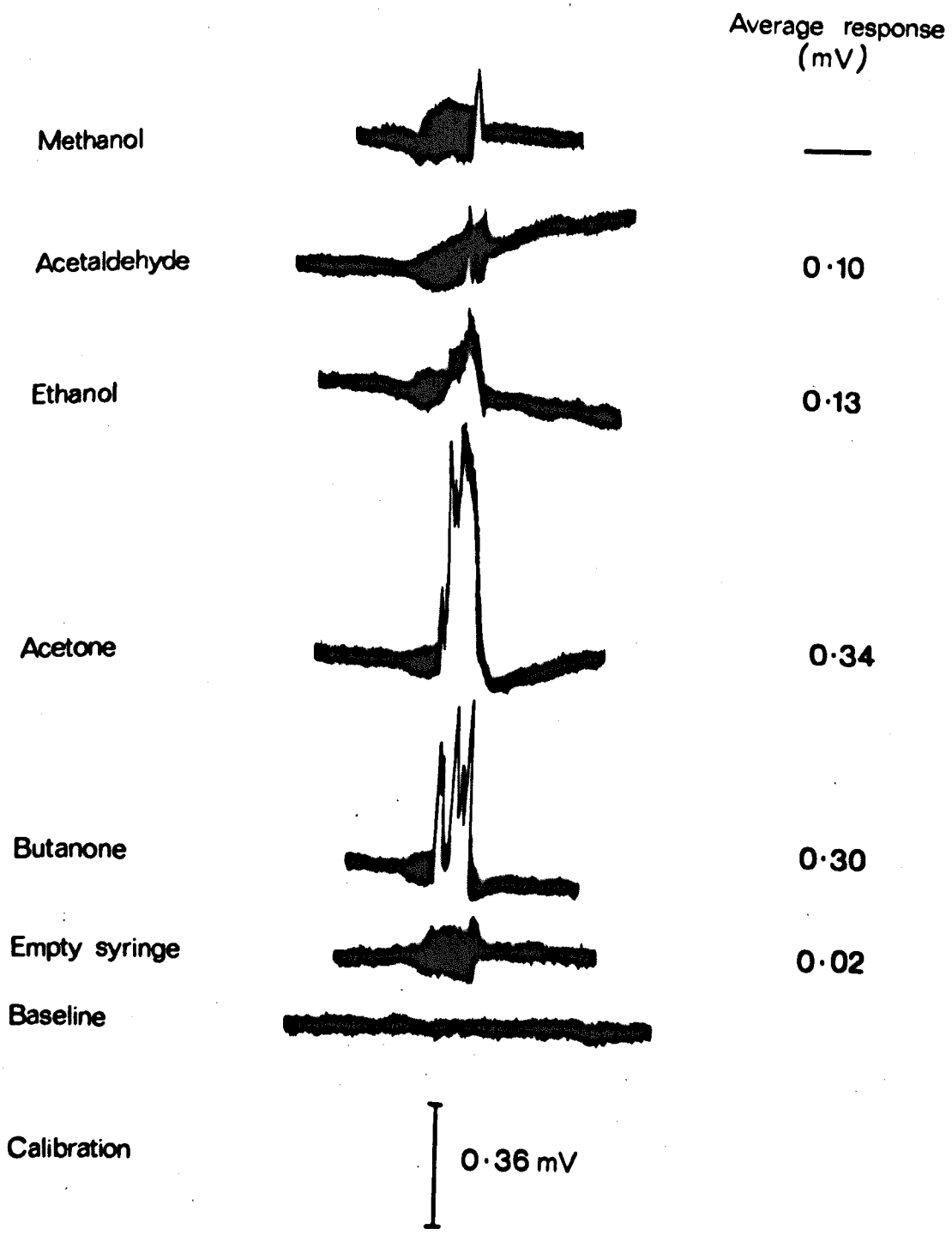
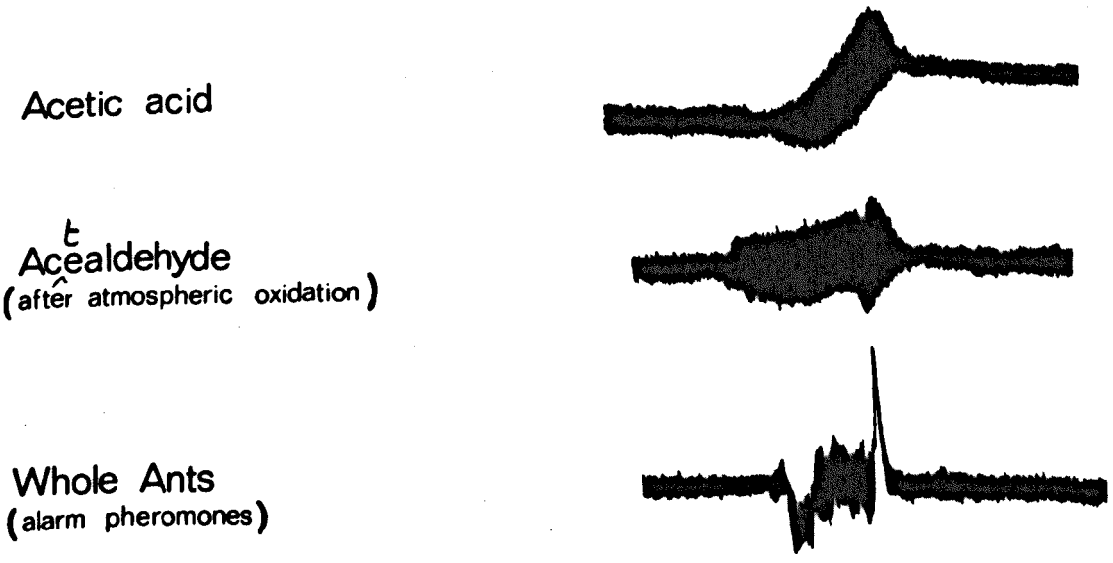
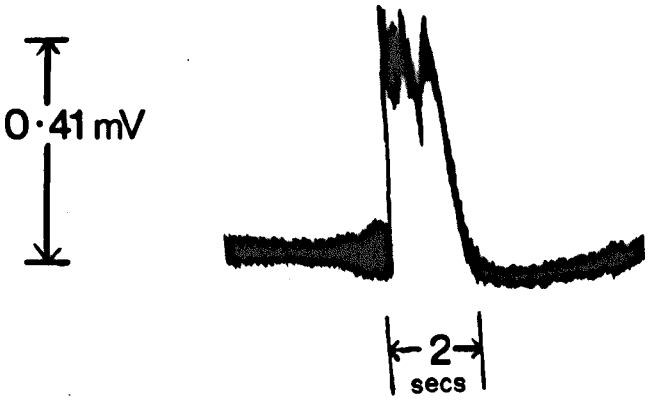


Fig.29

Electroantennograph responses to volatiles from M. rubra L.



Dimensions of response to Acetone.



ringer solution and have been shown to diminish with distance from the nerve[60]. The size of the EAG response will be greatest for any one compound, therefore, when the pipettes are positioned adjacent to the nerve. Unfortunately it did not prove possible to systematically insert the electrodes near to the nerve with our EAG system.

In virtually every case propanone was found to give the greatest response of the compounds tested, followed closely by butanone. The results were averaged for five different specimens and were compared with the response to air as a control (Fig.28) to assess any tactile response from the antenna.

In order to investigate the possibility that the activity of ethanal was due to its atmospheric oxidation to ethanoic acid, samples of this acid were tested. These gave no response but caused a characteristic shift of baseline potential. Samples of ethanal, which had been allowed to oxidise in the air, gave little or no EAG response and in some cases produced a baseline shift.

The dimensions of the EAG's recorded for the compounds tested (Fig.29), are comparable with those reported by Adler[59] for the cockroach Periplaneta americana L. where an isolated antenna stimulated with amyl acetate was found to give responses of about 1 mV amplitude and 1 second duration.

Bioassay of volatiles (reported studies not performed by author)

The bioassay of the compounds identified in M. rubra was conducted in the University of Brussels by M.-C. Cammaerts-Tricôt working in collaboration with us. The laboratory of animal and cellular biology in Brussels have been studying, for many years the recruitment and defensive behaviour of M. rubra. One series of ethological studies had specifically involved

the effect of the poison gland and Dufour's gland extracts on worker ants of this species. The behavioural activity associated with each gland was assessed by observation of the locomotive behaviour of ant workers when perceiving the gland contents. In this way the poison gland was found to contain the trail pheromone, whereas the secretion of Dufour's gland is deposited by workers whilst walking around or returning to the nest after perceiving an enemy[61]. This latter secretion attracts workers and induces them to walk rapidly with characteristic sinuous or wandering exploratory movements[26].

To enable an investigation of the individual compounds identified in Dufour's gland, the movements associated with the gland secretion were quantified by the measurement of three basic movement characteristics; attraction at a distance, augmentation of linear speed and changes in sinuosity of the ant workers.

The four compounds ethanal, ethanol, propanone and butanone when mixed together and presented to foragers induced all three movement characteristics and in addition caused workers to deposit their Dufour's gland secretion on their foraging area.

It was recognised that many chemicals unfamiliar to the ants might induce certain of these behavioural reactions if presented in too high a concentration. Even the natural pheromone of the ants may induce behaviour which is highly concentration dependent. This effect has been observed in a number of other species: for example, Conomyrma pyramica[62] are attracted by a low concentration of 2-heptanone, produced by their anal glands, but only show alarm activity when they perceive a high concentration of the pheromone. At centres of high pheromone concentration, however, workers are repelled or may begin a curious digging behaviour. Similarly, 1-hexanol

is an attractant to Oecophylla longinoda[63] at a distance of ten cms. but a repellant at a few mms. from the source.

Trail pheromones have also been reported to be concentration dependent for both Atta texana[36] and Solenopsis sp.[17], where too high a concentration was found to be repellant.

To avoid these problems the volatiles identified in the Dufour's gland of M. rubra were presented to foragers either singly or mixed in proportions and total quantities similar to those present in the gland contents. Reactions were obtained for example with 10 µl of a solution of propanone diluted 10^8 times in liquid paraffin. This is equivalent to 10^{-10} cm^3 of propanone, which at a density of 0.77 gms cm^{-3} would weigh:-

$$10^{-10} \times 0.77 \text{ gms} = 77 \text{ pg}$$

A single Dufour's gland contains an average of 27.4 ng of propanone, so that the amount presented to foragers represented 0.3% of the amount of this component present in the whole gland. We suggest that an ant possibly secretes 1% to 10% of its Dufour's gland content to elicit normal behaviour, so that the threshold concentrations of the substances tested are of the right order.

Each compound was diluted in liquid paraffin from 10^4 times to 10^{11} times, and 10 µl of each dilution was placed on a filter paper between the ants' nest and their food. In each experiment the movement characteristic investigated was measured for 30 foragers and the medians and quartiles of the values obtained were calculated. The results were analysed statistically by comparing them with those obtained with the hydrocarbon solvent alone in each case[48].

Ethanal was found to be the only attractant, the threshold dilution being 10^5. Furthermore since this compound is easily oxidised in the air to ethanoic acid, the latter was tested and found to have no significant attractive power at 10^5 dilution.

This result is consistent with that obtained by EAG, which showed that ants could not detect this acid. The four components together were found to be still attractive at 10^8 dilution and this was shown to be due to synergism of the ethanal by ethanol, the two components (98:2 v/v respectively) being attractive down to 10^9 dilution. The ketones had no synergistic effect on ethanal. We thus concluded that the attractive component of Dufour's gland is the ethanal synergised by the ethanol.

The ants' linear speed was only increased by propanone and this compound was found to be as active in increasing the ants' linear speed as the total mixture.

The change in sinuosity of movement was found to be significant only for ethanol (at 10^8 dilution), but the whole mixture was also active at 10^8 dilution, although it contained only 1% ethanol. This was not found to be an additive effect from the weak activities of the other components, since ethanal, propanone and butanone when presented together produced no change in sinuosity. However, ethanol and butanone (9:91 v/v) were active down to 10^{10} dilution showing that the activity of the mixture with respect to increased sinuosity was due to ethanol synergised by butanone. This mixture, like the total mixture, was also found to induce the deposition of Dufour's gland secretion by foragers.

The methanol, methylpropanal, butenone and butanol components of Dufour's gland, identified in capillary extracts of the gland, were also tested, at 10^4 and 10^6 dilution. None of the substances tested had any attractive effect on foragers even at 10^4 dilution. Methanol and butenone had no significant effect in any of the other tests even at 10^4 dilution. Both methylpropanal and butanol increased the linear speed and sinuosity of foragers slightly when presented at 10^4 dilution, but at

10^6 dilution these responses were lost[64].

The activity of Dufour's gland is not due to the methanol, methylpropanal, butenone and butanol components therefore, but to ethanal, ethanol, propanone and butanone.

Trail following activity

The morphology of the sting apparatus of M. rubra, revealed that the poison vesicle and Dufour's gland share a common duct to the sting lancet. The poison vesicle has a muscular sphincter but Dufour's gland has not. The Dufour's gland contents could therefore, be released separately from or together with the poison vesicle contents. The possibility existed therefore, that the trail-following activity associated with the poison vesicle was due to contamination with Dufour's gland contents from the adjacent duct. Even when the poison vesicle contents were removed by the capillary extraction technique, we recognised that cross-contamination from Dufour's gland could occur. This happened from time to time but was easily recognised by the presence of butenone (Fig.25a), unique to the Dufour's gland, in the chromatogram. The later identified volatiles of Dufour's gland, which had not been found to induce the movements associated with that gland, could then have produced the trail following activity associated with the poison vesicle. In order to eliminate this possibility methylpropanal, butenone and butanol were tested on a circular trail. Butenone readily polymerises on keeping and so its polymer was also tested. The bioassay used to assess trail pheromone activity was that developed by Pasteels and Verhaeghe[65]. Using this technique, none of the above volatiles was found to elicit trail following behaviour.

The trail following activity elicited by the poison vesicle

contents cannot be explained by any of the components which it shares with the Dufour's gland, although it should be borne in mind that unidentified components i and j and the methylbutanal present in the poison vesicles but absent from Dufour's gland have not yet been tested.

The active components of the trail pheromone have been isolated by Cammaerts-Tricôt by thin layer chromatography of some 2,000 poison vesicles. The bands on the silica gel plates were visualised by spraying with phosphomolybdic acid and heating to 150°C. Six bands were visible, which were presumably unsaturated lipid material, and the trail following activity was associated principally with band three. Recovery of this band from the plate with ether yielded, after careful distillation, a highly active extract. However we were unable to find any significant component in this extract by gas chromatography, although any very volatile component would have been obscured by the solvent peak. Capillary extracts of poison vesicles consistently showed no peaks of higher molecular weight than methylbutanal (Fig.6), so that we must conclude that the trail pheromone is a very minor component of the poison vesicle.

Cammaerts-Tricôt found that queens of M. rubra did not possess any trail pheromone in extracts of their poison glands and it was tempting to compare the chemical composition of queen poison vesicles with those of workers. This, however, did not prove possible as in queens the poison vesicle is often concealed by many eggs during dissection and if found, the gland is semi-collapsed with no liquid contents which could be extracted with a capillary.

Contents of queen Dufour's glands

In dissecting out the poison vesicles queens were found to

have large distended Dufour's glands and it was thought interesting to compare their chemical composition with those of workers. Several chromatograms consistently showed that queens had much less of the oxygenated volatile fraction and the high molecular weight hydrocarbons present in workers (Fig.30). The high molecular weight compounds, which were in much lower concentration in queens, corresponded to the bishomofarnesene and nonadecene components and some unidentified compounds of longer retention time.

The only qualitative difference found between between queens and workers was a component of retention time 2.8 mins present only in workers. This component, not found in workers or queens of M. scabrinodis, was found to have the retention time of undecane on the PEGA phase. The rôle of undecane in M. rubra is not known as it was previously found to be inactive by Wadhams[23].

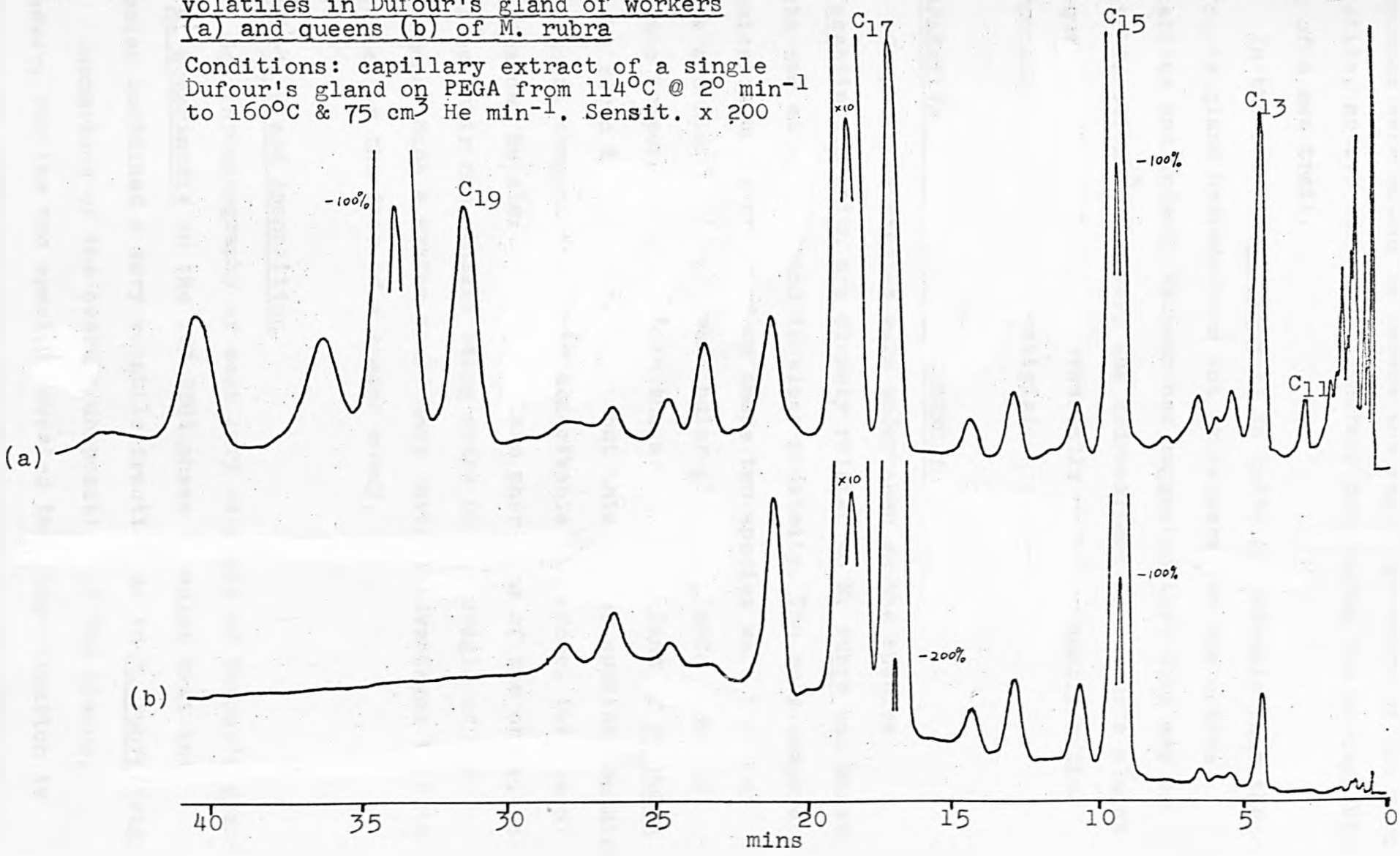
The larger volume of queen Dufour's glands compared with those of workers is accounted for by the presence of larger quantities of heptadecane and heptadecene.

The sedentary life of queens makes it tempting to postulate that the volatiles and high molecular weight compounds present in workers, but absent from queens, are concerned in foraging activities. The presence of the C_{13} to C_{17} alkanes and alkenes in queens as well as workers would suggest then, that these components are pheromonally inactive.

Other workers have suggested that pheromonally inactive substances act as 'keepers' of minor pheromonal components. Brand et al.[27] for example, have found two different heptylbenzenes and cyclohexylbenzene in Crematogaster peringueyi which were themselves pheromonally inactive, but which enhanced the activity of the active extract. They suggested that these

Fig.30 Gas chromatography of hydrocarbons and volatiles in Dufour's gland of workers (a) and queens (b) of M. rubra

Conditions: capillary extract of a single Dufour's gland on PEGA from 114°C @ 2° min^{-1} to 160°C & 75 cm^3 He min^{-1}. Sensit. x 200



compounds were acting to reduce the vapour pressure of the more volatile, active components, thereby prolonging the perceptibility of a new trail.

In the case of M. rubra it is quite conceivable that the Dufour's gland hydrocarbons act as keepers for the active volatiles and indeed, Wadhams had suggested that this may be their function[23]. However, the hydrocarbons in Dufour's glands of queens, where there are apparently no pheromonally active components are possibly vestigial.

COMPARATIVE STUDIES ON M. SCABRINODIS

Comparative studies were undertaken on the species M. scabrinodis which are closely related to M. rubra and whose nests are at times found in close proximity. The only comparative chemical studies reported on these two species were those of Crewe and Blum[25] on the mandibular gland contents (Table 2). All the components present in the mandibular gland of M. rubra may be found in M. scabrinodis, but this latter species contains additional components. It is conceivable therefore, that each species may be alerted by the alarm pheromone of the other, as well as their own, whilst being aware of the origin of the pheromone. Such a system could have obvious advantages to both colonies in the face of a common enemy.

Dufour's gland composition

Gas chromatography of capillary extracts of Dufour's gland of M. scabrinodis on the 10% PEGA phase revealed that this species contained a very volatile fraction as in M. rubra (Fig.7).

Comparison of the overall composition of the glands, however, for the two species revealed that the situation is reversed in comparison with the mandibular glands. That is to

say, all the components present in M. scabrinodis may be found in M. rubra, but M. rubra contains many components not present in M. scabrinodis.

The identity of the higher molecular weight components in M. scabrinodis has now been confirmed as the sesquiterpenes, (E,Z)-α-farnesene, a homofarnesene and a bishomofarnesene[66].

The very volatile fraction of Dufour's gland in M. scabrinodis was analysed as before on the Poropak Q column and the components submitted to reaction gas chromatography. The results (Fig.31), showed that this fraction was qualitatively identical to that from M. rubra. Quantitatively (Table 11), M. scabrinodis contains on average 114 ng of volatiles, whereas M. rubra contains on average 126 ng (Table 5). In terms of percentage composition the only difference between the two species, is that M. scabrinodis contains significantly more butanone, the minimum percentage of this component being higher than the maximum value found for M. rubra. Finally the average volume of the Dufour's gland in M. scabrinodis was calculated to be about 4 nl (Table 13), in comparison with 7.5 nl for M. rubra. This result is consistent with the microscopic examination of freshly dissected glands from both species, the gland from M. scabrinodis being perceptibly smaller. The Dufour's gland of M. scabrinodis like that of M. rubra, contains no water.

Poison vesicle composition

As in M. rubra, the poison vesicle of M. scabrinodis is largely aqueous with no volatile components other than the very volatile fraction (Fig.7).

The volatiles were analysed by reaction gas chromatography on Poropak Q (Fig.32), and found to show no qualitative differences from those in M. rubra. The poison vesicle is

Fig.31 Gas chromatography of volatiles in Dufour's gland of M. scabrinodis after passage through boric acid (a), no treatment (b) and borohydride reduction (c)

Conditions: capillary extracts on Poropak Q @ 167°C & 50 cm^3 He min^{-1}
Sensitivity x 100

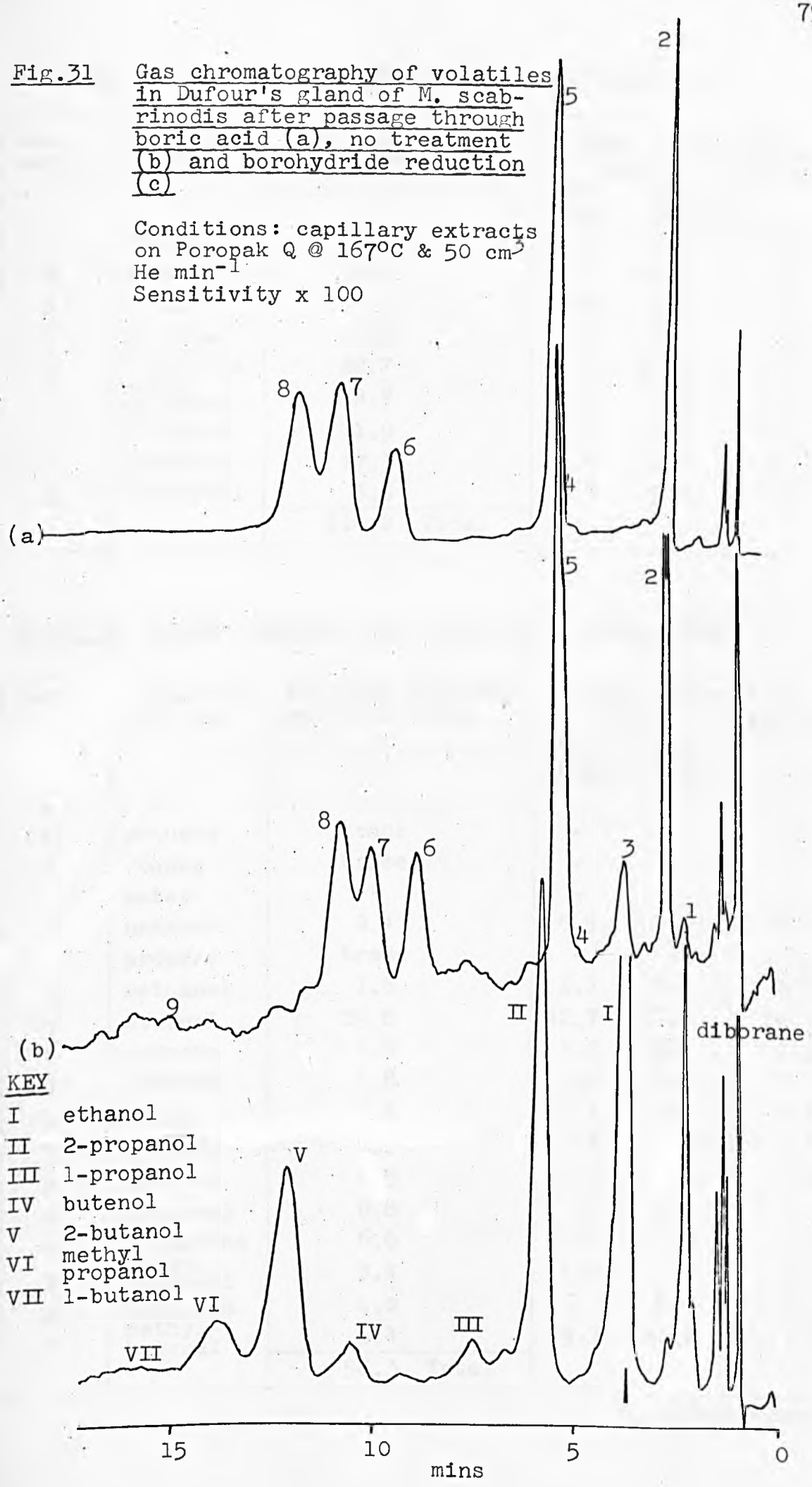


TABLE 11 Dufour's gland components of M. scabrinodis

Peak number	Component identity	Absolute quantity per gland (ng)	%age composition		
			Range Min. %age	Max. %age	Average
1	methanol	2.9	2.0	2.8	2.5
2	ethanal	38.9	22.2	48.5	34.1
3	ethanol	4.2	2.6	5.0	3.7
4	propanal	0.9	0.6	1.1	0.8
5	propanone	22.7	18.3	21.5	19.9
6	methyl propanal	9.9	7.8	9.8	8.7
7	butenone	11.9	9.0	13.6	10.4
8	butanone	17.3	11.8	18.5	15.1
9	1-butanol	5.5	3.8	5.6	4.8
		114.2 Total			

TABLE 12 Poison vesicle components of M. scabrinodis

Peak letter	Component identity	Absolute quantity per gland (ng)	%age composition		
			Range Min. %age	Max. %age	Average
a	air	-	-	-	-
b	methane	trace	-	-	-
c	ethane	trace	-	-	-
d	water	*	-	-	-
e	unknown	0.4	0.5	1.0	0.7
f	propane	trace	-	-	-
g	methanol	1.6	2.1	3.4	2.7
h	ethanal	29.6	42.7	57.8	50.8
i	unknown	1.0	1.0	2.1	1.7
j	unknown	1.8	1.8	4.0	3.1
k	butane	1.4	2.1	2.6	2.4
l	ethanol	0.8	0.8	1.6	1.4
m	unknown	0.5	0.5	1.0	0.9
n	propanal	0.8	0.6	3.0	1.4
o	propanone	6.6	10.0	14.0	11.3
p	methyl-propanal	3.4	5.2	7.2	5.8
q	butanone	4.0	6.1	8.0	6.9
r	methyl-butanal	6.4	9.7	12.6	11.0
		58.3 Total			

* not estimated

Fig.32 Gas chromatography of poison vesicle volatiles of M. scabrinodis after various treatments

Conditions: capillary extracts on Poropak Q @ 167°C & 50 cm^3 He min^{-1}
Sensitivity x 100

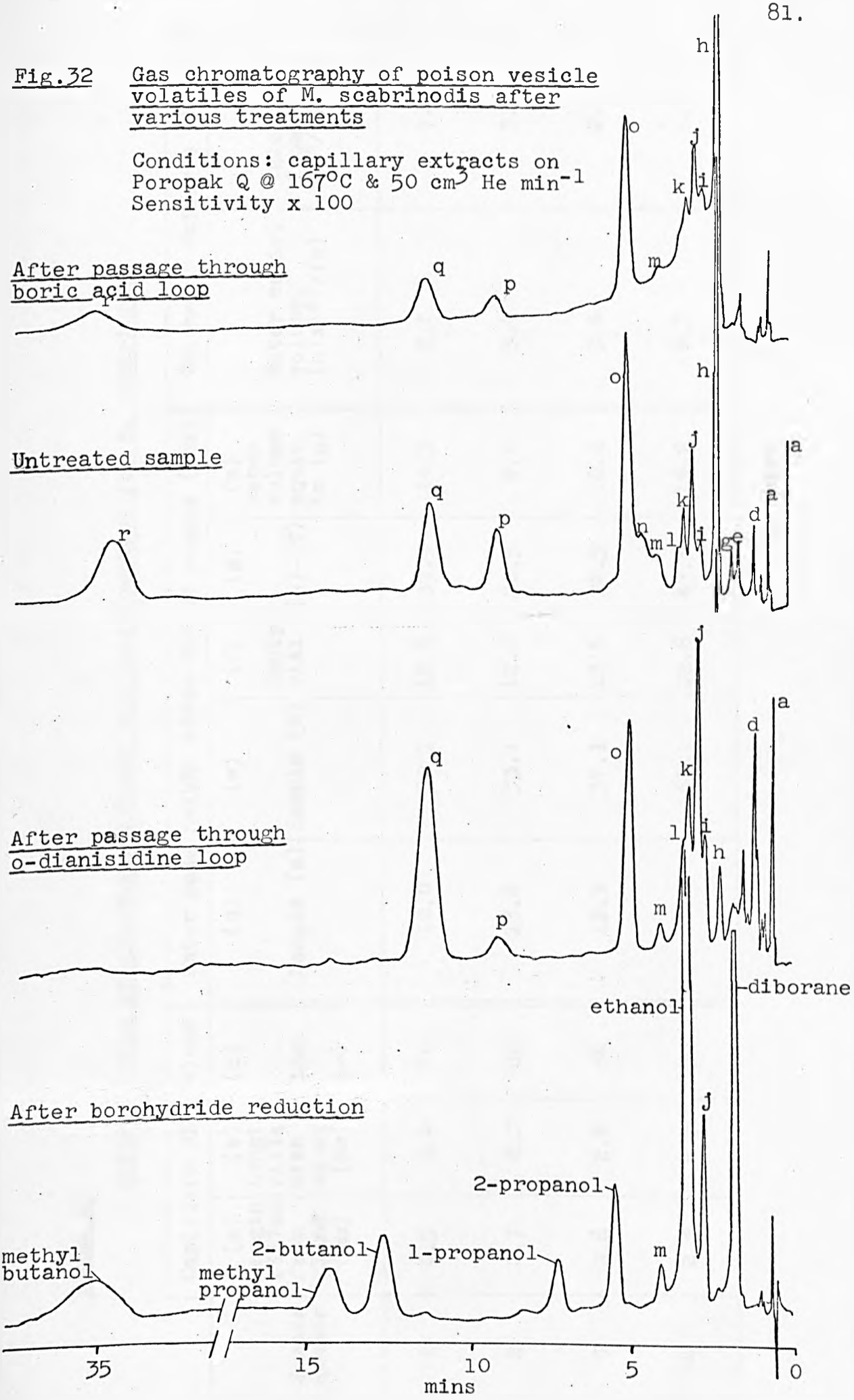


TABLE 13

Determination of glandular volumes and water content for M. scabrinodis

	Capillary dimensions			Water peak height after GLC of sample (cms)					Volume of Dufour's gland (nl)	
Sample number	(a) Length filled from gland (mm)	(b) Length filled with water (mm)	(c) Diam. (μm)	(d) Sample (a)	(e) Sample (b)	(f) Empty vial	(g) (e)-(f)	(h) Water volume equiv. to (g)	Water equiv. volume (h)x(a)/(b)	Calculated from $\pi(c/2)^2(a)$
1	0.5	3.4	70	10.0	52.1	12.6	39.5	14.3	2.1	1.9
2	0.7	2.1	80	13.8	33.1	12.6	20.5	9.0	3.0	3.5
3	0.8	2.4	64	12.3	37.1	12.6	24.5	10.2	3.4	2.6
4	2.3	4.5	56	11.0	60.1	12.6	47.5	16.2	8.3	5.7
								Averages	4.2	3.4

approximately 12 nl in volume for M. scabrinodis and contains on average 58 ng of volatiles (Table 12) in comparison with 72 ng in M. rubra. This quantitative difference is accounted for in part by the presence of less butanone and methylbutanal in M. scabrinodis than in M. rubra. The relative percentages of butanone in the two species (5:11) are interestingly the converse of those found in the Dufour's gland (15:9) where M. scabrinodis contains more butanone than does M. rubra.

The ratios of the total absolute quantities of volatiles found in the two species are similar for both glands (114 ng : 126 ng or 0.9 for the Dufour's glands and 58 ng : 72 ng or 0.8 for the poison vesicles). These ratios do not correspond to the volume differences found between the same glands in the two species (4 nl : 7.5 nl or 0.5) suggesting, within the limits of the measurements, that the volatiles in M. scabrinodis are more concentrated than those present in M. rubra. Furthermore the molar concentration of the volatiles present in Dufour's gland is over five times greater than that in the poison vesicles of both species (Table 14).

TABLE 14

Relative concentrations of the volatiles in the species M. rubra (M.r.) and M. scabrinodis (M.s.)

	Weight of volatiles per gland (ng)		Volume of gland (nl)		Concentration of volatiles (gm L^{-1})	
	M.r.	M.s.	M.r.	M.s.	M.r.	M.s.
Dufour's gland	126	114	7.5	4.0	16.8	28.5
Poison vesicle	72	58	22.5	12.0	3.2	4.8

General discussion of results

The occurence of aldehydes, ketones and alcohols in the same gland suggested that acetals and ketals may be produced. To investigate this possibility, samples of the volatiles were sealed in air-tight bottles in both water and hexadecane solution, and in the approximate concentrations found in the respective glands. Analysis of these samples at weekly intervals revealed that no additional peaks were produced: thus it was concluded that acetals and ketals are not produced at these concentrations or are decomposed on analysis.

The ubiquitous nature of the compounds identified immediately led us to investigate the occurence of these compounds in other parts of the ant body. Samples of haemolymph were analysed via the capillary extraction technique and showed a few small peaks corresponding in retention times to ethanal, ethanol, propanone and butanone. This certainly suggested that these compounds present in the ampoule distillate had not originated entirely from the sting apparatus, but at least in part from the haemolymph.

Ant tissue, when pyrolysed in the solid sampling device, showed a vast array of peaks on chromatography including the large methanol component previously observed in the ampoule distillate.

Removal of the individual gland contents, however, by the capillary extraction technique, enabled us to compare the concentration of the volatiles in the glands with those in the haemolymph. The volatiles in Dufour's gland were found in this way to be about 100 times more concentrated in their hydrocarbon solution than those in the aqueous haemolymph. Even in the poison vesicle, where the volatiles are in aqueous solution, they are still some 20 times more concentrated than in the

haemolymph. Clearly the presence of these volatiles in the gland is not merely a partition phenomenon and the volatiles are thus true glandular contents.

Further evidence for the authenticity of these glandular volatiles is provided by the constancy of their composition. The composition of the haemolymph was found to be rather variable, whereas the glandular contents were independent of diet and even showed little variation between laboratory colonies and freshly collected colonies from different areas. These findings indicate an active regulation of the composition of the glandular volatiles by the ant.

Our final concern was that these compounds might be thermal decomposition products of higher molecular weight compounds present in the ant. In the case of propanone this could indeed be so, since it could readily be produced by the decomposition of acetoacetate. Similar mechanisms for the formation of other compounds, however, are not obvious and are indeed very unlikely in the case of butane for example.

Little data is available on the concentration of volatiles in biological fluids for comparison with our results. The concentration of propanone may be contrasted with that found in the blood of normal humans[67], where the concentration is less than 0.5×10^{-3} gms per 100 gms of blood corresponding to about 0.05 ng in 10 nl of fluid. In the sting apparatus of ants the lowest concentration found is in the poison vesicle of M. rubra equivalent to an average of 3.5 ng per 10 nl of fluid, and in the Dufour's gland of M. scabrinodis the value is around 40 ng per 10 nl of fluid. Thus the concentration of propanone in ants is relatively high.

Occurence of volatiles elsewhere

Simple aldehydes, ketones and alcohols have been systematically studied in foodstuffs where their contribution to flavour is of considerable commercial interest. These compounds have been collected, by sampling the head-space gasses, over cooked meats, bread, roasted barley, tea, boiled potatoes and above the unheated samples of butter, wine, cheese and urine. The abundance of the volatiles from these typical sources allowed their analysis by gas chromatography-mass spectrometry.

No report of the natural occurence of butenone has been found, although other α,β-unsaturated carbonyl compounds such as propenal and butenal have been reported in the literature[68].

None of these compounds identified in Myrmica sp. have been reported in any other hymenopteran to our knowledge. There are however a few reports of their occurence in the Insecta in general, where they appear to be used as defensive secretions.

The Garden Tiger moth, Arctia caja L.[69], was found to produce a number of volatiles which were separated by gas chromatography on dinonyl phthalate. The most rapidly eluting component had the approximate retention time of methanol, but no positive identification was made. However this species has a distinct smell of stinging nettles, which it is believed to use as a defensive secretion. On analysis the nettles were found to contain methanol.

The carbonyl compounds are widely distributed, it appears, in bugs of the sub-order Heteroptera, where they are also used as defensive secretions.

Ethanal has been identified in the reservoir of the metathoracic gland of the adult Cotton Stainer bug Dysdercus intermedius[70] together with oct-2-en-1-al, octanal and a $C_{10}H_{16}$ terpene. This gland like Dufour's gland in the Myrmica sp. is

believed to be anhydrous.

The Bedbug Cimex lectularius[71] has a similar composition to the latter species with hex-2-en-1-al as the major component together with oct-2-en-1-al, ethanal and butanone.

Butanone has also been reported[72] as one of the twenty components present in the scent storage gland of the Green Vegetable bug, Nezara viridula. This gland also contains other higher aldehydes, ketones and esters, along with undecane, dodecane and tridecane. Unlike the Dufour's gland of the Myrmica sp. these heteropteran scent glands contain two phases: one pale yellow containing 70% tridecane and the other dark yellow, containing the more polar compounds.

In another heteropteran, Scaptocoris divergens[68], the yellow secretion of the stink glands contains a fungicidal mixture in which propanal has been identified. The mixture also contains propenal, butenal, pentenal, hexenal, heptenal, and octenal along with furans and quinones, and is again believed to be used in defence against predators.

The methylbutanal present in the poison vesicles of the Myrmica sp. could not be positively identified although it is most likely to be 3-methylbutanal. This compound forms the aromatic principal of the Stinking Water beetle Gyrinus natator[73] The pygidial glands of this species contain a yellow emulsion composed principally of gyrinial. One pair of these glands were found to contain 2.5 µg of 3-methylbutanal.

Most of the above secretions are yellow pungent liquids due to the presence of considerable quantities of α,β-unsaturated aldehydes. In contrast, the Dufour's gland of both Myrmica sp. studied contained a pale yellow, almost colourless liquid, the only discernible smell being due to the sesquiterpene components present in microgramme amounts. The poison vesicle is both

colourless and odourless, there being no volatiles present in greater than nanogramme quantities.

One interesting observation is that the unsaturated carbonyl compounds in the heteropterans above, exist, as far as one can tell, in non-aqueous environments and in the presence of other unsaturated compounds. This is consistent with the occurence of butenone in the unsaturated anhydrous environment of Dufour's gland in both Myrmica sp. studied; whereas the aqueous poison vesicle, apparantly containing no unsaturated compounds, does not contain any butenone either. We have also found that aqueous solutions of butenone rapidly polymerise even in the fridge and in dilute solution. We have not been able to study the effect of storing butenone in pure sesquiterpenes.

Possible biochemical origins of the volatiles

The biochemical origins of simple carbonyl compounds and alcohols are varied. Ethanal is an intermediate in the degradation of pyruvate to ethanol, so that both are metabolites of carbohydrates. Butanol, butenone and propanone are all derivable biochemically from acetoacetic acid. Both isomers of methylbutanal are possible biochemically, being derived from the degradation of the amino acids leucine or isoleucine.

Suggested function of the secretions

The Dufour's gland of M. rubra contains some nine very volatile components, although the sequence of behaviour released by the gland contents is due to only four components. Thus ethanal synergised by ethanol releases the attractive component of the behaviour, propanone causes foragers to walk more rapidly, and ethanol, synergised by butanone, induces them to walk sinuously. Cammaerts-Tricôt has informed us that freshly crushed

Dufour's glands of M. scabrinodis induce the same behaviour in M. rubra as its own glands do, although the similarity of behaviour is lost after three minutes. M. scabrinodis too, shows the same behavioural traits when foraging which may be released either by its own Dufour's gland secretion or by that of M. rubra. Once again the behaviour released by the secretions of the two species diverges after about three minutes. These observations can be explained by the known differences in composition of Dufour's gland of these two species (Fig.7). The very volatile fraction is essentially identical in both species and releases the behaviour associated with the total secretion of the gland. This volatile fraction rapidly disperses and reaches its threshold concentration in about three minutes, leaving a more persistent and species-specific odour.

Many multicomponent pheromones have been reported in ants, although the releaser properties of the components have rarely been studied.

In Conomyrma pyramica[62] propanone and butanone have been tested separately as releasers of alarm activity, but were found to be inactive. However the anal glands of this species normally produce 2-heptanone and have not been found to contain propanone or butanone.

In the case of the African Weaver ant, Oecophylla longinoda a study has been made[63] which parallels our own in M. rubra. The mandibular gland secretion of O. longinoda was found to contain some 33 components which together released a sequence of aggressive behaviour. In this case the behaviour was attributable to four compounds of differing volatility. Hexanal was found to alarm and alert the workers but ants did not orientate themselves towards a source of it. The attractive component was found to be 1-hexanol, although in high concentrations, near to its

source, this component was repulsive to workers and induced alarm. As in M. rubra the initial behaviour associated with the secretion was changed after a few minutes due to the dispersion of the volatile components. After this time had elapsed the alarm was replaced with a biting behaviour attributable to the less volatile, more persistent components, 3-undecanone and 2-butyl-2-octenal.

The function of the Dufour's gland in the Myrmicinae is not fully understood, although in Pheidole fallax[74] and the genus Solenopsis[17], this gland is the source of the trail pheromone.

In the Formicinae the Dufour's gland hydrocarbons are released with formic acid from the poison vesicle to produce alarm. The hydrocarbons themselves may release alarm behaviour as in Camponotus ligniperda[21] and Formica rufa[75] and can also act as a spreading agent for the formic acid from the poison vesicle, as in Acanthomyops claviger[76]. In Oecophylla longinoda the combined effect of the undecane from the Dufour's gland and the formic acid from the poison vesicle was found to be greater in releasing alarm than that of the individual components. This was generally found to be the case in other Formicinae too.

Maschwitz has reported that M. rubra possesses an abdominal alarm pheromone[13], although this may well correspond to the attraction and rapid sinuous movements released by the volatiles present in the sting apparatus. The true alarm pheromones of this species are almost certainly located in the mandibular glands[25], and not in the Dufour's gland.

M. rubra workers have been found to deposit droplets of Dufour's gland secretion on their foraging area. This observation, made independently in our laboratories and in Brussels, led us to consider the possibility that the Dufour's gland secretion is used as a territorial marking pheromone in this species.

Cammaerts-Tricôt investigated this phenomenon[77] by measuring the rate at which successive workers crossed a newly presented foraging area, as a function of their familiarity with the territory. The first foraging worker on an untreated area moved rather slowly (9 mm sec^{-1}) but the following worker and successive workers all moved more rapidly (19 mm sec^{-1}). Pre-treatment of the foraging area with poison gland extract did not change the speed of the initial worker, which remained at 10 mm sec^{-1}. Pre-treatment of the area with Dufour's gland extract, however, had the same effect as allowing a forager to cross the area, so that even the first forager moved rapidly (25 mm sec^{-1}) after this treatment. These results indeed suggested that the Dufour's gland secretion is laid down by foragers to mark their territory. Further experiments showed that territories marked with the Dufour's gland extract and left for fifteen minutes before testing, still induced foragers to move rapidly (21 mm sec^{-1}) although not as rapidly as with a freshly marked territory. Thus the very volatile components contribute only slightly to this role of the Dufour's gland: clearly the less volatile components form a more persistent odour and are responsible for the greater part of the behaviour observed.

We suggest, therefore, that the role of the volatiles is rather that of a recruitment pheromone, attracting foragers to a source of food, a freshly laid odour trail or an enemy, whilst the hydrocarbons present help maintain the concentration of the pheromone by acting as 'keepers'.

In both Atta texana and Solenopsis invicta[28] a freshly laid trail is more attractive to workers than an old well-established trail, suggesting that the trail pheromones of these species contain, apart from the persistent trail following substance, a very volatile attractant which functions to recruit workers

rapidly.

The morphology of the sting apparatus in M. rubra (Fig.5) results in the release of Dufour's gland contents along with those of the poison vesicle. Thus the Dufour's gland secretion may be laid alone for recruitment to an enemy, for example, or along with the poison vesicle contents. In this latter case trails of M. rubra laid with the poison gland contents have an attractive component associated with them due in part to the presence of Dufour's gland volatiles.

The poison vesicle contains some 16 volatiles, although none of these induced the trail following behaviour associated with secretions of this gland. The four ethologically active compounds present in Dufour's gland are also present in this gland, but in about five times lower concentration, and the propanone and ethanol components form a much lower percentage of the volatiles present. These components are present in essentially the same amounts in M. scabrinodis although they appear to be more concentrated in this species.

At first sight it seems unnecessary for the poison vesicle to contain the recruitment pheromone when the secretion is deposited together with the Dufour's gland secretion. However, nothing is known of the relative quantities released from the respective glands and it is possible that very little of the Dufour's gland contents are released with the poison vesicle contents, so that the latter secretion contains its own recruitment component. If this is so, then the low percentages of propanone and ethanol in the poison vesicle may reduce the rapid and sinuous movements released by these components, whilst leaving the attractive component at the same level as that found in the Dufour's gland secretion. Such a mechanism would give workers means of controlling the pheromone

composition which they release. It is tempting to postulate that the poison vesicle secretion alone, or with a small amount of material from Dufour's gland, provides principally information concerning orientation, and is used as an attractant to a freshly laid trail. The secretion of Dufour's gland alone, on the other hand, laid after perception of an enemy, for example, induces congeners to search rapidly as well as attracting them. This latter secretion also induces successive workers to deposit their Dufour's gland secretion on the foraging area, thus maintaining the concentration of the pheromone and ensuring that the territory is thoroughly foraged.

Suggestions for further work

It would be interesting to investigate the composition of the Dufour's gland and poison vesicle of other Myrmica sp. for comparative purposes, and to study the corresponding behavioural movements of these species.

High molecular weight components in Dufour's gland, of longer retention time than nonadecene, have been found in some nests of both M. rubra and M. scabrinodis. These compounds, which are virtually absent from queens, may be diterpenes and could be responsible for the persistent component of the territorial marking secretion deposited by workers. Analysis of these components would allow them to be bioassayed for territorial marking activity.

Finally, further work is still required to identify the trail pheromone present in the poison vesicle.

EXPERIMENTAL

EXPERIMENTAL

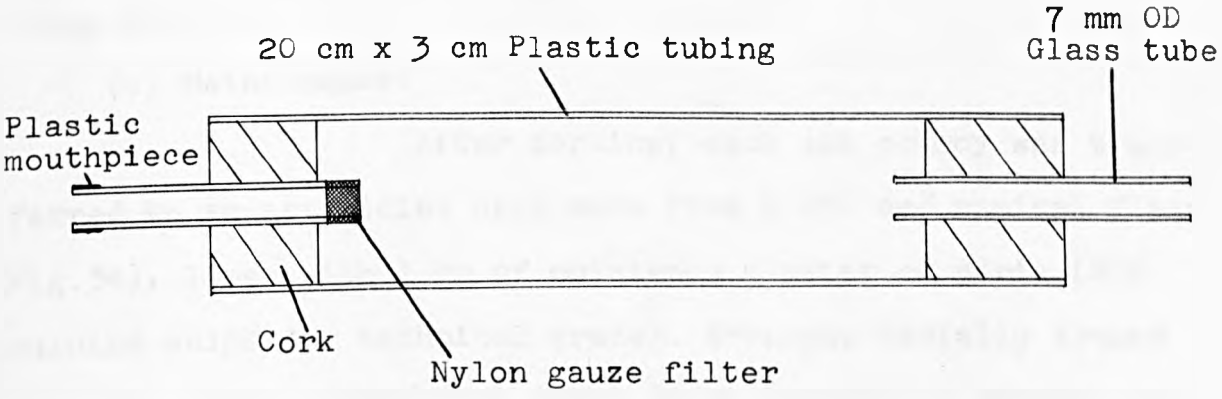
(1) COLLECTION, IDENTIFICATION AND MAINTENANCE OF ANT COLONIES

(a) Collection:

Ants of the species *Myrmica rubra* L. and *M. scabrinodis* NYL. were collected mainly from Chesterton and Apedale, North Staffordshire and Cardingmill Valley, Church Stretton, South Shropshire; nests of *M. rubra* were also collected from the Goyt Valley, Derbyshire and the Cambridgeshire Fens.

Whole nests were dug up and transported to the laboratory in large polythene bags. The soil was then spread on a tray and eggs, larvae, workers and queens were separated from the soil with a small aspirator held in the mouth (Fig.33).

Fig.33 Aspirator for the collection of ants



The aspirator contents were transferred to a plastic washing bowl provided with a damp tissue pad covered by an inverted plastic cup with edge notched, so the ants could enter. The colony quickly accumulated under the tissue, freeing it of the last traces of soil.

(b) Identification:

The ant species were originally identified by sending samples to C.A. Collingwood, Government Buildings, Lawnswood, Leeds. Ants of the genus Myrmica could subsequently be distinguished in the field by removing their antennal scapes using the nails of the thumb and forefinger, and examining the degree of curvature of their bases with the naked eye. With experience, an accurate preliminary identification could be made on this basis.(Plate 1). Identification was confirmed in the laboratory by close examination under a binocular microscope (Mag. x 30) of the antennal scapes, epinotal spines and petiole of workers[46].

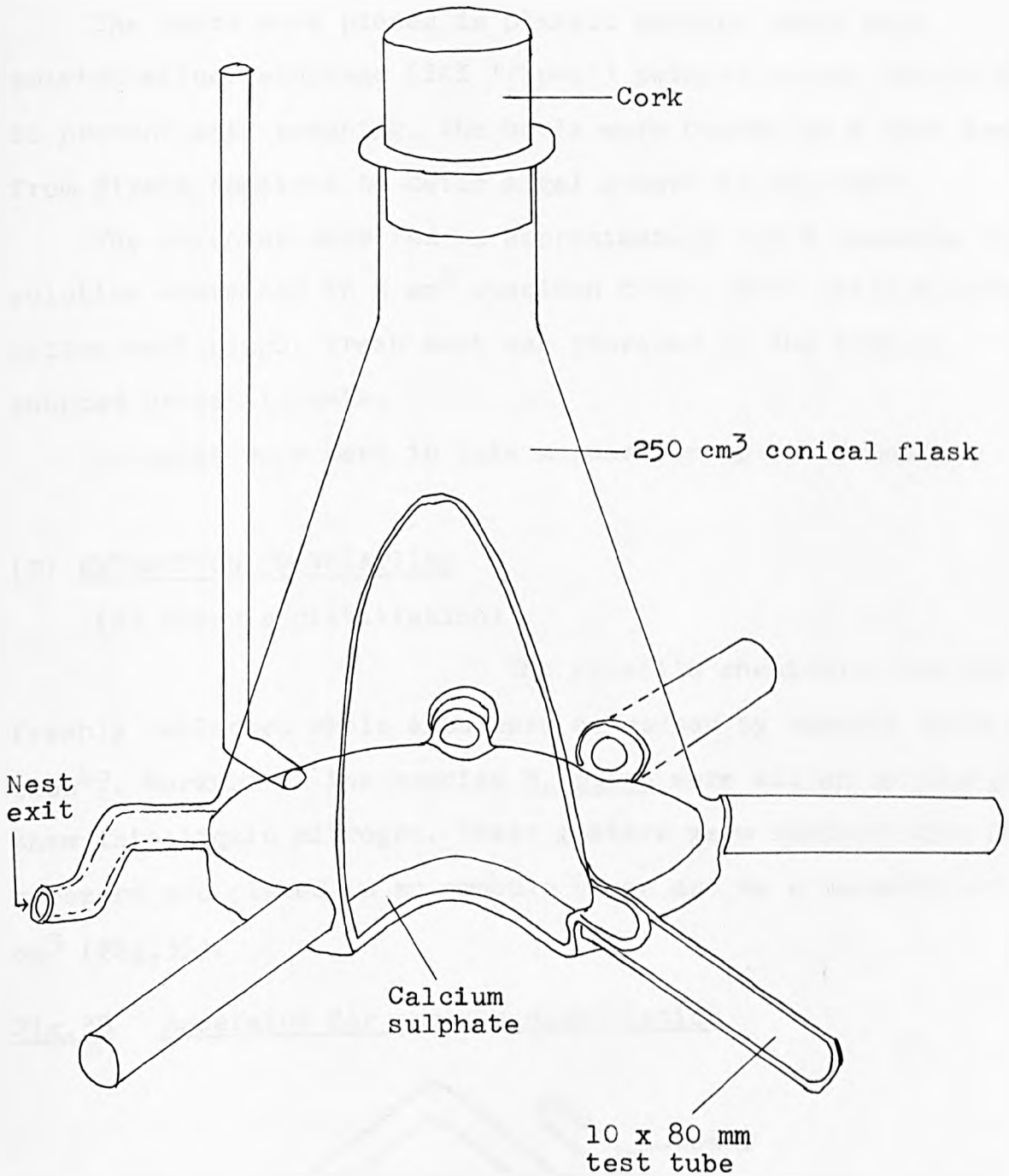
The species M. rubra and M. scabrinodis were also distinguished by the gas chromatographic profile of their Dufour's glands on a 9 ft x $\frac{1}{8}$ in OD glass column packed with 10% PEGA on Diatomite M-AW-HMDS, temperature programmed from 114°C to 158°C at 2° min^{-1} and N_2 flow rate of 75 cm^3 min^{-1} (Figs.6 & 7).

(c) Maintenance:

After sorting, each ant colony was transferred to an artificial nest made from a 250 cm^3 conical flask (Fig.34), lined with 1 cm of moistened plaster of paris (BDH calcium sulphate, technical grade). Arranged radially around the base of the flask were seven short lengths of tubing, four of which were connected with polythene tubing to 10 x 80 mm test tubes, covered to form darkened brood chambers for the queens. Two of the other tubes were extended to form nest exits and the remaining tube which opened below the level of plaster of paris, was extended vertically, as shown in Fig.34, to enable the nest to be moistened periodically. The humidity of the nest was maintained by closing the top of the flask with a cork.

Fig.34 The artificial nest

(after Wadhams[23])



Ant colonies were initially sealed in their nests over night by plugging the exits with cotton wool. This allowed the new environment to acquire the colony odour and prevented queens leading their brood out of the artificial nest.

The nests were placed in plastic washing bowls with polytetrafluoroethylene (ICI 'fluon') painted on the inside wall to prevent ants escaping. The bowls were housed on a rack away from direct sunlight to deter algal growth in the nests.

The colonies were fed on approximately 0.5 M demarara sugar solution contained in 5 cm^3 specimen tubes, with daily-moistened cotton wool plugs. Fresh meat was provided in the form of chopped desert locusts.

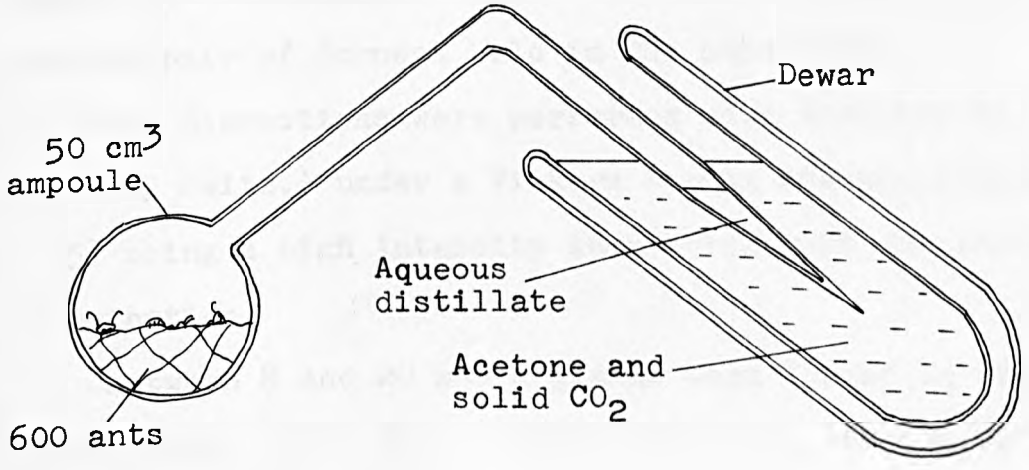
Colonies were kept in this manner for up to 18 months.

(2) EXTRACTION OF VOLATILES

(a) Ampoule distillation:

The volatile chemicals from 600 freshly collected whole ants were extracted by ampoule distillation[49]. Workers of the species M. rubra were killed by plunging them into liquid nitrogen. Their gasters were removed with fine tweezers and placed in an ampoule blown out to a capacity of 50 cm^3 (Fig.35).

Fig.35 Apparatus for ampoule distillation



Surface moisture was removed from the gasters by hand warming the ampoule under vacuum. The ampoule was then sealed off under reduced pressure (10^{-3} Torr), the bulb heated in a detergent bath at 200°C for 30 mins and the drawn-out end chilled in propanone and solid CO_2. An aqueous solution of volatiles (0.5 cm^3) was collected, the ampoule broken open and the solution removed with a 10 µl syringe for analysis by gas-liquid chromatography (GLC).

(b) Dissection of glands and solid sampling:

To ensure that all the volatiles in the distillate originated in the sting apparatus, the glands themselves were dissected out and analysed by the solid sampling technique described by Morgan and Wadhams[42]. Briefly the glands were sealed in glass vials and inserted into the heated zone of the injection port heater of a Pye model 64 gas chromatograph, modified to accomodate a Hewlett-Packard solid sample injector (Fig.36).

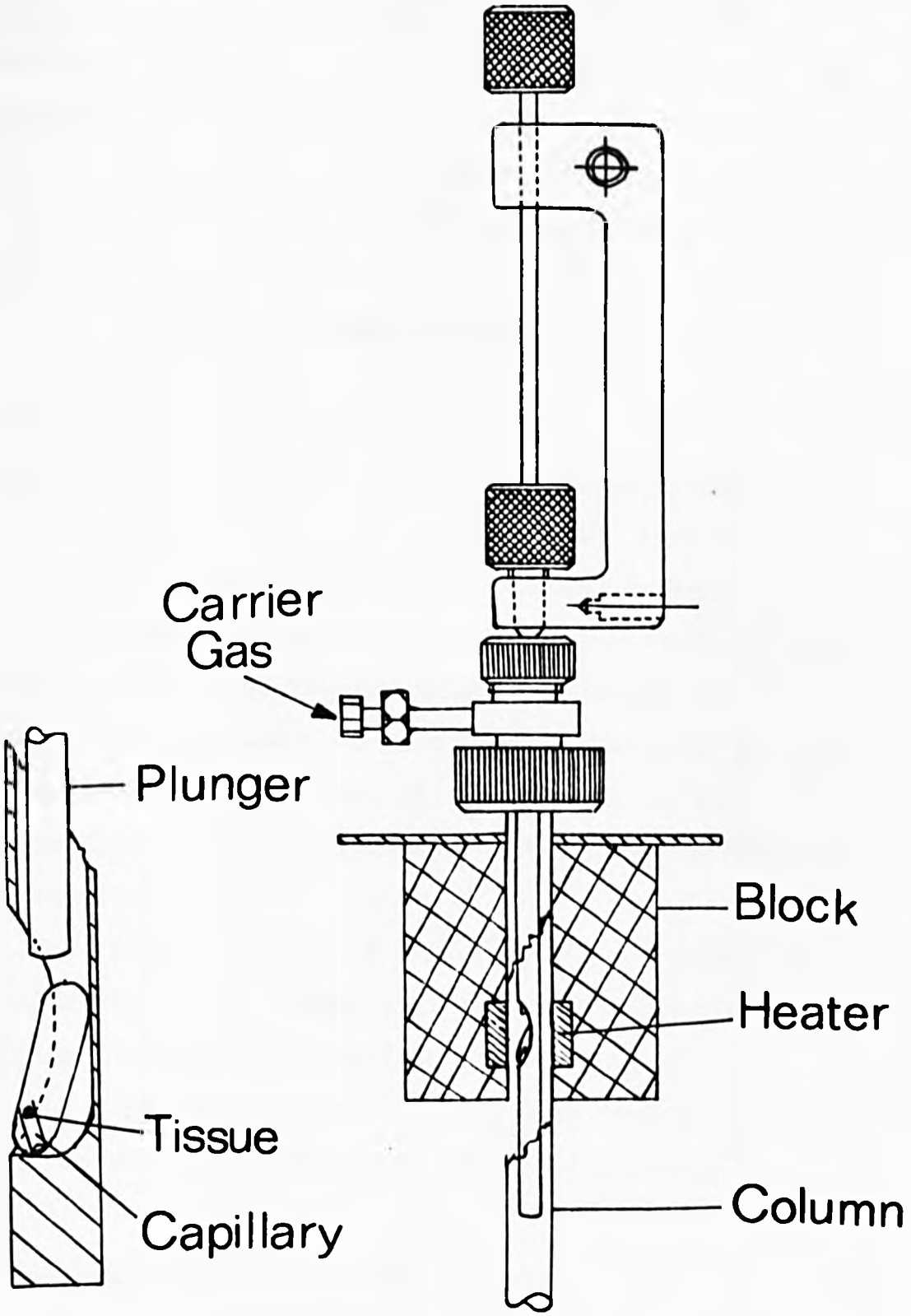
Workers were killed by plunging them into liquid nitrogen for one second, this being sufficient time to cause extrusion of the sting without disruption of the glandular sacs.

The Dufour's gland and poison vesicle with its two associated glandular filaments were removed by holding the ant by the petiole with fine forceps, held in one hand, and pulling gently on the extruded lancet of the sting apparatus with another pair of forceps held in the other hand.

All dissections were performed with Idealtek No.4 forceps (Trady's, Switz.) under a Vickers Zoomax stereomicroscope (Mag. x 15) using a high intensity lamp attachment for incident illumination.

Between 8 and 20 whole glands were lodged in fine glass capillaries, which were dropped into 2 cm lengths of melting

Fig.36 Solid sample injector (after Wadhams[23])



point tube (2 mm OD soda glass tubing). The sample tubes were held gently between thumb and forefinger and sealed by slowly rotating the open end in a small gas and oxygen flame. When completely sealed the end of the vial began to swell and the vial was then quickly removed from the flame.

Specimens prepared in this way were analysed using the solid sampling technique.

2(c) Capillary extraction of glandular contents:

To avoid contamination of samples with cell wall pyrolysis products, the contents of the glandular sacs were removed for analysis.

The Dufour's gland and poison vesicle were dissected from the gaster contents after removal of the terminal tergite with the fine forceps. The surfaces of the glands were dried with paper tissue and the liquid contents withdrawn by capillary action after piercing them with a 50-70 μm pyrex glass capillary. It was possible to see when all the liquid had been removed from the glandular sacs by adjustment of the angle of incident illumination.

The filled portions of capillary were dropped into solid sampling vials, sealed and analysed by GLC using the solid sampling technique described in section 2(b).

This capillary extraction technique enabled the separate glands to be analysed with no cross-contamination.

(3) GAS-LIQUID CHROMATOGRAPHY

3(a) Instrumentation:

Analyses were performed using a Pye series 104, model 64 gas chromatograph fitted with injection port heaters and dual heated hydrogen flame ionisation detectors (HFID). A Katharometer detector was used in the estimation of

glandular volumes and water content. Results were recorded on a Servoscribe 1s potentiometric recorder (Smiths Inds.,England).

Analyses were performed on the following columns:
The ampoule distillate was analysed on a 5 ft x $\frac{1}{4}$ in OD glass column packed with Poropak Q 120-150 mesh (Waters Associates) and a 5 ft x $\frac{1}{4}$ in OD glass column packed with Chromosorb 102, 100-120 mesh (Johns-Manville).

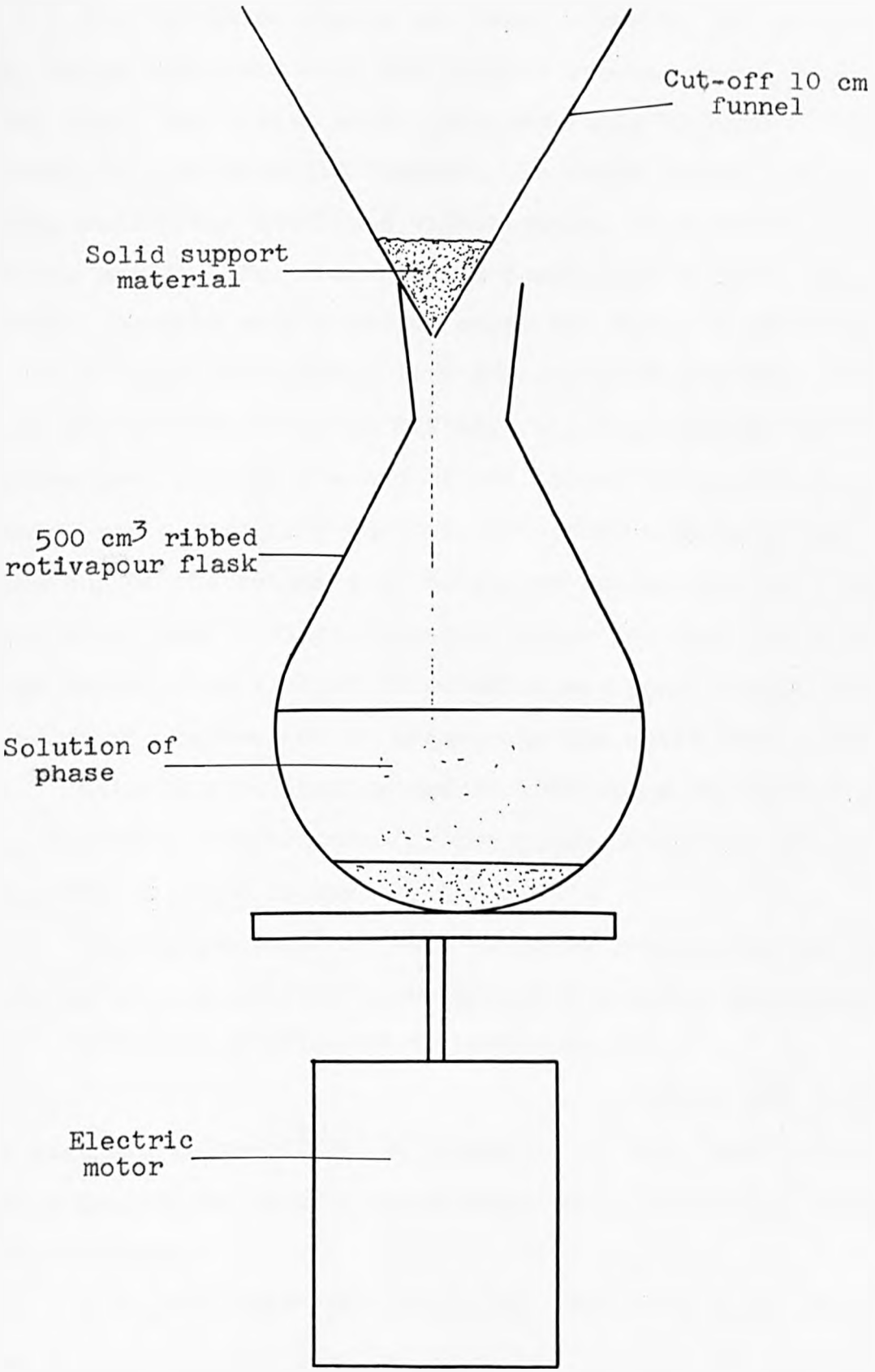
Whole glands were also analysed on a 5 ft x $\frac{1}{8}$ in OD stainless steel column packed with Poropak Q 120-150 mesh and a 9 ft x $\frac{1}{4}$ in OD glass column packed with 10% PEGA on Diatomite M-AW-HMDS 100-120 mesh; and a 9 ft x $\frac{1}{4}$ in OD glass column packed with 7% SE-30 on Chromosorb P.

The liquid extracted from glandular sacs was also analysed on a 9 ft x $\frac{1}{4}$ in OD glass column packed with Chromosorb 104, 100-120 mesh and a 5 ft x $\frac{1}{4}$ in OD glass column packed with 65% Chromosorb 102 and 35% Chromosorb 104.

3(b) Preparation of column packings:

Solid support material and liquid phase were weighed out in the required proportions, allowing 3 gms of column packing material per foot of $\frac{1}{4}$ inch OD column. The liquid phase was dissolved in the solvent recommended by the manufacturers in a 500 cm^3 ribbed rotivapour flask. The flask was supported on a cork ring placed slightly off centre on an electrical turntable in such a way that when rotated the mouth of the flask described an eliptical orbit. The solid support was added to the solution slowly through a fine hole in a 10 cm cut-off funnel supported above the flask (Fig.37), so that it collided with the mouth of the flask at one point in its orbit, knocking a small amount of support material through the fine hole in the funnel into the solution once every revolution. This system allows the solid support

Fig.37 Apparatus for the prepatation of column packings



material to be added slowly and evenly to the solution of liquid phase.

The resultant slurry was transferred in the ribbed flask to a rotary evaporator and the solvent removed slowly under vacuum. The flask was turned slowly and carefully by hand to avoid 'bumping', which would fragment the solid support material. Evaporation was continued with heating, if necessary, until the phase was dry. The free-flowing powder was poured onto a watch glass, covered with a filter paper and dried at 60°C for 12 hrs.

Columns were packed with the phase by applying a vacuum at one end through a column fitting, which prevented the silanised glass wool plug in the end of the column being sucked out. The phase was poured into the column through a funnel, sealed to the top of the column with polythene tubing and vibrated around the coils with a Pifco vibrator. After filling, the column top was sealed with 1 cm of silanised glass wool leaving an 8 cm gap at the column top to accomodate the solid sample injector.

Columns were conditioned at 10°C below the maximum isothermal operating temperature for the phase, using N_2 carrier gas for 48 hrs prior to use.

Columns prepared in this way were found to perform well giving no peak tailing under normal operating conditions.

3(c) Purification of distilled water:

Water was chosen as a suitable solvent for the injection of pure standards, as its poor ionisation in a HFID prevents the occurence of a large solvent peak.

Distilled water was prepared, free of organic impurities, by successive distillation from alkaline potassium permanganate in glass apparatus. The purity of the product was assessed by GLC analysis on Poropak Q.

3(d) Flame ionisation detector calibration:

10 µl of each authentic sample were measured out with a 10 µl Terumo syringe and injected into 1 cm^3 of distilled water sealed in a 1 cm^3 specimen jar with a rubber serum cap. The resultant solution was thoroughly agitated and chilled to 5°C in a refrigerator. 10 µl of this solution was drawn into a different 10 µl syringe and injected into 1 cm^3 of distilled water as before. 1 µl of this last solution contained approximately the same amount of each volatile as four Dufour's glands. The exact quantity of material present per 1 µl of solution was calculated from the density of the corresponding authentic compound at 20°C in each case (0°C for ethanal). In the case of propanone, for example, (density 0.77 gms cm^{-3} at 20°C) 1 µl of the final solution contained 0.77×10^{-7} gms propanone ie. 77 ng μl^{-1}. The solutions of standards were stored for reference purposes in the fridge when not in use.

Peak areas were measured by photocopying the GLC traces, cutting out the peaks from the photocopy and weighing them to the nearest 10^{-5} gm on a microbalance.

3(e) Retention data:

Aqueous solutions of authentic samples of the compounds under study were prepared as described in section 3(d). 1 µl of each resultant solution was injected onto each phase under the same conditions as the glandular extracts. Retention times were measured to the nearest millimetre.

Retention indices were calculated for the Poropak Q 120 -150 mesh column by the injection of nanogramme quantities of C_1 to C_8 normal alkanes (Aerosol calibration gases, Phase Sep. Ltd.). A graph of carbon number versus $\log_{10}$ retention times corrected for the dead-space of the column (0.4 mins) was drawn

for the alkanes and the Kovats' indices of the compounds under study calculated from it.

(4) REACTION GAS CHROMATOGRAPHY

(I) PRE-COLUMN REACTIONS

4I(a) Aqueous sodium borohydride reagent:

1 µl of the ampoule distillate followed by 1.5 µl of borohydride reagent (0.5 gm cm^{-3} aqueous sodium borohydride stabilised with two drops of 2M sodium hydroxide solution), were drawn into the barrel of a Terumo 10 µl syringe. The syringe contents were drawn right up into the top of the barrel and then thoroughly mixed by moving the plunger up and down, being careful not to expel any liquid from the syringe.

Aldehydes and ketones present in the distillate were partially reduced to their corresponding alcohols in 1.5 mins; complete reduction had occurred after 5 mins.

Between successive reactions with the ant distillate the viability of the reagent was assessed with authentic compounds in dilute aqueous solution, prepared as in section 3(d).

4I(b) Boric acid pre-column:

A mixture of 1:1 boric acid and Poropak Q 120-150 mesh was packed in a 5 cm x 2 mm OD melting point point tube and retained by small silanised glass wool plugs. This pre-column was suspended inside the top of the $\frac{1}{4}$ in OD analytical column and sealed in place with a silicone rubber 'O' ring, which fitted around the pre-column but inside the analytical column.

Injection of aqueous authentic standards prepared as described in section 3(d), through this pre-column, resulted in the retention of the alcohol components[54]. 1 µl of the ampoule

distillate was also injected to investigate the presence of alcohols there.

However, after continual use of this pre-column the analytical column became contaminated with boric acid, resulting in the elution of additional peaks after each injection and the partial absorption of alcohols.

4I(c) Solid sodium borohydride reagent:

The liquid from 4 to 10 glands was removed by the capillary extraction technique and transferred to a solid sampling vial, lined on its base with about 5 mg of sodium borohydride, finely crushed in situ with a fine, clean stainless steel rod.

The vial was sealed as described in section 2(b), inserted into a drilled cork, dropped into a centrifuge tube and spun at 3000 rpm for 5 mins. This treatment ensured contact between the liquid glandular contents and the finely crushed reagent. The vial was then inserted into the gas chromatograph by means of the solid sample injector and heated in the injection port heater to 220°C for 5 mins before crushing to release its contents onto the column. Under these conditions aldehydes and ketones were completely reduced to their corresponding alcohols.

Authentic samples of the compounds under investigation and other closely related compounds could be treated in a similar way by drawing approximately 10 nl of an aqueous or hexadecane solution (containing 10 μl cm^{-3} of each standard) into a fine capillary and transferring the filled section of capillary to the reaction vial. The sample was then sealed and analysed under the same conditions as the glandular samples.

4I(d) Solid sodium hydroxide reagent:

Employing the previous technique and under exactly the same conditions, sodium

hydroxide was substituted for sodium borohydride, resulting in the complete subtraction of straight chain aldehydes and unsaturated ketones, and the partial subtraction of ∝-branched aldehydes.

4I(e) Aqueous sodium hydroxide reagent:

Fine capillaries containing 10 to 50 nl of aqueous sodium hydroxide solution were substituted for the solid reagents in sections 4I(c) & (d). The reagent and sample were centrifuged together as before, resulting in the removal of base sensitive-compounds without causing polymerisations to occur.

4(II) POST-COLUMN REACTION LOOPS

The use of post-column loops enabled the small quantities of volatiles extracted from the glands to be submitted to further reaction gas chromatography. The loops were connected to the analytical column with a $\frac{1}{4}$ in Swagelok coupling bored out completely to permit glass to glass contact between the $\frac{1}{4}$ in OD tubing.

4II(a) o-dianisidine loop[51]:

Supersorb 80-100 mesh (3.8 gms) was added slowly with gentle stirring to a chloroform solution of o-dianisidine (0.2 gms in 25 cm^3) in a 100 cm^3 ribbed round -bottomed flask. The solvent was carefully removed by hand-held rotivation as described in section 3(b). The resultant 5% phase was dried at 60°C on a clock glass in a drying cabinet. A 6 in x $\frac{1}{4}$ in OD glass loop was packed by suction and vibration with 3 in of Poropak Q 120-150 mesh.

The loop was connected to the analytical column with the Poropak Q nearest to the detector to prevent substrate bleed into the HFID.

The column was operated isothermally at 167°C and 50 cm^3 He min^{-1}.

The absorption of C_1 to C_5 aldehydes and ketones was quantified by injecting them in ng to µg quantities in aqueous solution, prepared as described in section 3(d).

The specificity of the post-column for the selective absorption of aldehydes was assessed by the injection of an aqueous solution of the different compounds under investigation in the approximate quantities found in four Dufour's glands.

Dufour's gland and poison vesicle contents of both Myrmica species under investigation were removed by capillary extraction and analysed by GLC using the solid sampling technique. The presence of aldehydes in these samples could be assessed by their removal on passing the column effluent through the post-column loop.

4II(b) Boric acid loop:

Alcohols were removed by passing the column effluent through a 6 in x ¼ in OD glass loop packed with 20% w/w boric acid and Poropak Q 120-150 mesh, thoroughly mixed together as powders.

The analytical column was operated isothermally at 167°C and 50 cm^3 He min^{-1}.

Authentic samples were prepared and injected as described in the previous section. On passing through the post-column loop all saturated alcohols were completely absorbed in quantities from ng to µg per run.

(5) CALCULATION OF GLANDULAR VOLUMES AND WATER CONTENT

Analyses were performed using the Poropak Q column at 187°C and 45 cm^3 He min^{-1}. A Katharometer detector was used for the detection of water with a bridge current of 240 mA.

Absolute ethanol was dried over molecular sieve 4A and used to prepare ethanolic solutions of water, ranging from 1 to 20 µl water cm^{-3} ethanol.

1 µl of each of the resultant solutions containing from 1 to 20 nl of water, was injected onto the Poropak column and the corresponding water peak heights measured to the nearest mm from the recorder trace. These heights were corrected for the residual water content of the solvent ethanol by subtraction of the water peak heights obtained by injection of 1 µl of the 'dry' absolute ethanol.

Using the corrected data, a calibration graph of water peak height versus volume of water injected was compiled (Fig.27)

The volume of the poison vesicle or Dufour's gland was calculated in two ways, both of which involved measurement of the length of liquid drawn into a capillary from the respective gland. Both methods took advantage of the uniformity of the glass capillaries over a few centimetres of their length.

The liquid was drawn from the gland under investigation into the fine capillary. It could be clearly seen under the microscope when all the liquid from the glandular sac had been removed. The length of liquid drawn into the capillary was measured to the nearest 0.5 mm under the microscope and the filled section of capillary broken off and discarded. In one technique the internal diameter of the immediately adjacent capillary was measured under a microscope using a graticule and blue transmitted light source to reduce abberation.

In this way the volume of the cylinder of liquid drawn up from the glandular sac could be calculated from the formula $\pi r^2 h$, where r is the internal diameter of the capillary and h is the length of liquid drawn up into the capillary from the gland.

In another technique an approximately equal and measured

length of distilled water was drawn into the adjacent capillary, the filled portion broken off and sealed into a solid sampling via vial as described in section 2(b). The sample was introduced into the GLC by the solid sampling technique and the resultant water peak height measured from the recorder trace. The water content of the vial was then calculated by reference to the calibration graph relating water peak height to volume of water injected (Fig.27).

The volume of the uniform capillary per mm of its length was calculated and hence the volume of the measured length of liquid withdrawn from the glandular sac.

The volume of the Dufour's glands and poison vesicles of both M. rubra and M. scabrinodis were calculated in this way.

The results obtained by the two above methods were in good agreement.

The water content of the Dufour's glands and poison vesicles of both species was estimated by removing the liquid from the respective gland by the capillary extraction technique, transferring the filled section of capillary to a solid sampling vial and analysed by GLC using the solid sampling technique. The water peak height on the recorder trace was measured to the nearest 0.5 mm.

Empty vials were also sealed and their water content assessed by the above technique.

The water peak height of the sample of liquid from the glandular sac was corrected for the water content of an empty vial by subtraction of the average water peak height from empty vial samples. The volume of water originating from the respective glands was then calculated from the calibration graph relating water peak height to water volume.

(6) ELECTROANTENNOGRAM (EAG)

The electrophysiological response of the ant M. rubra to the volatiles identified in the ant distillate was investigated by recording their electroantennogram.

6(a) Electrode preparation:

Responses were recorded with ringer-filled glass micropipettes drawn from Phoenix (borosilicate) glass tubing 1.55-1.8 mm OD; 0.9-1.1 mm ID.

The tubing was cleaned by a conventional method of soaking in chromic acid, followed by washing in distilled water, sodium bicarbonate solution and finally boiled in filtered distilled water.

10 cm lengths were drawn on a Palmer puller (Palmers, Eng.) to give a pair of micropipettes with 2-5 μm tips and 0.5 cm shanks.

The micropipette dimensions were found to be critical: the short shank reduced the impedance of the pipette to permit the recording of potentials down to 0.1 mV; the electrode tip was large enough to permit recording of a summated action potential, without being too large to allow seepage of ringer solution into the specimen, causing tissue damage.

The settings required to produce these pipette dimensions with the Palmer puller were furnace 0.8, final pull 10, travel on microswitch 1 mm and extracellular setting.

The pipettes were back-filled with Hoyle's 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 and NaH_2PO_4 0.48 gms dissolved in 1 litre of distilled water.

The ringer solution was injected from a plastic syringe through a millipore No. 4 filter and fine needle cut off to a square end. the needle was inserted into the stem of the pipette

such that the tip was filled first. After filling, any bubbles of air remaining in the pipette tips were tapped out or teased out with a glass whisker.

The pipette tips were stored under distilled water prior to use to prevent damage caused by the crystallisation of salts in and around the tips.

Glass insulated electropolished metal microelectrodes of platinum, silver and tungsten, whilst suitable for recording fast motor pulses (AC) were found to polarise rapidly when used to record the slower action potentials (DC) encountered in the antennae of ants.

The problems of polarisation were overcome by the use of non-polarisable Ag/AgCl electrodes inserted into the electrolyte in the stem of the glass micropipette[78]: As the Ag/AgCl electrodes were not inserted into the ant directly, wire of 0.012 in was used, providing a relatively large surface area and hence a low current density: under these conditions the electrodes behaved reversibly and the composition of the electrolyte and hence the metal-electrolyte junction potential remained constant, minimising recorder baseline drift.

The Ag/AgCl electrodes were prepared from 0.012 in silver wire. Surface oxide was removed with 'Duraglit' metal polish, the electrodes washed in propanone and then anodised in 0.1 M HCl using a silver cathode and 2 V lead acid ~~acetate~~ accumulator as the power source.

Deposition of AgCl was continued until the wires were uniformly grey/white. To prevent atmospheric oxidation the electrodes were then stored in ringer solution in the dark prior to use.

6(b) Sampling and amplification:

The micropipettes were

supported as shown in Plate 2.

The distal 7 segments of the antennal funiculus were excised, the antennal tip carefully pierced with a razor blade and the micropipette tips inserted with the aid of a pair of micromanipulators (De Fonbrune, series B).

The responses of the antenna to methanol, ethanal, ethanol, propanone, butanone and ethanoic acid were tested by puffing 0.5 cm^3 of vapour of the pure compounds with 0.5 cm^3 of air from a 1 cm^3 ground glass syringe over the antennal preparation. The response to pure air puffed from another syringe was tested between samples. No attempt was made to measure the concentration of volatiles in the vapour, but they represented less than the saturated vapour pressures of the components in air at ambient temperatures.

Detection of the volatiles by the antennal receptors resulted in the generation of action potentials in the range 0.1 -1.0 mV, which were fed into one imput of a Fenlow AD 55 differential amplifier (Gain x 1000) via low-noise miniature screened lead. The other amplifier imput was connected to a millivolt source powered by a mercury (Mallory) cell.

The standing potential associated with the electrode system was backed off using this millivolt source and the zero control on the amplifier.

The imput impedance of the amplifier was in the order of 2×10^5 MΩ to avoid drawing large currents which would damage the preparation.

The antennal preparation lasted from 10 mins to 2 hours, the point of death being indicated by erratic responses to the puffs of air administered between samples.

Whole ant preparations lasted much longer, the ant being stapled to the cork and the electrodes inserted into the

antennal tip and into the head at its union with the base of the scape. However, any action potentials generated were obscured by continuous impulses of amplitude about 1 mV and duration 4 mS (Plate 3). These impulses had the dimensions of motor spikes produced in the brain for control of antennal movement.

Amplified responses were displayed on a Tectronix storage oscilloscope or recorded on a Bryans-Southern instrument series 10-350 UV recorder. Responses were quantified by calibration with the millivolt source.

The pre-amplifier and specimen were screened from extraneous electrical noise by enclosing them in a solid aluminium box earthed, to avoid loops, to the electrical mains.

The system was stabilised on a $\frac{1}{4}$ in plate glass sheet with Tico rubber pads.

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APPENDIX

Plate 1

Antennal scapes of *M. sabuleti* (a), *M. scabrinodis* (b) and *M. rubra* (c).

Plate 2

The electroantennogram.

Plate 3

A motor impulse of amplitude 1 mV and duration 4 mS.

APPENDIX

PLATE 1

PLATE 2

PLATE 3