ASPECTS OF STEROL METABOLISM IN CRUSTACEA

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ABSTRACT.

The sterol content of various species of Crustacea has been subject to comparatively few investigations. Previous work has been mainly confined to members of the Decapoda. To date, few studies have been made to determine the capacity of crustaceans to synthesise and metabolise sterols.

In the present work, an initial survey of representative members of the main crustacean orders was made. The results obtained were similar to already published work, with the exception that two previously unreported classes of sterols, the 4α-methyl and 4,4'-dimethyl sterols, were identified in certain organisms.

In order that meaningful metabolic studies could be carried out it was necessary to develop a suitable experimental system. This involved:

a. The selection of an experimental organism that fulfilled certain criteria.

b. The selection of a food organism that would support growth of the chosen crustacean from embryo to adult in a comparatively short time.

c. The selection of a compatible, chemically defined culture medium that would support growth of both food organism and crustacean.

The crustacean chosen for experimentation was the Californian strain of the brine shrimp, Artemia salina; the food organism a marine green alga, Dunaliella primolecta. The culture medium finally selected was modified from previously published work.

Analyses of the sterol composition of Artemia salina grown

under such conditions indicated that cholesterol is the major sterol throughout the life cycle of the organism.

It was established, using radiolabelled acetate and mevalonate, that Artemia salina is incapable of synthesising sterols or squalene but is capable of the synthesis of isoprenoid pyrophosphates, up to and including farnesol. This inability is present throughout the life cycle.

Neither ^{14}C-squalene nor ^{3}H-squalene-2,3-oxide was converted into sterols. It was concluded that at least three enzyme systems of the sterol biosynthetic pathway were missing; squalene synthetase, squalene oxidase and squalene-2,3-oxide cyclase.

Results are presented which show that Artemia salina is able to convert a wide variety of dietary 4-desmethyl sterols (stigmasterol, desmosterol, poriferasterol and β-sitosterol) into cholesterol, various steroid hormones and into material tentatively identified as 'ecdysones'. Similar conversions of the 4α-methyl sterol, cycloeucalenol, and the 4,4'-dimethyl sterols, cycloartenol, 24-methylenecycloartanol, lanosterol and 24,25-dihydrolanosterol did not occur. It was concluded that, although Artemia salina possessed the necessary enzymes to metabolise and utilise a wide range of dietary 4-desmethyl sterols, the enzymes necessary for the conversion of 4α-methyl and 4,4'-dimethyl sterols into cholesterol were not present.

FOREWORD.

I would like to extend my thanks and gratitude to:

The many members of the academic and technical staffs of the School of Environmental Sciences, Plymouth Polytechnic, who have given me assistance and advice throughout the course of this work.

Dr. L. J. Goad of the Department of Biochemistry, University of Liverpool, for the provision of many sterol standards and also for providing the facility for mass spectrometry.

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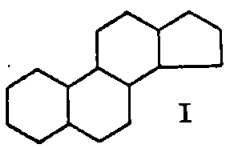
CHAPTER I.

INTRODUCTION.

I. Structure, stereochemistry and nomenclature of sterols.

Steroids are a group of compounds which, structurally, may be regarded as derivatives of cyclopentanoperhydrophenanthrene (I). Those steroids which have an hydroxyl group at position 3 are known as sterols. The sterol structure is typified by that of the major sterol of animal tissues - cholesterol (II). Although all steroids of this type are known as sterols, the sidechains may differ; extra methyl groups may be present at positions 4, 14 and 24; the 5,6 double bond may be absent and other double bonds may be present.



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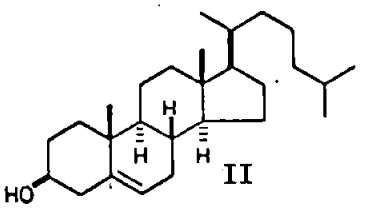

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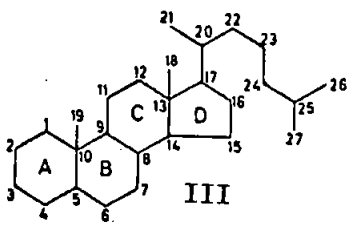

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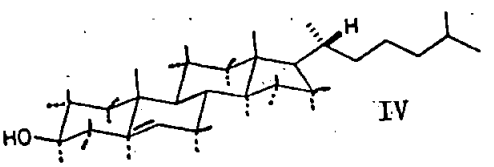


IV

The nomenclature and numbering used in this thesis follow the lines recommended by IUPAC (see IUPAC 1968), though trivial names in accepted usage are used in place of full systematic names.

The sterol nucleus can be considered as three fused cyclohexane rings (A, B and C) plus a terminal cyclopentane ring (D). Each of these can assume a 'chair' or 'boat' configuration, but a conformation in which all are 'chair' has been shown to possess the lowest entropy since interaction between atoms and groups is then the least (IV).

When the sterol structure is orientated as in II, an α position is defined as projecting into the plane of the paper and a β position outwards. Rings A, B, C and D are all fused in the trans configuration so that atoms or substituents at positions 8, 9, 10, 13 and 14 (III) become 8β, 9α, 10β, 13β and 14α respectively.

Most naturally occurring sterols have the hydroxyl at C-3 in the β-configuration. Some sterols contain a cis A/B ring junction, as in coprostanol, and hence the stereochemistry at position 5 is usually defined in the nomenclature, but in this thesis all sterols are assumed to be 5β unless otherwise stated. The 18 and 19 angular methyls and the sidechain are all of the β configuration. Sterols of plants differ in that they may have a cyclopropane ring at $9\beta,19$, unsaturation at C-22 and possess an alkyl or alkylidene group at C-24.

Substitution at position 24 produces two possible epimers. These, under the Plattner convention, were described as α or β (Fieser & Fieser 1959) and this convention is used in this thesis. This is a deviation from the IUPAC convention, which requires that the configuration at C-24 of alkylated sterols should be defined by the revised sequence rule (Cahn, Ingold & Prelog 1966).

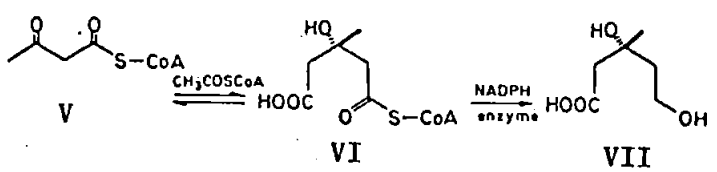
II. The biosynthesis of sterols.

The mechanisms by which terpenes and sterols are biosynthesised

have been the subject of many recent reviews (see, for example, Bloch 1965; Clayton 1965a, 1965b; Frantz & Schroepfer 1967; Goodwin 1971; Mulheirn & Ramm 1972; Rees & Goodwin 1972; Sih & Whitlock 1968). The initial part of the pathway has been shown to involve the formation of a C_{30} triterpene, squalene, by the consecutive condensation of 'active isoprene units'. A brief outline of the reactions involved will be given below.

i. Formation of squalene.

Acetate was known for years to be a precursor of sterols. It was not until 1956, however, with the discovery of mevalonic acid as a growth factor for Lactobacillus acidophilus (Skeggs et al. 1956) that it was realised that this compound was a specific precursor for isoprenoids. The biochemical importance of mevalonate was quickly realised. Its intermediacy in the biosynthesis of numerous terpenoids, carotenoids, sterols and polyisoprenoids was demonstrated. Only 3R-mevalonic acid is metabolically active and it appears to be utilised solely in terpenoid biosynthesis.

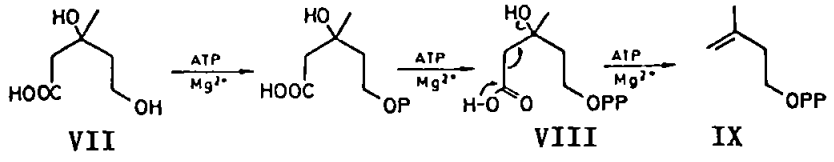


V VI VII

The biosynthetic pathway of mevalonate (VII) formation is from acetyl CoA via acetoacetyl CoA, β-hydroxy-β-methylglutaryl CoA (VI) and some form of mevaldic acid. Mevaldic acid has not been isolated in vivo and it is suggested that it is enzyme-bound as the hemi-thioacetal with coenzyme A (Retey et al 1970).

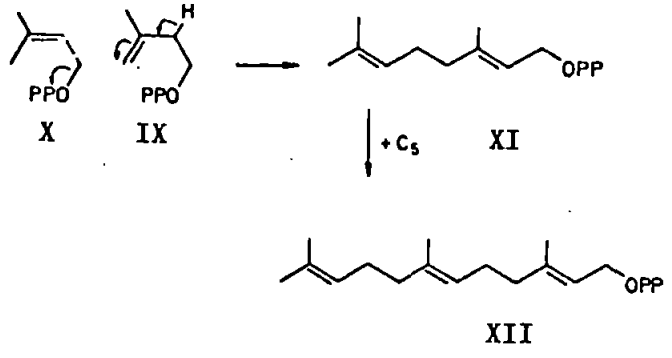
The enzymatic conversion of mevalonate into squalene requires adenosine triphosphate and some of this is utilised in the formation of

mevalonic pyrophosphate (VIII).



VII VIII IX

The next step, which also requires adenosine triphosphate, is a combined decarboxylation and dehydration of mevalonic pyrophosphate, leading to the formation of 3-methylbut-3-enyl pyrophosphate (the 'active isoprene unit' - isopentenyl pyrophosphate - IX). The mechanism of this step is believed to be a concerted trans-elimination process (as shown), probably involving the 3-phosphate, though no such intermediate has ever been isolated.

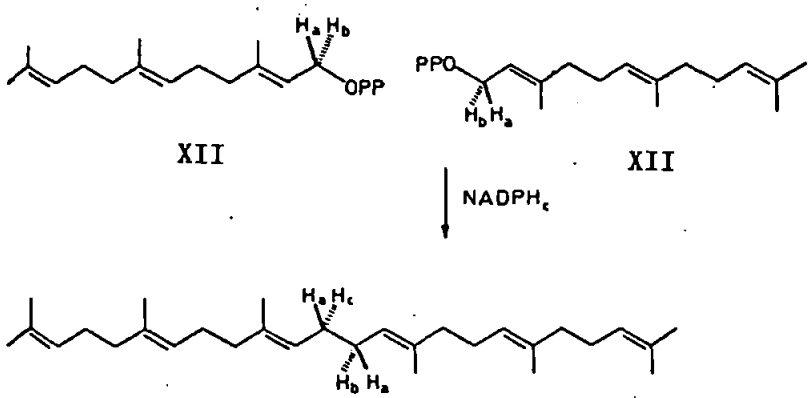


X IX XI

XII

An enzyme catalysed prototropic shift first converts isopentenyl pyrophosphate into its highly reactive isomer, 3-methylbut-2-enyl pyrophosphate (dimethylallyl pyrophosphate - X). Condensation of these two compounds, with the elimination of a pyrophosphate grouping, leads to trans-geranyl pyrophosphate (XI). The reaction proceeds in two distinct stages: a trans addition followed by a trans elimination (Cornforth 1968; Cornforth et al 1966a, 1966b). Geranyl pyrophosphate readily condenses with another molecule of the dimethylallyl pyrophosphate to give trans-farnesyl pyrophosphate (XII). The tail to tail

condensation of two farnesyl pyrophosphate molecules produces squalene (XIII).



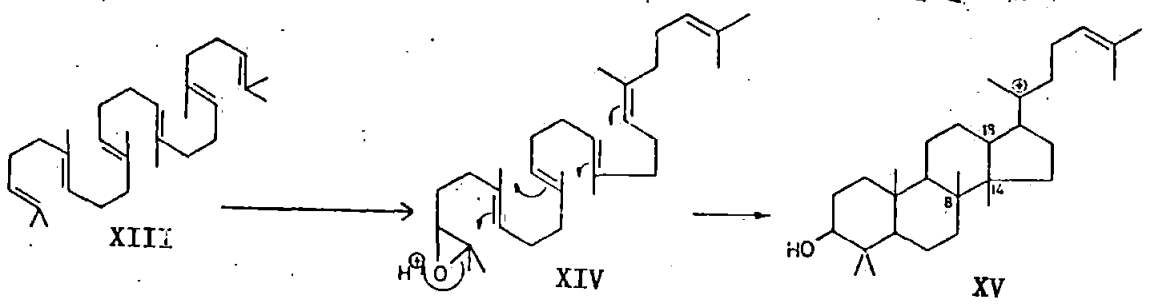
XII XII

XIII

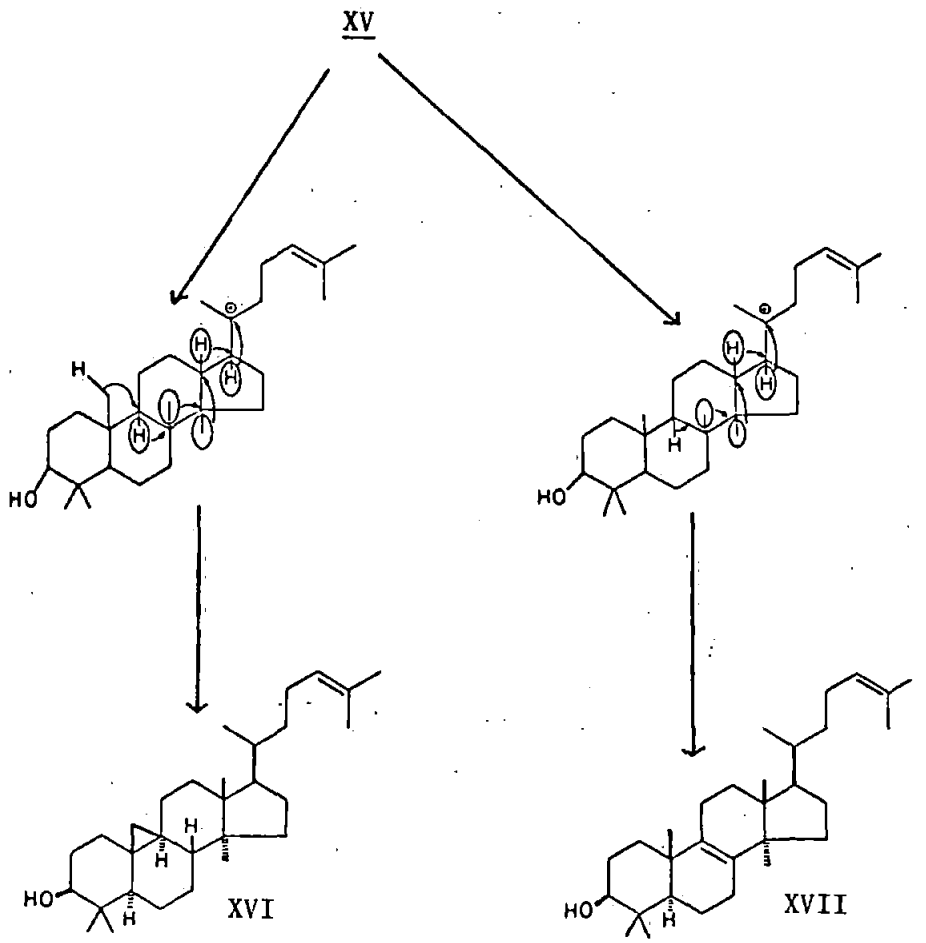
During this condensation, one of the hydrogen atoms on C_1 of farnesol is removed and a hydrogen from the B side of NADPH inserted (Popjak & Cornforth 1966). A C_{30} precursor of squalene, presqualene pyrophosphate, is almost certainly involved in this reaction, but the exact mechanism is unknown (Edmond et al 1971; Epstein & Rilling 1970). The stereochemistry of these various reactions has been studied and elucidated by Popjak & Cornforth with the aid of stereospecifically labelled mevalonic acids (Popjak & Cornforth 1966; Cornforth 1969). The formation of squalene in plant tissues has been shown to proceed by mechanisms identical to those in animals (Benveniste et al 1970; Green & Baisted 1971).

ii. Cyclisation of squalene.

The cyclisation of squalene requires, besides the appropriate enzyme systems, molecular oxygen and reduced pyridine nucleotide. The first product has been identified as squalene-2,3-oxide (XIV - Willett et al 1967; Van Tamelen et al 1966, 1967). This is then converted by electrophilic attack to the carbonium ion (XV), which may be stabilised either by loss of a proton from C-19 to form cycloartenol (XVI - in plants) or from C-9 to form lanosterol (XVII - in animals).



As regards the methyl migrations which must accompany the conversion of the carbonium ion into lanosterol, it has been shown that the transformation is accomplished by two consecutive 1,2 shifts rather than by a single 1,3 shift of a methyl group from C-8 to C-18. This is accompanied by loss of a proton from C-8 to give the 8,9 double bond of lanosterol.



In the case of cyclisation to cycloartenol, stabilisation of the carbonium ion occurs by loss of a proton from C-19, not C-8, so that a 9β,19-cyclopropane ring is formed (Rees et al 1968a, 1968b).

iii. Conversion of lanosterol into cholesterol.

The further metabolism of lanosterol into cholesterol involves a number of modifications which can be summarised as follows:

a. Removal of the 4,4'-gem-dimethyl and 14α-methyl groups.

b. Transposition of the 8,9 double bond to the 5,6 position.

c. Reduction of the 24,25 double bond.

These processes have been subjects of extensive reviews (Rees & Goodwin 1972; Clayton 1965a, 1965b; Frantz & Schroepfer 1967; Moss 1972; Schroepfer et al 1972).

a. Demethylation reactions.

It is considered that elimination of the 4- and 14-methyl groups proceeds by conversion of the methyl group, via an alcohol and aldehyde, to the carboxylic acid, which undergoes decarboxylation (Bechtold et al 1972; Miller et al 1967, 1971; Miller & Gaylor 1970; Rahimtula & Gaylor 1972). Prior oxidation at C_3 would facilitate the decarboxylation at C_4 and tritium labelling experiments suggest that this in fact does take place (Lindberg et al 1963; Swindell & Gaylor 1968). The C-14 methyl group appears to be removed before the C-4 methyl groups (Frantz & Schroepfer 1967).

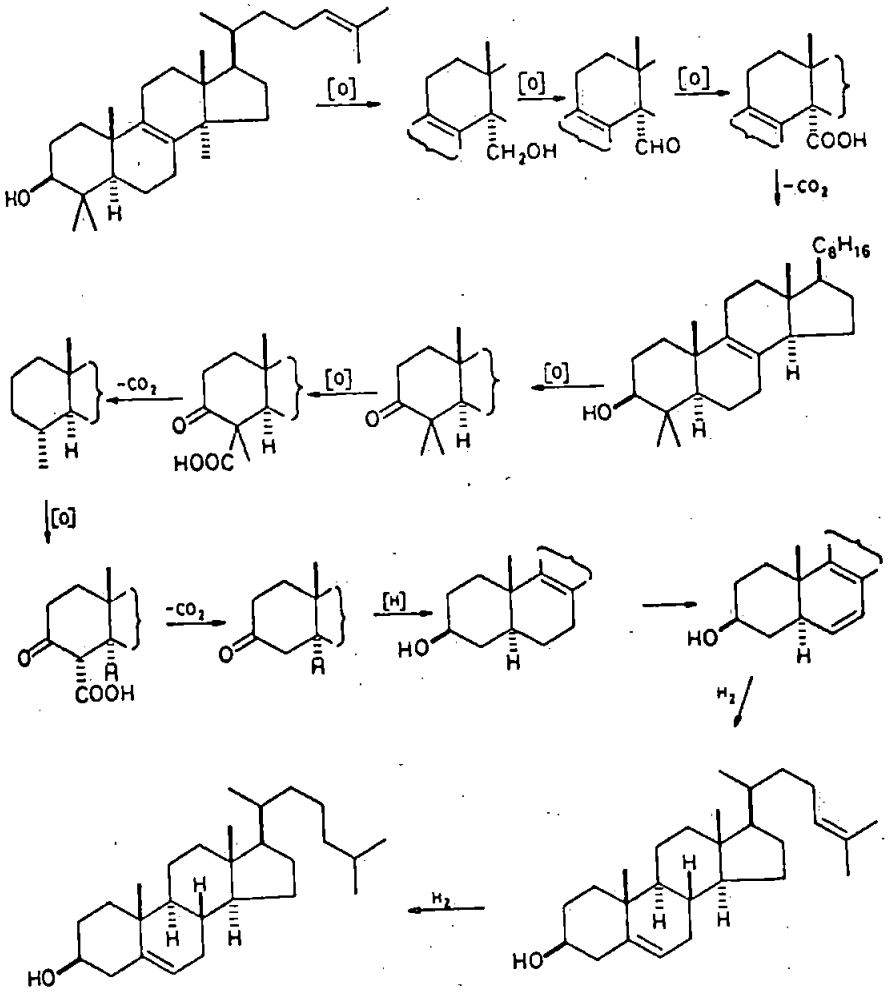
b. Transposition of the double bond.

The reaction sequence for the transposition of the 8,9 double bond to the 5,6 position is still not fully elucidated but the sequence $\Delta^{8(14)} \rightarrow \Delta^{8,14} \rightarrow \Delta^{8} \rightarrow \Delta^{7} \rightarrow \Delta^{5,7} \rightarrow \Delta^{5}$ is normally considered to be the main route (see reviews quoted above).

c. Reduction of the Δ^{24} bond.

The Δ^{24} double bond can be saturated at various stages during cholesterol biosynthesis (Frantz & Schroepfer 1967), but the point in the sequence at which this reaction occurs may vary with the tissue. In human brain tissue, for example, the preferred pathway may be via Δ^{24} intermediates (Paoletti et al 1971). It has been concluded that the reduction of this olefinic bond in rat liver involves the cis-addition of hydrogens at C-24 and C-25 (Duchamp et al 1971).

One feasible scheme for the overall conversion of lanosterol to cholesterol is shown below.



It must be emphasised that, although each step individually is fairly well established, the exact sequence in which these steps occur, if indeed there is an invariable sequence, is uncertain. Mention has already been made of the fact that reaction sequences may well vary from tissue to tissue. It is thought that a number of pathways can and do operate and it is likely that the enzymes which catalyse the various steps are non-specific. Frantz & Schroepfer (1967) concluded that 'present evidence does not permit any conclusions as to which pathway predominates'. This conclusion was strengthened by the recent listing of 42 compounds as possible intermediates in the enzymic formation of cholesterol from lanosterol (Schroepfer et al 1972).

III. Conversion of cycloartenol to phytosterols.

If campesterol and β-sitosterol are taken as being representative of typical plant sterols, then the reactions by which these compounds are synthesised from cycloartenol may be summarised as follows:

a. Removal of the 4,4'-gem-dimethyl and 14α-methyl groups.

b. Transposition of the 8,9 double bond, resulting from cleavage of the cyclopropane ring, to the 5,6 position.

c. Reduction of the 24,25 double bond.

These reactions are analogous to those previously described for the lanosterol to cholesterol sequence and are thought to proceed by similar mechanisms (see reviews quoted above).

d. Alkylation at C-24.

e. Cleavage of the 9β,19 cyclopropane ring.

f. Introduction of a C-22 double bond.

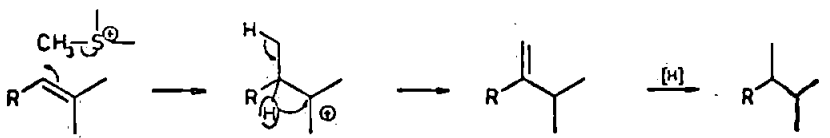
Reactions d $\longrightarrow$ f are unique to phytosterol biosynthesis and have been subject to a number of excellent reviews (Clayton 1966a,

1965b; Anding et al 1972; Goodwin 1971; Knights 1973; Lederer 1969). Only a cursory description will be given here.

i. Alkylation at C-24.

It is well established that alkylation occurs by successive transmethylations from S-adenosylmethionine to give the 24-methyl and 24-ethyl sterols (Lederer 1969). The intermediacy of 24-methylene and 24-ethylidene sterols was first postulated by Castle et al (1963) and many such sterols have been isolated from plant sources (Goodwin 1971).

Firstly, a methylation of a Δ^{24} sterol can occur to yield a 24-methylene compound (Akhtar et al 1966a; Barton et al 1966; Katsuki & Bloch 1967; Lederer 1964) with a concomitant hydrogen ion migration from C-24 to C-25 (Akhtar et al 1966b; Stone & Hemming 1967). Saturation of this compound then yields the 24-methyl derivative.

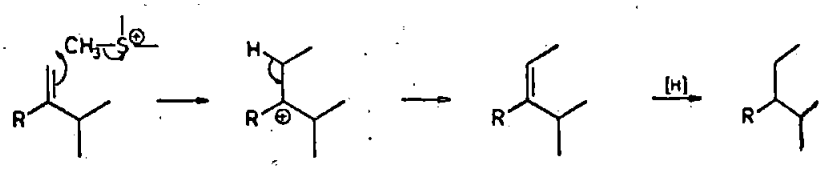


The intermediacy of a 24-methylene compound in the biosynthesis of 24-methyl sterols is indicated by the fact that only two of the three hydrogens from the methyl group of methionine are retained in ergosterol biosynthesis (Jaureguiberry et al 1965).

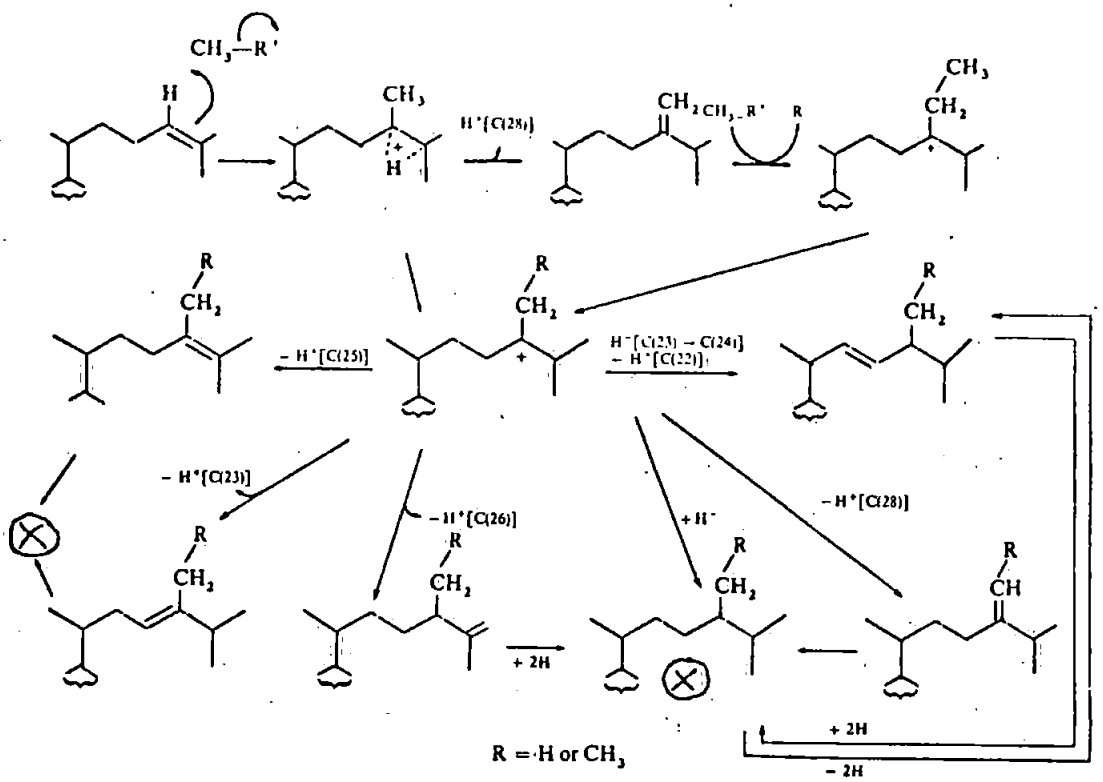
Instead of stabilisation of a 25-carbonium ion by the formation of a 24-methylene compound, there are a number of theoretically possible alternative schemes involving the introduction of a second methyl group from S-adenosylmethionine. These alternatives are shown overleaf.

There is evidence for more than one mechanism for the addition of a second methionine methyl group during the biosynthesis of the 24-ethyl and 24-ethylidene sterols. In some organisms, eg. Ochromonas malhamensis and Larix decidua, it seems likely that a 24-methylene

sterol accepts a further methyl group from S-adenosylmethionine and the resulting 24-carbonium ion is stabilised by loss of a proton from C-28 to give a 24-ethylidene sterol (Goad et al 1966; Smith et al 1967).



That 24-methylene and 24-ethylidene sterols can act as precursors of the 5,22-ethyl sterol, poriferasterol, has been demonstrated using Ochromonas malhamensis (Knapp et al 1971; Lenton et al 1971). Van Aller et al (1969) have shown that 24-ethylidene sterols can be precursors of 24-ethyl sterols in pea seedlings.



It is also possible for stabilisation of the 24-carbonium ion to occur by expulsion of a proton from either the C-26 or C-27 methyls to form a Δ^{25} bond and this has been reported to be the case in the

synthesis of cyclolaudenol and 24-ethylcholesta-5,22,25-trien-3β-ol (Bolger et al 1970; Ghisalberti et al 1969).

In some organisms alkylation can occur without the intermediacy of an ethylidene sterol, possibly through stabilisation of the carbonium ion formed at C-24 by Δ^{22}, Δ^{23} or Δ^{24} bond formation, eg. a $\Delta^{24(25)}$ compound is indicated during the synthesis of stigmasterol by Nicotiana tabacum and Dioscorea tokoro (Tomita & Uomori 1970).

Although a Δ^{24} bond is regarded as a prerequisite for alkylation, Nicotiana tabacum and Ochromonas malhamensis alkylate saturated sidechain sterols, presumably after the introduction of unsaturation at C-24 (Tso & Cheng 1971; Beastall et al 1972).

Reduction of the $\Delta^{24(28)}$ bond of 24-methylene and 24-ethylidene sterols produces chirality at C-24 and so epimers are produced, eg. campesterol and 22-dihydrobrassicasterol, the α and β epimers of 24-methylcholesta-5-en-3β-ol. The stereochemistry of the sidechain alkylation reactions appears to be rigorously controlled and is of some taxonomic significance. In higher plants those sterols possessing a C-24 ethyl or methyl group have, with few exceptions, the 24α configuration, eg. campesterol, stigmasterol and β-sitosterol (Goad 1967). One exception is brassicasterol. Sterols of the opposite 24α configuration, eg. ergosterol, poriferasterol and clionasterol, occur in algae and fungi (McCorkindale et al 1969; Patterson 1971).

ii. Cleavage of the 9β,19-cyclopropane ring.

The cleavage of the 9β,19-cyclopropane ring appears to result in the formation of a Δ^{8} double bond and a C-19 methyl group. There are few reports in the literature concerning this aspect of the phytosterol pathway.

Scheme 1.1. Proposed pathway for the biosynthesis of Phytosterols. (Goad 1970).

Cyc = Cycloartenol

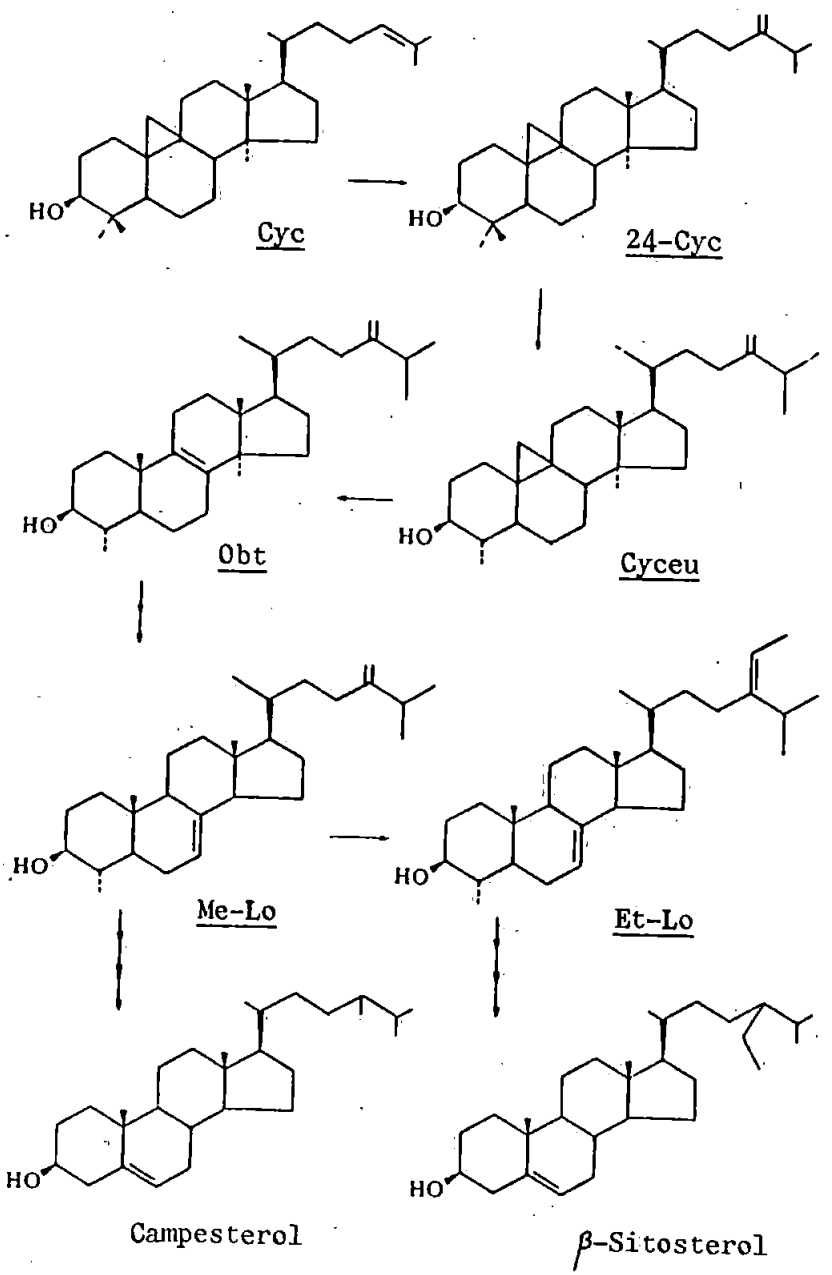
24-Cyc = 24-Methylenecycloartanol

Me-Lo = 24-Methylenelophenol

Obt = Obtusifoliol

Cyceu = Cycloeucalenol

Et-Lo = 24-Ethylidenelophenol



iii. Introduction of a C-22 double bond.

Phytosterols often possess a feature not found in animal sterols, a Δ^{22} bond. It has been claimed that the introduction of Δ^{22} unsaturation can be linked to C-24 alkylation. In most organisms, however, formation of the Δ^{22} bond is probably unrelated to alkylation (Goodwin 1971).

The order in which the various modifications occur is uncertain. One plausible scheme for phytosterol biosynthesis is shown in Scheme 1.1. (Goad 1970) but the structural features of many naturally occurring sterols make other diverging pathways equally possible (Anding et al 1972). Evidence to support Scheme 1.1 has been provided in recent years (Baisted et al 1968; Benveniste 1968; Benveniste et al 1906; Goad et al 1967; Hall et al 1969; Kemp et al 1968; Lenton et al 1971; Malhotra & Nes 1972) and a full description of this work and that of identifying other phytosterol intermediates, can be found in the reviews cited above.

It is evident that, although the broad pattern of phytosterol biosynthesis resembles that of cholesterol, there are some differences. In animals, the overwhelming dominance of cholesterol is striking. In plants, the reactions would seem to be more random than those of cholesterol biosynthesis; either because of a great multiplicity of enzyme production or of the low specificity of some plant enzymes. The net result of the random nature of phytosterol biosynthesis has been the detection of almost every possible intermediate between squalene and the phytosterols. Whether the predominance of any of the intermediate phytosterols seen in some species has any significance in terms of plant taxonomy remains to be seen (Knights 1973).

IV. Distribution of sterols.

Sterols are found in all eucaryotic organisms and have been reported in geological sediments (Attaway & Parker 1970; Mattern et al 1970). Until recently it was generally believed that a point of metabolic resemblance between bacteria and the Cyanophycaea lay in their mutual inability to synthesise sterols. In recent years, however, there have been several reports of low levels of sterols in bacteria (Schubert et al 1968) and blue-green algae (DeSouza & Nes 1968; Reitz & Hamilton 1968; Nadal 1971; Teshima & Kanazawa 1972a). Most plants contain sterols alkylated at C-24; some contain cyclopropane rings and are unsaturated at C-22.

The most primitive animals, such as the sponges (Erdman & Thompson 1972), contain the most diverse collection of sterols. Cholesterol is included in this diverse collection but it is neither the most typical nor the most prominent sterol of lower invertebrates. Higher on the evolutionary ladder, other sterols disappear and cholesterol becomes more prominent until, in vertebrates, it is the principal sterol. Bergmann (1962) has suggested that the reduction in the number of closely related compounds performing closely related functions to a few compounds, or to one compound, is a general feature of biochemical evolution. Cholesterol may thus be viewed as representing 'the survival of the fittest sterol'.

V. Sterols of Crustacea.

Dorée first isolated cholesterol from the edible crab, Carcinus maenas, in 1909 (Dorée 1909). Since that time, with few exceptions, analyses of crustacean sterols have been confined to the decapods, the most advanced true invertebrates and the most economically important

crustaceans. Early workers (Leulier & Policard 1930; Abernethy & Vilbrandt 1931; Collin et al 1934; Kind & Fasolino 1945) were able to identify only cholesterol. No recent worker, however, has suggested that cholesterol occurs alone in Crustacea, but it does appear to be the predominant sterol (Austin 1970; Idler & Wiseman 1971a; Thompson 1964; Kritchevsky et al 1967; Pihl 1957; Teshima & Kanazawa 1971a; Yasuda 1973 and references cited therein).

There are slight deviations from this pattern. Fagerlund & Idler (1957) reported that desmosterol accounted for 34% of the sterol of the barnacle, Balanus glandula. Whitney (1967) showed that desmosterol is the major sterol in Balanus nubilis. A high percentage of desmosterol, however, is not characteristic of Cirripedia alone since large amounts have been found in all classes of the Decapoda (Idler & Wiseman 1968, 1971a; Teshima & Kanazawa 1971a; Yasuda 1973) and in single members of the Mysidacea (Neomysis intermedia) and Amphipoda (Caprella sp.; Teshima & Kanazawa 1971a). Brassicasterol has been identified as a major component (37% of the total sterol) of the edible crab, Cancer pagurus (Kritchevsky et al 1967). The claim of Collin et al (1934) to have demonstrated ergosterol in the copepod, Calanus finmarchicus, was based on circumstantial evidence from vitamin D potency studies and has not been substantiated.

Apart from the few organisms in which it is reported to be the sole sterol, cholesterol is accompanied by minor amounts of other sterols. Those identified so far (see Chapter IV) are 22-dehydrocholesterol, fucosterol, 28-isofucosterol, campesterol, 24-methylenecholesterol, stigmasterol, 22,23-dihydrobrassicasterol, brassicasterol and β-sitosterol.

Whilst it is generally accepted that all vertebrates and plants are able to synthesise sterols from simple precursors, not all invertebrates are capable of such transformations. Arthropods in general are unable to synthesise sterols. The absence of sterol synthesis in insects has been extensively investigated and is well documented (see Robbins et al 1971 and Thompson et al 1972b for reviews). Similar results have emerged from studies with an arachnid, Avicularia avicularia, and a myriapod, Graphidostreptus tumuliporus (Zandee 1967). The absence of sterol synthesis in the fourth arthropod class, the Crustacea, is also well documented and is dealt with in greater detail in Chapter V (Whitney 1969, 1970; Zandee 1964, 1966, 1967; Teshima & Kanazawa 1971c, 1971e; Walton & Pennock 1972).

Fossil remains do not demonstrate either the origin of Arthropoda or of its classes. The question arises as to whether arthropods are monophyletic, that is have arisen from an animal that was an arthropod itself, or polyphyletic, that is the features which they share in common and which distinguish them from all other phyla have been acquired independently in several evolutionary lines. The wide range of morphologies seen in this family complicates a taxonomy based on structural similarities or differences. Biochemical studies, such as those on sterol biosynthesis, will shed some light on the origin and evolution of the arthropod classes and their relationships with other invertebrates.

CHAPTER II.

MATERIALS AND METHODS.

I. Materials.

Unless otherwise stated, all solvents and chemicals were obtained from British Drug Houses Ltd., Poole, Dorset.

i. Solvents.

Acetone - redistilled from potassium permanganate and dried with anhydrous sodium sulphate.

Diethyl ether - referred to as ether - was obtained from May & Baker Ltd. It was dried over sodium wire and redistilled from reduced iron powder to remove peroxides.

Petroleum ether (bpt. $40/60^{\circ}$) - referred to as petrol - was dried over sodium wire and redistilled prior to use.

Toluene, benzene, cyclohexane and n-hexane were dried over sodium wire and redistilled prior to use.

All other solvents were AnalaR grade and were used as received.

ii. Reagents.

Adenosine triphosphate - Sigma Chemicals.

Agar (No. 3) - Oxoid Ltd.

Aluminium isopropoxide - freshly redistilled ($140-150^{\circ}$ at 12mm Hg) and stored in a desiccator.

Antifoam Emulsion A - Sigma Chemicals.

Chromic acid reagent - prepared by the method of Djerassi et al (1956). Chromium trioxide (26.72g) was dissolved in concentrated sulphuric acid ($23cm^3$). This was diluted to $100cm^3$ with water.

Cyclohexanone - redistilled and stored in a desiccator.

Glucose-6-phosphate dehydrogenase (from bakers' yeast) - Sigma Chemicals.

Malt extract - Oxoid Ltd.

Merthiolate (Thiomersal) - Sigma Chemicals.

Nicotinamide adenine dinucleotide phosphate - Sigma Chemicals.

Nystatin (mycostatin) - Calbiochem Ltd.

Penicillin G - Sigma Chemicals.

Pyridine - dried by refluxing with and redistilling from potassium hydroxide pellets.

Sodium borohydride - Sigma Chemicals.

Streptomycin sulphate - Sigma Chemicals.

Yeast extract - Oxoid Ltd.

iii. Terpenes.

Dimethylallyl alcohol (3-methyl-2-buten-1-ol) - Aldrich Chemical Co.

trans, trans-Farnesol - separated from the commercial mixture obtained from Ralph Emmanuel Ltd. by TLC on silica gel with 20% ethyl acetate in benzene as developing solvent (McSweeney 1965).

Geraniol - Koch-Light Chemicals.

Isopentenyl alcohol (3-methyl-3-buten-1-ol) - Ralph Emmanuel Ltd.

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexene) Sigma Chemicals. This was purified via the thiourea adduct before use.

iv. Steroids.

The following steroids were obtained from Sigma Chemicals.

11α-Hydroxyprogesterone	Δ^4-pregnen-11α-ol-3,20-dione.
Progesterone	Δ^4-pregnen-3,20-dione.
Hydrocortisone	Δ^4-pregnen-11β,17α,21-triol-3,20-dione.
Cortisone	Δ^4-pregnen-17α,21-diol-3,11,20-trione.
Oestrone	$\Delta^{1,3,5(10)}$-oestratrien-3-ol-17-one.

17β-Oestradiol $\Delta^{1,3,5(10)}$-oestratrien-3,17β-ol.

Testosterone Δ^{4}-androsten-17β-ol-3-one.

v. Sterols.

5α-Cholestane - Sigma Chemicals.

Cholest-5-en-3β-ol (cholesterol) - British Drug Houses Ltd.

Cholest-5,22-dien-3β-ol (22-dehydrocholesterol) - Dr.L.J.Goad, University of Liverpool.

24β-Methylcholest-5,22-dien-3β-ol (brassicasterol) - synthesis detailed elsewhere.

Cholest-5,24-dien-3β-ol (desmosterol) - Supelco Inc.

24α-Methylcholest-5-en-3β-ol (campesterol) - impurity in commercial -sitosterol.

24-Methylenecholest-5-en-3β-ol (24-methylenecholesterol) - ex. Tapes philippinarum, Dr.S-I.Teshima, Kagoshima University.

24α-Ethylcholest-5,22-dien-3β-ol (stigmasterol) - Koch-Light Chemicals.

24α-Ethylcholest-5-en-3β-ol (β-sitosterol) - Koch-Light Chemicals.

24β-Ethylcholest-5,22-dien-3β-ol (poriferasterol) - ex. Ochromonas malhamensis, Dr.A.R.H.Smith, Plymouth Polytechnic.

24E-Ethylidenecholest-5-en-3β-ol (fucosterol) - Dr.L.J.Goad, University of Liverpool.

24Z-Ethylidenecholest-5-en-3β-ol (28-isofucosterol) - Dr.L.J.Goad, University of Liverpool.

24β-Methylcholest-5,7,22-trien-3β-ol (ergosterol) - Sigma Chemicals.

4α-Methyl-24-ethylidenecholest-7-en-3β-ol (24-ethylidenelophenol) - Dr.L.J.Goad, University of Liverpool.

4α-Methyl-24-methylenecholest-7-en-3β-ol (24-methylenelophenol) - Dr.L.J.Goad, University of Liverpool.

4α,14α-Dimethyl-9β,19-cyclocholest-24(28)-en-3β-ol (cycloeucalenol) - ex. Musa sapientum (Knapp & Nicholas 1969).

4α,14α-Dimethyl-9β,19-cyclocholest-24-en-3β-ol (31-norcycloartenol) - Dr.L.J.Goad, University of Liverpool.

4α,14α-Dimethyl-9β,19-cyclocholest-25(26)-en-3β-ol (31-norcyclolaudenol) Dr.L.J.Goad, University of Liverpool.

4,4,14α-Trimethyl-9β,19-cyclocholest-24-en-3β-ol (cycloartenol) - Dr.L.J.Goad, University of Liverpool.

4,4,14α-Trimethyl-24-methylene-9β,19-cyclocholest-3β-ol (24-methylene-cycloartanol) ex. Musa sapientum (Knapp & Nicholas 1969).

4,4,14α-Trimethylcholest-8-en-3β-ol (24,25-dihydrolanosterol) and 4,4,14α-Trimethylcholest-8,24-dien-3β-ol (lanosterol) - commercial lanosterol (Koch-Light Chemicals) contained approximately 40% 24,25-dihydrolanosterol. These were separated by TLC of the mixed acetates on 10% $AgNO_3$/silica gel plates with 40% benzene in hexane as developing solvent. The free sterols were generated by saponification with ethanolic potassium hydroxide, purified by TLC on silica gel with chloroform as developing solvent and recrystallised from methanol.

vi. Column packings for Gas Liquid Chromatography.

Column packings used throughout this work were 3% OV-17 and 1% SE-30 on GasChrom Q 80/100 mesh (Applied Sciences Laboratories Inc.). Column conditions are specified in the text.

II. Methods.

i. Chromatographic techniques.

a. Column chromatography.

The non-saponifiable lipid was dissolved in a small volume of petrol and chromatographed on Woelm aniontropic alumina, deactivated to Brockmann grade III. 1g of alumina was used per 10g of non-saponifiable lipid. The column was developed by stepwise elution with increasing percentages of ether (E) in petrol (P) - $10cm^3$ of solvent per g of alumina - as follows: 0, 2, 6, 9 and 20% E/P. Column fractions were monitored by TLC on silica gel with chloroform as developing solvent. 4,4'-Dimethyl sterols appeared in the 6% E/P fractions, the 4α-methyl sterols in the 9% E/P fractions and the 4-desmethyl sterols in the 20% E/P fractions (Goad & Goodwin 1966). For experiments using Artemia salina, the amounts of tissue generally obtained were not sufficiently large to warrant the use of column chromatography as a general method.

b. Thin layer chromatography.

Thin layer chromatography (TLC) was carried out on 20cm square glass plates coated with silica gel (Kieselgel G; E.Merck. A-G., Darmstadt, Germany) to a thickness of 0.5mm. The dry adsorbent was mixed to a slurry with twice its weight of water and applied to the plates using a 'Quickfit' thin layer spreader. The plates were dried at room temperature and then activated by heating in an oven at 120° for one hour. Samples to be chromatographed were dissolved in small volumes of solvent (usually ether or cyclohexane) and strip-loaded approximately 2cm from the foot of the plate. Usually not more than 15mg per plate was applied. Greater amounts markedly affected the separations. Where necessary, marker compounds were chromatographed as spots along with the unknown sample. The developing solvent used for any particular

separation is quoted where appropriate in the text.

In separations involving sterols and squalene, the adsorption indicator, Rhodamine 6G, was dissolved to a concentration of 0.2% in the water used to prepare the silica gel slurry. After development, plates were examined in ultra-violet light. The sterols and squalene appeared as dark zones. The required areas were then scraped off the plates into a sintered funnel containing a small quantity of alumina. The material was eluted from the silica gel with ether. The alumina served to adsorb any indicator eluted by the ether.

Isoprenol alcohols were located by exposure to iodine vapours.

Argentation TLC was employed to separate sterols differing in their degree of unsaturation. This was performed on silica gel plates impregnated with silver nitrate (10:1 w:w). The techniques for preparation of the plates were as described above, except that a 5% silver nitrate solution instead of distilled water was used. Material was located by spraying, after development, with a 1% solution of Rhodamine 6G in acetone. Developing solvents are quoted in the text.

c. Gas liquid chromatography.

Gas liquid chromatography (GLC) was carried out on a Pye 104 gas chromatograph, fitted with flame ionisation detectors and connected to a Honeywell Electronik 194 chart recorder. 5' or 7' 1/4" id. glass columns were used throughout. Stationary phases and solid supports are given in the text. Oxygen-free nitrogen was used as carrier gas. Samples were injected on column in cyclohexane solution.

For preparative GLC an effluent splitter, with an approximate split ratio of 10:1, was inserted between the column and the detector. The bulk of the component peaks were condensed at ambient temperatures in glass capillary tubes. By repeated injection of samples, sufficient

material could be usually trapped for further analysis. At high levels of loading, the efficiency of sample trapping was markedly lowered.

ii. Isolation procedures.

a. Extraction of non-saponifiable lipids.

Tissues were refluxed for two hours with one volume of ethanol and 0.5 volumes of 60% aqueous potassium hydroxide, to which pyrogallol had been added to a final concentration of 1.5% to act as an antioxidant. Approximately 3 volumes of water were then added and the mixture extracted with ether (x3). The ether extracts were bulked, washed to neutrality with water and dried over anhydrous sodium sulphate. The lipid material was finally recovered by removal of the solvent by evaporation under reduced pressure.

In experiments involving the separation of steroids, chloroform was used in place of ether for the extraction of the non-saponifiable lipids from the saponification mixture.

b. Isolation of sterols.

The non-saponifiable lipid fractions were strip-loaded onto silica gel plates which were developed twice in chloroform. This effected a good separation between 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterols.

c. Isolation of squalene and squalene-2,3-oxide.

The non-saponifiable lipid fractions were strip-loaded onto silica gel plates which were developed in hexane. In this solvent, squalene-2,3-oxide remains at the origin (Goodfellow & Liu 1972). This area of the silica gel was removed, the lipid material eluted and rechromatographed on silica gel, with 5% ethyl acetate in benzene as developing solvent (Rees et al 1968a).

In those experiments involving the concurrent isolation of squalene and sterols, the non-saponifiable lipid was first fractionated into crude sterol and hydrocarbon fractions. The material was strip-loaded onto silica gel plates and developed with 1% ether in hexane. This solvent carries the hydrocarbons to the solvent front, the sterols remaining near the origin. After marking the position of the band corresponding to a squalene marker, the sterols were separated by redeveloping the plate in chloroform. The bands eluted from this system were designated 'crude sterol' and 'crude squalene' fractions. If necessary, these crude fractions were purified by further TLC using chloroform and hexane as solvents to separate sterols and squalene respectively.

d. Isolation of steroids.

The chloroform extractable non-saponifiable lipid material was strip-loaded onto silica gel plates and developed in chloroform. The areas of silica gel corresponding to a marker mixture were scraped off and the material eluted rechromatographed on silica gel plates with cyclohexane/acetone/chloroform 75:25:20 v:v:v as developing solvent (Sallam et al 1969).

e. Isolation of isoprenol alcohols.

Prenol pyrophosphates are stable to alkaline hydrolysis (Goodman & Popjak 1960). These compounds were therefore contained in the aqueous residues remaining after tissue saponification and extraction of the non-saponifiable lipids. Hydrolysis of the compounds was accomplished by addition of concentrated hydrochloric acid to these residues to give a pH less than 2, followed by incubation at 60° for three hours. After this time, 60% potassium hydroxide solution was added to give a pH greater than 10 and the isoprenols were extracted

with ether (4 volumes). This crude fraction was chromatographed on silica gel using 20% ethyl acetate in benzene as developing solvent (McSweeney 1965). Areas between isoprenol alcohol markers were removed and the material eluted rechromatographed on silica gel plates using ethyl acetate/benzene/hexane 12:25:63 v:v:v as developing solvent (Chayet et al 1973).

iii. General methods.

a. Preparation of sterol acetates.

The sterols to be esterified were dissolved in pyridine (0.5 - 2.0cm^3) and an equal volume of acetic anhydride added. The mixtures were left overnight and then water (25cm^3) was added and the mixture left for ten minutes to ensure the hydrolysis of excess acetic anhydride. The acetates were extracted into petrol and the petrol solution washed with dilute hydrochloric acid, 10% sodium bicarbonate solution, then with water to neutrality. The extract was then dried over anhydrous sodium sulphate and the sterol acetates recovered by rotary evaporation.

b. Purification of sterols via the digitonides.

Sterols were purified via the formation of the digitonides, as detailed by Rees et al (1968a). The sterols were dissolved in a minimum quantity of boiling aqueous 90% (v/v) ethanol and to this was added a boiling solution containing a 3.5-fold excess of digitonin dissolved in the minimum quantity of aqueous 95% (v/v) ethanol. Boiling was continued until a slight turbidity was seen and precipitation was completed by standing at 0-4^{o} overnight. In calculating the amount of digitonin to be used, the material was regarded as cholesterol. The precipitated digitonides were collected by centrifugation. In the

cases of 4α-methyl and 4,4'-dimethyl sterols, the precipitation was repeated. The collected precipitates were washed twice with ether and dried.

Sterols were regenerated by dissolving the digitonides in hot pyridine. The digitonin was then precipitated by the addition of ether. The precipitate was collected by centrifugation and the process repeated twice. The combined ether extracts were washed with dilute hydrochloric acid and water (x3). The extract was then dried over anhydrous sodium sulphate and the sterols recovered by evaporation of the solvent.

c. Purification of squalene via the thiourea adduct.

Squalene was routinely purified via the formation of the thiourea adduct. Squalene (100mg) was dissolved in butan-1-ol ($2cm^3$) and a saturated solution of thiourea in methanol ($8cm^3$) added (Goad & Goodwin 1966). The squalene-thiourea adduct was allowed to crystallise overnight at $0-4^{\circ}$ and collected by centrifugation. Squalene was recovered from the adduct by the addition of water and extracting the squalene with ether.

d. Production of emulsions.

Emulsions of sterols and squalene were prepared in the manner of Hsu et al (1970). Approximately 5mg of the sterol or squalene was dissolved in a small quantity of chloroform. To this was added $5cm^3$ of a chloroform solution containing Tween 80 (100mg). The solutions were thoroughly mixed and the chloroform removed by evaporation under a jet of nitrogen, leaving a solution of the compound in Tween 80. An aqueous emulsion was constituted by the addition of a small volume of sterile distilled water, followed by vigorous shaking.

e. Infra-red absorption spectroscopy.

Samples of the dry compound to be examined were ground with

dry potassium bromide (1mg/200mg KBr) and compressed into discs under 8 tons p.s.i. of pressure. Liquids were examined as thin films on sodium chloride discs. Infra-red spectra of the discs were recorded on either a Pye Unicam SP200 or a Perkin-Elmer Type 357 spectrophotometer. The spectra were calibrated against a polystyrene film.

f. Melting points.

Melting points were determined with a Reichert hot stage microscope.

g. Mass spectra.

Mass spectra were determined on a AEI MS12 instrument at the Department of Biochemistry, University of Liverpool, under the kind auspices of Dr.L.J.Goad.

h. Liquid scintillation counting.

Samples were assayed for radioactivity by liquid scintillation counting using a Phillips P100 Liquid Scintillation Analyser. Counting efficiency for each sample was determined by external standardisation and reference to quench correction curves. All observed count rates, counts per minute (cpm), were corrected to disintegrations per minute (dpm).

Samples to be assayed for radioactivity were dissolved in a known volume of solvent, almost invariably cyclohexane, and aliquots transferred to counting vials containing the appropriate scintillant. Background count rate due to cosmic rays, temperature variations, ^{40}K in the glass of the counting vials and internal electrical signals was measured for each vial before the addition of samples and was subtracted from the count rate recorded after the addition of the sample.

For organic solutions, the scintillant mixture used was 0.5% butyl-PBD in toluene. For aqueous samples, Unisolve (Koch-Light

Chemicals) was used, with a sample volume made up to 0.5cm^3 with distilled water. The volume of scintillant used in both cases was 10cm^3.

Specific radioactivities were calculated by dividing the dpm obtained by the weight of the compound counted.

i. Monitoring GLC effluents for radioactivity.

The GLC systems previously described were used with the effluent splitter fitted. The effluent was trapped at one minute intervals in glass capillary tubes. The contents of each tube were washed out with a known volume of cyclohexane into counting vials and assayed for radioactivity by liquid scintillation counting. In figures in this thesis, the radioactivity of the effluent is shown as a histogram superimposed on the GLC trace.

j. Autoradiography of crude steroids.

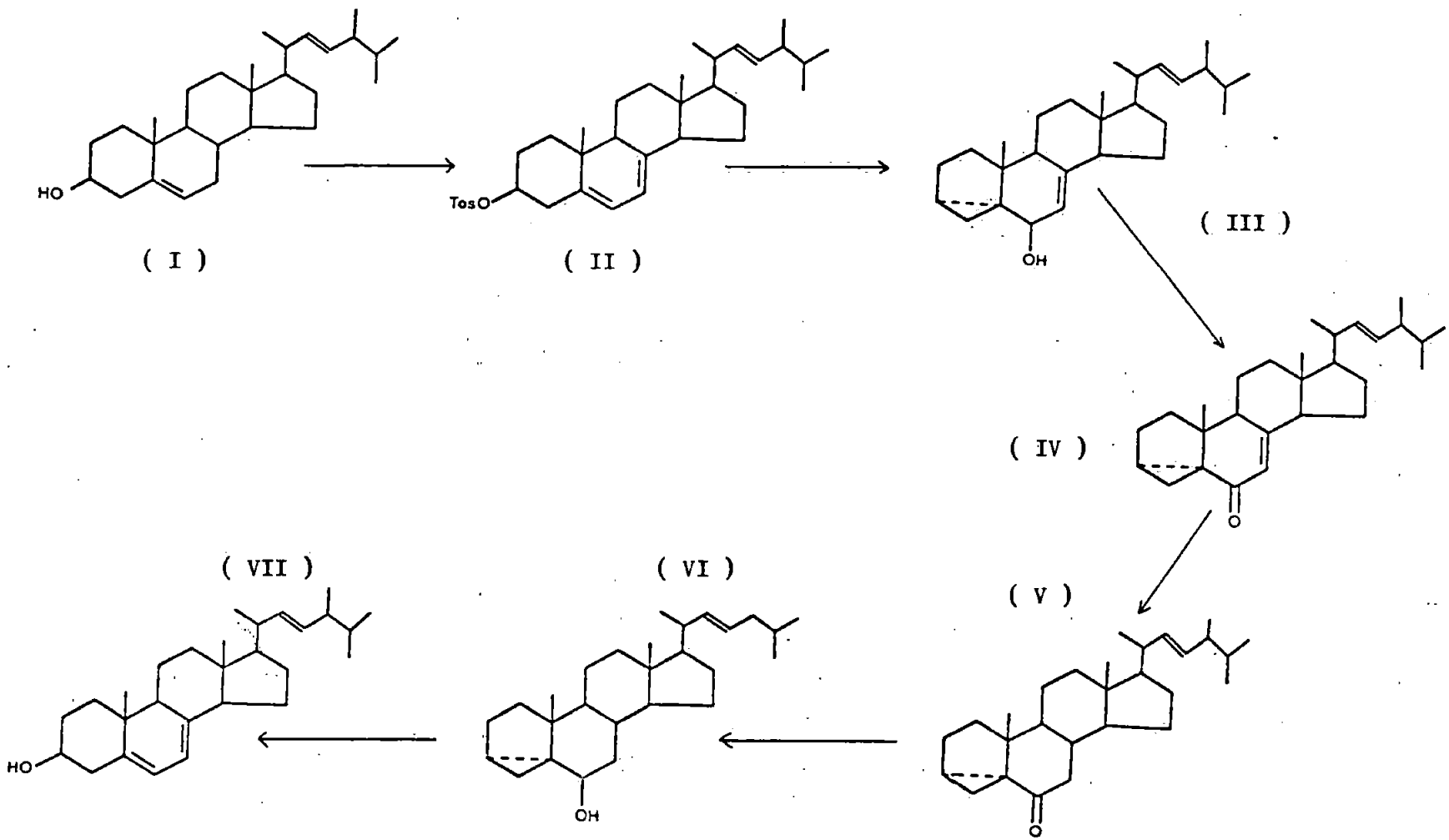
After the removal of an aliquot for liquid scintillation counting the steroid fraction was strip-loaded onto a silica gel TLC plate and the plate developed in the appropriate solvent system as described previously. After development, the plates were placed in contact with X-ray film (Agfa-Gevaert FP1: contrast 2) and left in the dark for six weeks. After this exposure, the films were developed in Phenisol (4 minutes: 20^o), fixed in Amfix (4 minutes: 20^o), washed and dried.

iv. Synthetic methods.

a. Synthesis of brassicasterol.

Brassicasterol for use as a GLC standard was synthesised by the method of Thompson et al (1965). The reaction sequence is outlined in Scheme 2.1.

Scheme 2.1. Reaction scheme for the synthesis of brassicasterol.



(I) (II) (III) (IV) (V) (VI) (VII)

i-Ergosterol (3,5-cycloergosta-7,22-dien-6β-ol).

To 2.5g of dried, recrystallised ergosterol (I) was added a solution of p-toluenesulphonyl chloride (3g) in pyridine ($15cm^3$). The mixture was stirred in the dark overnight. After this time the reaction mixture was poured into crushed ice and water ($250cm^3$). The crystalline ergosterol tosylate (II) was filtered and washed with ice cold 2% potassium carbonate solution and ice cold water. The crude wet tosylate was dissolved in acetone ($250cm^3$) and added, over a five minute period, to a boiling solution of potassium carbonate (1.75g) in acetone ($525cm^3$) and water ($220cm^3$). The mixture was refluxed for 15 minutes and then distilled at atmospheric pressure until approximately $750cm^3$ of acetone had been collected. The mixture was diluted with water ($750cm^3$) and kept overnight at 0^o. The semi-crystalline solid was collected by filtration, dissolved in ether, dried over anhydrous sodium sulphate and the ethereal solution evaporated in vacuo. The residue was dissolved in hexane ($50cm^3$) and adsorbed onto a column of hexane-washed alumina (100g). Fractions were eluted as follows:

Hexane/benzene	3:1	$200cm^3$.
Hexane/benzene	1:1	$200cm^3$.
Hexane/benzene	1:3	$800cm^3$.
Benzene		$200cm^3$.

The fraction eluted with hexane/benzene 1:3 was taken to dryness in vacuo. Recrystallisation from acetone/water gave i-ergosterol (1.6g) as white needles, mpt. 134-135^o (lit. value 132-133^o: Thompson et al 1965).

3,5-Cycloergosta-7,22-dien-6-one.

To $20cm^3$ of dried, redistilled pyridine, chromium trioxide (1.6g) was added, with magnetic stirring, over a five minute period.

A solution of i-ergosterol (III: 1.6g) in pyridine ($20cm^3$) was then added to this mixture. The mixture was left overnight at room temperature and then diluted with ether (one volume) and filtered through a bed of alumina. The crude 3,5-cycloergosta-7,22-dien-6-one was recovered by evaporation of the filtrate. This was recrystallised from acetone/water to give 0.17g of white plates of 3,5-cycloergosta-7,22-dien-6-one (IV), mpt. 166-167° (lit. value 168-169°: Thompson et al 1965).

3,5-Cycloergosta-22-en-6-one.

A solution of 3,5-cycloergosta-7,22-dien-6-one (90.3mg) in dry ether ($10cm^3$) was added rapidly, and with vigorous stirring, to a solution of lithium (40mg) in dry liquid ammonia ($20cm^3$). Stirring was continued for three minutes when any excess lithium (indicated by a deep blue colour) was destroyed by the addition of ammonium chloride. The reaction mixture was allowed to come to room temperature, diluted with an equal volume of water and extracted with ether (x3). The ethereal solution was dried over anhydrous sodium sulphate and the solvent removed by evaporation in vacuo. The residue obtained was dissolved in the minimum amount of hexane and adsorbed onto a column of hexane-washed alumina (10g). The column was then eluted with hexane ($150cm^3$) and the residue obtained after removal of this solvent in vacuo, was recrystallised from acetone/water to give 3,5-cycloergosta-22-en-6-one (V:47mg) as white plates, mpt. 111-112° (lit. value 108-110°: Thompson et al 1965).

3,5-Cycloergosta-22-en-6α-ol.

3,5-Cycloergosta-22-6-one (47mg) was dissolved in dry ether ($10cm^3$), solid lithium aluminium hydride (40mg) was added and the mixture refluxed for two hours. Excess lithium aluminium hydride was

destroyed by the addition of ethyl acetate. The mixture was diluted with an equal volume of water and extracted with ether (x3). The ether extracts were bulked, washed with water and dried over anhydrous sodium sulphate. Filtration and evaporation of the ethereal solution in vacuo gave a crystalline residue. Recrystallisation of this from methanol gave 3,5-cycloergosta-22-en-6α-ol (20mg: VI) as white plates, mpt. 95-96° (lit. value 92-93°: Thompson et al 1965).

Ergosta-5,22-dien-3β-ol (brassicasterol).

A mixture of 3,5-cycloergosta-22-6α-ol (20mg), fused zinc acetate (0.5g) and acetic acid ($20cm^3$) was refluxed, with stirring, for two hours. The solution was cooled, diluted with an equal volume of water and filtered to give the crude acetate. The crude acetate was saponified by refluxing with 2% ethanolic potassium hydroxide ($10cm^3$). The saponification mixture was diluted with an equal volume of water and extracted with ether (x3). The combined ether extracts were bulked and washed with water until neutral to phenolphthalein. The ethereal solution was dried over anhydrous sodium sulphate and the solvent removed by evaporation in vacuo. A crystalline residue of crude brassicasterol (VIII) was obtained.

Purification and identification of brassicasterol.

A preliminary purification was achieved via the digitonide. After regeneration, the free sterol was chromatographed on silica gel plates with chloroform as developing solvent. Recrystallisation of the material eluted from these plates from acetone/water produced 4.7mg of white needles, mpt. 147-148° (lit. value 148°: Thompson et al 1965). The acetate gave a melting point of 157° (lit. value 149-151°: Thompson et al 1965).

The mass spectrum obtained for brassicasterol acetate (Figure

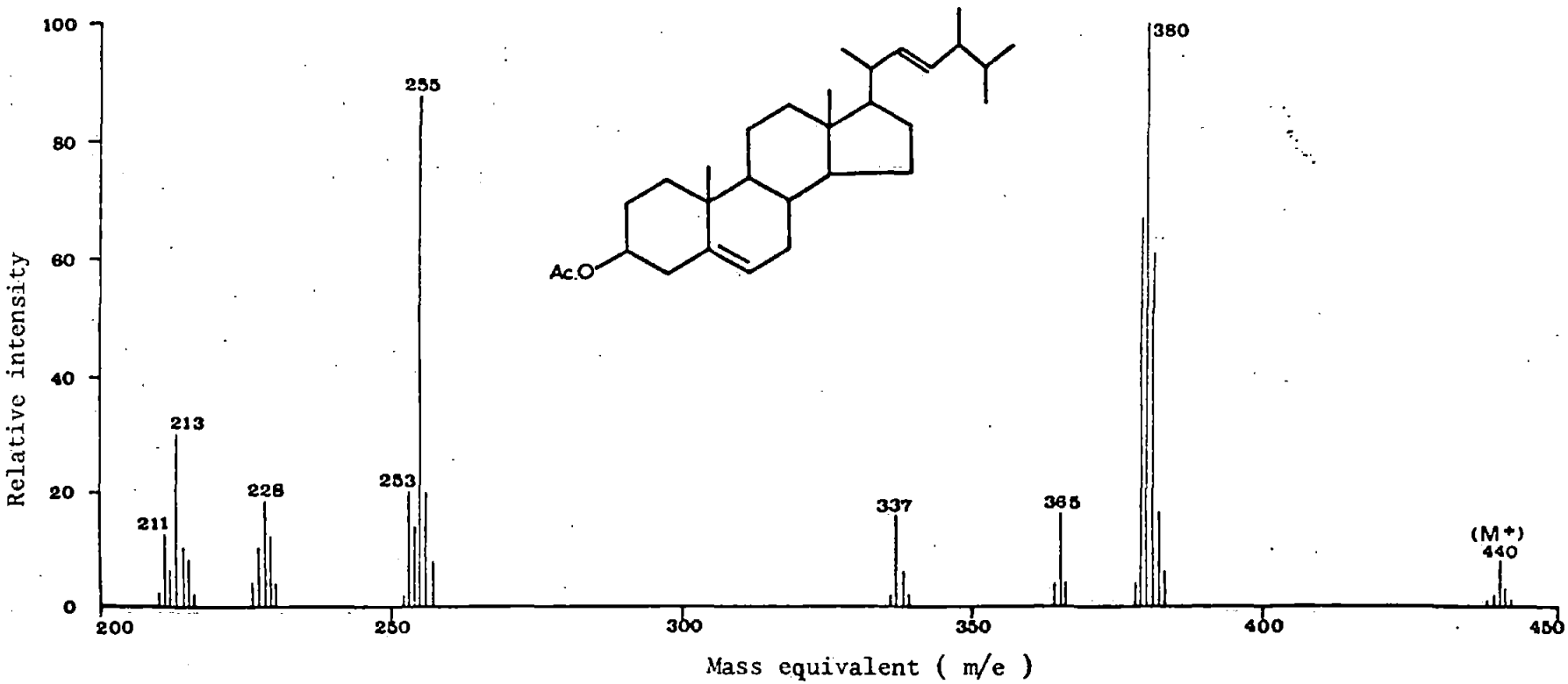


Figure 2.1. Mass spectrum of brassicasterol acetate.

Table 2.1. Mass spectrum of Brassicasterol Acetate.

Peak at m/e	Relative Abundance %	Fragmentation
440	8	M^+
380	100	M^+ - (Acetate)
365	16	M^+ - (Acetate + Methyl)
337	16	M^+ - (Isopropyl)
255	88	M^+ - (Acetate + Sidechain)
253	20	M^+ - (Acetate + Sidechain + 2H)
228	18	M^+ - (Acetate + Sidechain + 27)
213	30	M^+ - (Ring D fission + Acetate + Sidechain)
211	12	M^+ - (Ring D fission + Acetate + Sidechain + 2H)

M^+ = Molecular ion peak for brassicasterol acetate.

2.1 & Table 2.1) is consistent with the structure and agrees with previously published data (Teshima & Kanazawa 1972a).

b. Synthesis of squalene-2,3-oxide.

N-Bromosuccinimide (0.125g) was added in small portions, over a period of ten minutes, to a solution of squalene (0.25g) in tetrahydrofuran ($4.2cm^3$) and water ($1.2cm^3$: Willett et al 1967). The addition of reactants was made under nitrogen, which was maintained while the solution was stirred at 0^o for a further sixty minutes. After evaporation of the major portion of the solvent under reduced pressure, the residual aqueous mixture was poured into ice water and extracted with hexane (x3). The combined hexane extracts were dried over anhydrous sodium sulphate to give the crude bromohydrin (0.27g). The crude product was dissolved in the minimum volume of petrol and applied to a column of silica gel (10g: 15% water) made up in the same solvent. Unchanged squalene and non-hydroxylic bromination products were eluted with petrol ($75cm^3$), crude 2-hydroxy-3-bromosqualene was eluted with 50% benzene in petrol ($100cm^3$). This product was further purified by TLC on silica gel with 15% ethyl acetate in benzene as developing solvent. The 2-hydroxy-3-bromosqualene (0.11g) was transformed into racemic squalene-2,3-oxide by hydrolysis with 2% ethanolic potassium hydroxide ($5cm^3$) at 45^o for 30 minutes (Rees et al 1968b). Squalene-2,3-oxide was extracted with ether (x3), the ether extracts were bulked and washed with water until neutral to phenolphthalein. The ethereal solution was dried over anhydrous sodium sulphate and the solvent removed by rotary evaporation. The squalene-2,3-oxide so obtained was purified by TLC on silica gel plates with 5% ethyl acetate in benzene as developing solvent. An infra-red spectrum of the material eluted from these plates was in

agreement with the assignments of Willett et al (1967). Absorptions due to epoxide were prominent at 1250cm^{-1} and to non-conjugated alkene at 1670cm^{-1}. Other peaks were prominent at 1380, 1450, 2850, 2925 and 2960cm^{-1}.

CHAPTER III.

DEVELOPMENT OF AN EXPERIMENTAL SYSTEM.

I. Introduction.

i. General considerations.

Before meaningful metabolic studies on the various aspects of sterol biosynthesis and metabolism in a crustacean could be carried out, a suitable system had to be derived. It was necessary to be able to culture a crustacean under sterile conditions so that any extraneous sterol metabolism was eliminated. This involved the selection of:

a. An experimental organism.

b. A food organism.

c. A compatible, chemically defined medium.

ii. The selection of an experimental organism.

Certain criteria had to be satisfied for a crustacean to be considered suitable for a study of the metabolism and biosynthesis of sterols.

a. The organism had to be suitable for culture under laboratory conditions and small enough to allow statistically valid numbers to be used for each experiment.

b. The eggs had to be easily obtainable and sterilizable.

c. The time taken for maturity to be attained had to be comparatively short.

d. The organism had to have a comparatively simple sterol profile.

These criteria precluded the use of any member of the Decapoda. Artemia salina, the brine shrimp, has been used in many investigations

(see, for example, Clegg 1964; Croghan 1958; Ewing & Clegg 1972; Hsu et al 1970; Kayama et al 1963; Littlepage & McGuiley 1965). Teshima (1971a) reported that A.salina reared on the marine yeast, Cryptococcus albidus, contained only cholesterol. Thus A.salina had a simple sterol profile and, on the basis of the evidence presented in the papers cited above, appeared to satisfy all the conditions imposed.

iii. The life cycle of Artemia salina.

The eggs of A.salina are, in fact, dried encysted gastrulae (Finamore & Clegg 1969). The fertilised egg undergoes cleavage to form the blastula; gastrulation follows. Environmental conditions affect the fate of the gastrulae. Favourable conditions lead to continued development whilst adverse conditions lead to dehydration and dormancy. Rehydration of the dormant gastrulae induces further development. A certain amount of pre-emergence development takes place before the embryo is liberated from the egg membranes as a free-swimming pre-nauplius larva. In the life cycle of A.salina, this stage is termed the metanauplius III. Morphogenesis and differentiation leads, through a range of different intermoult forms, to the adults. The adult males have long claspers and the females have a slender head and conspicuous egg pouches below the last pair of limbs. There is no common system of nomenclature for the various life stages of A.salina and throughout this work, that of Provasoli & D'Agostino (1969) is used.

iv. Selection of a suitable food organism.

Ideally one would have liked to have reared A.salina on a completely defined medium. Little progress has been made in the

development of artificial media for small filter feeders such as Artemia salina and there is only one report in the literature of such a medium (Provasoli & D'Agostino 1969). These workers derived a complex defined medium capable of supporting growth from nauplii to adults. This medium comprised of a liquid phase containing mineral salts, six amino acids, five nucleic acid components, eight vitamins, two sugars, a pH buffer and a fine particulate phase consisting of precipitated albumin, gelled rice starch and cholesterol. Numerous experiments with this system were unsuccessful, A.salina failing to reach the adult stage and generally dying at the metanauplius stage. A recent paper by Jones et al (1974) indicates that microencapsulation of food represents a feasible approach towards the development of artificial diets for filter feeders. These workers have shown that encapsulated U-^{14}C-glucose and U-^{14}C-starch were assimilated by A.salina and incorporated into various amino acids. A complete diet has not yet been formulated.

As an alternative to an artificial medium, it was decided to rear A.salina on an axenic culture of some microorganism. There are reports of the maintenance of cultures of A.salina on yeast (Bowen 1962; Teramoto & Kinoshita 1961) and various algae (eg. Mason 1963; Reeve 1963). As the natural food of A.salina is algae, it was obvious that such an organism could be used as a food source when grown on a chemically defined medium. The organism chosen had to satisfy certain criteria:

a. It should support the growth of A.salina from juvenile to adult.

b. It should not be fastidious in its growth requirements.

c. It should be easily maintained in axenic culture.

d. It should not metabolise sterol emulsions.

There have been numerous reports in the literature of the growth of A.salina on various species of the marine chlorophyte Dunaliella, namely D.tertiolecta (Mason 1963), D.salina (Gibor 1956) and D.viridis (Provasoli et al 1959). Trial experiments using Dunaliella primolecta grown on Erdshreiber medium were successful. Cultures did not metabolise sterol emulsions and egg-bearing females were noted in approximately 14 days. It was decided that the experimental system to be finally adopted would involve the inoculation of growing cultures of D.primolecta with A.salina nauplii.

Because of the nature of the experimental system selected, it was necessary to select a defined culture medium for the algae which was compatible with the growth of A.salina. The requirements of such a medium have been listed by Taub & Dollar (1964):

a. It should support the growth of the algae.
b. It should permit unlimited A.salina survival (to starvation) both as the unused and spent medium.
c. It should be chemically defined and essentially inorganic.
d. It should be reproducible.
e. It should be heat stable and remain in solution on autoclaving.
f. It should be relatively simple and convenient to prepare.

Erdshreiber medium is typical of those generally used for the growth of marine algae in that it contains both natural sea water and a soil extract. It was unsatisfactory for the present work on two counts: a. natural sea water has to be filter-sterilised or pasteurised as precipitates are formed on autoclaving and b. the soil extract used is neither chemically defined nor reproducible, its composition varying with the flora and fauna of the soil used. The medium finally selected

for use, the composition of which is shown in Table 3.1, was a modification of that of Kanazawa et al (1971a).

Dunaliella primolecta was obtained from the Cambridge Culture Centre for Algae and Protozoa, culture no. 11/34. The culture was received as an agar slope. Material from this slope was transferred to 100cm^3 of sterile growth medium contained in a 250cm^3 conical flask to form a stock culture. Stock cultures were maintained on an orbital shaker at a temperature of 15-24^o with constant illumination from a bank of four 3' 40W Ascot fluorescent lights. They were subcultured approximately every four weeks.

All cultures were screened regularly for contaminants by the following methods:

a. Microscopic examination.

b. Plating techniques.

i. Bacterial contaminants were detected by streaking samples onto plates of yeast extract agar of the following composition:

Yeast extract	0.5
K_2HPO_4	0.1
$MgSO_4\ 7H_2O$	0.05

(g/100cm^3 distilled water).

The pH was adjusted to 7.5 and agar (1.5g) added. The mixture was autoclaved for 15 minutes at 15psi. Inoculated plates were incubated at 30^o. If no growth was noted after three days, the culture was assumed to be bacteria-free.

ii. Fungal contaminants were detected using agar plates

Table 3.1. Growth medium for Dunaliella primolecta.

NaCl	30g[1]
$MgCl_2\ 6H_2O$	5.30g
$Na_2SO_4\ 10H_2O$	4.45g
$CaCl_2\ 2H_2O$	0.073g
KCl	0.33g
$NaNO_3$	0.30g
K_2HPO_4	0.01g
$NaHCO_3$	0.20g
DiSodium EDTA	12.5mg
Vitamin B_{12}	0.015µg
$FeSO_4\ 7H_2O$	3mg
Citric acid	3mg
Boric acid	1.5mg
$MnCl_2\ 4H_2O$	1mg
$ZnSO_4\ 7H_2O$	22µg
$CuSO_4\ 5H_2O$	79µg
$(NH_4)_6Mo_7O_{24}\ 4H_2O$	15µg
NH_4VO_3	23µg
$CoCl_2\ 6H_2O$	15µg

[1] Weights per litre of distilled water.

To those cultures which were aerated, a few drops of Antifoam A emulsion were added prior to autoclaving.

containing malt extract (2g/100cm^3 distilled water). The pH was adjusted to 5.5 and agar (1.5g) added. The mixture was autoclaved for 15 minutes at 15psi. The inoculated plates were incubated at 25°. If no growth was detected after 10 days, the culture was assumed to be fungi-free.

II. Final experimental system.

i. Preparation of culture medium.

The following system was found to be satisfactory. The culture vessel used was a 5 litre flat-bottomed flask containing 3 litres of growth medium. The flask was fitted with a gassing tube and a tube of 2.9cm id. through which additions could be made. The whole system was sterilised by autoclaving for 30 minutes at 15psi. The system was inoculated with 250cm^3 of fully grown starter culture of D.primolecta and growth continued for about 12 days, with constant illumination and aeration at a temperature of 15-24°. After this time, the cultures were inoculated with sterile A.salina nauplii.

At the same time as setting up the main culture vessel, a further 1.5 litres of sterile growth medium, contained in a 2 litre conical flask, were inoculated with D.primolecta. These were incubated in the same way as the stock cultures and acted as a food reserve which could be added to the main culture vessel if the A.salina grazed the algae too rapidly.

ii. Preparation of sterile nauplii.

Brine shrimp eggs are heavily contaminated with debris and various microorganisms, some of which are closely associated with the shell and cannot be removed by washing with sterile saline. Provasoli

& D'Agostino (1969) used merthiolate (ethylmercurithiosalicylate) to achieve sterility and the method adopted was a modification of their procedure.

A.salina eggs (5g: of San Francisco origin and purchased from K.Barraclough, 568 Great Horton Road, Bradford 7.) were shaken for five minutes with sterile growth medium to which a few drops of Tween 80 had been added as wetting agent. After leaving to settle, broken and non-viable eggs could be removed by careful decanting. This treatment was followed by three washings and separations with sterile medium. The eggs were then sterilised by shaking for 10 minutes in a solution of merthiolate ($1mg/cm^3$) in sterile medium. This was repeated and followed by a further three washings and separations with sterile medium to remove all traces of the merthiolate. The eggs were then transferred to $100cm^3$ of sterile medium in a $250cm^3$ conical flask containing the following antibiotics (concentrations/cm^3): penicillin G, 50iu; streptomycin sulphate, 0.05mg and nystatin, 10µg. These concentrations were well within the tolerance limits of A.salina (D'Agostino & Provasoli 1968). The medium containing the eggs was incubated on an orbital shaker at a temperature of 15-24°. Rupture of the shell was seen after 12 hours and the embryo, contained in an enveloping membrane, was released. Rupture of this membrane to liberate the free-swimming nauplii took approximately a further 24 hours. There was a variation in the hatching rates of the eggs and so the egg inoculum used was not synchronous. The nauplii were therefore added to the culture vessel 48 hours from the commencement of the incubation of the eggs.

iii. Growth of Artemia salina on Dunaliella primolecta.

When the food organism in the culture vessel had reached a

suitable density, sterile nauplii were added. The size of the nauplii inoculum varied and consequently the rate at which the algae were grazed also varied. Usually it was necessary to add additional food organisms. Using this system, adult A.salina (indicated by the presence of females bearing egg pouches) could be obtained within approximately 14 days after the addition of the nauplii to the culture vessel.

The adult animals were collected by passing the culture medium through a nylon net (0.5mm mesh). The filtered organisms could then be washed free of algae with sterile medium.

In most experiments it was necessary to void the contents of the gut of A.salina and this was achieved in the following manner. A.salina were left in the nylon net, which was now suspended in a sterile container containing sterile hatching medium. A suspension of rice starch particles was added ($10cm^3/100cm^3$ medium). The cultures were left in the dark for 24 hours to prevent algal growth. After this time, the alimentary tracts were visibly cleared.

The rice starch particles were prepared in the following manner (Provasoli & D'Agostino 1969): insoluble rice starch (2g) with some glass beads was sterilised in a hot air oven at 180° for two hours. After cooling, sterile medium was added ($100cm^3$) and the rice starch suspended by vigorous shaking.

III. Sterol composition of the experimental system.

i. Sterol composition of Artemia salina eggs.

Artemia salina is a branchipod, ubiquitous to salt ponds and salt lakes. With few exceptions, all salterns and salt lakes which do not have predatory invertebrate and vertebrate life, support large populations of A.salina. Because of the diverse physiochemical

characteristics of the isolated niches of the ecosystems which this organism inhabits, one would expect physiological and morphological divergencies between geographically isolated populations. Early biologists noticed certain morphological differences between different strains of A.salina and great efforts were made to find qualitatively distinct morphological differences between the strains which would permit the definition of species. Criteria suggested were, for example, length and number of abdominal segments, number of setae on the telsic furca and character of the peripheral ridge of the males' claspers (Kellogg 1906; Kuenen 1939). It has long been assumed that slight qualitative differences between the same and different strains may have been induced by variations in the physiochemical characteristics of the habitat, such as salinity, temperature, pH and nutrition (Bond 1932; Heath 1924; Kuenen 1939). Some authors have found it impossible to generalise about the influence of external factors on the body form of different strains of A.salina (Gilchrist 1960; Weisz 1946) and no clear picture has yet emerged from such studies. Most zoology texts follow Daday's monograph where he pools all known variants into one collective genus and species, Artemia salina Leach (Daday 1910).

Despite this apparent lack of constant and distinctive morphological characters between the different strains, cytological data suggest that a degree of divergency does exist. Some populations of A.salina are bisexual whereas others consist only of females which reproduce parthenogenetically. Some populations are diploid, others have been reported to be triploid, tetraploid, pentaploid or octaploid on the basis of cytological studies (Barigozzi 1939, 1944, 1957; Bowen 1964; Goldschmidt 1952).

Evidence from morphological, cytological and physiological

studies suggest that there may be distinct species of A.salina. Recent biochemical analyses have shown there to be differences in the fatty acid and nucleic acid base compositions in the eggs of A.salina from different sources (W.Barton - personal communication). Eggs of A.salina from two sources were examined for differences in sterol composition. The eggs examined originated from San Francisco Bay, California and the Great Salt Lake, Utah.

The eggs (5g) were ground and the non-saponifiable lipid extracted by direct saponification of the ground mixture. From this the sterols were isolated, separated into 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions (Table 3.2) and characterised by GLC in the usual way.

Although there were no visible bands, areas corresponding to 4α-methyl and 4,4'-dimethyl sterol markers were removed from the plates and eluted. Subsequent GLC analysis did not reveal any components.

When examined by GLC, material eluted from the band co-chromatographing with a 4-desmethyl sterol marker was found to be separated into five components. The GLC traces are shown in Figures 3.1 and 3.2. The retention times of the components of this mixture, together with the retention times of sterol standards, are shown in Tables 3.3 and 3.4. On the basis of this evidence, the components of the 4-desmethyl sterol mixture obtained from both Utah and Californian eggs were identified as cholesterol, 24-methylcholesta-5,22-dien-3β-ol, 24-ethylcholesta-5,22-dien-3β-ol, 24-methylcholesterol and 24-ethylcholesterol. As Table 3.2 shows, the sterol composition of A.salina eggs from both sources was almost identical. Cholesterol was the major component in both cases, accounting for 99+% and 97% of the total sterols

Table 3.2. 4-Desmethyl sterol content and composition of Artemia salina eggs from two sources.

	Sources	
	Utah	California
Dry weight (g)	5.23	5.44
Non-saponifiable lipid		
Weight (mg)	20.1	27.0
%[1]	0.38	0.49
4-Desmethyl sterols		
Weight (mg)	0.6	0.8
%[1]	0.01	0.01
Composition of desmethyl sterols[2]		
Cholesterol	97	99+
24-Methylcholesta-5,22-dien-3β-ol	trace	trace
24-Methylcholesterol	trace	trace
24-Ethylcholesta-5,22-dien-3β-ol	trace	trace
24-Ethylcholesterol	3	trace

[1]Expressed as a percentage of the dry weight.

[2]Expressed as a percentage of the total sterols.

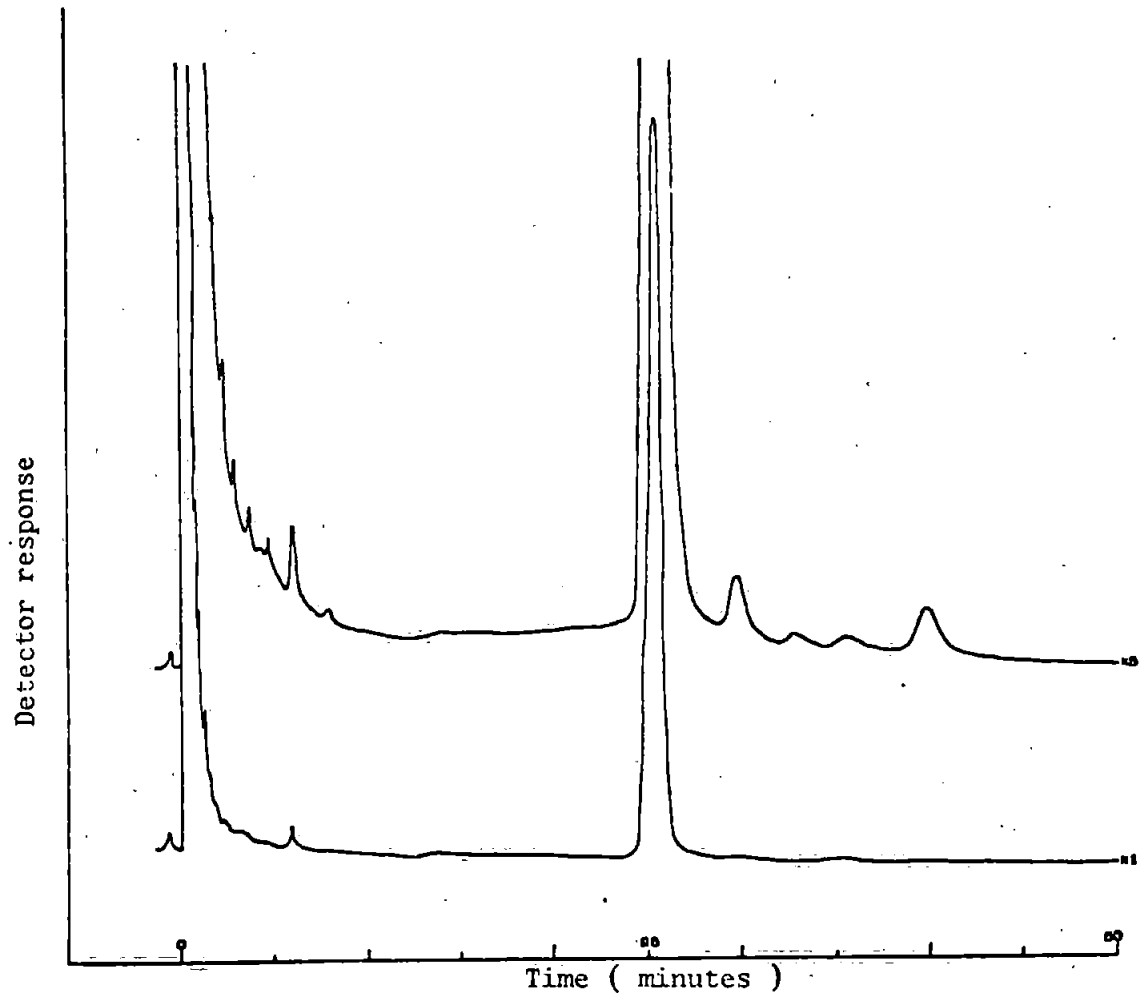


Figure 3.1. GLC analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (California) eggs.

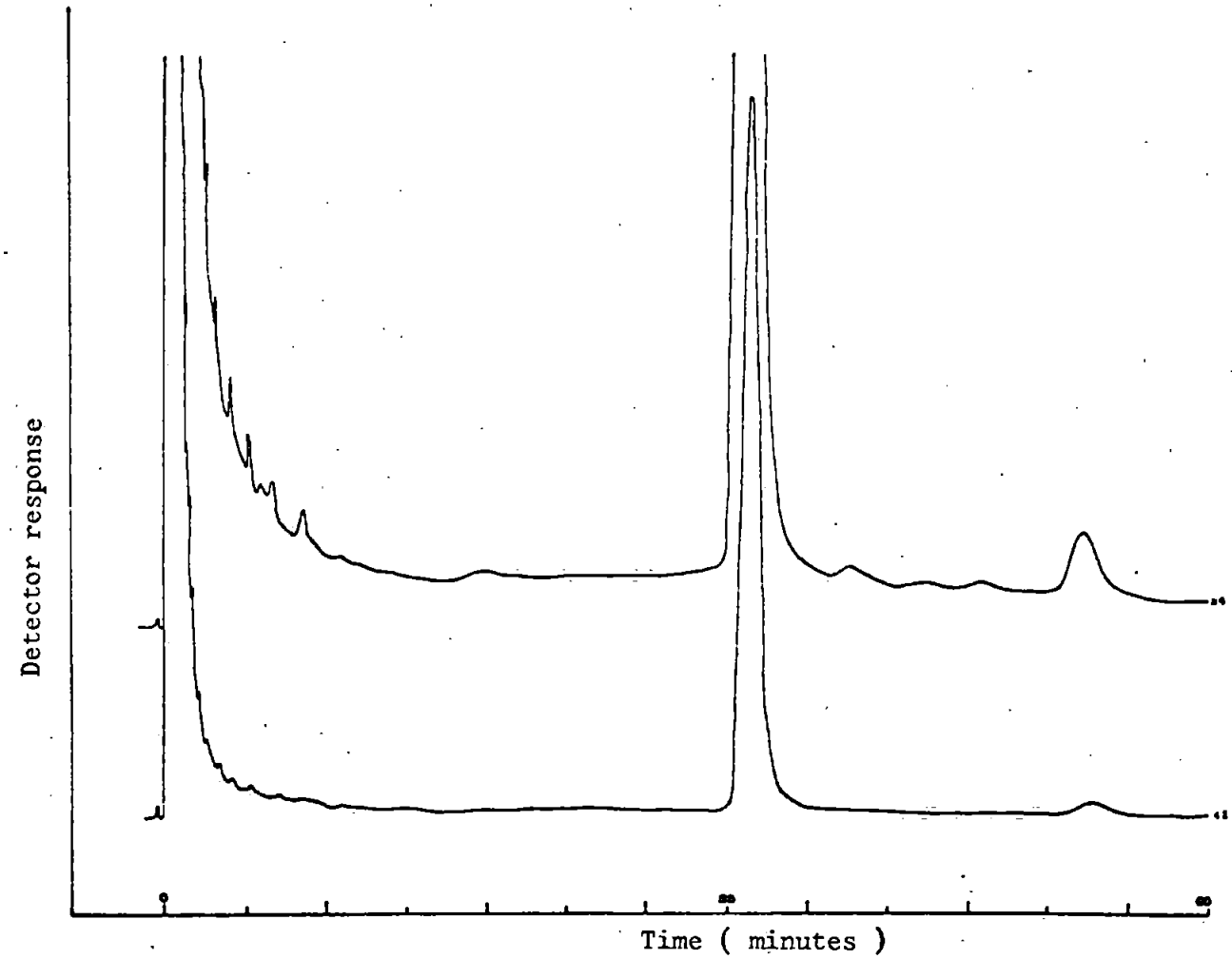


Figure 3.2. GLC analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (Utah) eggs.

Table 3.3. 4-Desmethyl sterol content of Artemia salina eggs.

Relative retention times of standard sterols.

	Relative retention times[1]			
	Column			
	1% SE-30[2]		3% OV-17[3]	
	Sterol	Acetate	Sterol	Acetate
Cholesterol	1.86	2.68	2.33	3.03
24-Methylcholesta-5,22-dien-3β-ol	2.10	2.92	2.63	3.44
24-Methylcholesterol	2.46	3.44	2.98	3.94
24-Ethylcholesta-5,22-dien-3β-ol	2.68	3.74	3.19	4.24
24-Ethylcholesterol	3.09	4.30	3.61	4.80

[1]Relative to 5α-cholestane (1.00).

[2]Oven temperature 242.5°; Detector temperature 255°; gas flow (N_2) 30cm^3/min.

[3]Oven temperature 280°; Detector temperature 290°; gas flow 25cm^3/min.

Table 3.4. Desmethyl sterol composition of Artemia salina eggs as determined by GLC analysis.

	Relative retention times				
	Column				
	1% SE-30[1]		3% OV-17[1]		
Source	Sterol	Acetate	Sterol	Acetate	Identification
Utah	1.86	2.68	2.33	3.03	Cholesterol
	2.12	2.92	2.73	3.42	24-Methylcholesta-5,22-dien-3β-ol
	2.46	3.42	3.00	3.92	24-Methylcholesterol
	2.69	3.73	3.26	4.24	24-Ethylcholesta-5,22-dien-3β-ol
	3.09	4.30	3.66	4.81	24-Ethylcholesterol
California	1.86	2.67	2.31	3.02	Cholesterol
	2.12	2.92	2.69	3.44	24-Methylcholesta-5,22-dien-3β-ol
	2.45	3.44	2.98	3.94	24-Methylcholesterol
	2.70	3.76	3.18	4.22	24-Ethylcholesta-5,22-dien-3β-ol
	3.07	4.30	3.63	4.81	24-Ethylcholesterol

[1]Column conditions as for Table 3.3.

in Californian and Utah eggs respectively. With the exception of 24-ethylcholesterol accounting for 3% of the sterols in the Utah eggs, the other sterols were present in trace amounts only. The Californian eggs gave a marginally simpler sterol spectrum and work was continued with the eggs from this source.

ii. Sterol composition of Dunaliella primolecta.

Dunaliella primolecta was grown in 3 litre cultures under the conditions previously described. The cells were harvested by centrifugation and the non-saponifiable lipid extracted by direct saponification of the pellet. From this, the sterols were isolated and separated into 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions (Table 3.5).

Table 3.5. Sterol content of Dunaliella primolecta.

Dry Weight	Non-saponifiable lipid	Sterols
0.654g	25.9mg (3.96%[1])	2.3mg (0.35%[1])

[1]Expressed as a percentage of the dry weight.

Although there were no visible bands, areas corresponding to 4α-methyl and 4,4'-dimethyl sterol markers were removed from the plates and eluted. Subsequent GLC analysis did not reveal any components.

When examined by GLC, material eluted from the band co-chromatographing with a 4-desmethyl sterol marker was found to be separated into six components. The GLC trace obtained is shown in Figure 3.3. The retention times of the components of this mixture, together with the retention times of sterol standards, are shown in Tables 3.6 and 3.7.

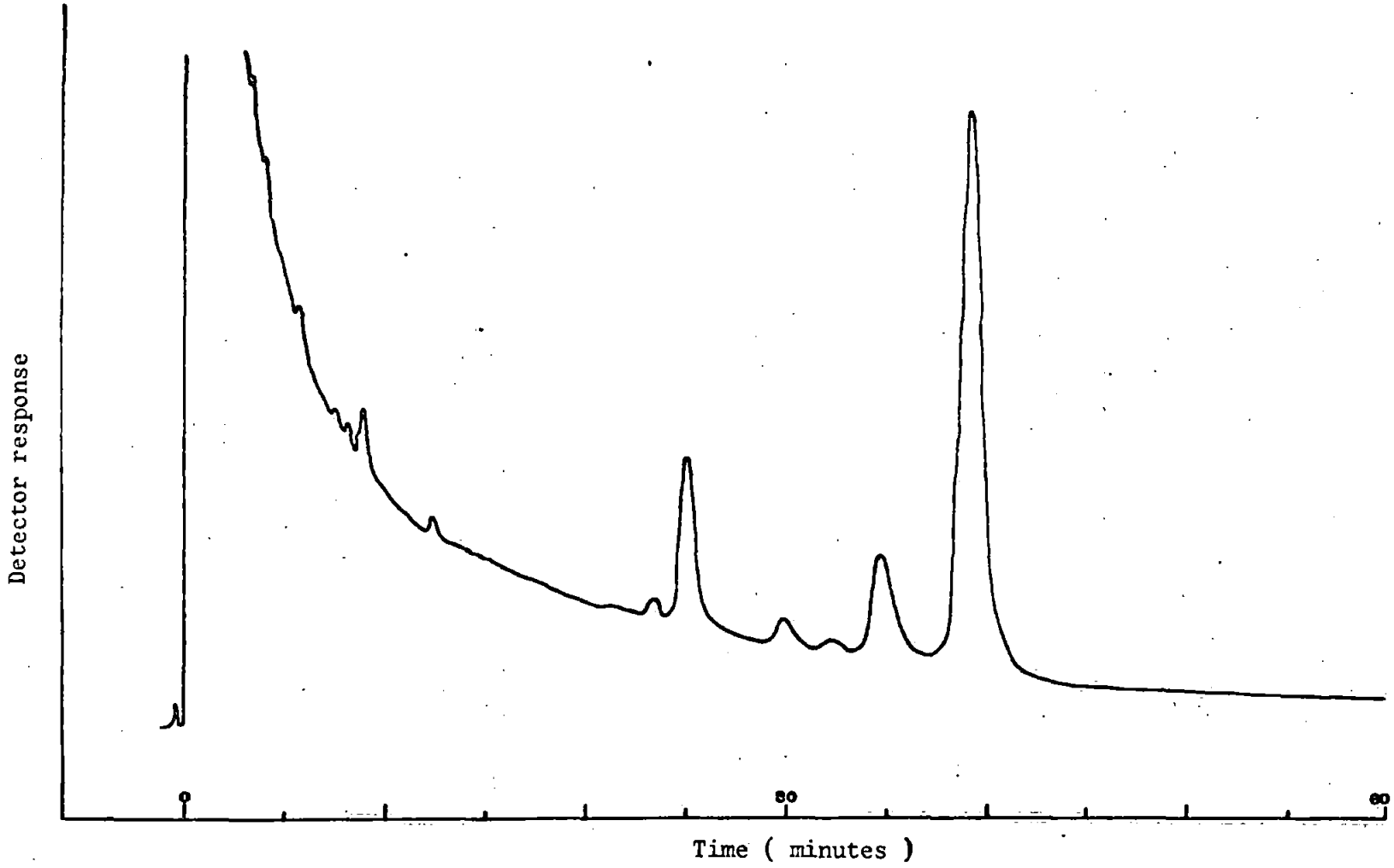


Figure 3.3. GLC analysis (3% OV-17) of 4-desmethyl sterols of Dunaliella primolecta.

Table 3.6. 4-Desmethyl sterol composition of Dunaliella primolecta.

Relative retention times of standard sterols.

	Relative retention times[1]			
	Column			
	1% SE-30[2]		3% OV-17[3]	
	Sterol	Acetate	Sterol	Acetate
22-Dehydrocholesterol	1.69	2.39	2.17	2.84
Cholesterol	1.85	2.47	2.30	3.04
24-Methylcholesta-5,22-dien-3β-ol	2.10	2.92	2.76	3.46
24-Methylcholesterol	2.45	3.14	2.96	3.94
24-Ethylcholesta-5,22-dien-3β-ol	2.65	3.74	3.20	4.24
24-Ethylcholesterol	3.06	3.90	3.60	4.82

[1]Relative to 5α-cholestane (1.00)

[2]Oven temperature 242.5°; Detector temperature 255°; Gas flow (N_2) 30cm^3/min.

[3]Oven temperature 280°; Detector temperature 290°; Gas flow 25cm^3/min.

Table 3.7. 4-Desmethyl sterol composition of Dunaliella primolecta as determined by GLC analysis.

	Relative retention times				
	Column				
	1% SE-30[1]		3% OV-17[1]		
	Sterol	Acetate	Sterol	Acetate	Percentage Composition[2]
22-Dehydrocholesterol	1.69	2.38	2.17	2.84	trace
Cholesterol	1.85	2.46	2.30	3.02	13.5
24-Methylcholesta-5,22-dien-3β-ol	2.12	2.92	2.76	3.46	3.2
24-Methylcholesterol	2.44	3.14	2.97	3.92	trace
24-Ethylcholesta-5,22-dien-3β-ol	2.65	3.74	3.20	4.24	10.8
24-Ethylcholesterol	3.04	3.90	3.60	4.81	70.6

[1]Column conditions as for Table 3.6.

[2]Expressed as a percentage of the total sterol.

On the basis of this evidence, the components of the 4-desmethyl sterol mixture obtained from Dunaliella primolecta were identified as cholesterol, 24-methylcholesta-5,22-dien-3β-ol, 22-dehydrocholesterol, 24-ethylcholesta-5,22-dien-3β-ol, 24-ethylcholesterol and 24-methylcholesterol. 24-Ethylcholesterol was the major component of this mixture, accounting for 70.6% of the total sterols. Cholesterol (13.5%), 24-ethylcholesta-5,22-dien-3β-ol (10.8%) and 24-methylcholesta-5,22-dien-3β-ol (3.2%) were present in smaller amount, with trace amounts of 22-dehydrocholesterol and 24-methylcholesterol.

iii. Sterol composition of Artemia salina reared on a diet of Dunaliella primolecta.

A.salina hatched from California eggs, were reared from nauplii to adults on a diet of D.primolecta. Individuals at the metanauplius IV and adult stages were collected and the non-saponifiable lipid extracted by direct saponification. From this the sterols were isolated and separated into 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions (Table 3.8).

Table 3.8. Sterol content of A.salina reared on D.primolecta.

Life stage	Dry Weight	Non-saponifiable lipid	Sterols
Metanauplii IV	0.36g	21.6mg (6.0%[1])	1.1mg (0.21%[1])
Adult	0.13g	9.0mg (7.1%[1])	0.5mg (0.39%[1])

[1]Expressed as a percentage of the dry weight.

Although there were no visible bands, areas corresponding to 4α-methyl and 4,4'-dimethyl sterol markers were recovered from the plates

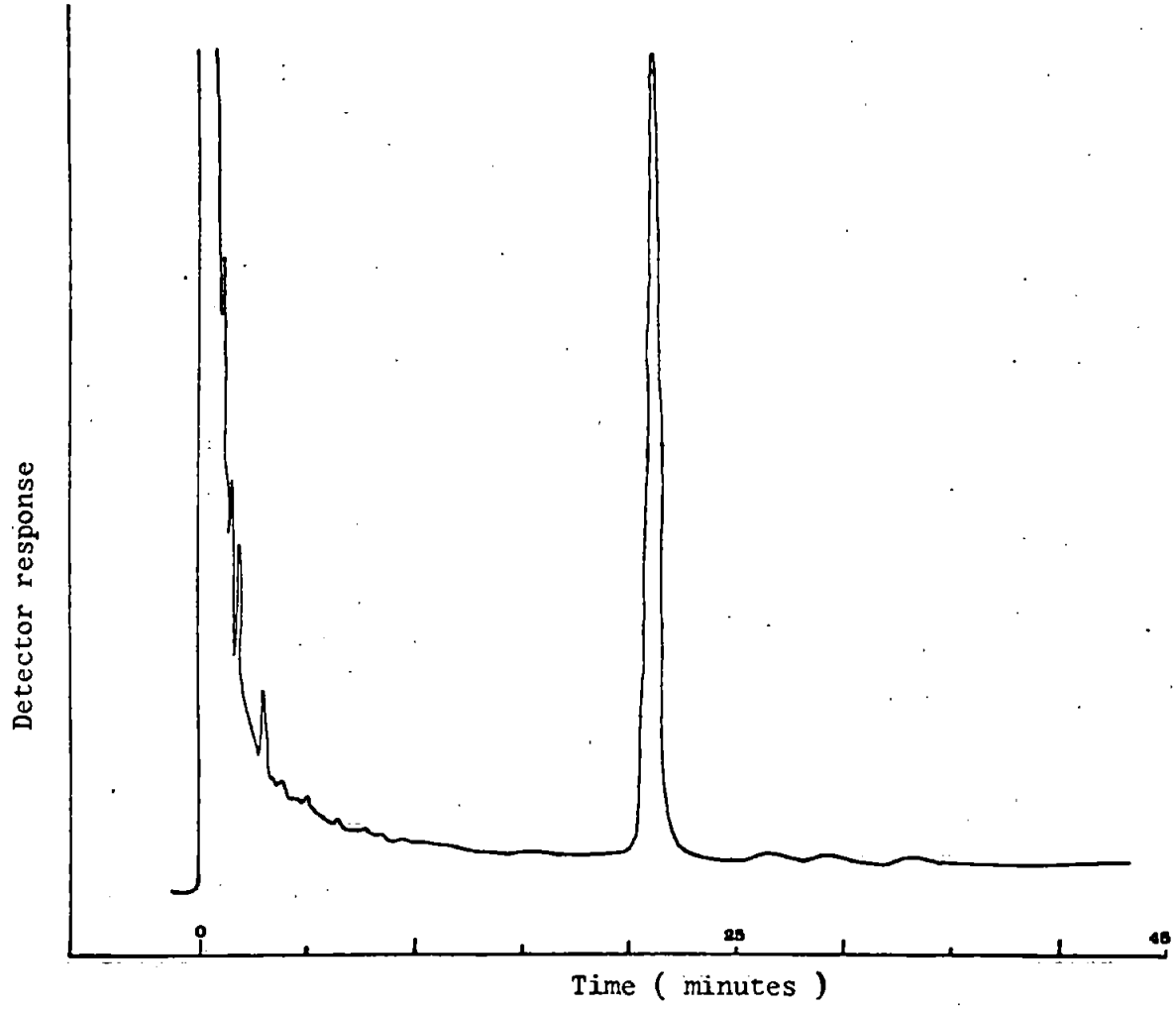


Figure 3.4. GLC analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (metanauplii IV) reared on Dunaliella primolecta.

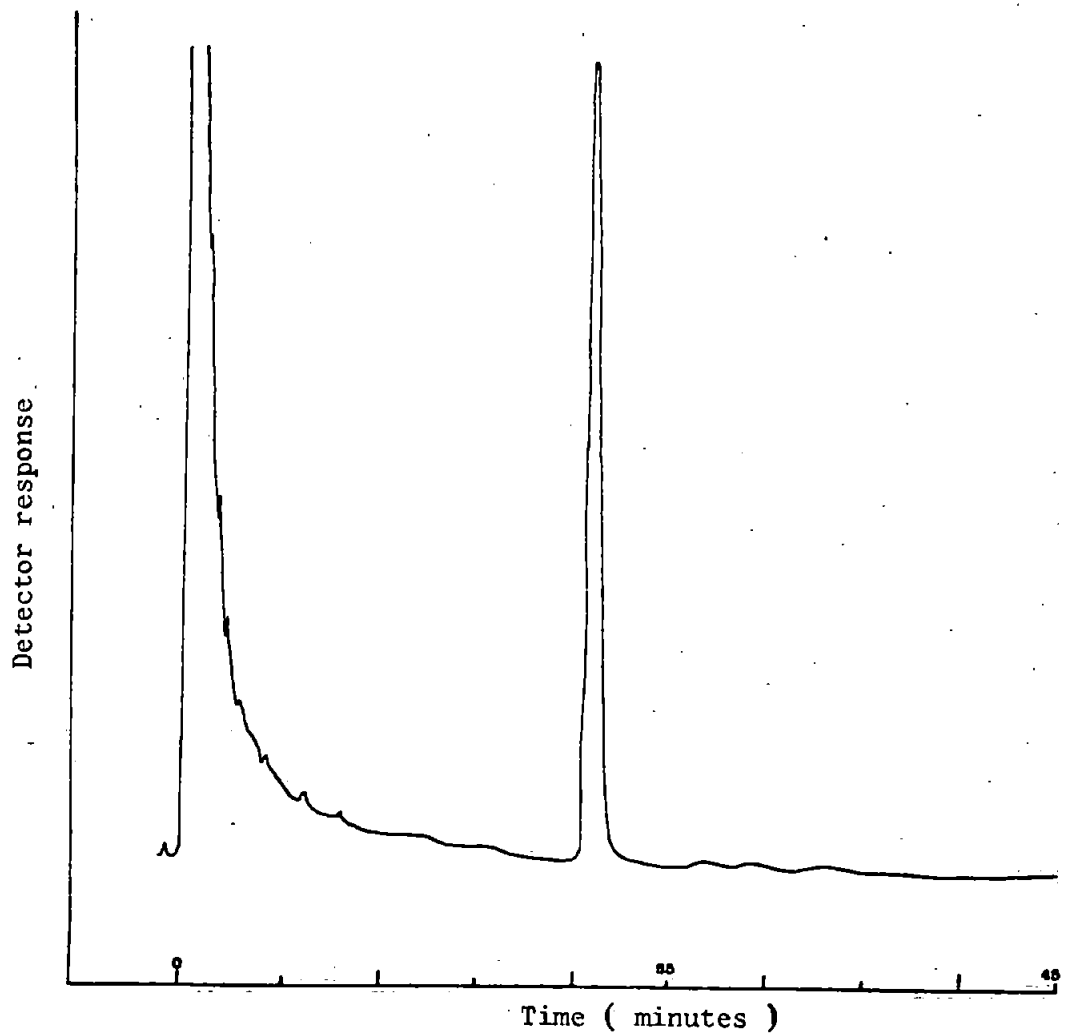


Figure 3.5. GLC analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (adult) reared on Dunaliella primolecta.

Table 3.9. 4-Desmethyl sterol composition of A.salina reared on D.primolecta. Relative retention times of standard sterols.

	Relative retention times[1]			
	Column			
	1% SE-30[2]		3% OV-17[3]	
	Sterol	Acetate	Sterol	Acetate
Cholesterol	1.86	2.47	2.30	3.04
24-Methylcholesterol	2.45	3.14	3.00	3.94
24-Ethylcholesta-5,22-dien-3β-ol	2.65	3.74	3.24	4.24
24-Ethylcholesterol	3.08	3.90	3.68	4.82

[1]Relative to 5α-cholestane (1.00)

[2]Oven temperature 242.5°; Detector temperature 255°; Gas flow (N_2) 30cm^3/min.

[3]Oven temperature 280°; Detector temperature 290°; Gas flow (N_2) 25cm^3/min.

Table 3.10. 4-Desmethyl sterol composition of Artemia salina reared on Dunaliella primolecta.

	Relative retention times					
	Column					
	1% SE-30[1]		3% OV-17[1]			
Life stage	Sterol	Acetate	Sterol	Acetate	Identification	Percentage Composition[2]
Metanauplii IV	1.85	2.47	2.30	3.04	Cholesterol	99+
	2.46	3.12	3.00	3.94	24-Methylcholesterol	trace
	2.65	3.75	3.23	4.26	24-Ethylcholesta-5,22-dien-3β-ol	trace
	3.04	3.90	3.68	4.80	24-Ethylcholesterol	trace
Adult	1.85	2.47	2.30	3.04	Cholesterol	99+
	2.45	3.12	3.00	3.96	24-Methylcholesterol	trace
	2.65	3.75	3.23	4.24	24-Ethylcholesta-5,22-dien-3β-ol	trace
	3.06	3.90	3.68	4.80	24-Ethylcholesterol	trace

[1]Column conditions as for Table 3.9.

[2]Expressed as a percentage of the total sterol.

and eluted. Subsequent GLC analysis did not reveal any components.

When examined by GLC, the material eluted from the bands co-chromatographing with 4-desmethyl sterol markers was found to be separated into four components. The GLC traces obtained are shown in Figures 3.4 and 3.5. The retention times of the components of these mixtures, together with the retention times of sterol standards, are shown in Tables 3.9 and 3.10. On the basis of this evidence, the components of the 4-desmethyl sterol mixtures obtained from A.salina metanauplii and adults reared on D.primolecta were identified as cholesterol, 24-ethylcholesta-5,22-dien-3β-ol, 24-methylcholesterol and 24-ethylcholesterol. In both cases, cholesterol accounted for 99+% of the total sterols, with 24-ethylcholesta-5,22-dien-3β-ol, 24-methylcholesterol and 24-ethylcholesterol being present in trace amounts.

IV. Discussion.

A review of our present knowledge of the sterols of algae has shown that the red and brown algae are quite predictable in their sterol composition (Patterson 1971). Fucosterol has been shown to be the principal sterol of every brown alga examined to date and cholesterol is by far the dominant sterol of red algae, with the exception of Porphyridium cruentum where 22-dehydrocholesterol is the major sterol (Beastall et al 1971; Kanazawa et al 1972a). Generalisations about the sterols of the green algae, however, are more difficult to make. It has been suggested (Gibbons et al 1968) that 28-isofucosterol is the sterol characteristic of the order Ulvales. A wide variety of sterols have been found in other green algae.

Epimers at position 24 in the sidechains of sterols are difficult to distinguish. β-Sitosterol (24α-ethyl) and clionasterol

(24β-ethyl) can be distinguished only by the melting points of their acetates (Thompson et al 1972a). Stigmasterol (24α-ethyl-5,22-dien) and poriferasterol (24β-ethyl-5,22-dien) can be distinguished by NMR and by the melting points of the free sterols (Thompson et al 1972a). From the data available thus far it appears that most, if not all, the algal sterols have the 24β configuration. Several recent papers have identified stigmasterol and β-sitosterol in algae (Avivi et al 1967; Collins & Kalnins 1969) but these authors have not reported any data to distinguish between the 24α and 24β epimers. In another member of the green algae, Chlorella pringsheimii, poriferasterol and clionasterol have been identified (Patterson 1974). On the basis of this and other analyses (Dr.L.J.Goad & Dr.I.Rubenstein - personal communication) the sterols of D.primolecta were tentatively assigned the β configuration. Thus the alga contained, in addition to cholesterol and 22-dehydrocholesterol, 22-dihydrobrassicasterol, brassicasterol, poriferasterol and clionasterol.

Analysis of the sterols of A.salina eggs from California and Utah showed cholesterol to be the major component. Data from the analyses of metanauplii IV and adult stages show that this sterol composition is constant throughout the life cycle of the Californian strain of A.salina reared on D.primolecta. Other workers (Payne & Kuwahara 1972; Teshima & Kanazawa 1971e) have also shown cholesterol to be the main sterol of A.salina. Payne & Kuwahara (1972) demonstrated the presence of cholestanol by mass spectrometry. On the GLC systems employed in this present study, cholesterol and cholestanol are not completely separated but mixtures of both can be identified as such. No cholestanol was detected. It is interesting to note that the relative amount of sterol present increases as the organism grows. This is possibly due to an increased demand for sterol as precursor of hormones etc. in the

metabolic processes associated with metamorphosis.

The stability of the sterol composition of A.salina throughout its life cycle suggests two possibilities. Either A.salina is capable of synthesising sterols or they are obtained by the modification of dietary sterols. The remainder of this work is devoted to an investigation of the capacity of A.salina to a. synthesise sterols and b. to modify dietary sterols.

CHAPTER IV.

STEROLS OF CRUSTACEA.

I. Introduction.

Despite the ubiquitous distribution of Crustacea, these organisms have been subject to comparatively few investigations of their sterol composition. The work to date (reviewed in Chapter I) has, in the main, been confined to the more economically important members of the family; the decapod crabs, lobsters, prawns and shrimps. A fairly uniform sterol spectrum has emerged from such studies; cholesterol has been shown to be the major crustacean sterol, accompanied by varying amounts of minor sterols.

The present investigation was undertaken to determine the sterol composition of some common British shore crustaceans and to extend the survey to include several orders of Crustacea which had been hitherto ignored.

II. Materials.

The organisms which were investigated are listed in Table 4.1. With the exception of Artemia salina, all organisms were collected from the foreshores of Plymouth Sound during the Spring and Summer of 1971. Organisms were maintained in the laboratory for 24 hours without feeding before extraction of the lipids, in order to void the gut contents. Artemia salina were purchased as a freeze-dried preparation from a local aquarium supplies store. This was a product of Miracle Pet Products Inc. of Jersey City, New Jersey 07302, U.S.A. and originated from Taiwan.

Table 4.1. Classification of Crustaceans studied.

FAMILY: CRUSTACEA.

Class: Branchiopoda.

Artemia salina (Brine shrimp)

Chirocephalus spp. (Fairy shrimp)

Class: Copepoda.

Tigriopus brevicornis

Class: Cirripedia.

Chthalamus stellatus (Acorn barnacle)

Class: Malacostraca.

Sub-class: Eucarida.

Order: Decapoda.

Sub-order: Brachyura.

Carcinus maenas (Common shore crab)

Sub-order: Caridea.

Leander serratus (Common prawn)

Sub-class: Pericarida.

Order: Isopoda.

Ligia oceanica (Shore slater)

Order: Amphipoda.

Gammarus spp. (Sand hopper)

III. Methods.

After starvation, organisms were dried by vacuum desiccation. With the exception of C.stellatus, whole organisms were then ground to a powder and this powder exhaustively Soxhlet-extracted with a mixture of chloroform/methanol 2:1 v:v. The total lipid recovered on evaporation of the solvent was then saponified and the non-saponifiable lipid extracted and subjected to the fractionation procedures detailed in Chapter II. Due to the presence of a unicellular flora in the shell-plates of C.stellatus, the soft internal tissues were separated from the external plates before extraction.

IV. Results.

The sterol content and composition of the organisms investigated are shown in Table 4.2.

i. 4-Desmethyl sterols.

The identities of the various 4-desmethyl sterols isolated were established by comparison of the retention data obtained from the free sterols and their acetates on two GLC systems with reference compounds (Tables 4.3, 4.4 and 4.5). GLC traces are shown overloaded only when the overloading produced further information.

ii. 4α-Methyl sterols.

The identities of the various 4α-methyl sterols indicated by GLC retention time data (Tables 4.6 and 4.7) were further established by mass spectrometry.

a. 31-Norcycloartenol.

This compound was identified by GLC in Chirocephalus and

Gammarus. Samples from both sources were collected by trapping of the GLC effluent and the resultant mass spectrum was in accord with the identity of the substance as 31-norcycloartenol (Table 4.8 and Figure 4.1). The molecular ion peak of 31-norcycloartenol acetate was present at m/e 454. Other fragments were visible at m/e 394 for loss of acetate, at m/e 339 for fission of ring A resulting in the loss of carbons 2, 3 and 4 and at m/e 289, a fragment characteristic of the cleavage of the 9β,19-cyclopropane ring. This is shown in Scheme 4.1b (Audier et al 1966; Benveniste et al 1966).

b. 24-Ethylidenelophenol.

24-Ethylidenelophenol was identified in Gammarus. The fragmentation pattern shown in Table 4.9 and Figure 4.2 is in accord with the identity of the substance as 24-ethylidenelophenol. The molecular ion peak was present at m/e 468, with peaks at m/e 453 and m/e 227 representing loss of methyl and fission of ring D respectively. The fragment at m/e 310 arises from the fragmentation pattern shown in Scheme 4.1a and is characteristic of a 24-alkylidene group (Audier et al 1966; Goad & Goodwin 1967).

c. 24-Methylenelophenol and 31-norcyclolaudenol.

These compounds were identified in T.brevicornis, Chirocephalus and Gammarus. They could not be satisfactorily separated on the GLC systems used and so were trapped and analysed by mass spectrometry as a mixture (Table 4.10 and Figure 4.3). Molecular ion peaks were present at m/e 454 and m/e 468, representing the parent ions of 24-methylenelophenol acetate and 31-norcyclolaudenol acetate respectively. Other peaks were seen

at m/e 310 representing the fragmentation of the sidechain of 24-methylenelophenol in a manner analogous to that outlined in Scheme 4.1a for 24-ethylidenelophenol and at m/e 300 representing the fragmentation of the 9β,19-cyclopropane ring of 31-norcyclolaudenol and the production of fragment A, as in Scheme 4.1b. The spectra are in agreement with the identity of the compounds as 24-methylenelophenol and 31-norcyclolaudenol (Audier et al 1966; Benveniste et al 1966).

iii. 4,4'-Dimethyl sterols.

4,4'-Dimethyl sterols were identified in Gammarus and Ligia oceanica (Tables 4.11 and 4.12). The identities of these sterols were established by GLC and mass spectrometry.

a. Cycloartenol.

Samples from both organisms were trapped and the mass spectrum obtained (Table 4.13 and Figure 4.4). The molecular ion peak was present at m/e 468, with other peaks visible at m/e 286, originating from 9β,19-cyclopropane ring cleavage, m/e 357 for the loss of the sidechain and m/e 339 for the fission of ring A. The pattern obtained was consistent with the identity of this compound as cycloartenol (Audier et al 1966; Benveniste et al 1966).

b. Lanosterol.

The molecular ion peak was present at m/e 468 (Table 4.14 and Figure 4.5). Other peaks were visible at m/e 408 for the loss of acetate, m/e 453 for the loss of methyl and m/e 340 for the fission of ring A. This pattern is consistent with the identity of the compound as lanosterol (Benveniste et al 1966).

c. 24,25-Dihydrolanosterol.

The mass spectrum of this compound substantiates its identification as 24,25-dihydrolanosterol (Table 4.15 and Figure 4.6). The molecular ion peak is seen at m/e 470, with other peaks visible at m/e 410, 395 and 342, representing the loss of acetate, acetate plus methyl and ring A fission respectively.

V. Discussion.

i. Chthalamus stellatus.

C.stellatus, a cirriped, contained only 4-desmethyl sterols. Cholesterol was the major sterol in this organism (80.8% of the total sterol). High proportions of desmosterol (12.1%), 22-dehydrocholesterol (3.4%) and 24-methylenecholesterol (2.1%) were also present. 24-Methylcholest-5,22-dien-3β-ol, 24-methylcholesterol, 24-ethylcholesterol, fucosterol and 28-isofucosterol were present in trace amounts.

There have been two previous reports of sterol analyses of cirripedes, both showing cholesterol and desmosterol as being the major components. Fagerlund & Idler (1957) showed that desmosterol accounted for 34.2% of the total sterols in Balanus glandula, cholesterol being the major component (59.8%). In Balanus nubilis, however, desmosterol was present in greater amount (42.4%) than cholesterol (37%), small amounts of β-sitosterol, campesterol and stigmasterol were also identified (Whitney 1967).

ii. Gammarus.

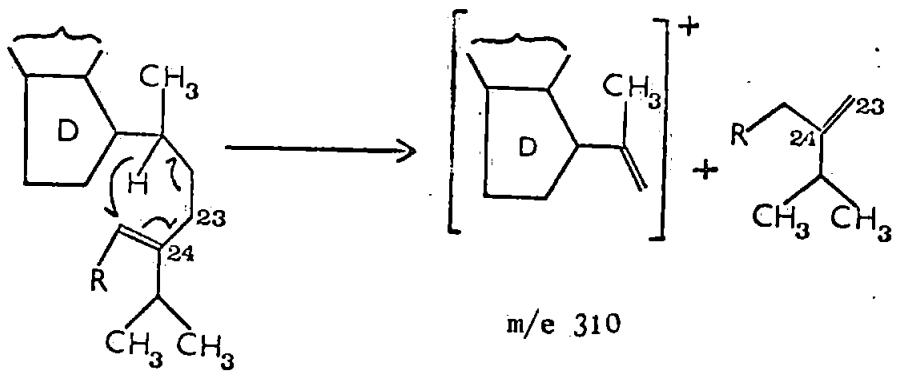
Gammarus, an amphipod, contained 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterols (Figures 4.8, 4.9 and 4.10).

Scheme 4.1.

Mass spectral fragmentation patterns.

4.1a. Fragmentation of compounds containing a C-24 alkylidene group.

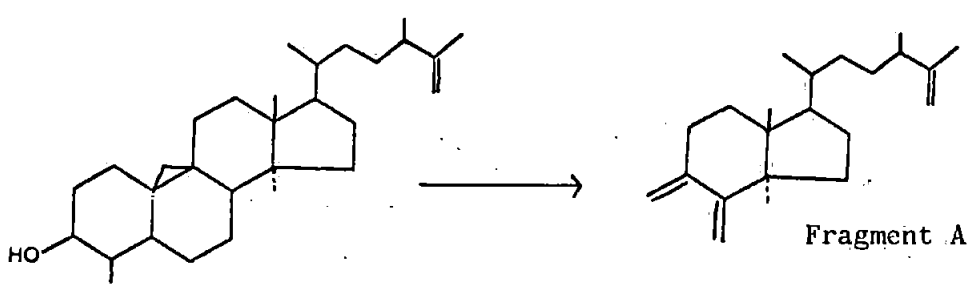
eg. 24-methylenelophenol and 24-ethylidenelophenol.



m/e 310

4.1b. Fragmentation of compounds containing a 9β,19-cyclopropane ring.

eg. 31-norcyclolaudenol.



Fragment A

31-norcyclolaudenol ⟶ m/e 300

31-norcycloartenol ⟶ m/e 286

Cycloartenol ⟶ m/e 286

Table 4.2. Sterol content and composition of marine Crustacea.

Species	Dry weight	Non-saponifiable lipid		Total sterols		4-desmethyl sterols		4α-methyl sterols		4,4'-dimethyl sterols	
	g	g	%[1]	mg	%[1]	mg	%[2]	mg	%[2]	mg	%[2]
A. salina	2.70	0.04	1.48	3.7	0.14	3.7	100	———		———	
C. maenas	38.81	0.16	0.41	14.9	0.04	14.9	100	———		———	
Chirocephalus	7.04	0.09	1.28	11.6	0.16	10.3	88.8	1.3	11.2	———	
C. stellatus	40.02	1.71	4.27	20.8	0.05	20.8	100	———		———	
Gammarus	1.73	0.013	0.75	3.2	0.18	2.1	65.6	0.4	12.5	0.7	21.9
L. serratus	4.08	0.13	3.19	2.8	0.07	2.8	100	———		———	
L. oceanica	3.91	0.30	7.67	3.2	0.08	2.5	78.1	———		0.7	21.9
T. brevicornis	2.22	0.05	2.30	2.0	0.09	1.5	75.0	0.5	25.0	———	

[1] expressed as a percentage of the dry weight.

[2] expressed as a percentage of the total sterol.

Table 4.3. Relative retention times of 4-desmethyl sterol standards.

Standard sterols[1]	Relative Retention Time[2]			
	Column			
	1% SE-30		3% OV-17	
	Sterol[3]	Acetate[3]	Sterol[4]	Acetate[4]
5α-cholestane	1.00	1.00	1.00	1.00
Cholest-5-en-3β-ol	1.85	2.68	2.46	3.03
Cholest-5,22-dien-3β-ol	1.68	2.39	2.29	2.85
24-Methylcholest-5,22-dien-3β-ol	2.07	2.92	2.77	3.44
Cholest-5,24-dien-3β-ol	2.02	2.86	2.93	3.62
24-Methylcholest-5-en-3β-ol	2.26	3.44	3.18	3.94
24-Methylenecholest-5-en-3β-ol	2.38	3.34	3.26	4.03
24-Ethylcholest-5,22-dien-3β-ol	2.70	3.74	3.55	4.24
24-Ethylcholest-5-en-3β-ol	3.05	4.30	3.89	4.80
24E-Ethylidenecholest-5-en-3β-ol	3.10	4.36	4.15	5.06
24Z-Ethylidenecholest-5-en-3β-ol	3.16	4.43	4.37	5.30

[1] Trivial names as in Chapter 2. [2] All retention times are relative to 5 -cholestane.
[3] Oven temperature 280°; Detector temperature 290°; Gas flow (N_2) 25cm^3/min.
[4] Oven temperature 242.5°; Detector temperature 255°; Gas flow 30cm^3/min.

Table 4.4. 4-Desmethyl sterol composition of marine Crustacea as determined by GLC.

Species	Relative Retention Time Column[1]				Peak[2]	Identification
	1% SE-30		3% OV-17			
	Sterol	Acetate	Sterol	Acetate		
L. serratus	1.72	2.40	2.34	2.86	A	Cholest-5,22-dien-3β-ol
	1.87	2.66	2.48	3.02	B	Cholest-5-en-3β-ol
	2.43	3.40	3.25	4.02	F	24-Methylenecholest-5-en-3β-ol
	2.72	3.70	3.50	4.25	G	24-Ethylcholest-5,22-dien-3β-ol
	3.12	4.32	3.90	4.79	H	24-Ethylcholest-5-en-3β-ol
Chirocephalus	1.67	2.40	2.28	2.81	A	Cholest-5,22-dien-3β-ol
	1.82	2.65	2.42	3.00	B	Cholest-5-en-3β-ol
	2.07	2.96	2.76	3.38	C	24-Methylcholest-5,22-dien-3β-ol
	2.66	3.74	3.15	4.01	E	24-Methylcholest-5-en-3β-ol
	2.38	3.36	3.24	3.85	F	24-Methylenecholest-5-en-3β-ol
	3.07	4.36	3.46	4.17	G	24-Ethylcholest-5,22-dien-3β-ol
			3.90	4.81	H	24-Ethylcholest-5-en-3β-ol

Table 4.4: continued.

C.maenas	1.69	2.42	2.29	2.85	A	Cholest-5,22-dien-3β-ol
	1.86	2.64	2.45	3.00	B	Cholest-5-en-3β-ol
	2.04	2.90	2.91	3.40	D	Cholest-5,24-dien-3β-ol
	2.26	3.44	2.73	3.60	C	24-Methylcholest-5,22-dien-3β-ol
	2.69	3.72	3.15	4.01	E	24-Methylcholest-5-en-3β-ol
	3.06	4.36	3.65	4.22	G	24-Ethylcholest-5,22-dien-3β-ol
	3.18	4.46	3.89	4.81	H	24-Ethylcholest-5-en-3β-ol
			4.08	5.07	I	24E-Ethylidenecholest-5-en-3β-ol
			4.31	5.29	J	24Z-Ethylidenecholest-5-en-3β-ol
Gammarus	1.68	2.39	2.33	2.84	A	Cholest-5,22-dien-3β-ol
	1.85	2.62	2.45	3.00	B	Cholest-5-en-3β-ol
	2.03	2.88	2.92	3.61	D	Cholest-5,24-dien-3β-ol
	2.37	3.40	3.16	3.92	E	24-Methylcholest-5-en-3β-ol
	2.73	3.78	3.49	4.20	G	24-Ethylcholest-5,22-dien-3β-ol
	3.05	4.36	3.90	4.74	H	24-Ethylcholest-5-en-3β-ol
			4.15	5.02	I	24E-Ethylidenecholest-5-en-3β-ol

Table 4.4: continued.

C.stellatus	1.70	2.40	2.30	2.87	A	Cholest-5,22-dien-3β-ol
	1.86	2.66	2.48	3.04	B	Cholest-5-en-3β-ol
	2.05	2.90	2.87	3.45	D	Cholest-5,24-dien-3β-ol
	2.34	3.38	2.79	3.58	C	24-Methylcholest-5,22-dien-3β-ol
	3.08	4.32	3.28	3.91	F	24-Methylenecholest-5-en-3β-ol
	3.16	4.44	3.19	4.03	E	24-Methylcholest-5-en-3β-ol
			3.94	4.81	H	24-Ethylcholest-5-en-3β-ol
			4.17	5.07	I	24E-Ethylidenecholest-5-en-3β-ol
			4.41	5.32	J	24Z-Ethylidenecholest-5-en-3β-ol
T.brevicornis	1.71	2.37	2.24	2.84	A	Cholest-5,22-dien-3β-ol
	1.87	2.68	2.38	3.00	B	Cholest-5-en-3β-ol
	2.10	2.90	2.75	3.45	C	24-Methylcholest-5,22-dien-3β-ol
	2.45	3.50	3.28	4.01	F	24-Methylenecholest-5-en-3β-ol
	2.72	3.76	3.11	3.94	E	24-Methylcholest-5-en-3β-ol
	3.11	4.34	3.49	4.23	G	24-Ethylcholest-5,22-dien-3β-ol
			3.89	4.81	H	24-Ethylcholest-5-en-3β-ol

Table 4.4: continued.

L.oceanica	1.66	2.38	2.35	2.87	A	Cholest-5,22-dien-3β-ol
	1.89	2.60	2.49	3.04	B	Cholest-5-en-3β-ol
	2.07	2.94	2.96	3.45	D	Cholest-5,24-dien-3β-ol
	2.40	3.30	2.80	3.58	C	24-Methylcholest-5,22-dien-3β-ol
	2.67	3.74	3.24	4.03	F	24-Methylenecholest-5-en-3β-ol
	3.02	4.32	3.90	4.81	H	24-Ethylcholest-5-en-3β-ol
			4.21	5.07	I	24E-Ethylidenecholest-5-en-3β-ol
A.salina	1.67	2.39	2.26	2.83	A	Cholest-5,22-dien-3β-ol
	1.89	2.63	2.55	3.00	B	Cholest-5-en-3β-ol
	2.07	2.94	2.86	3.40	D	Cholest-5,24-3β-ol
	2.42	3.36	2.80	3.55	C	24-Methylcholest-5,22-dien-3β-ol
	2.66	3.76	3.16	3.88	E	24-Methylcholest-5-en-3β-ol
	3.05	4.36	3.44	4.22	G	24-Ethylcholest-5,22-dien-3β-ol
			3.88	4.81	H	24-Ethylcholest-5-en-3β-ol

[1] Column conditions are as specified in Table 4.3.

[2] Peaks refer to those in the GLC tracings given.

Table 4.5. Percentage composition of 4-desmethyl sterols of marine Crustacea.

Sterol	Organism/Percentage Composition[1]							
	A	B	C	D	E	F	G	H
Cholest-5,22-dien-3β-ol	4.1	trace	3.0	1.9	3.4	trace	trace	trace
Cholest-5-en-3β-ol	81.1	99+	77.0	96.3	80.8	98.2	97.8	99+
24-Methylcholest-5,22-dien-3β-ol	trace	trace	1.8	1.0	trace	trace	——	——
Cholest-5,24-dien-3β-ol	3.9	trace	——	——	12.1	trace	——	trace
24-Methylenecholest-5-en-3β-ol	——	——	9.6	trace	2.1	trace	——	trace
24-Methylcholest-5-en-3β-ol	9.3	trace	8.3	trace	1.2	——	——	trace
24-Ethylcholest-5,22-dien-3β-ol	trace	trace	trace	trace	——	——	trace	trace
24-Ethylcholest-5-en-3β-ol	1.7	trace	trace	trace	trace	trace	trace	trace
24E-Ethylidenecholest-5-en-3β-ol	——	trace	——	——	trace	1.0	——	trace
24Z-Ethylidenecholest-5-en-3β-ol	——	trace	——	——	trace	——	——	——

[1] Expressed as a percentage of the total 4-desmethyl sterols.

A. A.salina; B. C.maenas; C. Chirocephalus; D. T.brevicornis; E. C.stellatus; F. L.oceanica; G. L.serratus; H. Gammarus.

Table 4.6. Relative retention times of 4α-methyl sterol standards.

Standard sterols	Relative Retention Time			
	Column			
	1% SE-30[1]		3% OV-17[2]	
	Sterol	Acetate	Sterol	Acetate
31-Norcycloartenol	2.45	3.88	3.11	3.74
31-Norcyclolaudenol	3.00	3.97	4.02	4.84
24-Methylenelophenol	3.00	4.06	4.18	4.98
24-Ethylidenelophenol	3.90	5.03	5.53	6.23

[1] Oven temperature 280°; Detector temperature 290°; Gas flow 37cm^3/min.

[2] Oven temperature 235°; Detector temperature 250°; Gas flow 40cm^3/min.

Table 4.7. Identification and composition of 4α-methyl sterols of marine Crustacea as determined by GLC.

Species	Relative Retention Time Column[1]				Peak[2]	Identification	Percentage Composition[3]
	1% SE-30		3% OV-17				
	Sterol	Acetate	Sterol	Acetate			
T.brevicornis	2.99	3.88	4.02	4.84	L	31-Norcyclolaudenol	99+
		4.08	4.20	4.96	M	24-Methylenelophenol	trace
Gammarus	2.46	3.86	3.14	3.74	K	31-Norcycloartenol	8.2
	3.00	4.00	4.04	4.84	L	31-Norcyclolaudenol	38.4
	3.92	4.08	4.20	5.00	M	24-Methylenelophenol	12.3
		5.02	5.53	6.20	N	24-Ethylidenelophenol	41.1
Chirocephalus	2.44	3.88	3.12	3.76	K	31-Norcycloartenol	26.8
	2.99	3.96	4.05	4.84	L	31-Norcyclolaudenol	70.2
		4.06	4.18	5.00	M	24-Methylenelophenol	3.0

[1] Column conditions as specified in Table 4.6. [2] Peaks refer to those on GLC tracings given.

[3] Expressed as a percentage of the total 4α-methyl sterols.

Table 4.8. Mass spectrum of 31-Norcycloartenol acetate.

Isolated from Gammarus and Chirocephalus.

Peak at m/e	Relative Abundance %	Fragmentation
454	19	M^+
439	11	M^+ - (methyl)
412	3	M^+ - (isopropyl)
394	100	M^+ - (acetate)
379	41	M^+ - (acetate + methyl)
341	11	M^+ - (sidechain + 2H)
339	11	Fission of ring A
286	22	Fission of 9β,19-cyclopropane ring
281	20	M^+ - (sidechain + acetate + 2H)
271	8	Cyclopropane ring fission - methyl

M^+ = Molecular ion peak for 31-Norcycloartenol acetate.

Table 4.9. Mass spectrum of 24-Ethylidenelophenol acetate.

Isolated from Gammarus.

Peak at m/e	Relative Abundance %	Fragmentation
468	19	M^+
453	6	M^+ - (methyl)
408	2	M^+ - (acetate)
393	3	M^+ - (methyl + acetate)
370	100	M^+ - (part of sidechain)
327	51	M^+ - (sidechain + 2H)
310	5	M^+ - (part of sidechain + acetate)
269	10	M^+ - (sidechain + acetate)
267	4	M^+ - (sidechain + acetate + 2H)
227	8	Fission of ring D

M^+ = 24-Ethylidenelophenol acetate.

Table 4.10. Mass spectrum of 31-Norcyclolaudenol acetate and 24-Methylenelophenol acetate.

Isolated from T.brevicornis, Gammarus and Chirocephalus.

Peak at m/e	Relative Abundance %	Fragmentation
468	19	M_1^+
454	2	M_2^+
453	7	M_1^+ - (methyl)
439	1	M_2^+ - (methyl)
408	100	M_1^+ - (acetate)
394	13	M_2^+ - (acetate)
393	32	M_1^+ - (acetate + methyl)
379	5	M_2^+ - (acetate + methyl)
370	2	M_2^+ - (part of sidechain)
353	7	M_1^+: fission of ring A
343	8	M_1^+ - (sidechain)
327	2	M_2^+ - (sidechain + 2H)
310	3	M_2^+ - (part of sidechain + acetate)
300	19	M_1^+: cyclopropane ring fission
285	5	M_1^+: cyclopropane ring fission - methyl
283	18	M_1^+ - (sidechain + acetate)
269	3	M_2^+ - (sidechain + acetate)
267	3	M_2^+ - (sidechain + acetate + 2H)
227	4	M_2^+: fission of ring D.

M_1^+ and M_2^+ are the molecular ion peaks for 31-Norcyclolaudenol and 24-Methylenelophenol acetates respectively.

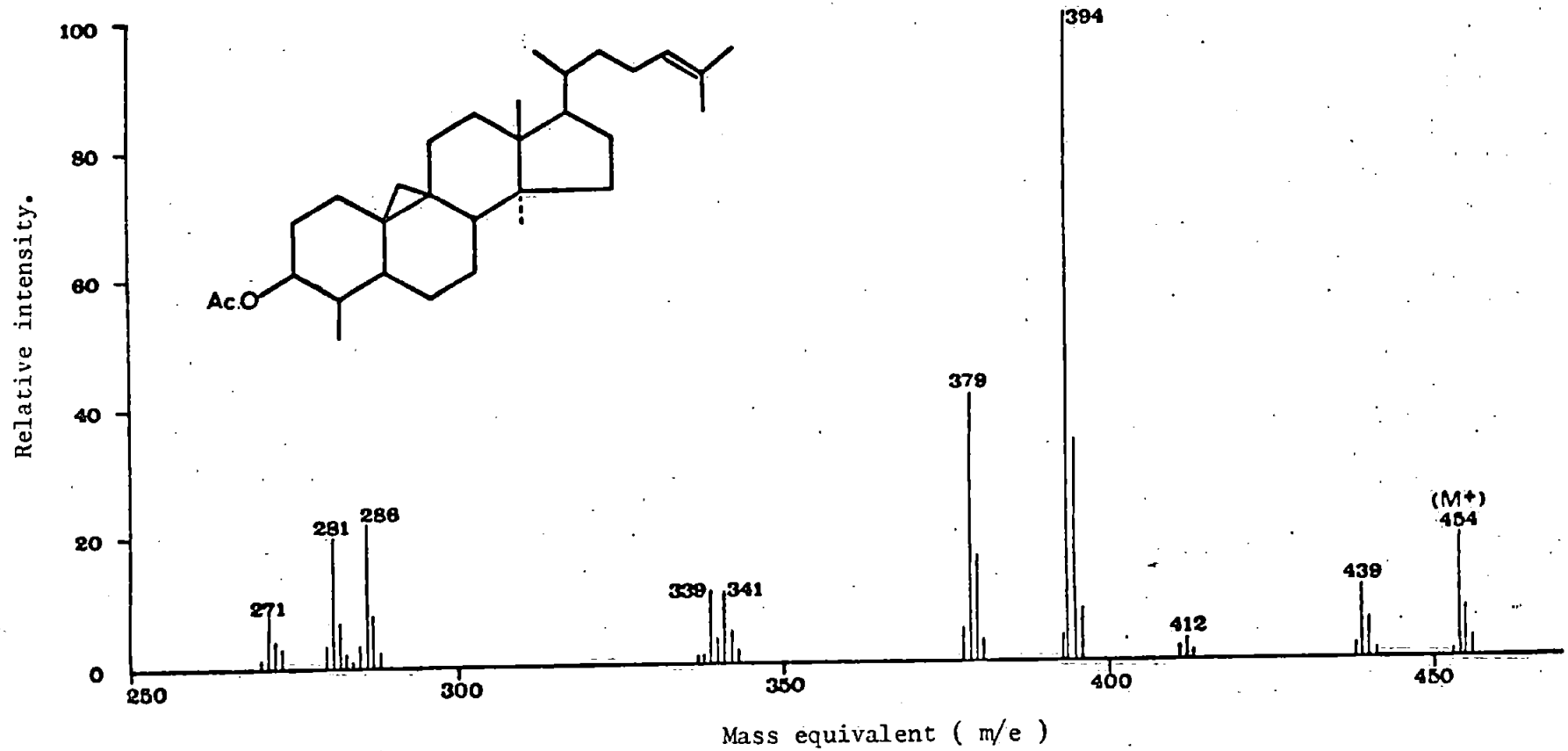


Figure 4.1. Mass spectrum of 31-norcycloartenol acetate.

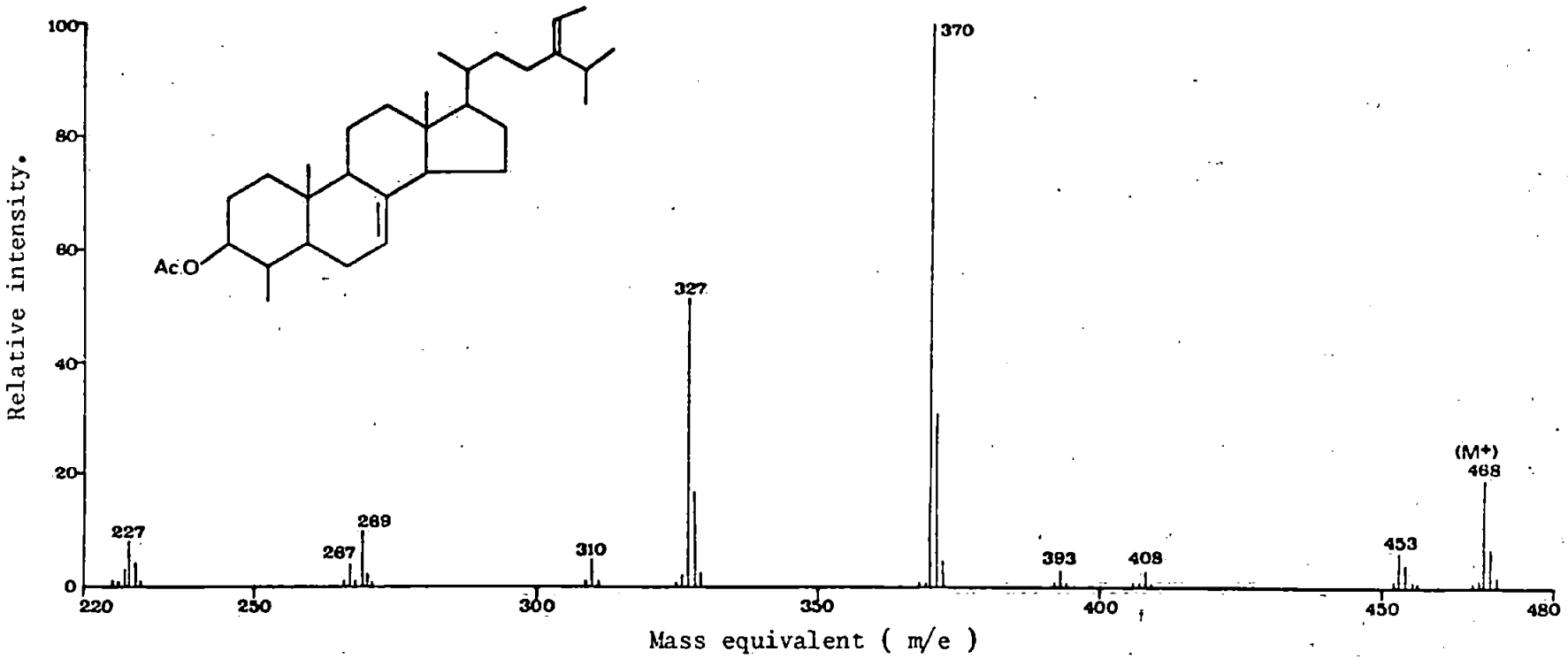


Figure 4.2. Mass spectrum of 24-ethylidenelophenol acetate.

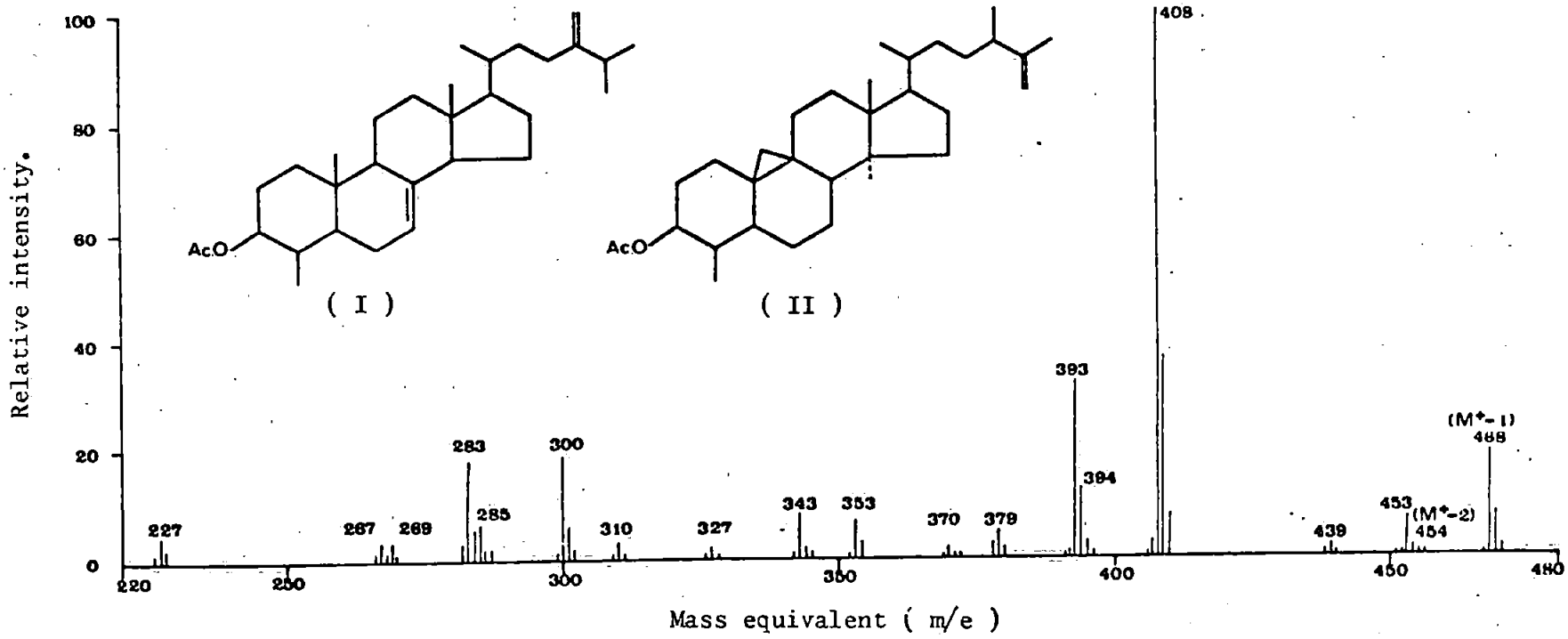


Figure 4.3. Mass spectrum of 24-methylenelophenol acetate (I) and 31-norcyclolaudenol acetate (II).

Table 4.11. Relative retention times of 4,4'-dimethyl sterol standards.

Standard sterols	Relative Retention Time			
	Column			
	1% SE-30[1]		3% OV-17[2]	
	Sterol	Acetate	Sterol	Acetate
24,25-Dihydrolanosterol	2.61	3.38	3.18	3.69
Lanosterol	2.85	3.66	3.81	4.40
Cycloartenol	3.19	4.18	4.38	5.20

[1] Oven temperature 285°; Detector temperature 295°; Gas flow 40cm^3/min.

[2] Oven temperature 250°; Detector temperature 260°; Gas flow 40cm^3/min.

Table 4.12. Identification and composition of 4,4'-dimethyl sterols of marine Crustacea as determined by GLC.

Species	Relative Retention Time Column[1]				Peak[2]	Identification	Percentage Composition[3]
	1% SE-30		3% OV-17				
	Sterol	Acetate	Sterol	Acetate			
L.oceanica	2.64	3.38	3.20	3.70	O	24,25-Dihydrolanosterol	2.1
	2.86	3.67	3.81	4.30	P	Lanosterol	23.3
	3.20	4.19	4.38	5.20	Q	Cycloartenol	74.6
Gammarus	2.64	3.36	3.18	3.69	O	24,25-Dihydrolanosterol	1.3
	2.86	3.67	3.81	4.40	P	Lanosterol	10.5
	3.20	4.19	4.39	5.20	Q	Cycloartenol	88.2

[1] Column conditions as specified in Table 4.11. [2] Peaks refer to those on GLC tracings given.

[2] Expressed as a percentage of the total 4,4'-dimethyl sterols.

Table 4.13. Mass spectrum of Cycloartenol acetate.

Isolated from L.oceanica and Gammarus.

Peak at m/e	Relative Abundance %	Fragmentation
468	45	M^+
453	16	M^+ - (methyl)
408	100	M^+ - (acetate)
393	54	M^+ - (acetate + methyl)
365	27	M^+ - (acetate + isopropyl)
357	12	M^+ - (sidechain)
339	22	Fission of ring A
297	22	M^+ - (sidechain + acetate)
286	51	Cyclopropane ring fission
271	22	Cyclopropane ring fission - methyl

M^+ = Molecular ion peak for Cycloartenol acetate.

Table 4.14. Mass spectrum of Lanosterol acetate.

Isolated from L.oceanica and Gammarus.

Peak at m/e	Relative Abundance %	Fragmentation
468	100	M^+
453	98	M^+ - (methyl)
408	2	M^+ - (acetate)
393	43	M^+ - (acetate + methyl)
340	15	Fission of ring A
314	3	Fission of ring D
297	2	M^+ - (sidechain + acetate)
254	4	Ring D fission fragment - acetate

M^+ = Molecular ion peak for Lanosterol acetate.

Table 4.15. Mass spectrum of 24,25-Dihydrolanosterol acetate.

Isolated from L.oceanica and Gammarus.

Peak at m/e	Relative Abundance %	Fragmentation
470	100	M^+
455	2	M^+ - (methyl)
410	38	M^+ - (acetate)
395	16	M^+ - (acetate + methyl)
357	2	M^+ - (sidechain)
342	3	Fission of ring A
314	3	Fission of ring D
297	7	M^+ - (sidechain + acetate)
254	3	Ring D fission frgment - acetate

M^+ = Molecular ion peak for 24,25-Dihydrolanosterol acetate.

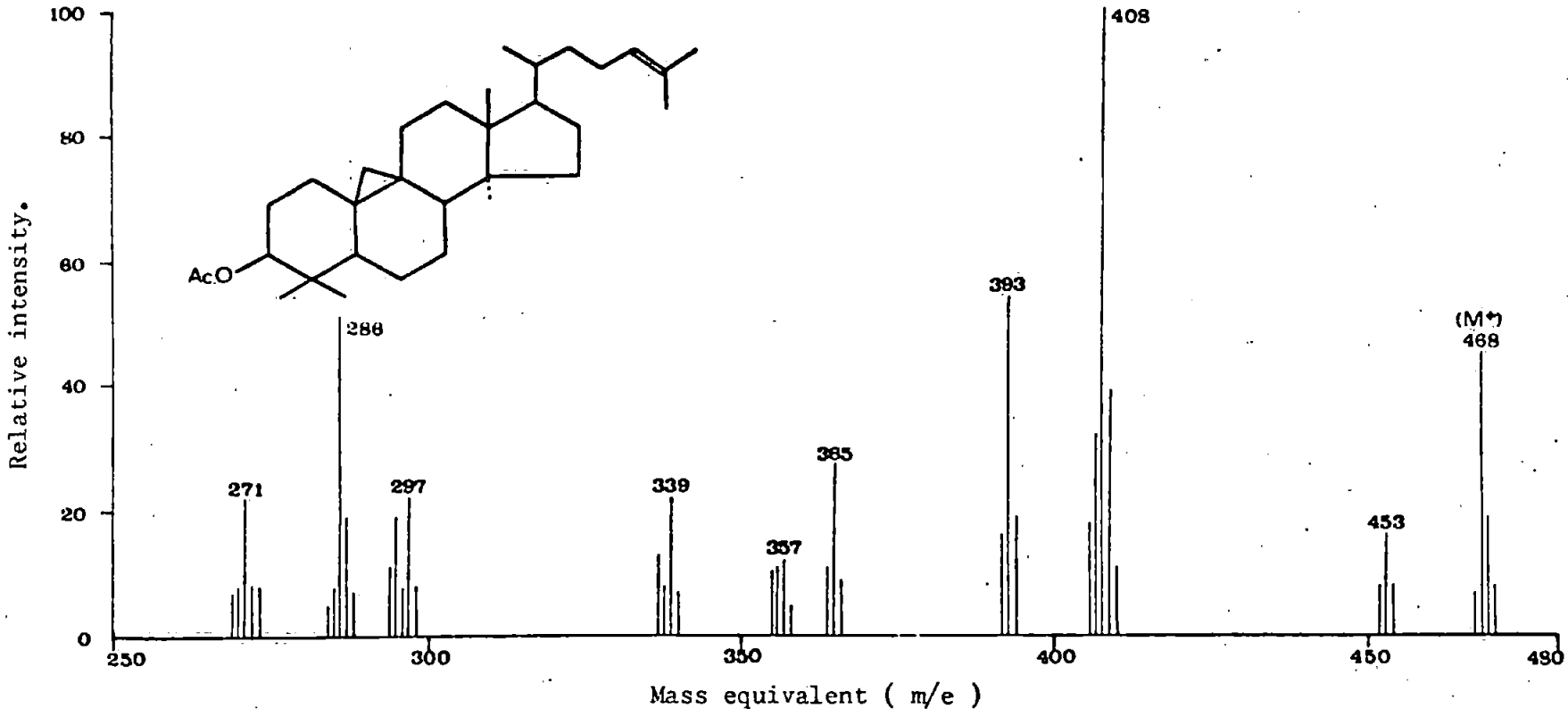


Figure 4.4. Mass spectrum of cycloartenol acetate.

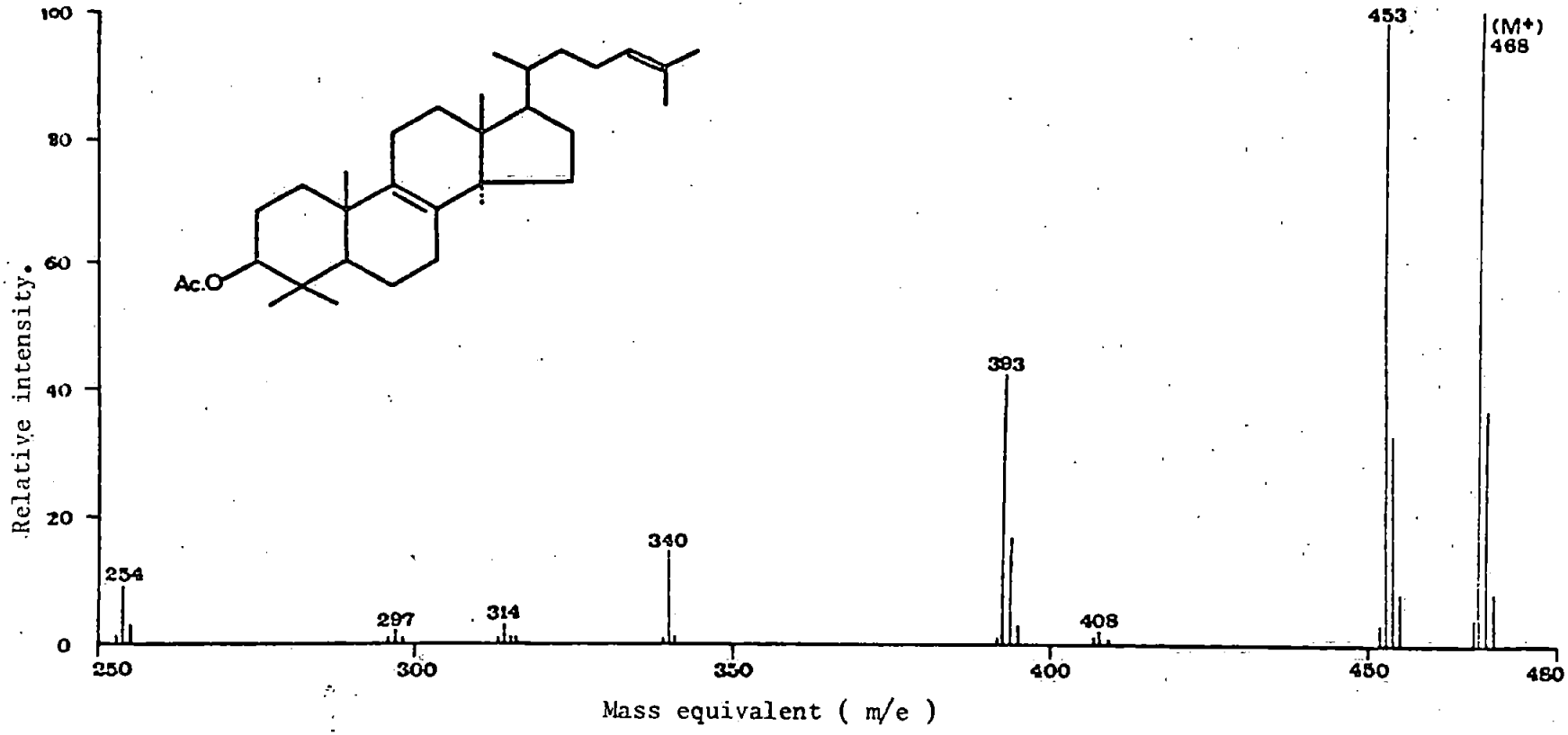


Figure 4.5. Mass spectrum of lanosterol acetate.

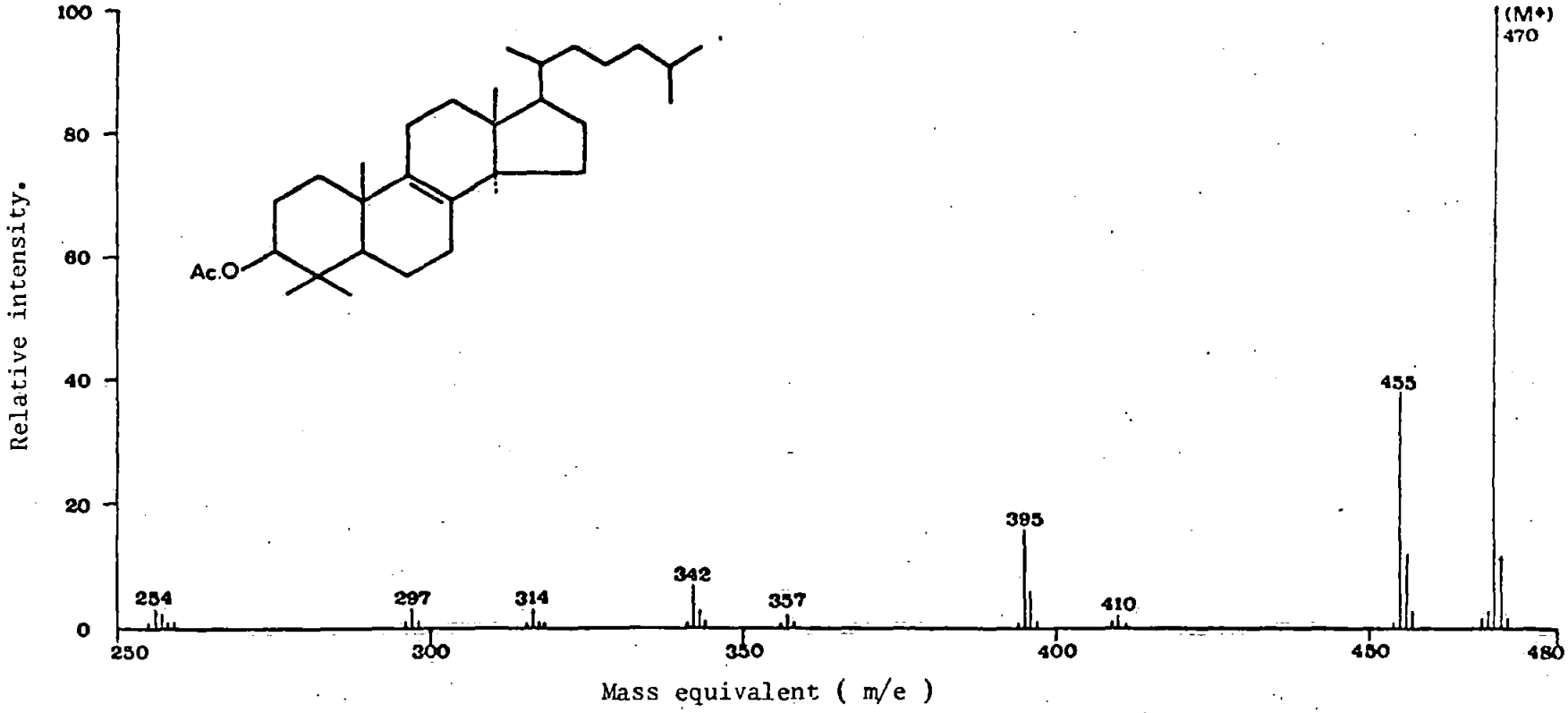


Figure 4.6. Mass spectrum of 24,25-dihydrolanosterol acetate.

The 4-desmethyl sterols consisted predominantly of cholesterol (99+% of the total 4-desmethyl sterols), with trace amounts of desmosterol, 22-dehydrocholesterol, 24-ethylcholest-5,22-dien-3β-ol, fucosterol, 24-methylcholesterol and 24-ethylcholesterol.

The 4α-methyl sterol fraction accounted for 12.5% of the total sterols found in Gammarus. Of these, 31-norcyclolaudenol (38.4% of the total 4α-methyl sterols) and 24-ethylidenelophenol (41.1%) were present in greatest amount; there were significant amounts of 24-methylenelophenol (12.3%) and 31-norcycloartenol (8.2%).

21.9% of the total sterols were present in the 4,4'-dimethyl sterol fraction. Cycloartenol accounted for 88.2% of this fraction, lanosterol 10.5% and 24,25-dihydrolanosterol 1.3%.

The only other amphipod whose sterol content has been reported is the ghost shrimp, Caprella (Teshima & Kanazawa 1971a). In this organism, cholesterol was found to account for 73% of the total sterols. Desmosterol was present in large amount (22%) with smaller amounts of 22-dehydrocholesterol (4%) and 24-methylenecholesterol (1%). Only 4-desmethyl sterols were reported and the authors do not mention any attempts at identifying either 4α-methyl or 4,4'-dimethyl sterols.

c. Tigriopus brevicornis.

T.brevicornis, a copepod, contained 4-desmethyl and 4α-methyl sterols (Figures 4.11 and 4.12).

The major 4-desmethyl sterol was cholesterol (96.3% of the total 4-desmethyl sterol fraction). There were trace amounts of 24-methylcholest-5,22-dien-3β-ol, 24-ethylcholest-5,22-dien-3β-ol, 22-dehydrocholesterol, 24-methylenecholesterol and 24-ethylcholesterol.

25% of the total sterols were present in the 4α-methyl sterol

fraction, which was comprised predominantly of 31-norcyclolaudenol (99+% of the total 4α-methyl sterols). The remainder consisted of 24-methylenelophenol.

Calanus finmarchicus is the only other copepod to have been studied. Both Belloc et al (1930) and Collin et al (1934) reported the occurrence of ergosterol. The evidence presented in both studies was based on vitamin D potency experiments and has not been substantiated.

d. Ligia oceanica.

To the author's knowledge, this is the first report of the analysis of the sterols of a member of the Isopoda. Ligia oceanica contained both 4-desmethyl and 4,4'-dimethyl sterols (Figures 4.13 and 4.14).

Of the 4-desmethyl sterols, cholesterol was by far the most prominent, accounting for 98.2% of the total 4-desmethyl sterols. Fucosterol was present to 1%, accompanied by trace amounts of 24-methylenecholesterol, 24-methylcholesterol, 24-ethylcholesterol, desmosterol and 22-dehydrocholesterol.

The 4,4'-dimethyl sterols accounted for 22% of the total sterols present in L.oceanica and consisted of cycloartenol (74.6% of the total 4,4'-dimethyl sterol fraction), lanosterol (23.3%) and a small amount of 24,25-dihydrolanosterol (2.0%).

e. Leander serratus.

L.serratus contained only 4-desmethyl sterols (Figure 4.15). Cholesterol accounted for 97.8% of the total sterols present. There was a small amount of 24-methylenecholesterol (1.1%) and trace amounts of 24-ethylcholest-5,22-dien-3β-ol, 22-dehydrocholesterol and 24-ethylcholesterol.

L.serratus is a caridean prawn and few of these have been subject to rigorous analysis. Cholesterol has been reported in several members of this group, some identified, eg. Penaeus aztecus and Penaeus setiferus (Thompson 1964) but also in some unidentified species, designated simply as 'shrimp' or 'prawn' (Pihl 1957; Abernethy & Vilbrandt 1937; Kritchevsky & Tepper 1961; Kritchevsky et al 1967). Only two named species have been analysed by GLC. Kanazawa et al (1971b) demonstrated that Penaeus japonicus contained predominantly cholesterol (90% of the total sterol), with significant amounts of 22-dehydrocholesterol (3%) and 24-methylenecholesterol (7%). Desmosterol and β-sitosterol were present in trace amounts. An analysis of this species by Teshima & Kanazawa (1971a) produced almost identical figures, with the exception of trace amounts of stigmasterol in place of desmosterol. Yasuda (1973) failed to identify 24-methylenecholesterol in the freshwater shrimp, Paratya compressa compressa, and reported this caridean to contain cholesterol (98.6% of the total sterol), 24-methylcholesterol (1.1%), 24-ethylcholesterol (0.3%) and trace amounts of 24-norcholesta-5,22-dien-3β-ol.

f. Carcinus maenas.

C.maenas, a brachyuran crab, is a decapod and most of the work on the sterol content of crustaceans has been carried out on members of this group.

In this present study, C.maenas was found to contain solely 4-desmethyl sterols (Figure 4.16) and of these cholesterol was dominant (99+% of the total sterol). This compound was accompanied by trace amounts of eight other desmethyl sterols, namely 22-dehydrocholesterol, 24-methylcholest-5,22-dien-3β-ol, 24-ethylcholest-5,22-dien-3β-ol,

fucosterol, 24-methylcholesterol, 24-ethylcholesterol, 28-isofucosterol and desmosterol.

Idler & Wiseman (1971a) analysed the sterol contents of seven species of marine brachyuran crabs. Cholesterol was the major sterol in all, with amounts of desmosterol ranging from less than 0.1% of the total sterol in the deep sea red crab, Geryon quinquedens, to 11% in the toad crab, Hyas araneus. 24-Methylenecholesterol and 22-dehydrocholesterol were present in all species investigated and 28-isofucosterol was identified in G.quinquedens. One or two species contained small amounts of brassicasterol and β-sitosterol. Yasuda (1973) extended his coverage of brachyuran crabs to encompass both marine and freshwater types. In the five species studied, cholesterol was again the dominant sterol. Desmosterol and 24-methylenecholesterol were present in the marine crabs (Chionecetes opilio, Erimacrus isenbeckii, Hemigrapsus sanguineus) but not in the freshwater species. The freshwater species (Potamon dehaani, Ilyoplax pusillus) did, however, contain 24-methyl and 24-ethylcholesterol whereas the marine crabs did not. There have been no other analyses of freshwater crabs for purposes of comparison. Idler & Wiseman (1968) presented analyses of Chionecetes opilio which agree with those of Yasuda (1973). The Alaskan king crab, Paralithodes camtschatica, shows a very high percentage of desmosterol (31.1% of the total sterol: Idler & Wiseman 1968), cholesterol accounts for 62.3%. There are minor amounts of 24-methylenecholesterol, brassicasterol, 22-dehydrocholesterol, fucosterol and β-sitosterol. Cholesterol has been shown to be the sole sterol of Portunus trituberculatus (Teshima & Kanazawa 1971a).

g. Chirocephalus.

Prior to this investigation, Artemia salina was the only branchipod to be investigated.

Chirocephalus, the fairy shrimp, contained both 4-desmethyl (88.8% of the total sterols) and 4α-methyl sterols (11.2%: Figures 4.17 and 4.18).

The 4α-methyl sterols comprised of 31-norcycloartenol (26.8% of the total 4α-methyl sterols), 31-norcyclolaudenol (70.2%) and 24-methylenelophenol (3.0%).

Cholesterol was the major 4-desmethyl sterol, accounting for 77% of the total. There were high levels of 24-methylenecholesterol (9.6%) and 24-methylcholesterol (8.3%), smaller amounts of 22-dehydrocholesterol (3.0%) and 24-methylcholest-5,22-dien-3β-ol (1.8%) and trace amounts of 24-ethylcholesterol and 24-ethylcholest-5,22-dien-3β-ol.

h. Artemia salina.

Artemia salina contained only 4-desmethyl sterols (Figure 4.19). Again cholesterol was the major sterol (81.1% of the total). There were smaller amounts of 22-dehydrocholesterol (4.1%), 24-methylcholesterol (9.3%), 24-ethylcholesterol (1.7%) and desmosterol (3.9%), with trace amounts of 24-ethylcholest-5,22-dien-3β-ol and 24-methylcholest-5,22-dien-3β-ol.

The sterols of A.salina have been analysed by two other groups. Teshima & Kanazawa (1971c, 1971f, 1972c) have analysed A.salina fed on various algal and yeast diets and reported that cholesterol was the sole sterol. Payne & Kuwahara (1972) concluded that the sterol fraction of A.salina from Mono Lake is composed primarily of cholesterol, with a significant percentage of cholestanol also present.

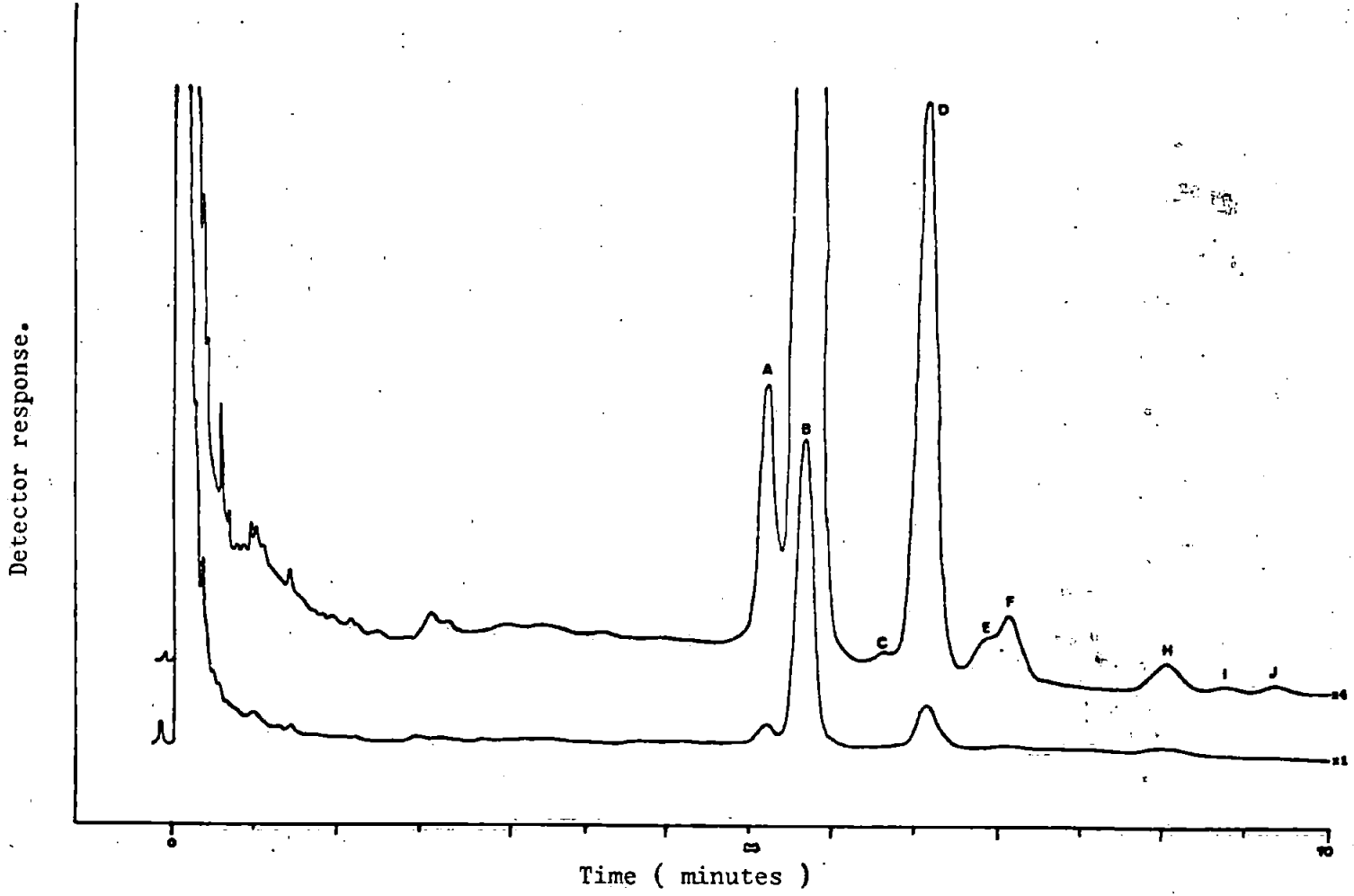


Figure 4.7. GLC analysis (3% OV-17) of 4-desmethyl sterols of Chthalamus stellatus.

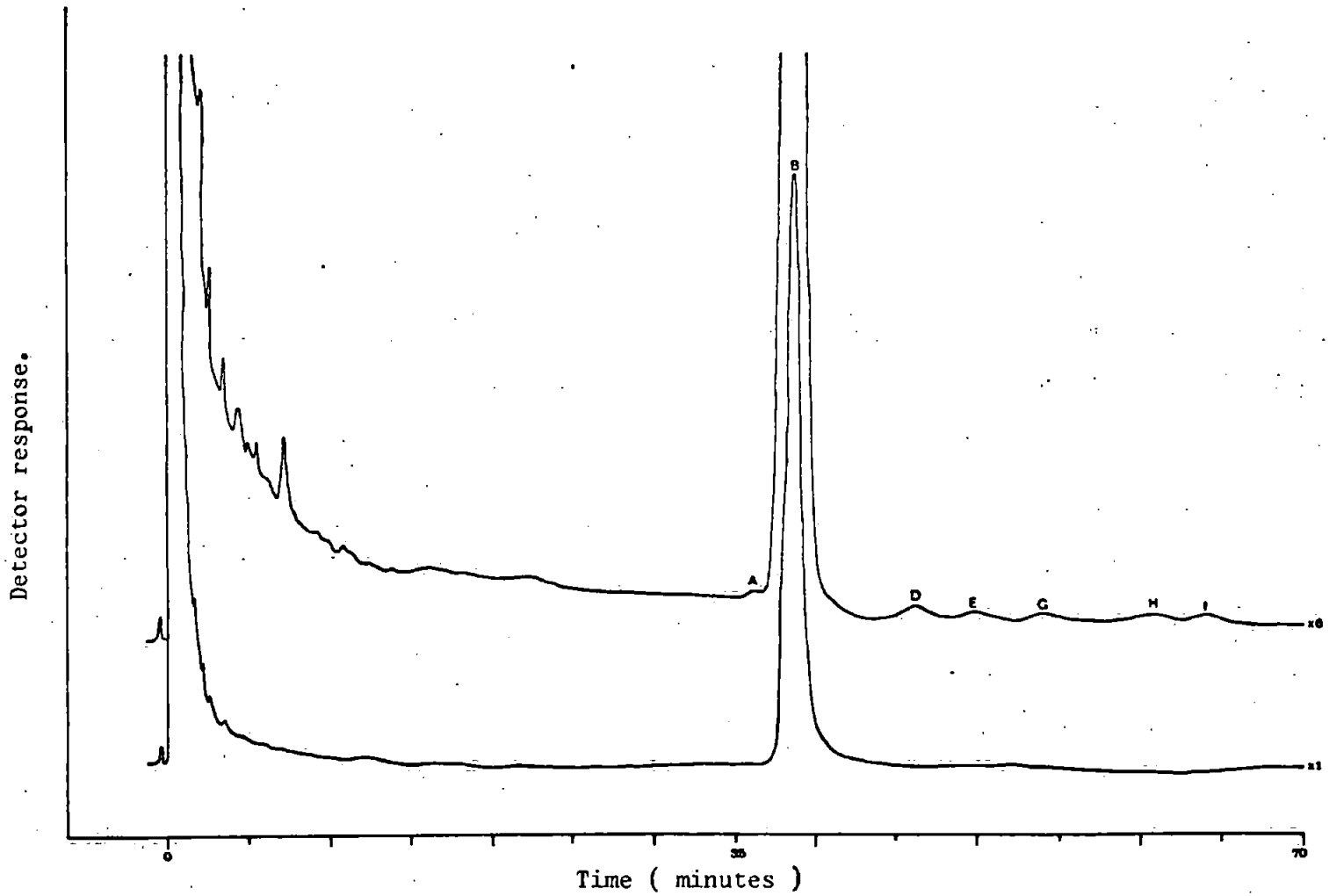


Figure 4.8. GLC analysis (3% OV-17) of 4-desmethyl sterols of Gammarus.

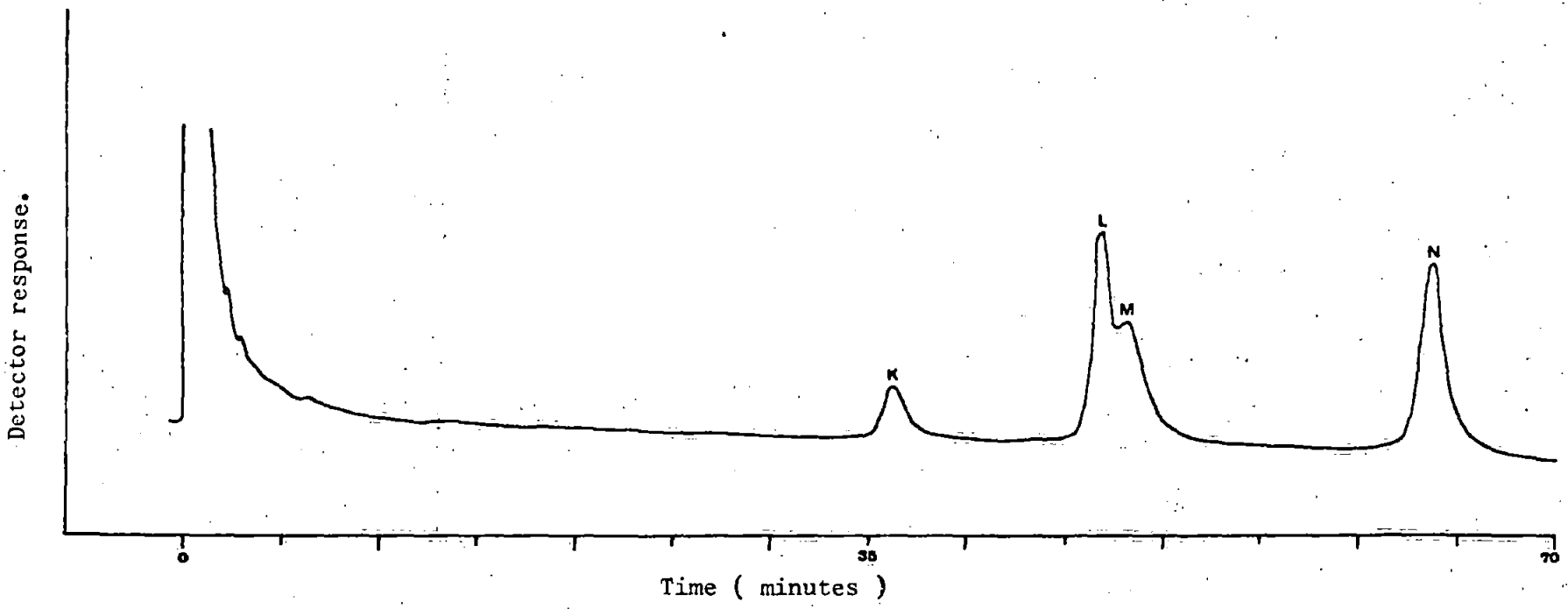


Figure 4.9. GLC analysis (3% OV-17) of 4α-methyl sterols of Gammarus.

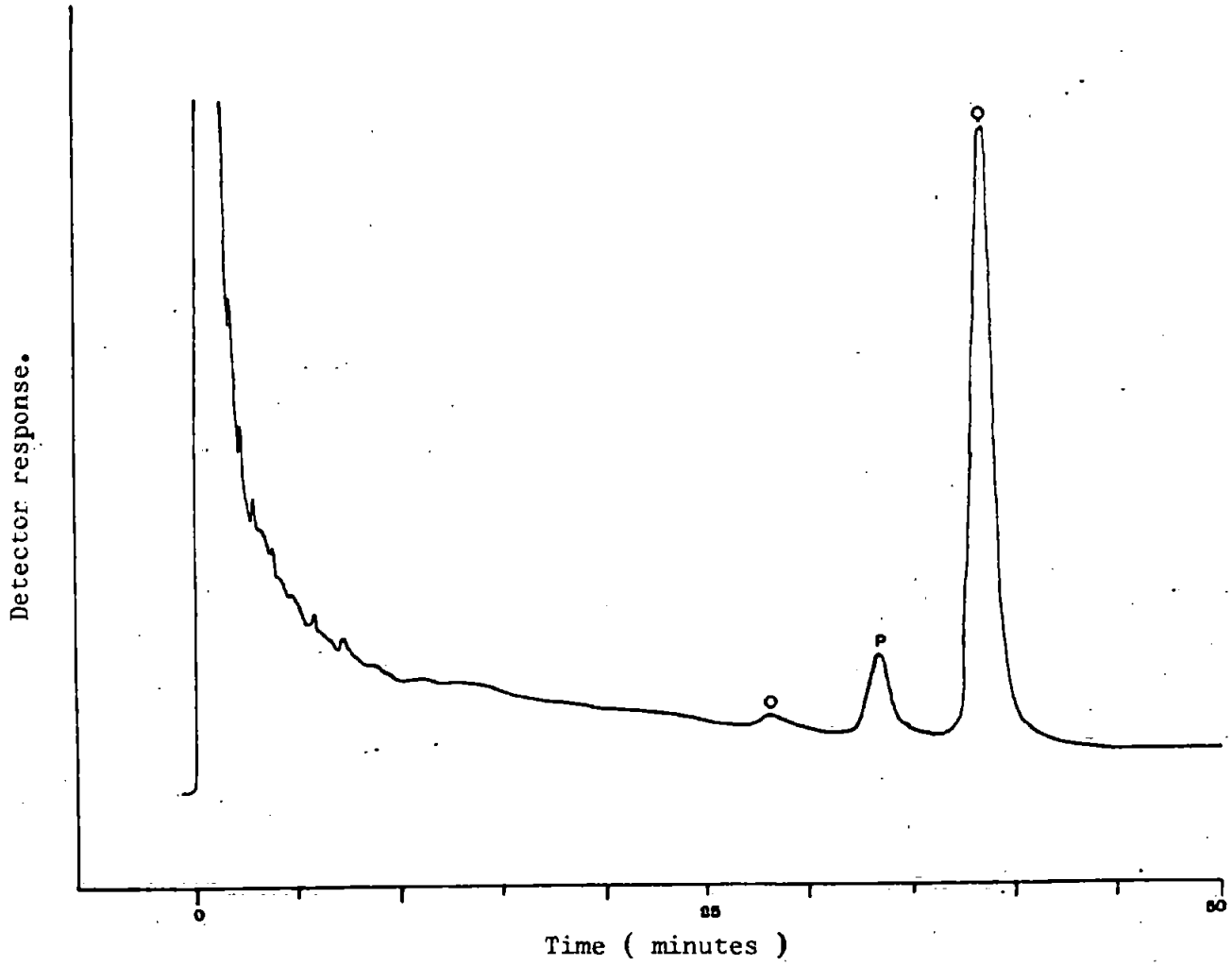


Figure 4.10. GLC analysis (3% OV-17) of 4,4'-dimethyl sterols of Gammarus.

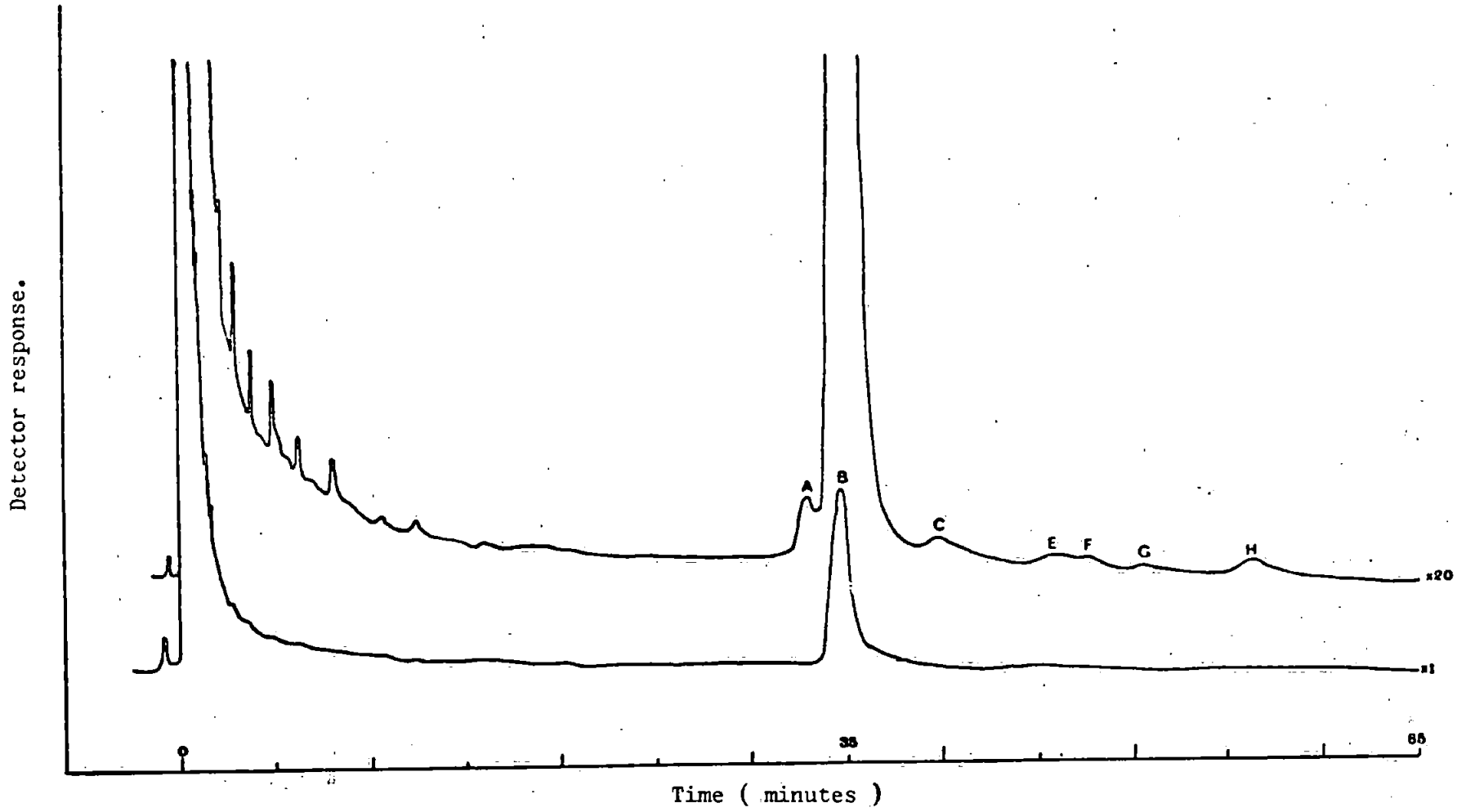


Figure 4.11. GLC analysis (3% OV-17) of 4-desmethyl sterols of Tigriopus brevicornis.

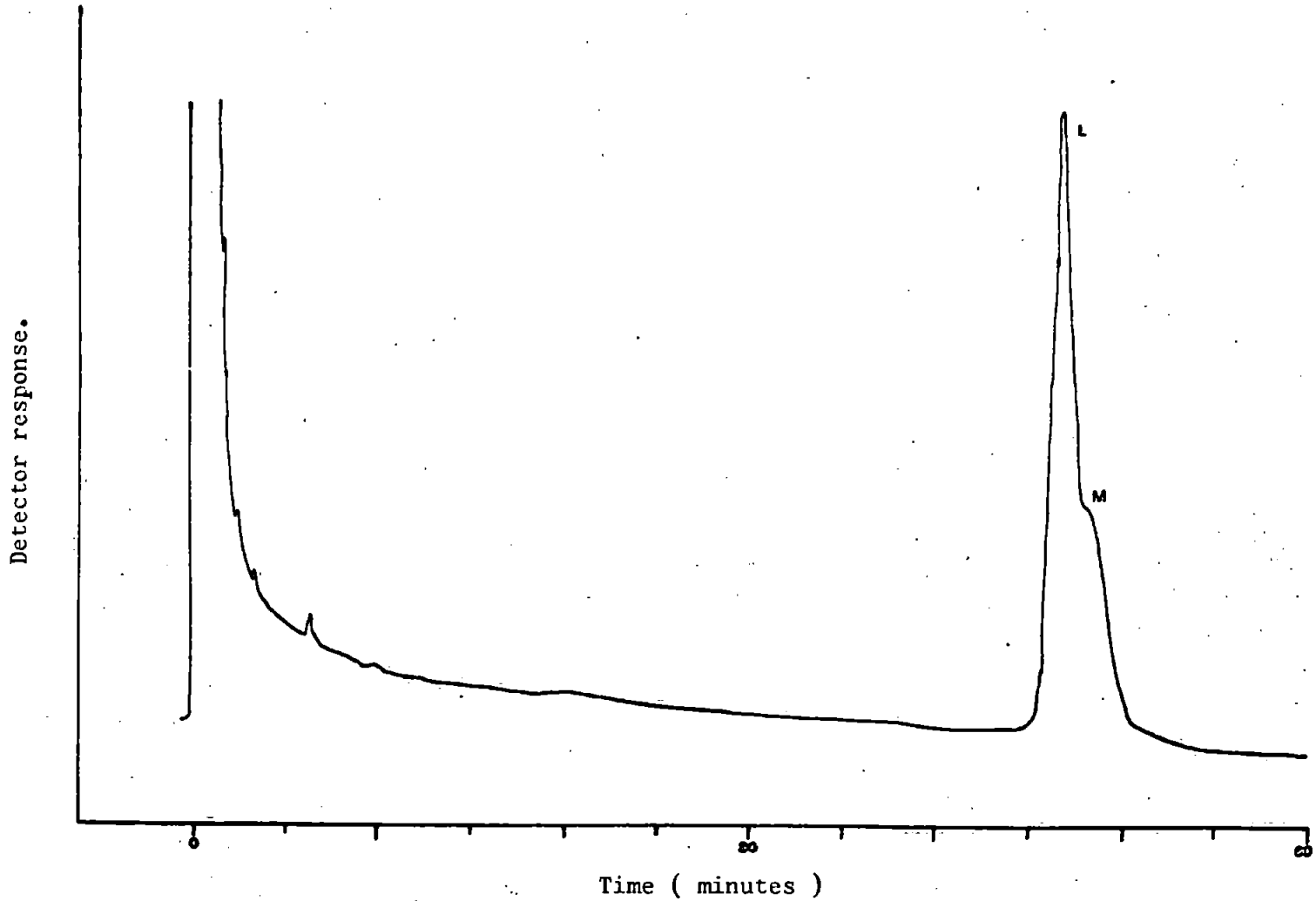


Figure 4.12. GLC analysis (3% OV-17) of 4α-methyl sterols of Tigriopus brevicornis.

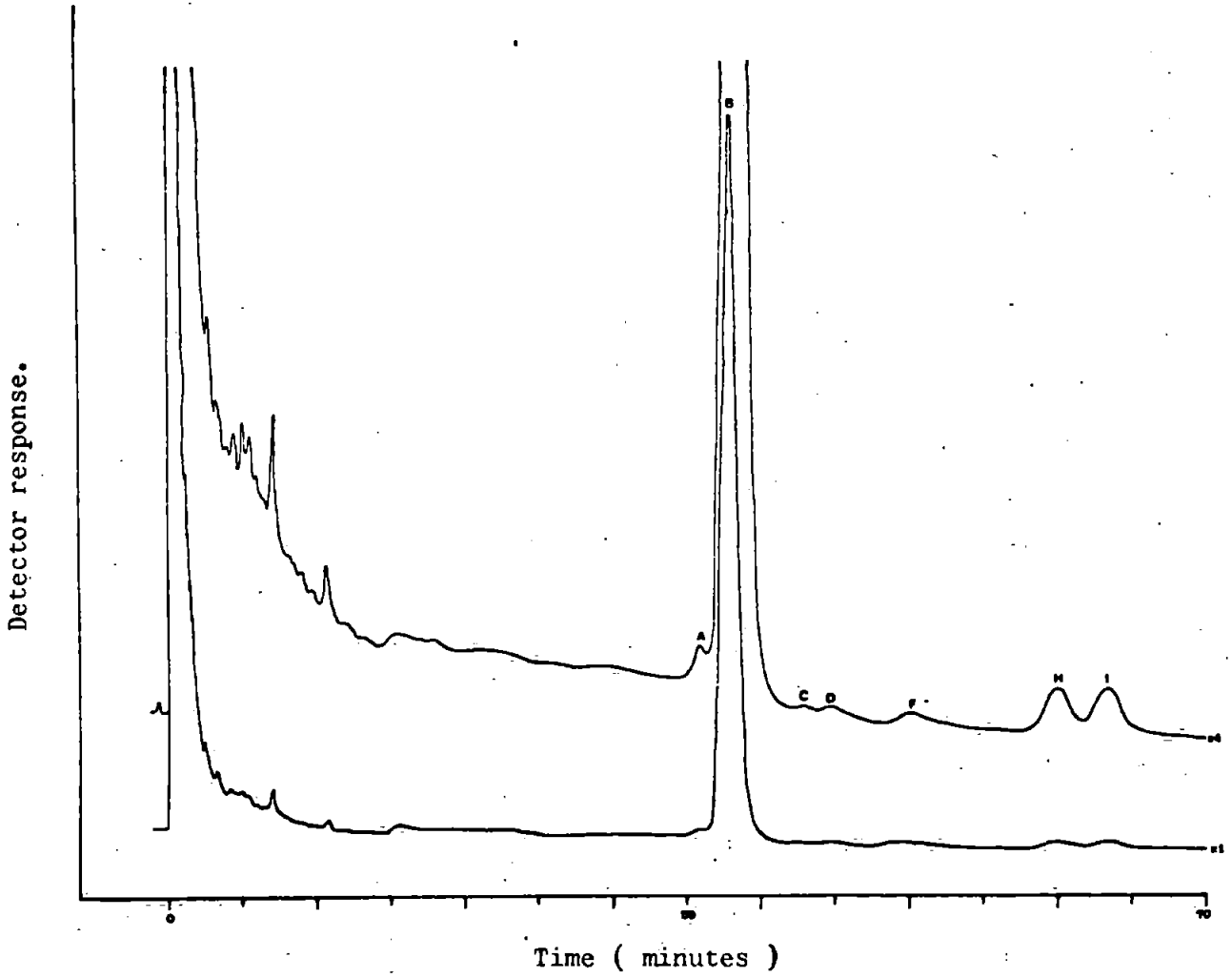


Figure 4.13. GLC analysis (3% OV-17) of 4-desmethyl sterols of Ligia oceanica.

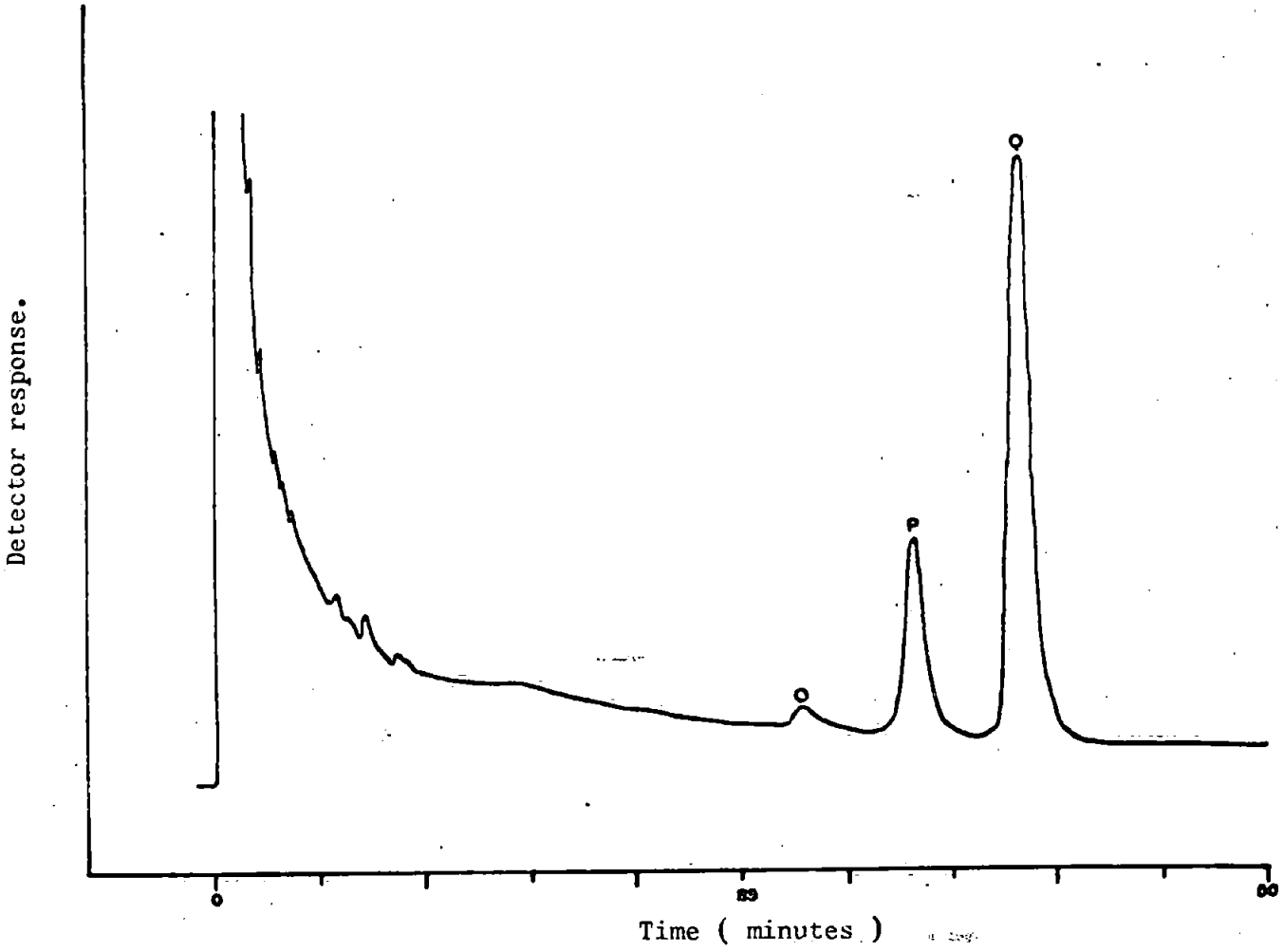


Figure 4.14. GLC analysis (3% OV-17) of 4,4'-dimethyl sterols of Ligia oceanica.

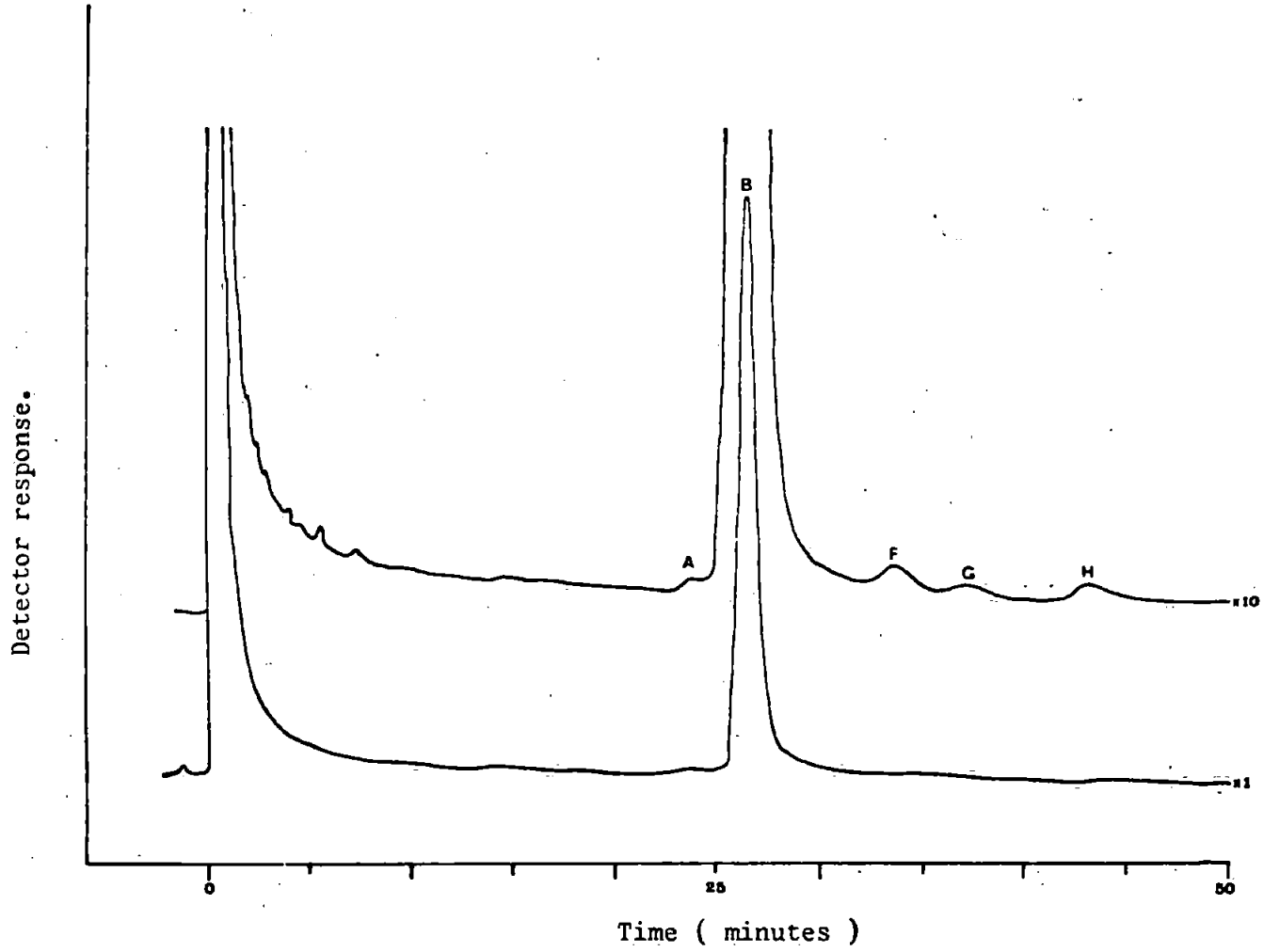


Figure 4.15. GLC analysis (3% OV-17) of 4-desmethyl sterols of Leander serratus.

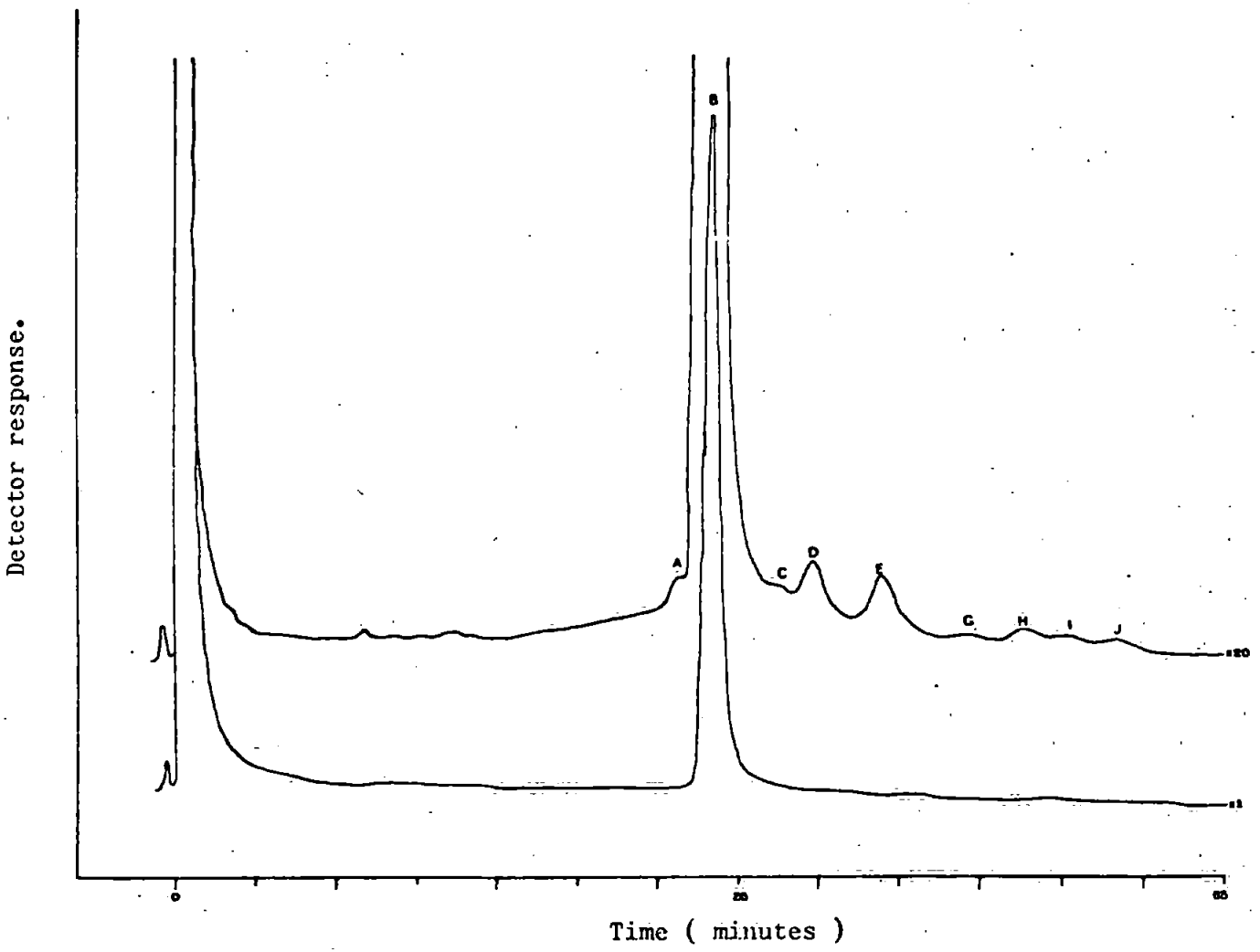


Figure 4.16. GLC analysis (3% OV-17) of 4-desmethyl sterols of Carcinus maenas.

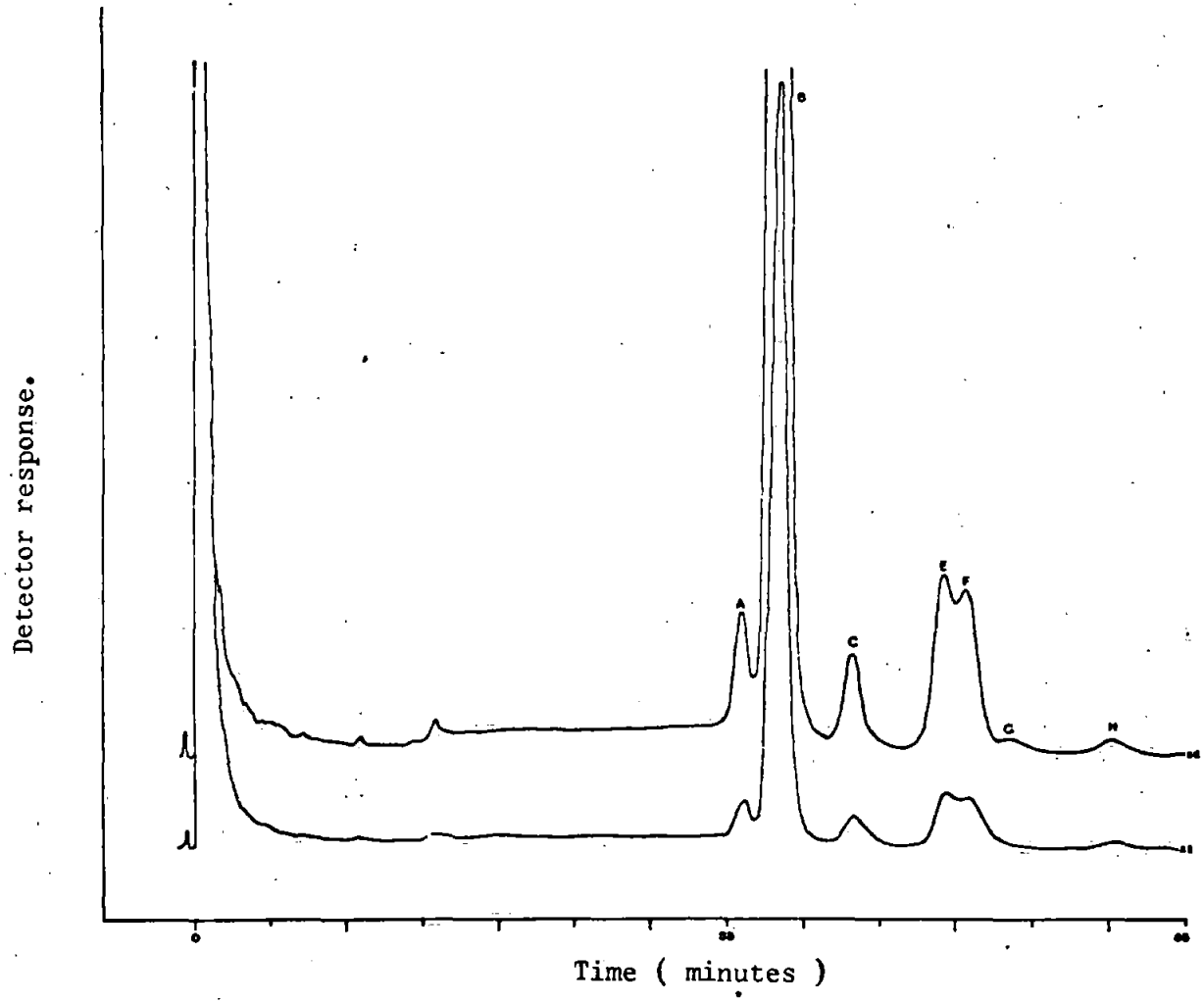


Figure 4.17. GLC analysis (3% OV-17) of 4-desmethyl sterols of Chirocephalus.

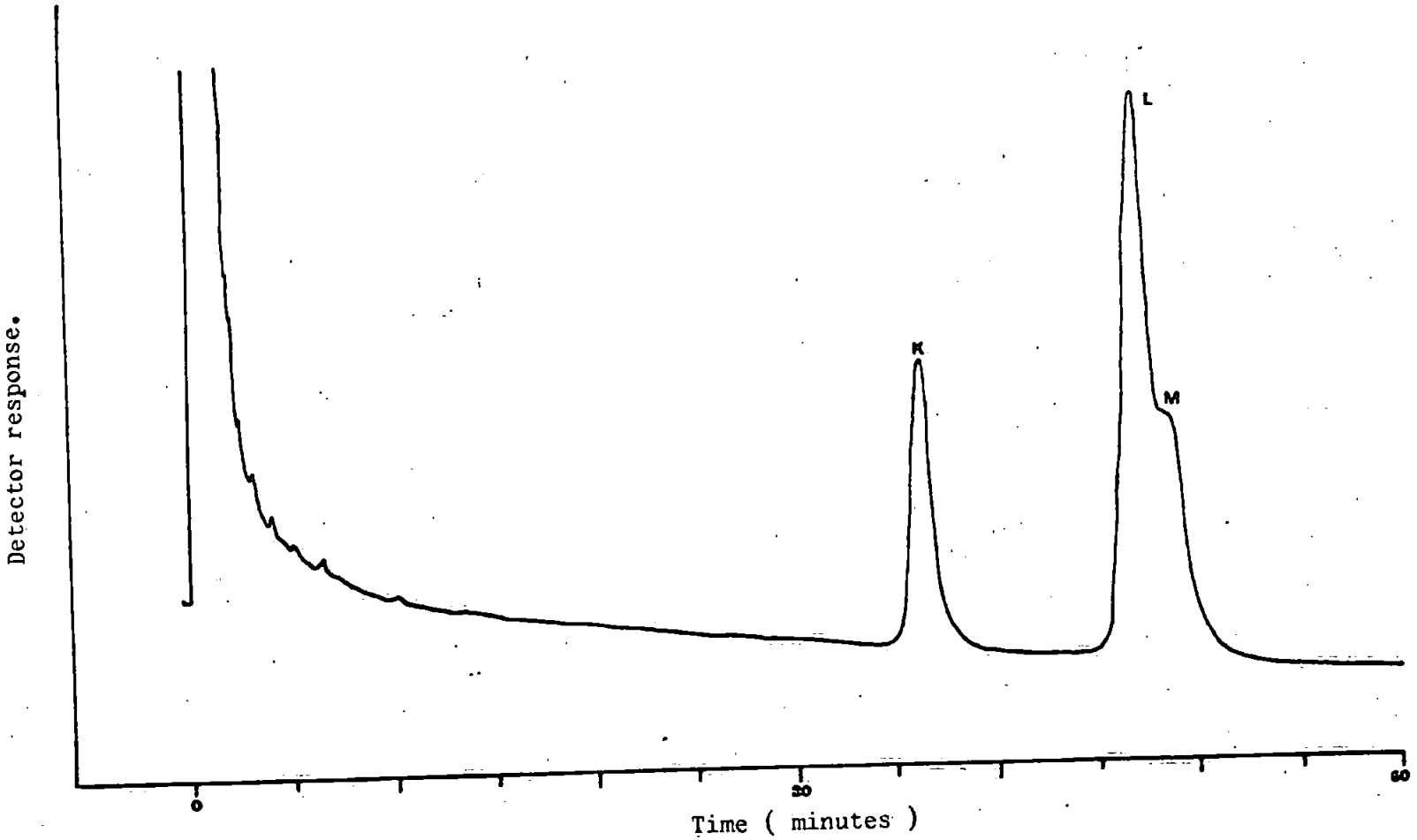


Figure 4.18. GLC analysis (3% OV-17) of 4α-methyl sterols of Chirocephalus.

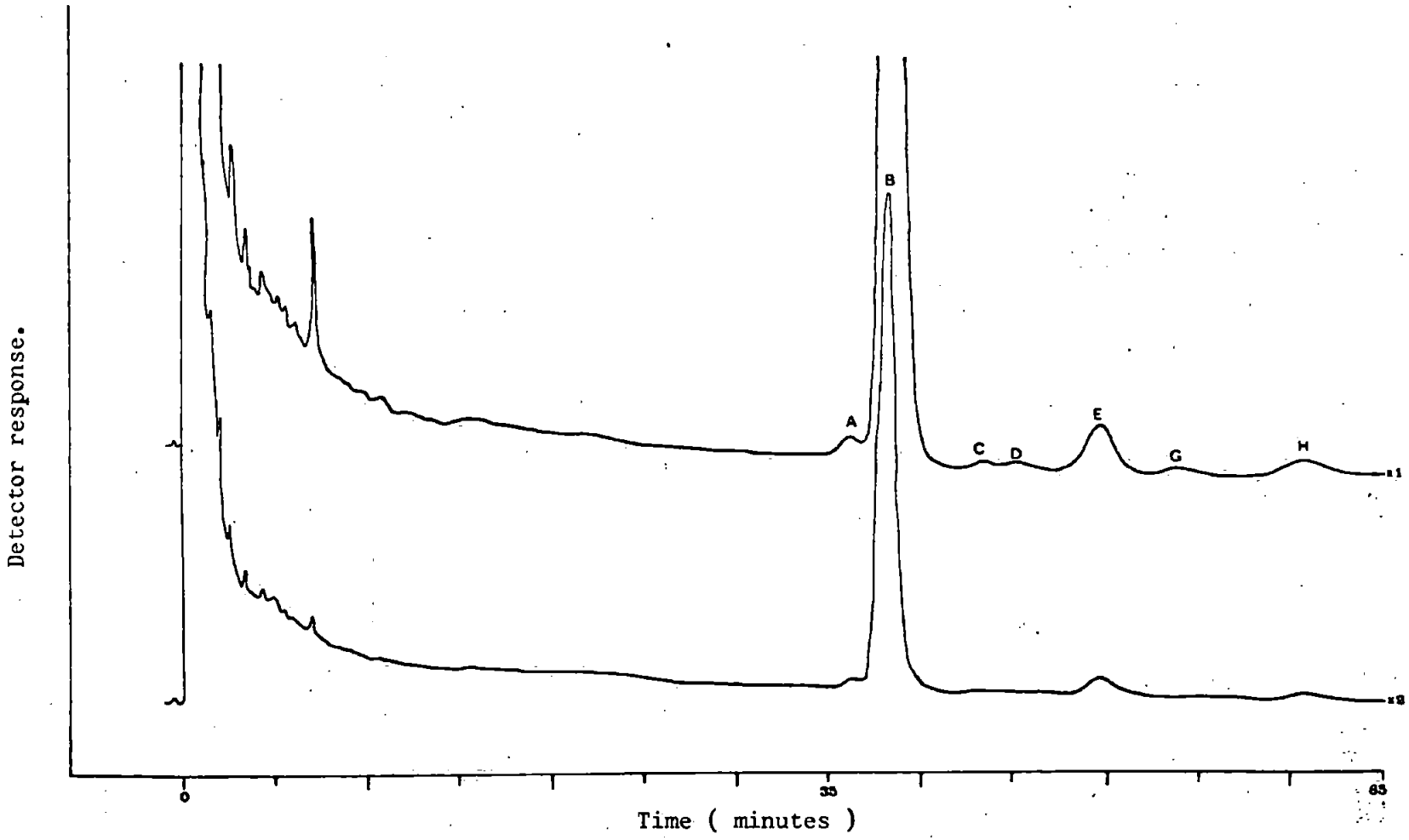


Figure 4.19. GLC analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina.

VI. General discussion.

The sterol compositions of the Crustacea reported here are consistent with the results obtained by other workers (Austin 1970; Idler & Wiseman 1971a; Teshima & Kanazawa 1971a; Yasuda 1973). With one exception, cholesterol is the major sterol in all crustaceans studied to date. It is usually accompanied by a mixture of minor sterols. The one exception to the pattern is the finding of Whitney (1967) that the barnacle, Balanus nubilis, contained more desmosterol (42.4% of the total sterol) than cholesterol (37%). It emerges from these studies that, unlike many other groups of marine invertebrates, Crustacea do not have a characteristic sterol other than cholesterol. Those sterols which are major components of other marine organisms are not so in crustaceans, eg. 24-methylenecholesterol and 22-dehydrocholesterol in molluscs (Idler & Wiseman 1971b, 1972; Teshima & Kanazawa 1972b) and the Δ^7 and $\Delta^{5,7}$ sterols of the Echinodermata (Goad et al 1972).

The high percentage of desmosterol found in Chthalamus stellatus and reported in other barnacles, is not characteristic of the Cirripedia alone since large amounts have been found in various members of the Decapoda (Idler & Wiseman 1971a; Yasuda 1973), Amphipoda and Mysidacea (Teshima & Kanazawa 1971a).

Crustacea, along with the insects, are the most highly developed invertebrates and it is seen that the progression through the invertebrate classes, from the most primitive sponges to Crustacea, is accompanied by a reduction in the number of sterols accompanying cholesterol. This is in accord with the view of Bergmann, that an increase in the complexity of the organism should be accompanied by an increase in the relative amount of cholesterol (Bergmann 1962).

There have been few reports of 4α-methyl and 4,4'-dimethyl

sterols in marine invertebrates. Although the methods used by other workers would detect sterols of these groups, the presence of 4α-methyl and 4,4'-dimethyl sterols is not recorded. From the relevant papers, it would appear that the possible occurrence of these sterols was not investigated and, if this is the case, then 4α-methyl and 4,4'-dimethyl sterols may well be of wider occurrence than hitherto indicated. Goad et al (1972) identified six 4,4'-dimethyl sterols in the echinoderm, Asterias rubens. Cycloartanol and lanosterol were major components; cycloartenol being a minor constituent. Cycloartenol has been tentatively identified in the holothuroid, Stichopus japonicus (Nomura et al 1969a). Lanosterol has been identified as a normal constituent of the lipids of the free-living nematode, Panagrellus redivivus (Willett & Downey 1973). The absence of de novo sterol biosynthesis in nematodes has been demonstrated (Cole & Krusberg 1968; Hieb & Rothstein 1968) and nutritional studies have shown that nematodes require an exogenous source of sterols for normal growth and reproduction (Hieb & Rothstein 1968). Three 4 -methyl sterols were identified in Asterias rubens (Goad et al 1972), 31-norcycloartenol constituting by far the greatest amount.

The sterols present in Crustacea may be derived from:

a. Biosynthesis by the organism.

b. Absorption and modification of the sterols present in dietary organisms.

c. A combination of a and b.

Evidence presented in this thesis (Chapter V) and by other workers (reviewed in Chapters I & V) indicates that those crustaceans studied to date do not have the ability to synthesise sterols.

The crustaceans studied in this investigation were collected from the seashore and were all omniverous. Marine algae and smaller

marine invertebrates would comprise the bulk of their diets. In neither of these groups of organisms is cholesterol the sole or predominant sterol (see references cited in this context in Chapter I). Such a diet would lead to the ingestion of a wide variety of sterols, eg. fucosterol and 28-isofucosterol from Fucales, ergosterol, poriferasterol, haliclonasterol and 24-methylenecholesterol from Ulvales and Chlorococcales (Patterson 1971). In addition to these major sterols, the various compounds which are present in the dietary organisms as intermediates of their own sterol biosynthesis would also be ingested and possibly absorbed.

The 4,4'-dimethyl sterol, cycloartenol, and the 4α-methyl sterols, 31-norcycloartenol and 31-norcyclolaudenol, which contain the 9β,19-cyclopropane ring, together with the 4α-methyl sterols, 24-methylene and 24-ethylidenelophenol, have been isolated from a number of plant sources (see Goodwin 1971 and Rees & Goodwin 1972 for reviews). Cycloartenol is believed to replace lanosterol as the first stable tetracyclic triterpenoid product of squalene-2,3-oxide cyclisation in plants; the other 4,4'-dimethyl and 4α-methyl sterols mentioned are believed to be intermediates in the conversion of cycloartenol to the various phytosterols. Lanosterol has been established as the product of squalene-2,3-oxide cyclisation in animals. Its presence has been reported in some algae (Rees & Goodwin 1972) and a few members of the Euphorbiacea (Goad 1970). Beastall et al (1972) reported the occurrence of 24,25-dihydrolanosterol in the red alga, Porphyridium cruentum, and suggest that it arises from the metabolism of cycloartenol.

If the crustaceans studied were capable of metabolising 4,4'-dimethyl and 4α-methyl sterols, these compounds in the diet could also serve as sources of cholesterol. No animal has yet been found,

however, in which any of the 9β,19-cyclopropane sterols are precursors of 4-desmethyl sterols. It has been shown that rat liver homogenates are incapable of opening the 9β,19-cyclopropane rings of such sterols (Gibbons et al 1971). They are, therefore, probably absorbed from the diet in these crustaceans and not further metabolised. Nomura et al (1969b) concluded that cycloartenol tentatively identified in Stichopus japonicus was derived from the diet. The other sterols with a lanostane derived skeleton could possibly be precursors of 4-desmethyl sterols if extensive sterol metabolism takes place.

Such a diversity in the sterol spectrum of the diet contrasts with the predominance of cholesterol seen in Crustacea. From this one may conclude that crustaceans, as they do not appear to be able to synthesise sterols, must be capable of converting a variety of dietary sterols into cholesterol. It is possible that the exact sterol composition of crustaceans is not fixed but depends on the nature of the sterols present in the diet. In this case, some of the compounds identified may not be of dietary origin but may be intermediates in the conversion of dietary sterols into cholesterol. It was with these considerations in mind that the capacity of Artemia salina to synthesise and metabolise sterols was investigated.

CHAPTER V.

INCORPORATION AND UTILISATION OF ACETATE, MEVALONATE, SQUALENE AND SQUALENE-2,3-OXIDE BY ARTEMIA SALINA.

It is generally accepted that all mammals can synthesise squalene and the various sterols they require from acetate via mevalonate (Bloch 1965). Not all lower animals are capable of such transformations and arthropods in general are incapable of making sterols (see, for example, Clayton 1964; Walton & Pennock 1972). Investigations into the capacity of crustaceans to synthesise sterols have demonstrated that they are unable to incorporate acetate or mevalonate into squalene or sterols (Whitney 1969, 1970; Gosselin 1965; Teshima & Kanazawa 1971c, 1971e; Van den Oord 1964; Zandee 1964, 1966, 1967). It may be presumed that this is due to the loss of one or more of the enzymes of the normal biosynthetic sequence. With one exception, there have been no attempts to locate the site of this blockage. It has been demonstrated that, although Carcinus maenas and Eupagurus bernhardhus failed to incorporate ^{14}C-mevalonate into squalene and sterols, label was incorporated into the isoprene alcohols, farnesol, geranylgeraniol and, tentatively, geraniol (Walton & Pennock 1972). These workers concluded that the occurrence of such intermediates indicated a functional isoprenoid biosynthetic pathway and that the defect in sterol biosynthesis in these organisms occurred at the level of formation of squalene from farnesol pyrophosphate. Similar results have been obtained with the fly, Sarcophaga bullata (Goodfellow et al 1972).

This, however, may not be the only point of blockage in the sterol biosynthetic pathway in such organisms. It has been shown that

the terrestrial annelid, Lumbricus terrestris, is able to synthesise squalene but cannot cyclise this into sterols (Wooton & Wright 1962) and that Sarcophaga bullata, although able to convert squalene into a squalene-2,3-oxide like compound, exhibits no significant level of sterol biosynthesis (Goodfellow & Liu 1972). The free-living nematode, Panagrellus redivivus, has been shown to convert squalene-2,3-oxide to lanosterol but not to cholesterol or other sterols (Willett & Downey 1974).

The initial aim of this present work was therefore:

a. To establish the absence of squalene and sterol biosynthesis in Artemia salina by radiotracer techniques.

b. To identify the location of the blockage(s) in the biosynthetic pathway.

A. Incorporation of ^{14}C-acetate and ^{14}C-mevalonate by A.salina.

The life stages of A.salina used in these experiments were identified according to Provasoli & D'Agostino (1969) and were:

a. Mature adults.

b. Metanauplii IV big.

c. Metanauplii III big.

(See Life Cycle of A.salina in Chapter III).

I. Incubation procedures.

i. Whole organisms.

a. Adults and Metanauplii IV.

The size of these stages enabled them to be separated by sieving and washing in the usual manner. The organisms (approximately 200mg wet weight per culture vessel) were then suspended in the sterile

medium used for voiding the gut ($250cm^3$) as described in the previous chapter. Either ^{14}C-mevalonate (1μCi) or ^{14}C-acetate (2μCi) was then added. It has been suggested that the rate of formation of sterols in invertebrates may be much slower than in mammals (Wooton & Wright 1962). For this reason, incubations were allowed to proceed for 48 hours. Algal growth was prevented by carrying out such incubations in the dark. After this time, the A.salina were thoroughly washed and dried in vacuo. Aliquots of the labelled medium were taken for liquid scintillation counting before and after incubation.

b. Metanauplii III.

A.salina eggs were hatched under the usual conditions of sterility. On leaving such cultures to settle, it was possible to separate metanauplii from egg-cases by aspiration.

$100cm^3$ of medium containing ^{14}C-acetate (2μCi) was autoclaved; the antibiotic mixture and a known volume of sterile metanauplii were then added. Incubation was continued for three days. After this time the yolk of the egg would have been fully absorbed by the metanauplii and they had commenced to feed (Provasoli & D'Agostino 1969).

^{14}C-Mevalonic acid lactone (1μCi) was dissolved in sterile potassium carbonate solution and transferred with sterile medium ($100cm^3$) to a sterile Buchner flask, via a membrane filter. After the addition of antibiotics and a known volume of sterile metanauplii, incubation was continued for three days.

In both cases, the metanauplii were collected by passage through a nylon net (0.5mm mesh), thoroughly washed and dried in vacuo. Aliquots of the labelled medium were taken for liquid scintillation counting before and after incubation.

ii. Cell-free tissue preparations.

a. Preparation of tissue homogenates.

Metanauplii IV and adults were filtered from algae, washed with sterile medium and suspended in a nylon net in the medium used for voiding the gut contents described previously. They were left in this medium for 24 hours. Metanauplii III were separated from the egg-cases by settlement and removing the metanauplii from the surface by aspiration. These were filtered through a nylon net (0.5mm mesh) and washed well with sterile medium. Cell-free tissue homogenates were prepared in the following manner:

The A.salina were homogenised in tris HCl buffer, pH 7.5, enriched with $MgCl_2$ (5mM), $MnCl_2$ (2mM) and nicotinamide (30mM). The homogenate was centrifuged to remove cell debris (800g for 20 minutes at 0°). A thick layer of red oily pigment was invariably obtained, this being particularly pronounced with the metanauplii III. This layer was removed and the supernatant beneath used for the incubations. $5cm^3$ of tissue homogenate were incubated with the following additions: ethanol (6µmoles), ATP (3µmoles), NAD^+ (1µmole), $NADP^+$ (1µmole), glucose-6-phosphate (3µmoles) and glucose-6-phosphate dehydrogenase (10 IU). Radioactive substrates were added as specified in the text. The reaction mixtures were incubated aerobically at 37° in a shaking water bath. A three hour incubation period was used, with further additions of the cofactors (with the exception of enzyme and radiolabelled substrate) at the end of the first and second hours. At the end of the incubation period, reaction was stopped by the addition of 60% aqueous potassium hydroxide ($1cm^3$).

b. Incubations with cell-free tissue preparations.

Tissue homogenates were prepared as described above and incubations were carried out with either ^{14}C-mevalonate (1μCi) or ^{14}C-acetate (1μCi).

II. Results and discussion.

Non-saponifiable lipid was extracted by direct saponification of the cell-free homogenates or dried whole A.salina. Squalene, sterols and isoprenoid alcohols (derived from their pyrophosphates) were isolated by methods previously described in Chapter II.

The results obtained from such incubations are detailed in Tables 5.2 to 5.8. At all stages, the whole organisms assimilated both ^{14}C-acetate and ^{14}C-mevalonate (Table 5.2). The levels of ^{14}C-mevalonate assimilation were less variable than the levels of ^{14}C-acetate assimilation. Small amounts of this assimilated activity were found to be present in the non-saponifiable lipid fraction (Table 5.3) but this was associated with neither squalene nor sterols (Table 5.5). A much greater assimilation of the substrates into the isoprenoid alcohols was seen (Table 5.3) Separation of these alcohols and elution of the material from TLC plates showed that farnesol, geraniol and the isomeric pentenols had incorporated label (Table 5.4).

Similar results were obtained from incubations with cell-free preparations. Assimilation of ^{14}C-mevalonate and ^{14}C-acetate into the non-saponifiable lipid fraction and distribution of activity between the isoprenoid alcohols were comparable with those obtained from incubations with whole organisms. The similarity in the figures from both incubations would indicate that the uptake of the radiolabelled substrate is not the limiting factor, rather the utilisation of the compound.

As stated previously, the incorporation of label from ^{14}C-mevalonate into farnesol, geraniol and geranylgeraniol (as free alcohols) in the non-saponifiable lipid fractions from two decapod crustaceans has been demonstrated (Walton & Pennock 1972). These workers also located label in the ubiquinone, UQ_{10}, and in various dolichols. In these present experiments, the small amount of radioactivity present in the non-saponifiable lipid was not associated with squalene or sterols and it is possible that it had been incorporated into ubiquinone and dolichol fractions, although positive localisation was not attempted.

A.salina is capable of incorporating both mevalonate and acetate into the isoprenoid pyrophosphates up to, and including, farnesol. The formation of these labelled isoprenoids show that there is a functional pathway for the biosynthesis of isoprene units in A.salina. The failure to incorporate label into squalene would indicate the lack of the enzyme system synthesising squalene from farnesol pyrophosphate, that is squalene synthetase. There does not appear to be any accumulation of farnesol and this would suggest that it is not a 'dead-end' product.

Although, in general, young, rapidly growing animals have a greater rate of sterol synthesis than older animals (Gould 1958), there was no detectable synthesis of sterols or squalene throughout the life cycle of A.salina. If endogenous sterol synthesis did occur it should have been detected in the larval stages, where rapid growth and metamorphosis demand a supply of sterols. Even these stages, however, did not incorporate sterol precursors.

The next stage in the synthesis of sterols, the formation of squalene-2,3-oxide from squalene via the squalene oxidase system, was then investigated.

B. Incorporation of ^{14}C-squalene by adult Artemia salina.

I. Preparation of biosynthetically labelled squalene.

Squalene was labelled biosynthetically by the method of Bucher & McGarrahan (1956) as modified by Popjak (1965).

Adult male albino rats were killed by cervical fracture and the livers perfused *in situ* for a few seconds with ice cold potassium phosphate buffer solution (0.1M, pH 7.4) to which had been added $MgCl_2$ (5mM), $MnCl_2$ (2mM) and nicotinamide (30mM). The livers were removed and homogenised in a Potter homogeniser with the same buffer. Cell debris was then removed by centrifugation at 800g for 20 minutes at 0^o. The supernatant was separated and recentrifuged (10,000g for 30 minutes at 0^o). The supernatant from the second centrifugation was used for the incubations.

For maximum conversion of substrate to squalene, the following additions were made: ATP (3mM), $NADP^+$ (1mM) and glucose-6-phosphate (3mM). The mixture was thoroughly degassed at a water pump and saturated with oxygen-free nitrogen. The labelled substrate (1μCi per 5cm^3 of supernatant) was added under a stream of nitrogen. The mixture was then incubated with the exclusion of air for 3 hours at 37^o in a shaking water bath. Further additions of the cofactors were made at the end of the first and second hours. Squalene was extracted from the mixture by the usual methods and purified via the formation of the thiourea adduct.

For ^{14}C-squalene the substrate was DL-mevalonic acid-2-^{14}C and for ^3H-squalene the substrate was ^3H-methyl acetic acid. The total and specific activities of squalene prepared by the above methods are given in Table 5.1.

Table 5.1. Total and specific activities of biosynthetically labelled squalene.

Substrate	Total activity (dpm)	Specific activity (dpm/mg)
Mevalonate-2-^{14}C (5μCi)	3.5×10^6	8.75×10^5
^{3}H-Methyl acetate (10μCi)	6.7×10^6	1.26×10^6

II. Incubation procedures.

i. Whole organisms.

Emulsions of squalene were prepared in the manner described in Chapter II and were injected aseptically into cultures of A.salina. The cultures were harvested after complete grazing of the food organism and gut contents voided in the usual manner. Squalene, squalene-2,3-oxide, 4-desmethyl and 4,4'-dimethyl sterols were isolated by methods already described in Chapter II.

ii. Cell-free tissue preparations.

Cell-free tissue homogenates were prepared by and incubations carried out by methods previously described in this chapter. ^{14}C-Squalene was added as an emulsion. Squalene, squalene-2,3-oxide, 4-desmethyl and 4,4'-dimethyl sterols were isolated by the methods detailed in Chapter II.

III. Results.

The results obtained from such incubations are shown in Table 5.9. Whilst there was some uptake of ^{14}C-squalene by the whole organism, there was no conversion of squalene into any of its possible metabolic

products - squalene-2,3-oxide, 4-desmethyl or 4,4'-dimethyl sterols. Similar results were obtained with cell-free preparations. This demonstrates the absence of the squalene oxidase system.

Experiments were now performed to establish the presence or absence of the squalene-2,3-oxide cyclase system, which catalyses the conversion of squalene-2,3-oxide to lanosterol.

C. Incorporation of ^{3}H-squalene-2,3-oxide by adult Artemia salina.

I. Synthesis of ^{3}H-squalene-2,3-oxide.

^{3}H-Squalene was obtained by anaerobic incubation of ^{3}H-methyl acetic acid with rat liver homogenates as described in the previous section of this chapter.

N-Bromosuccinimide (2.64mg) was added to a solution of ^{3}H-squalene (6.7×10^{6} dpm) in tetrahydrofuran ($0.15cm^{3}$) and water ($0.05cm^{3}$). The reaction products were extracted with hexane and chromatographed on silica gel plates with 15% ethyl acetate in benzene as developing solvent. The material chromatographing immeadiately prior to a squalene marker was removed and eluted. The products obtained from the hydrolysis of this compound were separated by TLC on silica gel plates with 5% ethyl acetate in benzene as developing solvent. The material co-chromatographing with a squalene-2,3-oxide marker was removed, eluted and, after the addition of carrier squalene-2,3-oxide, was rechromatographed in the same system. A total activity of 1.25μCi was obtained.

II. Incubation procedures.

Incubations of ^{3}H-squalene-2,3-oxide with whole organisms and cell-free preparations were carried out by the same methods as described for ^{14}C-squalene.

III. Results.

The results obtained from such incubations are shown in Table 5.9. Whilst there was some uptake of ^{3}H-squalene-2,3-oxide by the whole organism, there was no conversion of squalene-2,3-oxide into any of its possible metabolic products - 4-desmethyl or 4,4'-dimethyl sterols. Similar results were obtained with cell-free preparations. This demonstrates the absence of the squalene-2,3-oxide cyclase system in Artemia salina.

D. Discussion.

It has been demonstrated that Artemia salina is unable to synthesise sterols from acetate, mevalonate, squalene or squalene-2,3-oxide. It appears that there are blockages at, at least, three sites in the pathway. Three enzyme systems are lacking - squalene synthetase, squalene oxidase and squalene-2,3-oxide cyclase. This substantiates a previous report that A.salina could not incorporate ^{14}C-acetate into squalene or sterols (Teshima & Kanazawa 1971c). Isotopic assay techniques have previously been used to demonstrate the absence of squalene and sterol biosynthesis in the crayfish - Astacus astacus (Zandee 1966) and Astacus fluviatilis (Gosselin 1965), the lobsters - Homarus vulgaris (Zandee 1964) and Homarus gammarus (Zandee 1967), the prawns - Penaeus japonicus and Panulirus japonica (Teshima & Kanazawa 1971e), the crabs - Cancer pagurus (Van den Oord 1964), Callinectes sapidus (Whitney 1970), Portunus trituberculatus (Teshima & Kanazawa 1971e), Carcinus maenas and Eupagurus bernhardus (Walton & Pennock 1972) and the barnacle - Balanus nubilis (Whitney 1970). The larvae of the mud crab, Rhithropanopeus harrisii, and of the spider crab, Libinia emarginata, have also been investigated and have yielded similar results (Whitney 1969).

With the exception of A.salina and B.nubilis, the crustaceans so far studied have been decapods and the absence of sterol biosynthesis in three classes of crustaceans does not preclude the possibility of other classes being able to carry out such syntheses.

With the exception of the experiments using the larval stages of R.harrisii and L.emarginata (Whitney 1969), previous studies have been concentrated on adult animals. Very little is known of the affect of age or moulting on sterol metabolism in crustaceans, although it has been demonstrated that constituents such as lipid, DNA and RNA do vary in amount with the moult cycle (see, for example, Armitage et al 1972; Cook & Gabbott 1972; Dagg & Littlepage 1972; Bollenbacher et al 1972; Humphreys & Stevenson 1973; Lynch & Webb 1973; Renaud 1949; McWhinnie & Kirchenberg 1962). Many crustaceans have a marked periodicity of moulting and reproduction. The photoperiod plays a major role in the timing of both moulting and ovarian development (Aiken 1969a, 1969b; Armitage et al 1973; Perryman 1969). Lipid metabolism has been shown to vary throughout the moult cycle (Rice & Armitage 1974). It is a well known feature of the marine ecosystem that many pelagic organisms undergo a diurnal vertical migration. Although there is a vast literature on the subject of depth distribution and migration (Cushing 1951; Banse 1964), relatively little is known about the physiological and biochemical consequences of such a rhythmic behaviour. It has been shown recently that there is a variation in the sterol ester content of a decapod, Acanthephyra purpurea, over the diurnal migration period (Morris 1973). Under the culture conditions employed for A.salina, the photoperiod has been eliminated and any natural diurnal migrations that A.salina might have were removed. Although the results presented above indicate that A.salina is unable to synthesise squalene and sterols

throughout its life cycle and that this inability is unaffected by age, it should be borne in mind that, in its natural environment, when A. salina is subjected to the interplay of the above mentioned systems, it is possible that there may be departures from this scheme.

The location of label in isoprenoid alcohols shows that A. salina has the enzymes necessary for isoprenoid biosynthesis and, judging by the rate of conversion of precursors to products in the three hour incubation periods used for the cell-free preparations, the results indicate that this biosynthetic pathway is functional. The significance of such syntheses to Crustacea is difficult to ascertain as reports of terpene biosynthesis in these organisms are non-existent. Similarly, it is difficult to use like reactions in insects for the purposes of analogy, for again terpenoid biosynthesis has been subject to few investigations in Insecta. The presence of many and varied terpenes and terpene-derived structures in insects is well documented (Gilbert 1967; Bernhardi et al 1967; Briggs 1961; Hughes 1973; Pavan & Dazzini 1971; Rios & Perez 1969; Wientjens et al 1973) but whether any of these terpenes are in fact synthesised by the insect is a matter of conjecture. These terpenoid structures have many roles in insects; they have been identified as sex pheromones, trail substances, population attractants, alarm and defensive secretions (Evans & Green 1973; Pavan & Dazzini 1971). The juvenile hormone is thought to be derived from farnesol (Bowers & Thompson 1963; Slama 1971).

No particular defensive substances are known as yet in crustaceans. Evidence has been presented for the existence of sex pheromones in some species of Crustacea (Katona 1973; Ryan 1966). The presence of chemoreceptors has been demonstrated in Crustacea but these have been investigated chiefly from the point of view of food detection

(Mackie 1973; McCleese 1970, 1973; Tazaki & Shigenaga 1974). Some hermit crabs and the shrimp, Saron marmoratus, may utilise 'grouping pheromones' (Hazlett 1966; Krushwitz 1967); these substances have been shown to be diffusible but the structures have not been elucidated. The crustacean eyestalk has yielded material with juvenile hormone activity when bioassayed in insect systems (Schneiderman & Gilbert 1958) and it has been shown that premature metamorphosis is induced in larvae of Balanus galeatus by synthetic juvenile hormones (Gomez et al 1973). No juvenile hormone, however, has yet been isolated from a crustacean.

These various aspects of crustacean physiology and biochemistry are largely unexplored but it is possible that mechanisms are present in Crustacea similar to those known to exist in insects. Whether these mechanisms are initiated through chemically similar compounds remains to be discovered.

Table 5.2. Incorporation of ^{14}C-mevalonate and ^{14}C-acetate by Artemia salina (whole organisms).

Life stage	Substrate	Initial Activity	Total dpm Incorporated	% Incorporation	Dry weight
Metanauplii III	Acetate	2μCi	541,000	12.3	0.47g
	MVA	1μCi	99,000	4.5	0.37g
Metanauplii IV	Acetate	2μCi	356,000	8.1	26.3mg
	MVA	1μCi	202,000	9.2	30.5mg
Adult	Acetate	2μCi	1,311,000	29.8	73.2mg
	MVA	1μCi	138,000	6.3	41.5mg

Table 5.3. Distribution of activity incorporated from ^{14}C-mevalonate and ^{14}C-acetate by Artemia salina (whole organisms).

Life stage	Substrate	Non-saponifiable lipid fraction		Isoprenol fraction[1]	
		Total dpm	%[2]	Total dpm	%[2]
Metanauplii III	Acetate	1569	0.29	14066	2.6
	MVA	812	0.82	4257	4.3
Metanauplii IV	Acetate	2884	0.81	22784	6.4
	MVA	808	0.40	11918	5.9
Adult	Acetate	51129	0.39	40641	3.1
	MVA	1518	1.10	5796	4.2

[1] Derived from isoprenoid pyrophosphates.

[2] Expressed as a percentage of the incorporated activity.

Table 5.4. Distribution of activity incorporated into isoprenoid pyrophosphates from ^{14}C-mevalonate and ^{14}C-acetate by Artemia salina (whole organisms).

Life stage	Substrate	Isoprenol alcohol fractions[1]					
		Farnesol		Geraniol		Pentenols	
		Total dpm	%[2]	Total dpm	%[2]	Total dpm	%[2]
Metanauplii III	Acetate	647	4.6	408	2.9	1223	8.7
	MVA	396	9.3	217	5.1	630	14.8
Metanauplii IV	Acetate	1663	7.3	1025	4.5	501	2.2
	MVA	1024	8.6	750	6.3	1071	9.0
Adult	Acetate	528	1.3	1382	3.4	2479	6.1
	MVA	243	4.2	417	7.2	567	9.8

[1]Derived from isoprenoid pyrophosphates.

[2]Expressed as a percentage of total isoprenoid fraction activity.

Table 5.5. Distribution of activity incorporated into non-saponifiable lipids from ^{14}C-mevalonate and ^{14}C-acetate by Artemia salina (whole organisms).

Life stage	Substrate	Non-saponifiable lipid fraction			Crude sterol fraction			Sterol fraction		Crude squalene fraction			Squalene fraction	
		Weight	Total dpm	Spec Act[1]	Weight	Total dpm	Spec Act	Weight	Total dpm	Weight	Total dpm	Spec Act	Weight	Total dpm
Metanauplii III	Acetate	12.6mg	1569	124.5	9.2mg[2]	0	0	0	0	8.5mg[2]	40	4.7	8.0mg	0
	MVA	6.2mg	812	130.9	7.6mg[2]	0	0	0	0	9.0mg[2]	0	0	0	0
Metanauplii IV	Acetate	20.9mg[2]	2884	137.9	8.4mg	99	11.8	7.9mg	0	8.9mg	0	0	0	0
	MVA	21.3mg[2]	808	37.9	7.9mg	0	0	0	0	9.3mg	103	11.6	8.8mg	0
Adult	Acetate	26.4mg[2]	51129	1936	8.7mg	123	14.1	8.1mg	0	11.1mg	55	4.9	9.9mg	0
	MVA	19.9mg[2]	1518	76.3	9.4mg	73	7.7	8.8mg	0	9.7mg	74	7.6	9.0mg	0

[1]Specific activity (dpm/mg).

[2]Approximately 10mg each of carrier cholesterol and squalene added.

Table 5.6. Incorporation of activity from ^{14}C-mevalonate and ^{14}C-acetate into non-saponifiable lipid and isoprenol alcohol fractions by Artemia salina (cell-free preparations).

Life stage	Substrate[1]	Non-saponifiable lipid		Isoprenol fraction[2]	
		Total dpm	%[3]	Total dpm	%[3]
Metanauplii III	Acetate	3080	0.14	50,600	2.3
	MVA	7100	0.32	129,800	5.9
Metanauplii IV	Acetate	5280	0.24	92,400	4.2
	MVA	1760	0.80	70,400	3.2
Adult	Acetate	440	0.02	59,400	2.7
	MVA	4180	0.19	41,800	1.9

[1]Initial activity of substrate was 1μCi in each case.

[2]Derived from isoprenoid pyrophosphates.

[3]Expressed as a percentage of the initial activity.

Table 5.7. Distribution of activity incorporated into isoprenoid pyrophosphates from ^{14}C-mevalonate and ^{14}C-acetate by Artemia salina (cell-free preparations).

Life stage	Substrate	Isoprenol alcohol fractions[1]					
		Farnesol		Geraniol		Pentenols	
		Total dpm	%[2]	Total dpm	%[2]	Total dpm	%[2]
Metanauplii III	Acetate	10192	3.2	21758	4.3	32384	6.4
	MVA	11163	8.6	8307	6.4	8177	6.3
Metanauplii IV	Acetate	45276	4.9	25872	2.8	44352	4.8
	MVA	39424	5.6	40832	5.8	67584	9.6
Adult	Acetate	9504	1.6	14256	2.4	19008	3.2
	MVA	15884	3.8	28424	6.8	28006	6.7

[1]Derived from isoprenoid pyrophosphates.

[2]Expressed as a percentage of total isoprenoid fraction activity.

Table 5.8. Distribution of activity incorporated into non-saponifiable lipids from ^{14}C-mevalonate and ^{14}C-acetate by Artemia salina (cell-free preparations).

Life stage	Substrate	Non-saponifiable lipid fraction			Crude sterol[2] fraction			Sterol fraction		Crude squalene[2] fraction			Squalene fraction	
		Weight	Total dpm	Spec Act[1]	Weight	Total dpm	Spec Act	Weight	Total dpm	Weight	Total dpm	Spec Act	Weight	Total dpm
Metanauplii III	Acetate	2.2[3]	3080	1400	8.6	0	0	0	0	10.6	0	0	0	0
	MVA	1.9	7100	3737	9.8	0	0	0	0	11.3	120	10.6	10.6	0
Metanauplii IV	Acetate	6.3	5280	838	9.9	0	0	0	0	11.0	0	0	0	0
	MVA	5.8	1760	303	9.1	24	2.6	8.8	0	10.8	96	8.8	10.1	0
Adult	Acetate	8.4	440	52	9.2	50	5.4	8.7	0	8.9	0	0	0	0
	MVA	8.7	4180	480	10.1	76	7.5	9.6	0	9.4	0	0	0	0

[1]Specific activity (dpm/mg).

[2]Approximately 10mg each of carrier cholesterol and squalene added.

[3]All weights given are in mgs.

Table 5.9. Incorporation and utilisation of ^{14}C-squalene and ^{3}H-squalene-2,3-oxide by Artemia salina (whole organisms and cell free preparations

	^{14}C-squalene		^{3}H-squalene-2,3-oxide	
	Whole organisms	Cell-free preparations	Whole organisms	Cell-free preparations
Substrate added	2,500,000[1]	750,000	2,300,000	650,000
Activity recovered in				
Non-saponifiable lipid fraction	36,890	727,500	27,640	639,000
Cholesterol[2]	94[3]	36[3]	210[3]	0
Lanosterol[2]	38[3]	0	150[3]	50[3]
Squalene-2,3-oxide[2]	0	0	26,175	608,967
Squalene[2]	33,939	690,000	0	0
% activity of non-saponifiable lipid[4]	92	94.8	94.7	95.3

[1]All activities given are total dpm.

[2]Refers to areas of TLC plates that correspond to reference compounds.

[3]Activity disappears when rechromatographed with added carrier.

[4]Activity recovered in the added substrate expressed as a percentage of the activity present in the non-saponifiable lipid fraction.

CHAPTER VI.

METABOLISM OF STEROLS BY ARTEMIA SALINA.

A. Introduction.

In Chapters III and V, it has been established that Artemia salina is incapable of the synthesis of sterols but can utilise and metabolise dietary sterols. It has been shown that various crustaceans are able to convert 4-desmethyl sterols into cholesterol (Kanazawa et al 1971b; Teshima 1971a, 1971b; Teshima & Kanazawa 1971c, 1971f, 1972c, 1972d). The conversion of ^{14}C-β-sitosterol to cholesterol has been demonstrated in the prawn, Penaeus japonicus, and in the crab, Portunus trituberculatus (Teshima 1971a; Teshima & Kanazawa 1971d). It has been shown that A.salina is able to convert brassicasterol (Teshima & Kanazawa 1971c), ergosterol (Teshima & Kanazawa 1971f), β-sitosterol and 24-methylcholesterol (Teshima 1971a) into cholesterol. A similar conversion from ergosterol and β-sitosterol has been demonstrated in Portunus trituberculatus (Teshima 1971b; Teshima & Kanazawa 1972d).

There have been few reports of the ability of crustaceans to synthesise steroids from exogenous precursors. In a recent study, the conversion of ^{14}C-cholesterol into 'polar steroids' by a variety of tissues from the prawn, Penaeus japonicus, was demonstrated. There were marked variations in such conversions over the moult cycle (Guary & Kanazawa 1973). Similar variations in the uptake and turnover of ^{14}C-cholesterol by the crab, Hemigrapsus nudus, have recently been shown (Spaziani & Kater 1973). These workers were able to identify products chromatographically related to α- and β-ecdysone. Kanazawa & Teshima (1971a) have demonstrated the in vivo conversion of ^{14}C-cholesterol into

steroids in various tissues of the spiny lobster, Panulirus japonicus. Using a series of chromatographic systems, these workers identified 17α-hydroxyprogesterone, progesterone, androstenedione and testosterone as products from the hepatopancreas, ovaries and blood. Corticosterone and deoxycorticosterone were detected in the hepatopancreas.

A series of experiments are described in this chapter which were carried out to investigate the capacity of A.salina to metabolise a variety of sterols which it would be likely to encounter in its diet. These sterols were cholesterol, desmosterol, poriferasterol, β-sitosterol and stigmasterol.

The identification of 4,4'-dimethyl and 4α-methyl sterols in various crustaceans, coupled with the widespread occurrence of such compounds in planktonic algae, where they are obligatory intermediates in sterol biosynthesis (Goodwin 1971), prompted an investigation into the capacity of A.salina to metabolise members of these groups of sterols. The 4α-methyl sterol, cycloeucalenol, and the 4,4'-dimethyl sterols 24,25-dihydrolanosterol, lanosterol, 24-methylenecycloartanol and cycloartenol were used.

B. Materials.

^{3}H-Sterols were prepared by exchange-labelling as detailed in the methods section below. 2-^{14}C-Cholesterol, 24-^{14}C-desmosterol and 2-^{14}C-β-sitosterol were purchased from the Radiochemical Centre, Amersham.

C. Methods.

Incubations of whole organisms and cell-free preparations with emulsions of radiolabelled sterols were carried out by methods identical to those used for ^{14}C-squalene and ^{3}H-squalene-2,3-oxide. These methods

are described in detail in the preceding chapter.

I. Preparation of ^{3}H-sterols.

i. Preparation of 2,4-^{3}H-stigmasterol.

The methods used for the exchange-labelling of sterols are described in detail for stigmasterol and are illustrated in Scheme 6.1. Other sterols were labelled in the same manner.

a. Preparation of 24α-ethylcholesta-4,22-3-one.

The 3-ketone of stigmasterol was prepared by an Oppenauer oxidation (Rees et al 1966). Stigmasterol (I: 53.7mg) was dissolved in dry toluene (10cm^3); cyclohexanone (0.5cm^3) was added and a small amount of toluene distilled off. Aluminium isopropoxide (30mg) in toluene (10cm^3) was added and the mixture refluxed for 45 minutes with the condenser protected by a calcium chloride tube. A concentrated solution of sodium potassium tartrate (5cm^3) was added and the mixture steam-distilled to remove cyclohexanone. The mixture was then extracted with ether (x3) and the combined ether extracts washed with concentrated sodium potassium tartrate solution, 3N sulphuric acid and water (x3). The ethereal solution was dried over anhydrous sodium sulphate. Most of the solvent was removed in vacuo and the residue taken to dryness under a jet of nitrogen. 24α-Ethylcholesta-4,22-dien-3-one (II) was separated from the other reaction products and starting material by TLC on silica gel with 5% ethyl acetate in benzene as developing solvent. The 24α-ethylcholesta-4,22-dien-3-one was eluted and crystallised from acetone/water to give 36.9mg of white plates, mpt. 124-125°. An infra-red spectrum showed no hydroxyl peak at 3500cm^{-1} and a strong peak at 1680cm^{-1} for an α, β -unsaturated ketone.

b. Exchange-labelling of 24α-ethylcholesta-4,22-dien-3-one.

The method used was that of Klein & Knight (1965). Basic aluminium oxide (10g) was kept at 120° for 16 hours. On cooling, a quantity of benzene/hexane 1:1 v:v was added. Tritiated water (10mCi: 2μl) was added and the mixture shaken on a Gallenkamp flask shaker for 24 hours. The alumina was packed in a glass column with 20 column volumes of benzene/hexane 1:1 v:v, followed by elution with five column volumes of benzene. The 24α-ethylcholesta-4,22-dien-3-one was washed onto the column in benzene solution and left on the column for approximately 4 hours. The mixture was then eluted with benzene ($200cm^3$: flow rate approximately $2cm^3$/min) and the benzene solution dried over anhydrous sodium sulphate. After filtering and evaporating the solvent in vacuo, a crystalline residue of 2,4-^{3}H-24α-ethylcholesta-4,22-dien-3-one (III) was obtained.

c. Preparation of 2,4-^{3}H-24α-ethylcholesta-3,5,22-trien-3-acetate.

2,4-^{3}H-24α-ethylcholesta-4,22-dien-3-one was dissolved in acetyl chloride ($10cm^3$) and acetic anhydride ($1cm^3$: Chaudhuri & Gut 1969). The mixture was refluxed for two hours, diluted with an equal volume of water and extracted with ether (x3). The ethereal solution was dried over anhydrous sodium sulphate and evaporated in vacuo to give the crude 2,4-^{3}H-24α-ethylcholesta-3,5,22-trien-3-acetate (IV).

d. Recovery of stigmasterol.

The crude acetylated residue from the above preparation was dissolved in absolute ethanol ($20cm^3$) containing two drops of pyridine. A solution of sodium borohydride (100mg) in absolute ethanol ($20cm^3$) was added and the mixture left stirring overnight at room temperature. Excess sodium borohydride was destroyed by the addition of dilute hydrochloric acid ($20cm^3$). Ethanol was removed by evaporation in vacuo and

the resultant solution extracted with ether (x3). The combined ether extracts were washed with water (x3) and dried over anhydrous sodium sulphate.

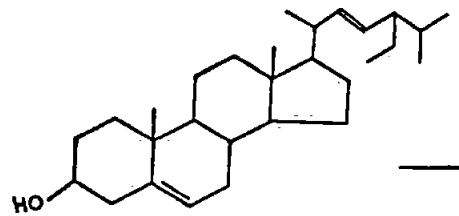
After a digitonin precipitation on this crude residue, 2,4-^{3}H-24α-ethylcholesta-5,22-dien-3β-ol (stigmasterol: V) was purified by TLC on silica gel with chloroform as developing solvent. 2,4-^{3}H-stigmasterol was eluted and recrystallised from acetone/water to give 15.1mg of white plates, mpt. 170-172° (lit. value 170-171°: Thompson et al 1970). Total activity was 4.7μCi, specific activity 0.31μCi/mg. The preparation cochromatographed with authentic stigmasterol on GLC (3% OV-17 and 1% SE-30).

ii. Preparation of 2,4-^{3}H-poriferasterol.

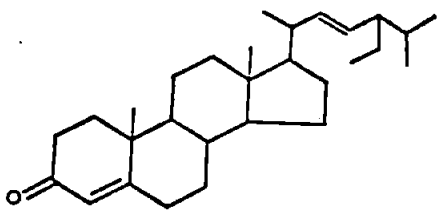
This was prepared in the same manner as stigmasterol. Poriferasterol (30mg) was oxidised to 24β-ethylcholesta-5,22-dien-3-one which was separated from the other reaction products and starting material by TLC on silica gel with 5% ethyl acetate in benzene as developing solvent. The 24β-ethylcholesta-5,22-dien-3-one was eluted and recrystallised from acetone/water to give 21mg of white plates, which gave a sharp melting point at 112°. The infra-red spectrum showed no hydroxyl absorption at 3500cm^{-1} but strong absorption at 1680cm^{-1} for an α,β-unsaturated ketone. After exchange-labelling by passage down an alumina column and reduction of the acetate with sodium borohydride, 2,4-^{3}H-24β-ethylcholesta-5,22-dien-3β-ol was precipitated via the digitonide. The crude sterol was purified by TLC on silica gel with chloroform as developing solvent and recrystallised from acetone/water to give 7.2mg of white needles, mpt. 155-157° (lit. value 154-155°: Smith 1969). Total activity was 3.1μCi, specific activity 0.43μCi/mg. The preparation

Scheme 6.1. General procedure for the preparation of 2,4-^{3}H-desmethyl sterols.

Stigmasterol as model compound.



(I)



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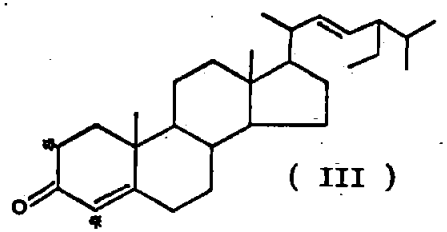
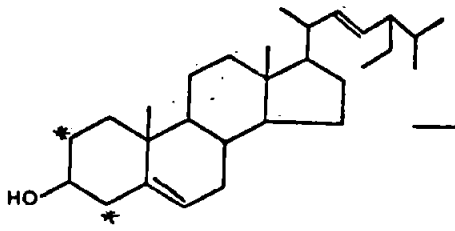
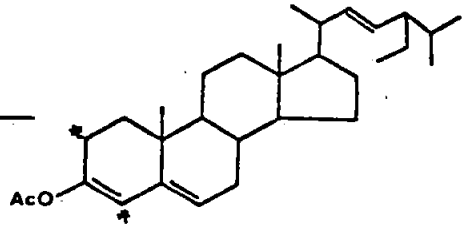


(III)

(V)



(IV)



cochromatographed with authentic poriferasterol on GLC (3% OV-17 and 1% SE-30).

iii. Preparation of 2-^{3}H-lanosterol.

A solution of lanosterol (20mg) in ice cold acetone (5cm^{3}) was bubbled with nitrogen and chromic acid reagent added dropwise until a faint brown colour persisted (Lenton et al 1971). The mixture was diluted with an equal volume of water and extracted with ether (x3). The ethereal solution was evaporated in vacuo to give a crystalline residue. The lanost-8,24-dien-3-one was separated from other reaction products and starting material by TLC on silica gel with chloroform as developing solvent. After exchange-labelling by passage through an alumina column, the recovered 2-^{3}H-lanosta-8,24-dien-3-one was refluxed with lithium aluminium hydride in anhydrous ether (20cm^{3}) for two hours. Excess lithium aluminium hydride was destroyed by the careful addition of ethyl acetate. The reaction mixture was poured into dilute sulphuric acid (25cm^{3}) and the 2-^{3}H-lanosterol extracted with ether. The 2-^{3}H-lanosterol was purified by TLC on silica gel with chloroform as developing solvent and recrystallised from acetone/water to give 6.6mg of crystals, which had a total activity of 27.4μCi and a specific activity of 4.15μCi/mg. The preparation cochromatographed with authentic lanosterol on GLC (3% OV-17 and 1% SE-30).

iv. Preparation of 2-^{3}H-24,25-dihydrolanosterol.

24,25-Dihydrolanosterol (20mg) was oxidised by the method outlined above to give, after TLC separation, 16mg of lanosta-8-en-3-one. This was exchange-labelled and the 2-^{3}H-lanosta-8-en-3-one produced reduced with lithium aluminium hydride. The recovered tritiated sterol,

2-^{3}H-24,25-dihydrolanosterol, was purified by TLC on silica gel with chloroform as developing solvent and recrystallised from acetone/water to give 6.4mg of white plates, which had a total activity of 21.38μCi and a specific activity of 3.34μCi/mg. The preparation cochromatographed with authentic 24,25-dihydrolanosterol on GLC (3% OV-17 and 1% SE-30).

v. Preparation of 2-^{3}H-cycloartenol.

Cycloartenol (40mg) was oxidised with chromic acid reagent by the procedure described above to give cycloartenone (32mg). This was labelled with tritium by exchange-labelling and the 2-^{3}H-cycloartenone produced reduced with lithium aluminium hydride to give, after purification by TLC (with chloroform as developing solvent) and recrystallisation from acetone/water, 27.6mg of crystals. These had a total activity of 210.2μCi and a specific activity of 7.6μCi/mg. The preparation cochromatographed with authentic cycloartenol on GLC (3% OV-17 and 1% SE-30). The principle mass spectral fragments are shown in Table 6.4 and Figure 6.3 and are consistent with published data (Benveniste et al 1966; Audier et al 1966).

vi. Preparation of 2-^{3}H-24-methylenecycloartanol.

24-Methylenecycloartanol (33mg) was oxidised with chromic acid reagent to give 24-methylenecycloartanone (27mg). This was exchange-labelled with tritium and the 2-^{3}H-24-methylenecycloartanone produced reduced with lithium aluminium hydride to give, after purification by TLC (with chloroform as developing solvent) and recrystallisation from acetone/water, 17.8mg of crystals. These had a total activity of 41.3μCi and a specific activity of 2.31μCi/mg. The preparation cochromatographed with authentic 24-methylenecycloartanol on GLC (3% OV-17 and 1% SE-30).

The principle mass spectral fragments are shown in Table 6.2 and Figure 6.1 and are consistent with published data (Knapp & Nicholas 1969; Audier et al 1966).

vii. Preparation of 2,4-^{3}H-cycloeucalenol.

Cycloeucalenol (11mg) was oxidised with chromic acid reagent by the procedure described above to give cycloeucalenone (6.7mg). This was exchange-labelled with tritium and the 2,4-^{3}H-cycloeucalenone produced reduced with lithium aluminium hydride to give, after purification by TLC (with chloroform as developing solvent) and recrystallisation from acetone/water, 3.4mg of crystals. These had a total activity of 9.4μCi and a specific activity of 2.76μCi/mg. The preparation cochromatographed with authentic cycloeucalenol on GLC (3% OV-17 and 1% SE-30). The principle mass spectral fragments are shown in Table 6.3 and Figure 6.2 and are consistent with published data (Knapp & Nicholas 1969; Audier et al 1966).

D. Results.

I. Incubations with 4-desmethyl sterols.

i. Incubations with whole organisms.

The results of incubations of adult Artemia salina with radiolabelled 4-desmethyl sterol emulsions are given in Tables 6.5 and 6.6. All sterols administered were assimilated into the non-saponifiable lipid fraction, but the levels of assimilation were very low, ranging from 0.43% (percentage of assimilated activity) for ^{14}C-cholesterol to 0.08% for 2,4-^{3}H-stigmasterol.

Table 6.1. Activities (total and specific) of 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterols exchange-labelled with tritium.

Sterol	Total activity	Specific activity
	μCi	μCi/mg
2,4-^{3}H-Stigmasterol	4.7	0.31
2,4-^{3}H-Poriferasterol	3.1	0.43
2-^{3}H-Lanosterol	27.4	4.15
2-^{3}H-24,25-Dihydrolanosterol	21.4	3.34
2-^{3}H-Cycloartenol	210.2	7.60
2-^{3}H-24-Methylenecycloartenol	41.3	2.31
2,4-^{3}H-Cycloeucalenol	9.4	2.76

Table 6.2. Mass spectrum of 24-methylenecycloartanol.

Peak at m/e	Relative Abundance %	Fragmentation
440	65	M^+
425	53	M^+ - (methyl)
422	100	M^+ - (water)
407	74	M^+ - (methyl + water)
397	6	M^+ - (terminal isopropyl)
379	38	M^+ - (terminal isopropyl + water)
364	9	M^+ - (terminal isopropyl + water + methyl)
353	29	
315	22	M^+ - (sidechain)
300	94	Fission of ring A
297	23	M^+ - (sidechain + water)

M^+ = Molecular ion peak for 24-methylenecycloartanol.

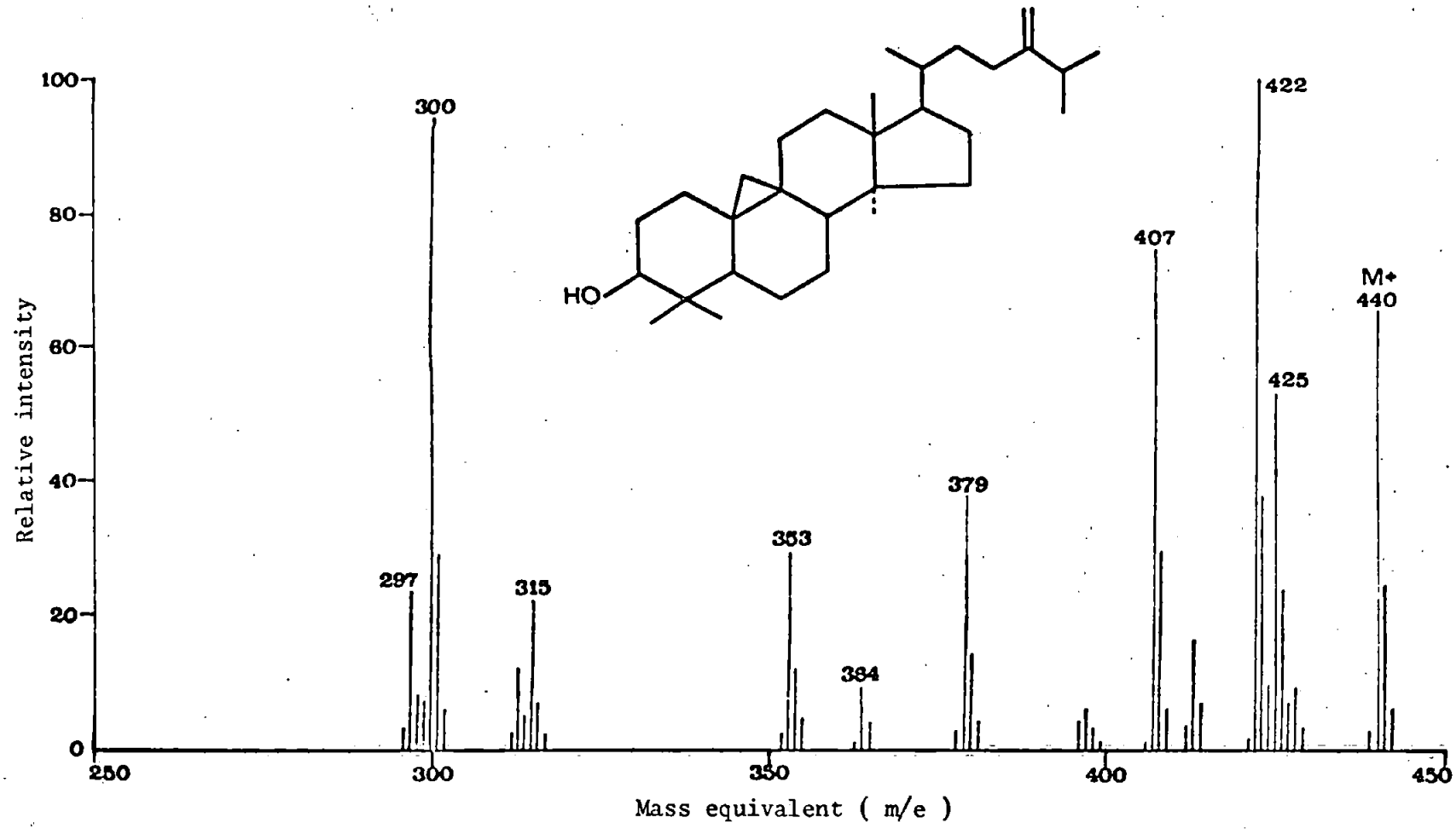


Figure 6.1. Mass spectrum of 2-^{3}H-24-methylenecycloartanol.

Table 6.3. Mass spectrum of cycloeucalenol.

Peak at m/e	Relative Abundance %	Fragmentation
426	47	M^+
411	47	M^+ - (methyl)
408	100	M^+ - (water)
393	63	M^+ - (methyl + water)
383	6	M^+ - (terminal isopropyl)
365	4	M^+ - (terminal isopropyl + water)
353	14	Loss of carbons 2, 3 and 4.
300	63	Fission of ring A
283	14	
285	18	

M^+ = Molecular ion peak for cycloeucalenol.

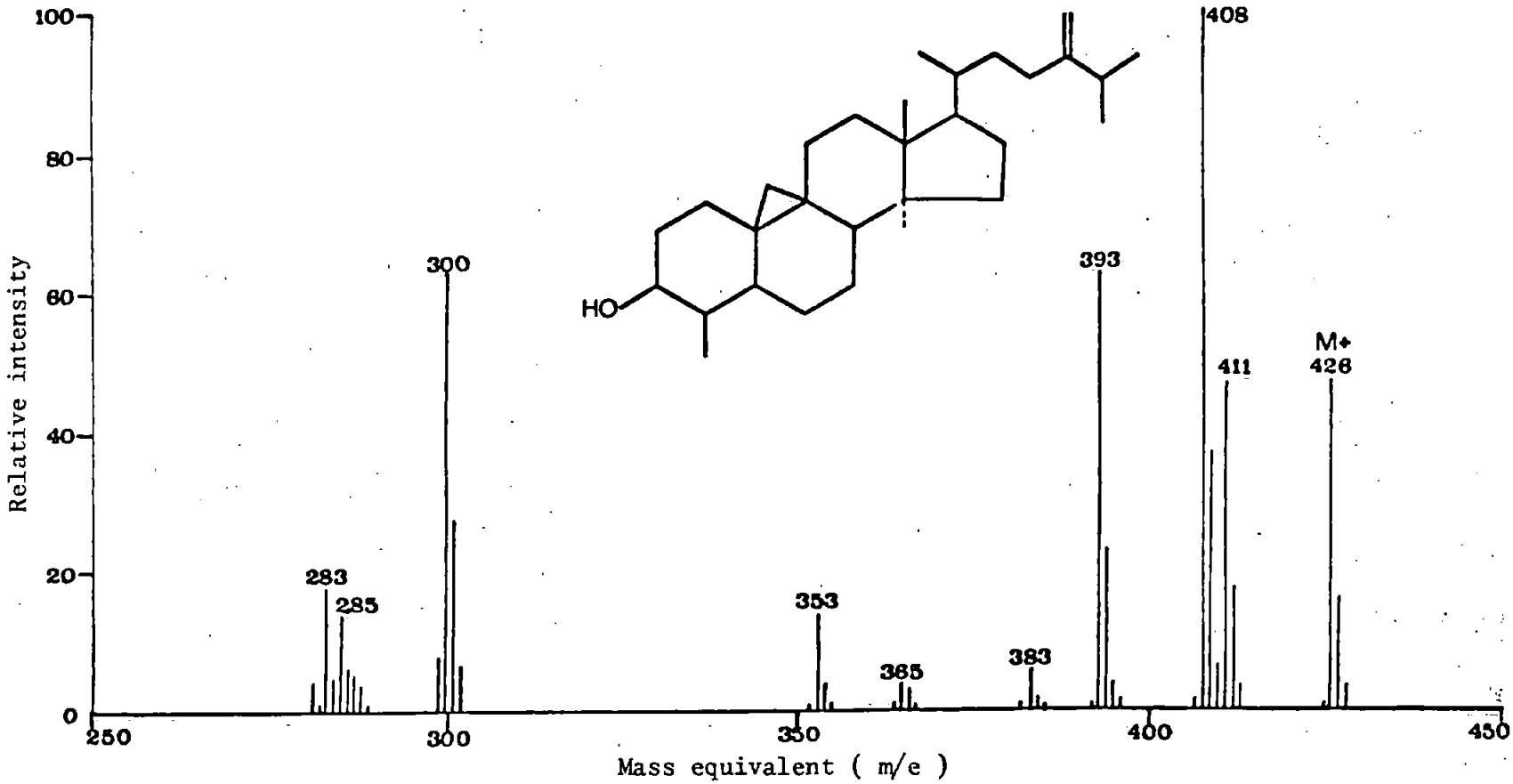


Figure 6.2. Mass spectrum of 2,4-^{3}H-cycloeucalenol.

Table 6.4. Mass spectrum of cycloartenol.

Peak at m/e	Relative Abundance %	Fragmentation
426	73	M^+
411	61	M^+ - (methyl)
408	100	M^+ - (water)
393	87	M^+ - (methyl + water)
365	38	M^+ - (terminal isopropyl + water)
357	7	
339	46	Loss of carbons 2, 3 and 4
315	12	M^+ - (sidechain)
297	15	M^+ - (sidechain + water)
286	98	Fission of Ring A
271	35	

M^+ = Molecular ion peak for cycloartenol.

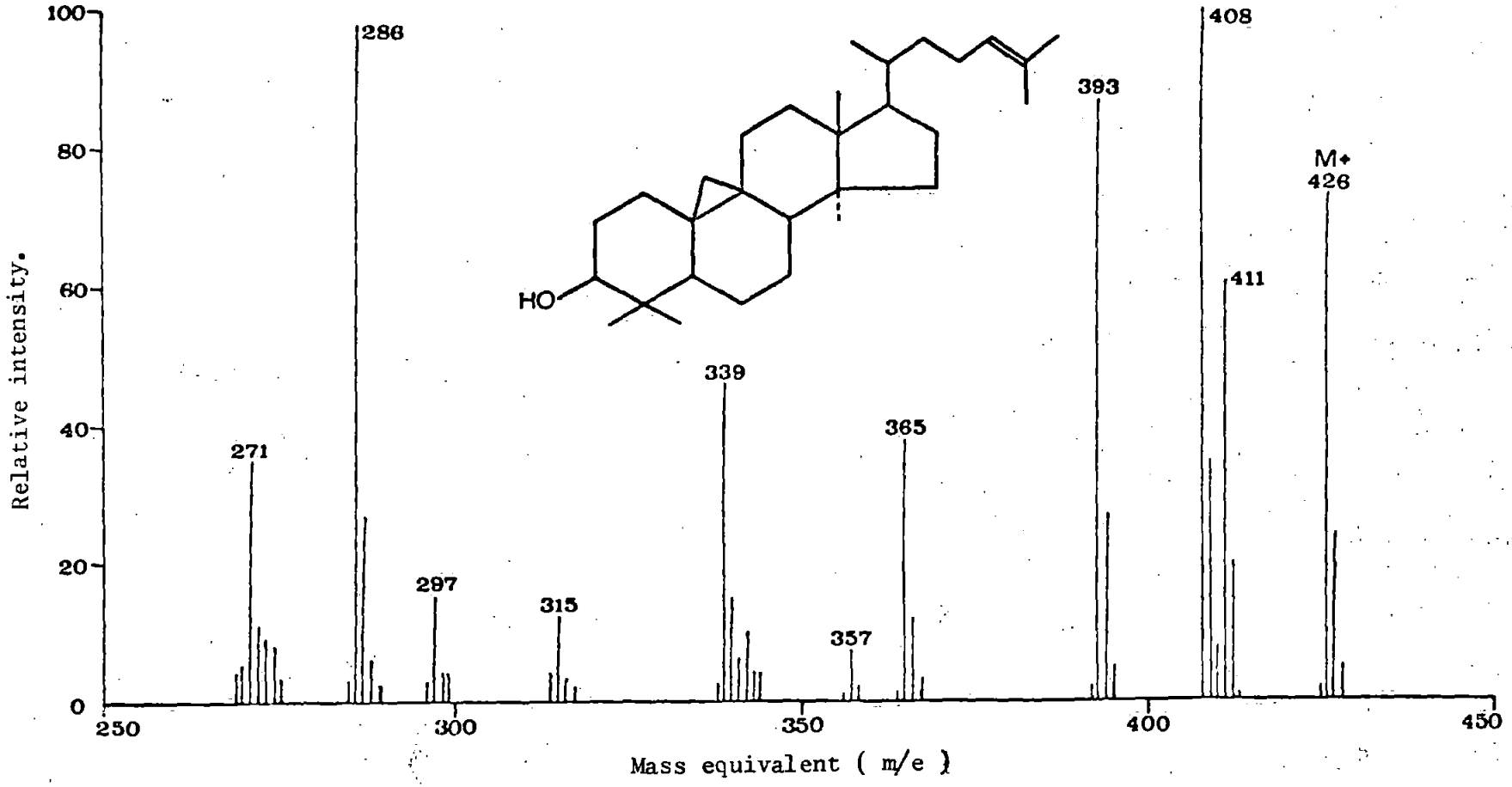


Figure 6.3. Mass spectrum of 2-^{3}H-cycloartenol.

Table 6.5. Incorporation of radiolabelled 4-desmethyl sterols by Artemia salina (whole organisms).

	^{14}C-Cholesterol	^{14}C-β-sitosterol	^{3}H-Poriferasterol	^{3}H-Stigmasterol	^{14}C-Desmosterol
Activity added (μCi)	1	2	2	2	2
Dry weight (mg)	64.4	93.9	104.0	83.0	90.5
Non-saponifiable lipid					
Weight (mg)	4.2	6.4	8.7	7.1	6.9
Total activity (dpm)	9460	7040	3960	3520	8640
% Recovery[1]	0.43	0.16	0.09	0.08	0.19

[1]Expressed as a percentage of the activity originally added.

Table 6.6. Distribution of activity incorporated into non-saponifiable lipid from radiolabelled 4-desmethyl sterols by Artemia salina (whole organisms).

	^{14}C-Cholesterol	^{14}C-β-sitosterol	^{3}H-Poriferasterol	^{3}H-Stigmasterol	^{14}C-Desmosterol
4,4'-dimethyl sterols					
Total activity (dpm)	0	0	0	0	0
4α-methyl sterols					
Total activity (dpm)	70	0	96	0	0
4-desmethyl sterols					
Total activity (dpm)	8295	6480	3541	3182	8010
% recovery[1]	87.7	92.0	89.4	90.4	92.7
Steroids					
Total activity (dpm)	654	370	352	213	198
% recovery[1]	6.9	5.2	8.9	6.0	2.2
Total % recovery[2]	94.6	97.3	98.3	96.5	95.0

[1]Expressed as a percentage of the activity present in the non-saponifiable lipid fraction.
[2]Expressed as percentage recovery of non-saponifiable lipid activity in 4-desmethyl and steroid fractions.

a. ^{14}C-Cholesterol.

^{14}C-Cholesterol was incorporated into the non-saponifiable lipid in greater amounts than any other sterol (0.43% incorporation). Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol zones contained ^{14}C-labelled material. Subsequent GLC analysis of the 4-desmethyl sterol fraction showed a single component cochromatographing with cholesterol; all radioactivity coincided with this peak (Figure 6.4). This component was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.7).

Autoradiography of the steroid fraction (after TLC separation) revealed prominent bands corresponding to progesterone, testosterone and cortisone markers (Figure 6.9). A less prominent band corresponding to 11α-hydroxyprogesterone and a faint area remaining at the origin were also seen.

b. ^{14}C-β-sitosterol.

Incorporation of ^{14}C-β-sitosterol emulsions into non-saponifiable lipid by A. salina were less than half as efficient (0.16% incorporation) as incorporation of ^{14}C-cholesterol. Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol zones contained ^{14}C-labelled material. Subsequent GLC analysis of the 4-desmethyl sterol fraction showed two components cochromatographing with cholesterol and β-sitosterol (Figure 6.5). Radioactivity was associated with both these compounds. The component identified as cholesterol was trapped and recrystallised to constant specific activity

with carrier cholesterol from acetone/water (Table 6.7).

Autoradiography of the steroid fraction (after TLC separation) revealed prominent bands corresponding to progesterone and testosterone, with less prominent areas being visible at the origin and corresponding to 11α-hydroxyprogesterone.

c. ^{14}C-Desmosterol.

24-^{14}C-Desmosterol was incorporated into the non-saponifiable lipid of A.salina to an extent of 0.19%. Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol fraction alone contained ^{14}C-labelled material. GLC analysis of the 4-desmethyl sterol fraction showed two components cochromatographing with desmosterol and cholesterol (Figure 6.6). Radioactivity was associated with both these compounds. The component identified as cholesterol was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.7).

The conversion of 24-^{14}C-desmosterol into steroids was the lowest of all the 4-desmethyl sterols used. Autoradiography of the steroid fraction (after TLC separation) revealed a single faint area remaining at the origin. The significance of this finding is dealt with more fully in the discussion which follows.

d. 2,4-^{3}H-Poriferasterol.

Incorporation of 2,4-^{3}H-poriferasterol into non-saponifiable lipid was very low (0.08%). Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol fractions contained

^{3}H-labelled material. GLC analysis of the 4-desmethyl sterol fraction showed two components cochromatographing with cholesterol and poriferasterol (Figure 6.7). Radioactivity was associated with both these compounds. The component identified as cholesterol was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.7).

Autoradiography of the steroid fraction (after TLC separation) revealed areas of activity corresponding to progesterone, testosterone and cortisone markers. A faint area remained at the origin (Figure 6.9).

e. 2,4-^{3}H-Stigmasterol.

Incorporation of 2,4-^{3}H-stigmasterol into non-saponifiable lipid, at 0.09%, was slightly greater than the incorporation of 2,4-^{3}H-poriferasterol. Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl fractions alone contained ^{3}H-labelled material. GLC analysis of the 4-desmethyl sterol fraction revealed two components cochromatographing with cholesterol and stigmasterol (Figure 6.8). Radioactivity was associated with both these compounds. The component identified as cholesterol was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.9).

Autoradiography of the steroid fraction (after TLC separation) showed a prominent band corresponding to testosterone, with less pronounced areas corresponding to progesterone and cortisone markers. A faint area remained at the origin (Table 6.9).

Table 6.7. Recovery of cholesterol from incubations of radiolabelled 4-desmethyl sterols with Artemia salina (whole organisms.

Material recovered from incubations with:	Specific activity of material + carrier cholesterol	Recrystallisation (dpm/mg) First	Second	Third	Fourth	Fifth
^{14}C-Cholesterol	350	341	349	345	-	-
^{14}C-β-sitosterol	276	264	260	254	259	-
^{3}H-Poriferasterol	90	95	93	-	-	-
^{3}H-Stigmasterol	120	110	105	108	-	-
^{14}C-Desmosterol	211	190	185	156	160	158

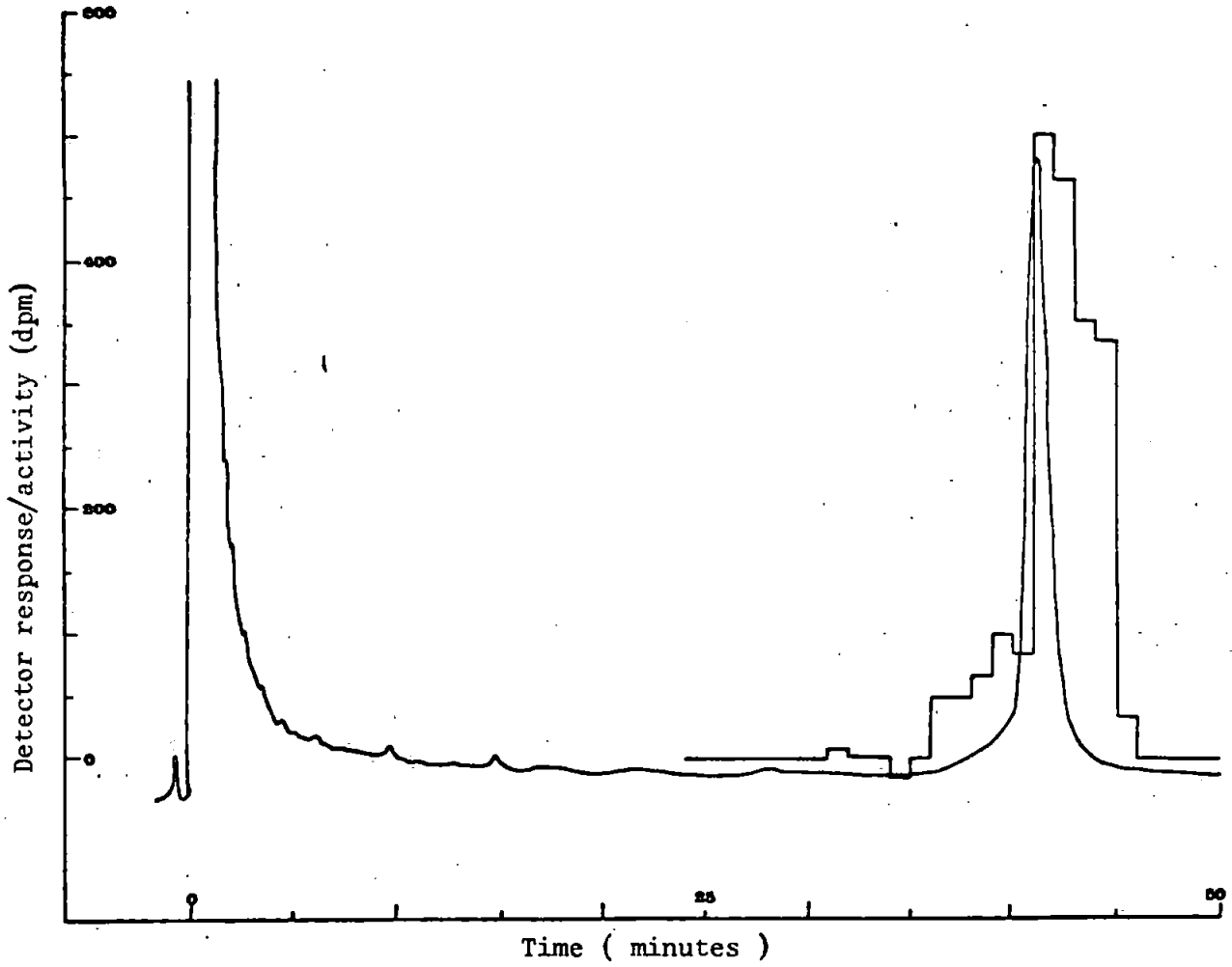


Figure 6.4. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (whole organisms) incubated with 2-^{14}C-cholesterol.

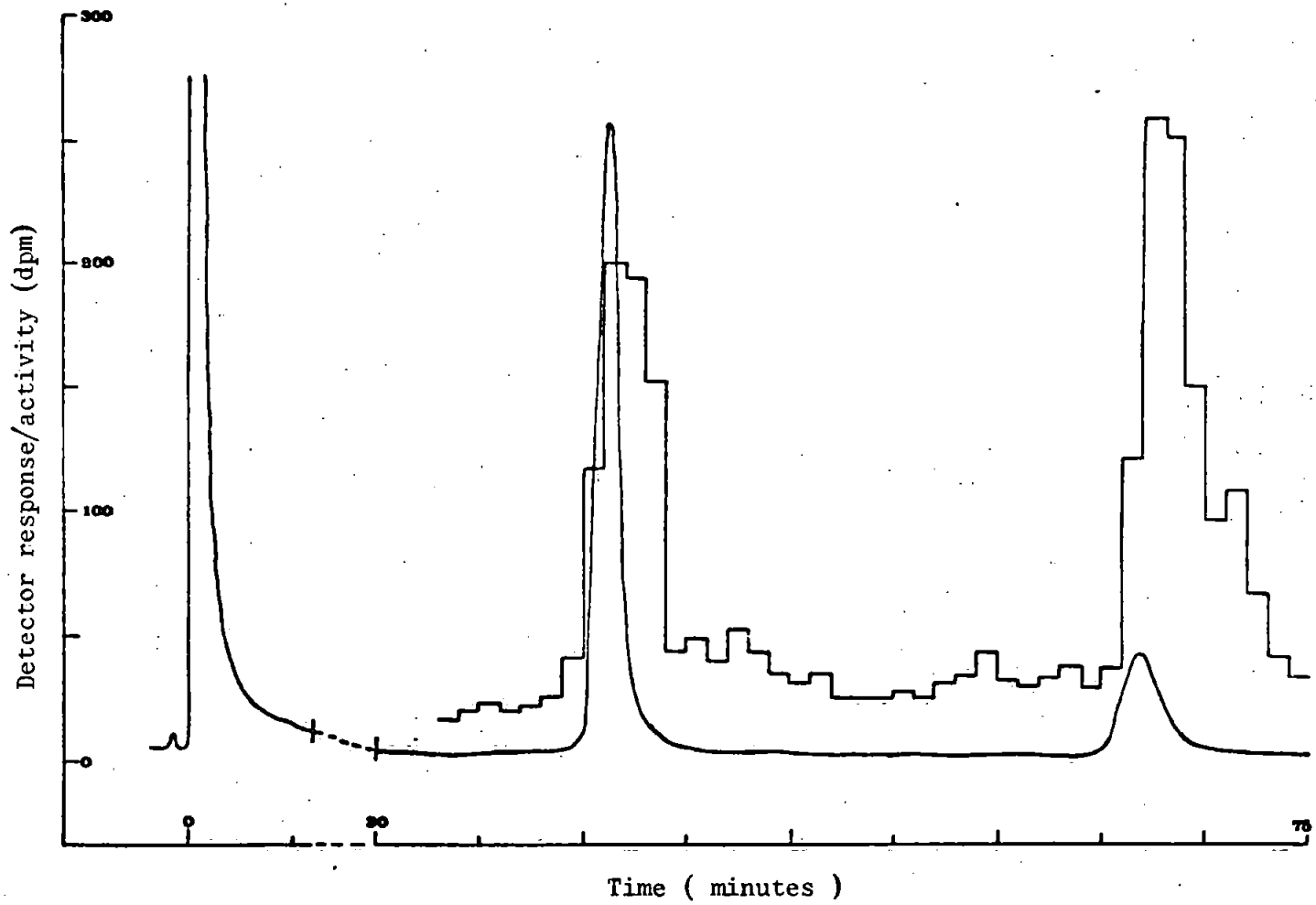


Figure 6.5. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (whole organisms) incubated with 2-^{14}C-β-sitosterol.

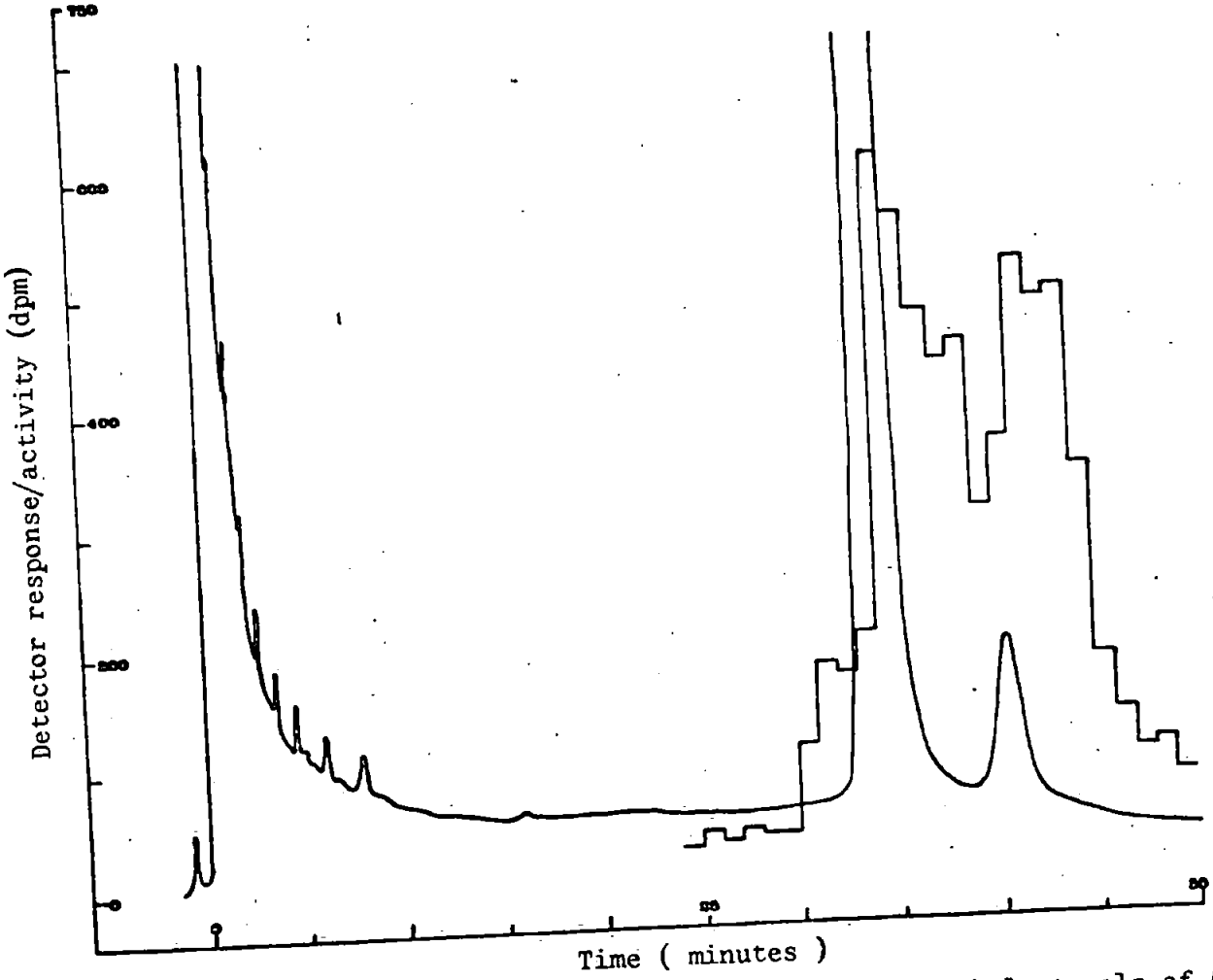


Figure 6.6. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (whole organisms) incubated with 24-^{14}C-desmosterol.

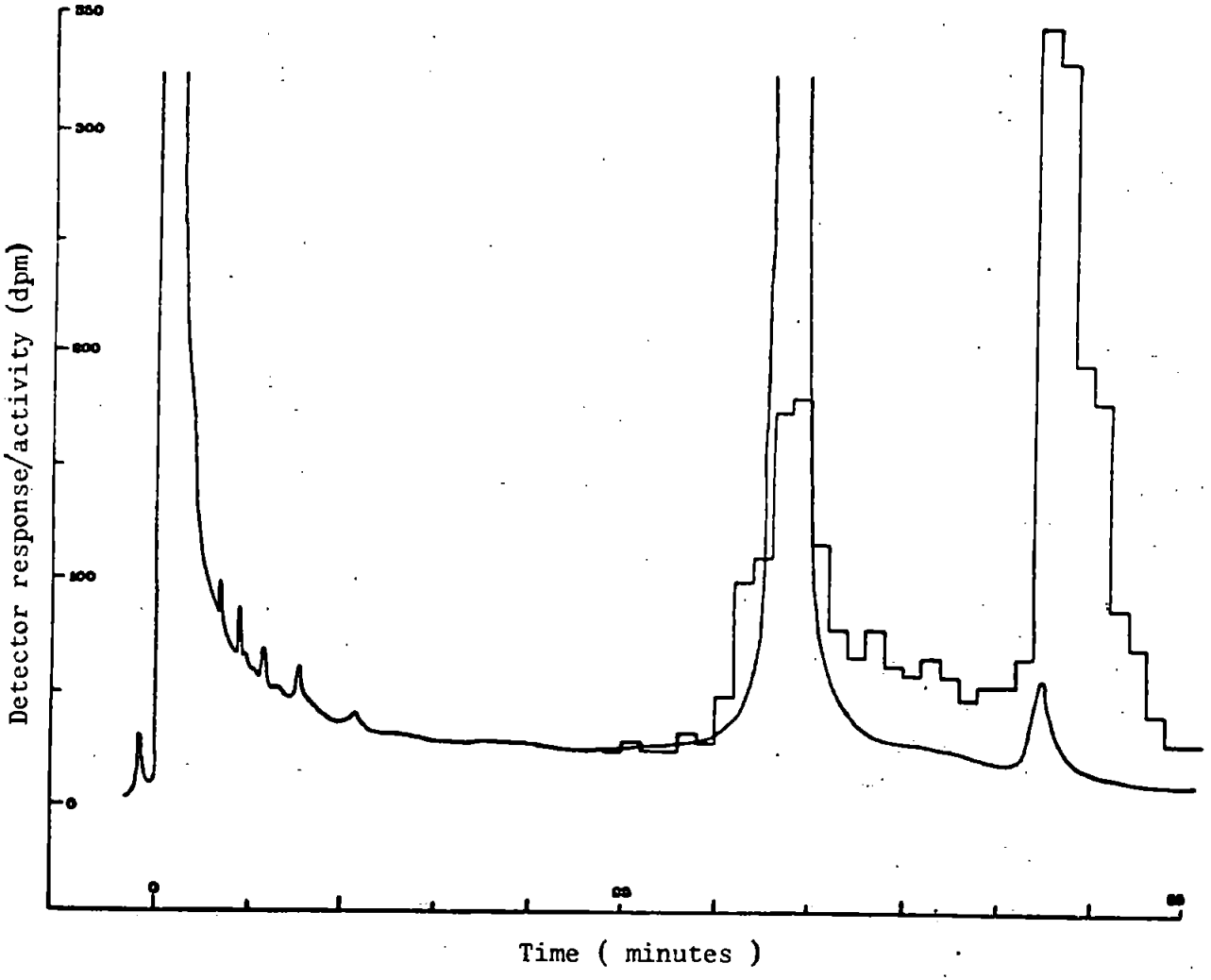


Figure 6.7. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (whole organisms) incubated with 2,4-^{3}H-poriferasterol.

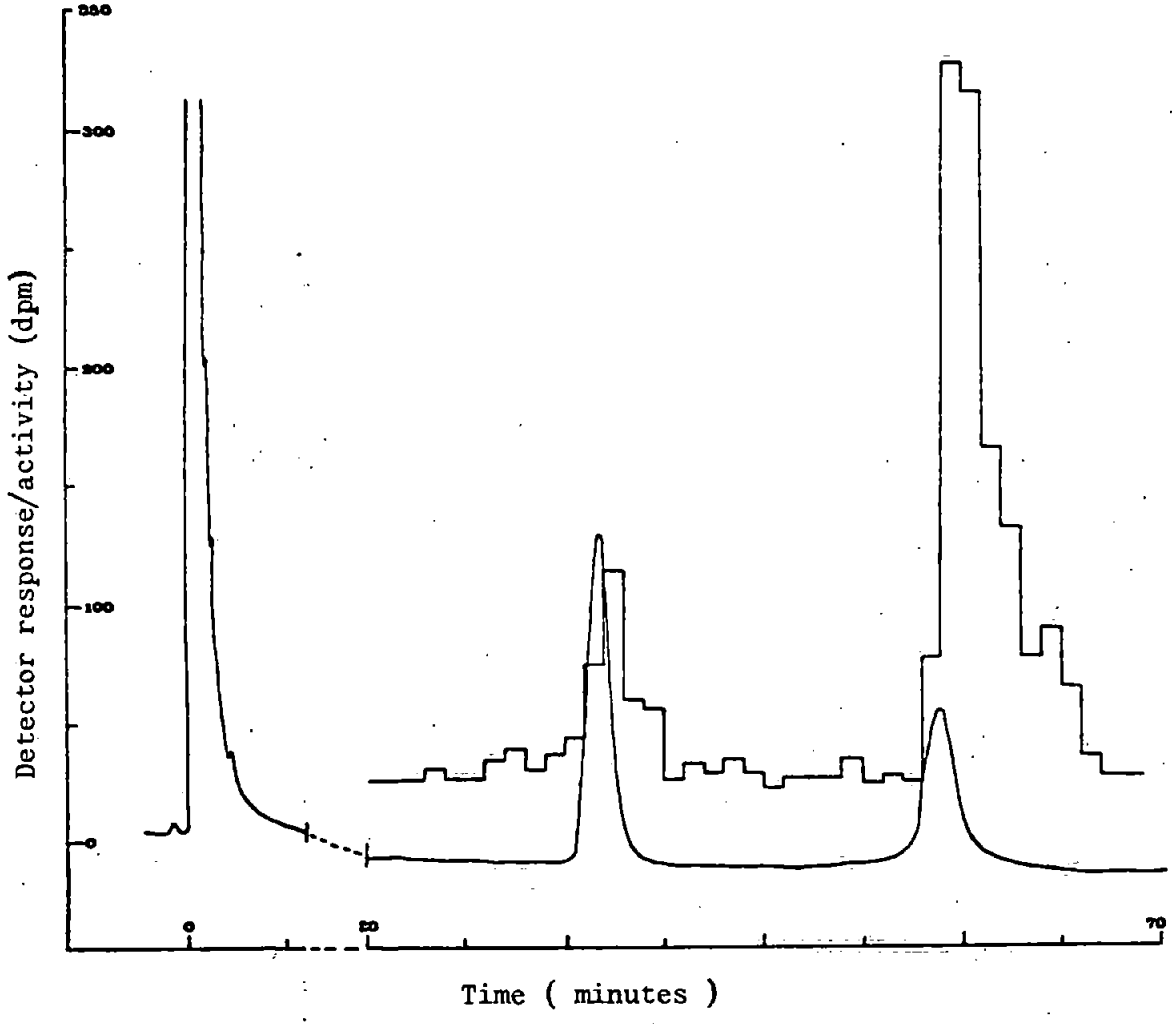
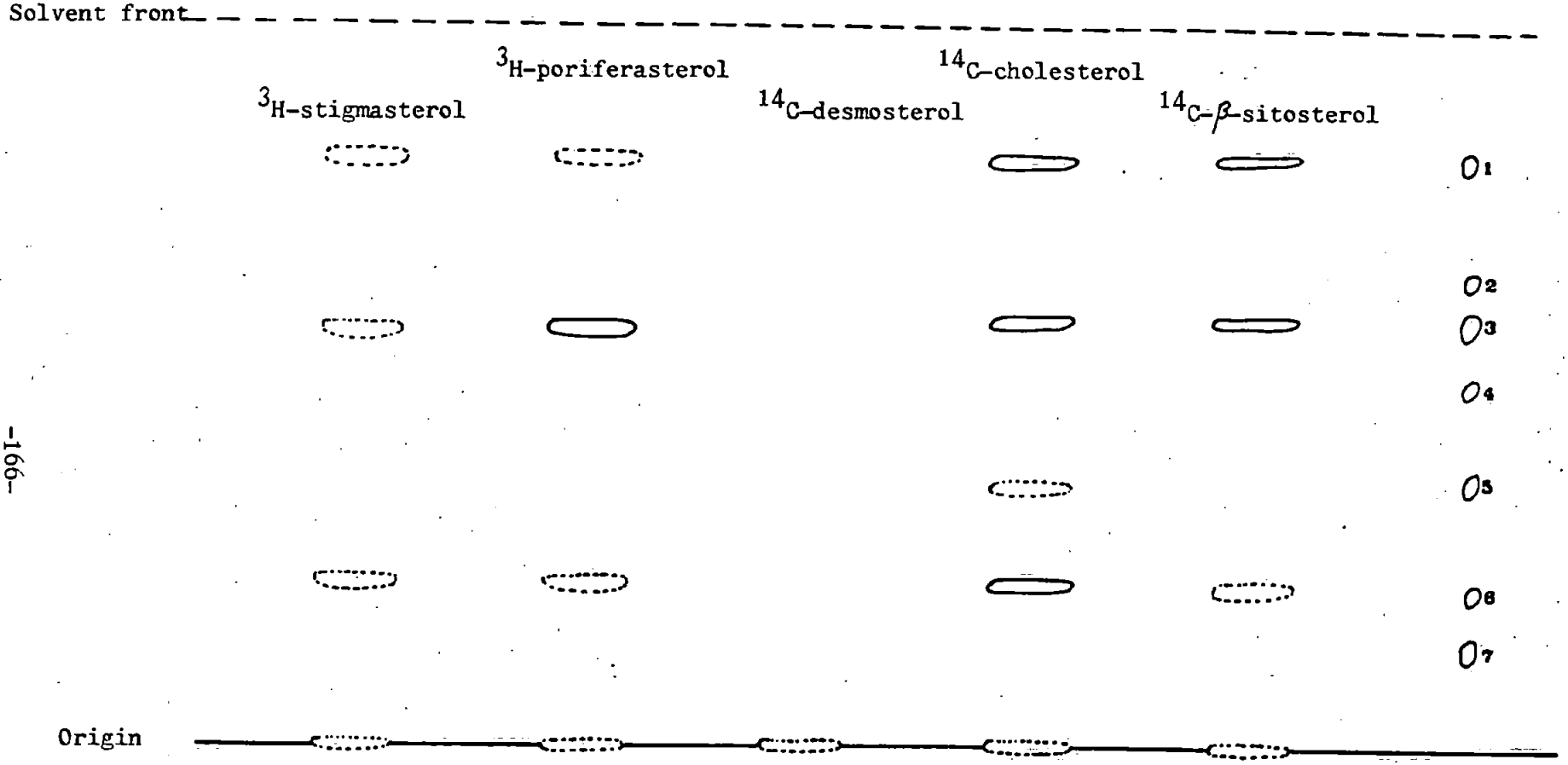


Figure 6.8. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of *Artemia salina* (whole organisms) incubated with 2,4-^{3}H-stigmasterol.



Key: 1 Progesterone; 2 Oestrone; 3 Testosterone; 4 β-Oestradiol; 5 11α-hydroxyprogesterone; 6 Cortisone; 7 Hydrocortisone (Faint areas are denoted by dotted lines).

Figure 6.9. Composite autoradiograph of steroid fractions from Artemia salina (whole organisms) incubated with radiolabelled 4-desmethyl sterols.

ii. Incubations with cell-free preparations.

The results of incubations of cell-free preparations of A.salina with radiolabelled 4-desmethyl sterol emulsions are given in Tables 6.8 and 6.9 and are detailed for each sterol below.

a. ^{14}C-Cholesterol.

Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol fractions alone contained ^{14}C-labelled material. 0.3% of the activity present in the non-saponifiable lipid was contained in the steroid fraction. GLC analysis of the 4-desmethyl sterol fraction revealed a single component cochromatographing with cholesterol. All radioactivity was associated with this compound (Figure 6.10), which was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.9).

Autoradiography of the steroid fraction (after TLC separation) revealed prominent areas of activity corresponding to oestrone, progesterone and cortisone markers, with less pronounced areas corresponding to 11α-hydroxyprogesterone and hydrocortisone. A faint area remained at the origin (Figure 6.15).

b. ^{14}C-β-sitosterol.

Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol areas alone contained ^{14}C-labelled material. GLC analysis of the 4-desmethyl sterols revealed the presence of two components cochromatographing with cholesterol and β-sitosterol. Radioactivity was associated with both these compounds

(Figure 6.11). The component identified as cholesterol was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.9).

Autoradiography of the steroid fraction (after TLC separation) revealed prominent areas of activity corresponding to progesterone, oestrone and cortisone; less prominent areas were seen at the origin and corresponding to β-oestradiol. There were several other areas of faint activity chromatographing between marker compounds (Figure 6.15).

c. 24-^{14}C-Desmosterol.

Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol fractions alone contained ^{14}C-labelled material. GLC analysis of the 4-desmethyl sterols revealed the presence of two components cochromatographing with cholesterol and desmosterol. Radioactivity was associated with both these compounds (Figure 6.12). The component identified as cholesterol was trapped and recrystallised from acetone/water to constant specific activity with carrier cholesterol (Table 6.9).

Autoradiography of the steroid fraction (after TLC separation) showed the presence of a single area of activity remaining at the origin (Figure 6.15).

d. 2,4-^{3}H-Poriferasterol.

Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol areas alone contained ^{3}H-labelled material. GLC analysis of the 4-desmethyl sterol fraction revealed the

presence of two components cochromatographing with cholesterol and poriferasterol. Radioactivity was associated with both these compounds (Figure 6.13). The component identified as cholesterol was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.9).

Autoradiography of the steroid fraction (after TLC separation) revealed prominent areas of activity corresponding to cortisone, progesterone, oestrone and β-oestradiol markers. A faint area remained at the origin (Figure 6.15).

e. 2,4-^{3}H-Stigmasterol.

Separation of the non-saponifiable lipid into steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl sterol fractions showed that the steroid and 4-desmethyl sterol areas alone contained ^{3}H-labelled material. GLC analysis of the 4-desmethyl sterol fraction revealed the presence of two components cochromatographing with cholesterol and stigmasterol. Radioactivity was associated with both these compounds (Figure 6.14). The component identified as cholesterol was trapped and recrystallised to constant specific activity from acetone/water with carrier cholesterol (Table 6.9).

Autoradiography of the steroid fraction (after TLC separation) revealed prominent areas of activity corresponding to cortisone, progesterone, oestrone and β-oestradiol markers. A faint area remained at the origin (Figure 6.15).

Table 6.8. Distribution and incorporation of activity from radiolabelled 4-desmethyl sterols into non-saponifiable lipid by Artemia salina (cell-free preparations).

	^{14}C-Cholesterol	^{14}C-β-sitosterol	^{3}H-Poriferasterol	^{3}H-Stigmasterol	^{14}C-Desmosterol
Activity added (dpm)	2.2×10^6	2.2×10^6	2.1×10^6	2.6×10^6	2.2×10^6
Non-saponifiable lipid					
Total activity (dpm)	2.05×10^6	2.10×10^6	2.02×10^6	2.49×10^6	2.08×10^6
% activity[1]	93.1	95.4	96.1	95.7	94.5
4-desmethyl sterols					
Total activity (dpm)	2013940	2057940	1977030	2427813	2039796
% activity[2]	99.70	99.90	99.85	99.91	99.99+
Steroids					
Total activity (dpm)	6060	2060	2970	2187	429
% activity[2]	0.30	0.10	0.15	0.09	0.0002
% recovery[3]	98.5	97.9	98.1	97.6	98.0

[1]Expressed as a percentage of the activity added. [2]Expressed as a percentage of the activity of the non-saponifiable lipid. [3]Total activity recovered in steroid and 4-desmethyl fractions as a % of non-saponifiable activity.

Table 6.9. Recovery of cholesterol from incubations of radiolabelled 4-desmethyl sterols with Artemia salina (cell-free preparations).

Material recovered from incubations with:	Specific activity of material + carrier cholesterol	Recrystallisation (dpm/mg) First	Second	Third	Fourth	Fifth
^{14}C-Cholesterol	3980	3935	3940	3940	-	-
^{14}C-β-sitosterol	198	220	210	215	-	-
^{3}H-Poriferasterol	356	350	335	340	-	-
^{3}H-Stigmasterol	268	275	280	263	269	-
^{14}C-Desmosterol	686	640	632	596	584	594

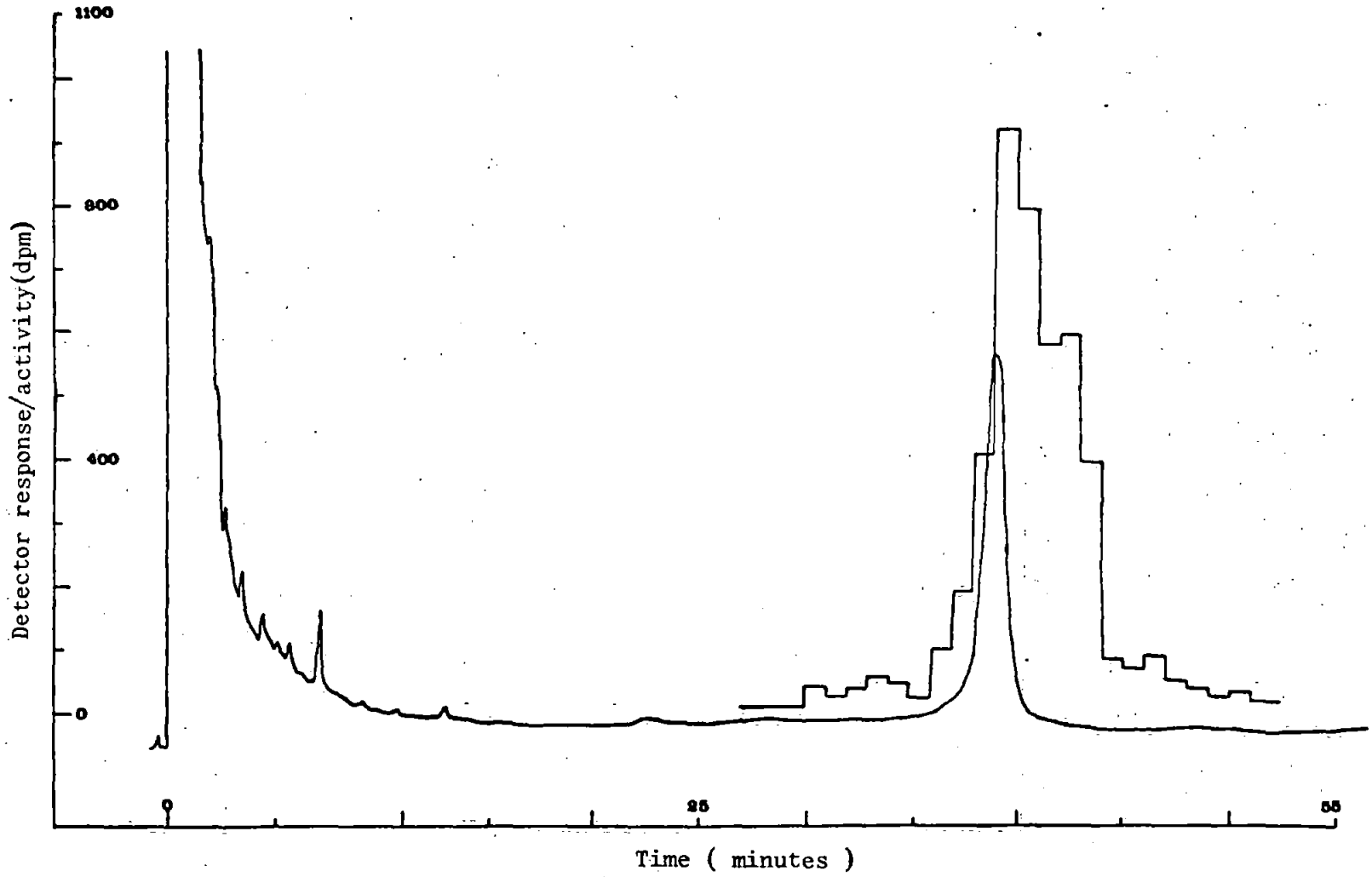


Figure 6.10. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (cell-free preparations) incubated with 2-^{14}C-cholesterol.

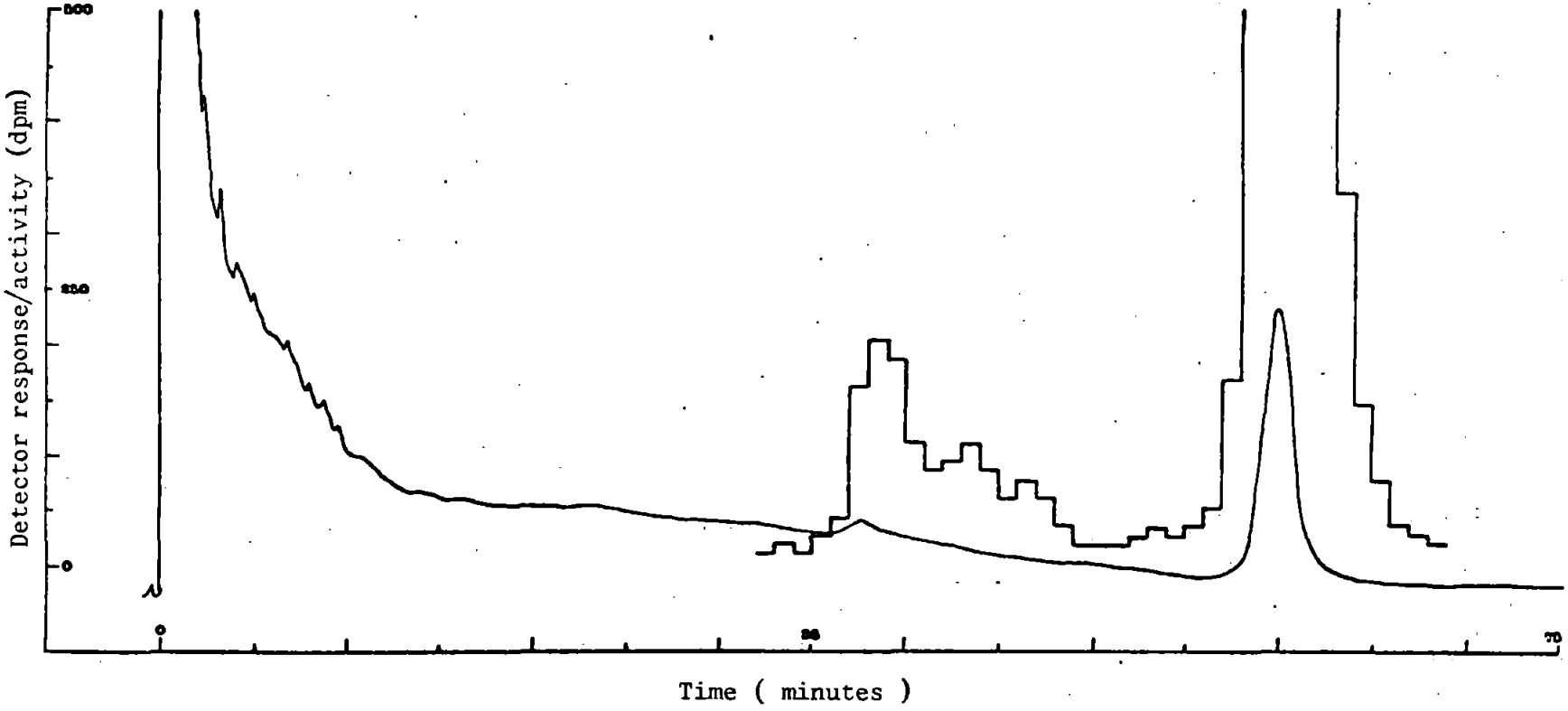


Figure 6.11. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (cell-free preparations) incubated with 2-^{14}C-β-sitosterol.

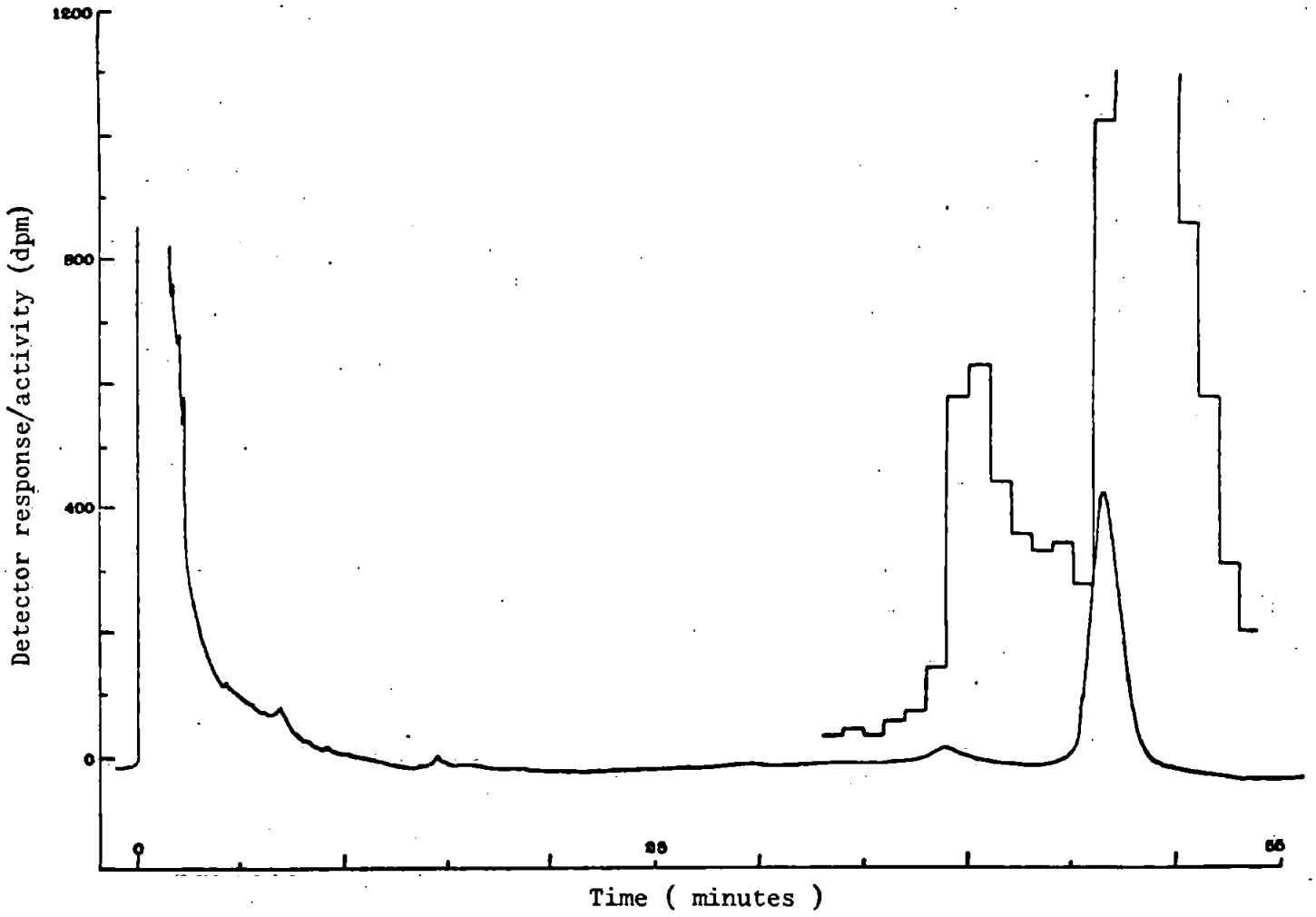


Figure 6.12. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (cell-free preparations) incubated with 24-^{14}C-desmosterol.

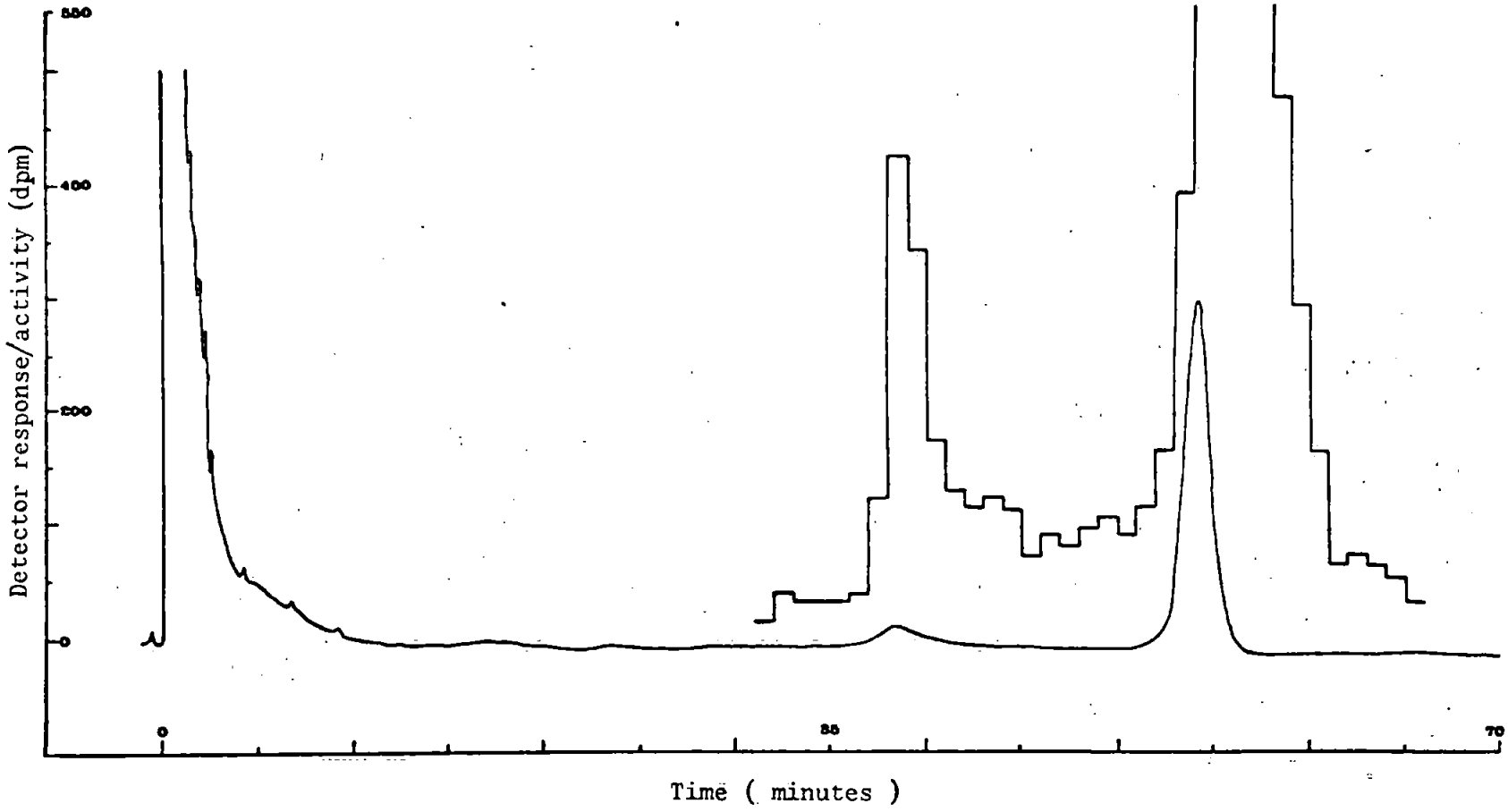


Figure 6.13. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (cell-free preparations) incubated with 2,4-^{3}H-poriferasterol.

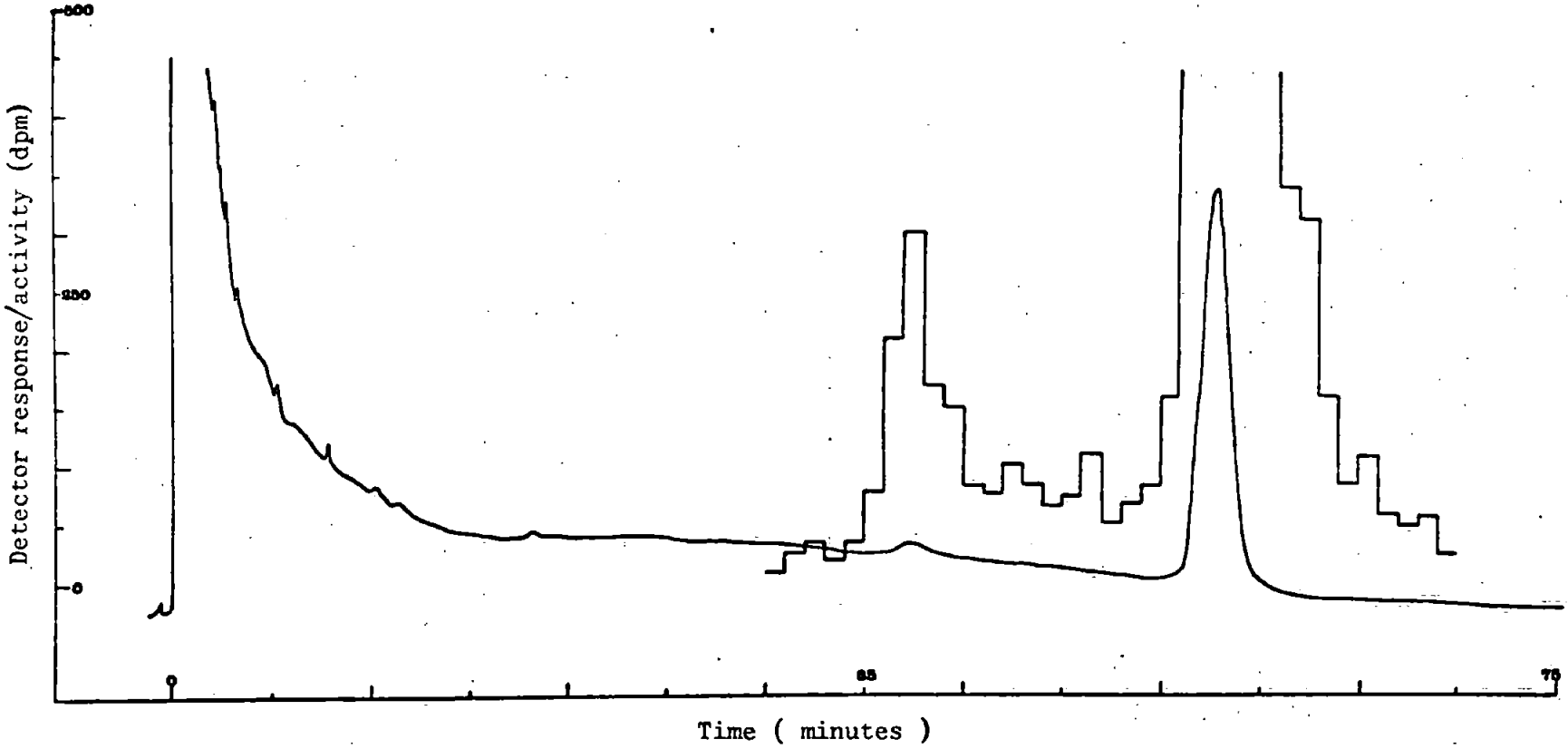
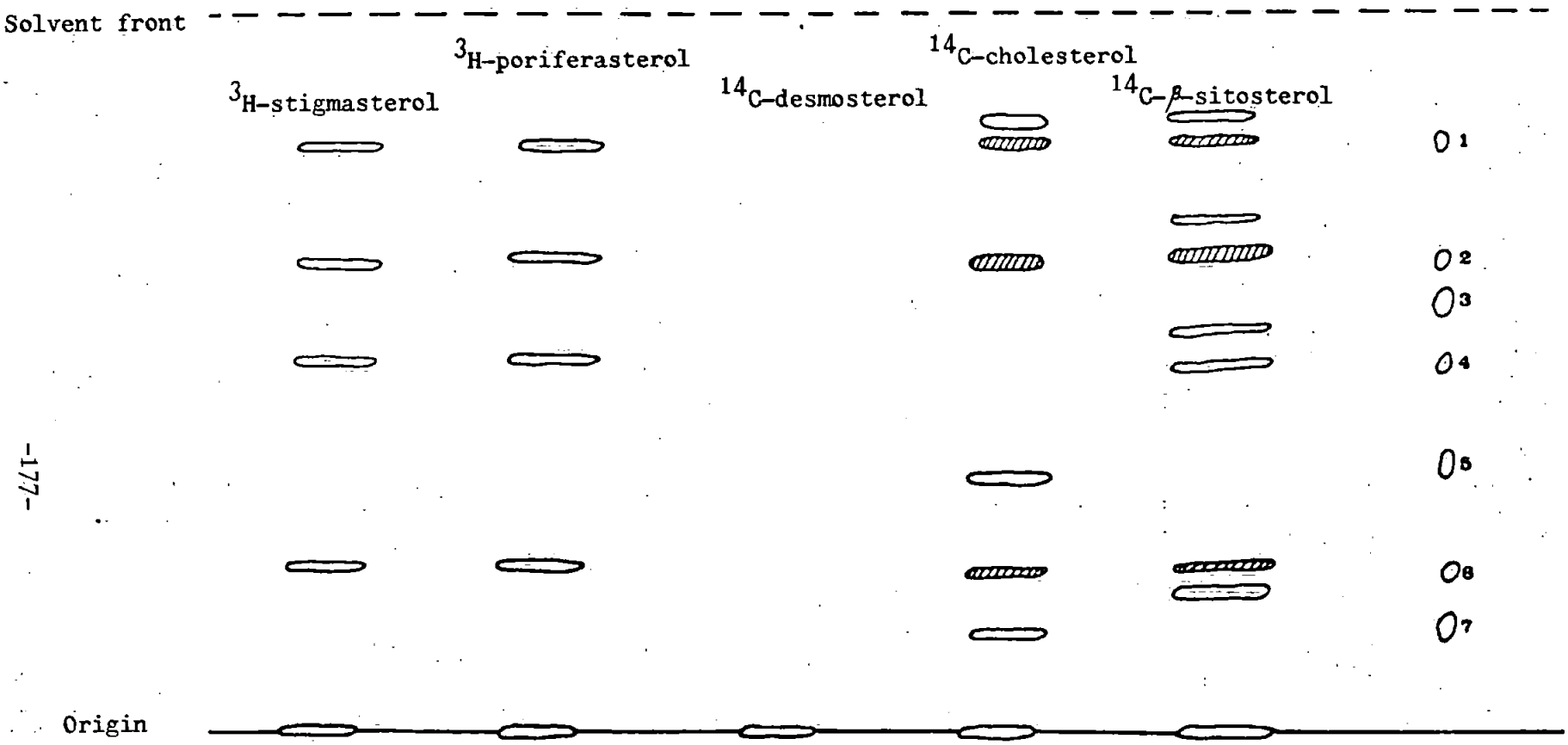


Figure 6.14. GLC/radio-analysis (3% OV-17) of 4-desmethyl sterols of Artemia salina (cell-free preparations) incubated with 2,4-^{3}H-stigmasterol.



KEY: 1 Progesterone; 2 Oestrone; 3 Testosterone; 4 β-Oestradiol; 5 11α-hydroxyprogesterone; 6 Cortisone; 7 Hydrocortisone. (Hatching denotes more prominent bands).

Figure 6.15. Composite autoradiograph of steroid fractions from Artemia salina (cell-free preparations) incubated with radiolabelled 4-desmethyl sterols.

iii. Discussion.

The conversion of poriferasterol, stigmasterol, desmosterol and β-sitosterol into cholesterol has been shown to occur, both in vivo and in vitro, in Artemia salina. Such conversions involve a number of mechanistic steps; the dealkylation of a C-24 ethyl group and the saturation of Δ^{22} or Δ^{24} double bonds. Both 24α (β-sitosterol) and 24β (poriferasterol) ethyl groups can be removed. These results indicate that A.salina possesses the enzymes necessary to metabolise a range of 4-desmethyl sterol sidechain types; certainly all those commonly encountered in its diet. It has been previously demonstrated that, in addition to the sidechain transformations mentioned above, A.salina is able to carry out a nuclear modification, the removal of the Δ^{7} bond of ergosterol (Teshima & Kanazawa 1971f).

GLC/radioanalysis of the 4-desmethyl sterol fractions did not reveal the presence of any labelled intermediates. This is possibly due to two factors; the low activities of the sterols employed and the extremely low incorporation levels obtained.

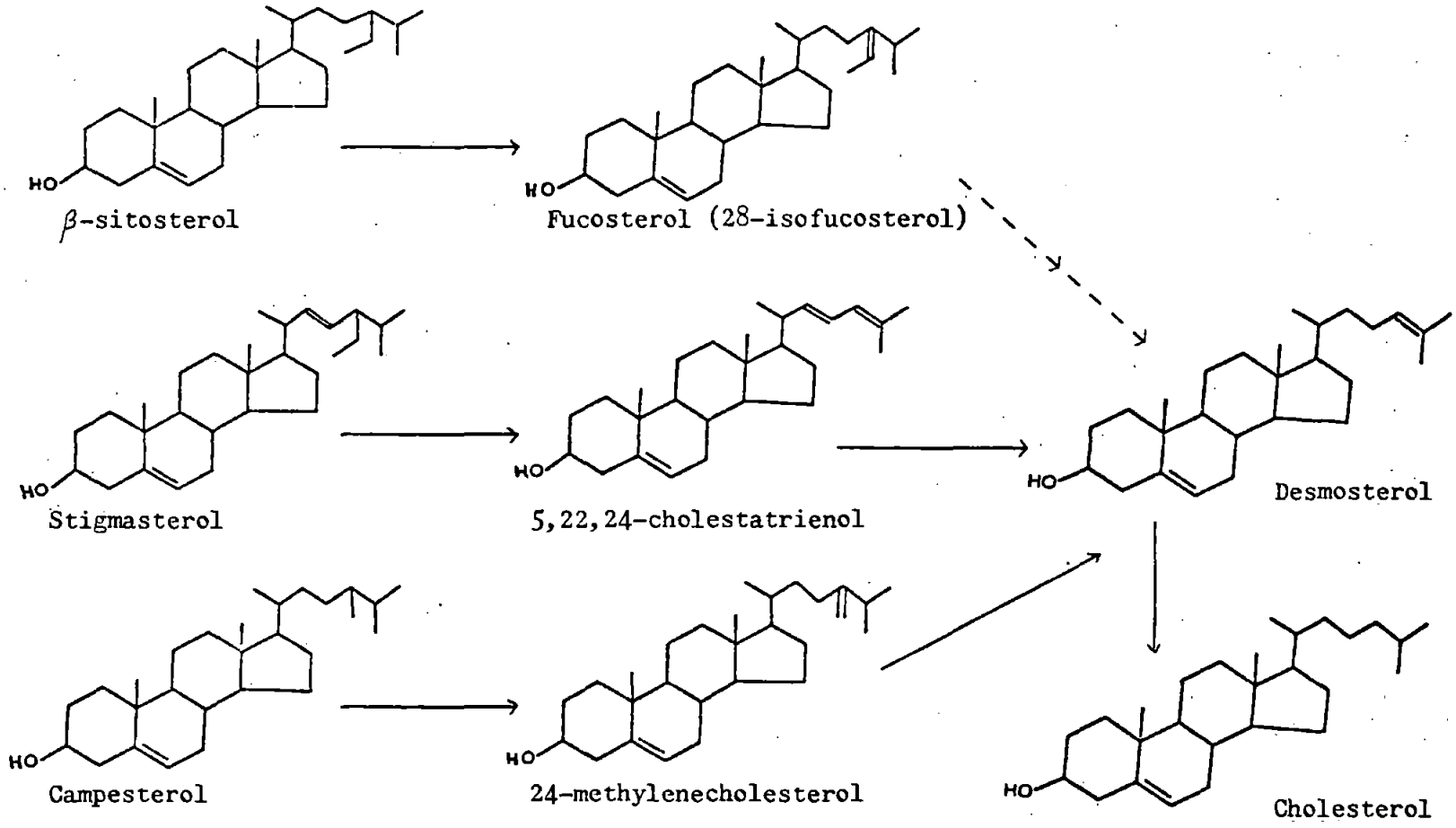
In view of the many biochemical similarities between insects and crustaceans, it is not unreasonable to assume that similar pathways to those elucidated for insects might exist in A.salina and other members of the Crustacea. The conversion of phytosterols to cholesterol by various insects has been well documented (see, for example, Robbins et al 1971; Thompson et al 1972). It has been reported that fucosterol is a metabolite of β-sitosterol and a probable intermediate in the conversion of this phytosterol to cholesterol in Locusta migratoria, Apis melifica (Allais et al 1971; Allais & Barbier 1971) and in the tobacco hornworm, Manduca sexta (Svoboda et al 1971). The latter group of workers have isolated and identified 24-methylenecholesterol as an

intermediate in the conversion of campesterol to cholesterol in the same organism (Svoboda et al 1972). 22-trans-5,22,24-Cholestatrien-3β-ol has been isolated and identified as an intermediate in the conversion of stigmasterol to cholesterol in several insects (Svoboda et al 1969; Hutchins et al 1970). The conversion of 28-isofucosterol to cholesterol via a postulated desmosterol intermediate has recently been demonstrated in the yellow mealworm, Tenebrio molitor (Randall et al 1972). These various conversions are summarised in Scheme 6.2 (Thompson et al 1972).

Evidence such as that presented above has lead to the suggestion that the process of C-24 dealkylation of sterols in insects may, at least in part, be the reverse of the C-24 alkylation mechanism of plants (Svoboda & Robbins 1968; Svoboda et al 1971, 1972). The possibility of a different pathway operating has been suggested by the discovery that fucosterol-24,28-epoxide is a probable intermediate in the conversion of β-sitosterol to cholesterol in the silkworm, Bombyx mori (Morisaki et al 1972).

It is interesting to note that both 24α and 24β ethyl groups are removed by A.salina. The results obtained indicate that sterols with these configurations at C-24 are utilised with the same degree of efficiency. Some degree of specificity for the orientation at C-24 has been observed in the tobacco hornworm in that the 24β methyls of brassicasterol and 22,23-dihydrobrassicasterol did not appear to be as readily removed as the 24α alkyls of β-sitosterol, stigmasterol, and campesterol (Svoboda & Robbins 1968). Such selectivity was suggested to be an adaptation to the sterol content of tobacco, the host plant of the tobacco hornworm, in which the latter three compounds are the predominant sterols. By similar reasoning, one may suggest that the ability of A.salina to dealkylate 24α and 24β substituents with apparent equal

Scheme 6.2. Conversion of 4-desmethyl sterols to cholesterol in Insecta.



efficiency is an adaptation to the varied sterol content of its diet. Dealkylation of the 24β substituents in insects is thought to proceed by the same mechanisms as those postulated for 24α-dealkylation (Ritter & Wientjens 1967; Svoboda et al 1972). If poriferasterol were to be dealkylated by the same mechanism as that illustrated for stigmasterol, 5,22,24-cholestatrien-3β-ol would be an intermediate. This could be easily tested by the administration to A.salina of poriferasterol labelled with tritium at C-25.

The in vivo conversion of cholesterol to various steroids has been shown in few crustaceans. Penaeus japonicus has been shown to convert ^{14}C-cholesterol into 'polar steroids' in a variety of tissues (Guary & Kanazawa 1973). Panulirus japonica is able to convert ^{14}C-cholesterol into corticosterone, deoxycorticosterone, testosterone, 17α-hydroxyprogesterone, androstenedione and progesterone (Teshima & Kanazawa 1971a). The results presented in this chapter represent the first report of the conversion of sterols other than cholesterol into steroids in a crustacean. In all cases a band corresponding to a progesterone marker was observed. Other steroids that were invariably present and more strongly labelled than any others were testosterone and cortisone. An area corresponding to a β-oestradiol marker was only present in incubations involving cell-free tissue preparations. It is significant that those steroids most prominently labelled are all 'end products' of a particular biosynthetic sequence. Incubations of tissue preparations with ^{14}C-cholesterol and ^{14}C-β-sitosterol resulted in more steroid bands being visible and these could possibly be intermediates in the biosynthesis of the more prominently labelled steroids. The biosynthetic degradation of cholesterol to form the various steroid hormones is complicated, is not well understood and shall not be dealt with in

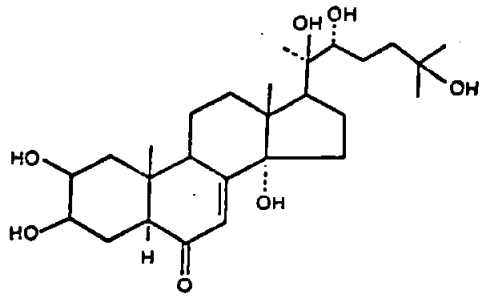
detail here. It is sufficient to say that, in animals, progesterone appears to be a central intermediate in the synthesis of steroids from cholesterol. As previously stated, in all incubations an area corresponding to a progesterone marker was present.

There have been many reports of *in vitro* interconversion of steroids in Crustacea and the identification of various intermediates in such conversions. Progesterone has been shown to be converted into a range of steroids by the sliced testes and ovaries of *Portunus trituberculatus*; amongst the products identified are testosterone, 17α-hydroxyprogesterone, deoxycorticosterone and 11-ketotestosterone (Teshima & Kanazawa 1970, 1971b, 1971d). Δ^5-Pregnenolone and progesterone are converted to 20α-hydroxyprogesterone, Δ^4-androstenedione, testosterone and 11-deoxycorticosterone by tissues of *Callinectes sapidus* (Tcholakian & Eik-Nes 1971). Δ^4-Androstenedione was converted to testosterone by tissues of the lobster, *Homarus americanus* (Gilgan & Idler 1967) and the crab, *Carcinus maenas* (Blanchet *et al* 1972). The latter authors have also demonstrated the interconversion of a variety of other steroids; amongst them oestrone to 17β-oestradiol, progesterone to 11-deoxycorticosterone and 17-hydroxyprogesterone to 11-deoxycortisol. With the exception of the identification of 3α and 3β-hydroxysteroid and 17β-hydroxysteroid dehydrogenases in *Astacus astacus* (Bjorkhem & Danielsson 1971) there have been no reports of detailed analyses of the enzymological aspects of such steroid transformations. Enzyme assignments have been made on assumptions derived from the steroid transformation products rather than on direct evidence. Lehoux & Sandor (1970) cautioned against regarding any steroid as a normal constituent of any invertebrate organism on the strength of evidence that the particular steroid was biosynthesised from exogenous precursors by

invertebrate tissue preparations in vitro. Unless such evidence is corroborated by in vivo data, it will only serve to indicate that the experimental tissue contained the necessary enzymes to effect the observed synthesis. On the other hand, if a tissue preparation is found to produce steroids from endogenous precursors under in vitro conditions, the chances are quite good that this metabolic activity will also take place in vivo and that the observed reactions were a natural and inherent capability of the tissue. In this present study, one may conclude on the basis of in vitro and in vivo data, that A.salina is able to convert a range of dietary sterols into a number of C_{18}, C_{19} and C_{21} steroids. The mechanisms involved must include sidechain cleavage and reduction and hydroxylation of the sterol nucleus.

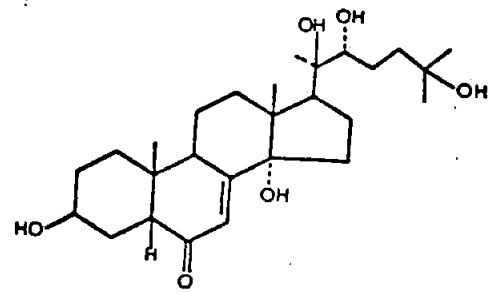
An area of activity at the origin was seen in all incubations and some clue to the possible identity of this area comes from the studies with 24-^{14}C-desmosterol. Desmosterol was the only 4-desmethyl sterol investigated that was labelled in the sidechain and autoradiography of the steroid fractions obtained from incubations with this compound showed a single area of activity at the origin. The occurrence of activity in this region is indicative of a highly polar fraction which had retained the sidechain of desmosterol. Ecdysones are polyhydroxylated C_{27} steroids and have been isolated from a number of crustacean sources (Gallego & Menendez 1969; Hampshire & Horn 1966; Horn et al 1966; Karlson & Skinner 1960), for example, crustecdysone (β-ecdysone structure I in Figure 6.16) from Jasus lalandei (Horn et al 1966) and Callinectes sapidus (Faux et al 1969); 2-deoxycrustecdysone (II) from Jasus lalandei (Galbraith et al 1968); callinecdysone A (III) and callinecdysone B (IV) from Callinectes sapidus (Faux et al 1969). Compounds of this nature would be expected to remain at the origin in the solvent

Figure 6.16. Ecdysones isolated from crustacean sources.

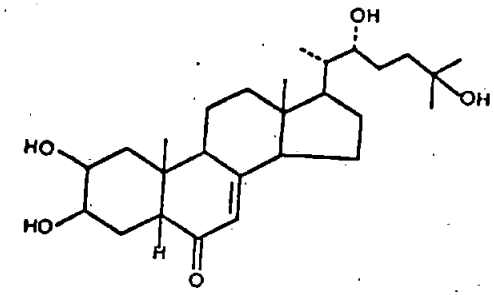


I. Crustecdysone

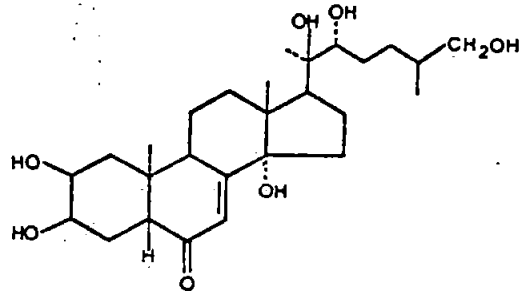
(β-ecdysone)



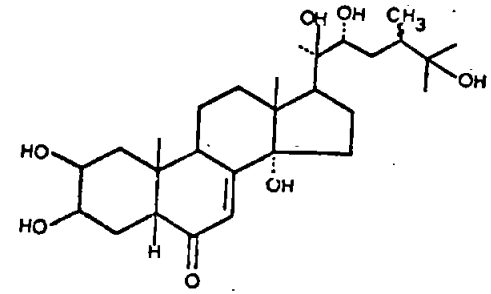
II. 2-Deoxycrustecdysone



III. Callinecdysone A.



IV. Callinecdysone B.



V. Ecdysone (α-ecdysone).

systems employed. Cholesterol has been shown to be a precursor of the ecdysones in insects (see Robbins et al 1971; Rees 1971 for reviews, plants (DeSouza et al 1970; Lloyd-Jones et al 1973) and of products chromatographically related to α and β-ecdysones in Hemigrapsus nudus (Spaziani & Kater 1973). If A.salina is capable of such transformations then it must possess the enzymes necessary for the hydroxylation of the sidechain and the nucleus, the introduction of a keto group at C_6 and the transposition of a C-5 double bond to C-7.

II. Incubations with 4α-methyl and 4,4'-dimethyl sterols.

i. Incubations with whole organisms.

The results of such incubations are given in Tables 6.10 and 6.11. It can be seen that, whilst there was some assimilation of both 4α-methyl and 4,4'-dimethyl sterols into the non-saponifiable lipid fraction of A.salina, there was no conversion of these into cholesterol or any other possible metabolic product. All activity recovered was limited to the sterol originally administered.

The sterols were isolated by TLC on silica gel (with chloroform as developing solvent) and the areas corresponding to steroid, 4-desmethyl, 4α-methyl and 4,4'-dimethyl markers eluted. The zone containing the sterol originally added was further investigated by TLC on silica gel with chloroform as the developing solvent and by GLC (3% OV-17 and 1% SE-30). The acetates were formed and separated by argentation TLC (10% silver nitrate) on silica gel with 40% hexane in benzene as developing solvent. In each system standard markers of possible metabolic products were also chromatographed but in all cases a single component was observed, cochromatographing with authentic starting material. In the cases of ^{3}H-lanosterol, ^{3}H-24,24-dihydrolanosterol

and ^{3}H-cycloartenol, the appropriate unlabelled sterol was added and the mixtures recrystallised to constant specific activity from acetone/water (Table 6.12).

ii. Incubations with cell-free preparations.

The results from such incubations are detailed in Tables 6.13, 6.14 and 6.15. It can be seen that there was no conversion of the administered 4α-methyl sterol or the 4,4'-dimethyl sterols to cholesterol or any other metabolic product. All activity was recovered as the originally added sterol. The areas containing the added sterols were investigated by the methods detailed above and allowed similar conclusions.

iii. Discussion.

The conversion of the administered sterols to cholesterol requires the removal of methyl groups at positions 4 and 14, the transposition of a C-8 double bond to C-5, the cleavage of a cyclopropane ring and various sidechain modifications. It would appear from the results presented that A.salina is unable to carry out any of these modifications. The sidechain transformations required are desaturations at positions 24(25) and 24(28). These have been demonstrated, in the first section of this chapter, to be within the capability of A.salina when metabolising 4-desmethyl sterols. Desaturation of lanosterol would form 24,25-dihydrolanosterol, of cycloartenol would form cycloartanol, of 24-methylenecycloartanol would form 24-methylcycloartanol and of cycloeucalenol would form 31-nor-24-methylcycloartanol. The presence of any of these compounds would have been detected by the systems employed but, in fact, were not. It has been generally assumed that the enzymes catalysing the modifications of the sidechains of sterols are non-specific in their actions; hence the variety of possible products

in the conversion of lanosterol and cycloartenol to cholesterol (see Chapter I). The evidence presented here, however, would indicate the presence of specific enzymes for the sidechains of 4-desmethyl sterols. The activities of such enzymes are inhibited by the structural characteristics of 4α-methyl and 4,4'-dimethyl sterols.

The finding that A. salina is unable to open the cyclopropane ring of cycloartenol, 24-methylenecycloartanol and cycloeucalenol is consistent with the fact that no animal has yet been reported which has this capability.

It was possible that the rate of conversion of these sterols was very slow and that a three hour incubation period was not adequate. In view of this, a 24 hour incubation period was used but similar results were obtained. Similar results were also obtained with a different buffer system (potassium phosphate 0.1M, pH 7.0, containing sucrose 0.25M and $MgCl_2$ 4mM).

From the evidence presented it is possible to conclude that Artemia salina is not capable of the metabolism of 4α-methyl and 4,4'-dimethyl sterols. This data supports the conclusion drawn in Chapter III, that the 4α-methyl and 4,4'-dimethyl sterols detected in Crustacea are probably accumulated from the diet.

Table 6.10. Incorporation of radiolabelled 4,4'-dimethyl and 4α-methyl sterols by Artemia salina (whole organisms).

	^{3}H-Lanosterol	^{3}H-24,25-Dihydrolanosterol	^{3}H-Cycloartenol	^{3}H-24-Methylene cycloartanol	^{3}H-Cycloeucalenol
Activity added (dpm)	4.6×10^6	3.9×10^6	4.2×10^6	5.2×10^6	4.4×10^6
Dry weight (mg)	93.3	105.2	79.9	110.4	83.0
Non-saponifiable lipid					
Weight (mg)	7.1	9.2	5.3	8.4	4.1
Total activity (dpm)	15950	37520	20860	43100	22900
% Recovery[1]	0.35	0.96	0.50	0.83	0.52

[1]Expressed as a percentage of the activity originally added.

Table 6.11. Distribution of activity incorporated into non-saponifiable lipid from radiolabelled 4,4'-dimethyl and 4α-methyl sterols by Artemia salina (whole organisms).

	^{3}H-Lanosterol	^{3}H-24,25-Dihydrolanosterol	^{3}H-Cycloartenol	^{3}H-24-Methylene cycloartanol	^{3}H-Cycloeucalenol
4,4'-dimethyl sterols					
Total activity (dpm)	15439	36432	19879	41333	0
% recovery[1]	96.8	97.1	95.3	95.9	0
4α-methyl sterols					
Total activity (dpm)	97	120	0	285	22100
% recovery[1]	0	0	0	0	96.5
4-desmethyl sterols					
Total activity (dpm)	0	0	0	90	560
Steroids					
Total activity (dpm)	0	0	0	0	0

[1]Expressed as a percentage of the activity present in the non-saponifiable lipid fraction.

Table 6.12. Incubation of radiolabelled 4,4'-dimethyl sterols with Artemia salina (whole organisms).

Recovery of 2-^{3}H-lanosterol.

Specific activity of 2-^{3}H-lanosterol + carrier: 520 dpm/mg.

Recrystallisation	Specific activity dpm/mg
First	490
Second	500
Third	490

Recovery of 2-^{3}H-24,25-dihydrolanosterol.

Specific activity of 2-^{3}H-24,25-dihydrolanosterol + carrier: 2560 dpm/mg.

Recrystallisation	Specific activity dpm/mg
First	2490
Second	2520
Third	2470
Fourth	2490

Recovery of 2-^{3}H-cycloartenol.

Specific activity of 2-^{3}H-cycloartenol + carrier: 230 dpm/mg.

Recrystallisation	Specific activity dpm/mg
First	250
Second	270
Third	250

Table 6.13. Incorporation of radiolabelled 4,4'-dimethyl and 4α-methyl sterols by Artemia salina (cell-free preparations).

	^{3}H-Lanosterol	^{3}H-24,25-Dihydrolanosterol	^{3}H-Cycloartenol	^{3}H-24-Methylene cycloartanol	^{3}H-Cycloeucalenol
Activity added (dpm)	1.14×10^{6}	8.72×10^{5}	9.05×10^{5}	1.62×10^{6}	9.35×10^{5}
Non-saponifiable lipid					
Total activity (dpm)	1.11×10^{6}	8.59×10^{5}	8.78×10^{5}	1.58×10^{6}	9.23×10^{5}
% Recovery[1]	97.4	98.5	97.0	97.5	98.7

[1]Expressed as a percentage of the activity originally added.

Table 6.14. Distribution of activity incorporated into non-saponifiable lipid from radiolabelled 4,4'-dimethyl and 4α-methyl sterols by Artemia salina (cell-free preparations).

	^{3}H-Lanosterol	^{3}H-24,25-Dihydrolanosterol	^{3}H-Cycloartenol	^{3}H-24-Methylene cycloartanol	^{3}H-Cycloeucalenol
4,4'-dimethyl sterols					
Total activity (dpm)	1.10×10^6	8.16×10^5	8.65×10^5	1.55×10^6	0
% recovery[1]	96.5	93.6	95.6	95.7	0
4α-methyl sterols					
Total activity (dpm)	679	942	126	490	8.92×10^5
% recovery[1]	0	0	0	0	95.4
4-desmethyl sterols					
Total activity (dpm)	0	330	415	0	250
Steroids					
Total activity (dpm)	0	0	0	0	0

[1]Expressed as a percentage of the activity present in the non-saponifiable lipid fraction.

Table 6.15. Incubation of radiolabelled 4,4'-dimethyl sterols with Artemia salina (cell-free preparations).

Recovery of 2-^{3}H-lanosterol.

Specific activity of 2-^{3}H-lanosterol + carrier: 1290 dpm/mg.

Recrystallisation	Specific activity dpm/mg
First	1300
Second	1280
Third	1310

Recovery of 2-^{3}H-24,25-dihydrolanosterol.

Specific activity of 2-^{3}H-24,25-dihydrolanosterol + carrier: 3500 dpm/mg.

Recrystallisation	Specific activity dpm/mg.
First	3450
Second	3510

Recovery of 2-^{3}H-cycloartenol.

Specific activity of 2-^{3}H-cycloartenol + carrier: 8650 dpm/mg.

Recrystallisation	Specific activity dpm/mg
First	8790
Second	8580
Third	8520
Fourth	8550

CHAPTER VII.

GENERAL SUMMARY.

Since the first discovery that the larva of the blow fly required a dietary source of sterol for normal growth and development (Hobson 1935), the sterol requirements of insects have been rigorously investigated. The sum of knowledge derived from these studies permits certain general conclusions (Robbins et al 1971):

a. Insects require a dietary or exogenous source of sterol for normal growth, metamorphosis and reproduction. The only exceptions are those insects in which a sterol source may be attributed to associated symbionts.

b. The dietary requirement for sterol results from a deficiency in the sterol biosynthetic mechanism in insects. Thus insects, along with related arthropods and certain other invertebrates, differ from plants and vertebrates which fulfil their sterol requirements through the endogenous biosynthesis of sterols from simple precursors.

c. In insects, as in mammals, the sterols serve a dual role; as structural components of cells and tissues and as precursors for essential steroid metabolites and regulators (eg. hormones).

d. Specific sterol structures are required by insects and not all sterols can be utilised. Insects, however, can modify dietary sterols and these metabolic modifications serve to provide structures appropriate to the specific physiological and biochemical functions of sterols in insects.

By comparison, related topics in Crustacea have received little attention. This probably stems from the fact that crustaceans lack the medical and economic significance of insects. Because there are so many available species, no one crustacean has received the attention necessary to produce a detailed knowledge of the biochemical steps of sterol biosynthesis and metabolism. This report represents a survey of the various steps involved in this pathway in Artemia salina. This summary is intended to relate the results obtained from this present study to results obtained by other workers on crustaceans in particular and arthropods in general.

That crustaceans require a dietary or exogenous source of sterol for normal growth is generally taken as understood but this supposition is not based on firm nutritional data. There have been few attempts to rear any crustaceans on completely chemically defined media. A series of semi-defined media have been developed for various prawns but studies with such diets have been mainly confined to comparative studies on growth rates (see, for example, Forster 1970, 1972; Forster & Gabbott 1971; Wickins 1972). To the author's knowledge, there has been but a single report of an investigation into the sterol requirements of a crustacean using an artificial medium. In this study, growth of the prawn, Penaeus japonicus, was normal only with a supply of sterol (Kanazawa et al 1971b). Optimum growth was obtained with cholesterol; though ergosterol, stigmasterol and β-sitosterol could replace cholesterol but resulted in growth rates inferior to those obtained with cholesterol. The sterol requirement of Artemia salina and Penaeus japonicus can be totally satisfied by cholesterol (Kanazawa et al 1970; Provasoli & D'Agostino 1969).

The dietary requirement for sterols in Crustacea results from an inability to synthesise sterols from simple precursors. Many workers have shown that acetate and mevalonate are not incorporated into squalene or sterols (this work has been reviewed in Chapters I and V). Results presented in this thesis show that A.salina is unable to synthesise sterols from acetate, mevalonate, squalene or squalene-2,3-oxide. It appears that there are blockages at, at least, three sites in the pathway. Three enzyme systems are lacking - squalene synthetase, squalene oxidase and squalene-2,3-oxide cyclase. A functional pathway for the biosynthesis of isoprene units is indicated and this agrees with a similar finding in the crabs, Carcinus maenas and Eupagurus bernhardus (Walton & Pennock 1972). Similar results have been obtained with the fly, Sarcophaga bullata (Goodfellow et al 1972). There is a great paucity of data concerning isoprene biosynthesis in insects; data concerning Crustacea is confined to the present report and that of Walton & Pennock (1972). Reference has already been made to the many and varied terpenes found in insects (Chapter V) and whether any of these terpenes are, in fact, synthesised by the insects is a matter of conjecture. Because of the close taxonomic relationship between insects and Crustacea it would be surprising if similar compounds with similar functions did not exist in crustaceans. This aspect of crustacean biochemistry, however, has attracted little research. Research into the various pheromones of insects was prompted by its economic and environmental importance. Synthetic pheromones have been used successfully for insect pest control, as artificial lures for traps, as insecticides and as 'mating disruptors'; whereby normal mating behaviour is disrupted by permeating the atmosphere with synthetic sex attractants (Evans & Green 1973). The increasing interest in the commercial farming of the larger

decapods may provide the stimulus needed by research into this aspect of crustacean biochemistry. The development of effective lures and 'grouping' compounds is of obvious economic importance in this context.

The functions of sterols, particularly of cholesterol as this is the most predominant sterol, in Crustacea are not well known. In other organisms, cholesterol functions as a constituent of biomembranes, as a direct precursor of bile salts and as a precursor of steroid hormones.

That cholesterol is an integral part of crustacean membranes has not been shown and there has not yet been found any kind of steroidal bile salt in crustaceans. The general structures for the emulsifiers in the intestinal juices of the Crustacea so far studied (exclusively decapods) have been shown to be fatty acyl taurine and fatty acyl sarcosyl taurine, with dodec-5-enoic acid as the main fatty acid (Holwerda & Vonk 1973; Van den Oord et al 1964, 1965; Yamasaki et al 1965). Cholesterol is thought to be involved in the tanning or sclerotization of the arthropod exoskeleton. In crustaceans, this may vary from a thin flexible structure, as in some Cirripedia and Copepoda, through a calcified but still flexible exoskeleton, as in Astacus, to a thick, massive, rigid and calcareous shell, as in the Decapoda. A sterol/protein complex has been implicated in the tanning process but conclusive data is not available (Hackman 1971).

The physiological aspects of the crustacean sex organs are well known and shall not be dealt with here (see Prosser & Brown 1966). The biochemistry of these organs has been largely ignored. Work to date has been concerned mainly with demonstrating the ability of crustacean tissues to perform steroid interconversions and there are a few reports

of the biosynthesis of steroids from cholesterol (see Chapter VI). The present study extends this knowledge and demonstrates that A. salina is capable of converting a number of dietary sterols into steroids. This is the first report of such transformations in a crustacean. There have been no documented attempts to exhaustively analyse the steroid content of any crustacean. The glandular origin and function of those steroids which have been identified in Crustacea have not been fully investigated. Even in insects steroid endocrinology is still at a very exploratory stage and investigations have progressed along much the same lines as those used for crustaceans, allowing analogous conclusions to be drawn. Thus far, the approach to the problem of steroid biochemistry in insects and crustaceans has taken the line of determining whether vertebrate-type steroid hormones can be found, anabolised or used by these organisms. This vertebrate-orientated thinking has, perhaps, clouded the possibility that steroid formation in arthropods might be accomplished by unknown mechanisms. As example of such mechanisms, one may cite the various enzymic steps necessary to synthesise the ecdysones. Steroids unknown in vertebrates have been found in some insects, eg. cybisterone (12α-hydroxy-4,6-pregnadien-3,20-dione) and cybisterol (20α-hydroxy-4,6-pregnadien-3-one: Schildknecht et al 1967a, 1967b; Schildknecht & Hotz 1967; Schildknecht & Korning 1968). Another possibility to be considered is that in arthropods steroid production might not necessarily be confined to distinct organs and their role might be much more general and basic than it is in vertebrates (Lehoux & Sandor 1970).

Although classical experiments have established that arthropod moulting is under humoral control, the exact means by which hormones initiate the complicated sequence of physiological and biochemical events

that culminate in ecdysis is not completely understood. It appears that crustaceans and insects utilise the same or similar molecules. Either α- or β-ecdysone is thought to be the moulting hormone and β-ecdysone has been isolated from a number of crustacean sources (see structures and references given in Chapter VI). α-Ecdysone has yet to be isolated from a crustacean source. Both α- and β-ecdysones have been shown to induce moulting in a variety of crustaceans, in a number of decapods (Hubschman & Armstrong 1972; Kanazawa et al 1972b; Kanga-Rao et al 1973; Krishnakumaran & Schneiderman 1968; Lowe et al 1968; McWhinnie et al 1972; Warner & Stevenson 1972), in the cirripede, Balanus balanoides (Tighe-Ford & Vaile 1972), in the amphipods Orchestria gammarella (Blanchet 1972) and Orchestria cavimana (Graf 1972) and in the isopod, Ligia oceanica (Maissiat & Maissiat 1973). It is generally seen that both α- and β-ecdysones are equally effective at inducing ecdysis though it has been suggested that the biological activity of α-ecdysone may be the result of its conversion to β-ecdysone (Moriyama et al 1970). This has raised the question whether α-ecdysone has a hormonal activity of its own or whether its activity is entirely the result of its conversion to β-ecdysone.

It is known that the ecdysones are biosynthesised from cholesterol, both in insects (Gersch & Sturzebecher 1971; Nakanishi et al 1972 and the review articles of Rees 1971; Robbins et al 1971) and in crustaceans (Kater & Spaziani 1971; Spaziani & Kater 1973; Guary & Kanazawa 1973). Results are presented in this thesis which show that A. salina is able to convert a wide variety of dietary 4-desmethyl phytosterols to substances tentatively identified as ecdysones. The steps leading from cholesterol to the ecdysones have so far defied elucidation. Because of the large cholesterol pool in arthropod tissues, radiolabelled

precursor becomes diluted to such an extent that it is impossible to isolate or even detect the minute amounts of radiolabelled intermediates formed. It has been suggested that cholesta-5,7-dien-3β-ol, which is formed from cholesterol in many insect species, may be a precursor of the ecdysones (Thompson et al 1972b) and this compound has been converted to ecdysterone (Galbraith et al 1970). Another postulated intermediate is 2β,3β,14α-trihydroxy-5α-cholesta-7-ene-6-one (Robbins et al 1971).

It is not possible at this stage to draw any general conclusions as to the structural requirements of crustaceans for sterols. The inability of A.salina to utilise 4α-methyl and 4,4'-dimethyl sterols indicates that the presence of methyl groups at positions 4 and 14 precludes the metabolism of a particular sterol. This results from the loss of one or more of the enzymes responsible for the oxidative removal of these methyl groups from the sterol ring nucleus. The presence of the cyclopropane ring of plant sterols is also inhibitory. Crustaceans, as typified by A.salina, can modify a range of dietary sterols and these metabolic conversions serve to provide structures appropriate to the specific physiological and biochemical functions of sterols in Crustacea.

Throughout this thesis, reference has been made to analogous reactions in insects. Although the majority of insects adhere to the biosynthetic schemes outlined previously, there are exceptions. Within the insect family there are variations in the ability of members to utilise sterols. For example, the cockroach, Blatella germanica, is not able to reduce the Δ^{22} double bond (Clark & Bloch 1959) and both the larva and the adult of the housefly, Musca domestica, are unable to

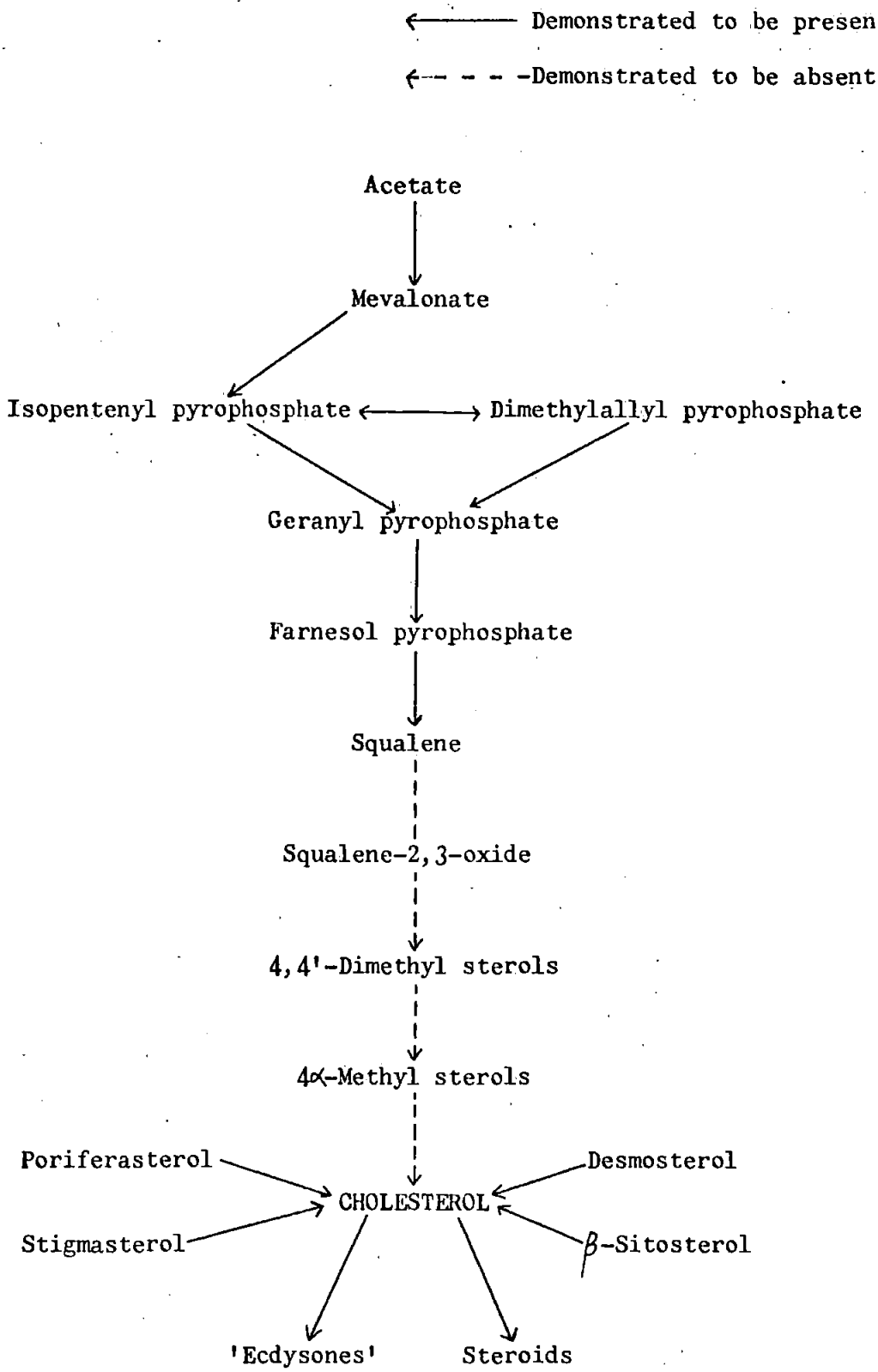
convert β-sitosterol to cholesterol (Kaplanis et al 1963, 1965). Similarly, there have been a few reports that some species of insects cannot utilise cholesterol. The sterol requirements of the Mexican cactus fly, Drosophila pachea, can only be satisfied by stigma-7-ene-3β-ol (a cactus sterol) or other Δ^7 or $\Delta^{5,7}$ sterols such as cholesta-7-ene-3β-ol and cholesta-5,7-dien-3β-ol (Heed & Kircher 1965). The beetle, Xyleborus ferrugineus, requires ergosterol or 7-dehydrocholesterol for normal growth and development; this is normally obtained from a gut fungal symbiont (Kok et al 1970). There are an infinitely greater variety of ecological niches occupied by insects than there are by crustaceans but it must be borne in mind that differences in the general pattern might also be present in those crustaceans which occupy highly specialised niches. The wood-boring gribble, Limnoria lignorum, and parasitic crustaceans, such as Sacculina, immediately spring to mind. One area in which a biochemical difference between crustaceans and insects has been found is the sex pheromone. Mention has already made to the steroidal nature of crustacean sex pheromones. In insects, the sex pheromones are either long chain fatty acids, long chain alcohols or terpenes (Cooke 1973; Klun et al 1973; Rogoff et al 1973).

The presence of dissolved sterols in sea water is well documented (Copin & Barbier 1971; Mathews & Smith 1968; Kanazawa & Teshima 1971b; Saliot & Barbier 1971). The recent finding that two marine invertebrates, the sea-anemone, Calliactis parasitica, and the oyster, Ostrea gryphea, are able to incorporate ^{3}H-fucosterol in sea water and convert this into cholesterol raises the question of whether such sterols are used by crustaceans. For free-swimming crustaceans it is unlikely that sterol ingestion by this method could satisfy the needs

of an organism. The metabolic demands of the more sedentary Crustacea are lower than for the free-living species and, in such organisms, dissolved sterols may have a significant role.

Evidence has been presented concerning the biosynthesis and metabolism of sterols in Artemia salina. It has been shown that this organism is unable to synthesise sterols from simple precursors but is able to convert dietary sterols into cholesterol and hence into steroid hormones and ecdysones. These findings are summarised in Scheme 7.1. It is readily apparent that certain portions of this scheme require further investigation, especially the early and terminal metabolites in the biosynthesis and metabolism of the ecdysones and steroids. A more serious deficiency, perhaps, is reflected in the complete lack of knowledge of the enzyme systems involved in these transformations in Crustacea. It is hoped that the expanding commercial interest in Crustacea will provide the stimulus for the further research needed.

Scheme 7.1. Metabolic scheme for the biosynthesis and metabolism of sterols in Artemia salina.



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With compliments

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Thesis returned as stated in letter of the 24th October.