Some Studies on the Biosynthesis of Ubiquinone, Isoprenoid Alcohols, Squalene and Sterols by Marine Invertebrates

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The ability of fourteen marine invertebrates to utilize [^{14}C]mevalonate for the biosynthesis of isoprenoid compounds was investigated. Several of the animals, in particular crustaceans, bivalve molluscs, a coelenterate and a sponge, were unable to synthesize squalene and sterols, whereas gastropod molluscs, echinoderms, an annelid and a sponge could. Regardless of sterol-synthesizing ability the animals (with the exception of a sponge) always made dolichol and ubiquinone, and thus a specific block in squalene and sterol synthesis was indicated in some animals. Radioactivity accumulated in relatively large amounts in farnesol and geranylgeraniol in those animals incapable of making sterols.

It is accepted that mammals can synthesize squalene and the various sterols that they require from acetate via mevalonate. However, not all lower animals can make sterols, and Clayton (1971) has discussed the sterol requirements of several invertebrates, particularly insects. All insects examined have been found to require sterols for growth (Lipke & Fraenkel, 1956) and arthropods in general are incapable of making sterols (Clark & Bloch, 1959; Clayton, 1960; Van den Oord, 1964; Whitney, 1970; Zandee, 1966, 1967*a*). The capacity of organisms to make sterols has been studied in other phyla, but only in molluscs and annelids in any detail, and in both these groups it has been found that whereas some members of the phylum can achieve the synthesis others cannot (Fagerlund & Idler, 1960; Voogt, 1967*a,b*, 1968*a,b*, 1969; Wootton & Wright, 1962; Zandee, 1967*b*). Clayton (1971) suggests that sterols are required by insects as components of membranes and as precursors of ecdysones.

If sterols are dietary essentials for some invertebrates it is of interest to know whether these organisms can synthesize other terpenoids such as ubiquinone and dolichol. In higher animals the terpenoid side chain of ubiquinone is made from mevalonate (see Glover, 1965), and dolichol similarly can be made from mevalonate in mammals (Butterworth *et al.*, 1963). As ubiquinone is an essential component of the mitochondrial electron-transfer system and is also known to be present in insects, it must be synthesized or be a vitamin (Crane, 1965). It has been reported that ubiquinone is a vitamin for the marine dinoflagellate *Oxyrrhis marina* (Droop & Pennock, 1971). Although it has not been shown that dolichol occurs in invertebrates, its presence seems likely, since it has been shown in mammals to be necessary for transferring sugars to an acceptor, possibly a

glycoprotein (Behrens & Leloir, 1970; Behrens *et al.*, 1971).

The purpose of the present paper is to determine whether there is any simple division in the invertebrate phyla with regard to sterol-synthesizing ability and to investigate whether ubiquinones and dolichol have biosynthetic or dietary origins in invertebrates. The results show that regardless of the ability to synthesize sterols all the invertebrates examined made ubiquinone and dolichol. Further, the accumulation of radioactivity (from labelled mevalonate) into farnesol and geranylgeraniol in animals incapable of making sterols, together with a lack of radioactivity in squalene, indicates that a major breakdown in sterol biosynthesis occurs between farnesyl pyrophosphate and squalene.

Experimental

Materials

Animals. These were obtained locally or from the University of Liverpool Marine Biological Station at Port Erin, Isle of Man, through Dr. R. G. Hartnoll, or purchased from the Marine Biological Association at Plymouth. They were maintained in sea-water aquaria at 8°C; the carnivorous animals were given *Littorina littorea*, and the herbivorous, *Fucus spiralis*, as food.

Methods

Incubation. The animals were usually starved for 2–3 days before incubation, and then 5–10 μCi of DL-[2- ^{14}C]mevalonate (The Radiochemical Centre, Amersham, Bucks., U.K.) in 0.01 M-sodium phosphate buffer, pH 7.4, was administered by a variety of

methods. Sea anemone, sea urchin and *Nereis pelagica* were injected through the mouth, gastropod molluscs through the foot muscle, bivalve molluscs into the mantle cavity, crustaceans through the leg joint, *Asterias rubens* and *Ophiocomina nigra* into the coelomic cavity. The labelled material was dropped on to moist Porifers and these were replaced in sea water after about 10–15 min. The animals were maintained in small aquaria for 24 or 40 h.

Preparation of unsaponifiable material. The animals were killed and then macerated in an equal amount (w/v) of methanol containing 5% (w/v) pyrogallol. With molluscs the shell was removed before maceration. After addition of 0.5 vol. of approx. 11 M-KOH the mixture was refluxed for 30 min and the lipids were extracted by the method of Pennock *et al.* (1962).

Column chromatography. The unsaponifiable lipids were fractionated on acid-washed alumina (M. Woelm, Eschwege, Germany) deactivated to Brockmann Grade 3, by using mixtures of diethyl ether and light petroleum (b.p. 40–60°C) for developing the column. Squalene and other hydrocarbons were eluted with light petroleum; the column was then developed with 2% (v/v) diethyl ether in light petroleum, and ubiquinone, dolichol, 4,4-dimethyl sterols and α -tocopherol were eluted with 6% (v/v) diethyl ether in light petroleum. Further development with 12% (v/v) diethyl ether in light petroleum yielded geranylgeraniol and farnesol, together with 4-monomethyl sterols and small amounts of 4-desmethyl sterols, and a final elution with 25% (v/v) diethyl ether–light petroleum gave the bulk of the sterols.

Thin-layer chromatography. A portion of the total unsaponifiable lipid was separated by two-dimensional t.l.c. on silica gel G (Pennock *et al.*, 1964). Fig. 1 is a composite diagram of a chromatogram showing the compounds identified. Reducing compounds were located with FeCl_3 – $\alpha\alpha$ -bipyridyl, quinones with leuco-Methylene Blue and all other lipids with phosphomolybdic acid (Krebs *et al.*, 1969). Authentic lipid markers were chromatographed with the lipid to confirm identifications.

The fractions from column chromatography (see above) were separated further by preparative t.l.c. on silica gel G (250 or 500 μm thick) as follows. Light petroleum was used as solvent for t.l.c. of the fraction eluted by light petroleum from alumina and in this solvent squalene had R_f 0.24. The chromatogram was scanned for radioactivity, the area corresponding to squalene was extracted with diethyl ether and the radioactivity of the extract counted in a scintillation counter.

The fraction eluted from the alumina column with 6% diethyl ether in light petroleum contained ubiquinone which was isolated by preparative t.l.c. in chloroform. The ubiquinone was then purified further by reversed-phase t.l.c. as follows. Thin layers of kieselguhr G (250 μm) were dipped in a

Fig. 1. Diagram of a two-dimensional thin-layer chromatogram showing the various compounds identified in marine invertebrates

For experimental details see the text. Solvent A, chloroform; solvent B, diethyl ether in light petroleum (b.p. 40–60°C) (1:4, v/v). The absorbent was silica gel G. 1, Squalene; 2, ubiquinone; 3, α -tocopherol; 4, dolichol; 5, 4,4-dimethyl sterols; 6, 4-monomethyl sterols; 7, 4-desmethyl sterols; 8, farnesol; 9, geranylgeraniol.

tank of 5% (v/v) paraffin in light petroleum (b.p. 40–60°C) and, after removal, the light petroleum was allowed to evaporate. The materials were applied in acetone solution and the chromatograms developed in aqueous acetone solvents (presaturated with paraffin). For optimum results it was necessary to pass the paraffin–light petroleum mixture through Brockmann Grade I alumina before use to remove congeners of unknown character present in the paraffin, which otherwise interfered with the chromatography. The chromatogram was developed with aq. 95% (v/v) acetone (Bolliger & König, 1969) and authentic ubiquinone isoprenologues were run as markers. The chromatogram was scanned for radioactivity and when it was established thus that no radioactive materials other than ubiquinone isoprenologues were present, the ubiquinones were eluted and counted for radioactivity in a scintillation counter.

Dolichol was also located in the 6% diethyl ether–light petroleum fraction from the alumina chromatography; it was isolated by t.l.c. of that fraction in chloroform, and a portion was counted for radioactivity. The dolichol fraction was separated further

by reversed-phase t.l.c. with aq. 99% (v/v) acetone as solvent (Dunphy *et al.*, 1967). The reversed-phase thin-layer chromatogram was scanned for radioactivity and usually material at the solvent front as well as dolichol was found to be labelled. The radioactivity in dolichol was determined from the radioactivity scan. Separation of dolichol isoprenologues (see the Results section) on reversed-phase t.l.c. was not sufficient for the radioactive scanner to differentiate between them. A radioautogram of the chromatogram was developed and this showed up radioactivity in individual isoprenologues clearly.

The 12% diethyl ether in light petroleum fraction from alumina chromatography was fractionated further by preparative t.l.c. with 20% diethyl ether in light petroleum and the area of plate corresponding to geranylgeraniol and farnesol markers was extracted. This fraction was chromatographed on reversed-phase t.l.c. with aq. 65% (v/v) acetone as solvent (McSweeney, 1965) and the plate scanned for radioactivity. In those animals that did not synthesize sterols, radioactivity was found associated with the farnesol and geranylgeraniol markers; however, only negligible amounts of radioactivity could be detected in the animals that synthesized sterols.

The three general sterol classes, 4,4-dimethyl, 4-monomethyl and 4-desmethyl sterols, were isolated by preparative t.l.c. of the 6, 12 and 30% diethylether in light petroleum fractions from alumina chromatography with chloroform as solvent. The fractions were purified further by chromatography on thin layers of silica gel G with 1% (v/v) methanol in benzene as solvent. Samples were counted for radioactivity in a scintillation counter. No attempt was made to separate the sterols further, although in some cases samples together with marker sterols were chromatographed on reversed-phase thin layers with aq. 75% (v/v) acetone as solvent. These plates were scanned for radioactivity and only small amounts of radioactivity were detected in materials other than sterols.

Radioactivity analysis was also carried out after two-dimensional t.l.c. (as described above) of a sample of the total unsaponifiable lipid. To approx. 1 mg of unsaponifiable lipid were added 10 μ g each of dolichol, ubiquinone-10, lanosterol (as a typical 4,4-dimethyl sterol) and a mixture of 4-monomethyl sterols, and after development of the chromatogram these materials were eluted and counted for radioactivity. The region readily identifiable as 4-desmethyl sterol was also removed and the lipid extracted and counted for radioactivity.

This analysis served as a check on the results obtained by column chromatography and t.l.c.

Detection of radioactivity. Three methods of detection were utilized, scintillation counting, scanning of chromatograms and radioautography. Samples for scintillation counting were dissolved in

5 ml of toluene containing 0.5% (w/v) 2,5-diphenyl-oxazole and 0.03% (w/v) 1,4-bis-(4-methyl-5-phenyl-oxazol-2-yl) benzene and were counted in a Beckmann LS 200 liquid-scintillation counter with an efficiency for ^{14}C of about 62%.

T.l.c. chromatograms were examined with a Panax Scanner, a minimum of 400 d.p.m. being required for detection on adsorption chromatograms and 1000 d.p.m. on reversed-phase chromatograms. As a general rule, after scanning of chromatograms and with all two-dimensional t.l.c. chromatograms, radioautographs were prepared on Royal Blue X-ray film (Eastman-Kodak Corp., Rochester, N.Y., U.S.A.). After 1–2 mg of unsaponifiable material containing about 5000 d.p.m. had been chromatographed in two dimensions on a thin layer of silica gel G a radioautograph showing the main radioactive areas could be obtained after about 4 days exposure, and after 2–3 weeks an extremely good film could be obtained.

Results and Discussion

Uptake of radioactivity

The incorporation of radioactivity into the unsaponifiable lipids of the marine invertebrates was surprisingly large in some cases (Table 1). The conversion of about 40% of the D-isomer into lipids by *Psammechinus* and *Asterias* represents extremely rapid utilization of the labelled mevalonate and the efficient uptake of material on injection. The difficulty of finding an efficient method of injection is reflected in some cases by a low incorporation; *Eupagurus* and *Cardium* for instance presented problems in administering the dose and there is a poor uptake of radioactive isotope in each case. The sponges too, particularly *Grantia*, showed a poor incorporation of label; this may be due in part to difficulties in administering the mevalonate, and also it was not easy to be sure whether parts of the sponge had died or not. All the other animals were in good condition after the incubation period.

Squalene and sterols

Values for the radioactivity detected in squalene and the various sterol fractions are shown in Table 2. The animals fall into two main classes, those which synthesize sterols and squalene from mevalonate and those which do not. Among the animals unable to make sterols and squalene are the crustaceans *Carcinus* and *Eupagurus*, thus confirming the earlier work on crustaceans by Van den Oord (1964) with the crab *Cancer pagurus*, Whitney (1970) on the blue crab *Callinectes sapidus* and the barnacle *Balanus nubilus*, and Zandee (1966, 1967a) on the crayfish *Astacus astacus* and lobster *Homarus gammarus*.

Table 1. *Incorporation of DL-[2-¹⁴C]mevalonate into the lipids of marine invertebrates*

For experimental details see the text.

Phylum	Species	Amount of DL-[2- ¹⁴ C]- mevalonate injected (μ Ci)	Number of animals injected	Duration of incubation (h)	Wet wt. of animals (g)	Wt. of unsaponifiable lipid (mg)	% Incorporation of the D-isomer
Porifera	<i>Grantia compressa</i>	5	50*	40	10.2	6.6	0.64
	<i>Suberites domuncula</i>	5	—†	40	96.4	106.3	1.47
Coelenterata	<i>Metridium senile</i>	5	13	24	17.7	58.4	3.60
Annelida	<i>Nereis pelagica</i>	5	24	40	9.6	33.0	9.20
Mollusca	<i>Nucella lapillus</i>	5	19	24	16.3	75.2	4.10
	<i>Littorina littorea</i>	10	30	24	26.9	84.5	2.25
	<i>Gibbula cineraria</i>	5	23	24	9.4	28.0	7.54
	<i>Mytilus edulis</i>	10	5	32	24.3	135.8	5.55
	<i>Cardium edule</i>	5	10	24	22.5	18.5	1.28
	<i>Asterias rubens</i>	5	2	24	46.5	107.1	41.0
Echinodermata	<i>Ophtocomina nigra</i>	5	3	24	14.1	32.3	3.20
	<i>Psammochinus miliaris</i>	5	2	24	19.8	22.4	38.6
	<i>Carcinus maenas</i>	10	3	24	160.2	248.5	12.8
	<i>Eupagurus bernhardus</i>	5	9	24	8.3	22.4	1.44
Crustacea							

* This is the number of 'purses' injected in this purse sponge.

† One large clump of sponge.

Table 2. Incorporation of label from DL-[2-¹⁴C]mevalonate into squalene and sterols

DL-[2-¹⁴C]Mevalonate (5 μ Ci) was injected into the animals and they were kept for a further 24h. The unsaponifiable-lipid fraction was isolated from the animals; this fraction was chromatographed on acid-washed alumina deactivated to Brockmann Grade 3; the various compounds were purified by adsorption and reversed-phase t.l.c. Radioactivity in the fractions was determined by scanning thin-layer chromatograms and by scintillation counting of materials eluted from thin-layer chromatograms.

Species	Squalene (total d.p.m.)	4,4-Dimethyl sterols (total d.p.m.)	4-Monomethyl sterols (total d.p.m.)	4-Desmethyl sterols (total d.p.m.)
<i>Grantia compressa</i> †	1301	2760	1460	1630
<i>Suberites domuncula</i> †	0	0	0	0
<i>Metridium senile</i>	0	0	0	0
<i>Nereis pelagica</i> †	258700	42130	21270	18950
<i>Nucella lapillus</i>	13960	38850	15870	10010
<i>Littorina littorea</i> *	54000	32670	14200	39500
<i>Gibbula cineraria</i>	159100	41820	32720	37780
<i>Mytilus edulis</i> *‡	0	0	0	0
<i>Cardium edule</i>	0	0	0	0
<i>Asterias rubens</i>	1387000	256200	139000	62920
<i>Ophiocomina nigra</i>	92320	1510	800	4442
<i>Psammechinus miliaris</i>	936900	458200	284500	106300
<i>Carcinus maenas</i> *	0	0	0	0
<i>Eupagurus bernhardus</i>	0	0	0	0

* 10 μ Ci of mevalonate used.

† Incubated for 40h.

‡ Incubated for 32h.

Similarly the inability of the plumose anemone *Metridium* to synthesize sterols agrees with the results of Van Aarem *et al.* (1964), who showed that acetate was not a precursor of sterols in the jellyfish *Rhizostoma* sp. All three echinoderms, the starfish *Asterias*, brittle-star *Ophiocomina* and small green urchin *Psammechinus*, convert mevalonate readily into squalene and sterols, although Salaque *et al.* (1966) were unable to show incorporation of acetate into squalene and sterols in the sea urchin *Paracentrotus lividus*. Voogt (1971) has shown that mevalonate is incorporated into the sterols of the mesogastropod mollusc *Natica cataena*, whereas acetate is not, and he suggests that this may show that sterol synthesis is controlled at the level of β -hydroxy- β -methylglutaryl-CoA reductase in these invertebrates. Biosynthesis of sterols when acetate is the precursor may be controlled, whereas mevalonate would by-pass this control and thus inconsistencies could arise.

The situation in the Mollusca is complicated. Certainly from the literature there are several reports of molluscs being capable of making sterols and also reports of others that cannot. The results of the present work add to the confusion. The gastropods in general can carry out the synthesis of sterols. Voogt has reported sterol synthesis by members of

the Archaeogastropoda (1968a) and Mesogastropoda (1969, 1971), and his work is supported by results obtained with *Gibbula* and *Littorina* respectively. *Buccinum undatum*, a Stenoglossan gastropod, cannot make sterols from acetate (Voogt, 1967a) and Voogt (1967a) suggests that the difference between this and other gastropods examined is that it is carnivorous and able to get cholesterol from its diet, whereas the others are herbivorous and need to synthesize sterols. This theory does not, however, fit the results obtained in the present work, as the carnivorous *Nucella* (also in the order Stenoglossa) shows sterol and squalene synthesis.

Results with *Mytilus* and *Cardium*, together with those of Salaque *et al.* (1966) on *Ostrea gryphea*, indicate that Lamellibranchs cannot make sterols. However, Fagerlund & Idler (1960) also working with bivalves *Mytilus californianus* and *Saxidomus giganteus* showed incorporation of acetate into digitonin-precipitable material. Thus it is difficult to come to any conclusions about the significance of the results, as they are conflicting not only within the phylum but also in the class and order. It is not easy to see how a mutation causing a deficiency in sterol synthesis could so split a phylum. In fact *Buccinum* is the only gastropod failing to synthesize sterols, and if a phenomenon similar to that found in

Natica (Voogt, 1971) occurred in *Buccinum* then perhaps it would be found that *Buccinum* could convert mevalonate into sterols and so fit in with the rest of the class. The inconsistencies in the bivalve series cannot be explained at the moment. One member of the class Cephalopoda, the cuttlefish *Sepia officinalis*, has been studied and is not capable of forming sterols from acetate (Zandee, 1967b).

Marine annelids have been investigated by Wootton & Wright (1962) and the two types used, the polychaetes *Nereis diversicolor* and *Arenicola marina*, have sterol-synthesizing capacity. This is confirmed in these studies with *N. pelagica*. No other studies have been carried out with Porifers as far as we can tell, but with the two species investigated the situation is straightforward; one is capable and the other incapable of making sterols. Several other sponges were used, but incorporation of radioactivity into the lipids was extremely low and little could be learned from them. Clearly the method of administration of labelled mevalonate, and perhaps the condition of the animals, was poor.

No attempt was made to separate the individual sterols in the three classes 4-desmethyl, 4-monomethyl and 4,4-dimethyl sterols, but it was apparent from radioautographs of the two-dimensional thin-layer chromatograms of total unsaponifiable lipids that most of the material in the 4-desmethyl spot was not labelled. The darkening on the radioautograph was generally confined to the more slowly running area of the spot (particularly in the second dimension). The main sterol in invertebrates seems to be cholesterol (see reviews by Bergmann, 1962; Austin, 1970), but the more primitive the animal the greater the variety of sterols. Perhaps it is these other sterols that are formed by the invertebrates examined here, at least in these relatively short-term incubations.

Short-chain isoprenoid alcohols

[^{14}C]Acetate has been used as a sterol precursor rather than [^{14}C]mevalonate by several investigators because the fatty acid fraction of the lipids can be examined for radioactivity. If the fatty acid fraction is labelled it is clear that the acetate has been actively metabolized even if sterols are not formed. In our studies with mevalonic acid we have examined other isoprenoid compounds, in particular ubiquinone and dolichol, and since these compounds have always been radioactive it is clear that the mevalonate has been taken up and utilized. However, it was found that these were not needed to prove uptake and usage of mevalonate in animals not making sterols, since several short-chain-length isoprenoid alcohols, in particular farnesol and geranylgeraniol, appeared in the labelled form.

In each of the six cases where sterols were not synthesized, radioautographs of two-dimensional

Fig. 2. Diagram of a radioactive scan of short-chain alcohols from *Carcinus maenas* on reversed-phase t.l.c.

A fraction containing short-chain isoprenoid alcohols was isolated from unsaponifiable lipids of *C. maenas* (which had been dosed with $10\mu\text{Ci}$ of DL-[2- ^{14}C]mevalonate) by column chromatography and adsorptive t.l.c. The fraction was chromatographed on paraffin-impregnated kieselguhr G with acetone-water (13:7, v/v) and scanned for radioactivity with a Panax Scanner. O, Origin; A, geranylgeraniol; B, farnesol; SF, solvent front.

thin-layer chromatograms indicated two spots in the general region of the 4-monomethyl sterols (see Fig. 1). The material corresponding to these spots was isolated and run on reversed-phase t.l.c. and scanned for radioactivity. Fig. 2 shows a scan of such a chromatogram; farnesol and geranylgeraniol are labelled. The labelled farnesol and geranylgeraniol were isolated by reversed-phase t.l.c. and chromatographed on silica gel G as the free alcohols and as acetates, and in each case the radioactive label had the same mobility as the marker compound. The compounds could only be detected by radioactivity and in no case were there signs of accumulated isoprenoid alcohol. With *Metridium* and *Eupagurus* there was radioactivity in the area of the chromatogram where geraniol would be found, although the identity of the compound was not confirmed. No accurate values for the radioactivity associated with these alcohols were determined, but radioautographs indicated that the amounts were comparable with those of ubiquinone and dolichol (see Table 3).

In each case the scans of radioactivity on reversed-phase t.l.c. indicated a labelled material at the origin and another migrating slightly more slowly than geranylgeraniol. The identities of these components are not known.

The presence of these alcohols indicates that the major defect in sterol synthesis in these animals

occurs at the level of formation of squalene from farnesyl pyrophosphate, although of course other enzymes may be missing. It is likely that there is an accumulation of radioactivity in these compounds as a result of the lack of squalene synthesis, since they were not readily detected in an animal synthesizing sterols. The compounds geraniol, farnesol and geranylgeraniol may, as pyrophosphates, play a role in the biosynthesis of the side chain of ubiquinone and of dolichol. One could visualize the formation of farnesyl and geranylgeranyl pyrophosphate on one multi-enzyme complex and the release of these compounds either for squalene synthesis or chain elongation with *trans*-isoprene units to give the side chain necessary for ubiquinone or *cis*-addition to give dolichol.

The blockage of sterol synthesis in these animals is clearly at the formation of squalene, but this is not likely to be the only blockage in sterol synthesis in invertebrates, since Wootton & Wright (1962) found that the terrestrial oligochaete *Lumbricus terrestris* converted mevalonate into squalene but not into sterols. In several examples shown in Table 2 the incorporation of the radioactive label into squalene was very much more (ten- or twenty-fold) than that

into 4-desmethyl sterol, and the possibility that Wootton & Wright (1962) had missed very small amounts of radioactivity in the sterols was investigated. *L. terrestris* was injected with radioactive mevalonate and the unsaponifiable lipids were examined in the usual way; in agreement with Wootton & Wright (1962) there was a very high incorporation into squalene, smaller incorporation into ubiquinone and dolichol but no radioactivity in the sterols.

Ubiquinone

Table 3 shows that all animals examined, except the sponge *Suberites*, synthesized ubiquinone from mevalonate. It is necessary to repeat the experiment with *Suberites* before ubiquinone could be postulated as an essential dietary factor for this animal (several ubiquinones were detected in the unsaponifiable lipids). The low incorporation of the radioactive label into *Suberites* together with the large amount of unsaponifiable lipid present gave a very low d.p.m./mg of lipid value in this organism compared with the other animals, and this contributed towards the failure to detect radioactivity in ubiquinone. Reversed-phase t.l.c. is a relatively sensitive technique and

Table 3. Incorporation of DL-[2-¹⁴C]mevalonate into ubiquinone and dolichol

DL-[2-¹⁴C]Mevalonate (5 μ Ci) was injected into the animals and they were kept for a further 24h. The unsaponifiable-lipid fraction was isolated from the animals and this fraction was chromatographed on acid-washed alumina deactivated to Brockmann Grade 3 and the various compounds were purified by adsorption and reversed-phase t.l.c. Radioactivity in the fractions was determined by scanning thin-layer chromatograms and by scintillation counting. t, Trace; n.d., not detected.

Species	Ubiquinone isoprenologues present as shown by staining			
	Ubiquinone (total d.p.m.)	(no. of isoprene units in side chain)	Radioactive ubiquinone isoprenologues detected on radioautography	Dolichol (total d.p.m.)
<i>Grantia compressa</i> †	550	10, 9	n.d.	210
<i>Suberites domuncula</i> †	0	10, 9, 8	n.d.	7650
<i>Metridium senile</i>	18310	10	10, 9(t), 8(t)	5920
<i>Nereis pelagica</i> †	1560	10	10	3660
<i>Nucella lapillus</i>	7570	9	9	5760
<i>Littorina littorea</i> *	6730	10	10	5140
<i>Gibbula cineraria</i>	2280	10	10	3300
<i>Mytilus edulis</i> *‡	39550	10	10	33380
<i>Cardium edule</i>	9165	10	10	3220
<i>Asterias rubens</i>	8300	10	10, 9(t)	21200
<i>Ophiocomina nigra</i>	770	10	10	2690
<i>Psammechinus miliaris</i>	11650	10, 9(t)	10, 9(t)	15060
<i>Carcinus maenas</i> *	575300	10	10	32610
<i>Eupagurus bernhardus</i>	2240	10	10	12020

*10 μ Ci of mevalonate used.

† Incubated for 40h.

‡ Incubated for 32h.

cannot be applied in the presence of large amounts of lipid without distorting the chromatogram, so only small portions of the ubiquinone fraction from *Suberites* could be run. However, if radioactivity was present it was extremely low.

The highest incorporation of label into ubiquinone occurred in *Carcinus*, with relatively high incorporations also by *Mytilus* and *Metridium*, and it is perhaps significant that all three do not synthesize sterols, indicating that a greater proportion of the pool of mevalonate in these animals is available for ubiquinone synthesis. In all cases but *Nucella* the main ubiquinone present was the decaprenyl homologue ubiquinone-10. With *Nucella* only ubiquinone-9 was detected. Small amounts of ubiquinone-9 were detected radiochemically in some of the animals and ubiquinone-8 was detected in *Metridium*. Both ubiquinone-9 and -10 have been found in invertebrates (Crane, 1965). These results indicate clearly that (apart from *Suberites*) ubiquinone is made even when squalene and sterols are not synthesized. Ubiquinone is not an essential dietary factor, as is the case with *Oxyrrhis* (Droop & Pennock, 1971).

Dolichols

As shown in Table 3 all organisms converted mevalonate into dolichol. Usually dolichols could be detected chemically as well as radiochemically, but there was insufficient to allow i.r. or n.m.r. spectroscopy to confirm the identity of the dolichols. However, the fact that the material identified as dolichol ran with marker dolichol from pig liver on two adsorption systems on t.l.c. as well as on reversed-phase t.l.c. makes the identification reasonable. Further, the fractions had very high extinctions at 186nm, as would be expected for compounds with a large number of isolated double bonds, and the chromatography on reversed phase is very characteristic. On reversed-phase t.l.c. dolichol can be separated into a series of homologues (Dunphy *et al.*, 1967). Pig liver dolichol can be separated into as many as seven homologues with chain lengths C₈₅, C₉₀, C₉₅, C₁₀₀, C₁₀₅, C₁₁₀ and C₁₁₅; the C₉₅ homologue is present in the highest amount and the C₁₁₀ and C₁₁₅ homologues are only in trace amounts.

All the dolichol fractions from the marine organisms separated into series of homologues corresponding more or less to those of the pig liver dolichol series. Some dolichol fractions tended to have predominantly higher-molecular-weight components, whereas others tended toward the lower-molecular-weight homologues. Usually, however, the spot corresponding to the C₉₅ or C₁₀₀ homologue of pig liver dolichol was present in highest amount and contained most radioactivity. Pig liver dolichol contains three internal *trans*-isoprene units, and the remainder are *cis*, with the hydroxyl terminal isoprene unit saturated

(Burgos *et al.*, 1963). Chemical work on larger amounts of dolichol from marine invertebrates is required before it can be certain that they have structures similar to those of pig liver dolichol. However, for the moment it is not an unreasonable assumption.

Study of the function of dolichol is only just beginning, but it seems likely that it plays a part in sugar transfer, possibly to a glycoprotein (Behrens & Leloir, 1970; Behrens *et al.*, 1971), and the presence and synthesis of dolichol in all the marine invertebrates investigated underline the fundamental nature of the role.

General conclusions

Clearly members of the Invertebrata are capable of synthesizing ubiquinone and dolichol, and although some species can synthesize squalene and sterols, others cannot. It is apparent that mutations during evolution affecting sterol synthesis must have occurred in several phyla at different times, since there is no clear-cut demarcation in sterol-synthesizing ability. It is impossible to account for the results if only one mutation had occurred during evolution. Therefore it appears that sterol biosynthesis is vulnerable to mutation, relative to, say, other terpenoid biosynthetic routes such as to ubiquinone and dolichol. An alternative suggestion is that if a defect in synthesis of the latter two compounds occurs it is impossible for the animal to survive. This might suggest that these two compounds are essential components, whereas sterols are not, or rather that the necessary sterols can be obtained readily from the diet whereas ubiquinone and dolichol cannot. Ubiquinone and dolichol are present in most diets, but both are large lipophilic compounds and absorption, from the gut may be very poor, and if biosynthesis is impaired insufficient material may be obtained to satisfy requirements.

Many more results are required before conclusions can be drawn about changes during evolution but there is little doubt that a full knowledge of the defects in terpenoid biosynthesis would be of great value in drawing up evolutionary relationships. For example, it is reasonable to assume in the first instance that animals on the main evolutionary line have full terpenoid-synthesizing ability and that when the ability is lost the class or phylum is on a branch. Thus the coelenterates are usually accepted as being close to the main line of evolution, but we must assume that although modern coelenterates have lost the ability to make sterols (if this always proves to be the case) primitive coelenterates could carry out the synthesis.

Finally, the accumulation of radioactivity in farnesol and geranylgeraniol when sterols are not synthesized may simply reflect the inability of the

animal to use farnesol for sterol biosynthesis, or it is possible that these compounds are intermediates in ubiquinone and dolichol synthesis. Another reason for the presence of farnesol may be as a precursor of several compounds found in marine fauna such as farnesene, terrestrol, dendrolasin and, of course, the juvenile hormones (see reviews by Herout, 1971; Pfiffner, 1971). There appear to be no similar compounds for which geranylgeraniol could be a precursor, but perhaps now that it has been established that this compound is a component of invertebrates and that it is synthesized from mevalonate more work will be carried out.

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