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# THE ABSENCE OF CHOLESTEROL SYNTHESIS IN THE CRAB, *CANCER PAGURUS* L.

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**Abstract**—1. The incorporation of acetate-1-$C^{14}$ and mevalonic acid-2-$C^{14}$ into lipids of the crab, *Cancer pagurus* L., was studied. No evidence for the incorporation of these compounds into squalene or cholesterol was obtained.

2. It was concluded that the crab lacks the ability to synthesize squalene or cholesterol and that cholesterol which is the predominant sterol present in the tissues is of dietary origin.

## INTRODUCTION

THE occurrence of sterols in marine invertebrates has been studied in detail (Bergmann, 1962). Whereas the metabolism of sterols has been investigated extensively in vertebrates (Popják & Cornforth, 1960; Grant, 1962), in insects (Clayton, 1964), and in micro-organisms (Hunter & Holz, 1962), little is known about the metabolism of sterols in marine organisms. Possible dietary requirements in marine invertebrates have not been elucidated as nutrition experiments are difficult to conduct with these animals. The mussel *Mytilus californianus* and the clam *Saxidomus giganteus* have been found to incorporate acetate-2-$C^{14}$ into a sterol (Fagerlund & Idler, 1960), and several marine Annelida have been shown to synthesize cholesterol from mevalonic acid (Wootton & Wright, 1962). The formation of 24-methylenecholesterol from cholesterol as well as dehydrogenation of cholesterol at $C_{22}$ and $C_{25}$ have been observed in the clam *Saxidomus giganteus* (Fagerlund & Idler, 1961a, b) and the starfish *Pisaster ochraceus* can convert cholesterol into cholest-7-en-3$\beta$-ol (Fagerlund & Idler, 1960). Crustacea, including the advanced types of decapods (crab, lobster, crayfish), which have cholesterol as their main sterol (Bergmann, 1962), have been studied with respect to sterol synthesis only for the freshwater crayfish, *Astacus astacus* L., which was found incapable of incorporating acetate-1-$C^{14}$ into squalene or cholesterol (Zandee, 1962). In the crab, *Cancer pagurus* L., substantial amounts of cholesterol are present in hepatopancreas (midgut-gland), haemolymph and muscle. However, it is not known whether the cholesterol is of exogenous or endogenous origin.

The present paper reports the absence of squalene and cholesterol biosynthesis in the crab, *Cancer pagurus* L. Acetate-1-$C^{14}$ and mevalonic acid-2-$C^{14}$ were administered to crabs, and cholesterol and a hydrocarbon fraction with chromatographic properties of squalene were isolated from several tissues of the crab and analysed for isotope content.

## EXPERIMENTAL

### *Animal experiments*

The crabs, mainly females, used in the acetate experiments were caught in November in the North Sea near the coast of Holland. The animals were kept in aquaria with running sea water at a temperature of 13°C. These experiments were carried out in the Netherlands Institute for Sea Research, Den Helder, Holland. Sodium acetate-1-$C^{14}$ (specific activity 0·114 mc/mg, obtained from Philips-Duphar, Amsterdam) in a solution made up to 1% with carrier acetate was injected into the haemolymph in the abdomen. During the experiment one series of crabs was fed with fish. For the mevalonate experiment the crabs were obtained from Uddevalla, Sweden, in October. The animals were kept in sea water at 4–6°C in aquaria. The sea water was percolated continuously through a sand filter and a jet of air was blown in. Mevalonic acid-2-$C^{14}$ (specific activity 25·8 mc/mg, Radiochemical Centre, Amersham, England) in a 1% solution of sodium chloride was injected into the hepatopancreas. Another group of animals were given acetate-1-$C^{14}$ (specific activity 0·147 mc/mg) in the same way. During the experiments no food was given to the crabs. Volumes injected never exceeded 0·5 ml.

### *Isolation of lipids*

Hepatopancreas, ovaries and, in one case, all soft tissues were isolated from the crabs, which were killed by freezing. The wet tissues were refluxed with 2 M potassium hydroxide in 70% methanol for 3 hr in an atmosphere of nitrogen. The saponification mixtures were then filtered and the non-saponifiable and the saponifiable fractions were isolated by extraction with ether. From crabs injected with mevalonic acid the haemolymph was collected by bleeding the animals after amputation of some legs. To the haemolymph and hepatopancreas (ground in a mortar) ethanol was added. The ethanol extracts were filtered and the residues on the filter papers were extracted thoroughly with ether. The combined ethanol and ether extracts were evaporated to dryness under reduced pressure, and the residues were then saponified as described above.

### *Analysis of lipids*

The non-saponifiable fractions were chromatographed on columns of aluminium oxide, grade II (Woelm, Eschwege, W. Germany). Aluminium oxide was used in a hundredfold excess and fractions of 2–3 ml/g of aluminium oxide were collected. The hydrocarbon fraction, eluted with hexane, was further separated by means of preparative thin layer chromatography (TLC) on 0·4–0·5 mm layers of silicic acid using heptane for development. Squalene was run as a reference compound. The band containing squalene was removed and extracted with petroleum ether. If necessary, the TLC procedure was repeated using heptane/hexane, 6:4, as solvent. Cholesterol was eluted from the aluminium oxide columns with 15–20% ethyl acetate in benzene and was crystallized from methanol. The purity was checked by TLC using ethyl acetate/benzene, 1:4, as moving phase. The same

system was used to test the presence of sterols in the other fractions from the aluminium oxide columns. The ether extract of haemolymph in the mevalonate experiments was chromatographed on a 4·5 g column of hydrophobic Hyflo SuperCel using phase system I (Danielsson, 1958). The fractions were analysed by counting of aliquots. Aliquots of the saponifiable fractions were esterified in methanol/sulphuric acid. The fatty acid methyl esters were chromatographed on columns of aluminium oxide, grade II, and were eluted with hexane/benzene, 1 : 1.

*Isotope determinations*

Radioactivity in aliquots of isolated fractions and of effluents from column chromatograms was counted in an I.D.L. liquid scintillation counter, type 2022. In the mevalonate experiments aliquots of isolated samples were counted as infinitely thin layers on alumina planchets in a gas-flow counter, Frieseke-Hoepfner.

## RESULTS

*Acetate-1-$C^{14}$ injection*

Two series of ten crabs were used in this experiment. Each crab in the first series was given 70 $\mu$c of acetate-1-$C^{14}$ in two portions with a 24 hr interval. Twenty-four hours after the second injection the crabs (series A1) were sacrificed. The animals in this series were not fed. In the second series (A2) each crab was given 80 $\mu$c in four doses with intervals of 48 hr. The animals were killed 12 days after the first injection. The hepatopancreas and the ovaries of several animals of both series were isolated. One male crab from the second series was examined in total except for the exoskeleton. Cholesterol and the "squalene" fraction were isolated from these tissues and analysed. Typical results are given in Table 1.

*Mevalonic acid-2-$C^{14}$ injection*

Two crabs were given each 0·05 mc of this compound. Twenty-four hours and 6 days after injection a specimen was sacrificed (M1 and M2, respectively). In a parallel experiment 0·1 mc of acetate-1-$C^{14}$ was given to two crabs. From the hepatopancreas cholesterol and the "squalene" fraction were isolated and analysed. The results are summarized in Table 2.

*Analysis of hydrocarbon and sterol fractions*

Results obtained from the aluminium oxide chromatograms are summarized in Tables 1 and 2. Upon elution of the columns with hexane, the hydrocarbon fraction was separated from a yellow pigment, which appeared in the 50% benzene in hexane effluent. The fractions eluted with benzene contained yellow and red pigments. Ethyl acetate, 10% in benzene, eluted yellow and brown pigments. The following fractions, 15–20% ethyl acetate in benzene, all contained several yellow pigments as shown by TLC and ultra-violet absorbance.

The part of the hydrocarbon fraction that had the same mobility as squalene in TLC was not further characterized as most of the "squalene" fractions isolated did not contain any radioactivity.

TABLE 1—INCORPORATION OF ACTIVITY INTO FATTY ACIDS AND NON-SAPONIFIABLE MATERIAL FROM HEPATOPANCREAS AND OVARIES OF CRABS INJECTED WITH ACETATE-1-$C^{14}$

| | | Series A1 | | | | Series A2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Hepatopancreas* (4 females) | | Ovaries (1 female) | | Hepatopancreas* (4 females) | | Ovaries (1 female) | | Whole animal (1 male) | |
| | | Weight mg | Activity cpm/mg | Weight mg | Activity cpm/mg | Weight mg | Activity cpm/mg | Weight mg | Activity cpm/mg | Weight mg | Activity cpm/mg |
| Wet tissue | | 121,000 | | 82,000 | | 170,000 | | 70,000 | | 267,000 | |
| Fatty acids | | 25,030 | 1162 | 4920 | 423 | 18,520 | 1120 | 3770 | 1200 | 6970 | 253 |
| Non-saponifiable material (NSM) | | 258 | 432 | 131 | 417 | 275 | 497 | 172 | 368 | 237 | 404 |
| $Al_2O_3$-chromatography of NSM: | | | | | | | | | | | |
| Fraction | Solvent† | | | | | | | | | | |
| 1 | H | 15·7 | 222 | 1·8 | 370 | 13·9 | 635 | 1·1 | 995 | 4·0 | 2132 |
| 2 | H/B 1:1 | 2·0 | 4721 | 0·8 | 304 | 5·4 | 7996 | 0·7 | 820 | 11·4 | 1997 |
| 3 | B | 8·2 | 36 | | | | | 3·4 | 7 | | |
| 4 | E/B 1:9 | 19·1 | 894 | 27·5 | 1341 | 14·0 | 343 | 9·3 | 445 | 5·7 | 2375 |
| 5 | E/B 1·5:8·5 | 152·6 | 65 | 91·0 | 0 | 129·4 | 72 | 114·9 | 143 | 166·0 | 28 |
| 6 | M/E 0·4:9·6 | 9·4 | 328 | | | 11·1 | 461 | | | | |
| "Squalene" from fraction 1 | | 1·0 | 0 | 0·06 | 0 | 0·5 | 0 | 0·15 | 0 | 0·42 | 0 |
| Sample of recrystallized cholesterol from fraction 5 | | 5·1 | 0 | 8·6 | 0 | 4·4 | 0 | 19·4 | 0 | 2·7 | 0 |

* Hepatopancreas from four crabs pooled.
† H, hexane; B, benzene; E, ethyl acetate; M, methanol.

TABLE 2—INCORPORATION OF ACTIVITY INTO FATTY ACIDS AND NON-SAPONIFIABLE MATERIAL OF CRAB HEPATOPANCREAS AFTER ADMINISTRATION OF ACETATE-1-$C^{14}$ AND MEVALONIC ACID-2-$C^{14}$

| | | Series A3 | | Series A4 | | Series M1 | | Series M2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight mg | Activity cpm/mg | Weight mg | Activity cpm/mg | Weight mg | Activity cpm/mg | Weight mg | Activity cpm/mg |
| Fatty acids | | 564 | 1330 | 1180 | 153 | 440 | 567 | 968 | 2820 |
| NSM* | | 103 | 1240 | 154 | 554 | 137 | 4450 | 163 | 6860 |
| $Al_2O_3$-chromatography of NSM: | | | | | | | | | |
| Fraction | Solvent | | | | | | | | |
| 1 | B/H 1:1 | 13 | 2390 | 19 | 1000 | 23 | 2400 | 9 | 7000 |
| 2 | B | 1 | 0 | 3 | 500 | 1·2 | 104,000 | 2 | 122,500 |
| 3 | E/B 0·5:9·5 | 2·5 | 2800 | | | | | | |
| 4 | E/B 0·5:9·5 | 6 | 1600 | | | | | | |
| 5 | E/B 1:9 | 2 | 5000 | 13 | 1300 | 11 | 13,600 | 6 | 66,000 |
| 6 | E/B 1:9 | 6 | 1250 | | | | | | |
| 7 | E/B 2:8 | 33 | 230 | 52 | 160 | 8 | 12,500 | 63 | 4750 |
| 8 | E/B 2:8 | 14 | 1040 | 7 | 2750 | 70 | 1850 | | |
| 9 | E/B 2:8 | 2 | 1000 | | | | | | |
| 10 | E/B 2:8 | 0·5 | 5000 | | | | | | |
| 11 | E | 4 | 1900 | | | | | | |
| "Squalene" from fraction 1 | | 0·15 | 0 | 0·42 | 8 | 0·10 | 5 | 0·18 | 0 |

* For abbreviations see Table 1.

Cholesterol was eluted from the columns with 15–20% ethyl acetate in benzene but was contaminated with yellow pigments. The pigments were removed upon repeated crystallizations of the cholesterol fractions. After two or three crystallizations the isolated cholesterol was devoid of radioactivity. A typical result of repeated crystallizations of a cholesterol fraction is given in Table 3. Cholesterol was identified by TLC, melting point (148°, uncorr.) and mixed melting point with authentic cholesterol. Elution of the aluminium oxide columns was continued with increasing concentrations of ethyl acetate in benzene. No sterols could be detected in these fractions.

TABLE 3—CRYSTALLIZATION OF CHOLESTEROL FROM FRACTION 7 OF THE ALUMINIUM OXIDE CHROMATOGRAM OF HEPATOPANCREAS M2

| | Weight (mg) | cpm/mg |
|---|---|---|
| Fraction 7 | 63 | 4750 |
| 1st crystallization | 45 | 1060 |
| 2nd crystallization | 36 | 220 |
| 3rd crystallization | 28 | 0 |

The non-saponifiable fraction from the haemolymph collected in the experiment with mevalonic acid was chromatographed with phase system I. All radioactivity was eluted with the solvent front. No polar sterols could be detected in this material.

The content of cholesterol in the tissues was found to be 0·7–1·5 mg/g wet weight.

## DISCUSSION

For comparison, the fatty acid fractions were isolated in some cases simultaneously with the non-saponifiable fractions. From Tables 1 and 2 it can be seen that acetate was readily incorporated into fatty acids with the highest incorporation in those from hepatopancreas. The tracer carbon of mevalonic acid was rapidly incorporated into the non-saponifiable fraction but more slowly into the fatty acids. The fractions which contained pigments were highly labelled in the acetate as well as in the mevalonate experiments. This may suggest that isoprenoid derivatives are present in these fractions. As it is generally accepted that carotenoids are synthesized only in plants (Goodwin, 1960), the radioactivity is possibly present in compounds like ubiquinones which are eluted from the column with the same solvent mixtures. The red pigment, which is responsible for the red colour of the ovaries and which is also present in hepatopancreas, most probably is a carotenoid. This pigment was isolated in the acetate experiments and was found to be unlabelled. Only small amounts of a hydrocarbon fraction with chromatographic properties of squalene were present and in several cases no "squalene" could be detected. The "squalene" fractions were found to be unlabelled.

Cholesterol was found present in the hepatopancreas in smaller amounts than found earlier (Vonk, 1960). The difference may be caused by influence of the sexual cycle of the season. The experiments were conducted in October–November because the crabs are in a vigorous condition and maintain themselves well during the experiments. In females, the ovaries are ripening and increase in size. The amount of cholesterol increases only when new tissues are formed (Vonk, 1960). For this reason the ovaries were examined for the presence of labelled cholesterol but none could be isolated. It was observed that in the females the hepatopancreas was reduced considerably in size and cholesterol could have been transported to the ovaries possibly explaining the low concentrations of cholesterol found in the hepatopancreas in these experiments.

In rats the biosynthesis of cholesterol is reduced on addition of large amounts of cholesterol to the diet (Langdon & Bloch, 1953). For this reason the crabs in one series of the acetate experiments and in the mevalonic acid experiment were not fed. The results were the same as with the fed crabs. Experiments were also conducted over a longer period of time, 24 days, with a continuous supply of labelled acetate in order to enable detection of a slow rate of formation of cholesterol. However, no evidence for any incorporation of isotope into cholesterol was obtained.

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