Synthesis in vitro of cholesterol by mitochondria in the shrimp Penaeus azteclus (Ives)

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MS received 11 December 1979; revised 12 March 1980

Abstract. The incorporation of [14C]-acetate, [14C]-mevalonate and [14C]-desmosterol into cholesterol in the muscle mitochondria of the brown shrimp Penaeus azteclus (Ives) is more as compared to that in hepatopancreas. [14C]-Desmosterol is more efficiently incorporated into cholesterol in comparison with [14C]-acetate. The muscle mitochondria from males incorporated more [14C]-mevalonate into cholesterol than those from females, while the converse is true in the hepatopancreatic mitochondria.

Keywords. Cholesterol biosynthesis; muscle mitochondria; Penaeus azteclus.

Introduction

Cholesterol biosynthesis is dependent on several endogenous and exogenous factors (Dempsey, 1974). Many dietary factors like linoleic acid, and β-sitosterol are known to retard or inhibit the synthesis in mammals (Goad, 1976). Earlier investigations (for review, see Goad, 1976) failed to demonstrate cholesterol synthesis in shrimp from simple precursors like acetate or mevalonate. In our laboratory, it was noticed that the brown shrimp Penaeus azteclus fed on algae-diet had reduced levels of cholesterol in tissues (Krishnamoorthy et al., 1979). These observations prompted us to believe that earlier investigators had overlooked some interfering factors in the biosynthesis of cholesterol. It is felt that experiments on the particulate system may obviate these interferences. This paper presents the data on the synthesis of cholesterol in vitro by mitochondria, isolated from the brown shrimp tissues.

Materials and methods

Substrates

(2,4-[14C])-Desmosterol (30 Ci/mmol), (2-[14C])-DL-mevalonic acid (10 Ci/mmol) and I-[14C]-sodium acetate (15 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts, USA. All other chemicals were obtained from Sigma Chemicals, St. Louis, Missouri, USA.

Experimental animals

Adult brown shrimp (90-95 mm length) P. azteclus (Ives) were obtained from Davis Bayou in Ocean Springs, Mississippi and maintained in tanks with running sea water.

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at 25°C. They were fed ad lib pelleted diet formulated in this laboratory (Gunter and Venkataramaiah, 1975). This diet had 1.157 mg cholesterol per g dry wt. After maintaining the animals under these feeding conditions for 15 days, they were starved for one week before they were killed for analyses.

**Preparation of mitochondria**

All operations were performed at 0-4°C. The tail muscles and hepatopancreas (the so-called liver) of 15-20 shrimp were excised. Ten g of each tissue was rinsed with the homogenizing solution (0.25M sucrose, 6 mM EDTA, 20 mM imidazole-HCl buffer, pH 6.8), briefly blotted and homogenized in 20 volumes of a solution containing 0.1% (w/v) sodium deoxycholate (Hendler et al., 1972) in a tissue grinder using 18 complete strokes at 600 g. The homogenate was filtered through a single thickness of cheese cloth and centrifuged at 600 g for 20 min in a Beckman J1-centrifuge. The sediment was discarded and the supernatant was then centrifuged at 18000 g for 45 min. After removal of the supernatant by aspiration, the mitochondrial pellet was resuspended in 20 ml of homogenizing medium, centrifuged at 18000 g and the pellet was collected. Finally an uniform suspension of the pellet in 5 ml phosphate (20 mM, pH 6.8) buffer was made. The protein content of the mitochondrial suspension was estimated colorimetrically (Lowry et al., 1951) using human serum albumin (500 µg/ml) as the standard.

The purity of the mitochondrial preparations obtained was tested assaying the marker enzyme activities like succinic dehydrogenase (EC 1.3.99.1), cytochrome c-oxidase (EC 1.9.3.1) and ouabain-sensitive Na⁺ K⁺-ATPase (EC 3.6.1.3) activity. Succinate dehydrogenase activity was determined spectrophotometrically with phenazine methosulphate and dichlorophenol indophenol as the electron acceptors as described by Arrigoni and Singer (1962). The activity was expressed as m units per mg protein; one unit being the amount of enzyme required to reduce 1 µ mol of the dye per min per mg protein at 25°C. Specific activity of cytochrome c oxidase (EC 1.9.3.1) was determined manometrically by measuring the oxygen uptake in the presence of added cytochrome c and a reagent to reduce the cytochrome c as it was oxidized (Slater, 1949) at 25°C. The activity was expressed as µ 1 of oxygen consumed by 1 mg protein in 1 min. Ouabain-sensitive Na⁺, K⁺-ATPase activity was assayed according to Towle et al. (1976).

**Incubations with labelled precursors**

An incubation mixture containing cofactors: 60 µmol glutathione, 17 µmol glucose-6-phosphate, 25 µ mol MgCl₂·6H₂O, 23 µ mol NAD⁺, 7 µ mol NADH, 3 µ mol NADPH, 13 µ mol ATP, 200 µ mol nicotinamide and 100 µ mol glucose was prepared according to Teshima and Kanzawa (1976). This mixture (1.5 ml) in a test tube was mixed with 0.5 µ Ci of solvent-free precursor suspended in 1.5 ml of phosphate buffer containing 0.1 mg streptomycin and 0.1 mg penicillin G.

Incubation was started by the addition of 0.5 ml mitochondrial suspension containing 1 to 1.5 mg protein equilibrated at 28°C in a thermostat. After 3 h, 10 ml of 1:1 acetone-alcohol mixture was added to stop the reaction and the sterols were isolated by digitonin method (Idler and Baumann, 1952). The digitonides were collected by centrifugation and washed with ether. Finally, they were dissolved in dimethyl sulphoxide, and the free sterols were extracted with 1 ml hexane.
Separation of sterols and autoradiography

The sterols in hexane (420 μl) was subjected to thin layer chromatography on silica gel with the developer containing petroleum ether: ethyl ether and acetic acid (87.5:12.5:1) (Guarry, 1973). Cholesterol was run as a standard along with the unknowns. The zones were visualized in iodine vapour, scrapped and transferred into counting vials. Radioactivity in the gel scrapped from the cholesterol zones of the thin layer plate was determined after dissolving the sterol in 150 μl absolute ethanol in 15 ml liqueflour and counted in a Packard Model 3375 scintillation spectrometer. The efficiency of counting was about 90%. Autoradiography was carried out by covering the thin layer chromatograms with x-ray film for 21 days.

Results

The results of table 1 shows that the present homogenization procedure for isolating mitochondria reduced the apparent mitochondrial contamination in the sarcoplasmic (i.e., supernatant) fraction. Another possible contamination in muscle mitochondria is the proteins due to disruption of sarcolemma (Azzone, 1963). This was tested by the activity of the sarcolemmal marker enzyme, the ouabain-sensitive (Na⁺, K⁺)-ATPase. In the mitochondrial fraction (see table 1), the ouabain-sensitive ATPase activity was very low (< 0.12 μmol P_i/mg protein/min at 25°C); hence the sarcolemmal contamination is minimum in the isolation process of the muscle mitochondria.

After the incubation of mitochondria, prepared from the muscle of hepatopancreas with the radioactive precursors, the incorporation of the radioactivity into unsaponifiable fraction of cholesterol was investigated. Figure 1 shows that significant radio-

<table>
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<tr>
<th>Enzyme</th>
<th>No. of observations</th>
<th>Cytoplasmic or sarcoplasmic fraction</th>
<th>Mitochondrial fraction</th>
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<tbody>
<tr>
<td><strong>Muscle</strong></td>
<td></td>
<td></td>
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<tr>
<td>Succinate dehydrogenase activity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>0.7±0.1 (1.1)</td>
<td>64±2.9 (100)</td>
</tr>
<tr>
<td>Cytochrome-c-oxidase&lt;sup&gt;c&lt;/sup&gt; activity</td>
<td>16</td>
<td>0.6±0.09 (1.2)</td>
<td>49.8±2.1 (100)</td>
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<tr>
<td><strong>Hepatopancreas</strong></td>
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<tr>
<td>Succinate dehydrogenase activity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>1.8±0.3 (3.0)</td>
<td>61±2.1 (100)</td>
</tr>
<tr>
<td>Cytochrome-c-oxidase&lt;sup&gt;c&lt;/sup&gt; activity</td>
<td>8</td>
<td>1.86±0.39 (4.0)</td>
<td>46.9±2.5 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup> mean ± s. d.  <sup>b</sup> m units (see text for details).  <sup>c</sup> μl of O₂ consumed per mg protein per min.  Number in parenthesis denote per cent purity.
activity was distributed in the cholesterol fraction obtained with muscle mitochondria. Only, the precursors mevalonate and desmosterol showed significant incorporation into cholesterol by the muscle mitochondria. Mitochondria of the hepatopancreas did not show significant radioactivity. The incorporation of acetate into cholesterol was poor in mitochondria from both the tissue.

The amount of labelled precursor incorporated into cholesterol was analyzed by counting the radioactivity of the cholesterol isolated by thin layer chromatography.
The results are presented in table 2. Percentage incorporation of radioactive acetate into cholesterol was very low compared to other precursors. Desmosterol incorporation was higher than that of mevalonate, and the muscle mitochondria were more efficient than the mitochondria of hepatopancreas in incorporating these precursors into cholesterol.

The mitochondria from male shrimp showed greater rates than those of the female \((P < 0.05)\).

**Discussion**

The ability of Crustaceans to synthesize sterols has been investigated by several authors, and hitherto it was established that they are incapable of \textit{de novo} biosynthesis. It has been demonstrated that \([1-^{14}C]\)-acetate was not incorporated either into squalene or sterols by a crayfish, \textit{Astacus astacus} (Zandee, 1962, 1964, 1966), a lobster \textit{Homarus vulgaris} (Zandee, 1964) and a prawn, \textit{P. japonicus} (Teshima and Kanazawa, 1971 a). It was also demonstrated that a brine shrimp \textit{Anemia salina}, a prawn \textit{P. Japonicus}; a lobster \textit{(Panulirus japonica)} and a crab \textit{Portunus tritabeculatus} failed to utilize labelled mevalonate for sterol elaboration (Teshima and Kanazawa, 1971 a, b). It was therefore believed that shrimp rely upon a dietary source of sterol or dealkylate dietary phytosterols into cholesterol (Teshima and Kanazawa, 1976; Teshima, 1972). Furthermore, in most of the earlier studies (Zandee, 1966; Teshima and Kanazawa, 1971a; Teshima, 1972), \([^{14}C]\)-acetate or mevalonate was either injected into the blood stream or incubated with tissue slices. In general when \([^{14}C]\)-acetate was injected or incubated, it was poorly incorporated into the sterols, and in a few cases it was apparently not incorporated at all into sterols, though some fatty acids were appreciably labelled showing that the animals were able to utilize the \([^{14}C]\)-acetate. The mevalonate was also not incorporated in many cases and hence, the authors deduced that cholesterol synthesis is absent in shrimp. In the light of these observations the present results demonstrating the occurrence of cholesterol synthesis \textit{in vitro} by the shrimp mitochondria are interesting.
The poor incorporation of [14C]-acetate into sterols as observed here is now rationalized on the basis of recent work on the role of β-hydroxy-β-methyl glutaryl-CoA reductase (EC 1.1.1.34) in the control of mammalian sterol synthesis (Brown et al., 1973). Clearly, if β-hydroxy-β-methyl glutaryl-CoA reductase is also the rate-limiting step in sterol synthesis in shrimp, then the incorporation of [14C]-acetate will depend upon the activity of this enzyme.

Starvation markedly reduces the activity of β-hydroxy-β-methyl glutaryl CoA reductase (Ramasarma, 1974). Probably, for this reason, low incorporation rates of [14C]-acetate, into sterols were obtained in the present study. When mavalonate (a product of the β-hydroxy-β-methyl glutaryl-CoA reductase) was incubated with mitochondria, the incorporation was not subjected to this regulation (Richard and Rodwell, 1974; Dempsey, 1974). Desmosterol, an intermediate in the de novo synthesis of cholesterol was converted into cholesterol. The specific conversion rates were more with desmosterol than with mevalonate. These results are not surprising as the conversions of desmosterol into cholesterol have been demonstrated in the prawn *P. japonicus* (Teshima and Kanazawa, 1971b).

β-Hydroxy β-methyl glutaryl-CoA reductase activity is controlled by a number of dietary factors (Brown et al., 1973; Goad, 1976). The activity may be suppressed by dietary cholesterol or β-sitosterol. Apparently, studies with mitochondria reduce the influence of such factors and therefore when incubated with mitochondria, mevalonate was easily incorporated. The results thus demonstrate that mitochondria of shrimp *P. aztecus* are capable of synthesising cholesterol.

Acknowledgements

The authors thank Mr John D. Valleau for his help in isotope experiments and Miss Patricia Biesiot for maintaining the animals.

References