Cholesterol Metabolism in *Asterias Rubens*

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Summary: [4-^{14}C] cholesterol, [26-^{14}C] cholesterol and [2-^{14}C] acetic acid were employed to study cholesterol metabolism in *A. rubens*. With [4-^{14}C] cholesterol highest radioactivity was found in free sterols (71%) and steryl esters (24%) and smallest in glycerides and free fatty acids. Sterol sulphate were also heavily labelled. Fatty acid methyl esters prepared from glycerides and free fatty acids contained negligible radioactivity.

Incorporation of [26-^{14}C] cholesterol also resulted highest label in free sterols (75%) and steryl esters (19%). Saponification of steryl esters did not yield activity into fatty acid while sterols were labelled. Again sterol sulphates were labelled heavily.

Incubation with [2-^{14}C] acetic acid recorded highest activity in triglycerides (72%) and steryl esters (16%) while free sterol were significantly labelled (5%). Moreover, situation of label in steryl esters and free sterols was opposite to that of [4-^{14}C] cholesterol.

Cholesterol catabolism did not produce labelled precursors or support re-utilization of carbon for lipid/cholesterol synthesis

Introduction

A very low level of sterols after spawning increased upto 200 times after dietary assimilation of sterols has been found in Asterias vulgaris [1]. Increased in the steroid level and sterol metabolism upon exposure to cadmium and zinc has been reported in *Asterias rubens* [2]. Cholesterol containing steroids and steroid esters have been identified in stomach and pyloric caeca of Acanthaster planci [3]. In Solaster borealis fifteen sterol constituents have been isolated [4]. Androgens metabolism in somatic and germinal tissues of Asterias vulgaris has been discussed [5]. Occurrence of radioactivity from either [26-^{14}C] cholesterol or [4-^{14}C] sitosterol into neutral and phospholipid and of significant amounts in particular in triglyceride, diglyceride, monoglyceride and free fatty acids has been described [6,7].

Moreover, extensive catabolism of dietary sterols to produce small units which are subsequently re-utilized for lipid biosynthesis and dietary Δ^5-sterols to C2 units can provide a source of carbon for the de novo synthesis of cholest-7-ene-3β-ol which is the major sterol of this animal [6,7].

The present work was undertaken to examine these proposals whether such extensive catabolism of ingested cholesterol and its carbon re-incorporation into various neutral and phospholipids was possible ?

Results and Discussion

[4-^{14}C]Cholesterol:

With [4-^{14}C] cholesterol the total lipids, neutral lipids and phospholipids were labelled (Table-1). The highest recoveries of the radioactivity were in the steryl esters (24%) and free sterols (71%). The small amount of radioactivity found associated with the glycerides and free fatty acids was in accord with the findings of Voogt and Van Rheenan [7]. However, the methyl esters of the free fatty acids and triglycerides fatty acid prepared contained negligible radioactivity. This indicated that sterol catabolism did not produce labelled precursors which could be used for fatty acid biosynthesis.

Among the phospholipids radioactivity was associated mainly in the sterol sulphates. The cerebrosides, phosphatidyl lecithin, phosphatidyl inositol and phosphatidyl serine were essentially unlabelled. This observation of *A. rubens* incubation with [4-^{14}C] cholesterol and thus appearing in sterol sulphates is obviously consistent with the previous

Table-1: Distribution of radioactivity from [4-^{14}C] cholesterol in lipids of *Asterias rubens*

S.No.	Lipid group	Radioactivity (dpm)	Distribution (%)
	A) Neutral Lipid		
1.	Steryl esters	2.5×10^6	23.6
2	Triglycerides	8.0×10^4	0.8
3	1,3-Diglycerides	8.0×10^4	0.8
4	1,2-Diglycerides	8.0×10^4	0.8
5	Monoglycerides	2.8 x 105	2.6
6	Free Fatty Acids	7.0×10^4	0.7
7	Sterols	7.5×10^6	70.8
	B. Phospholipids		
8	Cerbrosides	5.0×10^3	5.0
9	Sterol Sulphates	8.7×10^4	47.0
10	Phosphatidyl:		
	i. Ethanolamine	5.0×10^3	5.0
	ii. Lecithin	2.5×10^3	2.5
	iii. Inositiol	-	-
	iv. Serine	-	-

demonstration of sterol sulphation [14-16]. The phospholipids from *A. rubens* do not therefore represent only true phospholipids but they also contain the sterol sulphates and cerebrosides [14,15,18,19].

[26-^{14}C] Cholesterol:

In this incubation radioactivity was again found in both the neutral (6.5×10^6 dpm) and phospholipids (1.25×10^6 dpm) fractions (Table-2). The free sterols contained the highest proportion of the recovered radioactivity but the steryl esters were again very significantly labelled. Radioactivity in the various glycerides and free fatty acids was low and of the order observed in similar experiment by Voogt and Van Rheenan [7].

Table-2: Pattern of radioactivity from [26-^{14}C] cholesterol incubation of Sea-star.

S.No.	Lipid group	Radioactivity (dpm)	Distribution (%)
	A) Neutral Lipid		
1.	Steryl esters	1.0×10^6	18.7
2	Triglycerides	2.0×10^4	0.4
3	1,3-Diglycerides	2.0×10^4	0.4
4	1,2-Diglycerides	4.0×10^4	0.8
5	Monoglycerides	1.1×10^5	2.0
6	Free Fatty Acids	1.7×10^5	3.2
7	Sterols	4.0×10^6	74.6
	B. Phospholipids		
8	Cerbrosides	1.0×10^3	18.0
9	Sterol Sulphates	4.5×10^5	82.0
10	Phosphatidyl:		
	i. Ethanolamine	-	-
	ii. Lecithin	-	-
	iii. Inositiol	-	-
	iv. Serine	-	-

The purification of the fatty acids with added carrier cholesterol followed by methylation resulted in no label. These results therefore, show that probably the loss of the side chain of sterols does not play an important role in the production of precursors suitable for the biosynthesis of lipids.

Similarly all steryl ester classes saturated (11,000 dpm), mono-unsaturated (39,000 dpm), di-unsaturated (15,000 dpm) tri-unsaturated (13,000 dpm), tetra-unsaturated 5000 dpm) and poly-unsaturated (68,000 dpm) were found labelled. As poly-unsaturated steryl esters were heavily labelled, a fraction containing 8500 dpm was saponifide. Only the sterol moiety was found labelled having 8000 dpm, no significant radioactivity was recovered in the fatty acid portion. Among the phospholipids, only sterol sulphates were found to contain label no radioactivity was located or determined in the cerebrosides.

The present observations with [26-^{14}C] cholesterol also did not support the idea of an extensive sterol catabolism and re-utilization of the carbon for lipid synthesis.

[2-^{14}C] Acetic Acid:

With [2-^{14}C] acetic acid, just like the previous incubations, distribution of radioactivity between various components of the neutral and phospholipids was detected (Table-3). Among the neutral lipids most heavily labelled component was the triglycerides. Moreover, the steryl esters were more heavily labelled than the free sterols which was the opposite situation to the observed when *A. rubens* was incubated with [4-^{14}C] cholesterol.

Table-3: Results of incorporation of [4-^{14}C] acetic acid into lipids of Startfish.

S.No.	Lipid group	Radioactivity (dpm)	Distribution (%)
	A) Neutral Lipid		
1.	Steryl esters	5.0×10^5	16.4
2	Triglycerides	2.2×10^5	71.6
3	1,3-Diglycerides	6.7×10^4	2.2
4	1,2-Diglycerides	1.4×10^5	4.6
5	Sterols	1.6×10^5	5.3
	B. Phospholipids		
6	Cerbrosides	3.0×10^3	68.5
7	Sterol Sulphates	6.7×10^4	13.7
8	Phosphatidyl:		
	i. Ethanolamine	3.0×10^4	6.9
	ii. Lecithin	1.4×10^4	3.2
	iii. Inositiol	3.4×10^4	7.8
	iv. Serine	-	-

Saponification of a portion of the steryl esters (90,000 dpm) yielding labelled sterols (51000 dpm) and fatty acids (24,000 dpm) gave a positive indication that the fatty acid moiety was labelled. Moreover, it has also been shown that the rate of cholest-7-en-3β-ol synthesis from [2-^{14}C] acetic acid was also low [17].

The precursor to all the lipid classes is acetate [20]. All the neutral and phospholipid classes were found labelled. Among the neutral lipids most heavily component was the triglycerides. The fatty acid methyl esters prepared from the triglycerides revealed a high rate of incorporation of [2-^{14}C] acetic acid. Similarly steryl esters which were more heavily labelled than the free sterols after saponification, in contrast to the labelled cholesterol incubations, gave label both in the sterols (51000 dpm) and fatty acids (24000 dpm) respectively. In phospholipids all classes contained the radioactivity but the cerebrosides were heavily labelled which may be attributed to the incorporation of [2-^{14}C] acetic acid in the fatty acid component of these lipids.

The present work was undertaken to examine the idea of extensive catabolism of sterols into smaller units such as acetyl CoA which was then utilized to re-synthesize lipids but it produced interesting and contrasting results. These facts lead to the conclusion that there is probably no extensive catabolism of sterol in the sea-star, *A. rubens* of the magnitudes suggested by Voogt and Van Rheenan [7]. Most probably radioactivity observed in various lipid components was due to auto-oxidation and degradation of labelled sterols during incubations subsequent extraction, handling and exposure on TLC plates such as observed by Smith *et al.*, [21]. In order to check the purity of labelled cholesterol used in present studies at least 15 minor radioactive bands were observed, and it is not inconceivable that some of these would co-chromatograph with some of the lipid classes investigated.

Experimental

Solvents used were redistilled unless otherwise stated. Ether, Chloroform (May & Baker), Petroleum ether (Esso) Hexane, Dioxane, Methanol (BDH) Acetic acid (Gallenkamp Ltd.) Chemicals of high purity grade were obtained: Kieselgel 60 (35-70 mesh) and Kieselgel G60 (E. Merck, Germany), Berberine (Sigma Chemical Company). BF_3-methanolic complex (BDH).

Lipid markers:

Tripalmitin and phosphatidyl serine (BDH), phosphatidyl ethanolamine, phosphatidyl inositol and egg lecithin (lipid product South Nutfield).

Radiochemicals:

[4-^{14}C] cholesterol, specific activity 56 mCi/m mol, [26-^{14}C] cholesterol specific activity 58 mCi/m mole and [2-^{14}C] acetic acid 57.7 mCi/mmole were supplied by the radiochemical centre, Amersham, UK.

Radioautography:

Kodak Kodirex X-ray film was used which after exposure was developed in the dark room.

Liquid Scintillation Counting:

It was performed on an inter-techniques SL-30 instrument. Samples were dissolved in scintillation fluid and radio-activities were quoted in as disintegration per minute (dpm) after feeding parameters into the computer of the machine.

Twelve healthy specimen of starfish were selected and divided into three groups having 4-animals in each. One group was injected with [4-^{14}C] cholesterol (12.5 mCi), second with [26-^{14}C] cholesterol (5 mCi) and the third with [2-^{14}C] acetic acid (5mCi). Animals in each group after an incubation of 72 hours were killed, freeze dried and extracted with chloroform methanol (2:1 v/v) and then separated into neutral and phospholipids [8]. All neutral and phospholipid fractions after purification on TLC (petroleum ether:ether:acetic acid 90:10:v/v/v and chloroform:methanol: 2M-ammonia 65:25:4 v/v/v) respectively were assayed for radioactivity by liquid scintillation counting [9].

From [4 ^{14}C]-cholesterol, fatty acids of triglyceride and free fatty acid of neutral lipids were methylated with methanolic-HCl [10] and counted for radioactivity. Similarly phosphatidyl ethanolamine from the phospholipids was solvolyzed [11] checked on TLC and band running parallel to marker cholesterol was scraped and counted.

From [26-^{14}C]-cholesterol, free tatty acid fraction was further purified by adding cholesterol (1 mg) as carrier which separated into three bands using same TLC (petroleum ether:acetic acid 90:10:1 v/v/v). Thus bands corresponding to cholesterol, fatty acid and unknown material were eluted and assayed for radioactivity. The purified fatty acids obtained after purification were methylated with boron trifluorid methanol complex [12] and counted. Similarly steryl ester fraction was separated into individual classes on TLC using benzene:hexane 40:60 v/v [13]. After TLC scanning the individual ester fractions were assayed for counting. A portion of the sterols recovered was assayed for radioactivity. Likewise a fraction of the cerebrosides from the phospholipid class was also purified on TLC (chloroform:methanol: 2M-ammonia 65:25:4 v/v/v).

From [2-^{14}C] acetic acid, triglyceride fraction was inter-esterified with methanolic HCl [10], purified and assayed for radioactivity. Similarly a portion of the steryl esters fraction was separated into individual classes using TLC [13] which were assayed for radioactivity. While another portion of these esters was saponified into fatty acids and sterols and their counts were determined.

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