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METABOLISM OF CHOLESTEROL BY CALLUS CULTURE OF *HOLARRHENA ANTIDYSENTERICA*

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Key Word Index—*Holarrhena antidysenterica*; callus culture; cholesterol metabolism.

Abstract—A chemically defined medium was established for the growth of tissue cultures of *Holarrhena antidysenterica*. Administration of cholesterol-[4-^{14}C] to 10-day-old callus yielded radioactive 24-methylenecholesterol, 28-isofucosterol, sitosterol, stigmasterol, and conessine, thereby indicating that the conversion of cholesterol into sitosterol is mediated through 24-methylenecholesterol and 28-isofucosterol in this system.

INTRODUCTION

In the area of phytosterol biosynthesis, the nature of intermediates resulting from cyclization of squalene and the mechanism of side chain alkylation have been extensively investigated [1]. Cholesterol, a key intermediate in the biosynthesis of other plant steroids has been isolated from a number of plants [2] and its conversion into some 24-alkyl sterols has also been reported [3,4].

We have shown that cholesterol, 24-methylenecholesterol, 28-isofucosterol, stigmasterol and sitosterol are the most abundant sterols formed in the tissue cultures of *Holarrhena antidysenterica* [5]. The formation of 24-methylenecholesterol and 28-isofucosterol in this system suggested that they may possibly function as intermediates in the formation of sitosterol. In this communication evidence is presented for the conversion of cholesterol-[4-^{14}C] into different phytosterols and steroidal alkaloids by callus cultures of *H. antidysenterica*.

RESULTS

Coconut milk and casein hydrolysate have been shown to be essential for active growth of *Holarrhena* callus [5]. When these growth supplements were substituted by a mixture of 13 amino acids, the growth of the callus was sustained but not vigorous. Incorporation of the amino acids in single addition series showed that a mixture comprising of L-aspartic acid, L-glutamic acid, L-arginine, L-lysine, DL-valine and DL-threonine was best suited for active growth of the tissue [6]. TLC analysis of the steroids isolated from tissues grown on the synthetic medium and the one containing CM + CH showed no significant differences [6].

Callus culture treated with cholesterol-[4-^{14}C] was extracted and the extract was separated into acidic, basic and neutral fractions. Maximum radioactivity was found in the neutral fraction and low radioactivity in the basic and acidic fractions. From the water extract, low radioactivity was observed in the neutral fraction and none in the basic fraction (Table 1).

Column chromatography of the neutral fraction (methanol–benzene extract) using Si gel gave a radioactive sterol mixture. When an aliquot of this mixture was subjected to TLC (10% $AgNO_3$–Si gel G) followed by autoradiography, maximum radioactivity was observed in the region where sitosterol, stigmasterol and cholesterol appeared as one spot. The spots with R_f values corresponding to 24-methylenecholesterol and 28-isofucosterol were also radioactive, but their intensity was comparatively low. PLC of the sterol mixture gave chromatographically homogeneous 24-methylenecholesterol, 28-isofucosterol and the sitosterol, stigmasterol and cholesterol mixture (S). The former two were crystallised to constant specific radioactivity after dilution with inactive authentic samples. Incorporation of radioactivity into 24-methylenecholesterol and 28-isofucosterol was 0.43 and 0.72% respectively, whereas it was 36.73% in the sterol mixture (S) (Table 2). Further separation of the mixture (S) by acetylation and PLC on Anasil-B gave radioactive sitosterol acetate and stigmasterol acetate representing 0.87 and 2.02% incorporation (Table 2). These were diluted with cold samples and crystallised in methanol to constant specific radioactivity.

Sterols isolated from the water-soluble extract of the tissue showed low radioactivity associated with the sitosterol, stigmasterol and cholesterol mixture as evidenced by autoradiography. No ^{14}C was observed in the regions corresponding to 24-methylene-cholesterol and 28-isofucosterol. Further separation of the acetylated sterol mixture showed that sitosterol acetate, stigmasterol acetate and cholesterol acetate were labelled to the extent of 0.002%, 0.004% and 0.012% incorporation respectively.

Table 1. Radioactivity in fractions isolated from callus tissue incubated with cholesterol-[4-^{14}C]

Fraction	Wt (mg)	Radioactivity (dpm × 10^{-5})	% Administered radioactivity
(a) *Methanol extract*			
Neutral	407	1492	67 8
Basic	56	69	3.1
Acidic	86	29	1.3
Total	549	1590	72.2
(b) *Water extract*			
Neutral	34	2	0.1
Basic	38	Nil	Nil

Table 2. Radioactivity of sterols formed from cholesterol-[4-^{14}C]

	Radioactivity (dmp × 10^{-6})	% Administered radioactivity
24-Methylenecholesterol	9.52	0.43
28-Isofucosterol	1.58	0.72
Sitosterol, Stigmasterol, Cholesterol	80.8	36.73
Sitosterol acetate	1.91	0.87
Stigmasterol acetate	4.44	2.02

An aliquot of the basic fraction from the methanol extract subjected to TLC (alkaline Si gel) followed by autoradiography, showed six radioactive spots of which one corresponded to the R_f value of conessine. The radioactivity associated with unidentified Dragendorff-positive spots was significantly more than that observed for the spot corresponding to conessine. On subjecting the conessine to TLC in three solvent systems, radioactivity was retained.

DISCUSSION

For metabolic studies, it is necessary to formulate a chemically defined medium devoid of coconut milk and casein hydrolysate (CM + CH), since these contain sterols [7]. Similarities in growth and biosynthetic potential exhibited by the tissue on a synthetic medium and on medium containing CM + CH indicated that only six amino acids present in these natural growth adjuvants were essential for active growth of the tissue.

Administration of cholesterol-[4-^{14}C] to *Holarrhena* callus produced radioactive 24-methylenecholesterol, 28-isofucosterol, sitosterol, stigmasterol and conessine. The conversion of cholesterol into sitosterol demonstrates that the tissue is capable of a double alkylation reaction at the C_{27} level. The incorporation of radioactivity into 24-methylenecholesterol and 28-isofucosterol presents reasonable evidence for the intermediacy of these sterols in the conversion of cholesterol into sitosterol. Although sitosterol is a major constituent of the callus [5], radioactivity associated with stigmasterol was significantly more than that observed in sitosterol during the 20-day incubation period. This suggested a possible conversion of sitosterol into stigmasterol in the tissue as in *Digitalis lanata* [8].

Our previous studies have indicated a modification of steroid metabolism in the callus culture compared to the intact plant [5]. Low incorporation of radioactivity into conessine *vis-a-vis* other Dragendorff-positive components, further substantiates this view.

EXPERIMENTAL

The techniques adopted in tissue culture have been detailed previously [5].

Establishment of synthetic medium. The basal medium of Lin and Staba (modified) comprising inorganic salts, vitamins, Fe-EDTA, 2,4-dichlorophenoxyacetic acid (1 mg/l), kinetin (0.2 mg/l), mesoinositol (100 mg/l) and sucrose (2%) was supplemented with the major amino acids present in 200 mg of CH and 100 ml of CM in a single addition series in place of CM and CH [5]. The following amino acids were used for establishing synthetic medium: (mg/l) L-aspartic acid (200), L-glutamic acid (200), L-asparagine (10), L-glutamine (10), L-arginine (100), L-lysine (20), L-leucine (25), DL-valine (20), DL-threonine (10), *v*-aminobutyric acid (20), DL-alanine (20), DL-serine (10), L-phenylalanine (20). The amino acids which did not promote sustained growth were deleted from subsequent trials.

Extraction and isolation of steroids. Details of the procedure were reported previously [5]. They involved extraction of the dried callus with MeOH, MeOH–C_6H_6 and H_2O. The organic extract was refluxed with 5% KOH and subjected to acid–base separation. The aq. extract was hydrolysed [9] and separated into basic and neutral components. Isolation and purification of steroids was by chromatographic methods.

Incorporation of cholesterol-[4-^{14}C] into the callus. Tissues grown on synthetic medium for 10 days were injected with an ethanolic soln of 100 μCi of cholesterol-[4-^{14}C] (Cea, Department des Radio elements, Gif-sur-Yvette, France, specific radioactivity 35 mci/mM) so that tissue in each tube received 4 μCi. The tissues were incubated for a further period of 20 days, pooled, dried and extracted at the end of 30-days.

Radioactivity measurements. Aliquots of radioactive compounds were dissolved in 15 ml of scintillation solution containing 0.04% of BBOT (2,5-Di-(5-tert-butyl-2-benzoxazolyl) thiophene) in toluene.

Autoradiography. Autoradiographs were prepared by placing an X-ray film in close contact with the TLC plate for a period ranging from 30 to 45 days.

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