Phytochemistry, 1969, Vol. 8, pp. 1679 to 1686. Pergamon Press. Printed in England

THE METABOLISM OF ^{14}C-CHOLESTEROL BY *DIOSCOREA DELTOIDEA* SUSPENSION CULTURES*

S. J. STOHS, B. KAUL and E. J. STABA

College of Pharmacy, University of Nebraska, Lincoln 68508, U.S.A.

(*Received* 7 *March* 1969, *in revised form* 22 *April* 1969)

Abstract—Both 4-^{14}C- and 26-^{14}C-cholesterol were converted to labeled diosgenin in the undifferentiated cells of *Dioscorea deltoidea*, the extent of incorporation in both cases being nearly identical. In addition to diosgenin, sterols (β-sitosterol, stigmasterol, and possibly metabolites of cholesterol), and an unidentified fraction were also labeled. Differentiated (root) cell cultures showed little or no labelling of diosgenin from administered 4-^{14}C-cholesterol. The fact that both 4-^{14}C- and 26-^{14}C-cholesterol are incorporated into diosgenin in nearly equal amounts suggests that the entire cholesterol molecule is involved in diosgenin biosynthesis. No direct evidence for the incorporation of the side-chain of cholesterol in diosgenin has been previously demonstrated. The rates of incorporation of ^{14}C-cholesterol into diosgenin were much higher in our system than previously reported. After a 21-day incubation with 4-^{14}C-cholesterol, most of the radioactivity in *D. deltoidea* cell suspensions accumulates in the supernatant (cytoplasmic) fraction. However, mitochondrial and microsomal fractions also contain significant levels of radioactivity. Cell wall and nuclear fractions had relatively low levels of radioactivity.

INTRODUCTION

DIRECT evidence for the biosynthesis of cholesterol in plants at the present time is the isolation of radioactive cholesterol from *Dioscorea spiculiflora*,[1] *Solanum tuberosum*,[2] and *Digitalis purpurea* seedlings after the administration of 2-^{14}C-mevalonic acid.[3] *Dioscorea spiculiflora* seedlings have been shown to convert 4-^{14}C-cholesterol to the sapogenins diosgenin and kryptogenin. *D. lanata* leaves kept in a solution of 4-^{14}C-cholesterol glucoside, produced diosgenin and gitogenin having a higher specific activity than tigogenin.[5] Cholesterol is converted by *Lycopersicon pimpinellifolium* plants to tomatidine,[6] and to solanidine by *S. tuberosum*.[7] The incorporation of cholesterol into various steroidal sapogenins and alkaloids[8] and the entire subject of the biosynthesis of plant steroids have recently been reviewed.[9]

The present communication deals with the conversion of 26-^{14}C-cholesterol to diosgenin and related steroids by *D. deltoidea* suspension cultures. The authors are unaware of other published reports on the incorporation of 26-^{14}C-cholesterol in diosgenin. The subcellular distribution of ^{14}C from 4-^{14}C-cholesterol in *D. deltoidea* cell suspensions is also being reported. Few such investigations in plant cells have been reported. It has been shown in *Pythium* spp. (PRL-2142) that the majority of the administered cholesterol was taken up by the protoplasmic (cell) membranes and the remainder into the intracellular membranes.[10]

* Part II in the series "*Dioscorea* Tissue Cultures".

[1] R. D. BENNETT, E. HEFTMANN, W. H. PRESTON, JR., and J. R. HAUN, *Archs Biochem. Biophys.* **103**, 74 (1963).
[2] D. F. JOHNSON, E. HEFTMANN and G. V. C. HOUGHLAND, *Archs Biochem. Biophys.* **104**, 102 (1964).
[3] G. M. JACOBSOHN and M. J. FREY, *J. Am. Chem. Soc.* **89**, 3338 (1967).
[4] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 577 (1965).
[5] R. TSCHESCHE and H. HULPKE, *Z. Naturforsch.* **21b**, 494 (1966).
[6] E. HEFTMANN, E. R. LIEBER and R. D. BENNETT, *Phytochem.* **6**, 225 (1967).
[7] R. TSCHESCHE and H. HULPKE, *Z. Naturforsch.* **22b**, 791 (1967).
[8] E. HEFTMANN, *Lloydia* **30**, 209 (1967).
[9] E. HEFTMANN, *Lloydia* **31**, 293 (1968).
[10] J. H. SIETSMA and R. H. HASKINS, *Can. J. Biochem.* **46**, 813 (1968).

RESULTS AND DISCUSSION

Our results indicate that the side-chain of cholesterol is incorporated intact into diosgenin, since both 4-^{14}C- and 26-^{14}C-cholesterol were incorporated to the same extent in diosgenin by undifferentiated suspension cultures of *Dioscorea deltoidea* (Table 1). Ten-day-old suspension cultures of undifferentiated *D. deltoidea* cells in 500-ml Erlenmeyer flasks were each incubated with 1 μc of either 4-^{14}C- or 26-^{14}C-cholesterol. Cultures treated with 4-^{14}C-cholesterol were harvested in triplicate at 5-day intervals for 40 days, and five cultures were each harvested after 15 and 30 days of incubation for cultures treated with 26-^{14}C-cholesterol. The glycoside fraction was obtained by extracting the dried cells with chloroform (Extract II), hydrolyzing the extracted cells with acid and then reextracting with chloroform (Extract III). The steroids so liberated were separated by TLC and the radioactivity associated with each fraction was determined by liquid scintillation counting. Each point in Fig. 1 represents the average of three incubates. After 30 days of incubation with either 4-^{14}C- or 26-^{14}C-cholesterol, approximately 7·3 per cent of the total radioactivity administered was present in the diosgenin zone (Table 1). An unidentified zone that moved near the solvent front was also

TABLE 1. PERCENTAGE OF ADMINISTERED RADIOACTIVITY IN GLYCOSIDE FRACTION OF *D. deltoidea* CELL SUSPENSIONS AFTER INCUBATION WITH 4-^{14}C- AND 26-^{14}C-CHOLESTEROL

	After 15 days		After 30 days	
Zones	4-^{14}C- (%)	26-^{14}C- (%)	4-^{14}C- (%)	26-^{14}C- (%)
Diosgenin	4·99	7·94	7·14	7·43
Sterols	7·02	10·10	11·65	10·17
Unidentified	7·41	6·30	8·31	9·21

10-day-old cultures of undifferentiated *D. deltoidea* cell suspensions in 500-ml Erlenmeyer flasks were incubated with 1 μc of either 4-^{14}C- or 26-^{14}C-cholesterol. Each value represents the average of three flasks for the 4-^{14}C-cholesterol and five flasks for the 26-^{14}C-cholesterol.

highly labelled (Fig. 1). It contained approximately 9·0 per cent of the total radioactivity administered as either 4-^{14}C- or 26-^{14}C-cholesterol. In the solvent system employed for TLC, sapogenins such as tigogenin and kryptogenin move toward the solvent front with R_f values similar to that of the unidentified zone. The latter may contain one or more of these sapogenins, and is presently under investigation. Bennett and Heftmann[4] have isolated radioactive kryptogenin in addition to diosgenin after feeding 4-^{14}C-cholesterol to *D. spiculiflora* seedlings.

The R_f of a third zone referred to as the sterols (Fig. 1), corresponds to that of cholesterol and other sterols, such as β-sitosterol and stigmasterol. These sterols do not separate in the solvent system employed for TLC, and may contain glycosides of cholesterol. After 30 days of incubation with 26-^{14}C-cholesterol, the sterol zone contained 10·1 per cent of the administered radioactivity, and after incubation with 4-^{14}C-cholesterol it contained 11·7 per cent (Table 1). After 40 days of incubation, 16·2 per cent of the administered 4-^{14}C-cholesterol was present in the sterol zone (Fig. 1).

The change in radioactivity in the glycoside fraction (Extract III) with time is shown in Table 2 for undifferentiated and differentiated (root) cultures. Most noteworthy is the fact that the differentiated (root) cultures contained only 2·5 per cent of the total extractable

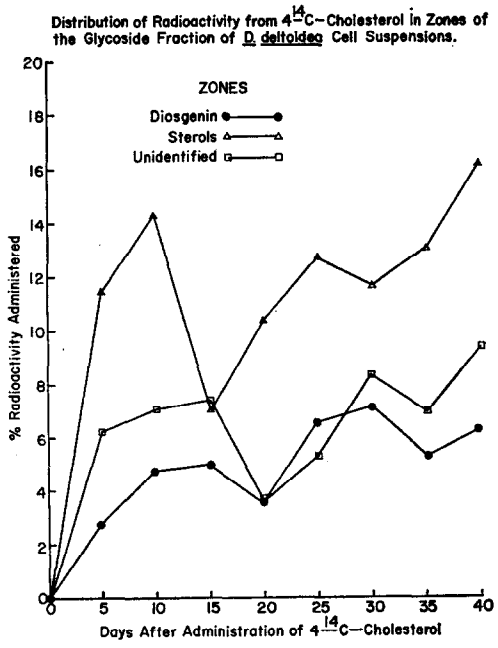

FIG. 1. THE DISTRIBUTION OF RADIOACTIVITY FROM 4-^{14}C-CHOLESTEROL IN ZONES OF THE GLYCOSIDE FRACTION OF UNDIFFERENTIATED *D. deltoidea* CELL SUSPENSION CULTURES.

1 μc of radioactivity was added to each culture in a 500-ml Erlenmeyer flask. Each point represents the average of three flasks. The results are expressed as the percentage of the total radioactivity administered in each of the three major zones of the glycoside fraction (Extract III) obtained by TLC.

TABLE 2. PERCENTAGE TOTAL EXTRACTABLE RADIOACTIVITY IN THE GLYCOSIDE FRACTION OF *D. deltoidea* CULTURES INCUBATED WITH EITHER 4-^{14}C- OR 26-^{14}C-CHOLESTEROL

Incubation time in days	Undifferentiated cell suspension cultures		Differentiated (root) cultures
	4-^{14}C- (%)	26-^{14}C- (%)	4-^{14}C- (%)
5	52·6	—	18·3
10	54·8	—	3·7
15	43·9	46·8	3·2
20	40·0	—	2·8
25	44·0	—	2·5
30	44·8	52·4	—
35	42·2	—	—
40	42·1	—	—

10-day-old undifferentiated cell suspensions of *D. deltoidea* in 500-ml Erlenmeyer flasks were incubated with 1 μc of either 4-^{14}C- or 26-^{14}C-cholesterol. 10-day-old differentiated (root) cultures were incubated with 1 μc of 4-^{14}C-cholesterol. Each value represents the average of the total extractable radioactivity in the glycoside fractions of three flasks for the 4-^{14}C- in undifferentiated cell suspensions, and five flasks for the 26-^{14}C- with undifferentiated cells as well as 4-^{14}C- given to root cultures.

radioactivity in this fraction as compared to 40–50 per cent for undifferentiated cultures given either 4-^{14}C- or 26-^{14}C-cholesterol for 20–30 days. In order to establish the radiochemical purity of diosgenin, it was diluted with carrier diosgenin and recrystallized to constant specific activity from acetone–methanol solution (Table 3).

TABLE 3. CRYSTALLIZATION OF RADIOACTIVE DIOSGENIN TO CONSTANT SPECIFIC ACTIVITY

Crystallization	Cpm/mg
First	$2{\cdot}59 \times 10^3 \pm 150$
Second	$3{\cdot}35 \times 10^3 \pm 260$
Third	$2{\cdot}88 \times 10^3 \pm 170$
Fourth	$2{\cdot}93 \times 10^3 \pm 190$

Radioactive diosgenin isolated by TLC was diluted with carrier diosgenin and recrystallized to constant specific activity from acetone–methanol solution.

When the culture medium of undifferentiated cells treated with 4-^{14}C-cholesterol was extracted, significant radioactivity was found only in the sterol zone. Approximately 25 per cent of the administered radioactivity could be recovered from this zone after 30 days' incubation. The change of radioactivity in the medium with time is shown for the differentiated (root) and undifferentiated cells in Table 4. With maximum growth of undifferentiated

TABLE 4. PERCENTAGE TOTAL EXTRACTABLE RADIOACTIVITY IN THE MEDIUM OF *D. deltoidea* CULTURES INCUBATED WITH EITHER 4-^{14}C- OR 26-^{14}C-CHOLESTEROL

Incubation time in days	Undifferentiated cell suspension cultures		Differentiated (root) cultures
	4-^{14}C- (%)	26-^{14}C- (%)	4-^{14}C- (%)
5	23·8	—	17·9
10	7·4	—	12·0
15	15·6	11·3	12·2
20	20·8	—	11·3
25	18·8	—	11·4
30	24·6	20·4	—
35	30·6	—	—
40	32·2	—	—

10-day-old undifferentiated cell suspensions and differentiated (root) cultures of *D. deltoidea* in 500-ml Erlenmeyer flasks were incubated with 1 μc of either 4-^{14}C- or 26-^{14}C-cholesterol. Each value represents the average of the total extractable radioactivity in the medium of three flasks for the 4-^{14}C- in undifferentiated cell suspensions, and five flasks for the 26-^{14}C- in undifferentiated cells as well as 4-^{14}C- given to root cultures.

cell suspensions occurring at approximately 14 days,[11] the increase in radioactivity in the medium after 10 days may be due in part to liberation of soluble products by lysed tissues. After undifferentiated cell suspensions were incubated with 26-^{14}C-cholesterol for 30 days, 42·4 per cent of the radioactivity in the medium remained at the origin of the thin-layer

[11] B. KAUL and E. J. STABA, *Lloydia* **31**, 171 (1968).

chromatogram, while 48·3 per cent was in the sterol zone (Table 4). Extraction of medium in control flasks incubated with 26-^{14}C-cholesterol showed that after 30 days 92·3 per cent of the radioactivity was present in the sterol area. These results suggest that some of the cholesterol is being metabolized, possibly by cleavage of the side-chain, to produce more polar materials that remain at the origin of the thin-layer chromatogram.

All tissues were extracted with chloroform (Extract II) prior to acid hydrolysis. The change of radioactivity with time in Extracts II of differentiated (root) and undifferentiated cell suspension is given in Table 5. The radiochromatograms of Extract II after the administration of 4-^{14}C-cholesterol to undifferentiated cultures revealed that not less than 90 per cent of the radioactivity remained in the sterol (cholesterol) zone, and that two minor radioactive areas occurred at the origin and solvent front. In Extracts II of the undifferentiated cells harvested on the 15th day after the administration of 26-^{14}C-cholesterol, 87·9 per cent of the

TABLE 5. PERCENTAGE TOTAL EXTRACTABLE RADIOACTIVITY IN THE PRE-HYDROLYSIS FRACTION OF *D. deltoidea* CULTURES INCUBATED WITH EITHER 4-^{14}C- OR 26-^{14}C-CHOLESTEROL

Incubation time in days	Undifferentiated cell suspension cultures		Differentiated (root) cultures
	4-^{14}C- (%)	26-^{14}C- (%)	4-^{14}C- (%)
5	23·0	—	63·4
10	38·9	—	84·2
15	41·8	44·8	88·7
20	38·1	—	85·7
25	40·8	—	85·9
30	33·6	29·4	—
35	25·0	—	—
40	24·8	—	—

10-day-old undifferentiated cell suspensions and differentiated (root) cultures of *D. deltoidea* in 500-ml Erlenmeyer flasks were incubated with 1 μc of either 4-^{14}C- or 26-^{14}C-cholesterol. Each value represents the average of the total extractable radioactivity in the pre-hydrolysis fraction of three flasks for the 4-^{14}C- in undifferentiated cell suspensions, and five flasks for the 26-^{14}C- in undifferentiated cells as well as 4-^{14}C- given to root cultures.

radioactivity was present in the sterol area and 2·5 per cent each near the origin and solvent front. In Extract II, of the cells harvested on the 30th day, 79·3 per cent was in the sterol fraction, 6·2 per cent near the origin, and 8·7 per cent at the solvent front. These results suggest that little of the cholesterol is metabolized by undifferentiated cells.

Differentiated (root) suspension cultures were incubated with 1 μc of 4-^{14}C-cholesterol per flask for 25 days and harvested every 5 days. After 25 days of incubation, 85·9 per cent of the total extractable radioactivity was present in Extract II (Table 5). No significant conversion was noted in this extract, virtually all radioactivity remaining in the sterol zone following TLC. After 25 days, 11·6 per cent of the radioactivity from the 4-^{14}C-cholesterol administered to these root cultures was present in the medium (Table 4). No major metabolites were present, with over 90 per cent of the radioactivity in the medium remaining in the sterol zone. Only 2·5 per cent of the total extractable radioactivity was present in the glycoside fraction (Extract III) after incubating 4-^{14}C-cholesterol with these differentiated (root) cultures for 25 days (Table 2). This compares with approximately 42·5 per cent of the ^{14}C present in this fraction for undifferentiated cells after 25 days' incubation with 4-^{14}C-cholesterol. The

glycoside fraction of the differentiated suspension cultures did not reveal any significant conversion of the administered 4-^{14}C-cholesterol to diosgenin or other metabolites. As previously reported,[11] these results demonstrate that the differentiated tissues of *D. deltoidea* in suspension culture have a poor biosynthetic potential for the conversion of cholesterol to diosgenin or the *de novo* production of diosgenin itself.

The work of Behnke[12] with *Dioscorea* and that of others with *Cucurbita*[13, 14] indicates that with increased differentiation, there is a reduction or loss in the number of mitochondria. Assuming that the mitochondria are intimately associated with sterol biosynthesis in plant cells, these observations may explain the marked difference in diosgenin production in differentiated and undifferentiated *D. deltoidea* tissue cultures. The differentiated cultures produce only trace amounts of diosgenin compared with significant amounts (1 per cent) for the undifferentiated cultures.[11]

The results of the subcellular distribution of radioactivity in *D. deltoidea* suspension cells, 21 days after the administration of 4-^{14}C-cholesterol, are summarized in Table 6. Most of

TABLE 6. SUBCELLULAR DISTRIBUTION OF ^{14}C 21 DAYS AFTER THE ADMINISTRATION OF 4-^{14}C-CHOLESTEROL TO *D. deltoidea* SUSPENSION CELLS

Fraction	Percentage total radioactivity in each fraction
Cell-wall material	8·41 ± 0·31
Nuclei	3·14 ± 0·17
Mitochondria	18·05 ± 1·61
Microsomes	12·80 ± 0·72
Supernatant	57·63 ± 1·87

To each of three suspension cultures, 2·5 μc of 4-^{14}C-cholesterol was added on the 10th day of culture growth. 5 g wet weight (0·374 g dry weight) samples of the cells were fractionated 21 days after the administration of the radioactive cholesterol.

the radioactivity resides in the supernatant (cytoplasmic) fraction (57 per cent). However, the mitochondrial and microsomal fractions also contain significant amounts (18 and 12 per cent, respectively) of the administered radioactivity. It is interesting to note that the cell wall and nuclear fractions have relatively low amounts of the radioactivity, namely 8 and 3 per cent, respectively. A qualitative and quantitative analysis of the sterols and sapogenins in the free and glycoside form associated with each subcellular fraction would lead to a greater understanding of the sites of synthesis and storage of diosgenin at the subcellular level. Since the cytoplasmic fraction contains most of the radioactivity, it is possible that the homogenization and fractionation techniques employed may have released certain hydrolytic enzymes, which in turn liberated various steroids from their glycosides. The results suggest that the major sites of metabolism and/or storage of cholesterol in *D. deltoidea* are the microsomes, mitochondria, and cytoplasmic fractions.

[12] H. D. BEHNKE, *Z. Pflanzenphysiol.* **53**, 214 (1965).
[13] K. ESAU and V. I. CHEADLE, *Botan. Gaz.* **124**, 79 (1962).
[14] R. KOLLMANN, *Phytomorphol.* **14**, 247 (1964).

EXPERIMENTAL

Inocula Preparation

Undifferentiated *Dioscorea deltoidea* cell suspensions, maintained as a friable suspension of cell aggregates for nearly 3 years, was used. 25 ml of an actively growing culture were aseptically transferred to 100 ml of fresh RT-.1 liquid medium[11] contained in 500-ml Erlenmeyer flasks. This culture was allowed to grow under the previously described conditions[11] for 14 days before being used as an inoculum for subsequent experiments. 25 ml of this 14-day-old culture were added to each sample flask. Similarly, 14-day-old differentiated (root) cultures were used in 100 ml of RT-.1 medium.

Administration of Cholesterol

Twenty-four 500-ml Erlenmeyer flasks of both 10-day-old differentiated (root) and undifferentiated cultures of *D. deltoidea* were each inoculated with 1 μc of 4-^{14}C-cholesterol (New England Nuclear Corp., Boston, Mass.) per flask, dissolved in 0·20 ml of 70% ethanol. Equal amounts of radioactive cholesterol were also added to two flasks containing only RT-.1 medium without cells. The cultures were allowed to grow under normal conditions as previously described.[11]

Beginning with the 5th day after administration of the radioactive cholesterol, five flasks of the differentiated and three flasks of the undifferentiated cells were harvested at 5-day intervals. The cells from each individual flask were filtered, washed, dried, and extracted according to the scheme described under Extraction, and then chromatographically resolved and examined for radioactivity.

For the incubation with 26-^{14}C-cholesterol, only 10-day-old cultures of undifferentiated cell suspensions were used. To each flask 1 μc of 26-^{14}C-cholesterol was added (New England Nuclear Corp., Boston, Mass.) in 0·20 ml of 70% ethanol. An equal amount of radioactive 26-^{14}C-cholesterol was added to two blank flasks containing only RT-.1 medium and no cells. The cultures and the blank flasks were incubated under normal conditions, and five flasks each were harvested on the 15th and 30th day after the administration of the 26-^{14}C-cholesterol.

Harvesting and Extraction

D. deltoidea suspension culture cells or roots were filtered separately and washed with 100 ml of 0·01 M phosphate buffer, pH 6·0, prior to being dried. The media from the undifferentiated or differentiated cultures were pooled at each harvest, whereas the cells from each flask were extracted and analyzed separately.

The pooled medium from each harvest was extracted with an equal volume of $CHCl_3$ and the extract was evaporated. The residue was redissolved in $CHCl_3$ to a total volume of 2·0 ml, and designated Extract I. The dried cells from individual flasks were extracted with $CHCl_3$ for 8 hr in a Soxhlet. The extract was evaporated and the residue redissolved in $CHCl_3$ to make 2·0 ml of solution, designated Extract II. The residual cells were air-dried, hydrolyzed with 30% v/v HCl for 4 hr on a steam bath, and finally extracted with $CHCl_3$ for 8 hr. The extract was evaporated, the residue redissolved in $CHCl_3$ to a final volume of 2·0 ml, and designated Extract III.

Chromatography

Aliquots of 100 μl of Extracts I and II, and 50 μl of Extract III were each applied as a spot on Adsorbosil-5 (Applied Science Labs., State College, Pa.) thin-layer plates. A reference ^{14}C-cholesterol spot was also applied to each plate. The plates were developed three times in benzene: ethyl acetate (10:1) for 30 min each time. The developed areas (2 m × 18 cm) of the radioactive TLC plates were divided into eighteen 1-cm zones and each individual area was placed into a liquid scintillation counting vial and counted as described below.

Subcellular Distribution of 4-^{14}C-Cholesterol

Three flasks of 10-day-old cultures of *D. deltoidea* were each given 2·5 μc of 4-^{14}C-cholesterol and incubated for a total of 21 days. The cells and medium were separated by filtration, and the cells were washed with 25 ml of isotonic sucrose solution containing 0·25 M sucrose, 0·005 M $MgCl_2$, 0·025 M KCl, and 0·050 M tris chloride, pH 7·4. Three 5-g wet weight of cell samples were taken for the determination of dry weight, total radioactivity, and cell fractionation. 5 g wet cells represented 0·374 g dry cells.

5-g samples of washed wet cells were mixed with 5 ml isotonic sucrose solution and 7·5 g washed white sand, and ground for 10 min in an ice-cold mortar with a pestle. The homogenate was filtered through four layers of cheese-cloth, and the residue was transferred to the mortar. The cheese-cloth was washed with 10 ml of the buffer and the washing mixed with the residue and homogenized for 5 min more. The mixture was again filtered through four layers of cheese-cloth. The residue was microscopically shown to be composed primarily of cell walls. The pooled filtrates were centrifuged for 3 min at 375 rev/min, which removed crushed sand, cell-wall material and cell debris. The supernatant was carefully decanted and the pellet resuspended in 5 ml of the buffer and recentrifuged. The latter supernatant was combined with the initial 375 rev/min supernatant and the pellet combined with the cell-wall fraction obtained by filtration.

The combined 375 rev/min supernatant fraction was centrifuged at 875 rev/min for 10 min to yield the nuclear fraction. The supernatant was decanted and the pellet was resuspended in 5 ml of the buffer and again centrifuged, giving a washed nuclear pellet.

The 875 rev/min supernatants were combined and were further centrifuged for 10 min at 10,000 rev/min. The supernatant was decanted and the mitochondrial pellet was resuspended in 5 ml of buffer and recentrifuged. The two supernatant fractions were combined.

The 10,000 rev/min supernatant was finally centrifuged at 40,000 rev/min (100,000 × *g*) for 60 min in a Spinco Model-L centrifuge, yielding the microsomal and supernatant (cytoplasmic) fractions.

Radioactivity Determinations

From each extract chromatographed on TLC plates, 18 samples were obtained. Each sample was scraped into a liquid scintillation counting vial, and 15 ml of toluene counting solution of the following composition was added: 100 mg dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene) and 3·00 g PPO (2,5-diphenyloxazole) per l. of toluene. Each sample was counted for at least 20 min in a Beckman liquid scintillation counter model LS-100, equipped with an external standard to determine counting efficiency.

In order to determine the distribution of ^{14}C from 4-^{14}C-cholesterol administered to *D. deltoidea* cultures, the whole cell sample and each of the isolated particulate fractions were partially dissolved by macerating in an aqueous solution of 0·1 N NaOH and 0·05% sodium lauryl (dodecyl) sulfate. The mixture was allowed to stand overnight. After vigorously shaking each sample, 0·25 ml aliquots in triplicate were taken from each fraction, including the supernatant fractions, placed in counting vials, 1·0 ml of solubilizer (Bio-Solv BBS-3) was added to each of the samples, and the samples were allowed to stand overnight. Occasionally some samples were warmed at 40–50° to aid solution of the sample by the solubilizer. To each solubilized sample 20 ml of dioxane-counting solution of the following composition was added: 0·750 g dimethyl POPOP, 7·50 g PPO, 120·0 g naphthalene, 400 ml xylene, and 500 ml 1,4-dioxane. A clear solution was obtained with this counting solution, and the external standard method was employed to calculate the counting efficiency in these samples.

Acknowledgements—This investigation was supported in part by a grant from the Nebraska Heart Association, Omaha.