Rapid screening of lipid metabolism in monolayer cell cultures

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Summary Monolayer cell cultures grown on coverslips in the presence of radioactive lipid precursors were embedded in silica gel layers for extraction and resolution of the labeled products directly by thin-layer chromatography. The method permits rapid screening of lipid metabolism in tissue cultures with a small number of cells.

Supplementary key words CHO · ether lipids · neutral lipids · phospholipids

Metabolic studies with cell cultures grown as monolayers are often difficult because of the large number of flasks and manipulations required. The usual methods have employed detachment of monolayers either by trypsinization or by scraping with a rubber policeman followed by extraction of lipids and subsequent analyses. Although useful, these methods require many steps that can be expensive and time-consuming when large numbers of samples are examined.

In this report we describe a practical procedure for monitoring lipid metabolism in monolayer cultures that is based on techniques that have been described for the extraction and chromatography of lipids from tissue slices (1) and microscopic sections of tissues (2–4) directly on thin-layer chromatograms. We used cells cultured as monolayers on microscope coverslips; the slips were subsequently attached to silica gel layers for extraction and analysis of the labeled products formed. The technique provides not only information on the extent to which labeled precursors are incorporated and on their metabolic fate, but it also permits one to evaluate and select suitable solvents for the quantitative extraction of the labeled products.

Materials and Methods

CHO cells (clone K_1), which have been previously described (5, 6), were maintained in Ham's F-12 medium (Grand Island Biological Company, Grand Island, N.Y.) supplemented with 10% fetal calf serum. For labeling experiments the serum was heat inactivated (56°C, 30 min) and dialyzed. All cultures were incubated at 37°C in a 100% humidified atmosphere containing 5% CO_2. Cells were routinely grown in 60 × 15-mm plastic culture dishes (Falcon Plastics, Oxnard, Cal.) at an initial density of 1 to 2×10^5 cells in 5 ml of medium. Cells were counted with a hemocytometer or with a Coulter counter.

Coverslips (9 × 22 mm, No. 1) were washed in Hemosol and then rinsed at least four times each in a sequence of hot water, cold water, distilled water, and 95% ethanol. They were stored in alcohol until used for cell cultures. The coverslips were transferred to sterile culture dishes by picking them up with microforceps, passing them through a flame, and finally placing them at the bottom of the dish. Six to seven coverslips were used in each dish. The density of cells from one coverslip to another may vary; therefore, unless proper care is taken to disperse cells uniformly at the time of plating, they will tend to localize at different areas on the slip, which can account for differences in radioactivity found with coverslips from the same culture.

[1,2-^{14}C]Ethanolamine·HCl (6.3 μCi/μmole), [1,2-^{14}C]choline chloride (7.8 μCi/μmole), and [1-^{14}C]-palmitic acid (54.3 μCi/μmole) were purchased from New England Nuclear, Boston, Mass. [1-^{3}H]Hexadecanol was prepared by reduction of hexadecanal (7) (Applied Science Laboratories, Inc., State College, Pa.) with NaB^3H_4 (8) (Amersham/Searle, Arlington Heights, Ill.).

Before adding the labeled compounds, spent medium was removed; cultures were then gently washed with 2 ml of prewarmed medium and replenished with 5 ml of fresh medium. Radioactive material was either added to fresh medium before replenishing the cultures or added as an aliquot of an ethanol solution (5–25 μl of 95% ethanol) to growing cells. After incubation, the old medium was removed and the monolayers adhering to the coverslips were gently washed twice with cold 0.85% NaCl. Individual coverslips could be picked up with microforceps if a blunt instrument such as a stirring rod were positioned beneath the coverslip. With slight pressure, the plastic culture dish deformed, permitting an edge of the coverslip to detach enough so the pointed end of the microforceps could be slipped beneath it. The coverslips with the cell side up were transferred to clean culture dishes and stored frozen until analyzed.

For chromatography the coverslips were positioned on a flat surface, cell side up, and allowed to air dry

Abbreviations: CHO, Chinese hamster ovary cells; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S, sphingomyelin; TLC, thin-layer chromatography.

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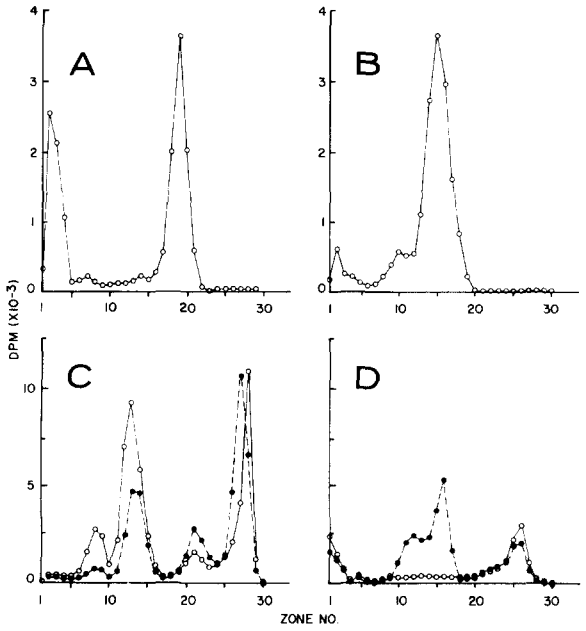

Fig. 1. Incorporation of ^{14}C (○ —— ○) and ^{3}H (● —— ●) into lipids of monolayer cultures of CHO cells grown on coverslips (5-mm zonal scans of thin-layer chromatograms). Scan A: cells incubated with [1,2-^{14}C]ethanolamine · HCL (0.2 μCi/ml) for 24 hr; zones 1–4 (nonlipid material) and zones 17–21 (PE). Scan B: cells incubated with [1,2-^{14}C]choline chloride (0.2 μCi/ml) for 24 hr; zones 8–13 (S) and zones 14–17 (PC). Scan C: cells incubated with [1-^{14}C]palmitic acid (0.125 μCi/ml) and [1-^{3}H]hexadecanol (0.279 μCi/ml) for 24 hr; zones 5–10 (S), zones 11–14 (PC), zones 19–22 (PE), and zones 25–29 ("neutral" lipids). Scan D: Same incubation conditions as C; zones 10–13 (ROH), zones 14–17 (unidentified), and zones 23–28 (mixture of triacylglycerols, alkyldiacylglycerols, and cholesterol esters plus wax esters). Unincorporated [1-^{14}C]palmitic acid migrates with the ROH in this system.

for 15–30 min. For attachment to the silica layers, coverslips were completely covered with 12–13 drops of silica gel HR–chloroform slurry (1:3, v/v). Care was taken to ensure that the slurry did not spill over the edges of the slip. Standard silica gel plates (5 × 20 cm, 250 μm layers) were gently lowered, gel side down, until the surface made contact with the slurry. Capillary action was sufficient to attach the monolayers and the coverslip to the gel surface. The entire assembly was then turned over to allow the slurry to dry (approx. 15 min). Coverslips were attached to silica gel layers so the lower edge of the coverslip was approximately 3.2 cm from the lower end of the plate, with the long dimension of the coverslip perpendicular to the direction of solvent migration.

Silica gel G layers for "neutral" lipids or silica gel HRB layers for phospholipids were developed in chloroform–methanol–acetic acid 98:2:1 (v/v/v) and chloroform–methanol–acetic acid–water 50:25:8:4 (by vol.), respectively (9). After development, coverslips were removed from the plates and the distribution of the radioactivity on the chromatograms was determined by zonal scanning using liquid scintillation spectrometry (10, 11). Zonal scans were initiated approximately 2 mm below the lower edge of the coverslip. The origin was defined as the center of the impression left by the coverslip.

In some experiments, cellular lipids were extracted with chloroform–methanol (12) containing 2% glacial acetic acid and then analyzed in the same thin-layer chromatographic systems used for analysis of the cells on the coverslips. Incorporation of radioactivity into ether lipids was based on chromatography of the products formed after Vitride[2] reduction of the total lipid extracts (13).

Results and Discussion.

Fig. 1 illustrates the labeling results obtained with the coverslip technique when monolayers of CHO-K_1 cells were incubated with four different lipid precursors. As expected, Fig. 1-A demonstrates that [1,2-^{14}C]ethanolamine was mainly incorporated into PE (47% of the total activity), whereas when [1,2-^{14}C]choline was the precursor (Fig. 1-B), most of the activity was incorporated into PC (73%) and S (7%). Some labeled material always remained at the origin or with cells on the coverslip when either labeled ethanolamine or choline was used. However, the radioactivity associated at the origin was sufficiently distant from the lipid components that it did not interfere with the analysis. The material remaining at the origin or with the cells on the coverslip appears to be nonlipid, since no radioactivity was observed at the origin with the lipid extracts obtained with the Bligh and Dyer procedure (12) and radioactivity could not be extracted from the cells on the coverslip after chromatography. Furthermore, when the solvent system for resolving phospholipids was used, ^{14}C-labeled internal standards (consisting of mixtures of PC, tripalmitoylglycerol, hexadecanol, and palmitic acid) were not associated with the coverslips or the origin, in either the presence or the absence of cells after radiochromatography. Also as predicted, only ^{14}C-labeled PC was found at the origin and on the coverslip when the chromatograms were developed in the solvent system for the "neutral" lipids.

The data presented in Fig. 1-C and -D are from a double-label experiment with [1-^{14}C]palmitic acid and [1-^{3}H]hexadecanol as precursors. Figure 1-C shows that palmitic acid preferentially labeled PC in comparison to PE or S, a labeling pattern that has been reported previously in studies of L-M fibroblast

[2] $NaAlH_2(OCH_2CH_2OCH_3)_2$ purchased from Eastman Organic Chemicals; see ref. 13 for procedure.

cultures (14). Also there was more ^{14}C than ^{3}H in the PC fraction and more ^{3}H than ^{14}C in PE; this too is consistent with the larger proportion of ether lipids (formed from fatty alcohols) found in the ethanolamine phospholipids.

Unincorporated [1-^{14}C]palmitic acid was virtually absent from the cells incubated for 24 hr (Fig. 1-D). Peak I was identified as unincorporated [1-^{3}H]-hexadecanol on the basis of its migration with a standard of hexadecanol; peak II was unidentified. The bulk of the ^{14}C was associated with peak III, which yielded ^{14}C-fatty alcohols and ^{3}H-labeled alkylglycerols after Vitride reduction. The component of peak III could be resolved into three peaks (triacylglycerols, alkyldiacylglycerols, and cholesterol esters plus wax esters) on silica gel G layers developed in hexane–diethyl ether–acetic acid 80:20:1 (v/v/v). In a separate experiment with [1-^{14}C]palmitic acid as the precursor, components of peak III were purified by thin-layer chromatography. Vitride reduction of the triacylglycerols produced only ^{14}C-labeled fatty alcohols, while saponification yielded fatty acids. When the purified alkyldiacylglycerols were reduced with Vitride, ^{14}C-labeled alkylglycerols and fatty alcohols were produced, whereas saponification gave labeled alkylglycerols and fatty acids. Reduction of cholesterol esters and waxes with Vitride produced ^{14}C-labeled fatty alcohols and saponification yielded ^{14}C-fatty acids.

When the cells on coverslips were extracted by the Bligh and Dyer procedure (12) approximately 55–60% less radioactivity was removed than by direct extraction with the chromatographic solvents. Presumably this poor recovery is due to losses of labeled material in the water phase when extracting lipids from very small samples, since when total lipids were extracted from pooled samples of monolayer cultures the results were similar to those obtained by direct extraction on the silica gel layers. For example, in a 2-hr labeling experiment with [1-^{14}C]-palmitic acid, 14.8% of the ^{14}C-label added to the incubation medium could be recovered in the Bligh and Dyer extract of the cells. Direct TLC of monolayer cultures grown on coverslips under similar conditions gave recoveries of 10.8–15.3%. When cells labeled for 24 hr with [1-^{14}C]palmitic acid were compared, 55.4% of the total ^{14}C was recovered from the Bligh and Dyer extract of the cells, while 49.3% of the ^{14}C incubated was recovered from the cells on the coverslips. Deviations from the mean values for measurements made on nine coverslip samples ranged from 6–14% for PC and from 2–13% for PE when [1-^{14}C]palmitic acid was used as a precursor. Our data show that regardless of the extraction technique used, the relative distribution of lipid classes on the thin-layer chromatograms is generally similar for all cells incubated under identical conditions.

Extracting labeled lipids and resolving them into classes by direct thin-layer chromatography of monolayers on coverslips is not only rapid, but is easily employed with a minimal number of cells and small quantities of radioactivity. Since coverslips can readily be removed from the culture dish, the effect of time on the incorporation of labeled material into lipids can be measured for cultures grown under the same conditions or with identical cells transferred to different environments. Although the extraction procedure has been simplified, one still needs to consider the possibility that different lipids can be extracted with different efficiencies, as with all lipid extractions.

Our results indicate that the coverslip method, in conjunction with thin-layer chromatography, is applicable to screening lipid metabolism in cell cultures grown as monolayers. Useful preliminary results obtained with this method can then be confirmed and extended by the more time-consuming and expensive conventional procedures of analysis.

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