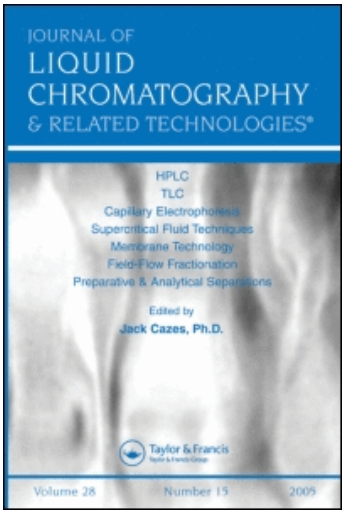

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A Review of Radiochromatogram Analysis Instrumentation

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A Review of Radiochromatogram Analysis Instrumentation

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ABSTRACT

The available techniques for the analysis of radiolabled TLC plates are described and compared, including the newest technique based on imaging proportional counters. The imaging systems offer a 100-fold improvement in sensitivity over conventional chromatogram scanners and can replace much of the analysis currently being done by plate scraping and scintillation counting. For most quantitative analysis, imaging systems offer superior speed and comparable or better accuracy when compared with scintillation counting. Large savings can be realized in sample preparation time, disposable supply costs, and liquid waste disposal.

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Introduction

TLC is a major analytical tool for both qualitative and quantitative applications. For radioisotope work, qualitative techniques are largely photographic such as autoradiography and spark camera analysis, while the quantitative techniques rely on nuclear detectors such as Geiger or proportional counters and scintillation counting. Often a combination of these techniques is used. The qualitative technique provides the location of the activity, and then scintillation counting is used to obtain accurate quantitative results.

A brief summary of the various radioisotope techniques and their capabilities including sensitivity, resolution, and quantitative accuracy will be discussed. Most of these techniques have been in use for many years, and were described by Touchstone and Dobbins (1). The exception is the radiochromatogram imaging system which has been commercially available for about two years. The techniques used for radioisotope analyses are:

1. Autoradiography - film recording of ionization produced by betas or X-rays. High spatial resolution, low sensitivity, poor quantitation.
2. Spark Camera - a gas filled spark chamber produces light along the ionization track which is then photographed. High sensitivity, poorer resolution than direct autoradiography, poor quantitation.
3. Radiochromatogram scanners - a collimated Geiger or proportional counter is scanned along the TLC lane. Output is generally a plot

of counts vs. position on the lane. Moderate sensitivity and spatial resolution, quantitation possible but not readily available from output plot.

4. Scintillation counting - active regions or sections at regular intervals are removed from the TLC substrate by scraping or cutting and then individual samples are eluted and prepared for standard scintillation counting. Poor resolution, excellent sensitivity and quantitation.
5. Radiochromatogram imaging systems - an imaging proportional counter which measures both the occurrence and location of each radioactive decay event is placed over the TLC lane. Output is generally in digital form with both CRT and paper histogram displays available as well as numerical peak integration features. High sensitivity, moderate spatial resolution, and excellent quantitation.

There are other requirements and concerns besides sensitivity, resolution, and quantitation which impact on the choice of radiochromatogram analysis technique. These include turn-around time (the time elapsed before results are in hand), labor and material costs, health hazards, and sample preservation. In general elapsed time is directly related to sensitivity and so is longest for autoradiography and shortest for spark cameras and radiochromatogram imaging systems. However, scintillation counting may also have a long elapsed time due to the large amount of preparation time required and counter backlogs. Labor and mate-

Table 1

Comparison of Radiochromatogram Analysis Instrumentation

	Sensitivity	Resolution	Quantitation	Turn-around Time	Cost/Sample	Health Hazard	Sample Preserved
Autoradiography	Poor	Ex.*	Poor	Poor	Low	Low	Yes
Spark Camera	Good	Mod.**	Poor	Ex.	Low	Low	Yes
Scintill. Counting	Ex.	Poor	Ex.	Mod.	High	High	No
Chrom. Scanner	Mod.	Mod.	Mod.	Mod.	Low	Low	Yes
Chrom. Imaging	Good	Mod.	Ex.	Ex.	Low	Low	Yes

*Ex = Excellent

**Mod = Moderate

rial costs are modest for all techniques except scintillation counting. To prepare a single TLC lane for counting in 20 1-cm segments may require 30 minutes or more of bench time and $3-5 in expendable vials, scintillation cocktail, and liquid waste disposal. Scintillation counting also presents the worst health hazard since it is the only technique in which there is a signi-

ficant chance of creating airborne silica particles and radioactivity during the scraping or cutting process. And finally, if sample preservation is required for recovery or further analysis, any of the techniques except scintillation counting can be used.

A summary of all techniques and the evaluation critieria discussed above is given in Table 1.

Quantitative Analysis

For the quantitative analysis of radiochromatograms, the choice of techniques is limited to conventional scanners, scraping and counting, and imaging systems. Conventional scanners and imaging systems are similar in that they measure the activity *in situ*, while scintillation counting requires the destruction of the original chromatogram. The *in situ* measurements, while preserving the chromatogram, are limited in their sensitivity by the absorption of betas by the chromatographic layer itself. The counting efficiency for tritium is generally in the range 0.5 - 2 percent depending on the type and thickness of TLC plate used. For ^{14}C, the efficiency is about 10 times greater. Scintillation counting can have efficiencies in excess of 30 percent for tritium. Conventional scanners suffer from the further inefficiency that they view a given portion of the chromatogram for only a small fraction of the total analysis time. They, as well as scintillation counters, perform a sequential analysis of the chromatogram so that for the same total counting time a scanner will generally have 50 times less sensitivity than scintillation counting. An imaging system views all portions of the chromatogram for the entire analysis time thereby recouping most of its

efficiency disadvantage when compared with scintillation counting.

In general, conventional scanners and imaging systems have similar characteristics except that imaging systems are 20-100 times more sensitive. Available scanners also suffer to some extent from being an older generation of equipment designed before sophisticated electronics and microprocessors were available. The imaging systems, therefore, have a considerable advantage in computational power which allows the user, with a few simple commands, to integrate the counts in peaks of interest, to obtain relative percents of total activity for each peak, and to automate the analysis of large numbers of similar chromatograms.

The quantitative limitations of scraping and scintillation counting and imaging systems are difficult to compare. The major limitations in scintillation counting are in the preparation of the samples. Errors can arise from a desire to scrape the minimum number of zones thus degrading the resolution of the chromatogram or missing some important areas of background or contaminants.

By contrast, the major quantitative limitation with imaging systems is often counting statistics due to the lower counting efficiencies. A 1000 disintegrations per minute (dpm) tritium spot would produce about 5 counts per minute (cpm) in the imaging system so that a 20 minute analysis would produce a total count of 100 with a statistical uncertainty of 10 percent. A 20 minute analysis in a scintillation counter would yield at least 6×10^3 counts with a statistical uncertainty of less than 2 percent. This error is much less than the average error introduced by the scraping process. Also, if the scraping were in zones of 1 cm (rather poor resolution), as many as 15 samples would be gene-

rated from a 15 cm plate development, and the total counter time required would be 5 hours for 20 minute counts and 1.25 hours for 5 minute counts from a single chromatogram. If all the samples were scintillation counted within the 20 minute span alotted for the imaging analysis, the 1000 dpm spot would produce 400 counts in 1.3 minutes, a statistical precision of 5 percent.

Because quantitative accuracy and spatial resolution are related in practical work, it is important to compare these characteristics. For tritium, imaging system resolution is about 2 mm or equivalent to 75 or more scraping zones on a standard TLC plate. For ^{14}C, the resolution is degraded somewhat, as explained below, to 3-5 mm or at least 30 scraping zones. In practice, users rarely scrape this many zones, and in each case it is important to assess what impact not having this resolution could have on the precision of the analysis. Often, the potential impact is important enough that some less quantitative, but higher resolution technique, such as autoradiography or conventional scanners, is used to validate the scraping and scintillation counting analysis.

A direct comparison of the quantitative results from scraping and scintillation counting and imaging system analysis was made by Baird et al.(2). Duplicate samples of carcinogen ([^{3}H]benzo(a)pyrene) metabolite separations were run and analyzed by the two methods (Figure 1). The scintillation counting results are shown above and the imaging system results below. The percentage of each metabolite calculated from the two methods of analysis agree to better than the 10-15 percent sample-to-sample variation found by scintillation counting alone. Thus, the quantitative results from the imaging system are at

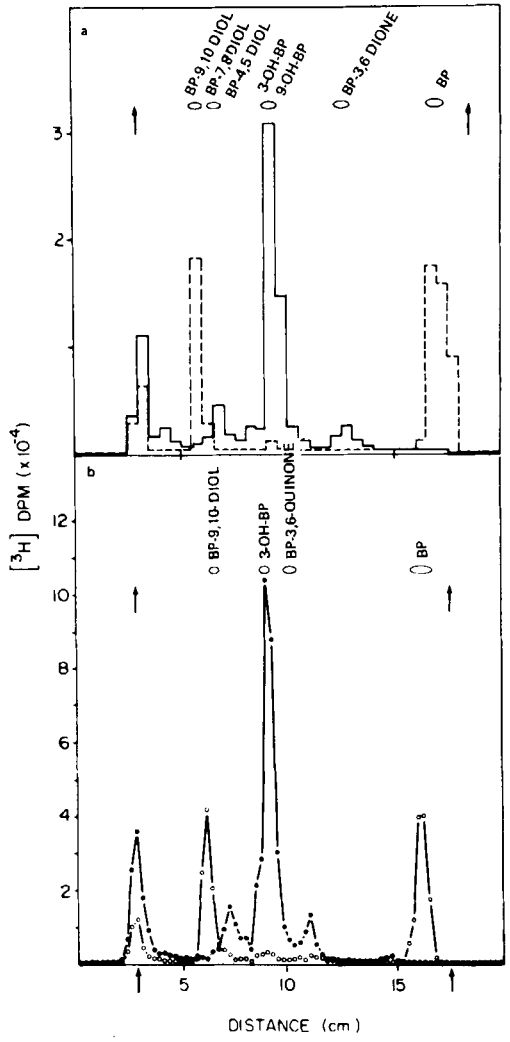

Figure 1 A comparison between scraping and scintillation counting (top) and the imaging system (bottom). The open and filled circles correspond to the dashed and solid lines, respectively. The imaging system analysis time was 20 minutes.

least as accurate as the experimental reproducibility in this type of work.

Overall, the radiochromatogram imaging system offers superior quantitative performance over the conventional technique of scraping and scintillation counting. It is faster, has better spatial resolution, and is less costly to use. However, scintillation counting may be the preferred technique with samples of such low activity that the required imaging system analysis time is longer than the scraping and preparation time. Typically, this point is reached with total activities below 500-1000 dpm of tritium which would require 0.5-1 hour analysis times with the imaging system. In most laboratory experiments, the imaging system will handle 90 percent or more of the work load with an average analysis time of 5-15 minutes per lane.

Radiochromatogram Imaging Systems

The functional characteristics of imaging systems have been discussed above. In this section, a more detailed explanation of the underlying principles is presented. Generally, these systems consist of three separate parts: an imaging proportional counter, a CRT and associated electronics for storing the data from the counter and presenting a display of the chromatogram, and a terminal for controlling the operation of the instrument and printing the final results.

The new element in this system is the imaging proportional counter. It is instructive to first review the principles of a standard proportional counter, Figure 2. The counter has a gas volume, usually circular or rectangular in cross-section, with a

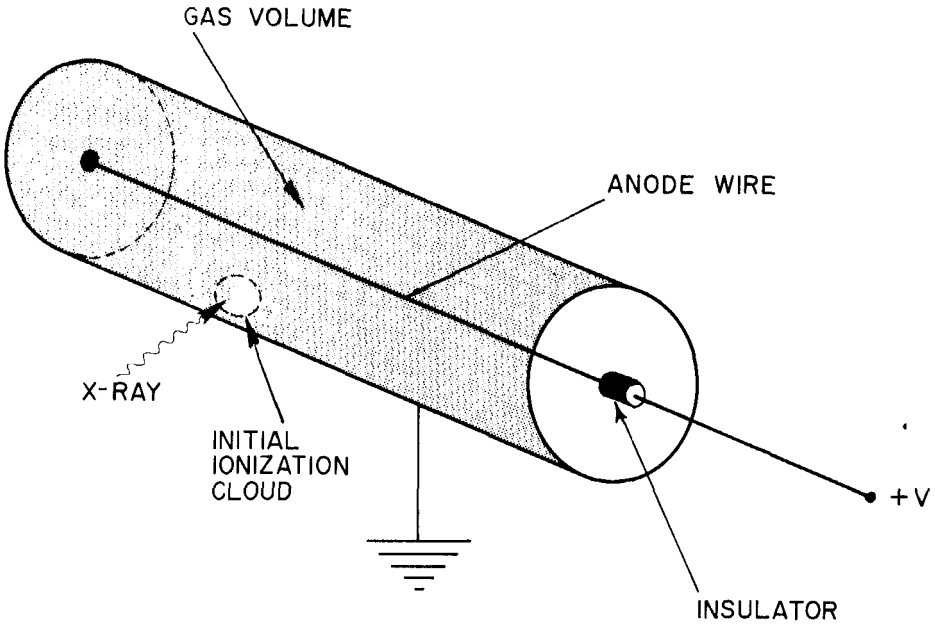

Figure 2 Schematic drawing of a conventional proportional counter.

high voltage anode wire running through it. Radiation, such as X-rays or beta particles, ionizes the gas and produces free electrons which are accelerated toward the anode wire. When the electrons enter the very high electric field near the wire surface, they are accelerated to sufficient energy to produce further ionization of the gas. This increase in the number of electrons produces a pulse on the anode wire of sufficient magnitude (1000 electrons or more) to sense with standard electronic amplifiers.

In counters designed to detect X-rays or high energy beta radiation, the gas volume is completely sealed, and the radiation enters through a window made of plastic, beryllium, or other

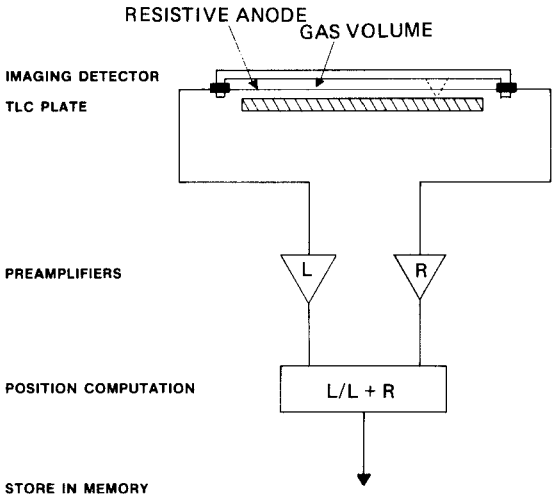

Figure 3 Schematic drawing of a resistance anode imaging proportional counter, its electronics, and the logical flow of event data.

material. In the case of low energy alpha and beta particle detection, the entrance aperture must be windowless, and gas lost to the surroundings must be continually resupplied to the detector volume. Many gases can be used, but the most common are mixtures of noble gases and hydrocarbons. A mixture of 90% argon and 10% methane (called P-10) is widely used and can be readily obtained from most gas suppliers.

To add imaging capability to the counters, the design must be altered to provide an electronic signal which varies as the position of the incident radiation varies over the sensitive area of the detector. One such scheme for obtaining position information in one dimension is shown in Figure 3. The metal anode of Figure 2 is replaced by a resistive anode made of a carbon coated

quartz fiber. A preamplifier is attached at both ends instead of only one end. When a pulse of electrons is collected at the resistive anode, it behaves like a current divider. Part of the pulse flows toward each end with the ratio of the two parts of the pulse determined by the amount of resistance between the original collection point and each of the preamplifiers. The two amplified pulses are then converted to a digital result on a pulse height scale, and the quotient shown is computed to give a numerical result for the position. A digital image can then be built up in a computer memory by adding 1 count to the total stored in the memory location which corresponds to a particular interval along the anode.

The resolution which can be achieved with this (or other) imaging scheme is on the order of 0.2-0.5% of the total detector length. For radiochromatograms, the detector length used is 25 cm, and the data is stored in 256 locations in the computer memory corresponding to 1 mm intervals. However, the main limitation on the resolution in practice is due to the finite depth of the detector and the omnidirectional nature of the radiation emanating from the sample. The dashed lines in Figure 3 illustrate two possible paths of betas radiated from the same location on the TLC plate. The betas will produce ionization along their paths, and the detector will measure the centroid of this ionization. In general, the spread (or defocussing) will be comparable to the detector depth for high energy betas, and will decrease as the energy and penertrating power of the beta decreases. Thus for P-32, the resolution will be limited to the detector depth of 5 mm, while for ^{14}C it will improve to about 3 mm, and for tritium it is 1-2 mm.

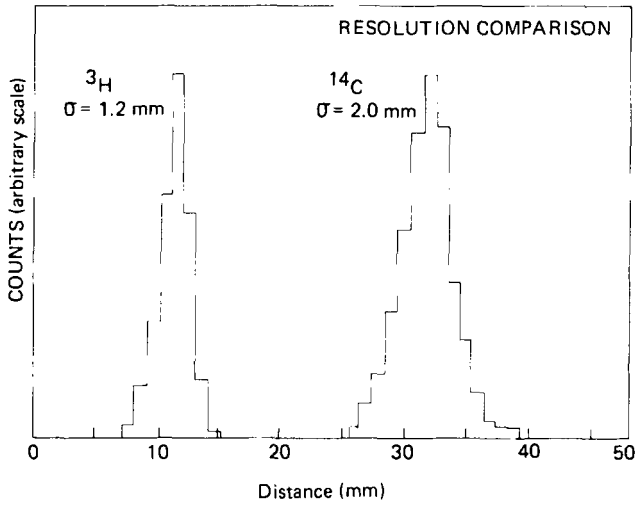

Figure 4 A resolution comparison between tritium and ^{14}C. The spots analyzed had intrinsic diameters of 1-2 mm.

The relative resolution performance for tritium and ^{14}C is shown in Figure 4. Radioactivity was spotted on a TLC plate with a micropipette, and then analyzed using an imaging system (Bioscan, Inc. BID SYSTEM 100). The spot sizes are 1-2 mm in diameter, so the actual detector resolution is somewhat better than the gaussian fit parameters shown.

Several groups (2,3,4,5) have investigated applications of imaging proportional counters to quantitative radiochromatogram analysis, and their work may be consulted for details.

A Radiochromatogram Imaging System Application

Radiochromatogram imaging techniques when combined with careful preparative and TLC techniques give excellent results.

As an example, the work of Bougnoux, Hoffman, and Herberman (6) as part of a study of membrane metabolism changes associated with the cytotoxicity of human peripheral blood cells acting against human tumor cells is cited.

The principal advantages of the radiochromatogram imaging system in this work are its quantitative accuracy, its sensitivity, and the very small amount of operator attention required. Plate scraping and scintillation counting would have been used if the imaging system were not available, and the researchers then estimate that the scraping and sample preparation would limit the rate at which experiments were performed and analyzed. With the availability of the imaging system, the time and effort required for accurate quantitative analysis is not a significant part of the total laboratory effort.

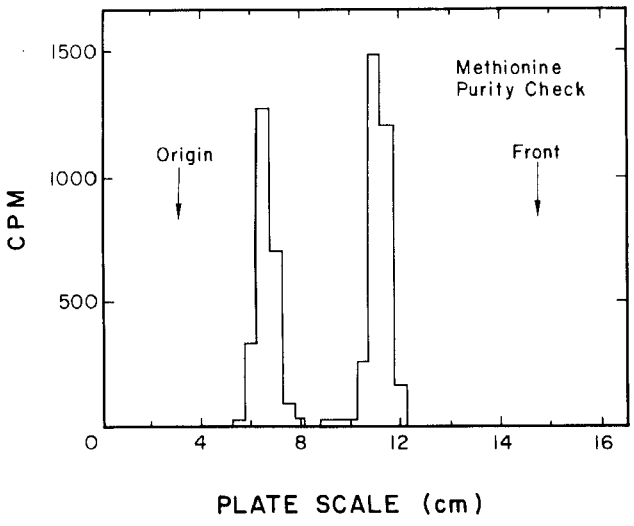

Figure 5 TLC separation of [Methyl-^{3}H]-L Methionine and principal impurity Methionine sulfoxide.

The first step in the careful procedures worked out for these separations is the reanalysis of the labeled reagents. An example is shown in Figure 5 of tritium labeled methionine used in kinetic studies of phospholipid methylation. In storage, the methionine (peak at 11.0 cm) is slowly oxidized to methionine sulfoxide (peak at 6.5 cm). The radiochromatogram imaging system required only 1 minute to acquire this data, and another minute of operator time at the terminal produced the numerical analysis that the sample is 43% inactive sulfoxide and 56% methionine. The total tritium activity on the plate is approximately 7×10^5 dpm.

The TLC separations of both neutral lipids and phospholipids are shown in Figure 6. The lipids were labeled with ^{14}C arachidonic acid. The TLC was done on Silica gel G plates using hexane/diethyl ether/acetic acid (60:40:1) to separate the neutral lipids (top) and chloroform/methanol/acetic acid/water (50:25:7:3) to separate the phospholipids (bottom). The success of the TLC techniques is complemented by the imaging system which gives a full display of the entire chromatogram. The user is able to verify instantly the success of his experiments by noting the symmetry of the peaks and the low background between peaks. When scraping and scintillation counting are used in similar circumstances, the tendancy is to scrape only one or two regions in the vicinity of each standard in order to reduce preparation and counting time. Often the resolution of the scraping procedure is inadequate to judge the extent of background smearing, peak asymmetry, and spurious peaks which might provide clues to problems in the experimental procedure.

The data shown in Figure 6 were obtained in a 5 minute analysis of each chromatogram. The neutral lipid separation

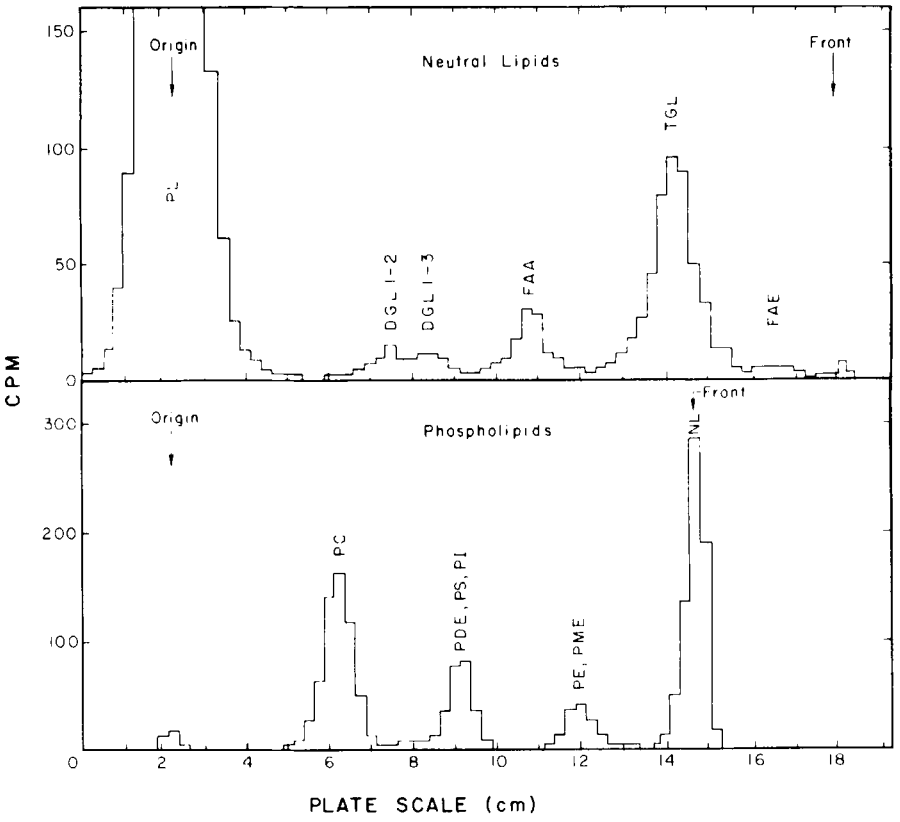

Figure 6 TLC separations of neutral lipids (top) and phospholipids (bottom) labeled with ^{14}C-Arachidonate.

(top) had a total of 5 x 10^4 dpm on the lane with approximately 81% of the activity in the phospholipids which remain at the origin. Of the remaining 19% labeled neutral lipid, 57% is in the triglyceride peak (TGL). The phospholipid separation (bottom) is from a different cell type and had a total of 2 x 10^4 dpm on the lane with 38% in the neutral lipids at the front. A total of 54% of the activity is in the three major phospholipid peaks.

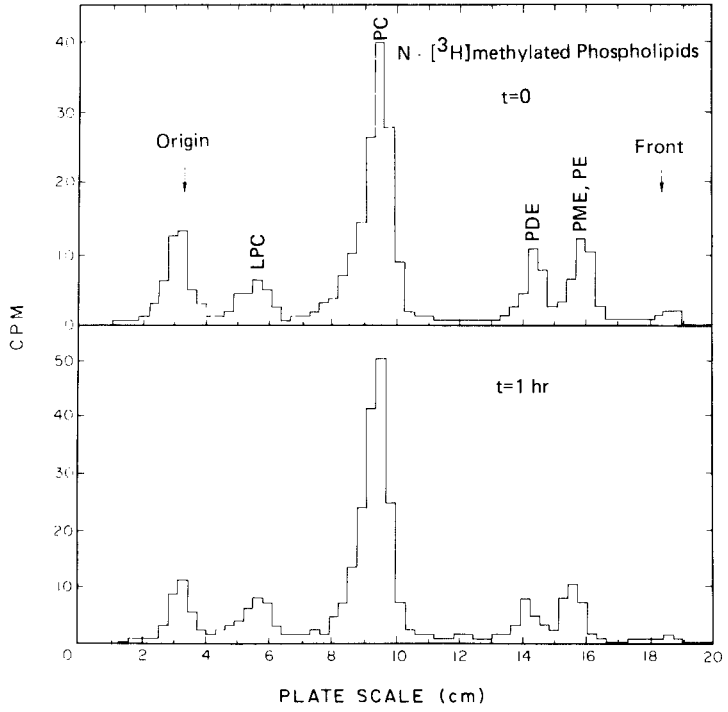

Figure 7 TLC separations of N-methylated phospholipids. The t=0 (top) data were obtained immediately after washing the cells to remove the [Methyl-^{3}H]-L Methionine labeling compound. The t=1 hour (bottom) data were obtained from cells harvested one hour after washing.

Another part of this work involves studies of the kinetics of phospholipid methylation. A technique using tritium labeled methionine has been worked out in which cell lipids can be labeled almost exclusively at the three methylation sites associated with the amine. The cells are washed so that all free label

is removed, and then the progress of methylation can be monitored at successive times by measuring the relative amounts of the monomethyl (PME), dimethyl (DME), and trimethyl (PC) phospholipids. An example of such a kinetics study is shown in Figure 7. Even though the unmethylated (PE) and monomethyl derivatives are not separated chromatographically, the unmethylated substance is not labeled and a complete separation of labeled methylated compounds is achieved. In this example the change after 1 hour is small but easily detected with the radiochromatogram imaging system. The PME and PDE derivatives continue to be methylated and are transferred into the PC peak. At the start (top) PME is 17% of the total and PDE is 14%. After 1 hour (bottom), PME is 13% and PDE is 10%. These results were obtained with a 15 minute analysis of each lane and a total tritium activity of approximately 6.5×10^4 dpm on each lane.

With the imaging system, quantitative results are reproducible to better than 1% from analysis to analysis so that these small changes can be measured reliably. Although scintillation counters give equally reproducible results, it is doubtful whether the entire process of plate scraping (or cutting of plastic backed plates) and sample elution can be performed reliably enough to measure such small changes.

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