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An Automated System for Sample Collection and Computer Analysis of Thin-Layer Radiochromatograms* †

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Summary

An automatic scraper for collecting minute zones of absorbent along thin-layer chromatographic lanes for subsequent radioisotopic or chemical measurements has now been combined with a direct data-acquisition system. Remotely located liquid-scintillation spectrometers transmit their data by means of a dedicated telephone circuit to an IBM 026 Card Punch in our data-processing center. Programs using an IBM 7090 computer and a Benson-Lehner Electroplotter have been developed, providing radiometric analysis of small (1- and 2-mm) and large TLC zones, and graphically producing exact-scaled replicas of the radioisotopic distribution along the chromatographic lanes from the punched-card data. The programmed analysis includes the conversion of counts per minute to disintegrations per minute, the computation of the radioisotopic distribution on chromatograms, recovery of activity from TLC plates, and determination of specific activity. Application of this system of analysis is illustrated for the separation of synthetic derivatives of ^{14}C-labeled alkyl glyceryl ethers and of products of a lipase-catalyzed reaction.

One of the salient features of thin-layer chromatography (TLC) is the speed by which complex mixtures of chemical classes of compounds can be resolved. There are many specific techniques

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† Work done under contract with the U.S. Atomic Energy Commission.

for quantitation of compounds isolated in this manner, and several reviews concerning chemical (*1,2*) and radioactive (*1–3*) measurements are available. However good these measuring techniques, all share the disadvantage of being very time-consuming.

Chemical mass and radioactivity of column-chromatographic effluents can be monitored quantitatively by several methods, yet no corresponding procedure has been reported for TLC. To fill this need we are developing a completely automated system for the quantitative analysis of chemical mass and radioisotopic distribution along a TLC lane. In our system, the chemical measurements are based on a liquid-charring spectrophotometric procedure (*4*) and the radioisotopic measurements on a liquid-scintillation assay procedure (*5*). TLC radioassay is facilitated by combining an automated zonal scraper and collector (*5*) with automated liquid-scintillation spectrometers, a direct data-transfer system, a card punch, a computer, and an electroplotter. This combined system leads to the elimination of time-consuming technical effort and tedious hand calculations in data analyses. In this report we shall illustrate the application of this automated radioassay system to the separation of the components of a model mixture containing ^{14}C-labeled alkyl glyceryl ethers and of products isolated during lipolysis of a ^{14}C-labeled triglyceride.

EXPERIMENTAL

Labeled Lipids

All 1-^{14}C-glyceryl alkyl monoethers (1- and 2-isomers of batyl alcohol) were synthesized in collaboration with Dr. Claude Piantadosi and Dr. Edward Oswald at the University of North Carolina. The molecular isomeric purity of these compounds was determined to be >98% by gas-liquid chromatography of their trifluoroacetate derivatives (*6*) on 5-ft × 1/8-in. glass columns containing 15% EGSS-X liquid phase. The radiopurity of the 1- and 2-isomers of batyl alcohol was determined by the TLC scans that are the subject of this report.

The radiopurity of a TLC-repurified tripalmitin-1-^{14}C (New England Nuclear Corp.) used in this study was >98% as determined by TLC zonal scans. The specific activity of the labeled tripalmitin used in the lipase reaction was adjusted to approximately 8000 dpm/mg triglyceride by the addition of unlabeled triolein purchased from the Hormel Institute.

Lipase Reaction

The pancreatic lipase (EC 3.1.13) was purchased from Nutritional Biochemicals, Inc. The lipolysis procedure, including the recovery of lipids, was carried out essentially according to Brockerhoff (*7*). Twenty milligrams of the diluted tripalmitin-1-^{14}C was used as substrate for the lipase.

TLC Separations

Silica Gel G mixed with water (1:2 w/v) was used to prepare 250-μ adsorbent layers (*8*) for all chromatographic separations. The 1- and 2-isomers of the glyceryl alkoxy monoethers were separated on layers impregnated with either boric acid (a 2.47% aqueous solution of H_3BO_3 was used to prepare the silica slurry) (*9*) or sodium arsenite (a 5.8% aqueous solution of $NaAsO_2$ in the slurry) (*10*).

The layers containing Silica Gel G alone and those containing arsenite ion were activated at 110°C for 30 min. The silica layers containing borate were activated at 110°C for 2 hr. All lipids were applied to the adsorbent layer in a 5-λ aliquot of chloroform removed from a stock solution. An identical aliquot, used as a recovery check, was pipetted into a scintillation vial and the chloroform was evaporated.

Three solvent systems, equilibrated in filter-paper-lined chambers, were used to develop the chromatograms; solvent system A, hexane/diethyl ether/acetic acid (80:20:1 v/v/v); solvent system B, hexane/diethyl ether/methanol/acetic acid (80:20:10:1 v/v/v/v); and solvent system B-2, chloroform/methanol (98:2 v/v).

After the chromatograms were developed in the solvent chambers, the separations were visualized by exposure of the chromatograms to iodine vapor. Nonradioactive standards of known purity were chromatographed on lanes adjacent to the labeled compounds so that all positions along the chromatographic lane were identifiable with iodine vapor. Samples were prepared for zonal scans by an automatic zonal scraper and collector (*5*). The scintillation solution used for radioassay is described in Table 1; the efficiency, 72% for ^{14}C, was essentially the same as that previously described for a dioxane/water scintillation solution (*11*). Neither the borate nor the arsenite ion exhibited any quenching properties in this scintillation system. The purpose of the water in the scintillation solution was to deactivate silica particles, thereby preventing

TABLE 1

Preparation of Scintillation Solution for Counting Scrapings of Silica

1. Mixture A	
BBOT[a]	4 g
Toluene	600 ml
Naphthalene	80 g
Methylcellosolve	400 ml
2. Add 1 ml glass-distilled water for each 30 ml of mixture A	

[a] 2,5-Bis-[5'-t-butylbenzoxazoly-(2')]-thiophene; fluorescence maximum: 435 mμ.

adsorption and reduction in counting efficiencies due to self-absorption (*11*).

Automated Transfer of Liquid-Scintillation Radioassay Data

The equipment required for the direct data-collection system includes a multiplexer,* a sending terminal,† a data phone (Western Electric 401A), a telephone line, a data receiver (Western Electric 401J), and a card punch (IBM 026 Model 5). Figure 1 is a schematic representation of this direct data-collection system. The sequence of the system's operation is as follows:

1. A batch of samples is loaded into the liquid-scintillation spectrometer. At this time, the operator keys certain indentification information concerning the batch into the sending terminal of the multiplexer (e.g., total number of samples in the batch, number of total-count samples to be used in calculating recoveries, experiment number, and the number of the machine into which the batch has been loaded). This information is transmitted to the card punch and one card is punched.

2. The liquid-scintillation spectrometer is then turned on, after which no further operator intervention is required. After the preset counting time for the first sample has elapsed (or a preset number of counts has been accumulated), the device emits a series of digits to the multiplexer. These digits comprise the

* Construction of multiplexer (an interface scanner) was done by our Technical Services Department under the supervision of Mr. H. Kimble of the Oak Ridge Associated Universities. A separate report describing the circuitry will be available in USAEC report ORINS-54.

† An IBM 1001 is compatible.

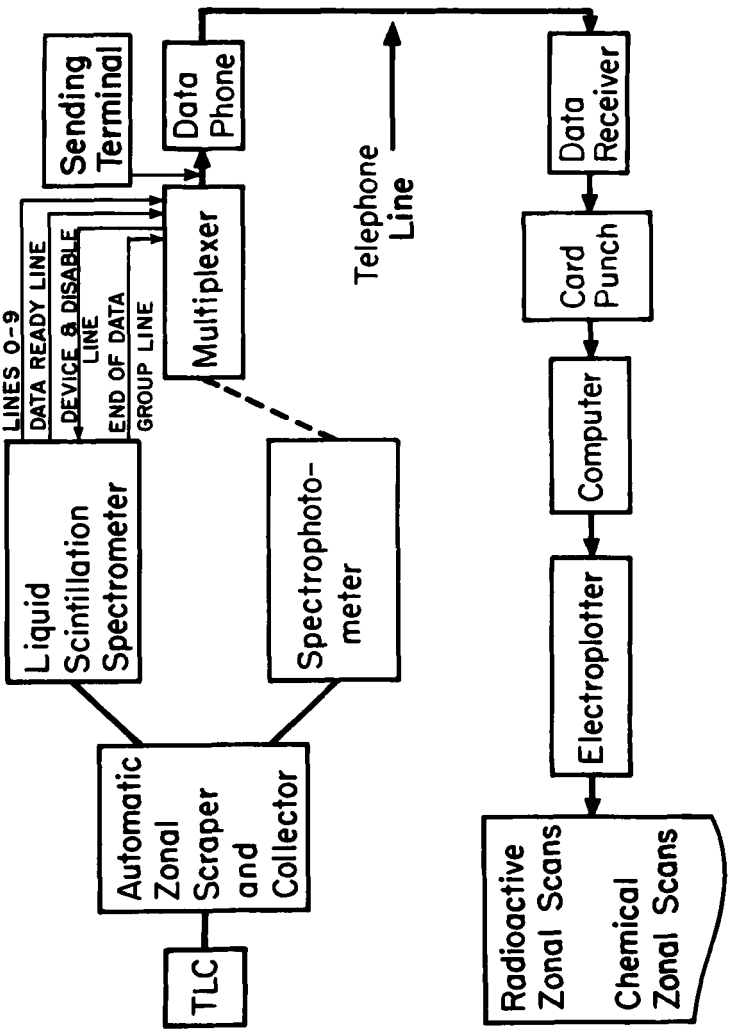

FIG. 1. Schematic diagram of the complete system for automated chemical and radiometric analysis of thin-layer chromatograms. Each input device is connected to the multiplexer by 13 circuit lines; ten digit lines, one line to indicate the presence of data, one line to disable the input device when another input device is transmitting data, and one line to indicate that a group of data has completed transmission. The automated spectrophotometric system is under development.

sample number within the batch, counting time, and counts accumulated from one or more isotopes. While data is being transmitted by the spectrometer, the multiplexer "locks out" the other input devices. The multiplexer contains the appropriate circuitry to temporarily disable the readout function of other spectrometers for the 2 to 3 sec required to complete data transmission from the input device which currently controls the telephone line. No data are lost, as any other spectrometers that have come to the "data-ready" condition during this time are allowed to transmit as soon as the line becomes available.

3. The multiplexer, after accepting the numbers describing the sample just counted, adds the identification number of the spectrometer. With minor modifications the multiplexer can service up to nine input devices simultaneously. The data phone serves to convert the data to a form suitable for transmission over the telephone line. After transmission, the data receiver converts the data to electrical impulses that drive the card punch, and then relinquishes the telephone line. After the punching of a data card, a blank card is automatically loaded by the card punch, which awaits the next readout.

4. The counting device automatically moves to the next sample. When all the machines have completed their batches, the card punch will contain an identification card describing each batch of samples and one data card for each sample of each batch. As the cards for each batch will be in sequence (although they may be interspersed with cards from other batches) and as each card has one digit identifying the machine from which it came, the cards can be sorted by machine number and placed with their identification card. The cards are now ready to be entered as data to the appropriate computer program. Figure 2 is a timing-sequence chart of the operations of the direct data-collection system for each group of data.

Several error-checking functions are built into the system. The data receiver checks the impulses arriving at the data-processing center to verify that they represent valid digits and to eliminate spurious data arising from electrical noise in the telephone line. Invalid codes will not operate the card punch. The card punch automatically counts the number of digits received in the readout cycle corresponding to each sample, and if the number is not correct, an error notch is punched into the card. (The readout cycle

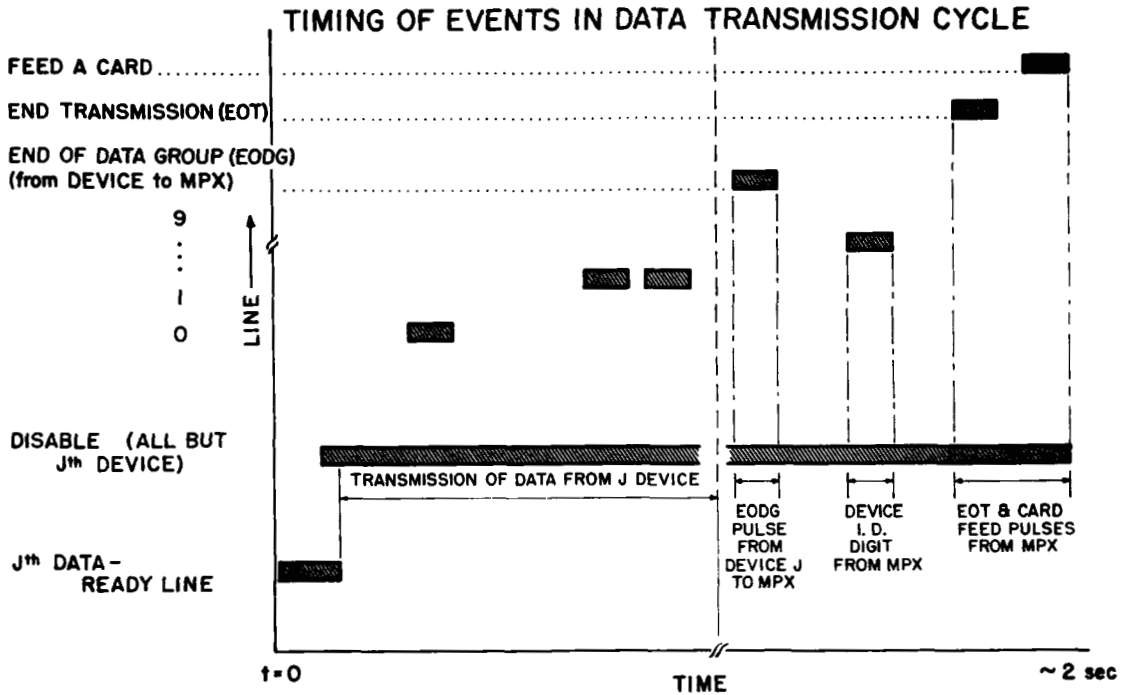

FIG. 2. Timing-sequence chart of the direct data-collection system. The shaded blocks represent the period for which each device is activated. The symbols J and MPX represent any one of a number of input devices and an abbreviation for multiplexer, respectively. The sequence as shown: data-ready-line pulsed; all input devices except device J disabled; data from device J, end of data pulse, input device number, and card-feed-pulse transmitted to card punch; all input devices enabled. Diagram prepared by Mr. Fred Brown, International Business Machines.

involves the same number of digits for every sample.) If a card with an error notch is found, the correct values can be obtained for that sample by inspection of the printed tape, which is generated at the transmitting site at the same time the data are read out. Also, the number of sample cards in each batch must correspond with the number punched into the batch-identification card at the time the machine was loaded.

Programmed Analysis and Graph Plotting

The data sets are utilized by a computer program written in Fortran IV for the IBM 7090, which performs the functions of correction of data, analysis, generation of reports, and plotting of graphs.

The data (counts per minute) are initially corrected by subtracting background counts and by correcting for the efficiency of the counting channel. If more than one isotope per plate is present, multiple isotope-correction factors are used to correct for counting-channel efficiencies and counting-channel overlays.

The data (disintegrations per minute) can be considered a function of TLC-plate position (centimeters), and the resulting graph consequently represents an exact-scaled replica of the radioisotopic distribution along the chromatographic lane. The area under the continuous curve can be found by numerical integration. As the area under the curve is directly proportional to the disintegrations per minute at any point, the numerical integration process is essentially one of summing the disintegrations per minute of the samples in the region of interest. With the use of this method in the programmed analyses, the total area under the continuous curve and the area under each peak of the curve are calculated. The start of a peak is defined as the point at which the activity becomes larger than two standard deviations above the background. The end of a peak is defined as either the point where the activity becomes less than two standard deviations above the background, or the point which is greater than two standard deviations of the background counts, but where the slope of the continuous curve becomes positive after being negative. Using this method, multiple peaks which overlap can be distinguished and analyzed. Other programmed analyses include computation of the percentage of the total area under each peak of the curve, the re-

covery of activity from the TLC plate, the specific activity, the mean, and the standard deviation of all samples.

An analysis report is generated for each set of data which represents a TLC plate. It includes a computer-generated listing of the original data, the corrected data, the total area under the continuous curve, the area under each peak of the curve, the percentage of the total area represented by each peak of the curve, the recovery of activity, the specific activity, the arithmetic mean, and the standard deviation of all sample data. It also includes a graph generated by the Benson-Lehner Electroplotter of data points (disintegrations per minute) as a function of plate position (in centimeters). The peaks are numbered in consecutive order on the graph, allowing reference to the printed analysis report for a particular peak. The comparison of the graph, scaled exactly to the TLC-plate length, with the actual plate furnishes a visual display of the amount of activity represented by any area on the TLC plate. This visual display is recorded with an MP-3 Polaroid Land camera. The 4×5 glossy prints can be used directly for publication or filed for future reference.

RESULTS AND DISCUSSION

The development of an automated zonal scraper and sample collector eliminated much of the technical effort necessary to obtain 1-, 2-, and 5-mm zones from TLC plates. A large portion of time was required for analyzing and plotting the data obtained from the large number of samples required for zonal-scan analyses. The direct data-collection system, computer analysis, and computer plotting described in this report now eliminate the use of technicians' time for routine data manipulation previously required in zonal-scan analysis. An alternate system that can be substituted for the multiplexer-phone transmission of data to the IBM 026 card punch is to install an adapter* on each scintillation spectrometer that is used to drive a standard IBM card punch. Either system used in conjunction with the automatic zonal scraper and collector eliminates all manual manipulation of quantitative radiometric TLC

* Model 540 card punch adapter available from Packard Instrument Co., Inc., Box 428, La Grange, Illinois.

procedures beyond the stage of chromatographic development, and it reduces human error and improves accuracy. Furthermore, the zonal scans obtained can be directly photographed for publication, eliminating the need for the services of an artist.

The total system is demonstrated here by the TLC separations of glyceryl ethers and their corresponding esters in solvent system A (Fig. 3). The ether bond, being less polar than the ester bond, migrates to the higher R_f. The resolution and sensitivity obtainable with 2-mm zonal analysis is illustrated in Fig. 3 for dipalmityl batyl alcohol-1-^{14}C, tripalmitin-1-^{14}C, palmitic acid-1-^{14}C, dipalmitin-1-^{14}C, and batyl alcohol-1-^{14}C (a peak height of 50 dpm for

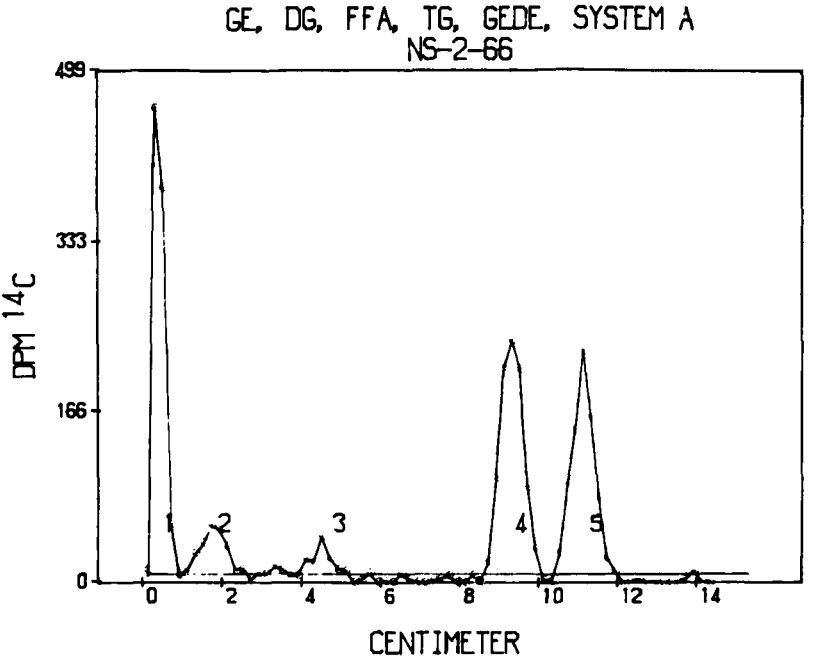

FIG. 3. A ^{14}C-zonal scan of a model mixture of labeled lipid components. Peak identification: 1, batyl-1-^{14}C alcohol (29.7%); 2, dipalmitin-1-^{14}C (7.9%); 3, palmitic acid-1-^{14}C (3.59%); 4, tripalmitin-1-^{14}C (29.0%); 5, dipalmityl chimyl alcohol-1-^{14}C (25.1%). The narrow line above the abscissa represents a calculated base line of two standard deviations above background. The illustration has been photographed untouched from the original electroplotter plot. Chromatographic development: solvent system A.

the palmitic acid area is easily detectable), and in Fig. 4 for the 1- and 2-isomers of batyl alcohol-1-^{14}C on borate- and arsenite-impregnated layers. The 1-isomer migrates at the lower R_f on the borate-impregnated layers, whereas the 1-isomer migrates at the higher R_f on the arsenite-impregnated layers. Arsenite and borate ions form complexes with the adjacent hydroxy groups (positions 2 and 3) of the glycerol moiety of the ether. The borate complex is more polar than the arsenite complex for reasons discussed elsewhere (*12*). The composition of a mixture of the 1- and 2-isomers as determined by calculation of the areas under peaks 1 and 2 obtained with borate and arsenite layers (Fig. 4) was essentially the same as the known percentage composition of the mixture when corrected for impurities as determined. by scans of the single compounds. Zonal scans can be used to determine quantitatively cross contamination in lipid pairs that trail or are difficult to resolve by the addition of high-specific-activity spikes to mixtures being chromatographed.

Perhaps the most significant aspect of the zonal-scan method is its ability to detect low-counting short-lived intermediates and products in biological systems. We have chosen the monitoring of a lipolytic reaction (Fig. 5) to illustrate the usefulness of zonal scans in a biological system. Pancreatic lipase is known to attack the 1 and 3 positions of triglycerides. The products at an early stage of a lipase-catalyzed reaction with tripalmitin would be expected to be tripalmitin, 1,2-dipalmitin, 2,3-dipalmitin, 2-monopalmitin (and its isomer), and palmitic acid. These ^{14}C-labeled products were, in fact, detected in the zonal scans we prepared on extracts of the products of the lipolytic reaction. Figure 4 vividly demonstrates that zonal scans are sensitive enough to detect even the limited quantities of diglycerides formed during the early stages of the reaction.

Determinations of specific activities are readily computed in the program analysis of the scan by including in the data to be analyzed by the computer program information of mass units obtained by photodensitometric (*13*), spectrophometric (*4*), or gravimetric analyses. A spectrophotometric system is being designed that is comparable to the zonal-scan liquid-scintillation analysis system described in this report, with automated input of pulses into the multiplexer as depicted in Fig. 1. The spectro-

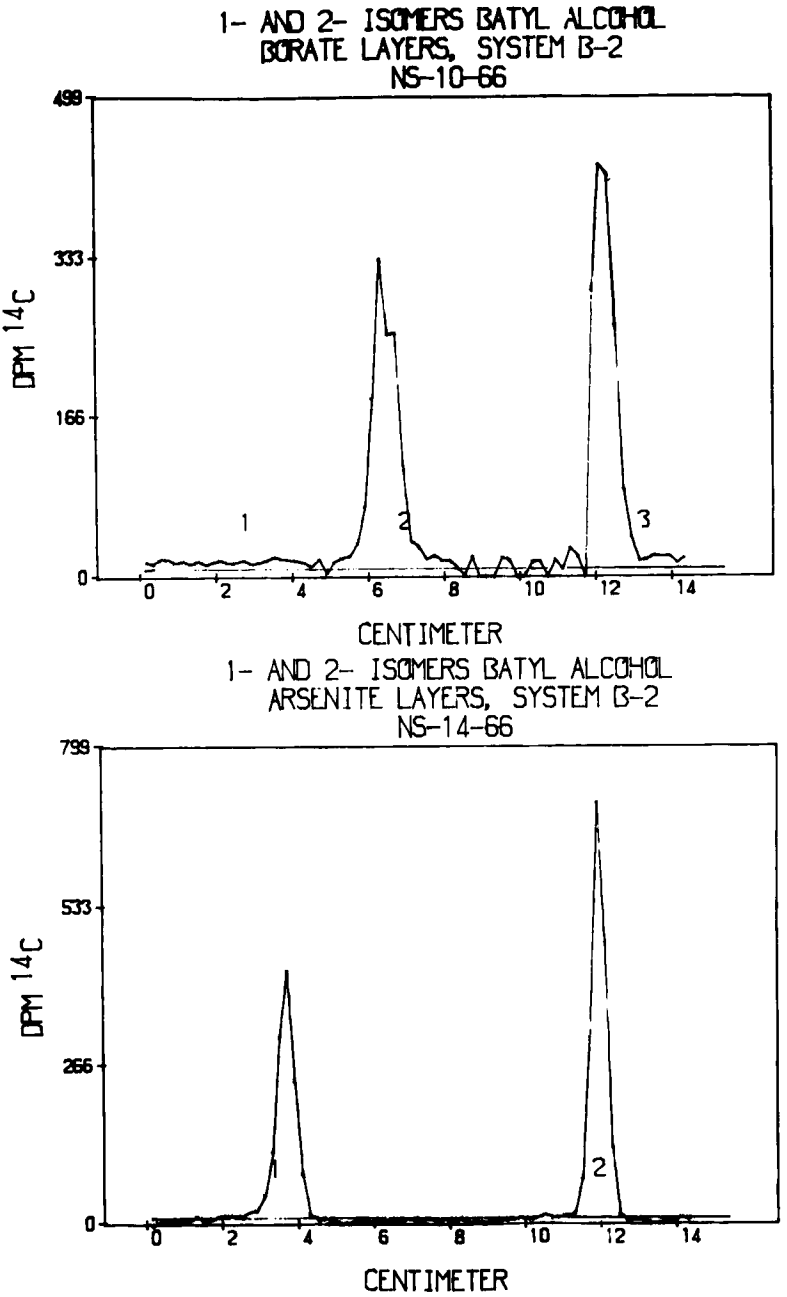

FIG. 4. A ^{14}C Zonal scan of 1- and 2-isomers of batyl alcohol-1-^{14}C separated on Silica Gel G layers impregnated with borate ion (upper) or arsenite ion (lower). The peak (1) at the lower R_f on the borate plate is the 1-isomer (50.0%), and the peak (2) at the higher R_f is the 2-isomer (45.4%). The peak (1) at the lower R_f on the arsenite plate is the 2-isomer (40.3%), and the peak (2) at the higher R_f is the 1-isomer (55.4%). The known percentage composition of the mixture was 52.3 for the 1-isomer and 47.2 for the 2-isomer. Chromatographic development: solvent system B-2.

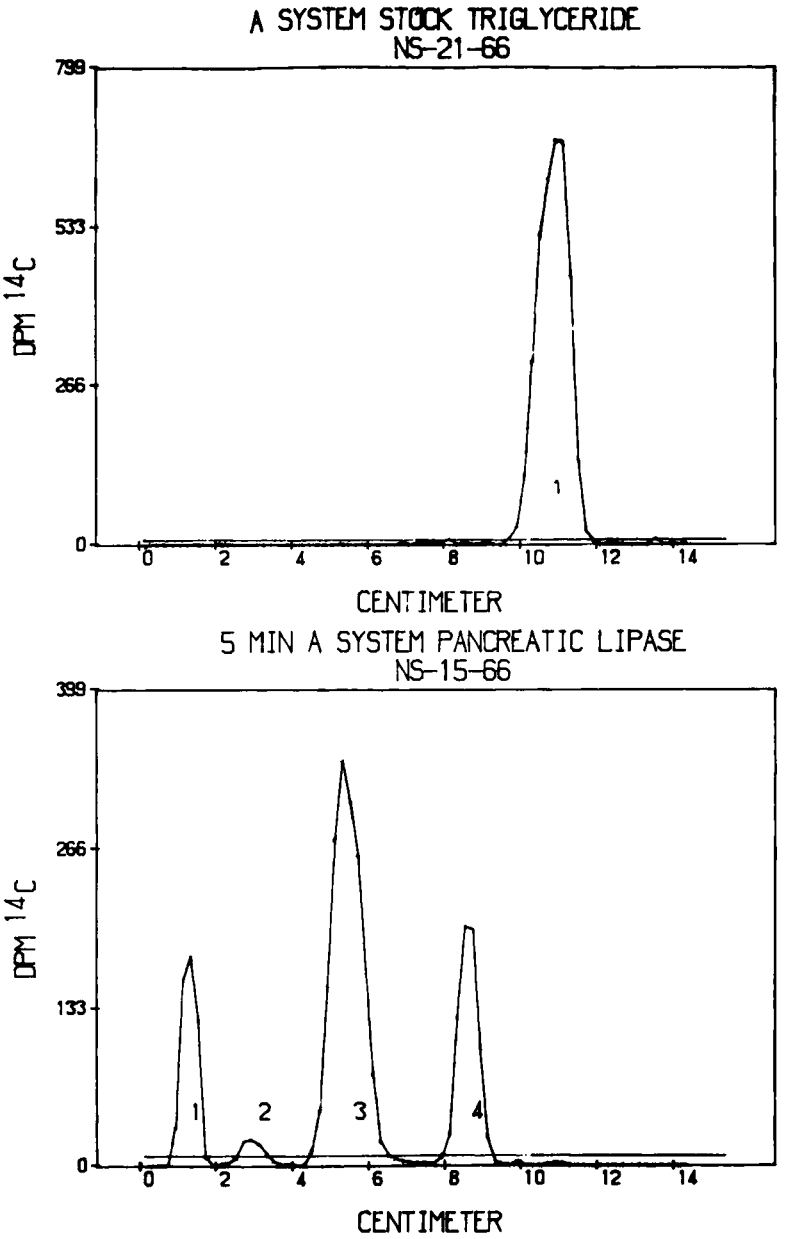

FIG. 5. ^{14}C Zonal scans of a lipase-catalyzed reaction with ^{14}C-carboxy-labeled tripalmitin. The upper scan shows the purity of the substrate before lipase attack. The lower scan shows the products obtained after a 5-min incubation at 37° were: 1, monopalmitin; 2, dipalmitin; 3, palmitic acid; 4, tripalmitin. Chromatographic development: Separations in upper scans were made in solvent system A, and separations in lower scans were made in solvent system B.

photometric system will provide analysis of curves from the zonal scans in a manner similar to that used for the radioassay system. The combined automated system for chemical and radioassay of zonal scans will provide the ultimate in quantitative measurements obtainable in TLC.

Acknowledgments

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