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HIGHLY SENSITIVE ON-LINE RADIOASSAY OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC EFFLUENTS

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SUMMARY

We previously described a procedure for highly sensitive radiochromatography that is based on depositing fractions of the high-performance liquid chromatographic effluent on non-wetting film, concentrating them, transferring them to filter paper, generating autoradiographs of the paper on photographic film, and quantifying by densitometry. The work reported here included design of: a modified procedure for assaying non-polar compounds in which the transfer solvent was changed and silica gel thin-layer chromatography plates were substituted for the filter paper; a method for detecting tritium by adding scintillator to the filter paper before autoradiography and for distinguishing ^{14}C and ^{3}H by comparing autoradiographs with and without added scintillator. Then, to decrease the time for assaying the fractions, we tested two different kinds of gas radiation detectors, using the equivalent of an array of detectors working simultaneously. Since these detectors permitted a number of fractions to be assayed at the same time, the time each was assayed could be extended and/or the same number of fractions could be assayed more rapidly.

Following the successful application of these detector arrays, we designed a system concept for using an array of detectors for increasing the sensitivity of on-line, flow-through detection. Fractions of the effluent are passed through a series of detectors sequentially. The counting rates of each counter for each time interval are recorded. The results are summed electronically to reflect the position of each sample in the array. The same resolution is achieved as in flow-through detection, with comparable convenience but much enhanced sensitivity, since each fraction is assayed for a much longer time.

INTRODUCTION

Because of the random nature of radioactive decay, the precision of any radioassay is a function of the number of radioactive events that contribute to the measurement. For any prescribed precision, the sample must therefore remain in the active volume of the detection system for the time needed for the required number of disintegrations to be detected. In radiochromatography the precision is related to the time each portion of the chromatogram or chromatographic effluent remains in the active volume of the detector. With a flow-through detector, time in the detector must

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be limited if the resolution of the chromatography is to be preserved. This limits the sensitivity of radiochromatography in on-line high-performance liquid chromatography (HPLC). If less radioactivity is present than can be measured with acceptable precision in up to *ca.* 20 s, flow-through detection is generally not useful. The usual alternative is to fractionate the effluent for subsequent radioassay. Here again, preserving the resolution of the chromatography requires that separate fractions be collected at least every 20 s. Many fractions must then be assayed, and this can occupy a counting device for many hours. For this reason, most workers find flow-through detectors more convenient to use and reserve fraction-collecting for those assays involving very low levels of radioactivity. Even then, most people tend either to limit the time each fraction is counted or to sacrifice resolution for sensitivity by collecting fewer fractions.

In work described previously, we attempted to address this problem by devising methods for making fraction collection more convenient and assaying their radioactivity more sensitive[1]. We found that we could deposit equal, small portions of HPLC effluents on non-wetting fluorocarbon film, concentrate each fraction to near dryness, transfer the fractions to filter paper where they form uniform 2-mm-diameter circular spots, and then assay all the spots for radioactivity simultaneously by autoradiography on photographic film followed by densitometry. ^{14}C at levels of > 50 dpm per fraction was measurable by an overnight exposure. We adapted an automatic fraction collector for this technology and could collect 80 equal fractions, reproducing in the record of the radioactive analysis the UV absorbance or fluorescence elution patterns[2].

This procedure proved less suitable for assay of water-insoluble compounds because they did not procedure spots on filter paper with uniform distribution of radioactivity, and therefore densitometry of the radioautograph was less quantitative.

In the first part of the work described here, we modified the procedure to make it more suitable for non-polar compounds by changing the transfer solvent and substituting silica gel thin-layer chromatography (TLC) plates for the filter paper. We detected ^{3}H by adding scintillator to the filter paper before the autoradiography and could distinguish ^{14}C and ^{3}H by comparing autoradiograms with and without added scintillator. Then, to decrease the time for assaying the fractions, we tested two diffeent kinds of gas radiation detectors, using the equivalent of an array of detectors working simultaneously. We considered the photographic film used in autoradiography to be similar in essence to a large number of radiation detectors operating simultaneously. Since the array of detectors permitted a number of fractions to be assayed at the same time, the time each was assayed could be extended and/or the same number of fractions could be assayed more rapidly.

Finally, we designed a system concept for using an array of detectors for increasing the sensitivity of on-line, flow-through detection.

EXPERIMENTAL

Material

Prostaglandin standards were purchased from Biomol (Plymouth Meeting, PA, U.S.A.), arachidonic acid from Sigma (St. Louis, MO, U.S.A.), [^{3}H]prostaglandins from Amersham International (Arlington Heights, IL, U.S.A.) and [^{14}C]arachidonic

acid and En^{3}hance Spray from New England Nuclear (Boston, MA, U.S.A.). Plastic-backed silica gel 60 TLC sheets (20 × 20 cm, 0.2 mm layer thickness) were bought from E. Merck (Darmstadt, F.R.G.).

Equipment

HPLC of prostaglandin precursors and metabolites was performed with a Constametric III pump (LDC, Riviera Beach, FL, U.S.A.), a Model 85 LC detector (Perkin-Elmer, Norwalk, CT, U.S.A.) with a 2-μl flow cell and a 7120 injector valve (Rheodyne, Cotati, CA, U.S.A.) with a solvent by-pass loop. The column, 30 × 4.6 mm I.D., packed with 3-μm C_{18}-coated silica was bought from Perkin-Elmer. Control of the step gradient of the mobile phase from 32 to 75% acetonitrile in 0.002 *M* hydrochloric acid was achieved by the Model 420 gradient microprocessor (Altex, Berkeley, CA, U.S.A.). The gradient was held at 32% for the first 6 min and at 75% for the next 12 min. The flow-rate was at 0.5 to 1 ml/min. The absorbance of the column effluent was monitored at 210 nm; the amplified detector signals were read out on a Model 4416 data system (Nelson Analytical, Cupertino, CA, U.S.A.).

Fraction collection

The fraction collector is based on the fluorocarbon film technology used to concentrate samples and to apply them to TLC plates by Fenimore and Davis[3]. For these experiments the fractionator was adapted for use with a Gilson fraction collector. The tubes were replaced by a hollow rectangular aluminum plate, 20.8 × 6.4 cm, with 80 wells (16 columns and 5 rows), 0.9 mm deep, milled on the upper surface, each supplied with a 1-mm hole at the bottom. The top surface of the plate was polished to mirror finish. The plate was constructed by cementing the 3-mm plate, machined as described, to a 1-cm-wide silicone rubber gasket, 1 mm thick, around its perimeter, which in turn was cemented to a second aluminum plate, 1.5 mm thick, provided with fittings for applying vacuum or pressure to the space between the plates formed by the gasket. A 20 × 3.75 cm, 100-W strip heater (Rama Industrial Heater, San Jacinto, CA, U.S.A.) was fixed to the bottom plate. Fluorocarbon-coated sample wells were formed by placing a sheet of 0.025-mm-thick fluorocarbon film over the plate and applying vacuum to the cavity. The column effluent was delivered by the fraction collector arm to each well through 15 cm × 0.002 in. I.D. stainless-steel capillary tube, fitted with a 2-mm long 1 PTFE sleeve at the end. From 20 to 80 μl of HPLC effluent were deposited in each well. In assays of amino acids, the final volume of each fraction after evaporation was limited by including 1% ethylene glycol in the mobile phase. For assays of lipids, 25 μl of 0.6% oleic acid in ethanol was added to each well. After evaporation, the residues in the oleic acid carrier were transferred to a plastic-backed silica gel sheet, 200 × 70 × 0.2 mm, by replacing the vacuum with 10 p.s.i. nitrogen pressure.

An autoradiogram was prepared by clamping the silica gel sheet against X-ray film in a Wolf X-ray cassette, 20 × 30 cm, and placing it in a freezer at −75°C. For assaying tritiated compounds, the TLC sheet was sprayed with En^{3}hance Spray scintillator solution before exposing it to the film. The film was then developed, and densitometry was performed with a Model CS-910 TLC (Shimadzu, Columbia, MD, U.S.A.). The scanner record was processed on an HP-3390 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Position-sensitive proportional counter

The efficacy of scanning the collected fractions with a position-sensitive proportional counter was assessed by scanning the radiochromatogram of amino acids that had previously been autoradiographed. The scanner was a Model RS Radio TLC scanner (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.).

The counter itself is a 20-cm-long, windowless proportional counter, used with "P-10" gas, and connected to a 1024-channel multichannel analyzer. With its associated aperture, it offers 1- to 2-mm resolution of ^{14}C. For assaying TLC plates, the counter moves over the plate stepwise in small increments at predetermined counting intervals. When the β-particle interacts with the gas in the counter, an electric discharge occurs in the volume where the interaction occurs. This induces a charge in an inductive delay line close to the wire. The charge travels as a pulse to the ends of this delay line at measurable speed. The position where the event occurs is sensed electronically by the time of arrival of the pulse at each end of the delay line. For the scan of the filter paper described here, the wire was set to count for 5 min at each of the 16 rows of spots on the filter paper, advancing 12 mm to the next row of spots automatically at the end of that time. The output was recorded in several ways, including the graphic representation of the counting rate across the row, as well as the total counts recorded at each location.

A second, similar experiment was performed on the same radiochromatogram of amino acids with the Vanguard Model 2001 1-D/2-D TLC plate scanner (Digital Diagnostic, Hamden, CT, U.S.A.). This scanner uses an array of ten 1 × 2.5 cm windowless flow-counters, operated in the Geiger mode with "Q-gas". The counters are fitted with metal collimators to achieve the required resolution in TLC scanning. To scan the filter paper with spots 0.5 in. apart, a collimator offering 0.5-cm diameter apertures was used. The counts along 10 columns of 5 spots were recorded for 10 min each.

Procedure

Metabolism of arachidonic acid by human platelets. A platelet concentrate (*ca.* 10 ×) was centrifuged at 250 *g* for 15 min to remove residual red cells. The supernatant was recentrifuged at 1800 *g* for 15 min. The resulting platelet pellet was washed with 10 ml of Tris buffer (pH 7.0–7.2), containing 1.5 m*M* EDTA. The washed platelets were then resuspended in 2 ml of Tris buffer without EDTA. This platelet preparation was incubated with $9 \cdot 10^5$ dpm of [^{14}C]arachidonic acid for 20 min at 37°C. The incubation was stopped by the addition of 0.5 ml glacial acetic acid, sufficient to bring the pH to 3 and the incubate was extracted twice with 3-ml aliquots of ethyl acetate.

The extract was evaporated to dryness on the contact spotter, and the residue was redissolved in 200 μl of ethanol. A 6-μl volume of the extract, containing approximately 24 000 dpm was injected into the HPLC column.

RESULTS

On a column packed with 3-μm particles, the prostaglandin metabolites could be separated in 15 min with a resolution comparable to that achieved much more slowly on columns containing larger particles. The effluent could be delivered to the fraction collector in the short length of microbore tubing described with minimal loss of

resolution, as judged by the peak widths on the record of the radioassay and that of the UV absorbance detector. In the analysis of amino acids, glycerol was added to the mobile phase to limit the volume of fractions after evaporation. Glycerol kept both the amino acids and the residual buffer salts in solution and facilitated delivery of the condensed fractions to the filter paper matrix. The autoradiograms were circular spots of apparently uniform density. When the same procedure was tried for transferring the residues from the prostaglandin analysis, the autoradiographic images were not uniform in density: they contained what appeared to correspond to particles of precipitate on the filter paper. When oleic acid was substituted for the glycerol, on the basis that it was liquid, had limited volatility, and should dissolve prostaglandins, the spots were uniform in density but more diffused than with the glycerol.

Substitution of the plastic-backed silica TLC sheet for the paper in radioassay of [^{14}C]arachidonic acid with oleic acid on the TLC sheet, yielded uniformly dense spots on the X-ray film that reproduced the elution pattern obtained with the UV detector (Fig. 1).

With the new spotting technique, quantitative radioassays of [^{3}H]thromboxane B_2 and [^{14}C]arachidonic acid were obtained. The radioactivity was proportional to the density of the spot on the exposed film at both high and low activity. The exposure was 7 h for 2000–8000, and 184 and 92 h for 20–120 dpm [^{3}H]thromboxane and [^{14}C]arachidonic acid, respectively.

Autoradiograms of [^{3}H]prostaglandins and [^{14}C]eicosanoids showed that ^{3}H is virtually undetected unless the paper or TLC plate was sprayed with scintillator before exposing it to the film. Chromatograms of standards yielded good separations (Figs. 2 and 3). In analyses of the products of arachidonic acid metabolism there was some loss of resolution, but this was difficult to quantify because of the relatively poor resolution of the many apparent metabolites present (Figs. 4 and 5). [^{14}C]Arachidonic acid was metabolized to several radioactive products, including thromboxane B_2, prostaglandin $F_{2\alpha}$ and hydroxyeicosatetraenoic acids.

Scanning with the two kinds of gas detectors gave similar results. The scan of fractions from an analysis of three amino acids, containing 39 000 dpm, approximately

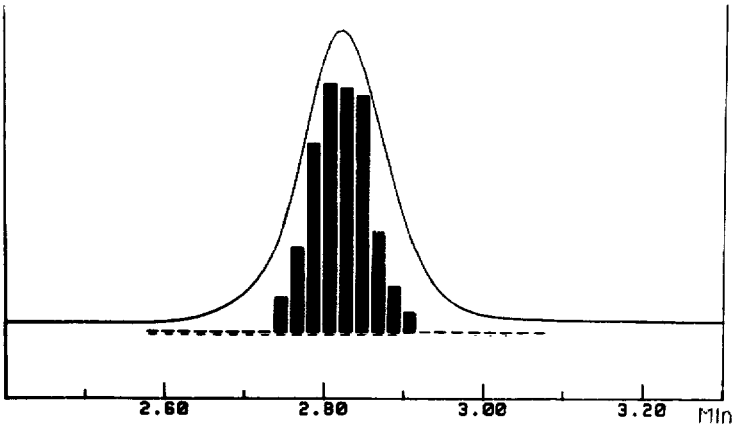

Fig. 1. Scans of the radioautographs (black bars) superimposed on the UV chromatogram of arachidonic acid.

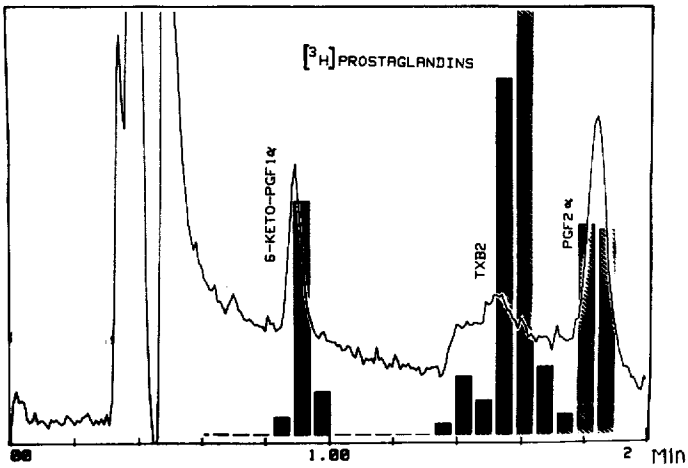

Fig. 2. Scans of the radioautographs superimposed on the UV chromatogram of the prostaglandins standards. Conditions: Perkin-Elmer reversed-phase C_{18} column 3 μm, 3 cm × 4.6 mm I.D. Mobile phase gradient from 32 to 75% acetonitrile in 0.002 *M* hydrochloric acid was controlled by the Altex Model 420 gradient microprocessor. The gradient was held at 32% for the first 6 min, and at 75% for the next 12 min. Flow-rate of the effluent was at 0.5 to 1 ml/min. The UV absorbance of the column effluent was monitored at 210 nm. 6-KETO-PGF 1α = 6-ketoprostaglandin $F_{1\alpha}$; PGF 2α = prostaglandin $F_{2\alpha}$; TXB2 = thromboxane B_2.

equally distributed among the three, gave a usable autoradiogram after 6 h of exposure to the film (Fig. 6). The maximum counting rate of an individual fraction was 1500 dpm. Counting the same fractions with the two gas counting systems gave gave usable results in less than 1 h of counting, even though the counting routines were not optimized.

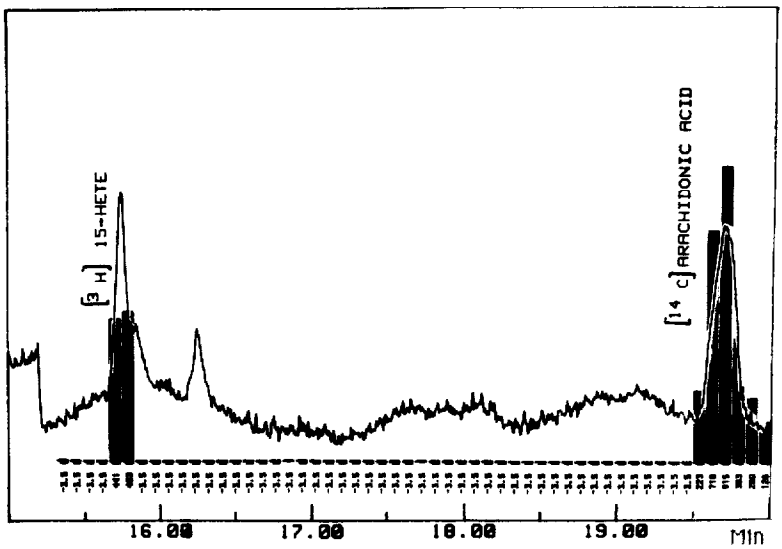

Fig. 3. Scans of the radioautographs superimposed on the UV chromatogram of the hydroxyeicosatetraenoic acid (15-HETE) and arachidonic acid. Conditions as in Fig. 2.

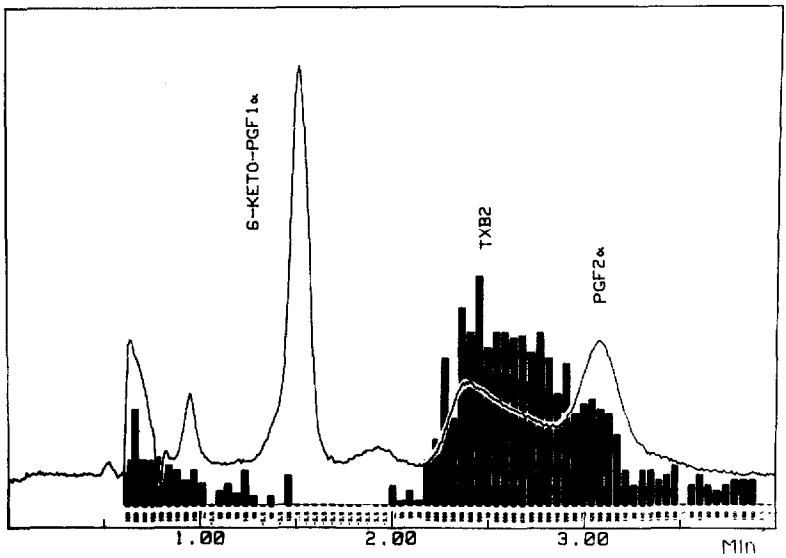

Fig. 4. Scans of radioautographs superimposed on the UV chromatogram of prostaglandin metabolites of platelets of arachidonic acid. Conditions as in Fig. 2.

The Geiger counters were 20 mm apart, and the fractions 12.7 mm (0.5 in.) apart, requiring that the counters scan the paper in order to cover the radioactive samples, thus spending at least part of the time away from the radioactivity. Allowing the counters to count for 10 min over each column of spots, with scanning, gave results that approximated those of the autoradiogram. The efficiency of the position-sensitive

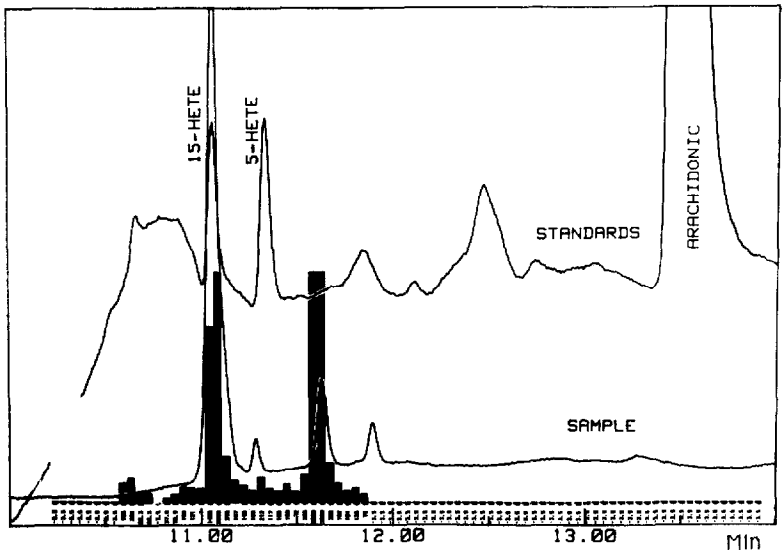

Fig. 5. Scans of radioautographs superimposed on the UV chromatogram of HETE metabolites of platelets of arachidonic acid. Conditions as in Fig. 2.

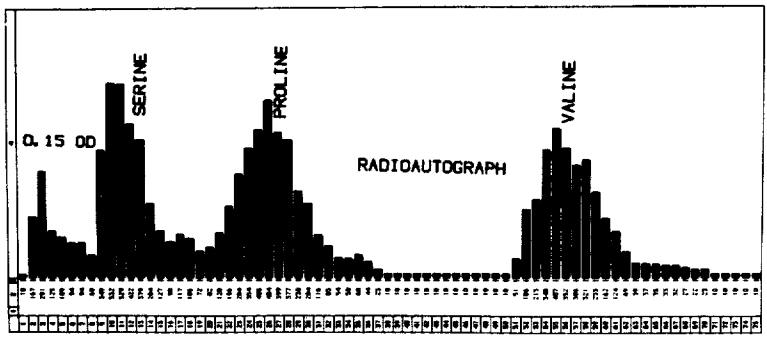

Fig. 6. Standard curves of scans of 10–120 dpm each of [^{3}H]thromboxane and [^{14}C]arachidonic acid.

proportional counter was similar. Here again, it was likely that the automatic scanner was not always directly over the radioactive spots during the counting intervals. With 5 min over each row of spots (rows were scanned, rather than columns, because of the size of the paper and the scanner), similar records were obtained.

DISCUSSION

It was apparent that the mechanical capabilities of the two scanners were not being fully utilized in scanning the evenly spaced, small, well-separated spots. Appreciably greater sensitivity was obviously available by coordinating the separation of the spots with the counter spacing or the settings of the position of the proportional counter.

All these procedures involved simultaneous radioassay by an array of detectors, which, for this purpose, includes both the position sensitive proportional counter and the photographic film, as well as the Geiger counter array. Upon noting the high sensitivity of off-line detection with this approach, we designed a method for extending it to achieving more sensitive on-line radiochromatography with approximately the same convenience as flow-through detection. One possible embodiment, employing a Geiger counter array, is as follows: fractions of the column effluent are deposited in pockets formed on a long strip of fluorocarbon film that are moved step-wise past the column exit. At the first, second and third steps in the movement of the film, the solvents are evaporated to dryness. The pockets containing the condensed radioactivity then travel past the array of detectors so that each spot is counted by each detector sequentially. The counts recorded by each detector during each time interval are stored in memory. The counting rates are summed continuously by a formula that takes into account the position of each sample passing through the array: during the third counting interval, for example, the output of the third detector is summed in the memory bank corresponding to the first sample, etc. This approach gives defined resolution, the same resolution as flow-through detection, but each sample is counted many times as long, (*e.g.* ten times with the ten-detector array) with corresponding increases in sensitivity and precision.

It is contemplated that either one of the gas detector systems described here could be used. The same approach could also conceivably be used with internal liquid

or solid scintillation counting, using a similar succession or array of detectors. For example, liquid scintillation fluid can be added to the HPLC column effluent and the combined stream then led to a long length of PTFE tubing. Baba and co-workers[4,5] described then delivering a stream of this kind through five scintillation counters in series, achieving the predicted increase in precision from summing the radioactivity. They also noted, however, that there was an appreciable increase in peak width from the first to the fifth detector, caused presumably by mixing of one part of the effluent with the next. This effect is particularly troublesome with detergent-containing scintillation fluids which tend to wet even PTFE tubing. Mixing can be reduced somewhat by segmenting the stream into distinct fractions with an immiscible liquid. Each segment could then be passed stepwise, every 15 s for example, through a series of light detectors where the scintillations of each during each counting interval are summed as described above.

Liquid scintillation counting generally offers higher sensitivity, particularly for ^{3}H, than external counting with a gas detector, because of reduced self absorption of the β-particles. However, since photomultiplier detectors are currently much more expensive, the cost of an array of pairs of light detectors, arranged for coincidence detection, or a scintillation camera such as is used nuclear medicine, would be comparatively high.

The increase in sensitivity achievable by passing 15- or 20-s portions of the column effluent through a succession of detectors can be estimated from the increased time of counting: the counts recorded in ten detectors, if a fraction spends 15–20 s in front of each, would predictably be ten times the number recorded if the sample passes in front of a single detector of the same kind for the same time period. On the other hand, if the sensitivity of an array of ten external gas detectors is compared with that of a single flow-through scintillation counter, that accepts the same size fraction of the effluent (*e.g.* a 15-s portion) a smaller improvement would be predicted. From consideration of geometry alone, and neglecting self-absorption of the β-particles in the sample or the carrier, the efficiency of external gas detectors reaches a maximum of only 50%, compared to *ca.* 80% in internal liquid scintillation systems. A counter placed above the sample does not "see" the β-particles that are directed downward. Some improvement might be obtained by employing opposing detectors, one on each side of the carrier strip, arranging the spacing so that electric discharges in one do not affect the other. It would probably be more straightforward to use additional detectors to increase the number of steps in the array, increasing the sensitivity in direct proportion to their number, even though this would increase the time of completion of the assay.

Even though the procedure described involves fractionating the effluent, it accomplishes the primary objective of on-line detection: obtaining the result at close to the same time each portion of the effluent leaves the column. With the system described here, the counts in each memory bank can be portrayed continuously. The counts in a fraction can be examined within a very short interval after it leaves the column. However, additional data continues to accumulate at succeeding times, increasing the precision of the assay: as the tenth portion in a sequence enters the detection array, counts are still being recorded from the first nine, etc. The assay is complete only a few minutes after the analysis. In this regard, the radioassay is as on-line as detection that uses a post-column reaction that requires several minutes for completion. Unlike other

forms of on-line detection, the fractions remain available for repeated analysis off-line, when even greater sensitivity is needed.

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