COMPUTERIZED CONTOUR MAPPING APPLIED TO TWO-DIMENSIONAL RADIOCHROMATOGRAMS

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SUMMARY

A one- or two-dimensional radiochromatograph employing a new type of electron multiplier detector has been described earlier as a means of reducing the necessary detection time for chromatograms containing weak β-emitters as labels. As an alternative to the usually applied film detection method, our method has now been adapted to a system for digital read-out of the count numbers which are simultaneously punched out onto paper tape. These are obtained during standard integration intervals with the counter in fixed positions. These positions are set by an automatic stepped-motion program which enables the complete chromatogram to be covered with a set of equally spaced parallel profile scans of intensity. The point intensity grid is thus obtained on a paper tape and can then be used as input to a computer which is programmed to apply a two-dimensional smoothing to the set of recorded intensities. The resulting smoothed activity chart can then be reproduced in the form of a contour map, sufficiently detailed and with a one-to-one scale. An approximate evaluation of the individual spot activities is possible, as well as an accurate positioning of the spots in the chromatogram. This allows the recovery, after cutting out and elution, of the various labelled materials.

INTRODUCTION

No simple, fast, and inexpensive method for the recording of two-dimensional radiochromatograms is known to exist. So far, photographic film detection has been used almost exclusively, but with this technique it is difficult to obtain satisfactory quantitative values for the tracer activities, although a useful qualitative picture is easily obtained. An additional drawback is that for such weak β-emitters as tritium and radiocarbon the necessary exposure time may be considerable.

Many other solutions to the problem have been suggested; see for example the review article by POCCHIARI AND ROSSI[1]. All the detection methods discussed, both one- and two-dimensional, can be applied to ^{14}C and other radionuclides with higher β-energies, but for tritium most of these methods have a very low counting efficiency. This is also true for a development described by HARIHARAN et al.[2], which is effective in locating ^{14}C-labelled compounds in two dimensions. This development

is based largely upon two older papers[3,4]. In one[3], a GM counter which is scanned in parallel tracks over the two-dimensional chromatogram is used. In the other[4], an array of GM counters is placed along the track of one-dimensional chromatograms. By using thirty counters spaced at 1-cm intervals, an activity profile 30 cm long and with a resolution of about 1 cm can be obtained thirty times as fast as when one single-scan detector is used.

A new principle of detection in two dimensions has quite recently been applied in two commercially developed systems, one by Panax Equipment Ltd., Great Britain, called a spark chamber, and one by Baird Atomics Inc., U.S.A., called a β-camera. The first, a relatively cheap model, provides a photograph of the spark pattern in a thin, flat ionization chamber located closely above the chromatogram. The other, a rather expensive unit, provides various choices of photographic and electronic read-outs. This version is based on the simultaneous use of 1622 individual windowless gas-flow GM counters.

However, we feel strongly that there is, in the field, a lack of instrumentation sufficiently cheap for medium-sized laboratories which is capable of sensitive quantitative radiotracer measurements in two dimensions. Towards this end a step scanner for the measurement of both one- and two-dimensional distributions of radionuclides in paper or thin-layer chromatography has recently been constructed and tested[5]. Different versions of a miniaturized channel electron multiplier were applied as detectors. A description was given of the principle of operation for this type of detector, the vacuum scanner, as well as some possible ways of data
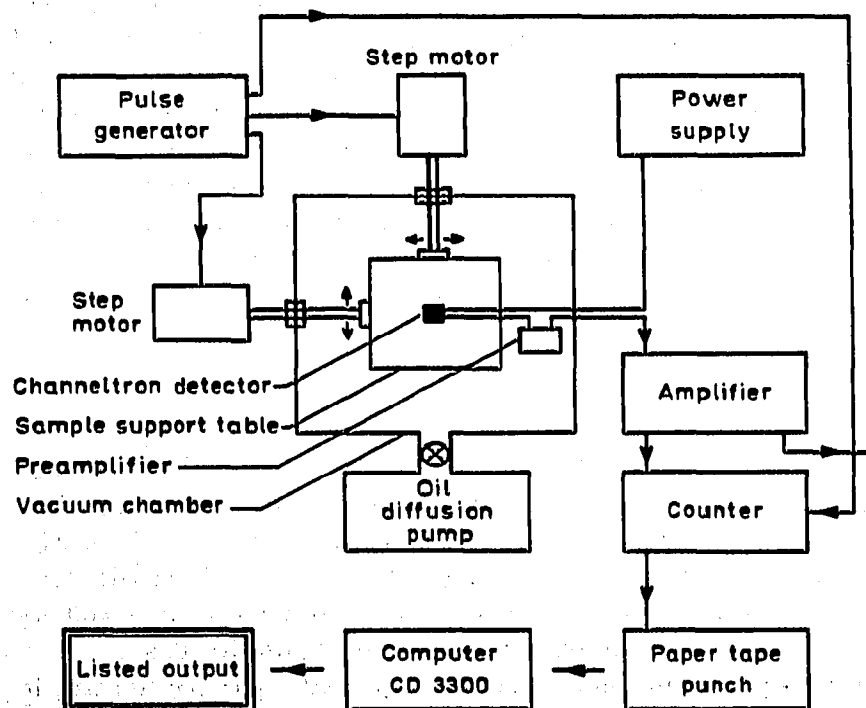

Fig. 1. Detector system shown schematically. Within the vacuum chamber the support table can be scanned in parallel scans and moved sideways. The detector signal is amplified and can be recorded, either as a ratemeter curve (single-scan operation)[5] or as a two-dimensional scaler output as a dot chart on the X–Y recorder,[6] (see text for comments on this way of operation. With the data logger described in this paper the detector pulses are emitted after amplification and fed to the counter.

presentation. The scanner and data logger used in the present work are shown schematically in Fig. 1.

In one dimension, the radiochromatogram strips may be scanned by a rate-meter recording of the variation of the count rate along the activity profile. A more useful type of display is achieved in the form of activity histograms, which can be obtained by the use of a special scaler connected to the detector. Our scaler counts for the pre-selected counting period and then at the end actuates the plotting of an X–Y recorder in which the Y-position is determined by a d.c. voltage proportional to the count number of the scaler. The scaler then is zeroed, the radiochromatogram table moved one step to the next position, and the detector starts counting again. The same pulse generator controls the step motor for the advancement of the chromatogram and plotting of the count number. Normally it is most suitable to obtain the activity profile in a 1:1 X-axis scale.

The results presented[5] were, however, somewhat preliminary, as the methods of detection and data presentation were tested to a satisfactory degree only in the case of one-dimensional scanning. The procedure for radiochromatograms where separation has been performed in two perpendicular directions is far more complicated. Our scanner is, however, suitable for this type of scanning. The whole chromatogram (20 × 20 cm) will, in the "auto" mode, be covered by parallel scans, the separation of which may be pre-selected. The difficulty lies in deciding upon the most useful way of presenting the activity readings.

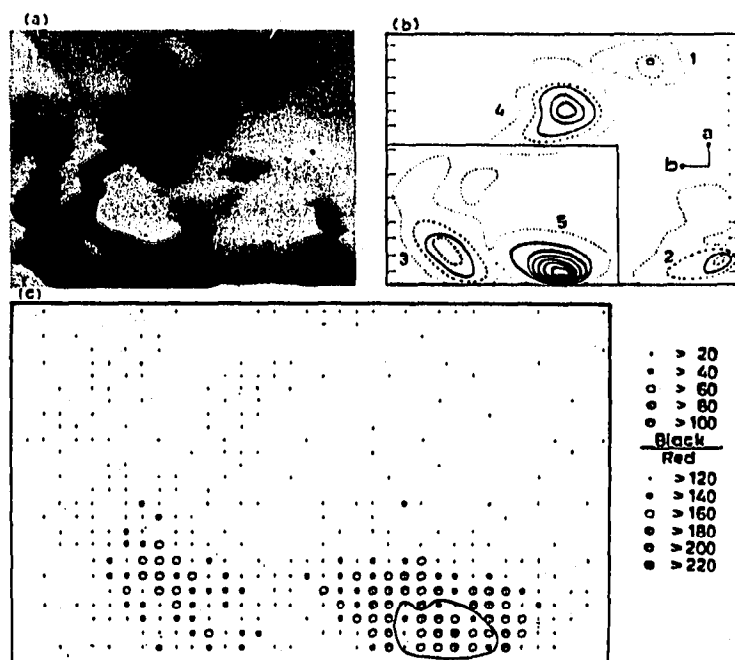A plotter has recently been constructed[6] which may be used in combination

Fig. 2. (a) Film autoradiogram from *Papaver somniferum*. The two-way chromatogram was made of an ethanolic extract after $^{14}CO_2$ incorporation[7]. The exposure time was 12 days. (b) Iso-activity chart obtained manually on the basis of a series of parallel scans. The scan time was 13 h. (c) Smaller part of the activity distribution chart as it would be obtained with the plotter. An iso-activity line at 120 c.p.m. is drawn on the chart, indicating that the higher activity signs are plotted originally in red. The signs and their values in this experiment are given beside the figure[6].

with any type of two-dimensional scanner, provided the detector is moved stepwise across the chromatogram along parallel lines. The number of detector pulses received over constant periods of time can be counted. Since it was desirable both to record this count number as accurately as possible using an X–Y plotter, and at the same time to cover a large range, a compromise was necessary. We chose to use a set of different signs, each indicating that a certain intensity level had been surpassed. In the actual construction, seven signs could be obtained, each occurring in one of the two colours, black or red, the latter colour being used for the highest intensities. In Fig. 2 a simulated plot of the activity distribution of a two-way chromatogram is reproduced. The plot (c) is simulated because the scaler which was required to produce the activation signals for the plotter had not yet been built. Part (b) of the figure shows a contour map obtained manually from a series of parallel scans.

The next obvious step is to consider the use of a computer to assist in the drawing of charts and the evaluation of spot activities. Count numbers can be recorded directly on magnetic or paper tape as they are measured, and the tape thus produced can then be used as input to the computer. We shall first describe an apparatus used for the direct recording of measurements on to paper tape and, secondly, a suitable program which both subtracts the background count rate, and performs a two-dimensional smoothing of the point-grid of recorded activities so as to eliminate the statistical fluctuations in the count numbers. The final computer results are obtained as a listed output of intensity distributions, and a one-to-one scale contour map may be obtained photographically if required.

MATERIALS AND APPARATUS

Materials

To illustrate the methods of computer processing of radiochromatographic data after an appropriate data transfer process, some measurements will be reported. Only [³H]glucose has been used as tracer material, as the difficulties of detection are at their greatest for this particular radionuclide. The spots were applied directly on to Whatman chromatographic papers in varying spot sizes and tracer activities from 0.1 nCi to 1 μCi per spot. Along one side of the chromatogram a series of spots were introduced containing the following activities: 10 nCi, 100 nCi, 1000 nCi, and 1 nCi. The tritiated glucose solution was purchased from the Radiochemical Centre, Amersham, Great Britain, in a strength of 1 mCi per ml solution.

Detector

The particular detector employed in this work is of the general type described in a recent work[5]. However, it is a "funneltron" rather than a "channeltron", the name indicating that the mouthpiece has been flared out into the form of a truncated cone. Hence, the small channel diameter compatible with the geometric requirements for optimal pulse amplification has been combined with a larger aperture, collecting more electrons from the spots to be measured. The noise pulse rate is only that caused by background radiation hitting the sensitive wall material of the mouthpiece and amounts to about 1 c.p.m.

The data logging system

Principally, the data logging system consists of a counter and a paper tape punch. As mentioned in the preceding section, the chromatogram is scanned stepwise with a counting time which can be set at anywhere from 1 to 60 sec. At the end of each counting period the count number is relayed in binary format from the counter to the punch, the punching is thus actuated, the counter is then zeroed, the detector moved to the next position, and the counting is then resumed.

A 15-bit counter is used, and the count number is punched in two columns across the 8-channel tape, the first column holding the lower digits and the second holding the more significant digits of the number. The sixteenth position on the tape is always perforated, thus guarding against reading errors which could otherwise result from a mixing of consecutive count numbers.

After one profile scan of the chromatogram has been transferred to the tape, the detector returns to the start position at one side of the chromatogram. While doing so it shifts sideways a pre-selected number of steps so that the next profile is scanned parallel to the former. In this way the whole chromatogram is covered by profile scans. While the detector is making its return sweep, about 20 cm of tape is fed through the punch without any figure being punched. This provides the computer with large areas of blank tape which separate the data from the individual profile scans.

DETECTION CRITERIA

In any radiochromatographic detection one wants to record the true label distribution; ideally this should be free from all types of statistical fluctuation and noise arising from the β-disintegration process, from the detection procedure, the data transfer, and finally from whatever method of visualization is chosen. A useful figure of merit for the overall imaging process is the resulting signal-to-noise ratio (S/N). This is also pertinent to the low-level limit of activity detection[5,8].

One obviously wants to use the most sensitive detector available. But in making the selection, another criterion to be kept in mind is the importance of the detector showing a low background count-rate. This rate may be due partly to a background of radioactivity in the surroundings, but it may also be inherent in the detector itself or in the electronic detection circuitry. Useful criteria for the choice of a counter, taking into consideration both its sensitivity and its noise level, have been described earlier[5,8].

In addition to increasing the label activity, the S/N ratio can be improved by lengthening the applied detection time. However, practical limits are imposed here by the usual laboratory set-up. The photographic exposure time used for ^{3}H-labelled chromatograms may in some cases extend to a month or more.

When all the above parameters have been optimized, one way of improving the recording still remains. This is by means of signal averaging. The averaging technique for one-dimensional recording is illustrated in Fig. 6 of ref. 5. Part (a) shows a histogram produced using definite, equally spaced, sampling positions along the scan. A smooth curve has been drawn by hand through the observed intensities. Part (b) shows a rate-meter recording. Here we see the influence of the time constant in the recording. Obviously too large an RC value introduces distortion, because

only *past* — and not *future* — signal intensities can influence the present reading. When the method of counting in fixed positions is used, no such tilting of the spot profiles is introduced.

Two-dimensional data logging, based on the digitization of a rate-meter signal obtained with an appropriate RC filter, would nevertheless improve the data by smoothing it in one dimension, (*viz.* along each of the scanned profiles). However, if count numbers are logged for a grid of sampling positions, one can then use a computer program to smooth the data in two dimensions, both across the direction of the scans as well as along each scan.

The S/N ratio affects the obtainable spatial resolution[9] as well as the low-level limit of detection. The resolution which may be obtained is influenced by the following factors: the field of view of the detector, the step length of the scans, and the distance chosen between the scanned profiles. Thus, when attempting to measure the activity distribution over a radiochromatogram, one should try to have a clear idea of what is the smallest required resolution. This is dependent upon either the smallest spot activity details or the smallest inter-spot distances which are to be visualized. The achievable resolution is set by the field of view covered by the detector, if complex computing is not used to limit this by an iteration deconvolution procedure. A step length or inter-profile distance much smaller than that dictated by the field of view is of little use in the scanning of the chromatogram. On the other hand, the sampling distance chosen must of course be small enough to give the wanted resolution.

Now, both the β-disintegration in the labels and the detector background count rate exhibit statistical fluctuations. For this reason it is desirable to smooth the recorded intensities. This procedure can serve as a very valuable and time-saving supplement or alternative to merely increasing the total recording time to overcome poor statistics.

The main part of the work reported here has been concerned with the application of such smoothing. A correlation- or influential-range has already been introduced by the overlap of the field of view of the detector occurring between the various adjoining positions. By smoothing, it is our intention to increase this range of influence until it approximates the required smallest distance of resolution.

It should be mentioned in passing that the common concept of a detector having a precise and sharply defined angular field of view is open to a slight amendment. In fact, one finds varying sensitivities over the field of view, the highest value normally being along the central line with the values then decreasing gradually in inverse correlation to the increase in the angle made with this line. If it should now happen that the field of view becomes greater than the step length, the scanning simultaneously introduces a moving, weighted-average response. The averaging range for a particular detector position is defined by the detector's field of view, and the weighting function by the angular dependency of the detector sensitivity. In the next section we shall concern ourselves somewhat more closely with the smoothing procedure.

COMPUTERIZED SMOOTHING

To begin with, we must estimate an appropriate size for the "influence area"

around a particular point which can then be used for the smoothing of the central point value. As stated earlier, this size must be compatible with, that is to say it must not be larger than, the wanted spatial resolution. Once this estimation has been made, the chosen smoothing procedure is applied successively to all points contained in the grid of observed activities or intensities.

SAVITZKY AND GOLAY[10] have described a method of smoothing for one-dimensional scans of various types of spectral intensity distributions, in which each point intensity is computed as a weighted mean of the neighbouring intensities, and gradually less attention is paid to the more distant points. This method might be extended to two-dimensional distributions where a smoothed intensity, I_s, at a particular point, can be computed as a weighted sum of all the intensities within a selected influence area around the particular point (i,j):

$$I_s(i,j) = \sum_{k=i-r}^{i+r} \sum_{l=j-r}^{j+r} c_{kl} \cdot I_{kl}$$

Where r is the range of influence in units of the chosen step length.

A nearly equivalent method of performing the summation of the weighted neighbouring contributions, to obtain the particular smoothed intensity for the point (i,j), might be preferred even though its performance is not so easily visualized since it is better suited to the computation facilities. With this method, which has been applied here, the smoothing procedure is applied in sequences, gradually widening the influential range around a particular point (i,j), thus gradually smoothing the intensity value at this point. As this procedure is applied to all points in the intensity grid, the smaller and purely statistically dependent details in the "landscape" can be made to disappear. With this method, a smoothed intensity can be represented by a somewhat more complex sum:

$$I_s(i,j) = \sum_n c_n \cdot I^{(n-1)}(i,j)$$

Where $I^{(n-1)}$ is the sum of weighted neighbouring intensities obtained after performing the averaging procedure successively $n-2$ times around the particular grid point. (The above expression will be further explained below.)

In both the above methods, the smoothing can be seen as the application of a mathematical low-pass frequency filtering, in which fluctuations of "wave-lengths" smaller than L — chosen equal to the minimum significant dimension in the activity distribution — are discarded, thus enhancing the S/N ratio in the computed distribution. In our case we have chosen an L equal to three times the sampling interval.

It can be shown[11] that the computation of a smoothed activity or intensity I_s in the particular grid position (i, j) can now be performed according to the following expression:

$$I_s = \frac{1}{216} |133 I^{(0)} + 225 I^{(1)} - 153 I^{(2)} - 69 I^{(3)} + 120 I^{(4)} - 48 I^{(5)} + 8 I^{(6)}|$$

Here the above sum has been expanded. $I^{(0)}$ is the directly measured count number or intensity at the point (i,j). $I^{(1)}$ is the mean value of the four intensities surrounding, (i,j) as nearest neighbours in the grid. By treating all points in the grid in this way a one-time smoothed intensity grid is obtained, in which the measured intensities

$I^{(0)}$ have been replaced by singly smoothed ones, $I^{(1)}$. The doubly smoothed intensity $I^{(2)}$ is reached by taking the arithmetic mean of the four nearest neighbours to the point (i,j) in the singly smoothed grid, etc.

PRINT-OUT OF THE RESULTS

There are two possible means of making the results of the computation available in a meaningful representation: one can select either the line-printer or the plotter. For thin-layer radiochromatograms, showing the smallest details, the plotter should be used in order to obtain the highest possible resolution. For paper chromatograms, an ordinary line-printer can give a satisfactory representation.

In both cases, a 1:1-scaled reproduction is the most useful. This can be obtained for large paper chromatograms by treating them section by section, which we had to do since our scanner cannot take areas larger than 20 × 20 cm. Afterwards, the print-outs can be mounted together and reduced to proper scale by a photographic technique. Activity distributions on thin-layer chromatoplates of the standard dimensions are directly obtainable in a 1:1 scale in the form of iso-activity contour maps produced as plotter output.
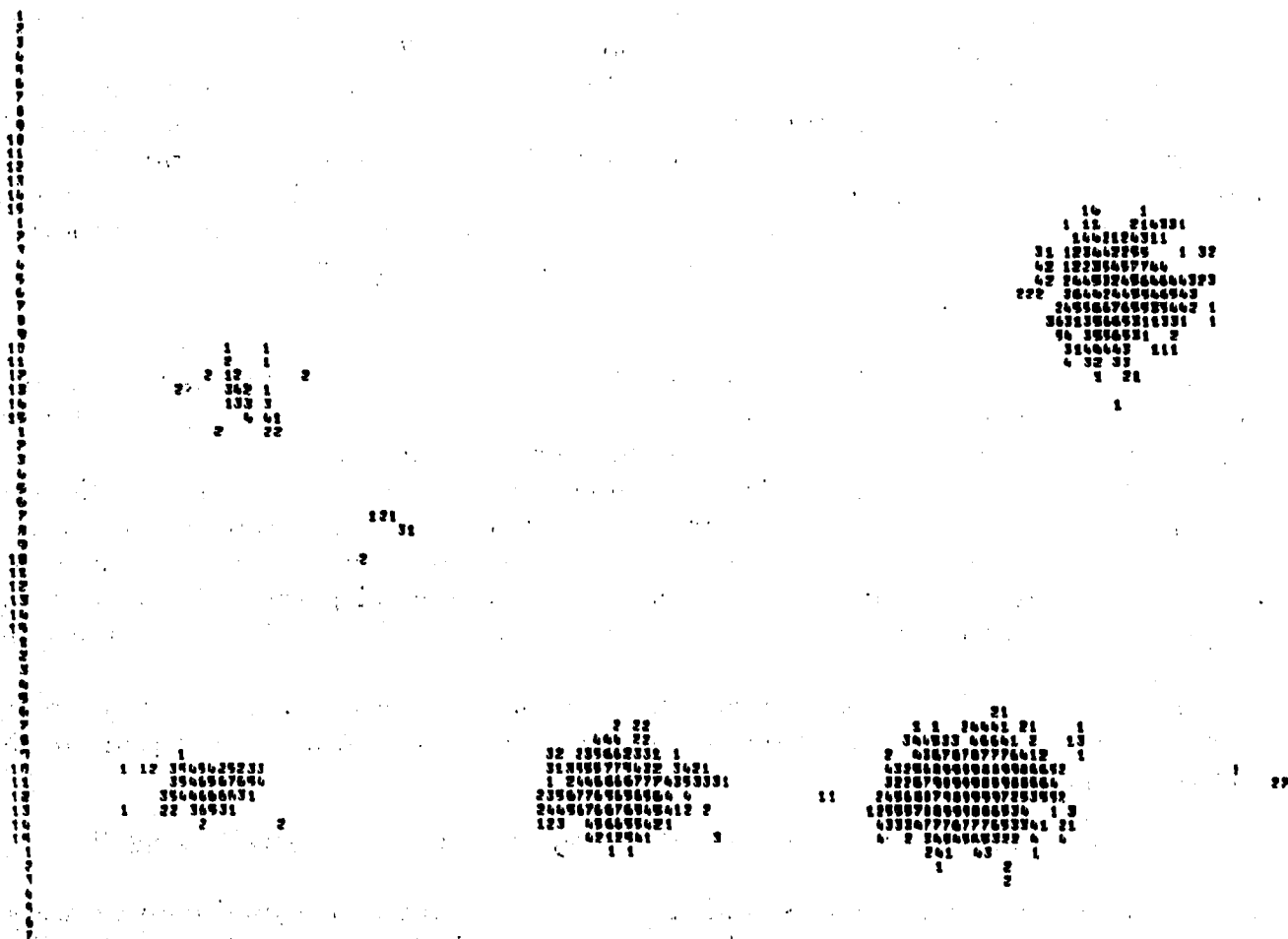

Fig. 3. The computer print-out of a section of a paper radiochromatogram produced by pipetting out small volumes of an aqueous solution containing definite activities of [³H]glucose. The lower row contains (from left to right) 10, 100, 1000, and 1 nCi per spot, respectively.

Since the main purpose of this paper is to draw attention to the possibilities for using computerized smoothing and reproduction of logged radiochromatogram data, we have included Fig. 3 as an example of a print-out of a simulated radio-chromatogram. Our types of output are certainly not the only, and possibly not the best, representations that could be achieved.

In the latter figure, there is a scale of ten activity levels. These are represented by the digits from 1 to 9 with zero as the maximum intensity. The first ten letters of the alphabet may be used in addition to make a scale with twenty levels. The use of this numbering system means that only a single print position is required to print out an intensity value, and thus the highest spatial resolution possible with a line-printer is obtained.

Our print-out intensities are drawn from a logarithmic scale which may be selected during the execution of the program from among a set of possible scales, the choice being based both on the scale's coverage of all the activities in the chromatogram and on its compatibility with maintaining the largest possible intensity resolution. By the use of a logarithmic scale, a fixed relative accuracy pertains to all values.

However, the main reason for choosing a logarithmic scale for the visualization of the activity distribution is that with such a scale it is possible to cover a large intensity range without loosing the intensity resolution in the print-out of low activities. This is exactly the property of a logarithmic scale as opposed to a linear one, namely to render a constant intensity resolution irrespective of the particular position on the scale.

Usually the highest intensity of the logged chromatogram is selected first. Then the range from zero up to this value is divided in ten (twenty) steps in such a way that the difference between the logarithms of every two succeeding levels is a constant value. In this way all intensities are grouped into ten (twenty) classes of magnitude, and in a particular position on the print-out chart the scale number pertaining to the class of magnitude to which the particular intensity belongs is printed.

Finally, it is possible in particular cases, when one or a few spots are very much more intense than the rest and where these are still of interest, to make a second print-out with the scale changed so as to pay full attention to the low-intensity spots, while ignoring the more intense ones. This technique is useful as long as the intensity steps are larger than or equal to the accuracy limits set by the counting statistics.

Once the smoothing has been performed, a computed mean background noise rate multiplied by the integration time spent in each position during the scanning is subtracted from all count numbers, so as to render the print-out intensities free on the average from the contributions from all types of background noise. If, on account of this subtraction, some point-values become negative due to a low-noise fluctuation having occurred in that position, nothing is printed.

Prior to an optional photographic alteration of the size proportions of the printed output, a set of iso-activity contours could easily be sketched in by hand on the basis of the printed symbols. In Fig. 3 we see a print-out where smoothing has been applied to a degree which is matched to the spatial resolution of the detecting equipment. However, if this smoothing should not be sufficient it can

easily be increased, either by increasing the "wavelength" limit L in our formula or by re-running the output data through the smoothing subroutine. Each time the results are displayed by the printer. One should then be able to choose from among the print-outs one showing the best noise filtering without blurring significant details too much.

The limit of the spatial resolution of a print-out is given directly by the available number of signs per cm, or put another way by the area occupied by each sign used for the indication of the activities. For our printer a 20 × 20 cm area would be filled with 48 lines each holding 80 signs. This corresponds to a spatial resolution of 4 mm in the one direction and 2.5 mm in the other. For cases where this resolution is insufficient, smaller areas can be expanded during the reproduction by a very simple computer operation. The practical resolution limit would then be set by the resolution obtained in the previous measurements. In our case this limit is about 1 mm.

CONCLUSION

The application of a computer-aided technique for two-dimensional radiochromatographic detection has been demonstrated. It should be noted that the noise level is extremely low for the applied type of channel electron multiplier, which implies that quite a high degree of sensitivity and resolution is obtainable with the combination of detection system and computer utilization described here. However, the main procedure described here, employing the particular type of data transfer and computing, is fully available for application in combination with any type of scanning detector.

We believe that, particularly in the case of large and medium-sized laboratories where radiochromatography finds many uses as a regular research tool, our findings may present a possible alternative to the reliance on photographic technique supplemented by liquid scintillation counting of eluates from scraped-off spot materials.

ACKNOWLEDGEMENTS

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