

## DETECTION OF TRITIUM ON PAPER AND THIN-LAYER CHROMATOGRAMS

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## INTRODUCTION

Tritium can be incorporated into a very large number of organic compounds using simple exchange techniques which have recently been developed<sup>1</sup>. Paper chromatography and thin-layer chromatography are powerful techniques for the separation of small quantities of very complex mixtures of compounds. It is therefore important to have methods for the detection of tritium on paper and thin-layer chromatograms. Many common isotopes important in biochemistry, for example <sup>14</sup>C, <sup>35</sup>S and <sup>32</sup>P, can be easily detected on thin-layer and paper chromatograms by laying a film on top of the chromatogram<sup>2</sup>. The  $\beta$ -particles pass into the emulsion of the film and on development their tracks leave darkened areas on the film which indicate the position of the radioactive compounds on the chromatogram. Tritium, however, emits only a very weak  $\beta$ -particle (maximum energy 0.018 MeV, mean energy 0.006 MeV) even the most energetic of which will only penetrate 2  $\mu$  in the photographic emulsion<sup>3</sup>. This presents a problem in that all commercial sheet film has a protective layer of gelatin covering the photographic emulsion. In the case of Kodak Royal Blue, for example, this overcoat is  $0.8 \pm 25\%$   $\mu$  thick<sup>4</sup>. For this reason special techniques must be adopted for the detection of tritium on thin-layer and paper chromatograms.

## DETECTION OF TRITIUM ON CHROMATOGRAMS

There are three general techniques which have been used for the detection of tritium on paper and thin-layer chromatograms.

*1. Radioautography*

The chromatogram is impregnated with liquid nuclear emulsion, allowing time for exposure and the whole is then developed in photographic developer. For the purpose of this article this procedure will be called radioautography. It has the advantage of being in principle very sensitive for water-insoluble materials which will not diffuse in the emulsion, but has the disadvantage of destroying the compounds involved so that they cannot be later used for co-chromatography. It has the further disadvantage that some compounds, for example reducing materials, will interact chemically with the emulsion and give "spots" even if they do not contain radioactivity. This

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method has been applied to paper chromatograms by ROGERS<sup>5</sup> and MARKMAN<sup>6</sup> and there seems to be no reason why it should not be applied to thin-layer chromatograms. SHEPPARD AND TSIEN<sup>7</sup> made their own photographic plates from liquid emulsion (*i.e.*, with no overcoat) and used these for radioautography on thin-layer chromatograms.

### 2. Sublimation autography

This technique involves the sublimation of some of the radioactive material from the chromatogram into the emulsion of the film where it produces an image. It can be used to detect not only volatile radioactive materials but also volatile materials which, because of their chemical properties, will develop the film. This technique has not been suggested as such by any workers in the field, but is clearly the mechanism involved in many of the suggested detection procedures. For example, MOSES AND CALVIN<sup>8</sup> claimed that tritiated water was incorporated predominantly into glycolic acid in a photosynthesising system. Our work has shown this not to be the case and the heavy spot due to glycolic acid was due to its sublimation into the emulsion where it was being detected with much greater efficiency than other much more heavily labelled compounds. Again, PARUPS, HOFFMAN AND JACKSON<sup>9</sup> suggested a technique for impregnating a paper chromatogram with anthracene crystals and exposing it to a light sensitive film. This is a valuable suggestion, as is discussed below, but PARUPS *et al.*<sup>9</sup> tested the procedure using nicotine, which is quite volatile and, from their published results, clearly distilled into the film.

More recently, RICHARDSON *et al.*<sup>10</sup> have detected tritium-labelled steroids by laying a sheet of X-ray film on top of thin-layer chromatograms. They claim that they obtain spots, but that with the interposition of a thin cellophane sheet (3.35 mg/cm<sup>2</sup>) only a faint spot was obtained. It is clear that the phenomena they were using was sublimation autography. They observed that estradiol gave a spot even although it contained no radioactivity. This is probably because it sublimed into the film and interacts chemically with the emulsion to produce an image. This phenomenon is well-known in conventional <sup>14</sup>C radioautography in paper chromatography. Here the "ghost" spots can be recognized by their "graininess."

Scintillation autography could be used to detect sublimable reducing material on paper and thin-layer chromatograms. The technique of sublimation autography could be a valuable additional tool used in conjunction with scintillation autography (described below) for the detection and identification of labelled compounds, for example, use with and without transparent screens.

### 3. Scintillation autography

In 1958 one of the present authors<sup>11,12</sup> suggested the technique which involves soaking a paper chromatogram in a liquid scintillator so that the energy of the disintegrating tritium atoms is converted into light which is detected by a fast photographic film. Thus the weak  $\beta$ -particle of tritium is converted into light quanta which can travel into the emulsion of the film to produce an image. It is proposed to call this technique "scintillation autography". This is a photographic technique and is, in principle, quite distinct from any radioautographic procedure discussed above; it is, however, only about one-tenth as sensitive as impregnating a paper chromatogram with nuclear emulsion, but has the advantage that the radioactive compounds are not

destroyed and can be used for co-chromatography. It has the disadvantage that it is of no use with compounds that are soluble in toluene. However, many can be rendered insoluble by turning them into their salts. In practice, it can be used directly with most biological materials, the principal exceptions being lipids and steroids. PARUPS *et al.*<sup>9</sup> suggested the modification of impregnating the paper chromatogram with anthracene crystals; this can be done by dipping or spraying, using anthracene or some other scintillator dissolved in a suitable solvent such as benzene. For the reasons discussed above, the figures as to sensitivity given by PARUPS *et al.*<sup>9</sup>, are not valid, but the procedure has been tested by us and shown to be one-tenth as sensitive as the liquid scintillator technique, but having the advantage of being usable with toluene soluble materials.

#### APPLICATION OF SCINTILLATION AUTOGRAPHY TO THIN-LAYER CHROMATOGRAMS

In our laboratories we use the technique of scintillation autoradiography for the detection of tritium-labelled compounds on thin-layer chromatograms. We have found that commercial silica gel is itself a scintillator and that it gives off light when bombarded with ionising radiation including the weak  $\beta$ -particles from tritium. Thus to detect tritium on thin-layer chromatograms where the stationary phase contains silica gel (*e.g.*, Merck silica gel G) all that is necessary is to lay a sheet of fast photographic film on top of the thin-layer chromatogram. In our work we use Kodak "Royal Blue", which has superseded Kodak "Blue Band" as one of the fastest films presently commercially available. For a thin-film chromatogram made from Merck silica gel G of thickness 0.2 mm, a spot of area 3 mm<sup>2</sup> having an activity of 0.01  $\mu$ C of tritium can be detected in seven days. The sensitivity can be doubled by spraying the thin-layer chromatogram with a saturated solution of anthracene (in benzene) until crystals of anthracene can be seen to be distributed evenly over the surface of the chromatogram.

In order to line up the image and the thin-layer chromatogram, the following procedure is used. Some tritium compound is placed on a piece of filter paper. The filter paper is then sprayed with anthracene or terphenyl and cut into segments with the radioactive material at the apex of each segment. A small amount of the stationary phase is scraped from two corners of the thin-layer chromatogram, exposing the glass plate, and two marker segments are taped onto the glass plate. The image of the apex of the marker segments can then be lined up with the apex of the marker segments after the film has been developed. If sublimation is suspected, then a thin sheet of a suitable transparent plastic is interposed between the film and the thin-layer chromatogram. This will only slightly reduce the light transmitted, but will reduce the rate of sublimation considerably. Scintillation autoradiography and sublimation autoradiography taken together can yield additional information of the physical and chemical properties of the compounds being investigated. Rates of sublimation depend on the heat of vaporization rather than on boiling point and some materials, for example glycolic acid, sublime very rapidly.

It should be emphasized that scintillation autoradiography, unlike autoradiography and sublimation autoradiography, is a photographic process and is therefore subject to reciprocity failure<sup>12</sup>. For a radioautographic process the darkening of the film is due to  $\beta$ -particle tracks and this darkening is approximately proportional to exposure time.

This is not the case for a photographic process. From these considerations it should be clear that it is not valid to select an exposure period, say 24 h, as has been done by CHAMBERLAIN *et al.*<sup>13</sup>, and compare the various techniques. The shorter the period taken, the better will a scintillation autographic technique appear with respect to any technique based on radioautography or sublimation autography.

#### ULTIMATE SENSITIVITY OF SUBLIMATION OR SCINTILLATION AUTOGRAPHY

Clearly, if one wishes ultimate sensitivity, techniques should be used to keep the spots as compact as possible. In this respect thin-film chromatograms have an advantage over paper chromatograms in that because they run quickly there is less diffusion and the spots are more compact. In principle, the sensitivity could be improved by placing a reflector behind the chromatogram. The authors have used sheets of tinfoil as reflectors behind paper chromatograms during scintillation autography and it does improve the sensitivity slightly.

In practice, if the spots produced on the film are not sufficiently dense or if the exposure time is inconveniently long, the simplest procedure is to use more activity on the chromatogram but, if this is not possible or leads to overloading, then resort must be made to other methods. We have found that improvements in film processing offer the easiest solution; for example, having selected the fastest film available, greater contrast and hence greater sensitivity can be obtained by developing the film for longer times at lower temperatures than recommended by the manufacturers. Very long development times, however, will bring up the background. Since there is emulsion on both sides of fast commercial sheet film and since sublimation or scintillation autography will only expose the emulsion on one side of the film, half this background can be avoided if the film to be developed is fixed to a supporting sheet by means of waterproof adhesive tape, with the unwanted emulsion layer in contact with the sheet. The supporting sheet may be a discarded piece of film and should be the same size as, or larger than, the film to be processed. Alternatively, when several sheets of film are to be processed, they may be taped together in pairs with their unwanted sides together. The development of the film is carried out as usual, washed, and placed in the fixer. During fixing the tape is stripped off the film, separated from its supporting sheet and the fixing continued until both sides of the film have cleared. Image intensifying procedures are also available if even greater sensitivity is desired<sup>14</sup>.

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#### SUMMARY

The techniques which have been used to detect tritium on paper and thin-layer chromatograms are reviewed and their theory of operation and experimental limitations discussed. A simple technique is described whereby small amounts of tritium-labelled compounds may be detected on thin-film chromatograms.

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