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THE APPLICATION OF CERENKOV COUNTING TO COLUMN CHROMATOGRAPHY OF ^{32}P LABELLED SUBSTANCES

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SUMMARY

In common with other energetic β-emitters ^{32}P in aqueous solution containing no scintillator, may be determined by counting the Cerenkov radiation in an unmodified liquid scintillation counter[1–4]. Although this has been known for some time, the value of this procedure for analysing ^{32}P labelled effluents from column chromatograms does not seem to have been generally realised. The purpose of this note is to show how Cerenkov radiation may be effectively applied to column chromatography using a modified fraction collector.

MATERIALS AND METHODS

A Packard liquid scintillation counter Series 3000 was used and, of course, the settings quoted apply only to this instrument. A Locarte Ltd. fraction collector was used for chromatography. Glass and polyethylene counting vials were purchased from Packard Co. DEAE-Sephadex was purchased from Pharmacia Ltd., DEAE-cellulose from H. Reeve Angel and Co. Ltd., pancreatic ribonuclease A from Worthington Biochemical Co., and the chromatographic columns from Aimer Ltd. The test solution of ^{32}P was obtained by rinsing a container of $^{32}PO_4^{3-}$ (PBS. 1 The Radio-Chemical Centre, Amersham) with 0.1 *M* phosphate buffer. This solution was diluted with the same buffer to give about 10^4 d.p.m./ml ^{32}P and an appropriate volume of this solution was placed in a counting vial. No other processing is required. At least 10^4 counts were collected for each measurement, except for background counts in which case the samples were counted for 100 min each.

Two channels of the liquid scintillation spectrometer were used for determining the characteristics of Cerenkov radiation and the best settings of the spectrometer controls for routine counting. One wide channel was fixed and the other varied. The ratio

$$\frac{\text{counts in variable channel}}{\text{counts in fixed channel}}$$

was used as a measure of the efficiency of counting in the variable channel. This simple device reduces the time usually required to attain a given statistical uncer-

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tainty in the efficiency by one order of magnitude with a reasonable fixed channel and has a wide application[5].

A fraction collector was modified so that the effluents from four chromatographic columns could be collected simultaneously into polyethylene counting vials, thus eliminating the need for test-tubes, which would become contaminated, and for subsequent transfers and/or sampling. A Locarte Ltd. fraction collector was chosen for

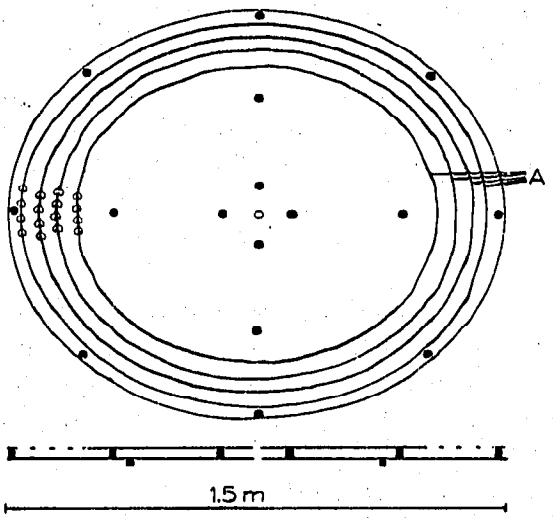

Fig. 1. Modification of a fraction collector. The rack consists of two perspex discs separated by spacers made from perspex rod. The upper disc is drilled to provide four rows of eighty holes each suitable for scintillator vials, A. Lugs made from perspex rod project from the bottom and fit in holes in the original fraction collector.

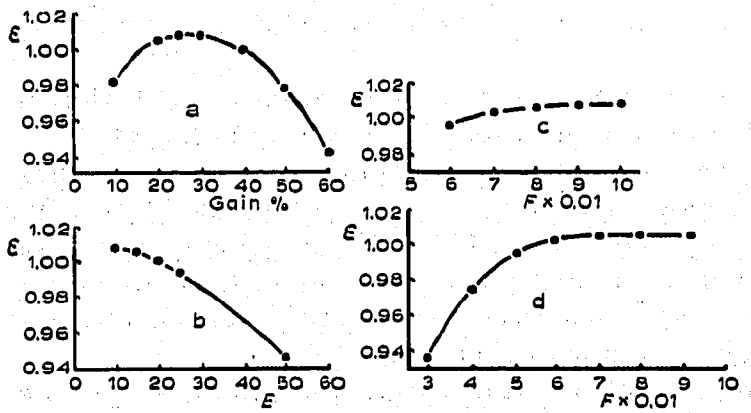

Fig. 2. Cerenkov counting of ^{32}P in aqueous solutions in polyethylene vials. The efficiency, ε, of counting ^{32}P in aqueous solution by Cerenkov radiation relative to the fixed channel (20 % gain; 50–1000 window) is plotted as a function of gain and discriminator settings in the channel to be used for Cerenkov counting. (a) Window 10–1000; gain varied. (b) Gain 25 %; upper discriminator 1000; lower discriminator (E) varied. (c) Gain 25 %; lower discriminator 10; upper discriminator (F) varied. (d) Gain 20 %; lower discriminator 15; upper discriminator (F) varied.

modification for several reasons. The fractions move in a circle and no movement of the liquid input tube is required. This property makes the collector easily adapted to multi-track operation. There are no projections on the apparatus above the top of

the rack for the fractions. Eighty fractions are collected per revolution of this rack, which allows a high capacity per track.

The holes for test-tubes in fraction collectors are too small for the standard polyethylene vials which are used in the Packard scintillation counter. So, a perspex disc was constructed with four concentric rings of eighty holes each suitable for the polyethylene vials. This was fitted to the top of the Locarte fraction collector and rotated with it. The platform was about 1.5 m in diameter and 5 cm high (Fig. 1). A Pasteur pipette was clamped above each row of vials and the column effluents were led into these and thence to the vials. The effluents from four columns were collected simultaneously in this apparatus, using a time switch to rotate the platform every 20 min. Every 24 h the polyethylene vials containing the fractions were simply capped, numbered, placed in the liquid scintillation counter, counted directly by Cerenkov radiation and then stored at —20°.

RESULTS

The characteristics of Cerenkov counting of ^{32}P in aqueous solution were investigated. Fig. 2 shows that the energy of the Cerenkov radiation observed by the photomultipliers is comparable to the energy of the scintillations produced by ^{14}C β-particles in moderately quenched scintillator solutions. ^{32}P Cerenkov radiation is thus suitable for measurement in most liquid scintillation counting systems. The maximum figure of merit, (efficiency)^2/background, was obtained for polyethylene vials at spectrometer settings: gain 20 %, window 15–500 divisions.

The reproducibility of measurements of Cerenkov radiation was excellent. Comparisons of the efficiency of Cerenkov counting under various conditions were made using one standard solution of ^{32}P. The activity of this solution was estimated by counting 0.1 ml of it in a homogeneous toluene blended scintillation system[5] at maximum efficiency, and taking this to be 100 % efficiency. This estimate is liable to a systematic error of $\pm$ 10 % but this does not affect the comparisons of efficiency of Cerenkov counting described below.

The efficiency of Cerenkov counting is a function of the volume of solution in the counting vial and of the material of the vial (Fig. 3). Measurements of these functions show that polyethylene vials give a maximum efficiency of about 32 % and that glass vials give a maximum efficiency of about 23 % (*cf.* ref. 4). A similar difference has also been found in Cerenkov counting of $^{90}Sr/^{90}Y$ solutions[1]. For both glass and polyethylene vials the efficiency for ^{32}P is nearly constant over a considerable volume range. Outside these ranges a correction for the volume effect is needed. The polyethylene vials are clearly superior to glass vials.

Weighing provides a convenient, accurate and rapid measure of volume, particularly with the polyethylene vials which have a mass of about 4 g only, although each vial must be weighed separately when empty if reasonably accurate measurements are required, as the masses of different vials are not exactly the same.

The automatic external standard fitted to the liquid scintillation counter provides an even more convenient method of volume measurement, up to 15 ml. Cerenkov counting is not susceptible to chemical quenching[4] and so the automatic external standard can be put to use not for quench determination in constant volume, but for volume determination in the absence of quenching. The samples must be colourless[1].

Fig. 3 shows how the automatic external standard count rate varies with the volume of liquid in the vial for polyethylene vials. Between 1 and 15 ml volumes this is steep and nearly straight providing a good calibration curve from which automatic external standard count rate may be converted into sample volume. This method is, of course, only applicable to those liquid scintillation counters in which the external standard count rate is a function of volume.

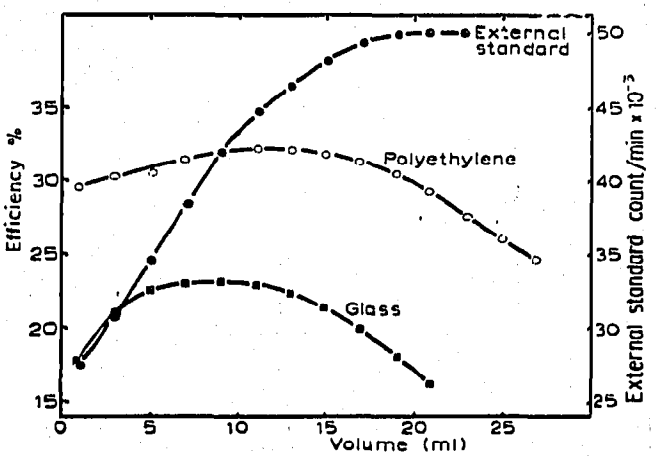

Fig. 3. The effect of sample volume. The efficiency of ^{32}P radio-activity determination was estimated roughly by counting a sample of the ^{32}P solution in a homogeneous toluene blended scintillator solution at maximum efficiency. The results in this figure are given as a per cent of this efficiency. 1 ml of the solution of $^{32}PO_4^{3-}$ in 0.1 *M* phosphate buffer was pipetted into a polyethylene counting vial and the Cerenkov radiation measured. 2 ml distilled water were added and the Cerenkov radiation measured again. This was repeated until the vial was full. The efficiency of ^{32}P activity measurements by Cerenkov radiation was plotted as a function of sample volume. The same experiment was carried out using glass vials. The same experiment was carried out using no internal radio-activity and polyethylene vials with the instrument's external standard source in the "in" position. The count rate in the Cerenkov counting channel was observed and plotted as a function of sample volume.

The system has been used extensively in column chromatography of ^{32}P labelled oligonucleotides. Two different applications are described as examples.

The system has been successfully used in nucleotide composition analysis of ^{32}P labelled oligonucleotides using BLATTNER AND ERICKSON'S[6] procedure. Each peak eluted from the column was collected by hand into a polyethylene counting vial. The volume of the eluant was made up to 18 ml with distilled water, using a vial containing 18 ml distilled water as standard. For accurate measurements, the volumes were determined very conveniently and precisely using a balance. The automatic external standard was not used here since the volumes were greater than 15 ml. The vials were then simply capped, labelled, and placed in the liquid scintillation counter ready for counting. Excellent quantitative results were obtained.

Cerenkov counting has also proved invaluable in the analysis of fractionations of the oligonucleotides in a complete pancreatic ribonuclease digest of $\mu 2$ viral RNA (ribonucleic acid). The fractionation was accomplished by a two-step procedure. The oligonucleotides were first separated into groups of components of a given chain length —isotichs or isopliths. The groups with chain lengths greater than six were each further fractionated according to nucleotide composition. The most interesting

components finally obtained each comprised some 0.1 % of the ^{32}P of the original digest in volumes approaching 100 ml.

In the initial isotich fractionation, Fig. 4, the analysis required an accurate measure of the amount of material in peaks where this amount differed by a factor of 100. High recovery of the material in the smaller peaks was also essential. In the latter fractionation, such as that in Fig. 5, high recovery and high sensitivity were required. Further, some 10^3 fractions were handled per run, so that convenience and the avoidance of contaminated glasswere were important.

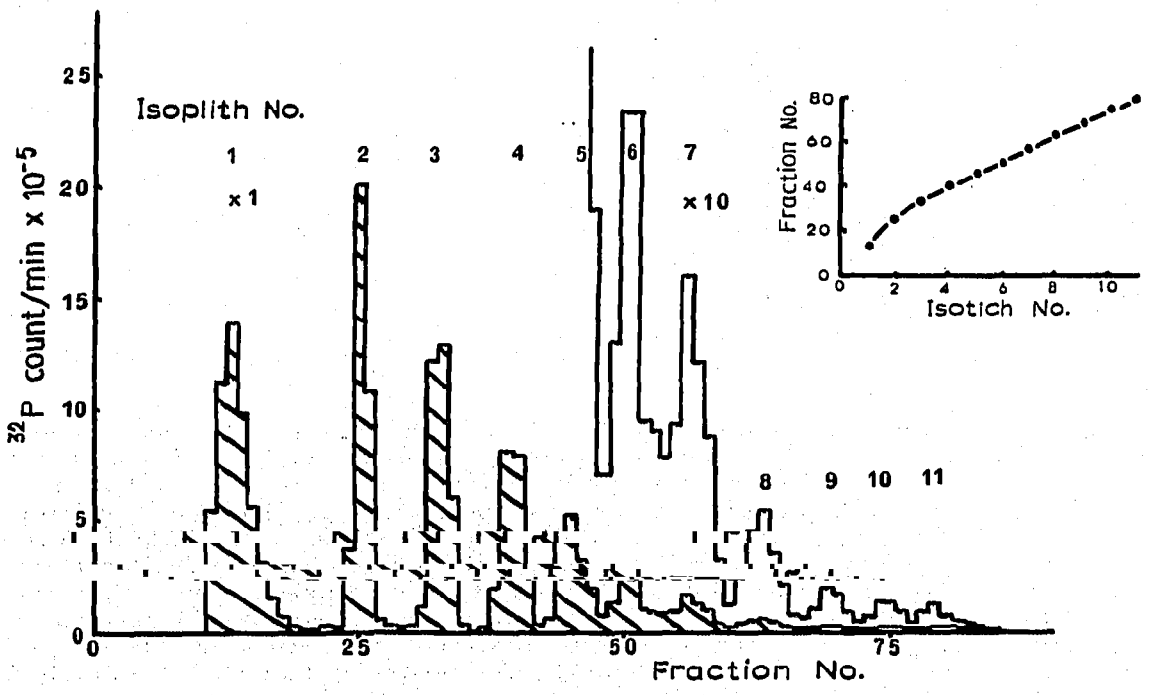

Fig. 4. Use of Cerenkov counting when the activity varies widely. DEAE-cellulose was washed in acid and alkali, equilibrated in the initial buffer and packed under pressure into a 1 cm × 50 cm siliconised glass column (Whatman Technical Bulletin IE2). 20 mg, about 10^6 count/min ^{32}P, of a ^{32}P labelled pancreatic ribonuclease digest of $\mu 2$ viral RNA was loaded onto the column and eluted at 20 ml/min with a 2 l salt gradient from the initial buffer, 0.1 *M* sodium acetate 10 m*M* tris 1 m*M* EDTA 7 *M* urea pH 7.9 to the final buffer, 1.0 *M* sodium acetate 10 m*M* tris 1 m*M* EDTA 7 *M* urea pH 7.9. The column effluent was collected by a time-actuated fraction collector in polyethylene counting vials and the radio-activity determined by Cerenkov radiation in the liquid scintillation counter.

No conventional method satisfies these requirements. However, with the use of a modified fraction collector to collect the column effluents directly into polyethylene counting vials (see methods section) and Cerenkov counting for ^{32}P determination, all the requirements were well satisfied. In addition, the automatic external standard was used to check for variations in fraction volume.

DISCUSSION

When the radio-active sample is in a small volume of aqueous solution conventional liquid scintillation counting techniques may be more efficient than Cerenkov counting, although they are still open to the objections that they need quench

correction and destroy the sample counted. Cerenkov counting is also unsuitable for counting low energy β-particles such as those from ^{14}C, ^{35}S and ^{3}H. It is useful, however, that ^{33}P, a common contaminant of ^{32}P (The Radio-Chemical Centre—personal communication), is not detected by Cerenkov counting so that it need not be taken into account when computing the decay of ^{32}P.

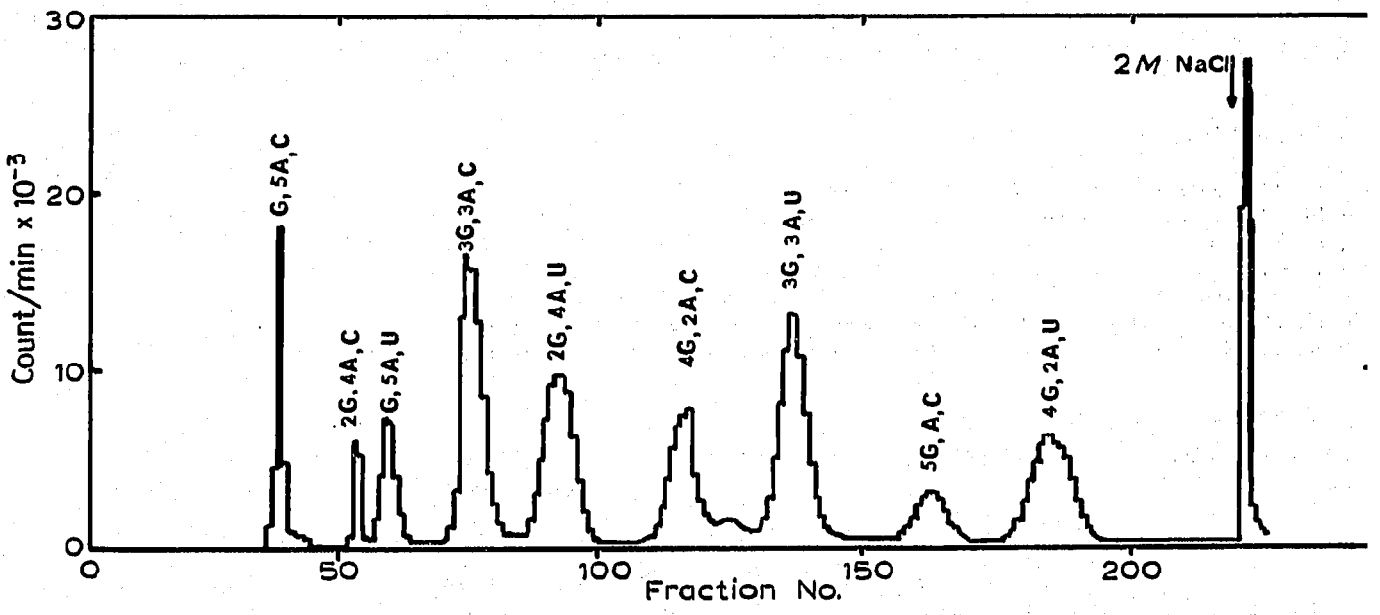

Fig. 5. Use of Cerenkov counting when high resolution is required. DEAE–Sephadex was washed in acid and alkali, equilibrated in the initial buffer and packed into a 1 cm × 50 cm siliconised glass column. 1 mg, about 10^5 count/min ^{32}P of a mixture of ^{32}P labelled heptanucleotides from a pancreatic ribonuclease digest of $\mu 2$ viral RNA was loaded onto the column and eluted at 20 ml/min with a 2 l linear salt gradient from the initial buffer, 7 *M* urea pH 2.7 to the final buffer, 0.2 *M* NaCl 7 *M* urea pH 2.7. The column effluents from this and three similar columns were collected by a time-actuated fraction collector in polyethylene counting vials and the radio-activity determined by Cerenkov radiation in the liquid scintillation counter.

Quench correction is a major problem in liquid scintillation counting: the internal standardisation method is time consuming and relatively expensive; the channels ratio method is unsuitable for low activity samples; and the automatic external standardisation method is less accurate and, like the channels ratio method, dependant on the stability of calibration curves[7]. Chemical quenching is eliminated by Cerenkov counting, and so no quench corrections are needed.

For both liquid scintillation counting and planchette counting with a Geiger–Müller tube, dilute aqueous samples must be concentrated. This process is time consuming and leads to inaccuracies. It can frequently be eliminated by the use of Cerenkov counting and, after Cerenkov counting, the entire sample is available for further analysis.

The effluents from chromatographic columns may be analysed using flow cells and digital or analogue recording. Such systems can be troublesome to operate and their sensitivity is usually limited. They are subject to error due to variations in column flow rate, and particularly with analogue recording are not accurate over very large ranges of activity. These difficulties are eliminated with Cerenkov counting. In particular, sensitivity can be made high, resolution can be adjusted to suit the

problem in hand, and wide variations in sample activity can be accepted. Use can also be made of reject facilities provided by the liquid scintillation counter.

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