

COUNTING RADIOACTIVE NUCLEOTIDES ON ANION EXCHANGE PAPER DISKS:
RECENT IMPROVEMENTS

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I. INTRODUCTION

Since the time Breitman (1963) and Furlong (1963) introduced the use of DEAE-paper in disk form for anion exchange and counting of radioactive nucleotides absorbed onto the disk, immersing it directly into the scintillation fluid, the method has found numerous applications in the assay of enzymes involved in ribo-, and deoxyribonucleotide metabolism (Bresnick and Karjala, 1964, Cheng and Prusoff, 1974, Ives *et al.*, 1969, Patel *et al.*, 1977, Stirpe and La Placa, 1971).

Although in the original method (Breitman, 1963) the counting efficiency was only 2% for (³H)-labelled thymidine nucleotides, the technique has also been adopted for assaying thymidine kinase in developing rat cerebellum (Weichsel, 1974). As can be expected, their values are low in comparison with those works in which thymidine nucleotides were separated by thin-layer chromatography (Yamagami *et al.*, 1972). Further-

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more, there have been large variations in cerebellar activity of thymidine kinase in normal rats of similar age and body weight. (Weichsel, 1974, Weichsel and Dawson, 1976).

Because of the speed and convenience of this technique we investigated the factors which seemed to be responsible for the apparently low and variable thymidine kinase activity. (Bendek and Patel, 1977).

II. MATERIALS AND METHODS

[2-¹⁴C]Thymidine 5'-monophosphate and [6-³H]thymidine were purchased from the Radiochemical Centre, Amersham. DE-81 (DEAE cellulose paper) disks, 2.3 cm diameter, were obtained from Whatman Biochemical Ltd., Maidstone. All other chemicals used were of analytical grade purchased from either BDH or Sigma, London.

Porton 12 day old rats were used in these studies. They were sacrificed by decapitation and the cerebellum and forebrain were quickly excised as described previously (Patel and Baláža, 1971) and processed separately. The brain parts were homogenized in 9 vol of ice-cold 0.25 M sucrose containing 20 mM Tris - HCl buffer, pH 7.4, and 4 mM MgCl₂ as described by Yamagami et al., (1972). The homogenate was centrifuge for 30 min at 35000 x g and the resultant supernatants were used as the source of thymidine kinase enzyme. The assay mixture (0.2 ml) contained (in final concentration 50 mM Tris - HCl buffer, pH 8.0, 10 μM [6-³H]thymidine (specific radioactivity 0.5 Ci/mmmole) 2.5 mM MgCl₂, 5 mM ATP, and 0.05-0.1 ml enzyme (boiled supernatants were used in blanks). After incubation at 37°C for 10 min the reaction was stopped by heating the tubes in a boiling water bath for 3 min followed by immediate chilling in ice. Denatured proteins were removed by centrifugation at 2500 x g for 15 min. In the supernatant the radioactivity present in thymidine nucleotides was estimated by three different methods.

A. Method 1. Three different portions of the supernatant (20, 50, and 100 μl) were applied on columns of anion-exchange resin (Dowex AG 1-X8, 200-400 mesh, Cl-form column size, 5.5 x 25 mm). The columns were then washed with 50 ml of water. The unconverted [6-³H]thymidine was fully recovered in the effluent and water wash. Next, the absorbed thymidine nucleotides were eluted with 30 ml of 2 M HCl. The eluate was evaporated to dryness, if necessary, and redissolved in distilled water before the estimation of radioactivity.

B. Method 2. The thymidine nucleotides from the supernatant of the enzyme reaction were separated using the method of Weichsel (1974). Briefly, the same aliquots of the supernatant as in Method 1 were applied onto Whatman DE-81 disks on a Millipore filter holder fitted into a suction flask. The disks were each washed twice with 10 ml of 1 mM ammonium formate and 10 ml of distilled water and once with 10 ml of absolute ethanol. After drying the disks were placed in a counting vial with 10 ml of scintillation fluid. Radioactivity was measured in a Nuclear Chicago Mark 2 Scintillation Spectrometer.

C. Method 3. The same aliquots of the supernatant as in Method 1 were processed for thymidine nucleotides separation as described in Method 2. The wet disks were placed in a counting vial and to elute the absorbed labelled thymidine nucleotides from the disks 1 ml of 1 M HCl containing 0.5 M NaCl was added to each vial. After 10 min of occasional swirling 10 ml of scintillation fluid was added, and the vials were vigorously shaken using a Vortex mixer. The elution from the disk of the labelled thymidine nucleotides, based on the recovery of standard [^{14}C] thymidine monophosphate, was 95-98%. The scintillation fluid had the following composition: 0.4 2,5-diphenyloxazole (PPO), in toluene - Triton X-100 (2:1, v/v); 1 ml aqueous sample was used with 10 ml of the Triton scintillant. The samples were counted in a Nuclear Chicago Scintillation Spectrometer (Mark 2), and correction for quenching was applied by the external standardization technique.

III. RESULTS AND DISCUSSION

The method for separation of nucleotides on DEAE-paper disks is simple to operate compared with anion-exchange or thin-layer chromatography, and a large number of samples can be handled simultaneously.

Using Weichsel's conditions we have found good linearity between the radioactivity counted directed on the disks and the incubation time up to 15 minutes and also with the enzyme quantity (data not shown). Unfortunately, applying different volumes of the same assay media onto disks, we were unable to find proportionality between the volumes and the counts (Table I). Compared to the values obtained from ion-exchange with AG 1x8 resin (Method 1), the counting efficiency of DEAE disk was higher with samples containing higher radioactivity (cerebellum vs. forebrain) but basically depended on the volume of

TABLE II. The Effect of Buffer and ATP Concentration of the Sample on the Binding of $/2-^{14}\text{C}/\text{TMP}$ to DEAE paper

Composition of sample ^a	Radioactivity /dpm. $\times 10^{-2}$ /	Recovery of $/2-^{14}\text{C}/\text{TMP}$ from DEAE disk /in % of sample value counted without disk/
ATP 1.25 mM ^b	265	94
2.5 mM ^b	255	91
5 mM ^b	228	82
TRIS-HCL, $p_{\text{H}}8$; 12.5 mM	271	97
25 mM	273	97
50 mM	271	97
ATP 1.25 mM+TRIS-HCL, $p_{\text{H}}8$; 12.5 mM	270	97
ATP 2.5 mM+TRIS-HCL, $p_{\text{H}}8$; 25 mM	260	93
ATP 5 mM+TRIS-HCL $p_{\text{H}}8$; 50 mM	251	90
Sample directly counted in 5 mM ATP+TRIS-HCL, $p_{\text{H}}8$; 50 mM, without disk	281	100

^aAll samples were of 100 microlitre volume and contained identical amounts of $/2-^{14}\text{C}/\text{TMP}$. The disks were washed and counted as in Method 3.

^bDissolved in unbuffered water, $p_{\text{H}} 6.4$

applied sample.

This drawback is probably due to the different quenching properties of the DEAE-paper, depending whether the radioactivity was absorbed on the surface of the paper or in its material at different depths. This quenching seems especially important in the case of low-energy tritium radiation. The quality and the quantity of solutes present in the sample also cause some variability in the method (Table II).

To overcome these difficulties we have tested a series of eluants to replace the radioactive anions in the disk. Adding 1 ml of 0.5 M NaCl dissolved in 1 M HCl to the disks in the counting vial and shaking well with the scintillant (see Method 3. in *Materials and Methods*), resulted in a 4-9 fold increase in counting efficiency (Table I) and the counts were in good agreement with the values of the resin ion-exchange. By this modification - with a single additional pipetting - one could fully utilize the advantages of this quick and easy method.

In the thymidine kinase assay (Yamagani et al., 1972) with a crude tissue extract radioactive TMP, TDP and TTP are produced simultaneously from the labeled thymidine in a 5 mM ATP containing system, and the triphosphates have the highest affinity to the anion-binding sites. In this competition - although the capacity of the DEAE paper is theoretically high enough (3-5 $\mu\text{equ}/\text{cm}^2$) to bind all the phosphates present - during the washing procedure some of the labeled TMP is washed away together with the unchanged thymidine, depending on the pH and the quantity of ATP. The loss depends on the sample volume, as the pH and the ATP concentration is constant in the same experimental series.

Table II shows the effect of these factors using identical sample volume and radioactivity with different concentrations of ATP and buffer. As this loss at 100 microlitre sample volume, 50 mM (pH 8) TRIS-HCl buffer and 5 mM ATP is 10% in our activity range, for comparison between a control and an experimental group it seems acceptable. When changing the experimental parameters the effect of this loss should also be born in mind.

A method similar in principle to that described here has recently been employed by Cheng and Prusoff (1974) for the estimation of deoxythymidylate kinase activity. The separation of (^{14}C)thymidine triphosphate from (^{14}C) thymidine monophosphate was achieved by washing the latter from DEAE-paper disks pre-treated with unlabelled monophosphate nucleotide by a solution composed of 4 M formic acid and 1 mM ammonium formate.

An earlier variation of the elution method was described in a detailed study by Ives et al., (1969). Using 0.1 M HCl - 0.2 M KCl eluant with H labelled nucleotides they successfully estimated the activity of some ribo-, and deoxyribonucleoside kinases and even that of various enzymes which hydrolyze nu-

cleoside monophosphates. They have found that Amberlite anion-exchange resin loaded paper circles (SB-2, Reeve Angel) were less fragile than DEAE-paper in the wet state and had a somewhat higher capacity and binding affinity for nucleotides, with the drawback of higher chemiluminescence in the first 24 hours, if TRITON-toluence scintillant was used.

In conclusion, our results showed that counting labeled nucleotides directly on dried DEAE disks produces erroneous results. The failure to recognize this is responsible for the variable and low thymidine kinase activities in the cerebellum reported in previous studies (Weichsel, 1974, Weichsel and Dawson, 1976). The elution of the labeled nucleotides with 1 ml of 1 M HCl containing 0.5 M NaCl in the counting vial in improved counting efficiency and reproducibility. Furthermore, it is not necessary to dry the disks, thus the overall method became less time-consuming.

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