BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 263–267 (1996)
ARTICLE NO. 1164

A New Method for Quantitative Determination of Tritium-Labeled Nucleoside Kinase Products Adsorbed on DEAE-Cellulose

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Received June 20, 1996

The counts of tritiated compounds—adsorbed to paper disks, paper chromatograms, electrophoretograms or TLC plates—can be strongly affected by extended self-absorption of tritium β-particles on the matrix, due to their low energy. Therefore fully quantitative results can be obtained only by elution of the substances or decomposition of the matrix and subsequent counting in homogeneous solution. In this study we describe a new method for fast and proper decomposition of cellulose matrices by cellulase digestion prior to scintillation counting. This new approach yields up to 98% recovery. For method validation recombinant herpes simplex type 1 thymidine kinase was characterised kinetically. The K_m of 0.2 μM remained the same as expected but V_{max} was considerably higher yielding 1050 pmol/μg/min.

The DEAE (Diethylaminoethyl)-Paper Disc Method represents a rapid assay technique for nucleoside kinases (1, 2). This anion-exchange paper retains labelled nucleotides but allows the unreacted precursor nucleosides to be washed away. The reaction products, still adsorbed on the paper, are then counted in a liquid scintillation counter.

We applied this technique to characterise the enzyme activity of Herpes Simplex Virus Type 1 thymidine kinase (HSV 1 TK) by detection of its tritiated reaction product thymidine monophosphate (TMP). In all preceding applications the cellulose-adsorbed tritiated TMP was either quantified directly or upon previous extraction from the paper disc with HCl/KCl (0.1/0.2M) (3, 4). Although the procedure with the direct measurement is quite satisfactory for the use with [^{14}C]-labelled nucleotides, it is considerably less reliable with tritium-labelled substrates because of the strong internal self-absorption of the week tritium β-radiation by the paper. Furthermore, it was found that even the orientation of the paper in the scintillation vial can lead to variable results (5, 6). On the other hand, the procedure with the elution of the nucleotides prior to scintillation counting often suffers in quantitative extraction. In this study we describe a more generally applicable quantification method based on cellulase digestion of the paper disc prior to the scintillation counting.

MATERIALS AND METHODS

Materials. The radioactively labelled Substrate: [methyl,1′,2′-^{3}H] thymidine (100-130 Ci/mmol) was purchased from Amersham. Cellulase and other chemicals were obtained from Sigma and Diethylaminoethyl (DEAE) substituted paper sheets (Whatman DE-81) from Inotech. The scintillation liquid (UltimaGold) was purchased from Packard. The plasmid pGEX2T was purchased from Pharmacia. The plasmid pBR322-TK containing the gene for HSV1 strain F TK was a gift from S. McKnight.

Expression and purification HSV1 TK. The bacterial expression vector pGEX2T-TK was constructed as described

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The abbreviations used are: HSV 1, herpes simplex virus type 1; TK, thymidine kinase; GST, glutathione *S*-transferase; TLC, thin-layer chromatography; DEAE-cellulose, diethylaminoethyl-cellulose; TMP, thymidine monophosphate.

0006-291X/96 $18.00

earlier (7). The purification was carried out by a one-step procedure as previously described (8). Briefly, competent cells of the TK-deficient *E. coli* strain KY895 were transformed with pGEX2T-TK. Gene expression was induced by the addition of IPTG (isopropyl β-D-thiogalactopyranoside). Bacteria were harvested by centrifugation, then frozen, thawed, resuspended and finally lysed by mild sonication in the presence of lysozyme, PMSF (phenylmethylsulfonyl fluoride), DTT (DL-dithiothreitol) and Triton X-100. The supernatant containing large quantities of soluble GST-TK fusion protein was applied to glutathione affinity chromatography. The fusion protein was subsequently cleaved with thrombin directly on the column. The resulting flow through was used for kinetic studies.

Sample preparation for method validation. Triple sets of reaction mixtures containing 50 mM Tris pH 7.2, 5 mM ATP, 5 mM $MgCl_2$, 2 mg/ml BSA, 1.1 ng enzyme and 3 different concentrations of [^{3}H]-thymidine (0.05, 0.25 and 1 μM) in a final volume of 330 μl were incubated at 37°C. After 10 minutes the entire reaction mixture was inactivated by heating to 95°C for 3 Minutes and centrifuged. Three corresponding blank reactions were carried out using 1 μM unlabelled instead of labelled thymidine in order to determine the background level. For determination of the total amount of labelled substrate, a triple set of 5 μl from each reaction mixture was pipetted directly into 8 ml of scintillation liquid. Three further triplets of 5 μl from each reaction mixture were transferred onto DEAE-cellulose squares (0.9 cm $\times$ 0.9 cm) placed on the bottom of the scintillation vials. After drying the paper squares at room temperature they were treated as follows:

1) 8 ml of the scintillation liquid were added without any further treatment in order to determine the amount of self-absorption of both, thymidine and TMP.

2) The second triplets of samples were digested with cellulase (as described below) prior to scintillation counting, intending quantitative recovery of the total amount of added radioactive material.

3) The paper squares were first washed (as described below) prior to cellulase digestion in order to evaluate the total amount of converted TMP. Furthermore, a triple set of 50 μl of each reaction mixture was transferred to 1.5 cm $\times$ 1.5 cm DEAE-paper squares, dried, washed and counted in order to determine the self-absorption losses of TMP.

Washing procedure. The DEAE-paper squares were washed twice with 8 ml 4 mM ammonium acetate and once with 8 ml methanol to remove surplus labelled thymidine. Afterwards the papers were dried at 70°C.

Cellulase digestion. 4 ml of 75 mM acetate buffer pH 5.0 containing 10 mg cellulase (10 units per mg solid) was added to the dried paper disk in the scintillation vial and shaked for 1 hour at 37°C. After completion of the digest, 8 ml of scintillation liquid was added to each vial and the samples were counted in a Beckman LS 6500 scintillation counter.

Enzyme kinetic. The kinetic constants of HSV1 TK were determined by measurement of initial velocities. Reaction mixtures containing 50 mM Tris pH 7.2, 5 mM ATP, 5 mM $MgCl_2$, 2 mg/ml BSA, 0.11 ng enzyme and various concentrations of [^{3}H]-thymidine (0.01-1 μM) in a final volume of 33 μl were incubated at 37°C. Samples of 5 μl were taken every 3 minutes and inactivated by transferring them immediately onto DEAE-cellulose paper squares (0,9 cm $\times$ 0,9 cm) placed on the bottom of the scintillation vials. After drying the DEAE-paper squares at room temperature they were washed, cellulase digested and counted as described above.

RESULTS

Evaluation of the Scintillation Process

The efficiency of the scintillation process was evaluated by determination of the two quench effects, by the shift in the pulse height spectrum of the particles to lower energy (H-number) and by reduction in the measured CPM of the sample (count efficiency). The Beckman quench monitor used in this study is based on the ''external standard'' method, which relies on the analysis of a specific region of the Compton spectrum generated by a [^{137}Cs] source. Table 1 shows the corresponding values of H-number and count efficiency of directly measured, DEAE-paper adsorbed and cellulase digested samples. The cellulase digested samples showed a slightly increased quench indicated by a increased H-number and a decreased count efficiency. Undigested DEAE paper discs do not appear to contribute much additional quenching compared to samples directly pipetted into the scintillation liquid which is congruent with the results from Ives et al. (3).

Each sample (background samples excluded) was counted to a 2-sigma (2σ) value of 2%. In other words, 95% of the measurements were within the range of 2% relative standard deviation. Thus, each sample was counted to the same count precision.

Validation of the Cellulase-Digestion Method

The values of the comparison between the methods without and with cellulase digestion of the DEAE-paper discs with adsorbed tritiated nucleotides, which results in a homogenous

TABLE 1
Quenching Effect of Different Sample Preparations Indicated by Count Efficiency and H-Number

	H-number	Count efficiency in %
direct (no disc)	48.3 ± 0.9	56.2 ± 0.2
on disc	49.0 ± 0.8	56.0 ± 0.2
digested disc	91.6 ± 2.2	46.4 ± 0.5
with previous washing to remove surplus thymidine (determination of TMP conversion)		
on disc	48.8 ± 0.7	56.1 ± 0.2
digested disc	92.2 ± 2.7	46.3 ± 0.6

solution when scintillation liquid is added, are shown in Table 2 and 3. The recovery of the tritiated thymidine and TMP (Tab.2) from the cellulase digested paper squares yielded up to 98%, whereas samples still adsorbed on undigested DEAE-paper show a value of about 11%. On the other hand, the precision of the two methods, namely direct paper-adsorbed determination and scintillation counting after cellulase digestion, correspond to each other indicated by the same range of relative standard deviation (S.D.) (1% - 6%). The results presented in table 3 indicates that the method using cellulase digestion is 12 time more efficient in recovering TMP, the targeted reaction product, than this without cellulase digestion.

Enzyme Kinetics

The modified DEAE-method with cellulase digestion of the paper disc prior to scintillation counting described in this study was applied for kinetic characterisation of Herpes Simplex Virus Type 1 thymidine kinase (HSV1 TK). Initial reaction velocities were recorded at fixed ATP and varied thymidine concentration in order to evaluate apparent K_m and V_{max}. The linear correlation of 5 samples within the time course of the corresponding thymidine concentration was between 0.998 and 0.977 (data not shown). As expected, the kinetic showed a hyperbolic velocity curve with a linear Lineweaver-Burk plot. The value for K_m was found 0.2 μM which

TABLE 2
Effect of Cellulase Digestion in Tritium Recovery without Washing Away the Unreacted Thymidine*

Reaction mixtures	Direct measurement	Adsorbed on disc	Recovery rate ''on disc'' in %	Digested disc	Recovery rate ''digested disc''
0.05 μM	46505 ± 1091	4967 ± 104	10.6	42544 ± 2838	91.5
[^{3}H]-thymidine	52801 ± 1502	5604 ± 44	10.6	49716 ± 2123	94.2
	45898 ± 575	4718 ± 210	10.3	40964 ± 1216	89.2
0.25 μM	211132 ± 4649	23499 ± 519	11.1	198878 ± 3872	94.2
[^{3}H]-thymidine	211327 ± 3418	24351 ± 477	11.5	206476 ± 9690	97.7
	209736 ± 9623	23700 ± 294	11.3	206939 ± 6790	98.7
1.0 μM	836312 ± 29430	98913 ± 1255	11.8	823975 ± 28363	98.5
[^{3}H]-thymidine	837479 ± 19324	98475 ± 944	11.8	801748 ± 10685	95.7
	835607 ± 31305	99136 ± 1673	11.9	808626 ± 22410	96.8
	25.0 ± 3.6	25.8 ± 0.9	—	38.7 ± 3.0	—
blank reaction	23.9 ± 1.7	27.3 ± 4.0	—	37.4 ± 3.0	—
	23.2 ± 2.2	27.7 ± 5.2	—	33.3 ± 1.4	—

* The presented values are the mean values out of 3 measurements.

TABLE 3
Effect of Cellulose Digestion in Thymidine Monophosphat Recovery

Reaction mixture	No.	Adsorbed on disc (**)	Digested disc	Factor of improvement
0.05 μM	1	1114.7 ± 37.0	13933 ± 649	12.5
[^{3}H]-thymidine	2	1246.0 ± 39.0	15493 ± 442	12.4
	3	971.9 ± 12.1	11850 ± 440	12.2
0.25 μM	1	3327.2 ± 171.5	36410 ± 3808	10.9
[^{3}H]-thymidine	2	3277.7 ± 52.7	39297 ± 1303	12.0
	3	3230.0 ± 64.1	40111 ± 662	12.4
1.0 μM	1	3997.9 ± 141.0	47457 ± 1127	11.9
[^{3}H]-thymidine	2	4016.4 ± 16.0	48139 ± 1527	12.0
	3	4077.2 ± 46.6	48249 ± 2446	11.8
blank reaction	1	21.8 ± 0.7	34.4 ± 4.5	—
	2	23.2 ± 0.7	35.8 ± 4.6	—
	3	21.8 ± 0.7	33.3 ± 1.9	—

** To allow a direct comparison, these data are extrapolated from a 50 μl sample (actually measured) down to a 5 μl aliquot as used in all other sample preparations.

corresponds to earlier reports (9, 10). V_{max} was found 1050 pmol/min/μg being considerably higher due to totally quantitative recovery of the reaction product TMP (10) (data not shown).

DISCUSSION

In this study we describe an improved method for total quantification of tritiated samples adsorbed on DEAE-paper discs. The procedure of DEAE-ion-exchange separation for nucleoside-kinase kinetic assays was first described by Furlong (1). In this ion-exchange paper disc method, samples of kinetic reaction mixtures were transferred to a DEAE-cellulose paper square which retained phosphorylated products. The residual substrate was removed by washing with 4 mM ammonium acetate before measuring the activity of the paper square sample. If ion-exchange paper techniques are used, the tritium labelled reaction product is firmly bound to the support media. This leads inevitably to self-absorption losses during the counting of the week β-emitter. Therefore it is recommended, whenever possible, to use [^{14}C] labelled substrate (2, 11), but even then, about 25% of the counts can be lost as a result of self-absorption by the paper (12). Nevertheless, tritium labelled substrates have been successfully used with different quantification techniques. The procedure with elution of the nucleotides prior to scintillation counting described by Ives et al. (3) often suffers in quantitative extraction. Interestingly, we could not reproduce the 6-fold increase in efficiency as reported earlier for this method. The combustion technique described by Schaumloeffel et al. (13) is a very precise but enormously time consuming and expensive procedure. The mechanical decomposition method (14) showed almost the same recovery rate (87 - 100%), but considerably high scattering samples made the procedure rather unreliable. Thus, the fast, simple and cheap cellulase-digestion method described in this study is the most adequate procedure for fully quantitative detection of tritiated materials adsorbed on cellulase matrixes.

The slight increase in quenching (tab. 1) is negligible compared to the tremendous increase in recovery rate from 11% up to 98% (tab. 2). Surprisingly, the recovery rate increases in the case of unwashed samples with increased substrate concentration in both, digested and undigested samples.

Ives et al. (3) reported a low reliability in the case of direct, paper-adsorbed determination with a S.D. value of 14%. Large deviations in the magnitude of self-absorption losses can

occur, if the scintillant partially elutes the active material. Therefore, it is essential to use a scintillant which will elute either all or none of the active material. As elution techniques are tedious and often not quantitative, it is customary to count samples in scintillants which do not elute the active material and to accept the large but reproducible loss of counts by self-absorption.

This new cellulase digestion method reveals several advantages compared with the previously applied techniques. It shows a high recovery yield and small standard deviations. The increase in sensitivity of the entire procedure by a factor of 12 reduces the consumption of tritiated substrate and thus, this new method is also superior in terms of pollution control and radiological protection.

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