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A novel assay of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity

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ABSTRACT

8-Oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) is a product of oxidative modification of dGTP, that can be misincorporated into DNA, causing AT→CG mutations. Cells are protected against 8-oxo-dGTP by 8-oxo-dGTP 5′-pyrophosphohydrolases (8-oxo-dGTP-ases) that convert it to 8-oxo-dGMP. Thus, inhibition of 8-oxo-dGTPases may lead to cancer. To elucidate the involvement of 8-oxo-dGTPases in carcinogenesis, an assay of the 8-oxo-dGTPase activity is required. This paper presents such an assay developed for Chinese hamster ovary (CHO) cells that can be applied to any biological material. It includes: (i) a convenient method for preparing 8-oxo-2′-deoxyguanosine 5′-phosphates; (ii) an HPLC/UV quantification of 8-oxo-dGTP hydrolysis products and (iii) separation of 8-oxo-dGTPase activity from interfering 8-oxo-dGTP phosphatase(s). The 8-oxo-dGTPase activity of CHO cells depends on magnesium, has a pH optimum of 8.5, K_m for 8-oxo-dGTP of 9.3 μM, and is inhibited by 8-oxo-dGDP, the product of interfering 8-oxo-dGTP phosphatases. The latter must be removed from the assayed samples by ultrafiltration through 30 kDa cut-off membranes. The method was used to test the inhibition by cadmium ions of the activity of 8-oxo-dGTPase in CHO cells. The cells cultured with 0.3–3 μM cadmium(II) acetate for up to 24 h had their 8-oxo-dGTPase activity suppressed in a Cd(II) concentration-dependent manner, down to 70% of the control value.

INTRODUCTION

The *mutT*⁻ mutants of *Escherichia coli*, the first discovered mutator strain of these bacteria (1), were characterized by at least a 1000-fold increase in frequency of a specific unidirectional A→C point mutation (2). The expression product of the wild-type *mutT* gene, an enzyme, hydrolyzes the canonical ribonucleoside and 2′-deoxyribonucleoside 5′-triphosphates to the corresponding nucleoside monophosphates and inorganic pyrophosphate, with great preference for dGTP (3). The enzyme hydrolyzes most specifically the products of dGTP and GTP oxidative modification, 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) (4) and 8-oxo-GTP (5,6). Thus, the high spontaneous mutation rate observed in *E.coli mutT*⁻ mutants results from the lack of 8-oxo-dGTP pyrophosphatase (8-oxo-dGTPase) activity.

8-Oxo-dGTP can be generated by endogenous oxidants arising in normal cell metabolism. Its mutagenicity results from mispairing properties of 8-oxoguanine (reviewed in 7,8). If not decomposed by MutT protein, 8-oxo-dGTP can be incorporated into DNA opposite cytosine or adenine (4,9,10). The 8-oxo-G:A mispair, if not repaired (11), may result in AT→CG transversion (12). *Escherichia coli* MutT protein also prevented DNA transcription errors by efficiently hydrolyzing 8-oxo-GTP that could be otherwise misincorporated into nascent RNA (6).

Activity of the mammalian homologues of MutT protein (13), demonstrating very similar kinetic properties (14), imply an antimutagenic function: their expression in *E.coli mutT*⁻ mutants reduces greatly (15) or reverts to normal level (16,17) the high rate of A→C mutations. Thus, one may expect that a knock-out mutation of the gene coding for 8-oxo-dGTPase should form a mutator eukaryotic cell. This, in turn, would lead to a high rate of point mutation and to high frequency of cell transformation. Also, a chronic exposure of cells (animals) to inhibitors of 8-oxo-dGTPase might elevate the point mutation rate. Such inhibitors might be carcinogenic in animals.

To investigate the potential involvement of 8-oxo-dGTPases in the mechanisms of mutagenic and carcinogenic processes, we must measure the level of the enzyme activity in cells exposed to different carcinogenic agents. Since, to our best knowledge, a reliable enzymatic assay of 8-oxo-dGTPase activity in cultured cells and animal tissues has not yet been proposed, we decided to develop such an assay. The assay was worked out on cultured Chinese hamster ovary (CHO) cells, but can be easily adapted to any biological material. The assay was then used to verify *in vivo* our former *in vitro* findings that certain carcinogenic metals, including Cd(II), inhibited the enzymatic activity of isolated bacterial (MutT) and human (MTH1) 8-oxo-dGTPases (18). We also describe synthesis and one-step purification of 8-oxo-2′-deoxyguanosine 5′-phosphates required in the procedure.

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MATERIALS AND METHODS

Chemicals

DEAE-cellulose DE52 was purchased from Whatman (England). Bovine catalase was from Boehringer Mannheim (Germany). Ascorbic acid, bovine serum albumin (BSA), dithiothreitol (DTT), sodium dihydrogen phosphate, disodium hydrogen phosphate, 2′-deoxyguanosine (dG) and its 5′- mono-, di- and triphosphates: dGMP (free acid), dGDP (disodium salt), and dGTP (trisodium salt), as well as disodium ethylenediaminetetraacetate (Na_2EDTA), hydrochloric acid, 30% (w/w) hydrogen peroxide, methanol (HPLC grade), phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, sodium chloride, triethylammonium bicarbonate (TEAB) and Tris-(hydroxymethyl)aminomethane (Tris) were from Sigma Chemical Co. (USA). Ham's F-12 nutrient mixture, fetal bovine albumin, glutamine, penicillin, streptomycin and Trypsin/Versene mixture were purchased from Biofluids Inc. (USA). Deionized, double-distilled water was used in all experiments.

Preparation of 8-oxo-2′-deoxyguanosine 5′-mono-, 5′-di- and 5′-triphosphates (8-oxo-dGMP, 8-oxo-dGDP and 8-oxo-dGTP, respectively)

All the 8-oxo-dG 5′-phosphates were prepared using the same procedure based on oxidation of the corresponding dG 5′-phosphates with H_2O_2 and ascorbic acid. Ascorbic acid (100 mg) and 50 mg of dGTP, dGDP or dGMP were dissolved in 40 ml of 20 mM phosphate buffer, pH 7.0, in a glass flask. Reaction was initiated by adding 0.5 ml 30% H_2O_2; the mixture was left in the dark at room temperature for 3 h. The reaction was terminated by adding 120 µg of catalase, followed by incubation at 37°C for 20 min with vigorous stirring. The reaction mixture was then filtered through a 0.2 µm Nylon membrane vacuum filtration unit (Nalgene, USA) and loaded on a DEAE-cellulose column (2.6 × 19.5 cm) equilibrated with 20 mM TEAB buffer, pH 8.5. The subsequent elution programs of the column at the flow rate of 2 ml/min and pH 8.5 were different depending on the 8-oxo-dG 5′-phosphate being isolated. Thus, for 8-oxo-dGTP, elution with 230 ml of 20 mM TEAB was followed by 2000 ml linear gradient of 20–160 mM TEAB and 1140 ml of 160 mM TEAB. For 8-oxo-dGDP, 190 ml of 20 mM TEAB was followed by 2000 ml linear gradient of 20–140 mM TEAB and 470 ml of 140 mM TEAB. 8-Oxo-dGMP was eluted with 80 ml of 20 mM TEAB, followed by 2000 ml linear gradient of 20–110 mM TEAB and 480 ml of 110 mM TEAB. The effluent was collected in 19 ml fractions whose UV absorbance spectrum was measured in the range of 200–320 nm. The fractions containing individual 8-oxo-dG 5′-phosphates eluted after the corresponding unreacted dG 5′-phosphate peak, exhibiting the characteristic UV spectrum of 8-oxo-dG, were pooled and evaporated to dryness at 45°C under vacuum. TEAB was removed from the preparations by co-evaporation (five times) with water/methanol (1:1). Each final 8-oxo-dG 5′-phosphate sample was dissolved in 2 ml H_2O and its concentration was determined spectrophotometrically using the molar absorbance coefficient for 8-oxo-dG and its phosphate derivatives, ϵ_{293} = 10 300 (19). The nucleotide solutions were stored at –20°C.

Cell culture and harvesting

The CHO cells, variant K1-BH4, were maintained at 37°C in 750 ml culture flasks, under air containing 5% CO_2, in 25 ml Ham's F-12 nutrient mixture supplemented with 5% fetal bovine serum, 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 µg/ml). Typically, the cells were grown to near confluence and harvested as follows: medium was removed from the flask and cells were washed three times with 15 ml of ice-cold 20 mM Tris-buffered saline, pH 7.4, and scraped to 10 ml of the same saline. The cells were recovered by 10 min centrifugation at 1500 *g* (4°C). Alternatively, the cells were harvested by trypsinization; after removal of the medium, the cells were treated for 1 min with 10 ml of Trypsin/Versene mixture, followed by 10 ml of Ham's medium. Detached cells were collected and washed three times with 15 ml of 20 mM Tris-buffered saline, each time with centrifugation at 1500 *g*, for 10 min, at 4°C.

Cell extracts for determination of 8-oxo-dGTPase activity

Typically, the cells from one culture flask were suspended in 0.25–0.5 ml of 20 mM Tris–HCl hypotonic buffer, pH 7.4, and lysed by three cycles of freezing and thawing in liquid nitrogen and water bath. Alternatively, 20 mM Tris–HCl, pH 7.4, containing 0.5 mM PMSF, 0.5 µg/ml leupeptin and pepstatin A, was used as a lysis buffer. The resulting cell lysates were ultracentrifuged for 3 h at 150 000 *g* (4°C). The supernatants, termed below as 'extracts', were stored at –70°C. To isolate the fraction of low molecular weight proteins, 200 µl of the extract was filtered through a 30 kDa cut-off, low protein-binding ultrafiltration membrane (Ultrafree-MC Filtration Unit, Millipore), with centrifugation at 3000 *g* until complete passage of the sample. The resulting through-fraction ('ultrafiltrate') was used for the 8-oxo-dGTPase activity determination either immediately or after several days storage at –70°C.

Determination of 8-oxo-dGTPase activity

The enzymatic assays were run in 0.5 ml capped polypropylene tubes in an incubation mixture of total volume of 60 µl containing 5–120 µM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl buffer (pH 6–10), other additives as specified in the Results, and 5–20 µl of the cell extract or its ultrafiltrate. In the developmental stage of the assay, 200 µg/ml of BSA and/or 5 mM DTT were also present in certain test solutions. The reaction was initiated by adding the extract or ultrafiltrate, carried out at 37°C for 30–120 min, and terminated by adding 20 µl of 50 mM Na_2EDTA. The reaction mixture was finally analyzed by HPLC. To determine the reaction time course, the volume of reaction mixture was scaled up to 600 µl, and 20 µl aliquots were analyzed by HPLC at 30 min intervals. The analyses were performed using Waters HPLC system consisting of two pumps (model 510), Waters Intelligent Sample Processor (model 710B), UV-VIS Photodiode Array Detector (model 996), Supelcosil LC-18-T column (250 × 4.6 mm, 5 µm grain; Supelco, Switzerland), controlled by a Millennium Chromatography Manager. The reaction mixtures (20 µl) were chromatographed isocratically with 100 mM NaH_2PO_4-NaOH buffer (pH 5.5)/methanol (95:5), at a flow rate of 1 ml/min. Solutions of known concentrations of 8-oxo-dGDP and 8-oxo-dGMP, ranging from 5 to 30 µM, were used for calibration. For quantitative analysis of the formed reaction products, chromatograms acquired at 293 nm were integrated.

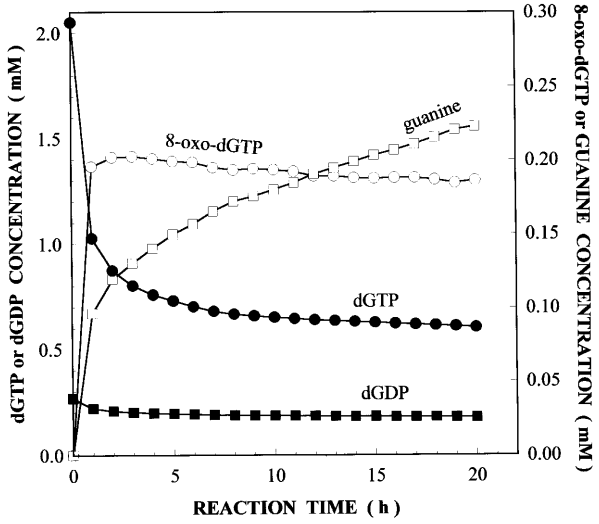

Figure 1. Formation of 8-oxo-dGTP and guanine from 2 mM dGTP reacting with 14.2 mM ascorbic acid and 133 mM H_2O_2 in 20 mM phosphate buffer, pH 7.0, at room temperature. Samples (20 µl) of the reaction mixture were analyzed by HPLC at 60 min intervals as described in Materials and Methods. The main nucleobase derivatives present in the mixture were quantified versus synthetic standards. The dGTP sample used for the reaction contained ~10% of dGDP.

Treatment of CHO cells with cadmium acetate

The CHO cells growing in 36 flasks were treated simultaneously at ~50% confluence with 1 ml of the culture medium (control group) or the same medium containing cadmium acetate to set the final cadmium concentration at 0.3, 1 or 3 µM level. Each group of cells consisted of nine separate cultures. After cadmium was added, the cells were allowed to grow for an additional 2, 6 or 24 h, at which points three cultures of each cadmium concentration were harvested with the cell scraper, lysed in 20 mM Tris–HCl, pH 7.4, and ultracentrifuged. The resulting extracts were ultrafiltered and assayed for 8-oxo-dGTPase activity.

Protein determination

Protein concentration in the cell extracts was determined in triplicate by the method with bicinchoninic acid (20). Crystalline BSA was used as a protein standard.

RESULTS

Synthesis of 8-oxo-dGTP, 8-oxo-dGDP and 8-oxo-dGMP

To establish the optimum reaction conditions for dGTP oxidation to 8-oxo-dGTP with the ascorbic acid/H_2O_2 system (14), the time course of the reaction and the effects of pH, as well as of dGTP and H_2O_2 concentrations on the product yield, were followed by HPLC. The 8-oxo-dGTP yield improved with increasing initial dGTP concentration, but only up to 2 mM dGTP. At 4 mM dGTP, the yield of 8-oxo-dGTP began to decline. H_2O_2 concentration in the reaction mixture of 100–150 mM was the best compromise between the 8-oxo-dGTP yield and reaction selectivity.

The oxidation of dGTP yielded two main UV-absorbing products, 8-oxo-dGTP and guanine (Fig. 1). Since the latter predominated in non-buffered reaction mixtures, preparatory reactions should be carried out in buffered neutral media. Under optimal reaction conditions (2 mM dGTP, 14.2 mM ascorbic acid, 133 mM H_2O_2 in 20 mM phosphate buffer, pH 7.0), the concentration of 8-oxo-dGTP reached its maximum after 3 h of incubation at room temperature (Fig. 1). The same reaction conditions were also used for oxidation of dGDP and dGMP. Dephosphorylation of nucleotides was not observed.

The individual 8-oxo-dG 5′-phosphates, formed in reaction mixtures during oxidation of dGTP, dGDP and dGMP, were purified by DEAE-cellulose column chromatography, using TEAB gradients for elution. Every 8-oxo-derivative being purified, eluted as the last UV-absorbing peak of the chromatographed mixture (Fig. 2A, B and C). This procedure allowed for obtaining high purity preparations of the nucleotides in a single chromatographic step (Figs 2 and 3). The purity of such prepared 8-oxo-dGTP, 8-oxo-dGDP and 8-oxo-dGMP was ascertained by HPLC and spectral analysis (Fig. 3). All three 8-oxo-2′-deoxyguanosine 5′-phosphate preparations had the same UV spectrum characteristic of 8-oxo-dG with the maxima at 245 and 293 nm (Fig. 3B). To further verify the identity of the synthesized 8-oxo-dGTP, it was subjected to hydrolysis by *E.coli* alkaline phosphatase, and the resulting reaction mixture was chromatographed by HPLC. This treatment led to sequential dephosphorylation of this nucleotide to 8-oxo-dGDP, 8-oxo-dGMP and finally to 8-oxo-dG, coeluting with genuine nucleotide and nucleoside standards. The yields were as follows: 8-oxo-dGTP, 6.1%; 8-oxo-dGDP, 10.4%; 8-oxo-dGMP, 4.1%.

Quantitative determination of 8-oxo-dGTP dephosphorylation products

Among the various HPLC techniques, we found reversed-phase chromatography on a Supelcosil LC-18-T column to be the best for fast resolution of 8-oxo-dGTP (retention time, t_R = 5.2 min) and all the products of its dephosphorylation, 8-oxo-dGDP (t_R = 6.3 min), 8-oxo-dGMP (t_R = 8.9 min) and 8-oxo-dG (t_R = 22.4 min), using isocratic elution with 100 mM NaH_2PO_4-NaOH buffer (pH 5.5)/methanol (95:5). The elution order of the compounds on the Supelcosil LC-18-T column depends on the number of phosphate residues in the molecules, 8-oxo-dGTP being the first and 8-oxo-dG the last eluting peak (Fig. 4).The detection limit at 293 nm for 8-oxo-dGMP was 5 pmol per injection, corresponding to 0.25 µM 8-oxo-dGMP when 20 µl of the reaction mixture was analyzed. It means that hydrolysis of 0.6% of the 40 µM 8-oxo-dGTP to 8-oxo-dGMP could be detected. The acquisition of the chromatograms at 293 nm ensured not only high sensitivity but also selectivity of 8-oxo-dG derivatives detection. No UV-absorbing compounds originating from the CHO cells were detected at this wavelength when typical enzymatic reaction mixtures were chromatographed.

Enzymatic hydrolysis of 8-oxo-dGTP by CHO cell extracts

Preliminary experiments revealed that at pH above 7.0, in the presence of 5 mM $MgCl_2$, 8-oxo-dGTP was hydrolyzed by the extract of CHO cells mainly to 8-oxo-dGDP (Fig. 4A). The 8-oxo-dGMP sought was a minor second hydrolysis product. To obtain the time course data for 8-oxo-dGTP hydrolysis, 40 µM 8-oxo-dGTP was reacted in the presence of 5 mM $MgCl_2$, 100 mM Tris–HCl, at pH 8.5, with the extract of CHO cells; total protein concentration in the reaction mixture was 132 µg/ml. As shown in Figure 5A, the predominant product was indeed 8-oxo-dGDP which after 330 min incubation at 37°C constituted 65% of both 8-oxo-dGDP and 8-oxo-dGMP. To determine if that 8-oxo-dGMP

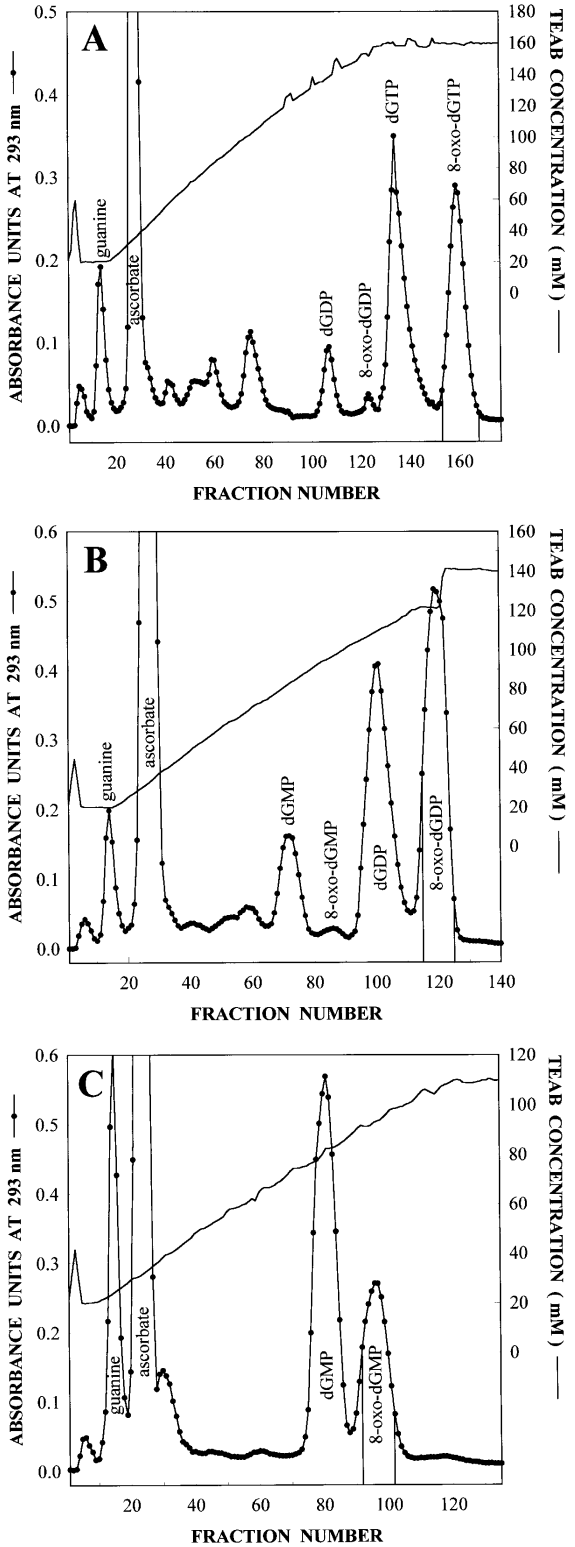

Figure 2. Purification of 8-oxo-dGTP (**A**), 8-oxo-dGDP (**B**) and 8-oxo-dGMP (**C**) by anion exchange chromatography on DEAE-cellulose column with TEAB gradient. Each 8-oxo-dG 5′-phosphate was prepared by oxidation of dGTP, dGDP or dGMP with the H_2O_2/ascorbate system. The reaction mixtures (40 ml) were loaded without preconcentration on the column equilibrated with 20 mM TEAB (pH 8.5), and eluted with gradient of TEAB (pH 8.5), as described in Materials and Methods. 8-Oxo-derivatives of the substrate nucleotides were eluted always as the last peak. Fractions of each 8-oxo-dG 5′-phosphate marked with vertical lines were pooled.

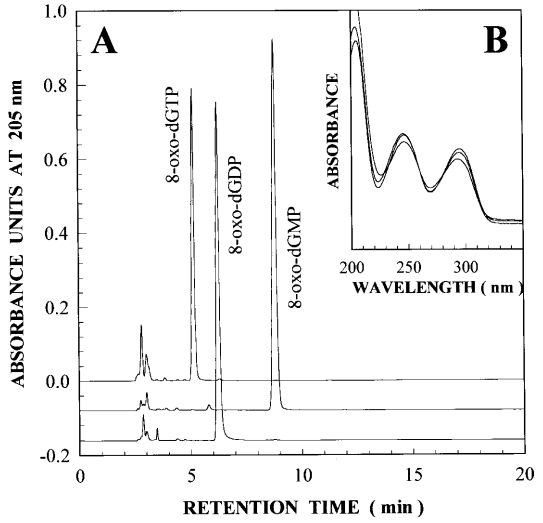

Figure 3. Purity check of the synthesized 8-oxo-dG 5′-phosphates. (**A**) Water solutions of the final preparations of 8-oxo-dGTP, 8-oxo-dGDP and 8-oxo-dGMP were chromatographed by HPLC as described in the Materials and Methods. Presented chromatograms were acquired by absorbance detector at 205 nm that ensured effective detection of UV-absorbing compounds. (**B**) UV spectra of water-diluted final preparations of the nucleotides measured in Beckman DU 68 spectrophotometer.

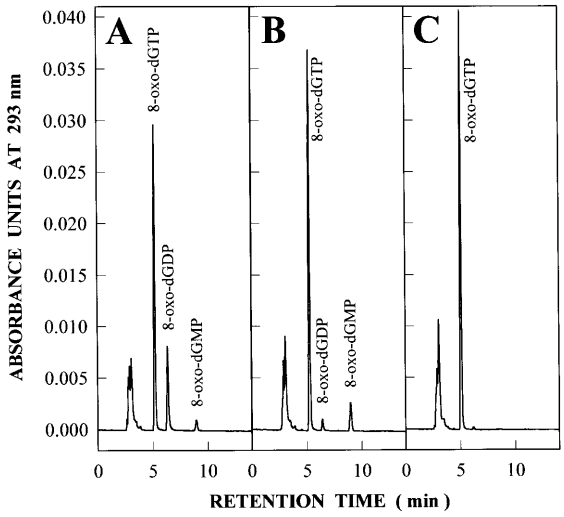

Figure 4. HPLC separation of 8-oxo-dGTP and its hydrolytic dephosphorylation products, 8-oxo-dGDP and 8-oxo-dGMP. (**A**) Chromatogram of 8-oxo-dGTP after incubation with extract of CHO cells. 8-Oxo-dGTP (40 μM), 5 mM $MgCl_2$ and 100 mM Tris–HCl, pH 8.5 were incubated with extract (7.9 μg protein) in total volume of 60 μl for 30 min. The main product of 8-oxo-dGTP hydrolysis in this case is 8-oxo-dGDP. (**B**) Chromatogram of 8-oxo-dGTP after incubation with ultrafiltrate of CHO cell extract used in (A). Reaction was carried out under the same conditions. 8-Oxo-dGMP is the main product. (**C**) Chromatogram of a control reaction mixture prepared as in (B) except that Na_2EDTA was added to the mixture before the ultrafiltrate. Hydrolysis of 8-oxo-dGTP was not detected. The peaks eluting near the column dead volume ($t_R \approx 3$ min) are produced mainly by EDTA.

originated from dephosphorylation of 8-oxo-dGDP or directly from 8-oxo-dGTP, 40 μM 8-oxo-dGDP was incubated at 37°C in the presence of 5 mM $MgCl_2$ with the same amount of CHO extract

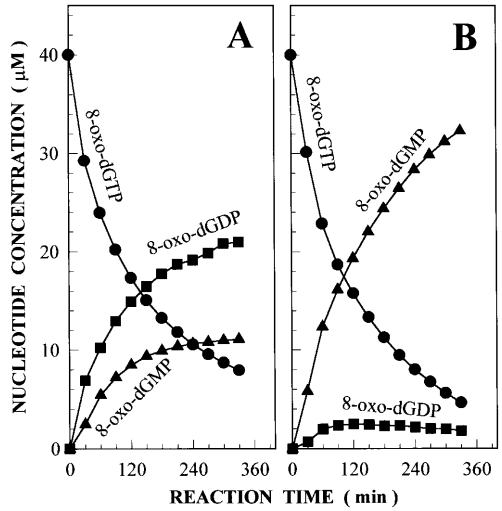

Figure 5. Time course of the hydrolytic dephosphorylation of 8-oxo-dGTP by the CHO cell extract (**A**) and its ultrafiltrate (**B**). Mixtures of 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 8.5 containing extract (100 μl, 79 μg protein) or its ultrafiltrate (100 μl) were incubated in a total volume of 600 μl. Aliquots of 20 μl were chromatographed every 30 min, as described in Materials and Methods.

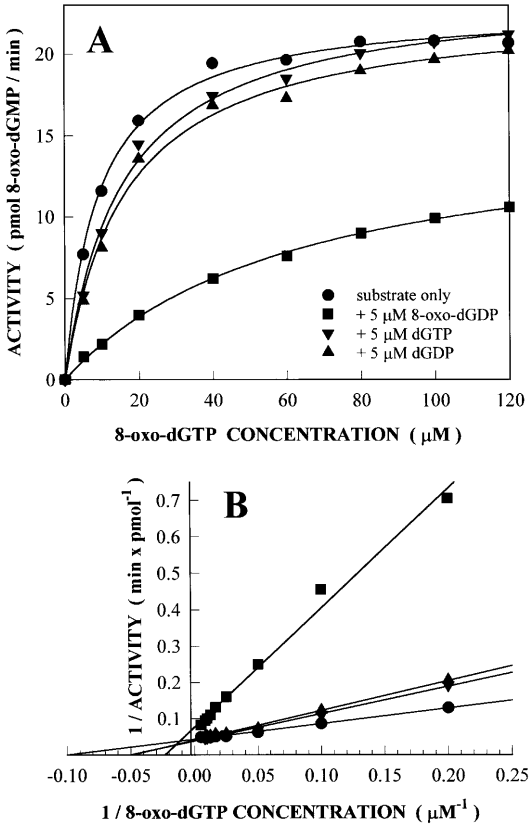

Figure 6. The substrate concentration dependence of the 8-oxo-dGTPase activity of CHO cells as determined in the absence or presence of 5 μM potential enzyme inhibitors: 8-oxo-dGDP, dGTP and dGDP. Ultrafiltrate of CHO cell extract (protein concentration in extract, 3.8 mg/ml) was incubated for 30 min at 37°C with 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 8.5 and 8-oxo-dGTP concentrations ranging from 5 to 120 μM. This set of reactions was repeated in the presence of 5 μM 8-oxo-dGDP, dGTP or dGDP. The reactions were terminated with 20 μl of 50 mM Na_2EDTA, and 20 μl aliquots of the mixtures were chromatographed to determine amount of 8-oxo-dGMP formed. (**B**) Lineweaver–Burk double reciprocal plot of the data.

as above, at pH 5–8.5. No decomposition of 8-oxo-dGDP was detected for several hours, meaning that the observed 8-oxo-dGMP was a product of pyrophosphohydrolase (8-oxo-dGTPase), and not just unspecific phosphatase activity of the CHO extract. Approximately 90% of the phosphatase activity catalyzing 8-oxo-dGDP formation was inhibited by the addition of EDTA [molar ratio EDTA/Mg(II) = 3.3].

Selective determination of the 8-oxo-dGTPase activity and kinetic properties of the enzyme from CHO cells

Upon incubation with the cell extract, 8-oxo-dGTP is rapidly consumed by the interfering phosphatase(s), making it impossible to measure reliably the 8-oxo-dGTPase activity (Figs 4A and 5A). However, 8-oxo-dGTPase activity may be separated from that of the phosphatase(s) by ultrafiltration through low protein-binding regenerated cellulose membranes with 30 kDa molecular cut-off pores, using Ultrafree MC Filtration Units, Millipore, USA (ultrafilters of some other companies gave poor results). As depicted in Figure 4B, an ultrafiltrate obtained from the CHO cell extract hydrolyzed 8-oxo-dGTP almost exclusively to 8-oxo-dGMP. Thus, the 8-oxo-dGTPase activity was associated with a small protein of molecular mass <30 kDa. This activity could be completely inhibited by adding EDTA, signifying its dependence on magnesium. Therefore, EDTA could be used for termination of the enzymatic reaction (Fig. 4C). After the addition of EDTA, neither generation of 8-oxo-dGMP nor loss of 8-oxo-dGTP could be detected in the reaction mixture for up to three days at room temperature. The pH optimum for 8-oxo-dGTPase activity was found to be 8.5.

To investigate the kinetics of the hydrolysis by CHO cell ultrafiltrate at optimum pH, 8-oxo-dGTP was incubated with ultrafiltrate prepared from the same extract used previously in the time course experiment, under the same conditions (compare Fig. 5A). As shown in Figure 5B, this time 8-oxo-dGTP was hydrolyzed predominantly to 8-oxo-dGMP, with only minor (5% in 330 min) formation of 8-oxo-dGDP. The formation of 8-oxo-dGMP was linear until ~40% of 8-oxo-dGTP was consumed. The reaction followed Michaelis–Menten kinetics (Figure 6), and the K_m value for 8-oxo-dGTP hydrolysis at pH 8.5 was 9.3 μM.

Standard reaction conditions for 8-oxo-dGTPase activity determination

The optimum reaction mixture for 8-oxo-dGTPase activity assay in CHO cells included: 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 8.5, and cell extract ultrafiltrate in total volume of 60 μl. The reaction was initiated by the addition of the ultrafiltrate (usually 10 μl of ultrafiltrate obtained from cell extract containing 0.5–3 mg protein/ml), carried out at 37°C for 30–60 min, and terminated by addition of 20 μl of 50 mM Na_2EDTA. Blank samples were prepared by adding Na_2EDTA before ultrafiltrate, followed by incubation at 37°C. Under these 'standard reaction conditions', 8-oxo-dGTPase was almost completely saturated with the substrate (Fig. 6A), and the substrate hydrolysis did not

exceed 25%. The activity unit (U) was defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per min under standard reaction conditions. The enzyme activity was linearly proportional to the amount of ultrafiltrate added to the reaction mixture. To compare different cell extracts, their activities determined in ultrafiltrates were expressed in relation to protein concentrations in the corresponding extracts (protein concentrations in the ultrafiltrates were too low for reliable determination). It means that activity of 10 µl of an ultrafiltrate was divided by the amount of protein (in mg) present in 10 µl of the extract from which the ultrafiltrate was prepared. The 8-oxo-dGTPase activity in stored ultrafiltrates was relatively stable in time. Not more than 20% loss of the enzyme activity was observed when the ultrafiltrate was left at room temperature for 64 h without addition of any protease inhibitors or protein stabilizers (data not shown).

The effects of dGTP, dGDP and 8-oxo-dGDP on the activity of 8-oxo-dGTPase

The influence of 5 µM dGTP, dGDP or 8-oxo-dGDP on the K_m and V_{max} parameters of 8-oxo-dGTP hydrolysis by the CHO cell ultrafiltrate was investigated. As found, both dGTP and dGDP were weak competitive inhibitors of 8-oxo-dGTPase activity, increasing the K_m value from 9.3 to 15.5 and 16.4 µM, and causing negligible changes of V_{max} from 22.9 to 23.9 and 23.0 pmol/min, respectively (Fig. 6). In contrast, 8-oxo-dGDP quite considerably affected both K_m and V_{max} of the 8-oxo-dGTPase activity. In the presence of 5 µM 8-oxo-dGDP, K_m increased to 61.7 µM and V_{max} decreased to 15.9 pmol/min (Fig. 6). The influence of different 8-oxo-dGDP concentrations on the activity is shown in Figure 7: 2.5 µM 8-oxo-dGDP caused a 50% and 40 µM 8-oxo-dGDP caused a 93% drop in the 8-oxo-dGTPase activity towards 40 µM 8-oxo-dGTP. Under the same conditions, 40 µM dGTP, an alternative substrate for 8-oxo-dGTPase (3), produced only a 4% inhibition. Hydrolysis of dGTP in this reaction mixture was undetectable.

Influence of cell harvesting methods, protease inhibitors and protein stabilizers on the recovery of 8-oxo-dGTPase activity

CHO cell cultures, grown in 10 flasks (started at 10^6 cells/flask), were terminated after 4 days and processed separately. The cells of five flasks were harvested manually with cell scrapers, and the remaining cells were harvested by trypsinization. The cells were further lysed in 20 mM Tris–HCl, pH 7.4, ultracentrifuged, ultrafiltered and analyzed for 8-oxo-dGTPase using standard reaction conditions. The mean enzyme activity in cells harvested by scraping was 1677 ± 185 SE (n = 5) and in cells harvested by trypsinization the activity was 865 ± 68 SE (n = 5) U/mg protein. Thus, the trypsinization procedure yielded only 50% of the 8-oxo-dGTPase activity that could be recovered from the same number of CHO cells by the scraping method (P = 0.015).

To compare the influence of protease inhibitors on the recovery of 8-oxo-dGTPase activity, 16 cultures of CHO cells were harvested by scraping. Eight of the collected cell samples were lysed by freezing and thawing in 20 mM Tris–HCl, pH 7.4, and the remaining samples were lysed in 20 mM Tris–HCl, pH 7.4, containing 0.5 mM PMSF and 0.5 µg/ml pepstatin A and leupeptin. The ultrafiltrates were analyzed for 8-oxo-dGTPase activity under standard reaction conditions. The mean activity in the cells lysed without protease inhibitors was 1206 ± 45 SE (n = 8) U/mg protein, while that in the cells lysed with PMSF, leupeptin and pepstatin A was significantly lower, 790 ± 49 SE (n = 8) U/mg protein (P = 0.0002). Hence, in our standard assays the inhibitors were not used.

Addition of 200 µg/ml of BSA and/or 5 mM DTT to reaction mixture had no significant effect on the 8-oxo-dGTPase activity.

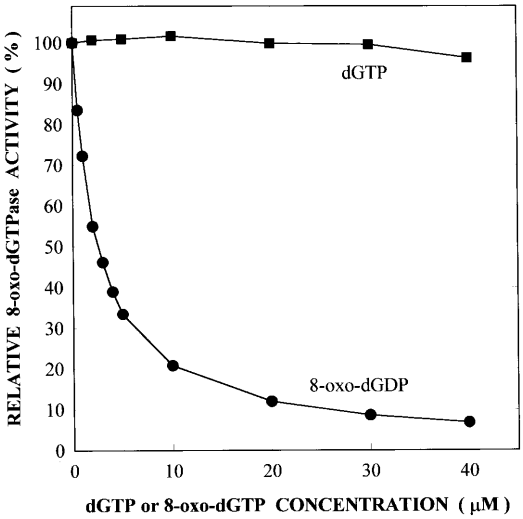

Figure 7. Inhibitory effect of 8-oxo-dGDP and dGTP on the activity of 8-oxo-dGTPase from CHO cells. The enzyme activity in the ultrafiltrate was determined under standard reaction conditions except that the reaction mixtures contained increasing concentrations of 8-oxo-dGDP or dGTP ranging from 0 to 40 µM. Neither 8-oxo-dGDP nor dGTP decomposed under these conditions.

Validation of the 8-oxo-dGTPase activity assay

The following conditions were found to be essential for the optimal analytical procedure of 8-oxo-dGTPase activity determination in cultured cells: (i) manual harvesting of the cells with cell scrapers; (ii) lysis of the cells in three cycles of freezing and thawing in the presence of hypotonic buffer, 20 mM Tris–HCl, pH 7.4; (iii) ultracentrifugation of the lysed cells (3 h at 150 000 *g*; 4°C) and determination of protein concentration in the resulting extract; (iv) ultrafiltration of the extract through 30 kDa molecular weight cut-off, low protein binding cellulose filters and (v) determination of the 8-oxo-dGTPase activity under standard conditions and quantitation of 8-oxo-dGMP formed by HPLC with UV detection.

The reproducibility of 8-oxo-dGTPase activity assay was investigated at different stages of the analytical procedure. Several independent experiments, based on seven repetitions (n = 7), were completed in order to estimate the contribution of a particular analytical step to the final standard error value of the assay. The results showed that the relatively least reproducible step of the entire procedure, determining the precision of the assay, was the ultrafiltration step (3.8% SE of the mean value), the HPLC analysis being the most precise step (0.18% SE). Overall, the standard error (n = 3) of the assay used to evaluate cadmium effects in CHO cells (below) varied from 1.6 to 15% of the mean values.

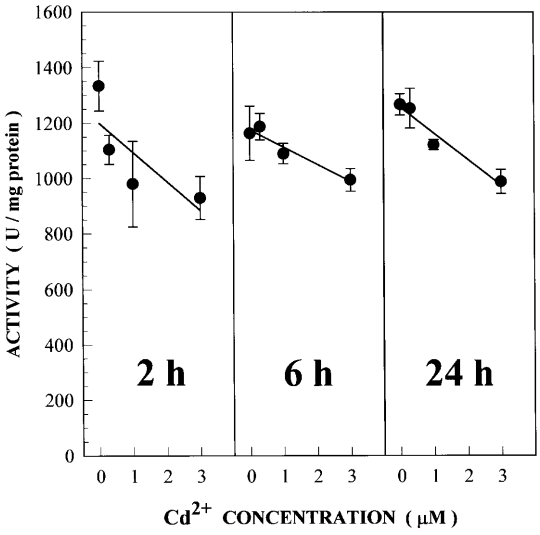

Figure 8. Activity of 8-oxo-dGTPase in CHO cells grown with 0, 0.3, 1 or 3 μM cadmium acetate for 2, 6 and 24 h. The error bars represent standard error values for three independent cultures treated in the same way. Linear regression lines were drawn to express the trend of activity to decrease with increasing cadmium concentration.

An example of the assay application: effect of cadmium on the activity of 8-oxo-dGTPase in CHO cells

Cadmium chloride has been shown previously to strongly inhibit isolated bacterial and human 8-oxo-dGTPases (18). To test this effect *in vivo*, the present assay was used to investigate the influence of cadmium ions on 8-oxo-dGTPase activity in cultured CHO cells. The cells were grown in the presence of 0.0, 0.3, 1.0 or 3.0 μM $CdCl_2$ in the culture medium for 2, 6 and 24 h, and then washed, harvested, and assayed for 8-oxo-dGTPase activity as described previously. As shown in Figure 8, an observed sequential decrease of 8-oxo-dGTPase activity correlated well with the increasing concentration of Cd(II). After the 2, 6 and 24 h incubation with 3 μM Cd(II), the enzyme activity decreased to 66, 89 and 78% of the control value (cadmium-free cultures), respectively. Analysis of variance (ANOVA) revealed that the observed cadmium concentration-dependent decrease of 8-oxo-dGTP pyrophosphatase activity was statistically highly significant for the 6 and 24 h treatments ($P = 0.013$ and 0.0006, respectively), and only weakly so for the 2 h exposure ($P = 0.076$).

DISCUSSION

8-Oxo-dGTPase is an enzyme hydrolyzing 8-oxo-dGTP, the strongly mutagenic (12) substrate for DNA synthesis. Thus, any factor causing a decrease of the 8-oxo-dGTPase activity could be potentially mutagenic and carcinogenic. To investigate the role of 8-oxo-dGTPase in carcinogenesis, a reliable assay of this enzyme's activity *in vivo* is essential. The analytical procedure for selective 8-oxo-dGTPase determination in CHO cells, developed in the present study, constitutes such an assay.

Pure preparations of the enzyme substrate and product are indispensable for any successful enzymatic activity determination. Two general approaches were reported for the 8-oxo-dGTP and 8-oxo-dGMP synthesis: a multistep chemical synthesis starting from 2′-deoxyguanosine (21) or a direct oxidation of dGTP and dGMP at the C8 position by oxygen radical-generating systems, such as ascorbic acid/EDTA/Fe^{2+} (12), ascorbic acid/O_2/Fe^{2+} (22), ascorbic acid/EDTA/Fe^{2+}/O_2 (23) or ascorbic acid/H_2O_2 (14). After trying the other approaches, we decided to adopt and optimize the method of dGTP and dGMP oxidation described by Mo *et al.* (14). In all methods based on oxidation of dG nucleotides, the main problem is the separation of their 8-oxo-derivatives from unreacted substrates and many other unidentified products. Previously described methods of 8-oxo-dGTP purification applied several steps of chromatographic separation, usually consisting of a combination of low-pressure anion exchange chromatography on DEAE-Sephadex followed by subsequent two steps of reversed-phase HPLC separation (12,22,23) or anion-exchange HPLC on Spherisorb SAX column with subsequent low pressure separation on DEAE-MemSep anion-exchange chromatographic cartridge (14). In contrast, our procedure allows purification of several milligrams of 8-oxo-dGTP, 8-oxo-dGDP or 8-oxo-dGMP in a single chromatographic run on DEAE-cellulose column (Fig. 2).

Another critical requirement of an enzymatic assay is precise quantification of the reaction product. The oldest (4,14) and most frequently used method of 8-oxo-dGTPase activity determination applies the radiolabeled [α-^{32}P]8-oxo-dGTP. The enzymatic reaction product ^{32}P-8-oxo-dGMP is separated from the substrate by thin layer chromatography and quantified autoradiographically (24). The inconveniences of this method include the synthesis of the short-lived radiolabeled and unlabeled 8-oxo-dGTP and low precision of autoradiographic quantitation. In a more recent HPLC method, 8-oxo-dGMP was separated on a Beckman Ultrasphere C_{18} column and measured electrochemically (21). However, the instability of the electrochemical detector made this method inconvenient for use with our reaction mixtures, containing many electrochemically-active constituents. In the present method we used the Supelcosil LC-18-T column, which ensures excellent separation of all the products of 8-oxo-dGTP hydrolysis. For detection and quantification of 8-oxo-dG phosphates, we used a photodiode array UV-VIS detector that offered very low background noise level of ~1×10^{-5} absorbance units, allowing very sensitive detection of 8-oxo-dG and its phosphates (minimum 5 pmol/injection).

The 8-oxo-dGTPase activity assays described to date can be successfully used only with purified enzymes and should not be applied to mammalian cell or tissue extracts. Upon incubation with such extracts, 8-oxo-dGTP is hydrolyzed to 8-oxo-dGDP. The presence of 8-oxo-dGTP phosphatase activity, hydrolyzing 8-oxo-dGTP to 8-oxo-dGDP in the presence of magnesium ions in alkaline solutions, was previously demonstrated in human (14,25) and mouse cells (17). This activity drastically lowers the substrate concentration for 8-oxo-dGTPase in reaction mixture. Moreover, we showed that 8-oxo-dGDP is a strong inhibitor of the 8-oxo-dGTPase activity. This observation also has another practical implication: the 8-oxo-dGTP preparation for 8-oxo-dGTPase assay should be virtually free of 8-oxo-dGDP. Because the latter is also produced by non-enzymatic decomposition of 8-oxo-dGTP in solution, long term storage of 8-oxo-dGTP preparation should be avoided. As little as 5% 8-oxo-dGDP in 8-oxo-dGTP preparations decreases the observed activity of 8-oxo-dGTPase by ~50%.

Considering the above, it is obvious that in order to be accurately determined, the 8-oxo-dGTP pyrophosphatase activity must be separated from 8-oxo-dGTP phosphatase activities. To achieve this, we propose the use of 30 kDa cut-off ultrafiltration

membranes for fast separation of both activities. Apparently, the molecular mass of the interfering phosphatase(s) is >30 kDa, in contrast with that of 8-oxo-dGTPase(s) having molecular mass of 18 kDa, as found in humans (14), mice (17) and rats (16). The following characteristic features confirm the identity of the CHO cell 8-oxo-dGTPase with other mammalian MutT homologues: (i) molecular mass below 30 kDa; (ii) total Mg(II)-dependence of the enzyme activity (Fig. 4C); (iii) maximum activity at pH 8.5 and (iv) K_m = 9.3 µM for 8-oxo-dGTP hydrolysis at pH 8.5 (Figure 6). In comparison, the purified human enzyme has been characterized as 18 kDa protein, demonstrating Mg(II)-dependent activity towards 8-oxo-dGTP with pH optimum of 8.0 and K_m = 12.5 µM (14). We also characterized the human enzyme from cultured fibroblasts: the pH optimum was 8.5 and K_m for 8-oxo-dGTP hydrolysis was 8.5 µM (unpublished results).

The present method of 8-oxo-dGTPase separation by ultrafiltration enables kinetic studies of the enzyme and estimation of the total enzyme activity in cells. It should be noted, however, that this method is not perfect since some of the interfering 8-oxo-dGTP phosphatase may get through the membrane (Figs 4B and 5B) and, possibly, the membrane may retain some of the 8-oxo-dGTPase present in cell extract. Both will result in underestimation of 8-oxo-dGTPase activity. In effect, the ultrafiltration step is a critical factor for reproducibility of the whole analytical procedure. Individual ultrafiltration membranes present some differences in their ultrafiltration capacity. Therefore, at least three separate ultrafiltrates for each enzyme activity determination should be prepared from an individual cell extract. Also, the extracts in which 8-oxo-dGTPase activity is to be compared should not differ substantially in total protein concentration because the ultrafiltration efficiency can be affected by total amount of macromolecules applied onto the ultrafiltration membrane.

In the formerly proposed assays, BSA and DTT were used in the reaction media as 8-oxo-dGTPase stabilizers (14,16,17). In our investigations, neither of these additives increased the observed activity of the CHO enzyme under standard reaction conditions. Also, the activity of 8-oxo-dGTPase in ultrafiltrates kept for days at room temperature without these stabilizers was stable. Therefore, there was no reason to include BSA and/or DTT in the cell lysis buffer or the reaction medium. We compared two methods of cell harvesting, scraping and trypsinization, for the recovery of 8-oxo-dGTPase from CHO cells. The results indicate that trypsinization should be avoided since it lowers by 50% the recovered enzyme activity in comparison with that obtained from cells harvested by scraping. PMSF, leupeptin and pepstatin A are protease inhibitors widely used as additives in cell lysis and homogenization buffers to preserve the activity of extracted enzymes. However, since a mixture of these three protease inhibitors decreased the recovery of 8-oxo-dGTPase activity by 35%, we did not use these compounds in our standard procedure of cell processing.

To demonstrate the usefulness of our 8-oxo-dGTPase activity assay, the assay was used to investigate the influence of *in vivo* treatment with cadmium ions on the enzyme activity in CHO cells. We observed a decrease in enzyme activity with increasing concentration of Cd(II) ions ranging from 0 to 3 µM after 2, 6 and 24 h of treatment. These results accord with previously published data (18) demonstrating the inhibition of purified *E.coli* and human 8-oxo-dGTPases by Cd(II) *in vitro*. The inhibitory effect of cadmium ions on the activity of 8-oxo-dGTPase provides one possible mechanism involved in the introduction of the 8-oxo-dG lesion into DNA, observed by others in cadmium treated cells (26) and thereby could explain the mutagenic and carcinogenic potential of this metal (27). This conclusion might be supported by the presence of the characteristic AT→CG transversions among the most frequent point mutations in CHO cells exposed to cadmium (28).

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