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UV Absorption as an Approximation for Cell Number in *In Vitro* Toxicity Testing

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Much of the early in vitro toxicological data were expressed in effect per Petri dish assuming that, initially, equal number of cells per dish was plated. For most of the toxicological studies, cell counting was done with a hemocytometer and cell plating by manually dispensing aliquots of a cell suspension unto dishes. Both procedures were known to have large inherent experimental errors.

In order to eliminate the large variation caused by uneven cell dispensing and plating, it is essential to express a toxic effect on a per cell basis. In addition to the use of a hemocytometer or an electronic cell counter, many other means have been described in literature to estimate cell numbers, such as using DNA content or measuring cellular protein content directly or indirectly. To measure DNA, a fluorescence technique is usually employed (Johnson-Wint and Hollis 1982; Sorger and Germinario 1983; Downs and Wilfinger 1983; Richards et al. 1985; West et al. 1985), while protein is measured either directly by the Lowry method (Lowry et al. 1951) or indirectly by measuring the protein-bound dye (Skehan and Friedman 1985; Borenfreund and Puerner 1985). Both approaches not only require a special reagent but are also time consuming. Therefore, a simple, rapid, and reproducible method is highly desirable for short-term in vitro toxicity testing.

In characterizing three different cell cultures biochemically, an UV absorption at 260 nm of a cell solution was found to be consistently proportional to the total cell number involved. It was found that cell growth curves monitored by A260 closely resembled those monitored by cell number or protein content or even to one monitored by the increase in the intensity of amide II infrared absorption band. When this parameter, A260, was incorporated in the determination of inhibition of DNA synthesis, the toxicity ranking of three direct ethylating agents and a group of inorganic salts was found to be comparable to what has been reported in literature.

MATERIALS AND METHODS

(Methyl-3H) thymidine (sp. act. 65 Ci/mmol) was obtained from ICN Chemical and Radioisotope Division (Irvine, CA). Sodium chloride, sodium citrate, sodium arsenite, potassium dichromate, glacial acetic acid, perchloric acid, and chloroform were purchased from J. T. Baker Chemical Co. (Phillipsberg, NJ). Potassium

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chromate was from Fisher Scientific Co. (Fairlawn, NJ). Nickel chloride, cadmium chloride, copper chloride, cobalt chloride, and zinc chloride were from Alfa Division Ventron Co. (Danvers, MA). N-ethyl-N-nitrosourea (ENU), ethyl methanesulfonate (EMS), and N-ethyl-N'-nitro-N-nitroso guanidine (ENNG) were from Pfaltz and Bauer, Inc. (Stamford, CT). Ribonuclease, proteinase K, sodium hydroxide, Trizma base, sodium dodecyl sulfate (SDS), and isoamyl alcohol were from Sigma Co. (St. Louis, MO). Methanol was from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Aquasol II was from New England Nuclear (Boston, MA).

Chinese hamster ovary (CHO) cells were obtained as a gift from Dr. A. W. Hsieh of Oak Ridge National Laboratory (Oak Ridge, TN). CHO cells were maintained at 37°C in Ham's F-12 nutrient medium (Flow laboratories, McLean, VA) buffered with 25 mM HEPES (Sigma) and supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT) and 5.0 μg/mL gentamicin (GIBCO Laboratories, Grand Island, NY).

Human lung fibroblasts (CCD-18 Lu) were purchased from American Type Culture Collection (Rockville, MD) at passage 3, then cultured in Earle's balanced salt solution supplemented with 1.5 x essential amino acids and 1.5 x BEM vitamins (Gibco). The medium was supplemented with 10% FBS and 0.1% gentamicin.

Normal human epidermal keratinocytes (NHEK) were obtained from Clonetics Corp. (Boulder, CO) and maintained in the keratinocyte growth medium CC-3001 (KGM, serum free, Clonetics).

Cells were grown in T-25 Corning flasks or Costar 35 mm six-well plates (Costar, Cambridge, MA) under 95% air and 5% CO_2 in a Napco 6200 Automatic CO_2 water-jacketed incubator (National Appliance Co., Portland, OR). Subcultures were carried out at one to three splits when cells reached a 85-95% confluency by 0.05% trypsin - 0.02% EDTA (GIBCO) in phosphate-buffered saline (PBS). NHEK cells were subcultured only once as recommended by the supplier.

Cells of various number, as described in Table 1, were collected in a centrifuge tube, washed twice with cold calcium magnesium free phosphate buffered saline (CMF-PBS), extracted twice with cold methanol : acetic acid (3:1) to remove free nucleosides and nucleotides, and solubilized with 0.2 N NaOH solution at million cells per mL. The solubilization was carried out at 37°C for 24 hours. UV scanning of each cell solution, from 200 to 320 nm, was accomplished with a Shimadzu 160 UV/Vis spectrophotometer. All spectra had an absorption maximum of 260 nm. The absorbance at 260 nm (A260) was also determined for each cell solution. For comparison, DNA of a CHO cell pellet was isolated by a modified 2 molar NaCl and isoamyl alcohol : chloroform method (Chang et al. 1979; Chilina et al. 1976) and dissolved in 0.05 M Tris pH 7.4 buffer and scanned. Also, in the process of DNA isolation, an aqueous solution of a RNA and DNA mix was obtained and scanned.

To monitor cell growth, CHO cells were seeded in a 24-well plate at a cell density of 1×10^4 cell/2 cm^2. The cell count of each well, 3 wells per day, was determined by trypan blue exclusion method using a hemocytometer for 8 days. Cells were

detached with 0.4 mL of a trypsin/EDTA solution which was then neutralized with 0.1 mL of the complete growth medium. In a parallel study, CHO cells were seeded at the same cell density in 35 mm dishes. Consecutively for 8 days, 3 dishes per day were terminated for the preparation of cell solution (1 mL/dish) as described. UV absorbance of each cell solution was determined at 260 nm (A260). Then, in duplicate, 0.3 mL of the same cell solution was used for protein determination by Lowry's method (1951).

In another cell growth study, a higher cell density was seeded. The growth was monitored hourly instead of daily. CHO cells at 2 x 10^5/2 mL were seeded on a germanium crystal and cell growth was monitored hourly in situ by Fourier transform infrared spectroscopy (FTIR) at 1546 cm^{-1}, the Amide II band, as described previously (Hutson et al. 1988). A parallel study was carried out in 35 mm dishes with an initial cell density of 2 x 10^5/8 cm^2. At hourly intervals, as shown in Figure 3, three dishes per point were processed and determined for A260.

To study inhibition of DNA synthesis, CCD-18 Lu, human lung fibroblasts were seeded in 6-well plates at a density of 2 x 10^5/well. Twenty-four hours later, cells in log phase were treated with various toxicants dissolved in saline for one hour before the addition of 3.0 μCi/well of 3H-thymidine for 45 minutes. Cells were then processed as described and dissolved in 1.0 mL 0.2 N NaOH. The A260 of each cell solution was determined. An aliquot of each cell solution was then neutralized with diluted acetic acid and assayed for radioactivity by a Beckman LS-7800 liquid scintillation counter using Aquasol-2 scintillant. 3H-Thymidine incorporation was expressed as cpm/A260. Inhibition was calculated as one minus 3H-thymidine incorporated for a treatment over its vehicle control.

RESULTS AND DISCUSSION

Aqueous DNA solution has an UV absorption maximum of 260 nm. In the process of DNA isolation, the addition of solid NaCl at a final concentration of 2M dissociated all of the cellular and nuclear proteins from the nucleic acids (Chilina et al. 1976). High speed centrifugation of 10,000 x g then separated the RNA and DNA from the protein - SDS complexes. An UV scan of the nucleic acids solution prepared from CHO cells is shown in Figure 1. Also shown in Figure 1 is the UV spectrum of a CHO cell solution dissolved in 0.2 N NaOH. Both spectra show a maximum absorption of 260 nm. A similar spectrum (not shown) was also obtained for the DNA solution. All of these strongly supported the intention to use UV absorbance at 260 nm (A260) as a measure of cell number. Similar type of spectra were also obtained for human lung fibroblasts and human epidermal keratinocytes.

When one, two, and three- million cells of CCD-18 Lu were extracted with methanol and acetic acid (3:1), and dissolved in one, two, and three mL of 0.2 N NaOH, the A260's were found to be 1.110, 1.080, and 1.088, respectively. The coefficient of variation (CV) was calculated to be 1.37%. In doing a triplicate of three- million CHO cells, the CV was found to be 8.80%. And a repeat of this CHO cell study gave a CV of 2.04%. Two similar studies were carried out with NHEK cells and the CV's were found to be 2.58% and 1.14%. These data are summarized in Table 1.

Table 1. UV absorption versus cell number

Cell No./x mL	A260	mean±SD	CV (%)
CCD-18 Lu			
1 x 10^6/mL	1.110		
2 x 10^6/2 mL	1.080	1.093±0.015	1.37
3 x 10^6/3 mL	1.088		
CHO			
I. 3 x 10^6/3 mL (n=3)		0.602±0.053	8.80
II. 3 x 10^6/3 mL (n=3)		0.588±0.012	2.04
NHEK			
I. 2 x 10^6/2 mL	1.174		
3 x 10^6/3 mL (n=2)	1.176; 1.235	1.201±0.031	2.58
4 x 10^6/4 mL	1.219		
II. 3 x 10^6/3 mL (n=3)		1.137±0.013	1.14

As shown in Figure 2, the growth curve depicted by A260 was almost parallel to the one depicted by μg protein per well. This was not surprising, since the protein determination was done on the same cell solution used for measuring A260. The third cell growth curve, started with an identical seeding density (1 x 10^4/2 cm^2) and obtained by counting cell number, also showed close resemblance to the protein and A260 curves. All three curves had an initial lag phase followed by an exponential growth and reached a stationary phase at about 5 days after seeding. All three curves showed a decline after day 6 post seeding. This was likely due to some aged cells, having become detached from the culture dish, being washed away in the initial PBS wash.

Shown in Figure 3 are two other CHO cell growth curves. Both were started with a higher seeding density of 2 x 10^5/2 mL than those shown in Figure 2. The open triangular growth curve was generated from one single culture of CHO cells grown on a germanium crystal and monitored hourly in situ by Fourier transform infrared spectroscopy (FTIR) at the amide II band of 1546 cm^{-1}. The open square growth curve was generated from separate CHO cultures grown in regular culture dishes and monitored hourly by A260. The two curves were largely superimposed.

In an attempt to rank the cytotoxic effect of various inorganic salts, a protocol of measuring inhibition of DNA synthesis (3H-thymidine incorporation) by dividing the radioactivity (in cpm) incorporated by the A260 of the cell solution, was developed against three frequently used direct ethylating agents i.e. ENNG, ENU, and EMS. The EC50 (the effective concentration to have an inhibition of 50%) of these three

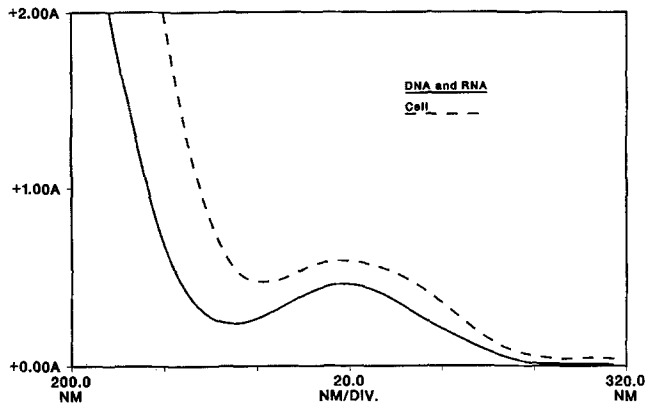

Figure 1. UV scanning spectra of a CHO-cell solution and a nucleic acids solution.

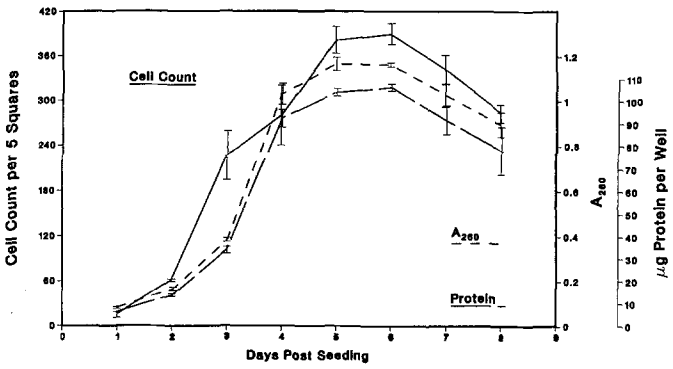

Figure 2. Growth curves of CHO-cell by monitoring the increase of (a) cell numbers, (b) protein content, and (c) UV absorbance at 260 nm.

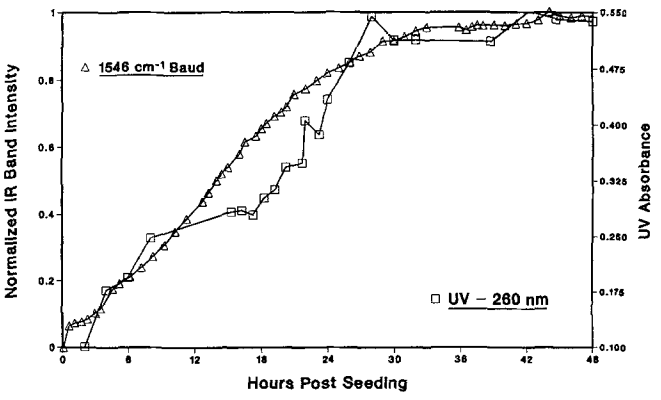

Figure 3. Growth curves of CHO-cell by monitoring the increase of (a) amide II band by FTIR and (b) UV absorbance at 260 nm.

ethylating agents was found in the decreasing order of EMS > ENU > ENNG. This was in agreement with the equalmolar toxicity reported in literature that ENNG was more toxic than ENU and ENU than EMS.

Presented in Table 2 are data of the vehicle control group of two 3H-thymidine incorporation studies. The table clearly shows that a normalization with A260 consistently decreases the CV.

By using cpm/A260 to measure 3H-thymidine incorporation, the following toxicity ranking was obtained for chloride of five divalent metals, Cd > Zn > Cu > Ni > Co and four oxy- anions, $Cr_2O_7^{-2} > CrO_4^{-2} > AsO_2^{-1} > As_2O_5$. These compared well with what was reported in literature studied by the neutral red method (Borenfreund and Puerner 1986) with one exception, that nickel chloride was less toxic than cobalt chloride. However, when cytotoxicity was evaluated by colony formation, nickel chloride was found to be more toxic than cobalt chloride (Hamilton-Koch 1986).

Table 2. Coefficient of variation for 3H-thymidine incorporation expressed as cpm/well versus cpm/A260

	cpm/well	CV, %	cpm/A260	CV, %
I.	17253	5.4	73335	1.5
	18925		72160	
	19047		74913	
II.	21552	16.7	85112	7.7
	15357		70359	
	19032		77924	

It was shown in this communication that a sodium hydroxide cell solution had a rather simple UV spectrum with a maximum absorption at 260 nm. The UV spectrum of a cell solution closely resembled the UV spectrum of its nucleic acids solution counterpart. This was true for all three cell types studied. At million- cell levels, the direct proportionality between cell number and A260 was demonstrated repeatedly (Table 1). At cell numbers one or two magnitude lower, as used in the begining of the cell growth studies (Figure 2 and 3), it was also shown that A260 was an adequate approximation to represent cell numbers.

Data presented in Table 2 strongly support the point discussed, that by doing an extra measurement of A260, the CV of the measured 3H-thymidine incorporation decreased. And usually, the smaller the CV's are, the more significant the difference will be between two study groups.

Conventionally, cells in the millions per sample were required in order to obtain a more accurate cell count using a hemocytometer or to obtain a more accurate DNA measurement by diphenylamine colorimetric method. The fluorometric quantification of DNA is much more sensitive than the A260 in representing cell numbers. An accurate measurement of DNA by fluorometric technique will consume about 5×10^3

diploid cells according to Downs and Wilfinger (1983), and about 2 to 5 x 10^4 diploid cells by Lowry's protein determination. And a one mL cell solution of about 5 x 10^4 diploid cells would give an accurate reading of A260 to approximately 0.055 (re. Table 1). If a microcuvette of a volume of less than 0.5 mL is employed, the sensitivity of A260 will increase at least two times.

In conclusion, A260 is a good approximation to represent cell number for in vitro study. The advantages to use A260 for in vitro toxicity evaluation are (1) there is no requirement of a special reagent, (2) a relatively small number of cells are needed per sample, and (3) the procedure is so simple that only one step is involved in obtaining an A260 reading versus multiple steps involved in a protein determination by the Lowry method or DNA by diphenylamine colorimetric method or by bisbenzimidazole or diamidinophenyl indole fluorometric methods.

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