IN VITRO DNA DAMAGE/ALKALINE ELUTION ASSAY FOR PREDICTING CARCINOGENIC POTENTIAL

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SUMMARY: Various noncarcinogens, ultimate, proximate and procarcinogens were evaluated for their ability to induce chromosomal damage using an *in vitro*/alkaline elution assay employing Chinese hamster V79 cells with or without a liver microsomal activation system. All of the ultimate carcinogens and most of the procarcinogens caused increased elution of DNA. None of the noncarcinogens induced DNA damage. In general, the extent of DNA damage was dose dependent.

The need for rapid and inexpensive assays to assess a compound's mutagenic and carcinogenic potential has become apparent with the increasing number of new chemicals being introduced into our environment. Investigations using the sensitive test strains of *Salmonella typhimurim* developed by Ames have indeed shown a good correlation between compounds of known mutagenicity and carcinogenicity and their ability to induce histidine revertents in the test strains (1,2). The addition of an exogenous rat liver microsomal preparation to activate procarcinogens to their ultimate form, greatly inhanced the reliability of the test. *In vitro* transformation and detecting DNA damage and repair represent the primary short term assays which employ mammalian cells. The former assay requires considerably more time than the Ames test and has been evaluated over a rather narrow range of compounds (3). *In vitro* exposure of mammalian cells to several carcinogens altered DNA sedimentation patterns in neutral and alkaline sucrose gradients (4) and induced repair synthesis of DNA (5).

We wish to report the use of the alkaline elution technique developed by Kohn, *et al* (6), to assess chromosomal damage caused by carcinogens in the

presence or absence of a liver microsomal activation system.

MATERIALS & METHODS

Materials: Eagle's minimum essential medium (MEM) and L-glutamine were obtained from Microbiological Associates. Fetal calf serum was purchased from Gibco, Inc. and [^{14}C] thymidine (TdR) was supplied by New England Nuclear Co. HEPE's buffer, NADPH, G-6-P, G-6-P dehydrogenase, Trypsin and EDTA were obtained from Sigma Chemical Co. Tetraethylammonium hydroxide (TEAH) was purchased from Aldrich Chemical Co. Other chemicals were reagent grade. Syringe filter holders (Cat. #4320-1) were obtained from the Gelman Instrument Co. which held 25 mm polyvinyl filters (2 μ) purchased from the Millipore Corp.

Methods: Crude rat or mouse liver microsomal preparation (S-9) was prepared as described by Ames (7), except the tissue was homogenized in 0.25M Sucrose containing 0.05M KPO_4, pH 7.4.

Chinese hamster lung fibroblast (V79) cells were cultured in 25 cm^2 flasks containing Eagle's MEM supplemented with 10% fetal calf serum, 2 mM L-glutamine and 20 mM HEPES, pH 7.4. The DNA was labelled by adding 0.05-0.1 μC [^{14}C] TdR. Radioactive medium was removed after 20-24 hours and the cells were incubated for 4-20 hours in non-radioactive medium. Cells were then exposed to test chemicals, either in growth medium, or in the presence of an activation system, pH 7.4, containing S-9 fraction (25 mg wet wt/ml), 1 mM NADPH, 1.5 mM G-6-P, 0.25 units/ml G-6-P dehydrogenase, and 25 mM $MgCl_2$. Cultures employing the activation system were gassed with 95% O_2. Following exposure to chemicals, the cells were washed with 5 mM phosphate-saline pH 7.4 (PBS) and trypsinized. Trypsinized cell suspensions were diluted with cold medium without calf serum (4.0 ml) and placed on ice. The cells were isolated by centrifugation, resuspended in growth medium, and frozen in liquid N_2 for up to 48 hours. As an index for cell viability, cellular ATP levels were assayed in selected cultures at the time of harvesting. A Lab-Line ATP photometer was used to measure the luciferin activity.

A modified procedure of Kohn, *et al* (6), was used to elute single stranded DNA. A single polyvinyl filter was placed in the syringe filter holder. The filter holder was filled with PBS and a 12 ml syringe was attached as a reservoir. Cells were rapidly thawed and an aliquot was resuspended in 1.0 ml of PBS and placed in the reservoir. The suspension containing 10^3-10^7 cells was pulled onto the filter using a peristaltic pump (2.0 ml/min.). An additional 2.0 ml of PBS and 6.0 ml of lysing solution containing 2M NaCl, 0.02M EDTA, and 0.2% triton X-100, pH 8.2, was pulled through the filter. Lysing solution was rinsed from the filters with 5.0 ml of 1 mM EDTA, pH 7.8, and single stranded DNA was then eluted from the filter by pulling 13.5 ml of 0.02M TEAH, 0.02M EDTA, pH 12.2, through the filter (0.5 ml/min.). Radioactivity in the last 15.0 ml of eluate and that remaining on the filter was determined. Recovery of radioactive DNA applied to the filter was greater than 95%.

RESULTS & DISCUSSION

The effect of direct exposure of V79 cells to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is shown in Figure 1. A 30-minute exposure to MNNG caused

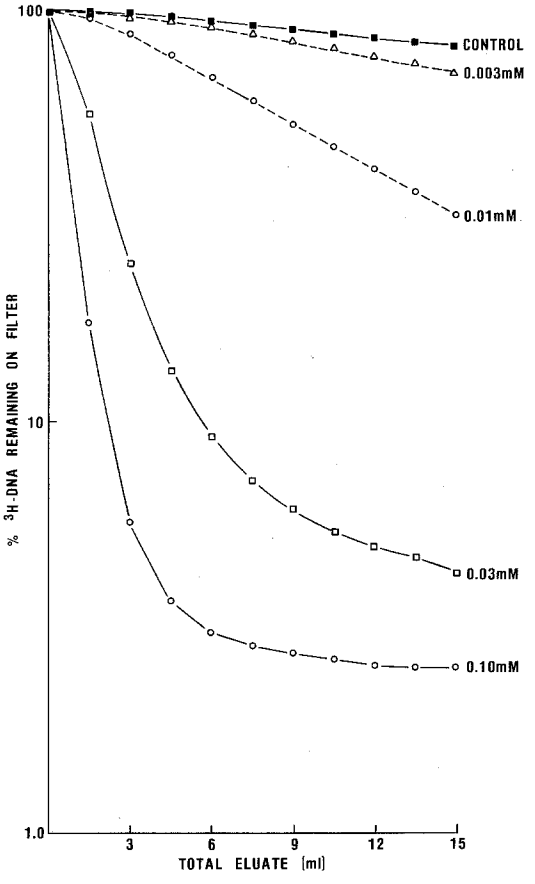

Figure 1. Rates of elution of [^{14}C] DNA from V79 cells exposed to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 minutes. Data is expressed as the per cent of the total recovered radioactivity.

dose dependent increases in the rates of [^{14}C] DNA elution from the filters. Similar increases in elution previously have been shown to be related to the size of the single stranded DNA (6). Exposure of cells to methylnitrosourea, ethylnitrosurea, methyl methanesulfonate, and ethyl methanesulfonate also gave dose related increases in the elution of [^{14}C] DNA (Figure 2a). Methylating agents caused greater elution than ethylating analogs. This increased elution does not necessarily reflect the relative carcinogenic potential of these compounds since ENU represents the most potent carcinogen shown in Figure 2a (8). In this regard, the alkaline elution assay represents

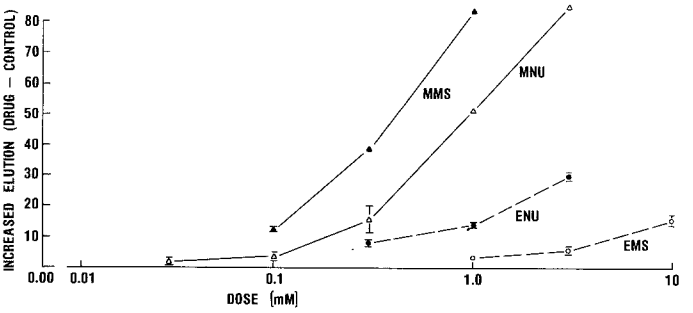

Figure 2a. Elution of [^{14}C] DNA from V79 Cells following a 2 hour exposure to alkylating agents. Methylnitrosourea (MNU) Δ——Δ; Ethylnitrosourea (ENU), •——•; Methylmethanesulfonate (MMS), ▲——▲; Ethylmethanesulfonate (EMS), ○——○. Data is expressed as the increase in elution over that observed in the controls.

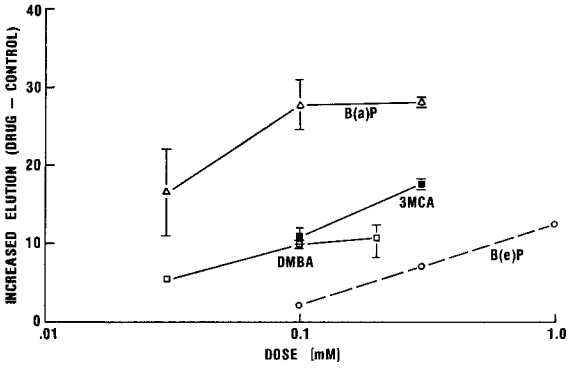

Figure 2b. Elution of [^{14}C] DNA from V79 cells following exposure to polycyclic aromatic hydrocarbons in the presence of the rat liver activation system. Benzo[a]pyrene [B(a)P] 2 hours, Δ——Δ; Benzo[e]pyrene [B(e)P] 4 hours, ○——○; 7,12-dimethylbenzanthracene (DMBA) 2 hours, □——□; 3-Methylcholanthrene (3MCA) 2 hours, ■——■. Data is expressed as in Figure 2a.

a qualitative rather than a quantitative assay for carcinogenic potential.

In the absence of the rat liver metabolic activation system, polycyclic aromatic hydrocarbons did not cause increased elution of [^{14}C] DNA. However, dose related increases in DNA elution were detected when known carcinogens of this class were tested in the presence of the activation system (Figure 2b). Two noncarcinogens, anthracene and phenanthrene, did not cause increases in the elution patterns.

Table 1: Alkaline Elution of DNA Following *In Vitro* Exposure of V79 Cells to Chemicals

Compounds	Exposure Time-Hr.	Activation System[a]	Results and Dose mM[b] 0.001	0.003	0.01	0.03	0.1	0.3	1.0	3.0	10.0
Alkylating Agents											
N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG)	0.5	-	-	+	+	+	+				
Methylnitrosourea (MNU)	1	-				-	+	+	+	+	
Ethylnitrosurea (ENU)	1	-				-	-	+	+	+	
Methyl Methanesulfonate (MMS)	1	-				-	+	+	+		
Ethyl Methanesulfonate (EMS)	1	-				-	-	-	+	+	+
β-Propiolactone	1	-				-	+	+	+	+	
Propyleneimine	1	-						+	+	+	
Dimethylnitrosamine (DMN)	2	M						+	+	+	+
Dimethylnitrosamine (DMN)	2	R						-	-	+ +	
Diethylnitrosamine (DEN)	2	M					-	+	+	+	+
Diethylnitrosamine (DEN)	2	R						-	-	+	+
Polycyclic Aromatic Hydrocarbons											
Benzo[a]pyrene [B(a)P]	2	R			-	+	+	+			
Benzo[e]pyrene [B(e)P]	4	R			-	-	+	+	+		
7,12-Dimethylbenz[a]anthracene (DMBA)	2	R			-	+	+	+			
3-Methylcholanthrene (3MCA)	2	R			-	-	+	+			
Phenanthrene	1,2,4	R			-	-	-	-			
Anthracene	1,2,4	R			-	-	-	-			
Amines											
Benzidine	2	R				-	+	+	+	+	
4-Aminobiphenyl	2	R				-	- +	+	+	+	
2-Acetylaminofluorene (2-AAF)	2	R				-	-	+	+	+	
N-Hydroxy-acetylaminofluorene (N-OH-AAF)	2	-		-	+	+	+	+	+		
N-Acetoxy-acetylaminofluorene (N-OAc-AAF)	2	-		-	+	+	+	+	+		
Analine	1,2,4	R				-	-	-	-	-	
para-Rosaniline	2	R						+	+	+	
Miscellaneous											
Aflatoxin B_1	2,4	R		+		+		+			
4-Nitroquinoline-1-oxide (4NQO)	1	R			-	+	+	+	+	+	
4-Nitroquinoline-1-oxide (4NQO)	1	-					-	-	+	+	
Safrole	1,2,4	R					-	-	-	-	
1-Hydroxysafrole	2	R,-						-	-	-	
1-Acetoxysafrole	2	-				+	+	+	+	+	
Captan	2	-			-	+	+	+	+		
Captan	2	R						-	-	-	
DDT	2,4	R					-	-	-	-	
Dieldrin	2,4	R					-	-	-	-	
Dinitrophenol	2	R,-							-	-	-
Cyclophosphamide	1,2,4	R						-	-	-	-
Ethionine	2	R						-	-	±	+
Methionine	2	R,-						-	-	-	-
Phenobarbital	2	R,-						-	-	-	-
Aspirin	2	R,-						-	-	-	-
Caffeine	2	-							-	-	-
EDTA	1,2,4	R,-							-	-	-
Other[c]	1,2,4	R,-									-

[a] R = Rat; M = Mouse; (-) = None. [b] (+) = Damage observed; (-) = No damage observed. [c] Found negative at higher concentrations: ethanol, acetone, dimethylsulfoxide, propylene glycol and benzene.

Increased elution of DNA was observed following exposure to additional ultimate, proximate and procarcinogens from other classes of chemicals. The results are shown in Table 1. While data are presented as positive or negative, dose responses were usually evident, *i.e.*, greater elution with increasing dose. None of the ultimate carcinogens required metabolic activation to induce DNA damage. 2-Acetylaminofluorine (2-AAF) required activation to induce DNA damage in the cells, whereas, the proximate and an ultimate form of the carcinogen, N-OH-AAF and N-OAc-AAF, caused increased elution of DNA in the absence of the activation system at doses 1/30th as high as 2-AAF.

Dimethylnitrosamine and diethylnitrosamine caused increased elution of DNA if either a mouse or rat liver activation system was employed. The mouse liver activation system gave dose related increases in elution for both carcinogens, whereas, the rat preparation caused significant increases in elution, but no dose response. As noted with direct acting methylating and ethylating agents, DMN caused greater elution of DNA than did DEN.

None of the noncarcinogens tested caused significant increases in the rate of DNA elution. All of the ultimate carcinogens and most of the procarcinogens induced sufficient DNA damage to be detected by the alkaline elution assay. This increased elution was detected at times when ATP levels of treated cells were similar to those of control cells. Several procarcinogens failed to cause increased elution. These include safrole, 1'-OH-safrole, DDT, dieldrin, and cyclophosphamide. In the presence of the rat liver activation system, safrole and cyclophosphamide caused increased elution. This was, however, only detected when cell viability was low. Under such circumstances the data cannot be interpreted, since endonuclease activity of dead cells can result in increased DNA elution. Direct exposure of V79 cells to Captan resulted in increased elution of DNA at concentrations of 0.03 mM and above. However, no increase in elution was detected when up to 3.0 mM levels of

Captan were assayed in the presence of the rat liver activation system.

The *in vitro*/alkaline elution assay represents a rapid, reproducible and inexpensive technique for detecting compounds with potential carcinogenic or mutagenic activity. With the exception of cyclophosphamide, compounds which have given false negatives in the alkaline elution assay have also been negative in the Ames system (1).

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