GENOTOXIC EFFECT OF HYDROQUINONE AND ELONE (MONO-METHYL-PARA-AMINOPHENOL SULFATE)

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INTRODUCTION

Since the origin of life, hydroquinone derivatives appear to be a popular group of organic chemical in the metabolic pathway of several aromatic amino acids in the first cell. As anaerobic cells evolved to aerobic or phototrophic cells, various hydroquinone derivatives are found to be very important co-factors for biological functions. Ubiquinone and Plastoquinone (Leonard et al. 1987; Weiss et al. 1987) are important coenzymes for the transport of electrons and protons across the membrane for ATP synthesis in the mitochondria and the chloroplasts. These stable and active chemicals occupy large proportions of the cell components and are expected to contribute to a large proportion of the biological waste material in the environment. In addition, both hydroquinone and its derivative, elone (Mono-methyl-para-aminophenol sulfate) are the most commonly used reducing agents in the photographic industries. It is a proposed anticancer drug and an antioxidant (Baehner et al. 1983: Packer et al. 1973: Green et al. 1973). Furthermore, hydroquinone derivatives are prevailing as popular in our living environment, pharmaceutical drug market, and important chemicals in our industries. The genotoxic safety tests of these chemicals have not been further studied. The current study is intended to investigate the toxic effect of hydroquinone and elon by 2 assay methods. The first method is to assay the quantity of DNA strandbreak by monitoring conformational change of SV-40 viral DNA in vitro (Alder et al. 1973). The second method is to assay the number of reverse mutants (revertants) of *Salmonella* test strains in vivo (Ames et al. 1975). Data obtained by the two assays will be employed for a more rational approach of estimating the effect of hydroquinone and elone to DNA and gene function.

MATERIALS AND METHODS

Bacterial Strain *Salmonella* Mutagenesis Test Strains TA98 was obtained from Dr Ames Laboratory, University of California at Berkeley.

Viral DNA *SV*-40 viral DNA was purchased from the Bethesda Research Laboratory, Life Technologies Inc. (BRL). The viral DNA was mostly in the form-I (more than 95%) and partially in the form-II (less than 5%) at the time of purchase. Electrophoresis purity grade Agarose powder was obtained from the Bio-Rad Laboratory.

DNA Strandbreak Assay

DNA strandbreak assay was performed by electrophoresis separation of SV-40 viral DNA into 3 forms: form 1, form 2 and form 3 for quantitative analysis under a densitometer (Alder et al. 1973). For electrophoresis, a TBE buffer containing 1.08% Tris-hydroxide-methyl-aminomethane (Tris-base), 0.55% Boric acid, and 0.0761% Ethylenediamine tetraacetic acid (EDTA) was prepared with pH adjusted to 7.5. A 0.8% agarose gel for electrophoresis was made by mixing 0.9 g of agarose in 115 ml TBE buffer and heated to the boiling point for complete dissolution of agarose in the solution. After the prepared agarose solution was cooled down to 60°C, it was poured into the mold (15 x 15 cm glass plate framed and sealed by tape) with a 20 well comb (teeth size for each well; 1 mm x 3 mm x 4 mm) hanging 1 mm above it. The prepared agarose plate was kept in a refrigerator until the time of use. For incubation of DNA with test chemicals, TBNE buffer containing 1.24% Tris-base, 0.2922% sodium chloride, and 0.38% EDTA was prepared. The pH of buffer was usually adjusted to 7.5 with 10 N NaOH. Fifty ml of the TBNE buffer was used to dissolve 0.4 g of hydroquinone or elone. Thereafter, the solution was diluted to the concentration of 0.2%, 0.1%, 0.05%, 0.025%, 0.0125% by sequential dilution of the original hydroquinone solution or elone solution. Ten μl of each sequentially diluted test samples were transferred into microcentrifuge tubes. After the addition of 10 μl DNA solution containing 1.7204 μg of DNA in various concentration of hydroquinone, the mixture was gently mixed by tapping and incubated for 8 hours in a refrigerator. Thereafter, 10 μl of loading medium containing 0.025% BPB, 0.025% Xylene cyanol, and 2.5% Ficol "Type 400" was added and mixed again. Finally, 15 μl of the final mixtures (sample mixed with loading medium) were gently and quietly added to the bottom of the wells in an agarose gel which was placed in a electrophoresis apparatus under TBE buffer solution. After electrophoresis for 4 to 6 hours at constant voltage of 80 DCV (40 mA), the gel was stained in Ethidium bromide solution (0.5 μg/ml water) for 4 hours or overnight in a tank. Following destaining with 1 mM magnesium sulfate for 20 to 30 minutes, the gel was placed on an ultraviolet transilluminator for fluorescent photography with the wave length of 300 nm. A Polaroid camera with Kodak #22 Wratten filter and a sensitive Polaroid film, type 55 P/N black and white film, was used for photography. A LKB Gelscan XL laser beam

densitometer was used for scanning, integration and data management of the fluorescent photograph.

Bacterial Mutagenic Assay

Bacterial mutagenic assay was performed according to the method of Ames et al (1975). A Salmonella mutant test strain, TA98 was streak and cultured in MG agar plate (1,5% agar, 2% dextrose, 0.00978% $MgSO_4$, 0.183% citric acid, 1% K_2PO_4, and 0.229% $NaHNH_4PO_4$) with the addition of histidine (260 μM), biotin (3 μM) and ampicillin (25 μg/ml) for maintenance. For preparation of frozen stocks, liquid culture of TA98 was made by shaking the culture from a single colony of tester strains to early stationary phase in 40 ml of the Oxoid Nutrient broth #2. The 40 ml culture was mixed with 7 ml glycerol and dispensed 1 ml per microtube for storage at -80°C in a deep freezer. For the mutagenecity test, one volume of frozen stocks was diluted into 80 volumes of fresh Nutrient broth #2 for culture in a shaker incubator. Following 5 to 6 hours of incubation at 30°C and 200 rpm in a shaker incubator, mid-logarithmic phase of the growth was obtained. 0.1 ml of the culture was mixed with various concentrations of hydroquinone or elone. Thereafter, 0.5 ml of salts mixture containing 0.1 mM phosphate buffer (pH 7.4), 33 mM potassium chloride and 8 mM magnesium chloride was added to the mixture. After the addition of top agar containing 0.5% saline solution, 0.6% agar, 0.5 mM histidine and 0.5 mM biotin, the mixture was poured onto the top of MG plate for incubation at 37°. The number of colonies formed by reverse mutation were recorded after 48 hours and 72 hours of incubation.

RESULTS

DNA Strandbreak Assay

Two DNA bands identified as form-1 DNA (closed circular, superhelix) and form-2 DNA (nicked circular) were obtained following electrophoresis of intact SV40 viral DNA. After densitometry, larger proportion of peak areas (more than 90%) were obtained for form-1 and smaller proportion (less than 10%) were obtained for form-2 in the intact SV40 viral DNA. As the concentration of hydroquinone or elone increased for incubation with the DNA, the proportion of form-1 DNA reduced and the proportion of form-2 DNA increased, suggesting the increase of single strand break. At higher concentration of chemicals, the new DNA band, form-3 (linear form) appeared and gradually increased its proportion suggesting the increase of double strandbreaks. The strand-breaking effect of hydroquinone was seen as the proportional increase of form-2 from 2.7% to 11.8%, and that of form-3 from 0% to 8.5%, as the concentration of chemical was increased from 71 μM to 9082 μM (Table 1). The concentration of hydroquinone estimated to reduce the form-1 to 75 % of the intact control was defined as the 25% SbED in this study. The 25% SbED for form-1 SV-40 was calculated as 24.293 mM for hydroquinone from its 2 nearest assay values in Table 1. The DNA strand-breaking effect of elone was seen during proportional increase of form-1 from 11.8 % to 83.4 %, and form-3 from 0 to 48.3 %, as the concentration of chemicals was increased from 91 μM to 5807 μM (Table 3). 25% SbED and 50% SbED for form-1 SV-40 was respectively calculated as 172 μM, and 3.149 mM for elone from its 2 nearest assay values in Table 3.

Bacterial Mutagenecity Assay

In the bacterial mutagenicity test, the least mutagenic dose of hydroquinone was estimated as the least concentration of chemical to cause the significant increase in the number of revertants. Since the increase of revertants from 20.4 (spontaneous revertants) to 31.0 followed the increase of hydroquinone from 0.0 μM (control) to 284 μM (Group 4, Day 2 revertants, Table 2) was significant. Therefore, 284 μM hydroquinone was estimated as the least mutagenic dose. Similarly, the least lethal dose of hydroquinone was estimated to be at the concentration level of 4.541 mM when the number of revertants was reduced from 20.4 (Group 1, spontaneous revertants) to 1.2 (Group 8, Day 2 revertants, Table 2). When *Salmonella* TA 98 was used for mutagenic assay of elone, a significantly increased number of revertants from 20.4 to 35.8 (mutageneic effect) was seen at 363 μM (Group 5, Day 2 revertant, Table 4). The least mutagenic dose was estimated to be 363 μM. The least lethal effect of elone was estimated to be at the concentration of 726 μM when the number of revertants was significantly reduced from 20.4 (spontaneous revertants) to 11.8 (Group 6, Day 2 revertants, Table 4).

DISCUSSION

Similar to earlier studies on restriction endonuclease (Adler et al. 1973), a very sensitive response to DNA strand-break was obtained by incubation of SV-40 DNA with either hydroquinone (Table 1) or elone (Table 3). In the radiation effect on DNA, formation of free radicals which attacked C-4' or other carbons of the deoxyribose residue was identified to be responsible for the single strandbreak (Dizdaroglu et al. 1977). As for hydroquinone, either enzymatic (Michaelis 1942) or nonenzymatic conversion (Borg 1972) of the chemical at the electron spin resonance state can lead to the formation of semi-benzoquinone radicals. It is possible that similar DNA strandbreak can be induced by the free radical formation in elone. Although DNA strandbreak can cause genotoxic effect demonstrated in mutagenesis or cell death, the capability of the chemical to go through the cell membrane to the gene sites and the affinity of the chemical to the nuclear base may play another important factor

affecting genotoxic effect. On the other hand, the effect of hydroquinone and elone to the DNA repair enzyme system can not be neglected. Because of those complicated factors affecting genotoxic effect, various indexes obtained for the DNA strand-breaking effect and genotoxic effect may not be parallel. Thus, the strand-breaking effect of elone is higher than hydroquinone [as can be seen from the lower 25% strandbreak effective dose, 25% SbED for elone (172 μM) and higher 25 % SbED for hydroquinone (24.293 mM)]. By contrast, the genotoxic effect of elone are estimated to be 363 μM in LMD (least mutagenic dose) and 726 μM in LLD (least lethal dose) for elone. These estimates are very comparable with the values which are observed for hydroquinone (284 μM in LMD and 4.541 mM in LLD). It is possible that the addition of Mono-methyl side chain with sulfate formation create the cation charge in the aminophenol for stronger binding to DNA for more active transfer of free radical formed by phenol to the DNA in vitro. With positive charge formation, elone will be less capable of penetrating through the membrane for genotoxic effect to the cell. Compared with positive control (Daunomycin), considerably lower mutagenecity of hydroquinone demonstrated in the current experiment may be considered as a physiological level of active metabolite activated free radical formation in the cell biochemical process. Nevertheless, the potential carcinogenecity of hydroquineon cannot be neglected at some extreme pathological condition. Animal experiments with similar doses of hydroquinone in the current study have induced tubal cell carcinoma of kidney in the male rat and mononuclear cell leukemica in the female rat (Kari et al. 1992).

SUMMARY AND CONCLUSION

Both hydroquinone (Para-dihydroxybenzene) and elone (Mono-methyl-para-aminophenol) are produced in large quantites in industrial concerns and consumed in the field of medicine. Varieties of hydroquinone are used as coenzymes for electron and proton transport for ATP synthesis by aerobic or phototrophic bioorganisms. Study of the genotoxic effects of hydroquinone appears to be important not only for understanding their biochemical mechanisms but also for the treatment of biological waste material in enviromental science. Two methods were employed for evaluation of toxic effects of hydroquinone and elone on the gene: (1) DNA strand-breaking effect; and (2) genotoxic effect. For the DNA strand-breaking effect, SV-40 viral DNA was used for assay in vitro. For studying the mutagenic effect, significant effects in increasing the number of revertants in the *Salmonella* mutagenecity test were assayed as mutagenic, whereas the significant effect in reducing the number of revertants following mutagenic effect were assayed as lethal effect. This study concludes that the 25% DNA SBED, the LMD, and the LLD of hydroquinone were at the levels of 24.293 mM, 182 μM, and 18.163 mM, respectively. With the same estimation, much lower 25% DNA SBED 172 μM was obtained for elone, suggesting a very high strand-breaking activity of elone to DNA. The genotoxic effect of elone, particularly lethal effect to cell, is estimated to be higher than hydroquinone with LMD 363 μM, and LLD 726 μM, respectively.

ACKNOWLEDGEMENTS

This research was supported by the Department of Energy under contract #DE-ACO#-76SF0098 (to University of California at Berkeley); subcontract #461710 to Jackson State University.

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Table 1. DNA strandbreaks induced by incubation of SV40 viral DNA (0.011μm) with hydroquinone (Incubation for 8 hours at 4°C and pH 7.5; Electrophoresis with 100 DCV and 46 mA).

Group Number	Concentration of chemical μM	(%)	Distribution of DNA (%) Form I	Form III	Form II
1	0	(0.0)	97.3	0.0	2.7
2	71	(0.000782)	94.6	1.8	3.6
3	142	(0.001563)	89.1	4.1	6.8
4	284	(0.003125)	86.5	5.0	8.5
5	568	(0.00625)	84.6	5.9	8.5
6	1135	(0.0125)	83.2	6.7	10.1
7	9082	(0.1000)	79.7	8.5	11.8

Effective dose to cause 25% strandbreak on form-1 DNA (25%SbED)=24.293 mM.

Table 2. Salmonella mutagenicity test of hydroquinone with TA98 test strains.

Group Number	Concentration of chemical μM	(%)	Number of revertants Day 2	Day 3	Obs. t** (Day 2)
1	0	(0.0)	20.4 ±3.0	35.1 ±6.0*	Control***
2	71	(0.000782)	16.8 ±2.5	29.3 ±4.8	
3	141	(0.001563)	17.5 ±2.7	33.3 ±6.2	t=-1.49
4	284	(0.003125)	31.0 ±5.4	43.5 ±8.8	t=1.99$
5	568	(0.00625)	10.3 ±2.4	20.8 ±4.4	
6	1135	(0.0125)	34.0 ±3.5	48.3 ±4.8	
7	2270	(0.0250)	17.5 ±2.5	33.8 ±4.9	t=-1.49
8	4541	(0.05)	0.0 ±0.0	0.0 ±0.0	t=-13.6$$
9	9082	(0.1)	0.0 ±0.0	0.0 ±0.0	
10	18164	(0.2)	0.0 ±0.0	0.0 ±0.0	
11	Daunomycin	(0.162 μg/100μl)	64.3 ±18.4	70.4 ±19.2	Control***
12	Daunomycin	(0.625 μg/100μl)	370.6 ±34.0	380.4 ±35.4	Control***

* Sample mean ± standard deviation = $\bar{x}_n \pm S_n$

** observed $t = [(\bar{x}_n - \bar{x}_1) - 0] / [Sp^2(1/n_1 + 1/n_n)]^{1/2}$, and
$Sp^2 = [(n_n - 1)S_1^2 + (n_1 - 1)S_2^2] / (n_n + n_1 - 2)$
observed t is compared with $t_{df=6,\ \alpha=0.05} = 1.9432$ for the t-test.

***Distilled water is used as the negative control; Daunomycin is used for the positive control.

$ Significant result for the mutageic effect.

$$ Significant result for the lethal effect.

Table 3. DNA strandbreaks induced by incubation of SV40 viral DNA (0.011 μM) with p-methylaminophenol hemisulfate. (Incubation for 8 hours at 4°C and pH 7.5; Electrophoresis with 100 DCV and 50 mA for 8 hrs).

Sample Number	Concentration of Chemical μM	(%)	Proportion of DNA (%) Form I	Form III	FormII
1	0	(0.0000000)	92.9	0.	7.1
2	91	(0.0015625)	88.2	0.	11.8
3	181	(0.003125)	67.7	10.7	21.6
4	363	(0.00625)	44.3	10.11	29.7
5	726	(0.0125)	35.0.	17.6	24.4
6	2904	(0.05)	49.2	18.1	33.3
7	5807	(0.1)	16.6	48.3	35.1

Effective dose to cause 25% strandbreak on form-1 DNA:
25% $SbED_{-}$=172.2 μM
Effective dose to cause 50% strandbreak on form-1 DNA:
50% $SbED_{-}$=3.149 mM

Table 4. Salmonella mutagenicity test of p-methylaminophenol hemisulfate with TA98 test strains.

Group Number	Chemic.concentrations μM	(%)	Number of Revertants Day 2	Day 3	Obs. t**
1	0	(0.00000)	20.4 ±3.0	35.1 ±6.0**	Control***
2	46	(0.00078125)	15.5 ±5.1	28.0 ±8.1	
3	91	(0.0015625)	18.3 ±2.7	33.8 ±4.4	
4	182	(0.003125)	21.3 ±5.4	33.0 ±7.5	t=0.29
5	363	(0.00625)	35.8 ±5.3	49.8 ±8.9	t=4.29$
6	726	(0.0125)	11.8 ±1.9	25.3 ±1.0	t=-4.22$$
7	1452	(0.025)	4.8 ±2.5	6.5 ±4.1	
8	2904	(0.05)	0.3 ±0.5	0.3 ±0.5	
9	5807	(0.1)	0.0 ±0.0	0.0 ±0.0	
10	11614	(0.2)	0.0 ±0.0	0.0 ±0.0	
11	Daunomycin	(0.162 μg/100ul)	64.3 ±18.4	70.0 ±19.2	Control***
12	Daunomycin	(0.625 μg/100ul)	370.0 ±34.0	380.0 ±35.4	Control***

* Sample mean ± standard deviation = $\bar{x}_n \pm S_n$
** observed $t = [(\bar{x}_n - \bar{x}_1) - 0] / [Sp^2(1/n_1 + 1/n_n)]^{1/2}$, and
$Sp^2 = [(n_n - 1)S_1^2 + (n_1 - 1)S_2^2] / (n_n + n_1 - 2)$
observed t is compared with $t_{df=6,\ -0.05} = 1.9432$ for the t-test.
***Distilled water is used as the negative control; Daunomycin is used for the positive control.
$ Significant result for the mutagenic effect.
$$ Significant result for the lethal effect.