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Genetic and Biochemical Effects of Dry Residue of Arno River *In vitro* and *In vivo*

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The majority of human cancers have been suggested to relate to environmental causes. Little is known about the effective environmental and occupational risk of cancer (to both the public and workers) despite the awareness of potential health hazards generated by technological and industrial processes has increased during the past few years. This is due, in part, to many possible interactions among the present mutagens, antimutagens, non mutagens, toxics, promoters, antipromoters, repair inhibitors, sensitizers and/or physical agents: they can modulate the effects on biological test systems used to monitor human risk. For this reason, considerable difficulties have been met in the search for specific carcinogens in complex mixtures employed in the work place. The above considerations can explain the fact that results on genotoxic damage in human beings, who are exposed to occupational chemicals, are lacking. Notwithstanding its complexity, considerable efforts are aimed in many laboratories at this issue.

An interaction of analytical and biological (e.g. epidemiological, mutagenic, cancerogenic, etc.) studies seems to be the better way to obtain important and necessary data. Some results of our multi-disciplinary program to study environmental genetic-carcinogenic risk, in a "Leather Industrial Area" near Florence (Tuscany), were previously reported (Bronzetti et al. 1983; Del Carratore et al. 1984). In 1981, 1982 and 1983 (on June-July) the Arno river, which flows through this area, appeared lacking of every form of aquatic life. "It was an awful sight the fishes dead heaped on the river bank". The present study continues our efforts to characterize the genetic toxicity associated with

effluents coming from "Leather Industrial Area". The ability of dry residue water of Arno river to induce "in vivo" and "in vitro" genetic alterations on the diploid D_7 strain of Saccharomyces cerevisiae with mitotic gene conversion, mitotic crossing-over and (point) reverse mutation as endpoints, and the "in vivo" effects on microsomal mono-oxygenase (cytochrome P-450 dependent) system of mouse liver (S9 fraction),were investigated. In order to explain the genetic and/or biochemical effects, the analytical studies of Arno river water were set out.

MATERIALS AND METHODS

Saccharomyces cerevisiae strain D_7, obtained from Prof. F.K. Zimmermann, was used to measure the frequency of mitotic gene conversion at the trp locus, mitotic recombination between the centromere and the ade 2 locus and point (reverse) mutation at the ilv locus (Zimmermann et al. 1975; Bronzetti et al., 1978, 1981).

Female Swiss Albino mice, CD1 strain, maintained on a standard laboratory diet of pellets, were starved the night before they were sacrificed. The animals were treated as follows:

a) for the preparation of liver S9 fractions (used in mutagenesis studies) with sodium phenobarbital (100 mg/Kg, half dose on the second day) for 3 days before being sacrified by cervical dislocation (24h after the final injection) and with ß-naphthoflavone (80 mg/Kg) 48h before the sacrifice (Matsushima et al. 1976).
b) for "in vivo" genetic and biochemical studies for 5 consecutive days with 1 ml of sterile water containing 50 or 80 mg of residue obtained from Arno river water after slow evaporation at 70°C. They were killed on the 5th days.

The livers were removed aseptically and homogenized in a Potter-Elvehjem homogenizer with a teflon pestle, at 4 ml/g, in 0,01 M Na^+/K^+ phosphate buffer (pH 7.4) containing KCl 1.15% (w/v). The S9 fraction was then prepared by centrifuging the homogenate at 9,000 x g for 20 min at 4°C. The post-mitochondrial supernatants were divided into equal portions and immediately frozen at -20°C. The frozen suspensions were stored in a deep freezer at -80°C. Aliquots of the frozen S9 fractions

were partially thawed at room temperature and were placed on ice for use in the daily experiments. Aminopyrine N-demethylase was determined by quantitating the CH_2O release by Nash reagent (Mazel, 1971) and protein concentration by the Lowry method with bovine serum albumin as standard (Lowry et al. 1951).

For the "in vitro" tests the livers were removed aseptically and homogenized in 3 vol. of ice-cold 0.1 M phosphate buffer (pH 7.4) in a Potter-Elvehjem homogenizer with a teflon pestle. This homogenate was centrifuged at 9,000xg for 20 min. The supernatant was pipetted off and used immediately. Into a 50 ml Erlenmeyer flask there were added: 0.1 ml of cell suspension (6×10^8 cells/ml buffer), 2.0 ml of 9,000 x g liver supernatant, 3.0 ml activation cofactors (54 mg KCl, 4.8 mg glucose 6-phosphate, 3.4 mg $MgCl_2$ $6H_2O$ and 2.5 mg NADPH per ml of 0.1 M phosphate buffer pH 7.4), and 0.1 ml of sterile water containing 40 or 80 mg of dry residue obtained from Arno river water. In experiments without metabolic activation 5.0 ml of 0.1 M phosphate buffer (pH 7.4) was substituted for the liver homogenate and cofactors. 5 tubes were used per dose. The tubes were incubated at 37°C for 2 h on a roller drum, after which some samples of mixture were plated on selective media for enumeration of *trp* convertants and *ilv* revertants, which were expressed as number of cells per 10^5 or 10^6 survivors. Other samples of mixtures were plated on complete medium for enumeration of *ade* recombinants per 10^4 survivors (Zimmermann, 1973).

Water of the Arno river was collected in the days when fishes died in great number. The dry residue at 70° of the water was 4.9 gr/L. Using a micromethod with the Coomassie brilliant blue G-250 reagent it was possible to determine proteins in the undiluted water and the concentration was 5.05 g/L, with a pH=7.2-7.6. The concentration of some ions was as follows: The dry residue was administered daily by gavage (40 or 80 mg in 0.5 ml of sterile water) for 4 days for a total dose of 160 or 320 mg/b.w. 0.2 ml of 2×10^9 yeast cells/ml 0.9% saline was injected into the retro-orbital sinus of the mice; this injection technique was adopted because the technique was easy to perform. The administration of the yeast immediately preceded the administration of the samples on the day of assay. The mice were killed 4h after injection of the yeast cells. The liver, lungs and

kidneys were aseptically removed. The various organs, pooled separately from 3 animals, were placed in 12.0 ml of 0.10 M Na^+/K^+ phosphate buffer (pH 7.4) and homogenized in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 2,000 rpm. The pellet was resuspended in 12.0 ml buffer, and plated as described above for the suspension test.

The concentration of Cr^{+++}, Mn^{++}, Cd^{++}, Cu^{++}, Ni^{++}, Zn^{++} and Pb^{++} were measured by Atomic Absorption Spectrophotometry after concentration of the samples, according to the method of Brown (1968).
The analytical methods for measuring the concentrations of NH_4^+, PO_4^{3-} and Cl^- were those presented in "Standard Methods" for the Examination of Water and Waste Water prepared and published jointly by: American Public Health Association, American Water Works Association, Water Pollution Control Federation.
Organics were not examined because, considering the type of industrial wastes, heavy metal contamination was considered of outstanding importance.

RESULTS AND DISCUSSION

The results of the suspension test, are provided in Table 1. It is evident a significant increase of both point (reverse) mutation and mitotic gene conversion occurred at the dose employed ($P < 0.01$). The *ilv* reversion frequency showed an increase at the 40 mg dose of 10-fold with respect to the 7.6-fold increase obtained with mammalian metabolic activation. The same increase was observed at the highest dose (21- and 14-fold increase with and without S9 fraction, respectively). Gene conversion at the *trp* locus appears to be less sensitive with both dose considered. In fact it increases over the background of 4.8- and 8.2 fold at 40 and 80 mg dose respectively.
No differences were observed in genotoxic responses when the S9 fraction was added.
Results of the intrasanguineous host-mediated assay are presented in Table 2. After multiple treatment with dry residue of Arno water by gavage (total dose of 160 or 320 mg/Kg b.w.), only weak genetic effects were seen in the yeast recovered from the liver, lungs and kidneys. The intrasanguineous assay was chosen as a reliable "in vivo" test fully taking into account the effect of the complete metabolism of the experiment animals.

Table 1. Induction of mitotic gene conversion and point mutation in Saccharomyces cerevisiae strain D7 by dry residue of Arno river.

Dry residue of Arno water	TRP^+ 10^5 Convertants/ survivors		ILV^+ 10^6 Revertants/ survivors	
Control	-S9	+S9	-S9	+S9
	0.35 ± 0.15	0.48 ± 0.13	0.15 ± 0.03	0.29 ± 0.17
40 mg	1.70 ± 0.27*	2.30 ± 0.45*	1.80 ± 0.81*	2.20 ± 0.78*
80 mg	2.90 ± 0.38*	3.90 ± 0.47*	3.20 ± 1.52*	3.80 ± 1.70*

The data are the mean $\pm$SD of 5 independent experiments.
* $P < 0.01$.

Table 2. Induction of mitotic gene conversion and point mutation in S. cerevisiae strain D_7 in the intrasanguineous assay in mice after repeated administrations of dry residue of Arno river for a total dose of 160[a] or 320[b] mg/Kg b.w. (see materials and methods section).

Organ	Number Expts.		TRP^+ 10^5 Convertants/ survivors	ILV^+ 10^6 Revertants/ survivors
Liver	Control	5	0.76 ± 0.14	0.24 ± 0.70
	Treated[a]	5	1.42 ± 0.22	0.96 ± 0.32
	Treated[b]	5	1.74 ± 0.06	1.25 ± 0.54
Lungs	Control	5	0.28 ± 0.18	0.23 ± 0.05
	Treated[a]	5	0.51 ± 0.15	0.53 ± 0.17
	Treated[b]	5	0.49 ± 0.08	1.03 ± 0.09
Kidneys	Control	5	0.28 ± 0.16	0.41 ± 0.13
	Treated[a]	5	0.72 ± 0.23	0.87 ± 0.31
	Treated[b]	5	1.07 ± 0.19	1.24 ± 0.50

The data are the mean $\pm$SD of 4-5 independent experiments.

As expected by our previous investigations (Bronzetti et al. 1983; Del Carratore et al. 1984) the analytical results are in agreement with our data about the presence of trivalent chromium (a well known mutagen) into the dry residue of Arno water (Table 3).

However, the high level of Mn^{++} concentration present in fluvial water, strongly suggest its additional effect on the mechanism of mutagenesis (El-Deiry et al. 1984). Obviously, the presence of other mutagenic substances cannot be ruled out, on the base of the above data, in the Arno's Water. Our work shows that this water clearly induces genetic and biochemical effects and some responsible agents are indicated.

Table 3. Analytical studies of Arno river water.

Cr^{+++}	0.06mg/L	Zn^{++}	0.02 mg/L
Mn^{++}	0.4 "	Pb^{++}	0.03 "
Cd^{++}	0.004 "	NH_4^{+}	2.6 "
Cu^{++}	0.184 "	PO_4^{3-}	1.3 "
Ni^{++}	0.04 "	Cl^{-}	78 "
		pH	7.2-7.6"

Finally, in Table 4 the "in vivo" effect of dry residue on microsomal mono-oxygenase activity system of mouse liver are reported. A marked increase of aminopyrine N-demethylation, with both doses used, was observed. The high percent of induction so obtained clearly indicate co-toxic and co-mutagenic effects of dry residue of Arno water, in addition to "in vitro" genotoxic effects.

Table 4. In vivo effects on microsomal mono-oxygenase activity system by dry residue of Arno river. Treatment by gastric gavage for a total dose of 160[a] and 320[b] mg/Kg b.w.

	Aminopyrine N-demethylase[a]	%Induction
Control	1.87±0.34	==
Treated[a]	2.72±0.36*	45.45
Treated[b]	2.90±0.41*	55.08

The data are the mean ± SD of five independent experiments.
a nmol x min^{-1} x mg^{-1}; * P<0.01.

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