

Cytogenetic effects of styrene and styrene oxide on human lymphocytes and *Allium cepa*

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MARJA SORSA, Ph.D.,² and HARRI VAINIO, M.D.²

LINNAINMAA, K., MERETOJA, T., SORSA M. and VAINIO H. Cytogenetic effects of styrene and styrene oxide on human lymphocytes and *Allium cepa*. *Scand. j. work environ. & health* 4 (1978): suppl. 2, 156—162. Styrene and styrene oxide induce cytogenetic effects already at very low concentrations (0.01 % v/v or even less); the effects are similar in both in vitro human lymphocytes and in vivo onion root tip cells (*Allium cepa* L.). It is characteristic that styrene treatment is more potent in causing chromosome breakage in both systems. In *Allium* styrene induced inhibition of mitotic spindle action as revealed by a strong c-mitotic effect. Also the number of micronuclei and nuclear bridges increased in both test systems, especially after styrene oxide treatment. Furthermore, the metaphase chromosome morphology in the cells treated with styrene oxide was strongly affected. In both systems, chromosome destruction was observed, or else the chromosome material was decondensed and resulted in a characteristic fuzzy appearance of *Allium* chromosomes or a banded appearance of human lymphocyte chromosomes. A specific effect of styrene oxide on the chromosomal proteins is thus suggested. The data obtained from the autoradiographic studies with *Allium* support the idea that [³H] styrene oxide binds irreversibly to the cytoplasmic and nuclear macromolecules.

Key words: chromosome morphology, c-mitosis, human lymphocyte tests, plant cytogenetic tests, styrene, styrene oxide.

Styrene oxide has been shown to induce mutations in bacteria (10), yeast, and Chinese hamster cells (4). It is also mutagenic in *Drosophila* (unpublished results of Sorsa et al.). In addition styrene has been found to be mutagenic in bacteria after metabolic activation (6, 16). In in vivo studies on the clastogenicity of styrene in man, the examination of lymphocyte chromosomes of reinforced plastic workers re-

vealed a clear increase in the frequency of chromosome breaks (7, 9). A significant increase in the rate of chromosomal aberrations was also observed in the bone marrow cells of rats after nine weeks of exposure to atmospheric styrene (8).

The present work was undertaken to examine further the cytogenetic and clastogenic effects of styrene and its metabolite, styrene oxide. In addition, we wanted to compare the cytogenetic effects of styrene and styrene oxide in two biologically different systems, human lymphocyte culture cells and growing root tip cells of *Allium cepa*.

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MATERIAL AND METHODS

Human lymphocyte cells

Whole blood microcultures were established according to conventional methods. Blood from the same healthy female donor was used throughout the experiments. An incubation time of 72 h was chosen so that a maximal number of the stimulated lymphocytes would reach mitosis. Styrene was added to the cultures in the beginning of the culture period. With styrene oxide also shorter treatment times were used. The air-dried slides were stained for 6 min with a 10 % solution of Giemsa.

Allium root tip cells

The cytogenetic tests with onion were carried out as described earlier (2). The treatment of the bulbs was performed at room temperature in the dark when the growing roots had reached a length of about 5 cm. The test chemicals were added in deionized water, and the treatment solutions were vigorously shaken before the bulbs were transferred to the solutions. The controls were treated similarly in deionized water. After treatment, restitution was carried out in tap water. Fixation was performed in 1:3 acetic ethanol. Staining was carried out by the total-Feulgen technique, after which four to six squash preparations were made of each bulb.

Test chemicals

The test chemicals used were: styrene monomer (phenylethylene) ≥ 99 % (GC) purum (Fluka AG/Buchs SG, Switzerland), stabilized with 40–50 ppm p-tert. buthylbrenzkatechin; styrene oxide (phenylethylene oxide) ≥ 97 % (GC) purum (Fluka AG/Buchs SG, Switzerland); and [7- ^3H] styrene oxide. (Radiochemical Centre, Amersham, England). The specific radioactivity of the labeled styrene oxide was 33 mCi/mmol. It was diluted in acetone, the activity of the solution being 1 $\mu\text{Ci}/\mu\text{l}$.

Cytogenetic aberrations

A total of 100 metaphases or anaphases per test concentration were examined for the occurrence of different types of cytogenetic aberrations. In addition, 1,000 interphase nuclei per concentration were examined for the presence of micronuclei and nuclear bridges. The statistical significance of the results was tested according to Fisher's exact probability test.

Tests with tritiated styrene oxide

Autoradiographic studies were performed with *Allium* root tip cells. The bulbs were radioactively labeled by the incorporation of [7- ^3H] styrene oxide. Acetone was evaporated from the solution by heating in a 60°C water bath for 10 min before the final treatment solutions were prepared. Treatment, restitution, and fixation of the bulbs were performed as previously described. After being fixed, half of the root tips was stained by the total-Feulgen technique, after which two to three squash preparations were made of each treatment. The root tips were squashed in 45 % acetic acid, and the coverslips were removed with the dry ice method, after which the slides were air-dried. Another half of the fixed root tips was hydrated by passing through ethanol (70 %, 30 %) and deionized water, after which they were macerated in 0.1 N hydrochloric acid for 15 min before being squashed in 45 % acetic acid. The coverslips were removed with dry ice, and the preparations were stained in 45 % acetic-orcein in 40°C for 30 min. After the slides were rinsed in deionized water, the preparations were air-dried. The autoradiograms were prepared according to the stripping film technique (14) with Kodak AR-10 stripping film. The exposure time was four weeks at 4°C. After exposure, the film emulsion was developed in Kodak Formula D 19b developer for 5 min, rinsed in deionized water for 30–60 s, and fixed in Kodak Unifix for 10 min. After being rinsed in gently running tap water for 5–10 min, the slides were air-dried, dipped into xylene, and mounted in Entellan (Merck Art. 7960).

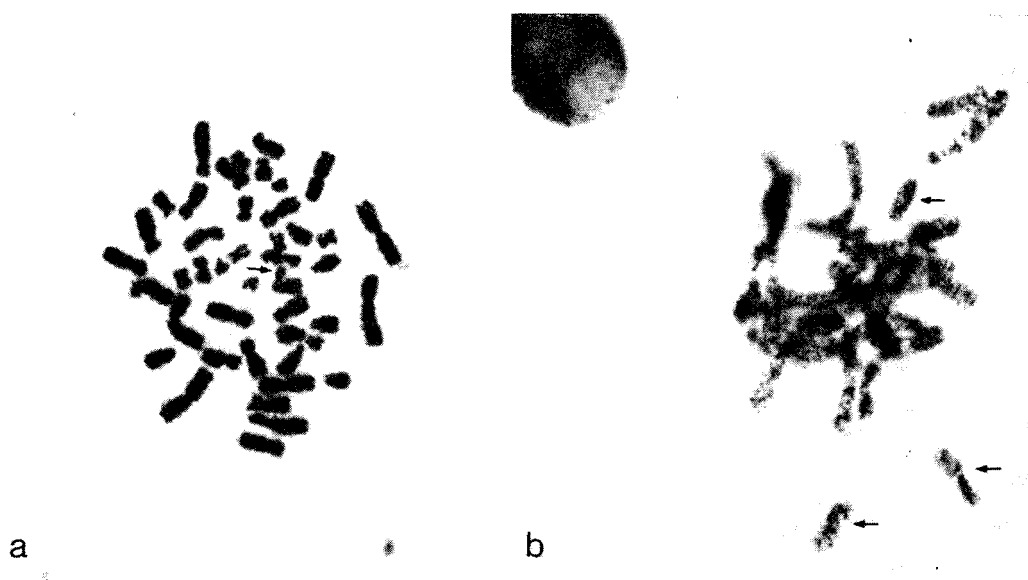


Fig. 1. Chromosome breaks after treatment with styrene: (a) human lymphocyte metaphase chromosomes (0.03 % v/v during the entire culture period), (b) *Allium* metaphase chromosomes (0.05 % v/v for 2 h and a restitution period of 24 h). *Allium* chromosomes are all large and metacentric normally.

Table 1. Occurrence of cytogenetic abnormalities in human lymphocyte cultures and in onion root tip cells after treatment with styrene and styrene oxide.

Treatment	Concentration % (v/v)	Time (h)		Breaks/100 metaphases	Micronuclei/1,000 interphase cells
		Treat- ment	Restitu- tion		
Styrene					
Lymphocytes	0.03	72	—	19***	10*
<i>Allium</i>	0.05	2	24	30***	0
Styrene oxide					
Lymphocytes	0.008	8	—	8*	38***
<i>Allium</i>	0.05	2	24	1	16***
Control					
Lymphocytes				1	2
<i>Allium</i>				0	2

* $0.01 < P < 0.05$ in Fischer's exact probability test.

*** $P < 0.001$ in Fischer's exact probability test.

RESULTS

Cytogenetic effects

The cytogenetic effects of styrene and styrene oxide were scored in toxic concentrations selected on the basis of an earlier described toxicity test (3). Styrene and styrene oxide induced cytogenetic effects,

which were similar in the two test systems. The cytogenetic aberrations observed in the cells treated with styrene were primarily chromosome breaks in both systems (fig. 1, table 1). Furthermore, in *Allium* cells, a strong c-mitotic effect and prolonged, disordered anaphases were observed which indicated inhibition of the mitotic spindle. The main aberration type after styrene oxide treatment was the

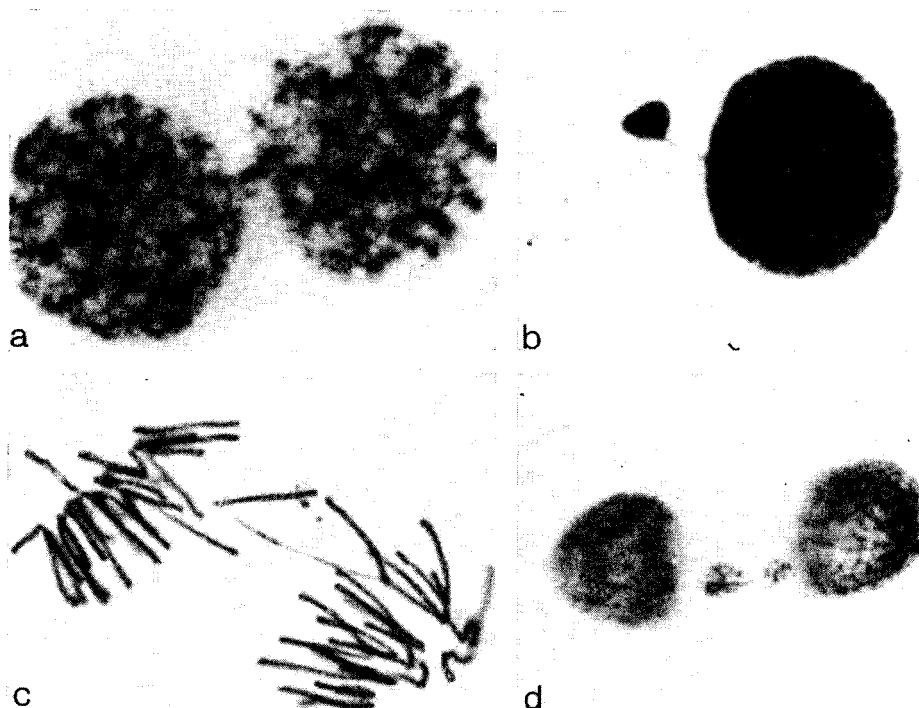


Fig. 2. Formation of micronuclei and chromatin bridges after treatment with styrene oxide: (a, b) human lymphocyte culture cells (0.008 % v/v for 8 h), (c, d) *Allium* root tip cells (0.05 % for 2 h).



Fig. 3. Styrene oxide causes specific changes in chromosome morphology: (a) occasional banded staining of human lymphocyte chromosomes (0.008 % v/v for 8 h), (b) decondensation of chromatin in *Allium* chromosomes (0.05 % v/v for 12 h).

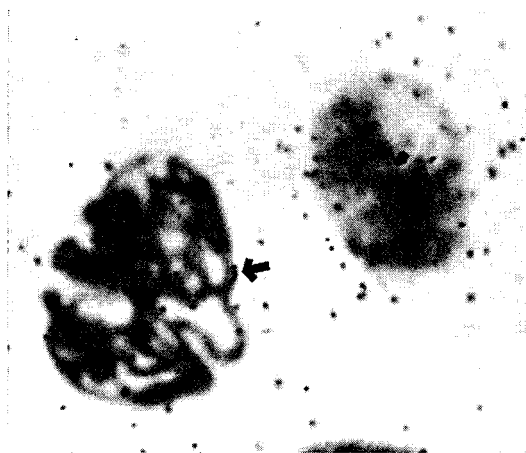


Fig. 4. Radioactive label in *Allium* root tip cells indicating irreversible binding of [7-³H] styrene oxide into the cells (3 μ Ci/ml for 2 h, total-Feulgen staining). The staining is DNA specific; only nuclei of the cells are stained.

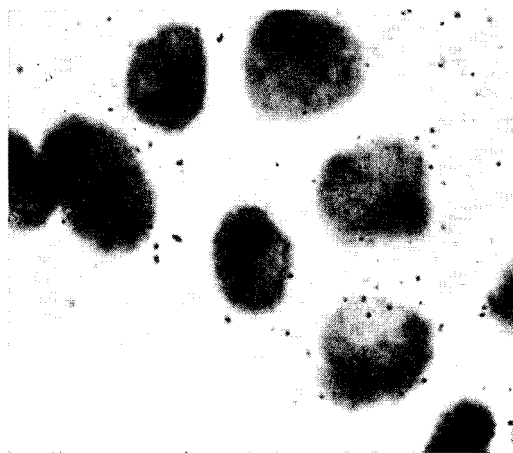


Fig. 5. After 20-h treatment with tritiated styrene oxide (3 μ Ci/ml) and a 6-h restitution period in tap water, radioactive label could occasionally be seen located along the chromatin in onion root tip cells (total-Feulgen staining).

formation of micronuclei and nuclear bridges in both systems (fig. 2, table 1). In *Allium* cells, bridges and fragments were frequently observed in the anaphases and obviously led to the formation of micronuclei in successive telophases and interphases (fig. 2). In human lymphocytes, treatment with 0.008 % v/v styrene oxide for 8 h also induced more severe chromosome destruction. Moreover, in many metaphases, an unspecific banded staining of the chromosomes was observed (fig. 3). Correspondingly, in *Allium* cells treated for 12 h with 0.05 % v/v styrene oxide, the chromosomes had a characteristic "fuzzy" appearance which indicated decondensation of the chromatin (fig. 3).

Autoradiographic tests

When the roots were treated with [7-³H] styrene oxide (3 μ Ci/ml, 0.1 mmol) for 2 h without restitution, the observed label was located mainly in the cytoplasm (fig. 4). After longer treatment periods (e.g., 20 h), some radioactivity could also be detected in the nucleus. After a restitution period of 6 h the label still remained in the cells. Occasionally, label was observed along the chromatin (fig. 5). After 24 h of restitution in tap water, almost all of the radioactive

label had disappeared from the cells; only a few clusters of grains remained, mostly in nuclei or micronuclei formed during the styrene oxide treatment.

DISCUSSION

The cytogenetic effects of styrene and styrene oxide were similar in the human lymphocyte cells and the onion root tip cells.

However, the cytogenetic abnormalities induced by styrene differed clearly from those induced by styrene oxide. Consequently we were not able to determine the extent to which the high amount of chromosomal breaks induced by the treatment with styrene was actually due to possible minor impurities in the chemical or its metabolic conversions in the lymphocyte culture and in the plant meristem. Gas chromatographic analyses made in our laboratory showed that the styrene used contained 0.60 ppm (v/v) ethylbenzene, 0.75 ppm (v/v) α -methylstyrene, and 0.5 ppm (v/v) styrene oxide. The mutagenicity of styrene in mammals has hitherto been explained on the basis of the metabolic

activation of styrene into styrene oxide. It has also been shown that enzymatic metabolic activation reactions are able to take place in human lymphocytes *in vitro* (11, 15). It is thus possible that the chromosome breaks observed were actually due to the metabolic conversion of styrene to styrene oxide, which is a relatively stable metabolite of styrene (half-time = 23 h 36 min in 0.1 M tris-HCL buffer, pH 7.5, according to the unpublished results of Pfäffli and Vainio). On the other hand, the aberrations induced by styrene treatment could also be due to 1-vinylbenzene-3,4-oxide, which is a possible metabolite of styrene with a very short lifetime [see the articles by Watabe et al. (17) and Pantarotto et al. (12) in this proceedings]. Furthermore, it is possible that a still unknown active metabolite exists. Neither can the biological activity of styrene itself be excluded.

Protein denaturation has been shown to play an essential role in the production of chromosomal banding (1). On the basis of this finding, the unspecific banded appearance of lymphocyte chromosomes and the decondensation of *Allium* chromosomes that we observed led us to suspect that styrene oxide has a specific effect on chromosomal proteins. This suggestion is supported by earlier obtained data (5), according to which styrene oxide was found to bind covalently to protein and nucleic fractions of rat liver *in vivo* and *in vitro*. In addition styrene has been shown to bind covalently to rat liver *in vitro* after metabolic activation (13, 17). The covalent binding of styrene oxide to chromosomal proteins was further supported by the results of our studies with tritiated styrene oxide.

ACKNOWLEDGMENTS

We wish to thank Mr. R. Mäkinen for providing the necessary facilities for carrying out the plant cytogenetic work.

The Finnish Cultural Foundation supported the study with a research grant to K. L.

REFERENCES

1. KATO, H. and MORIWAKI, K. Factors involved in the production of banded structures in mammalian chromosomes. *Chromosoma* 38 (1972) 105—120.
2. KIHLMAN, B. A. Root tips for studying the effects of chemicals on chromosomes. In: HOLLAENDER, A. (ed.), *Chemical mutagens* (vol. 2). Plenum Press, New York, N.Y. 1971, pp. 489—514.
3. LINNAINMAA, K., MERETOJA, T., SORSA, M. and VAINIO, H. Cytogenetic effects of styrene and styrene oxide. *Mutat. res.* (in press)
4. LOPRIENO, N., ABBONDANDOLO, A., BARALF, R., BARONCELLI, S., BONATTI, S., BRONZETTI, G., CAMMELLIN, A., CORSI, C., GORTI, G., FREZZA, D., LEPORINI, C., MAZZACCARO, A., NIERI, R., ROSSELLINI, D. and ROSSI, A. M. Mutagenicity of industrial compounds: Styrene and its possible metabolite styrene oxide. *Mutat. res.* 40 (1976) 317—324.
5. MARNIEMI, J., SUOLINNA, E.-M., KAARTINEN, N. and VAINIO, H. Covalent binding of styrene oxide to rat liver macromolecules *in vivo* and *in vitro*. In: ULLRICH, V., ROOTS, J., HILDEBRANDT, A., ESTABROOK, R. V. and CONNEY, A. H. (eds.), *Microsomes and drug oxidations: Proceedings of the third international symposium*, Pergamon Press, Berlin 1976, pp. 698—702.
6. MEESTER, C. DE, PONCELET, F., ROBERFROID, M., RONDELET, J. and MERCIER, M. Mutagenicity of styrene and styrene oxide. *Mutat. res.* 56 (1977) 147—152.
7. MERETOJA, T., JÄRVENTAU, H., SORSA, M. and VAINIO, H. Chromosomal aberrations in lymphocytes of workers exposed to styrene. *Scand. j. work environ. & health* 4 (1978): suppl. 2, 259—.
8. MERETOJA, T., VAINIO, H. and JÄRVENTAU, H. Clastogenic effects of styrene exposure on bone marrow cells of rat. *Toxicol. lett.* 1 (1978) 315—318.
9. MERETOJA, T., VAINIO, H., SORSA, M. and HÄRKÖNEN, H. Occupational styrene exposure and chromosomal aberrations. *Mutat. res.* 56 (1977) 193—197.
10. MILVY, P. and GARRO, A. J. Mutagenic activity of styrene oxide (1, 2-epoxyethylbenzene), a presumed styrene metabolite. *Mutat. res.* 40 (1976) 15—18.
11. PAIGEN, P., MINOWADA, J., GURTOO, H. L., PAIGEN, K., PARKER, N. B., WARD, E., THOMPSON HAYNER, N., BROSS, J. D. J., BOCK, F. and VINCENT, R. Distribution of aryl hydrocarbon hydroxylase inducibility in cultured human lymphocytes. *Cancer res.* 37 (1977) 1829—1837.
12. PANTAROTTO, C., FANELLI, R., BIODOLI, F., MORAZZONI, P., SALMONA, M. and SZCZAWINSKA, K. Arene oxides in styrene metabolism, a new perspective in styrene toxicity? *Scand. j. work environ. & health* 4 (1978): suppl. 2, 67—77.

13. PARKKI, M. G. and MARNIEMI, J. The covalent binding of styrene into rat liver *in vitro* (abstract). International conference on in vivo aspects of biotransformation and toxicity of industrial and environmental xenobiotics, 1977.
14. ROGERS, A. W. (ed.). *Techniques of autoradiography*. Elsevier Publishing Co., Amsterdam 1969, pp. 240—252.
15. TYRER, H. W., CANTRELL, T. T. and SWAN, A. G. Automated singlecell analysis of aryl hydrocarbon hydroxylase in human lymphocytes. *Life sci.* 20 (1977) 1723—1728.
16. VAINIO, H., PÄÄKKÖNEN, R., RÖNNHOLM, K., RAUNIO, V. and PELKONEN, O. A study on the mutagenic activity of styrene and styrene oxide. *Scand. j. work environ. & health* 2 (1976) 147—151.
17. WATABE, T., ISOBE, M., SAWAHATA, T., YOSHIKAWA, K., YAMADA, S. and TAKABATAKE, E. Metabolism and mutagenicity of styrene. *Scand. j. work environ. health* 4 (1978): suppl. 2, 142—155.

QUESTIONS AND ANSWERS

Question to Ms. LINNAINMAA

Dr. JENSSEN:

You show that styrene oxide gives rise to very high incidence of micronuclei, but almost no chromosome aberration in *Allium*. Have you any suggestion of how these micronuclei are formed?

Ms. LINNAINMAA:

I think that the chromatin bridges and fragments frequently observed in the anaphase stages in the *Allium* cells treated with styrene oxide may lead to the formation of micronuclei in successive telophases and interphases.