

FAILURE OF CHLOROFORM TO INDUCE CHROMOSOME DAMAGE OR SISTER-CHROMATID EXCHANGES IN CULTURED HUMAN LYMPHOCYTES AND FAILURE TO INDUCE REVERSION IN *ESCHERICHIA COLI*

D. J. KIRKLAND and K. L. SMITH

Toxicol Laboratories Limited, Bromyard Road, Ledbury, Herefordshire HR8 1LG

and

N. J. VAN ABBÉ

Beecham Products, Randalls Road, Leatherhead, Surrey KT22 7RX, England

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Summary—Two strains of *Escherichia coli*, WP2p and WP2uvrA⁻p, were treated with chloroform in the plate incorporation assay, and in liquid pre-incubation tests, and in both cases the chemical did not induce reversions. Chloroform, with metabolic activation (addition of S-9 mix) also failed to induce chromosome breakage or sister-chromatid exchanges in human lymphocytes, but benzo[a]pyrene, with and without S-9 mix, induced significant chromosome breakage and sister-chromatid exchanges in lymphocytes from the same donor. The relevance of these results in the light of other negative *in vitro* tests on chloroform, and positive and negative carcinogenicity tests, is discussed.

Introduction

Chloroform has been widely used over the last century as an anaesthetic, solvent, preservative and flavour additive, and an evaluation of the safety to humans of such a ubiquitous chemical is clearly important. Eschenbrenner & Miller (1945) reported induction of hepatomas in female Strain A mice after repeated oral administration of chloroform in olive-oil solution, but only when the doses were large enough to produce liver necrosis. The results of a study carried out by the National Cancer Institute (1976) on chloroform, given as a solution in corn oil, indicated a significant increase in kidney epithelial tumours in male Osborne-Mendel rats, and a significant increase in hepatocellular carcinomas in male and female B6C3F₁ mice. However, long-term administration of chloroform in a toothpaste vehicle to mice (Roe, Palmer, Worden & Van Abbé, 1979), rats (Palmer, Street, Roe, Worden & Van Abbé, 1979) and dogs (Heywood, Sortwell, Noel, Street, Prentice, Roe, Wadsworth, Worden & Van Abbé, 1979) produced no significant increase in neoplasms, except for a dose-related sex- and strain-specific increase in mainly benign kidney tumours in mice.

Chloroform has also been tested in *in vitro* mutagenicity tests. It was not mutagenic in Chinese hamster V79 cells at the 8-azaguanine locus (Sturrock, 1977). Neither was it mutagenic in *Escherichia coli* K.12 or *Salmonella typhimurium* TA1535 or TA1538 (Greim, Bimboes, Egert, Goggelmann & Kramer, 1977), nor in TA1537, TA98 or TA100 (Simmon, Kauhane & Tardiff, 1977), nor in TA1535, TA1537, TA1538, TA98 or TA100 (Daniel, Richold, Allen, Jones, Roe, Uttley & Van Abbé, 1980).

The purpose of the study reported in this paper was to extend the *in vitro* mutagenicity tests to include mutation in two other strains of *E. coli*, WP2p and WP2uvrA⁻p, using both plate incorporation and liquid pre-incubation, and to assess the damage done to human lymphocyte chromosomes and the induction of sister-chromatid exchanges by chloroform in the presence of rat-liver microsomes (S-9 mix).

Experimental

Reversion in *E. coli*. Two strains of *E. coli*, WP2p and WP2uvrA⁻p, kindly supplied by Dr S. Venitt of the Institute of Cancer Research, London, were used. Both are tryptophan auxotrophs and both carry the pKM101 Ampicillin resistance plasmid (McCann, Spingarn, Kobori & Ames, 1975). WP2p is fully DNA-repair proficient whereas WP2uvrA⁻p is deficient in excision repair at the *uvrA* locus. Both strains are reverted to prototrophy either by base change at the site of original alteration or by base change elsewhere in the genome so that the original defect is suppressed.

These two strains were treated with chloroform (Analar grade, BDH Chemicals Ltd, Poole, Dorset) in plate incorporation and pre-incubation tests both with and without rat-liver microsomes (S-9) prepared from Aroclor 1254-induced PVG/01a Hooded rats. In each case chloroform was administered at 10,000, 1000, 100, 10, 1 or 0.1 µg/plate using acetone as the diluent.

In the plate incorporation tests, the method was essentially that of Ames, McCann & Yamasaki (1975) for *S. typhimurium* in that 2 ml of molten top agar (0.6% Lab M agar containing 0.5% NaCl, 5% stan-

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dard nutrient broth and 5 µg L-tryptophan/ml) at 45°C were added 0.1 ml of a dilution of chloroform plus 0.1 ml (approximately 10⁸ organisms) of an overnight broth culture of the tester bacteria, and, where appropriate, 0.5 ml of S-9 mix containing 10% S-9 with standard co-factors (Ames *et al.* 1975). These ingredients were rapidly mixed on a Whirlimixer and poured onto prepared Vogel-Bonner agar plates (Vogel & Bonner, 1956). Each treatment was carried out in triplicate, and the three plates for each dose either with or without S-9 were separately packed in gas-tight containers to avoid leakage of the volatile substances. The plates were incubated at 37°C for 48 hr, after which revertant colonies were counted on a Biotran II automatic colony counter (New Brunswick Scientific, Edison, NJ, USA).

In the pre-incubation tests, 0.5 ml of a dilution of chloroform was mixed with 0.5 ml of an overnight bacterial culture and, where appropriate, 2.5 ml of 10% S-9 mix. These mixtures were shaken at 200 oscillations/min for 20 min at room temperature on a Braun orbital shaker, and aliquots were added to top agar: 0.2 ml were taken from non-S-9 tubes and 0.7 ml from S-9 tubes, in order to give the same final volumes and concentrations as in the plate incorporation test. The top agar mixtures were poured on to Vogel-Bonner plates (three per treatment), incubated at 37°C for 48 hr and then the revertants were counted.

Negative controls were included in each experiment by replacing the chloroform with acetone, and positive controls were also included: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Sigma London Chemical Co., Poole Dorset) was used at 1 or 10 µg/plate as a positive control without S-9 mix, and 2-aminoanthracene (2AA; Sigma Chemical Co.) was used at 5 or 50 µg/plate with 10% S-9 mix as a control chemical requiring metabolic conversion. Both plate incorporation and pre-incubation tests were repeated on separate days with fresh cultures, solutions and controls.

Chromosome breakage in human lymphocytes. Peripheral blood from a young, healthy volunteer with no history of chromosome fragility, no recent X-ray exposure and no recent virus infection was cultured in Hepes-buffered RPMI 1640 medium with glutamine, 20% foetal calf serum, and 100 units/ml each of penicillin and streptomycin (Gibco Europe Ltd, Paisley, Renfrewshire). Cultures (10 ml) were established in Sterilin disposable universal bottles and contained 0.4 ml blood, 0.1 ml phytohaemagglutinin (PHA; The Wellcome Foundation Ltd, Beckenham, Kent) and 0.1 ml preservative-free lithium heparin (Sigma Chemical Co.).

The cultures were incubated at 37°C for 24 hr, duplicate cultures were then treated with 0.1-ml aliquots of chloroform in acetone to give final concentrations of 50, 100, 200 and 400 µg/ml and 0.1 ml 10% S-9 mix was added to each culture. The treated cultures were incubated for 2 hr at 37°C. This method of treatment is a slight modification of that described by Thomson & Evans (1979). At the end of the treatment the cells were centrifuged at 800 rpm for 10 min, resuspended in fresh medium without PHA, and re-incubated for a further 22 hr to bring first cycle cells to mitosis after a total of 48 hr culture. One hour before harvest, colcemid (Gibco Europe Ltd) was

added to each culture at a final concentration of 0.5 µg/ml. Cells were collected and fixed, and slides prepared, stained with Giemsa and mounted using conventional methods.

Negative controls were cultures treated with 0.1 ml acetone and 0.1 ml S-9 mix. Positive controls were not included since the donor's lymphocytes had previously shown dose-related chromosome breakage after treatment with benzo[*a*]pyrene in the presence of S-9 mix.

Slides were coded by an independent observer, and 100 well-spread metaphases with 46 or more chromosomes were scored from each culture, making a total of 200 cells per treatment. Abnormalities were classified as previously reported (Kirkland, Lawler & Venitt, 1978), and, using the system of Bauchinger, Schmid, Einbrodt & Dresch (1976), each abnormality was expressed in terms of the theoretical number of breaks necessary to produce a given lesion: a chromatid break, a chromosome break, or an acentric fragment were designated as one break, a dicentric or a rearrangement was designated as two breaks, *except* where a dicentric and acentric occurred in the same cell, when the total number of breaks equalled two and not three.

Sister-chromatid exchanges (SCE) in human lymphocytes. Cultures of peripheral blood from the same donor were established as above, but contained 50 µM-bromodeoxyuridine (BUdR) and were kept in the dark to avoid photolysis of BUdR-substituted DNA. Treatments were again for 2 hr in the presence of S-9 mix, but were begun 48 hr after establishment of the cultures, fresh BUdR medium without PHA being used to replace the treatment medium for the final 22 hr. The levels of chloroform used were 25, 50, 75, 100, 200 and 400 µg/ml final concentration. The negative control cultures contained acetone, and positive controls were not included since the donor's lymphocytes had previously shown a dose-related increase in SCE after treatment with benzo[*a*]pyrene in the presence of S-9 mix.

Accumulation of metaphase spreads. Harvesting of cells and preparation of slides were as above. The dried slides were stored in the dark for at least 7 days after which the chromatids were differentiated by the method of Perry & Wolff (1974). The slides were again coded by an independent observer and, where possible, 50 well-differentiated metaphases with 46 or more chromosomes were scored from each culture, making a possible total of 100 cells per treatment.

Results

Reversion in E. coli

The results of the plate incorporation tests are shown in Table 1. It is clear that with chloroform all counts fall in the usual range expected for control counts. An analysis of variance on these figures confirmed that there was no dose-response. The bacteria and S-9 were not the cause of this lack of response since the positive control chemicals gave satisfactory reversion rates even though there was wide variation between the two experiments.

The results of the pre-incubation tests are shown in Table 2. With chloroform this method of treatment was more toxic than the plate incorporation method.

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Table 1. Plate incorporation assay of chloroform and positive control chemicals using *Escherichia coli* strains WP2p and WP2uvrA⁻p

		Mean number of revertants/plate in strain			
Chemical	Amount (μ g/plate)	WP2p		WP2uvrA ⁻ p	
		Without S-9 mix	With 10% S-9 mix	Without S-9 mix	With 10% S-9 mix
Experiment 1					
Chloroform	0	8.7	5.0	5.3	4.0
	0.1	7.7	7.0	6.3	1.7
	1	11.0	4.7	6.3	3.7
	10	11.3	8.7	5.3	3.0
	100	8.3	3.7	7.0	8.3
	1000	5.0	4.0	16.7	4.0
	10,000	9.0*	4.7*	6.3*	3.3*
MNNG	0	6.7	—	6.7	—
	1	57.3	—	58.5	—
	10	498.3	—	644.0	—
2AA	0	—	8.0	—	22.5
	5	—	15.3	—	289.0
	50	—	18.5	—	386.0
Experiment 2					
Chloroform	0	11.7	15.0	4.3	11.3
	0.1	8.3	8.3	9.7	20.3
	1	8.0	17.7	3.0	25.3
	10	9.3	14.7	6.7	17.3
	100	10.3	17.7	7.3	15.7
	1000	6.0	21.3	6.0	13.0
	10,000	16.0*	13.5*	4.0	14.0*
MNNG	0	12.0	—	14.7	—
	1	230.0	—	202.3	—
	10	1873.7	—	1091.7	—
2AA	0	—	27.7	—	51.7
	5	—	21.3	—	268.7
	50	—	34.7	—	752.3

MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine 2AA = 2-Aminoanthracene*The background lawn was sparse or absent on some or all of the plates.
Values are means for three plates.

and the control counts are generally higher, but there is no evidence of mutagenic activity. The positive controls were again satisfactory, but although there is variation between the experiments, both strains show better responses than the controls for the plate incorporation assay.

Chromosome breakage in human lymphocytes

The response of the donor's lymphocytes to chromosome breakage by benzo[*a*]pyrene treatment, with and without addition of S-9 mix, is shown in Fig. 1, in which all abnormalities have been calculated as theoretical lesions. It is clear that there is a dose-related increase in breakage with benzo[*a*]pyrene treatments, and this is confirmed by calculating correlation coefficients, which are 0.962 (without S-9) and 0.891 (with S-9).

The response of the same donor's lymphocytes to chloroform indicated random variation around the control value (Fig. 2). The highest breakage level was at 200 μ g chloroform/ml with 8 breaks/100 cells compared with 5.5 breaks/100 cells in the control. However, comparing these levels by using the chi-square test with Yates correction shows that this difference is not significant ($\chi^2 = 0.635$, $P > 0.25$). The random

nature of the breakage around control levels is confirmed by a correlation coefficient of 0.248.

SCE in human lymphocytes

The response of the donor's lymphocytes to SCE induction by benzo[*a*]pyrene is shown in Fig. 3. The control levels of SCE are quite high for this donor, being 16.375 SCE/cell without S-9 mix and 18.23 SCE/cell with the addition of S-9 mix. However, there is a significant increase in SCE with benzo[*a*]pyrene treatment and reasonable dose-response trends giving correlation coefficients of 0.782 (without S-9) and 0.995 (with S-9) over the range 0–10 μ g/ml.

The response of the same donor's lymphocytes to chloroform is shown in Fig. 4. The control level of SCE (18.35 SCE/cell) closely resembles that seen in the benzo[*a*]pyrene control with S-9 mix, but at only two treatment levels are there more SCE/cell than in the control. At one of these (50 μ g chloroform/ml) the SCE frequency is significantly different from the control ($\chi^2 = 11.78$, $P < 0.05$ with five degrees of freedom), but there is no dose-response trend, and at some doses (e.g. 25 μ g/ml) the SCE frequency is significantly lowered ($\chi^2 = 26.13$, $P < 0.001$ with five degrees of freedom). This lack of dose-response is con-

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Table 2. *Pre-incubation assay of chloroform and positive control chemicals using Escherichia coli strains WP2p and WP2uvrA⁻p*

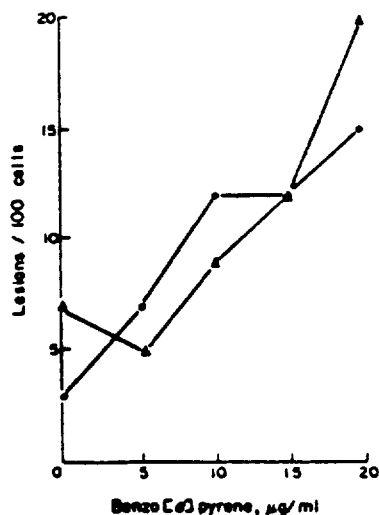
		Mean no. of revertants/plate in strain			
		WP2p		WP2uvrA ⁻ p	
Chemical	Amount (μg/plate)	Without S-9 mix	With 10% S-9 mix	Without S-9 mix	With 10% S-9 mix
Experiment 1					
Chloroform	0	24.0	17.7	33.3	40.0
	0.1	25.7	18.3	26.7	40.3
	1	17.7	17.7	2.3*	40.5
	10	22.7	18.3	NC*	42.3
	100	NC*	16.0	NC*	44.0
	1000	NC*	19.7	NC*	52.7
	10,000	NC*	NC*	NC*	NC*
MNNG	0	21.0	—	58.3	—
	1	878.0	—	455.7	—
	10	1403.3	—	1551.0	—
2AA	0	—	26.3	—	15.3
	5	—	52.0	—	152.3
	50	—	60.3	—	1239.3
Experiment 2					
Chloroform	0	16.7	46.0	45.0	77.3
	0.1	21.3	32.3	48.7	60.0
	1	19.3	19.7	47.3	113.7
	10	22.0	27.7	53.0	56.3
	100	24.0	30.3	53.0	51.7
	1000	NC*	15.3*	NC*	10.7*
	10,000	NC*	NC*	NC*	NC*
MNNG	0	18.3	—	84.0	—
	1	482.0	—	463.0	—
	10	2209.7	—	1672.7	—
2AA	0	—	35.7	—	139.3
	5	—	81.7	—	1360.7
	50	—	102.3	—	1990.0

NC = Not counted MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

2AA = 2-Aminoanthracene

*The background lawn was sparse or absent on some or all of the plates.

Values are means for three plates.

Fig. 1. Dose-related induction of chromosome breakage in human lymphocytes after treatment with benzo(a)pyrene with (▲) and without (●) metabolic activation (addition of S-9 mix). Abnormalities are expressed as the theoretical number of lesions necessary to produce the abnormalities according to the method of Bauchinger *et al.* (1976).

firmed by the correlation coefficient which is -0.206 for the whole dose range and is only 0.596 for the range $0-75 \mu\text{g}/\text{ml}$.

Discussion

In this study chloroform has been shown not to be mutagenic in three *in vitro* tests with different genetic endpoints. Since the start of this work, we have learned that chloroform was one of the substances

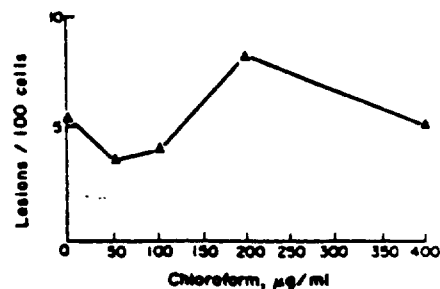


Fig. 2. Lack of effect of treatment with chloroform plus S-9 mix on chromosome breakage in human lymphocytes obtained from the same donor as for Fig. 1.

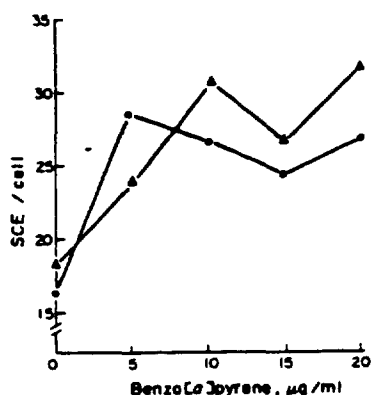


Fig. 3. Significant and dose-related induction of sister-chromatid exchanges (SCE) in human lymphocytes after treatment with benzo[a]pyrene with (▲) and without (●) S-9 mix.

assessed in the international programme for the evaluation of short-term tests for carcinogens (de Serres & Ashby, 1981), and out of 40 assays, 33 were negative, and seven gave positive results which were unreproducible, giving negative results on repeat. These tests included bacterial tests with *Salmonella* and *E. coli*, DNA repair in bacteria, mammalian cell transformation, unscheduled DNA synthesis in HeLa cells, the dominant lethal test, the micronucleus test and SCE induction in CHO cells.

The results reported here confirm the non-mutagenicity of chloroform in *E. coli*, even after pre-incubation, and extend the negative cytogenetic observations to breakage and SCE induction in human lymphocytes. This total failure of chloroform to cause any type of mutagenic event *in vitro* raises serious questions in relation to the prediction of cancer risk, particularly because in three animal studies (Eschenbrenner & Miller, 1945; National Cancer Institute, 1976; Roe *et al.* 1979) signs of carcinogenicity were observed when the dose levels greatly exceeded normal exposure levels in human use. However, in two of these studies, liver toxicity was observed and it may be that chloroform only acts as a carcinogen when it causes sufficient tissue damage to simulate partial hepatectomy. The development of kidney tumours under certain experimental conditions may likewise be directly

related (Moore, Chasseaud, Majeed, Prentice, Roe & Van Abbé, 1980) to the nephrotoxic action of chloroform at high doses. This may explain why with lower doses of chloroform that are insufficient to cause liver or kidney regeneration, there was no increase in tumours in the rats, mice and dogs treated with toothpaste (Heywood *et al.* 1979; Palmer *et al.* 1979; Roe *et al.* 1979).

An additional feature of the work reported here is that this appears to be the first experimental investigation of the response of human cells to chloroform. The view given in the IARC monograph on chloroform (IARC, 1979) that the compound should, for practical purposes, be regarded as if it presented a carcinogenic risk to humans, is not supported by the results of the studies reported here.

REFERENCES

- Ames, B. N., McCann, J. & Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutation Res.* 31, 347.
- Bauchinger, M., Schmid, E., Einbrodt, H. J. & Dresch, J. (1976). Chromosome aberrations in lymphocytes after occupational exposure to lead and cadmium. *Mutation Res.* 40, 57.
- Daniel, M. R., Richold, M., Allen, J., Jones, E., Roe, F. J. C., Uttley, M. & Van Abbé, N. J. (1980). Bacterial mutagenicity and cell transformation studies with chloroform. *Toxicology Lett.* Special Issue, p. 247.
- de Serres, F. J. & Ashby, J. (Editors) (1981). *Evaluation of Short-term Tests for Carcinogens. Report of the International Collaborative Program. Progress in Mutation Research.* Vol. 1. Elsevier/North-Holland Inc., New York.
- Eschenbrenner, A. B. & Miller, E. (1945). Induction of hepatomas in mice by repeated oral administration of chloroform with observations on sex differences. *J. natn. Cancer Inst.* 5, 251.
- Greim, H., Bimboes, D., Egert, G., Göggelmann, W. & Krämer, M. (1977). Mutagenicity and chromosomal aberrations as an analytical tool for *in vitro* detection of mammalian enzyme-mediated formation of reactive metabolites. *Archs Toxicol.* 39, 159.
- Heywood, R., Sortwell, R. J., Noel, P. R. B., Street, A. E., Prentice, D. E., Roe, F. J. C., Wadsworth, P. F., Worden, A. N. & Van Abbé, N. J. (1979). Safety evaluation of toothpaste containing chloroform III. Long-term study in beagle dogs. *J. env. Path. Toxicol.* 2, 835.
- IARC Working Group (1979). *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Some Halogenated Hydrocarbons.* Vol. 20, p. 401. International Agency for Research on Cancer, Lyon.
- Kirkland, D. J., Lawler, S. D. & Venitt, S. (1978). Chromosomal damage and hair dyes. *Lancet* II, 124.
- McCann, J., Spingarn, N. E., Kabori, J. & Ames, B. N. (1975). Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. *Proc. natn. Acad. Sci. U.S.A.* 72, 979.
- Moore, D., Chasseaud, L. F., Majeed, S. K., Prentice, D. E., Roe, F. J. C. & Van Abbé, N. J. (1980). Effect of dose and vehicle on early tissue damage and regenerative activity after chloroform administration. *Toxicology Lett.* Special Issue, p. 234.
- National Cancer Institute (1976). *Report on Carcinogenesis Bioassay of Chloroform.*
- Palmer, A. K., Street, A. E., Roe, F. J. C., Worden, A. N. & Van Abbé, N. J. (1979). Safety evaluation of toothpaste containing chloroform II. Long-term studies in rats. *J. env. Path. Toxicol.* 2, 821.

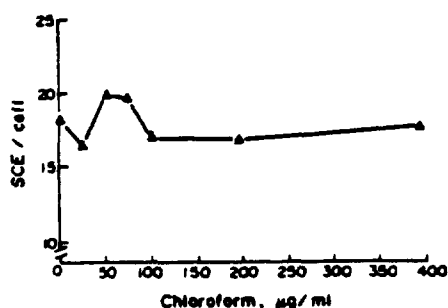


Fig. 4. Lack of effect of chloroform plus S-9 mix on the induction of sister-chromatid exchanges (SCE) in human lymphocytes obtained from the same donor as for Fig. 3.

- Perry, P. & Wolff, S. (1974). New Giemsa method for the differential staining of sister chromatids. *Nature, Lond.* 251, 156.
- Roe, F. J. C., Palmer, A. K., Worden, A. N. & Van Abbe, N. J. (1979). Safety evaluation of toothpaste containing chloroform I. Long-term studies in mice. *J. envir. Path. Toxicol.* 2, 799.
- Simmon, V. F., Kauhanen, K. & Tardiff, R. G. (1977). Mutagenic activity of chemicals identified in drinking water. In *Progress in Genetic Toxicology*, p. 249. Elsevier/North-Holland Biomedical Press, Amsterdam.
- Sturrock, J. (1977). Lack of mutagenic effect of halothane or chloroform on cultured cells using the azaguanine test system. *Br. J. Anaesth.* 49, 207.
- Thomson, V. E. & Evans, H. J. (1979). Induction of sister-chromatid exchanges in human lymphocytes and Chinese hamster cells exposed to aflatoxin B₁ and N-methyl-N-nitrosourea. *Mutation Res.* 67, 47.
- Vogel, H. J. & Bonner, D. M. (1956). Acetylornithase of *Escherichia coli*: partial purification and some properties. *J. biol. Chem.* 218, 97.