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The Sister Chromatid Exchange Test

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EXECUTIVE SUMMARY

The sister chromatid exchange test for the detection of mutagenicity is described. The potential use of the SCE test as a component of a battery of short term mutagenicity tests is discussed. A battery of suitable short term mutagenicity tests could be used to screen environmental samples for mutagenic activity.

THE SISTER-CHROMATID EXCHANGE TEST

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ABSTRACT

A short term in vitro bioassay for the detection of mutagenicity is described. The assay is based on the ability of mutagens to induce sister chromatid exchanges (SCE) in second metaphase mammalian cells; induced SCEs may be used to assess (i) the mutagenicity of analytical samples and (ii) the potential of samples to induce chromosome aberations by DNA breakage. With adaptation the SCE test should be suitable for use in the screening of environmental samples for mutagenicity: preferably as a component of a battery of short term tests. Some of the strengths and weaknesses of the SCE test are discussed.

Table of Contents

INTRODUCTION

Since the end of the nineteenth century the industrialized nations have waged an increasingly successful campaign against microbial diseases of humans. Many diseases that previously caused premature loss of human life in epidemic proportions have been brought under control: small pox, polio and tubercolosis are three examples. Although post infection patient treatment, usually with antibiotics, has been important in decreasing patient mortality rates, the most significant improvements in social health have resulted from advances in disease prevention. The preventative approach to disease control relies both on immunization techniques and on using scientifically acquired knowledge of disease agents and mechanisms to improve those sanitary, cultural and social practices that may predispose towards infection. Decreased infant mortality rates and increased longevity are two results of the foregoing advances.

Recent epidemiological data suggests that increases have occurred over the past 30 years, in the incidences of organ specific cancer and of deaths caused by cancers(2 to 9). Whether these data represent a current cancer epidemic or are merely the manifestation of conditions that previously were masked by other causes of premature death, has yet to be resolved(1). In either case, the fact remains that cancer has become a serious public health problem(9) and has replaced previous disease epidemics in the public's consciousness.

Experiences with other diseases indicate two approaches that should enable society to deal more effectively with cancer: disease treatment and disease prevention. Whereas, disease treatment is important in alieviating suffering and reducing loss of life among those who have already contracted cancer; history suggests that it is the latter course of action that is likely to have the most significant and long lasting effects on cancer incidence rates.

An understanding of cancer aetiology and the mechanisms whereby certain cancers can proliferate within the body is central to any serious effort to reduce or control cancer incidences. The concerted study of cancer aetiology commenced in the 1950's. There are many different causes of cancers(1,10); implicated agents include UV light, viruses, various forms of radiation, and carcinogenic substances(10). There is much debate concerning the relative contribution of the different carcinogen types to present cancer levels(1,9,11,12). The significance of non-voluntary and voluntary exposure to carcinogens is also a topic of some debate: environmentalists tend to stress the importance of the former factor, whereas representatives of the major multinational chemical corporations have stressed the latter(9).

Because of the paucity of reliable data available only approximations can be made of the proportion of cancers that are caused by environmental agents. Ames(13) states that it has been

estimated that 80% of human cancer is due to environmental causes. Using the term environment in its broadest sense, to include both the macro and micro environments, Higginson(14) used circumstantial evidence to estimate that 80 to 90% of all cancers are dependent on environmental factors; others have provided similar estimates(15). Whatever the final statistic, it is clear that environmental factors are considered important as causes of cancer.

The most widely accepted model of cancer induction proposes that the carcinogen acts either directly or indirectly at the subcellular level to induce aberrations in chromosomal genes: when such mutations lead to a loss of regulatory function the result may be tumor growth and cancer. Chemical carcinogens can also react with and alter the structure of subcellular macro molecules other than chromosomal DNA: mitochondrial DNA, RNA, and proteins are examples(23). Such reactions are usually between electrophilic carcinogens and nucleophilic reaction sites in the target molecules. The immediate and long term consequences of these reactions, however, are not yet understood. The observation that 90% of carcinogens can cause point mutations in bacterial genes(19) is often considered good circumstantial evidence that chemical carcinogens may act by causing local changes in DNA sequences. However, because critical target molecules have yet to be positively identified and experimentally confirmed for any carcinogens, the foregoing relationship is not necessarily causal;

thus, the mutagen-DNA model of carcinogenesis remains unsubstantiated. Critics(12) of the mutagen-DNA model have argued that it fails to explain many cancers, and have proposed alternative models based on mechanisms such as genetic transposition, or reaction of the carcinogen with a critical enzyme such as DNA polymerase. Clearly, the definitive carcinogenesis model has yet to be devised.

Because mutagens can induce chromosomal and genetic damage in humans, they can cause serious health problems other than cancer. Chromosome damage to young fetuses can cause gross abnormalities, often leading to spontaneous abortions or still births. Mutagenic chemicals can cause three forms of genetic damage in humans: (i) dominant mutations, which become immediately apparent as birth defects if they occur in either germ line cells or in the developing fetus; (ii) recessive mutations are the most common form of genetic damage, many of their effects are subtle and may escape detection for several generations; (iii) sex linked recessive mutations are expressed immediately in progeny, which explains why males are more prone than females to certain sex linked genetic defects. Genetic damage to the fetus can cause serious congenital defects. Recessive mutations in germ line cells can cause accumulations of mutations in the population pool with deleterious long term results.

Thus, mutagenic chemicals pose a dual problem when present in the environment: they are potential carcinogens and causal agents of genetic damage in humans.

Because many naturally occurring and synthetic chemicals are known to be carcinogenic and/or mutagenic(17) it would be desirable to identify problem compounds and to establish where they occur in the environment. Such data would enable environmental managers to reduce public exposure to genetically hazardous compounds.

This, however, is a formidable task as is illustrated by the following facts that were researched by Maugh(18): there may be as many as 50,000 chemicals in everyday use, not including pesticides, pharmaceuticals and food additives; there may be as many as 1,500 active ingredients in pesticides; there may be as many as 4,000 active ingredients in drugs and about 2,000 non-active ingredients. Estimates also suggest that there are about 2,500 additives used for nutritional value and flavoring and 3,000 chemicals used to promote productivity.

Expense and time factors render it unrealistic to use conventional mammalian tests to assess the carcinogenicity of each of the foregoing chemicals. For this reason, several short term tests have been developed for the purpose of screening suspect chemicals for mutagenic activity: according to the previously discussed mutagen-DNA

model for carcinogenesis, if a chemical is carcinogenic it should also be capable of inducing mutations in test cells; when properly selected such mutations are easier to detect than cancer which can take up to 20 years to develop. Also, by using test cell systems, much larger populations can be screened than is possible using conventional tests. The most popular of the short term tests is the Ames' test in which reverse mutations in the bacterium Salmonella typhimurium are used to detect mutagenicity in test chemicals: 90% of the carcinogens tested have yielded positive results in the Ames' test - an impressive correlation by any standards.

With so many mutagenic chemicals in everyday use, it is highly probable that many find their way into the aquatic environment; in fact, several mutagenic chemicals have been identified in natural freshwater systems(20). Monitoring effluents and receiving waters for the presence of individual mutagenic chemicals would be prohibitively expensive and time consuming. It should be possible, however, to adapt short term mutagenicity screening tests for the detection of mutagenic activity in effluent, water and sediment samples; positive samples could be further examined using analytical chemical techniques and where necessary, mammalian carcinogenicity tests. The Ames' test has already been used for this purpose in the Microbiology Labs at NWRI.

The Ames' test has associated limitations: it can, for instance, only detect those mutagens that cause specific point mutations in the Salmonella test strain. Substances that induce genetic alterations by other mechanisms, such as DNA breakage, may escape detection. For this reason other short term tests could provide useful complementary information if used in conjunction with the Ames' test in a battery of short term mutagenicity tests. The sister chromatid exchange test (SCE), which quantifies the formation of apparently reciprocal exchanges between homologus sections of sister chromatids, is one such test. Many mutagens cause SCEs in mammalian cells(24), however, the molecular basis for SCE formation is not yet fully understood(29).

In the SCE test mammalian cells are exposed to the base analogue 5-bromodeoxyuridine BRdU during two consecutive cell replications (Figure 1); the BRdU is differentially incorporated into the daughter chromosomes (Figure 1). The chromatid with BRdU substituted in both DNA strands is less condensed and stains weaker than the chromatid with the single substituted strand(25). After appropriate staining the sister chromatids are distinguished by their differential fluorescence (Figure 1) or staining(25) and SCEs can be readily identified(21). Because SCEs are thought to result from a DNA breakage mechanism, the SCE test should be sensitive to mutagens that cause genetic alterations by DNA breakage.

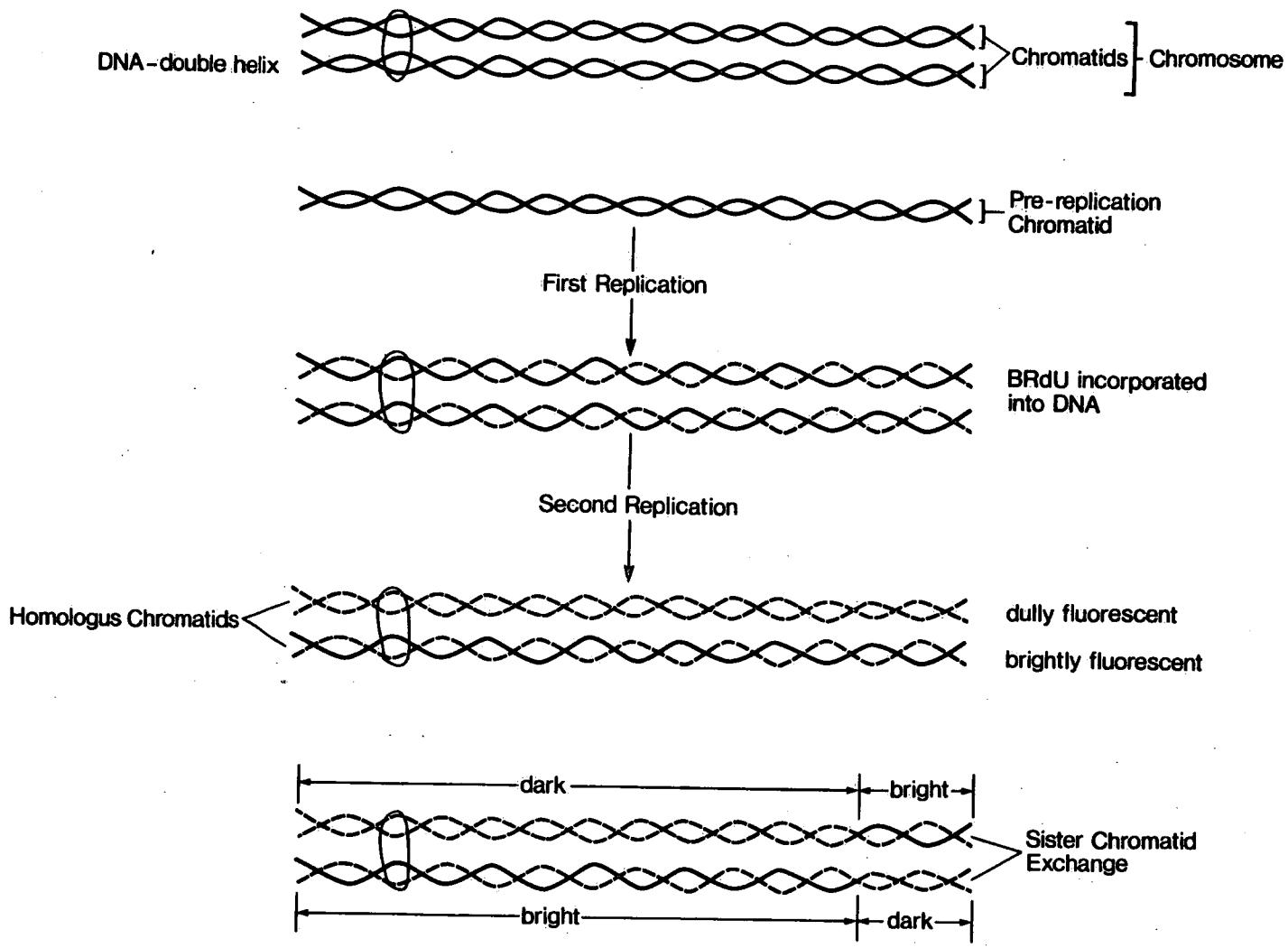

Figure 1 THEORETICAL BASIS OF SCE TEST

The SCE test has several properties that recommend it for use in a battery of short term mutagenicity tests: (i) the test system uses eukaryotic cells; (ii) SCEs are easily and rapidly detectable; (iii) because SCEs are common events in mutagen treated cells, a high degree of statistical accuracy can be achieved; (iv) a variety of mutagenic chemicals are detectable through SCE induction(24,29); (v) it can be adapted for use with a metabolic activation system; (vi) the SCE test gives few false postive results(29).

The SCE test however, is not without some shortcomings. Besides being an inhibitor of DNA synthesis(22) BRdU is also slightly mutagenic(21, 26); thus some positive samples may merely enhance the mutagenicity of BRdU rather than cause an actual mutagenic response. Further documented weaknesses of the SCE test have been considered by Raj and Heddle(28) and may be summed up in the observation that SCEs and chromosome aberrations arise by different mechanisms. Thus SCEs may be produced in the absence of chromosome aberrations; it has been established that aberrations can be produced without SCEs. The SCE test is known to produce false negative results(29).

The SCE test, as described in this report, should be readily adaptable for the detection of mutagenicity in environmental samples. It has been proposed(29) as a useful follow up test for samples that give positive responses in a simpler test such as the Ames' test.

Experimental investigation will be required to establish the optimum regime for the exposure of test cells to the sample matrix, and whether the incubation with BRdU should occur during or after cell treatment. As with other short term mutagenicity assays, considerable consideration must be given to the selection of a suitable sample preparation protocol so that the low mutagen levels associated with environmental water and sediment samples can be tested at detectable concentrations.

SCE TEST DESCRIPTION

1. Cell Cultures

Chinese hamster ovary cells (CHO) are suitable for general screening purposes(29). On receipt, the cell cultures are incubated overnight at 37°C and are then subcultured and reincubated at the same temperature. The cells are cultured in Minimal Essential Medium containg Earl Salts solution (MEM+; Appendix 1) with foetal calf serum (12.5%), L-glutamine (20 μM), sodium bicarbonate (0.3%), 0.1 g streptomycin, and penicillin (9.9×10^4 units) per litre. Fifteen mL portions of MEM+ in radiation sterilized tissue culture flasks (250 mL; surface treated) are inoculated and incubated in a humidified (100% relative humidity) incubator at 37°C. The incubator atmosphere is maintained at 5% CO_2: the CO_2 interacts with the bicarbonate in the growth medium to provide buffering capacity.

CHO subcultures are prepared using the following procedure:

(i) attach a sterile pasteur pipette to a suction pump and aspirate the spent growth medium from the tissue culture flask, the CHO cells will remain attached to the flask bottom.

(ii) Equilibrate the required solutions (HBSS, trypsin solution, and MEM+) at 37°C; surface sterilize the necks of the solution containers by flaming with alcohol before opening.

(iii) Serum, which can inactivate the tryprin enzymes, is removed from the tissue culture flasks by washing with Hank's Balanced Salt Solution (HBSS; Table 2): add 5.0 mL of HBSS to each flask; swirl the HBSS solution around the flask bottom and then aspirate it.

(iv) Add 5 mL of 0.25% trypsin solution to each flask: the trypsin solution is prepared in HBSS. Coat the sheet of cells on the flask bottom with the trypsin solution and then promptly aspirate it from the flask.

(v) Incubate the trypsin coated cells at 37°C.

(vi) After 5 minutes use an inverted microscope to determine whether the cells have detached from the flask surface; detached cells will have a rounded appearance.

(vii) When the cells have detached from the flask surface add 15 mL of MEM+ to the flask: the serum in the medium prevents cell damage by inactivating the Trypsin.

(viii) Suspend the cells in the MEM+ by pipetting vigorously and repeatedly.

(ix) Subculture the cells using a culture splitting procedure: add 5 mL aliquot of the CHO cell suspension to a 10 mL portion of fresh MEM+ in a 250 mL tissue culture flask; confluent growth will be attained after 3 days incubation at 37°C. Increase the splitting ratio (e.g. 1:25) to increase the incubation time required before cell growth becomes confluent. The mean doubling time for CHO cells under the described conditions is 18 to 20 hours.

(x) Each flask can be used to grow 2 cell crops.

(xi) Cultures should be sub-cultured as soon as growth has become confluent.

2. Determination of Viable Cell Count

The viable cell count determination method is based on the observation that viable cells do not take up Trypan Blue dye whereas nonviable cells do. Prepare a dilution of the cell suspension in Trypan Blue so that, when the cell suspension is added to a Hemacytometer, approximately 50 to 200 cells are observed. Use a standard procedure to count the number of unstained cells and then calculate the number of viable cells per mL of cell suspension.

3. Stock Cultures

Cell stock cultures are maintained by freezing.

(i) Prepare a 2.5 to 4.0×10^6 viable cell per mL suspension of log phase CHO cells in MEM+.

(ii) Cool the cell suspension in an ice water bath; add dimethyl sulfoxide (DMSO) to give a final concentration of 10% (v/v).

(iii) Using a syringe, transfer 1 mL of cell suspension to a sterile ampule; flame seal the ampule.

(iv) Place the ampule in a polystyrene box, seal the box and place it in a -70°C freezer for 2 hours.

(v) Store the ampule in liquid nitrogen.

Use the following procedure to resuscitate stock cultures:

(i) Remove an ampule containing frozen CHO cells from the liquid nitrogen.

(ii) Thaw the contents of the ampule by agitation in a 37°C water bath, rinse the ampule exterior with 70% ethanol and allow it to dry at room temperature.

(iii) Open the ampule and, using a sterile syringe with an 18 g needle, aseptically transfer the contents to a tissue culture flask.

(iv) Add 15 mL of MEM+ to the tissue culture flask.

(v) Incubate the inoculated flask at 37°C.

(vi) Replace the medium after 24 hours and reincubate the cells.

4. SCE Test Procedure

(i) Prepare a confluent culture of CHO cells.

(ii) Trypsinise the cells (as previously described).

(iii) Add 5 mL of MEM+ to the flask and suspend the cells. Remove the cell suspension and put it in a sterile, capped polystyrene tube.

(iv) Use a hemacytometer and a phase contrast microscope to determine the number of cells in the cell suspension.

(v) Add 5 mL of MEM+ to the required number [blank (X3), control (X3), analytical (X3)] of surface treated tissue culture dishes (100 mm x 20 mm; 55 cm^2 growth surface).

(vi) Add 10^5 CHO cells to each dish.

(vii) Incubate the inoculated cultures at 37°C for 24 hours. The cells should still be in the exponential phase of growth at this stage.

4.2 Exposure Procedure

(i) Aspirate the exhausted growth medium from the culture dishes.

(ii) Dissolve the test chemical in 0.2 mL DMSO and then dilute to the required concentration with MEM+ containing no serum.

(iii) Add 5 mL portions of the test chemical solution to 3 of the prepared culture dishes.

(iv) Prepare distilled water blanks and standards (methyl nitrosoguanidine) in the same way.

(v) Incubate the test culture dishes at 37°C in an air tight plastic box.

4.3 Exposure Termination Procedure

The cell treatments are terminated after 30 minutes, or an alternative exposure period (e.g. 1 hour, 29), using the following procedure:

(i) Remove the treatment solutions from the culture dishes.

(ii) Wash the treated cells in HBSS (10 mL per dish).

(iii) Add 10 mL of MEM+ containing 5-bromo-2'-deoxyuridine (BRdU) at a final concentration of 10 μM to each dish. This step is carried out under GE Gold Lamps in order to minimize photolysis of the BRdU which could cause increased levels of background SCES. BRdU substituted DNA is also subject to photodegradation(29).

(iv) Wrap the dishes in aluminum foil and incubate them at 37°C for 28 to 40 hours by which time second metaphase cells should have formed.

(v) Two hours before harvesting the cells add 0.1 mL of $2x10^{-4}$ M colchecine prepared in 0.85% saline to each culture. Colchecine inhibits spindle formation and causes an accumulation of metaphase cells.

4.4 Cell Harvesting

(i) Aspirate the exhausted growth medium from the culture dishes.

(ii) Remove the residual serum from the post treatment cells by washing with 5 mL portions of HBSS.

(iii) Trypsinise the cells as previously described (1(ii) to 1(vi)).

(iv) Terminate enzyme activity by the addition of 4 mL portions of MEM+ to each dish.

(v) Thoroughly suspend the CHO cells in the MEM+ and then transfer the cell suspensions to sterile, graduated, conical bottom centrifuge tubes (15 mL capacity).

(vi) Rinse each tube with MEM+ and transfer the rinsings to the appropriate centrifuge tube.

(vii) Cell clumps must now be broken up by rapidly and repeatedly flushing the cell suspensions with a pasteur pipette (approximately 20 cycles).

(viii) Centrifuge the cell suspensions at 1000 RPM for 5 minutes.

(ix) Aspirate each supernatant until the meniscus reaches the 0.1 mL graduation; resuspend the cells by vortex mixing, and then add 5 mL of hypotonic solution (Appendix 3) to each tube.

(x) Incubate the cells in the hypotonic solution for 12 minutes at room temperature; then, while mixing, add 3 to 5 drops of fixative solution (Appendix 4) to each tube; thoroughly vortex mix the tube contents.

(xi) Centrifuge the cell suspensions at 1,000 rpm for 7 minutes.

(xii) Aspirate each supernatant to the 0.1 mL graduation; resuspend the cells by vortex mixing; add 4 mL of fixative solution to each tube while vortex mixing the tube contents.

(xiii) Centrifuge the cell suspensions at 1,000 rpm for 7 minutes.

(xiv) Aspirate each supernatant to the 0.1 mL graduation; while vortex mixing the tube contents, add 4 mL of cold fixative (previously cooled in an ice water

bath); cap the tubes and store them overnight at 5°C.

4.5 Slide Preparation

(i) During the slide preparation procedure all cell suspensions should be stored on crushed ice.

(ii) Centrifuge the cell suspensions at 1000 rpm for 7 minutes.

(iii) Aspirate each supernatant, allowing 0.1 to 1.0 mL of cold fixative to remain in each tube.

(iv) Resuspend the cells by vortex mixing and then store the cell suspensions on crushed ice.

(v) Use a pencil to label 5 glass microscope slides per treatment tube.

(vi) a. Rinse a pasteur pipette with cold fixative solution.

b. Dip a glass slide in double distilled water, shake off any excess water.

c. Using the pre-rinsed pasteur pipette, allow 3 drops of cell suspension to fall from a height of 40 to 100 cm onto the glass slide. Five slide preparations should be made for each cell suspension.

d. Stand the slides at an acute angle and allow to dry overnight at room temperature.

(vii) Using a phase contrast microscope, examine the air dried slide preparations for the presence of metaphase cells.

This preliminary examination may reveal some technique short comings:

(a) The chromsomes are too tightly clumped: this condition suggests that the hypotonic treatment was too brief; correct by prolonging the incubation of cells in the hypotonic solution.

(b) The chromsomes are floating independently on the slide surface: this condition suggests that the hypotonic treatment was too severe; correct by

curtailing the incubation period of cells in the hypotonic solution.

(c) Many cells are overlapping; correct by increasing the cell suspension dropping height (step vi.c).

4.6 Staining Procedure

(i) Prepare a stock solution (500 μg/mL) of Hoechst stain in double distilled water (Hoechst 33258, Aldrich; Bisbenzimid H33258, Hoechst Pharamaceutical, Montreal, P.Q.).

(ii) Prepare a 10 μg/mL dilution of Hoechst in a plastic trough.

(iii) Place the slides in a stainless steel slide carrier.

(iv) Rinse the slides in distilled water (20 agitations) and then stand the slide carrier on a paper towel for 5 to 10 minutes.

(v) Imerse the slides in the Hoechst staining solution; stain for 20 minutes.

(vi) Rinse the stained slide preparations by imersion in tap water.

(vii) Repeat the rinsing procedure (vi) using distilled water.

(viii) Allow the slides to dry at room temperature.

(ix) Add 3 drops of Sorensen's buffer pH 8.0 (Appendix 5) to each slide.

(x) Cover each cell preparation with a coverslip; use a syringe to apply a seal of liquid rubber (Carter's Rubber Cement) around the edges of each coverslip to prevent evaporation.

(xi) Place the slides 15 cm under a bank of cool, white fluorescent light tubes; expose for 18 to 20 hours.

(xii) Remove the rubber cement and coverslip from the slides.

(xiii) Place the slides in the slide carrier, rinse them in distilled water, and then allow them to dry at room temperature.

(xiv) Prepare a 4% solution of Giemsa stain in 0.01M Sorensen's buffer, pH 6.8; remove the scum from the surface of the stain using a paper tissue.

(xv) Immerse the slides in the Giemsa stain for 8 minutes.

(xvi) Rinse the stained slide preparations in tap water.

(xvii) Repeat the rinsing procedure using distilled water.

(xviii) Allow the slides to dry at room temperature.

4.7 Slide Examination

(i) Place 2 drops of DPX mounting medium on each slide.

(ii) Examine 25 metaphase cells from each slide for SCE's.

(iii) Upon microscopic examination of the cells it will be apparent that the chromatids have stained differentially; the theoretical basis for this observation has been considered in the Introduction

to this report and is also discussed in greater depth by Perry and Wolff(25).

(iv) SCEs are recognized by the location of a dark stained chromatid segment on a lightly stained chromatid; a lightly stained chromatid segment will be located on the corresponding section of the dark chromatid.

(v) Each chromatid exchange is scored as a single event; where banding occurs, the exchanges at each end of the band are scored are separate events. Terminally located bands, however, are recorded as single SCEs (Figure 2).

(vi) Record the total number of SCEs on all the chromosomes of each cell examined.

(vii) Express the results as SCEs per cell.

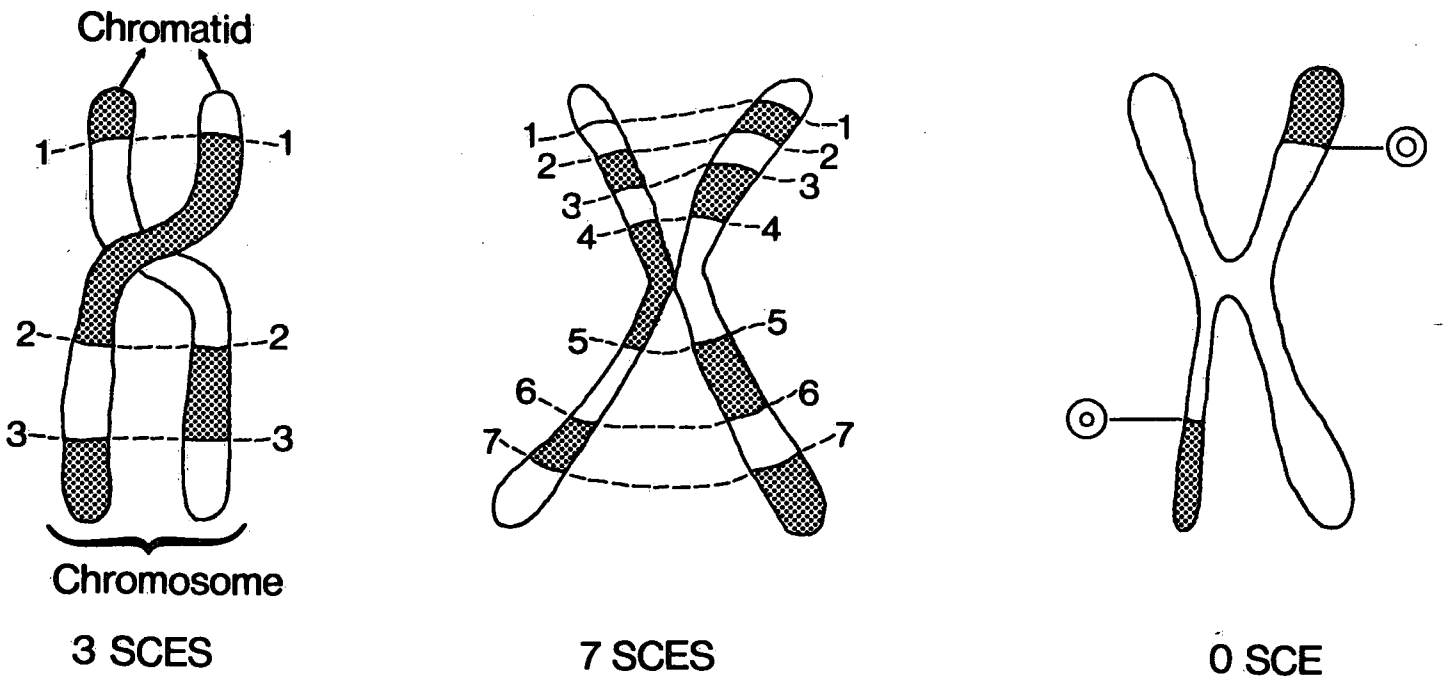

Figure 2 DIAGRAMATIC REPRESENTATION OF SISTER CHROMATID EXCHANGES

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APPENDIX 1

Ingredients of Minimum Essential Medium with Earl Salts (MEM+)

Available from Flow Laboratories Inc.

Ingredient	mg/L
$CaCl_2 \cdot 2H_2O$	264.90
KCl	400
$MgSO_4 \cdot 7H_2O$	200
NaCl	6,800
$NaH_2PO_4 \cdot H_2O$	140
Dextrose	1,000
Phenol Red, Na	17
L-arginine-Hcl	126.40
L-Cystine, Na_2	28.42
L-Histidine $\cdot$HCl $\cdot H_2O$	41.90
L-Isoleucine	52.50
L-leucine	52.50
L-lysine-Hcl	73.06
L-Methionine	14.90
L-phenylalanine	33.02
L-threonine	47.64
L-tryptophan	10.20
L-tyrosine	36.22
L-valine	46.90
D-Ca Pantothenate	1.00
Choline Chloride	1.00
Folic acid	1.00
i-inositol	2.00
Nicotinamide	1.00
Pyridoxal-HCl	1.00
Thiamin-HCl	0.10
Vitamin B_{12}	1.00
Hepes buffer	20 mM

Store at -20°C; shelf life is six months.

APPENDIX 2

Ingredients of Hanks Balanced Salts Solution (HBSS)

Ingredient	mg/L
KCl	400.0
KH_2PO_4	60.0
NaCl	8000.0
$NaHCO_3$	350.0
Na_2HPO_4	47.5
Dextrose	1000.0
Phenol Red, Na	17.0

Available from Flow Laboratories Incorporated

APPENDIX 3

Preparation of hyptonic solution.

Stock solution A: 0.075 MKCl

Stock solution B: MEM+ diluted 1:4 with sterile distilled water

Hypotonic solution contains 50 mL stock solution A + 12.5 mL stock solution B + 37.5 mL sterile distilled water.

APPENDIX 4

Fixative solution

30 mL absolute Methanol:10 mL glacial acetic acid

APPENDIX 5

Preparation of Sorensen's buffer

pH	M/15 Na_2HPO_4	M/15 KH_2PO_4
6.8	50	50
8.0	94.5	5.5

REFERENCES

1. Anon, 1981. Two views of cancer. Nature, 289:431-432.

2. Vital Statistics - Special Reports, U.S. DHEW, 43, 1956.

3. Vital Statistics of the U.S. 2:1970.

4. Pollack, E.S. and J.W. Horn, 1980. J. Natn. Cancer Inst. 64:1091.

5. Trends in Mortality, 1951 to 1975 (U.K. Office of Populations Censuses and Surveys, Series DH1, No. 3, 1978).

6. Third National Cancer Survey, 1969 to 1971 (NCl, Bethseda).

7. Schneiderman, M. 1978. Occupational Safety and Health Administration OSHA Docket 090.

8. Zdeb, M.S., 1977. Am. J. Epedemiol 106:6.

9. Epstein, S.S. and J.B. Swartz, 1981. Fallacies of Lifestyle Cancer Theories. Nature 289:127-130.

10. Reif, A.E. 1981. The causes of cancer. American Scientist 69:437-447.

11. Peto, R. 1980. Distorting the epidemiology of cancer: the need for a more balanced overview. Nature 284:297-300.

12. Cairns, J. 1981. The origin of human cancers. Nature 289:353-357.

13. Ames, B.N. and J. McCann, 1975. Carcinogens are mutagens: a simple test system. In: Screening Test in Chemical Carinogenics, eds. Montesano, R., H. Bartsch and L. Tomatis.

14. Higginson, J., 1976. Importance of environmental factors in cancer. Inserm Symposia Series 52:IARC Scientific Publications No. 13, 15 to 24.

15. Doll, R. 1967. Prevention of cancer - pointers for epidemiology. The Rock Carling Fellowship 1967 - Nuffield Provincial Hospitals Trust 1967, 1967 Whilefrears Press Ltd. London.

16. Doll, R., 1977. Strategy for detection of cancer hazards to man. Nature 265:489-596.

17. Ames, B.N., 1979. Identifying environmental chemicals causing mutations and cancer. Science 204: 587-593.

18. Maugh, T.H. 1978. Chemicals how many are there? Science 199, 162.

19. McCann, J., E. Choi, E. Yamasaki, and B.N. Ames, 1975. Detection of Carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. USA 72, 5135-5139.

20. Rokosh, D.A. and T.N. Lovasz, 1979. Detection of mutagenic activity; screening of twenty three organic compounds of industrial origin in the St. Clair River. Internal Publication, Microbiology Section, Laboratory Services Branch, Ontario Ministry of the Environment, Micro 7837, p. 31.

21. Soares, E.R., 1979. Genetic aspects of short-term testing and an appraisal of some in vivo mammalian test systems. In: Strategies for short term testing for mutagens/carcinogens, Editor B.E. Butterworth, CRC Press Inc., Florida, pp. 67-86.

22. Pollard, D.R., M.M. Baran and R. Bachvarova, 1976. J. Embryol. Exp. Morphol. 35:169-178.

23. Miller, E.C. and J.A. Miller, 1976. The metabolism of chemical carcinogens to reactive electrophiles and their possible mechanisms of action in carcinoginesis. In: Chemical Carcinogens. Ed. C.E. Searle. ACS Monograph 173. American Chemical Society, Washington, D.C. pp 737-762.

24. Perry, P. and H.J. Evans, 1975. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature 258:121-125.

25. Perry, P. and S. Wolff, 1974. New Giemsa method for the differential staining of sister chromatids. Nature 251:156-158.

26. Wolf, S. and P.E. Perry, 1974. Chromosoma 48:341-353.

27. Heddle, J.A. and C.F. Arlett, 1980. Untransformed xeroderma pigmentosum cells are not hypersensitive to sister-chromatid exchange production by ethyl methanesulphonate - implications for the use of transformed cell lines and for the mechanism by which SCE arise. Mutation Research, 72 (119-125).

28. Sudharsan Raj, A. and J.A. Heddle, 1980. Simultaneous detection of chromosomal aberrations and sister-chromatid exchanges; experience with DNA intercalating agents. Mutation Research, 78:253-260.

29. Latt, S.A., J. Allen, S.E. Bloom, A. Carrano, E. Falke, D. Kram, E. Schneider, R. Schreck, R. Tice, B. Whitfield, and S. Wolff, 1981. Sister chromatid exchanges: A report of the gene-tox program. Mutation Research, 87:17-62.