Sister chromatid exchange (SCE) frequencies were determined in
human amniotic fluid cells in vitro after exposure to Arochlor 1242
(A-1242) (10 ppm), infectious herpes simplex virus type 2 (HSV-2)
\(10^{-1}, 10^{-2}, 10^{-3}\) and \(10^{-4}\)PFU/cell), ultraviolet (uv) inactivated
HSV-2 and combinations of A-1242 and HSV-2. Mitomycin C (MMC) (4 ng/
ml) was included as a positive control; dimethyl sulfoxide (DMSO) (1%)
was included as a negative control. Short and long term toxicity of
A-1242 on these cells was also determined. Cells treated with 2.5 ppm
A-1242 for three weeks were analyzed for chromosomal aberrations.

Acute (short term) toxicity was not clearly defined after seven
days exposure of cells to 10, 25, 50 and 100 ppm of A-1242. Chronic
(long term) toxicity was evident after four weeks continuous exposure
to 2.5, 5 and 10 ppm of A-1242; it was dose dependent, indicative of
cumulative effects.

No increase in chromosome breaks or gaps was seen in cells
exposed to 2.5 ppm of A-1242 for three weeks. The number of chromo-
somes with accentuated secondary constrictions was greater than in
controls, but no predilection for any one chromosome was observed.

SCEs were determined after growth of cells for two replication cycles in the presence of $10^{-5} \text{M}$ and $4.5 \times 10^{-5} \text{M}$ of 5-bromodeoxyuridine (BrdU). A-1242 and infectious HSV-2 each induced a statistically significant increase in the number of SCEs at the higher BrdU level. A concentration of $10^{-4} \text{PFU/cell}$ HSV-2 produced no change in the number of SCEs over the background ($10^{-5} \text{M}$ BrdU) level; results with other concentrations of infectious HSV-2 at the lower BrdU level were ambiguous. Inactivated virus appeared to cause a decrease in the number of SCEs in the presence of $10^{-5} \text{M}$ BrdU; significance was at the 90% confidence level.

The combined effects of A-1242 and infectious HSV-2 at the lower BrdU concentration ($10^{-5} \text{M}$) showed no differences from either A-1242 or virus alone. At the higher BrdU concentration ($4.5 \times 10^{-5} \text{M}$) the number of SCEs was dependent on virus concentration. This implies that productive virions are required to induce an increase in SCEs.

Inactivated virus produced either no change over the background (BrdU) level or a decrease in the number of SCEs, depending on BrdU concentration. The combination of A-1242 and inactivated HSV-2 induced an increase in SCEs over virus alone, but no increase when compared to A-1242. This increase was not significant at the 95% confidence level.

The number of sister chromatid exchanges per chromosome within each of the treatment groups did not follow a Poisson distribution. The number of SCEs was not distributed according to chromosome length and did not fit patterns observed by others with BrdU alone indicating
that the smaller chromosomes had fewer than the expected number of SCEs (Chaganti et al., 1974; Latt, 1974). However, A-1242 with $4.5 \times 10^{-5} \text{M} \text{BrdU}$ had slightly higher than the expected number of SCEs in the F group chromosomes (numbers 19 and 20).

Densitometry scans of two homologous chromosomes from cells treated with MMC indicated that unequal exchanges possibly occur and may reflect subtle changes by such chemicals on chromosomes.

While HSV-2 may or may not be mutagenic, depending on whether productive or defective virions were involved in the increase in sister chromatid exchanges, the mutagenic potential of Arochlor 1242 appears detectable only in combination with another chemical or virus.

The combined effects of Arochlor 1242 and herpes simplex virus type 2 were neither synergistic nor additive; but an increase in the number of sister chromatid exchanges was observed; this increase was dependent on both BrdU and HSV-2 concentrations.
The Combined Effect of Herpes Simplex Virus Type 2 and a Polychlorinated Biphenyl (Arochlor 1242) on Human Chromosomes: Induction of Sister Chromatid Exchanges

by

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Human Amniotic Fluid Cells (H-AF) Grown for Two Replication Cycles with 10^-5M BrdU

Infectious HSV-2 (10^-3PFU/Cell), Acridine Orange

Arochlor 1242 (10 ppm) + Infectious HSV-2, Acridine Orange

Mitomycin C (4 ng/ml) + Infectious HSV-2

Endoreduplication. UV Inactivated HSV-2, Acridine Orange

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Mitomycin C (4 ng/ml) + HSV-2 (10^-1PFU/Cell)

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I. INTRODUCTION

Interactions among chemical, biological and physical agents are important in this era of environmental contamination; yet, little is known about such interactions. Of particular relevance are the synergistic or antagonistic effects of these agents in altering human cellular DNA. Two consequences of such effects are mutagenesis and carcinogenesis. Mutagens may affect germ cells, while carcinogens alter somatic cells, but a relationship between the two processes has been suggested. Ames (1975) believes that most, if not all, carcinogens are, in fact, mutagens. Using a bacterial system, he has shown that over 85% of the carcinogens tested cause mutations. Because of these results, Ames believes that carcinogens cause cancer through somatic mutation.

However, Berenblum (1972), who has reviewed the evidence for and against the somatic mutation theory of cancer, stated:

"far from finding a close correlation between mutagenesis and carcinogenesis, one is led to conclude that almost any chemically reactive compound, if tested by a sufficiently wide range of methods, is likely to prove mutagenic".

Whereas, animals are required to determine the carcinogenicity of most substances and factors (e.g., cost and facilities) limit the number of animals used, large numbers of bacteria can be used to test
for mutagenicity. J. A. Miller (1975) therefore believes that the problem of a correlation between carcinogens and mutagens is merely a statistical one.

Carcinogenesis involves a single individual, while a substance that induces mutations poses hazards for future generations. Which process -- carcinogenesis or mutagenesis -- poses the greatest risk to man? The answer to that question may be purely academic. Berenblum (1972) concluded that an attempt to correlate carcinogens and mutagens "becomes an exercise in theoretical speculation and of little relevance to the practical problem of human hazards".

One of the problems in determining whether a substance is mutagenic - or carcinogenic - is the necessity of using very large doses. The majority of the human population is not exposed to acute doses, but to lower concentrations over long periods of time. Thus, compounds that have widespread use and have weak mutagenic effects may be far more important than more powerful mutagens (Malling, 1972). At this point, we know little either about substances with weak mutagenic activity or about chronic exposure to such agents.

One way by which the mutagenic potential of a substance can be shown is to determine whether the agent produces an increase in chromosome breakage in vitro or in vivo. However, large concentrations of substances are required to induce breakage in vitro and the validity of chromosome breakage in vivo, as a parameter for mutagenic activity, has been questioned (Schinzel, 1976). Breakage may reflect only a small portion of mutations and, unless stable rearrangements occur, breaks may be repaired without permanent, detectable damage to the DNA.
Substances with weak mutagenic activity and substances causing mutations at the molecular level may not necessarily induce chromosome breakage.

Sister chromatid exchanges were first observed in mammalian cells by Taylor (1958) who noticed unequal labelling of sister chromatids after exposure of cells to tritiated thymidine. He concluded that DNA strand breakage had occurred and that there was a symmetrical exchange of material between sister chromatids.

In 1974 Latt demonstrated that Mitocycin C (10 ng/ml), a known mutagen and carcinogen, induced a significant increase in sister chromatid exchanges (SCE)\(^1\), while chromosome breakage remained within the normal range.

Recently Latt (1973) and others (Korenberg and Freedlender, 1974; Kato, 1974) demonstrated sister chromatid exchanges by growing cells for two replication cycles in the presence of 5-bromodeoxyuridine (BrdU), followed by staining with fluorescent dyes or with fluorescence plus Giemsa. Since then, numerous known mutagens, including N-methyl-N-nitro-N-nitrosoguanidine, ethylmethanesulfonate and 4-nitroquinoline-1-oxide, have been tested for their ability to induce sister chromatid exchanges (Perry and Evans, 1975). Of 12 mutagens and/or carcinogens studied, the dose that induced a significant doubling in the incidence of exchanges produced only a minimal number of chromosomal aberrations.

Spermatogonial cells of mice injected intraperitoneally with BrdU, then with low doses of Mitomycin C showed a significant increase in

---

\(^1\) See Appendix for complete list of abbreviations.
sister chromatid exchanges (Allen and Latt, 1976). Since chromosomal rearrangements induced by Mitomycin C in spermatogonia have been implicated in mutagenesis (Bempong and Trower, 1975), sister chromatid exchanges induced by various chemicals, including Mitomycin C, may also reflect an increase in mutations (Beck and Obe, 1975; Perry and Evans, 1975).

But what about interactions between two chemicals or between viruses and chemicals? Very few investigations have been conducted on the combined effects of two or more agents. A search for combined effects is important because no single chemical, biological or physical agent exists in isolation in the environment.

The work presented in this thesis was designed to seek evidence for mutagenic effects in human cells due to their interaction with low concentrations of herpes simplex virus type 2 (HSV-2) and low doses of one of the polychlorinated biphenyls (Arochlor 1242).

HSV-2 was chosen for several reasons: 1) it has been implicated in cervical cancer (Klein, 1972; Nahmias et al., 1971); 2) it passes through the placenta (Catalano and Sever, 1971); 3) it can remain in a latent or very slowly multiplying state in the dorsal root ganglia of the spinal cord (Walz et al., 1974; Baringer, 1975); and 4) it is a DNA virus that may be incorporated into the cell genome, thus altering the genetic code (Stich and Yohn, 1970; Wagner, 1974).

Polychlorinated biphenyls (PCBs) were selected because, like certain pesticides (e.g., DDT), they are present and persistent in the environment and may interact with other chemicals (Lichtenstein, 1972). PCBs have been used for over 40 years industrially in hydraulic and
lubricating fluids, as plasticizers for plastics, as ingredients of caulking compounds, printing inks, and adhesives, in flame retardants, and as extenders for pesticides (Fishbein, 1974). Their occurrence is worldwide and amounts varying from one part per billion to 600 parts per million (ppm) have been found in man, fish, birds and other animals (Lee and Falk, 1972).

To understand the possible combined mutagenic potential of Arochlor 1242 and HSV-2, one must keep in mind that the modes of action by which each agent may alter cellular DNA are probably different. While a great deal of information on chemical mutagenesis has accumulated (Kohlman, 1966; Hollaender, 1971; Hollaender, 1973), there is a relative lack of knowledge about viruses as mutagens.

Three important factors contribute to the effects of viruses on host cells, factors that have little, if any, influence on the action of chemicals on cells (Stich and Yohn, 1970): 1) a virus preparation may cause cell death, be mutagenic or have no effect depending on the host cell itself; 2) the virus population is not homogeneous, but is composed of both infectious and defective virions and the latter may vary in the degree of defectiveness; and 3) new genetic information carried by the viral genome may be added to that of the host. Infectious virus will not be mutagenic because it destroys the cells in which it replicates. However, defective virions are of concern

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2 In the United States PCBs are produced by Monsanto Company under the trade name "Arochlors".

3 The last two digits in the Arochlors represent the amount of chlorine present (i.e., Arochlor 1242 contains 42% chlorine).
because they may be incorporated into cellular DNA or, in some other manner, alter that DNA.
II. METHODS AND MATERIALS

A. Cells and Media

Cells from human amniotic fluid (H-AF) were obtained as a primary culture. At the second passage, cells were stored in liquid nitrogen in medium with 5% dimethylsulfoxide (DMSO). They were thawed as needed and used at either passage three or passage six. WI-38 human embryonic lung cells were obtained from the American Type Culture Collection at passage 17, stored in liquid nitrogen, and used at passage 24. A white New Zealand rabbit was the source of kidney cells. The animal was anesthetized before removal of kidneys. Capsule and medulla of the kidneys were removed; the cortex was minced and kidney fragments rinsed four times with Hanks' balanced salt solution (BSS). Trypsin (0.25% in BSS) was then added and kidney fragments were stirred for 15 minutes. Trypsin was discarded, replaced with fresh trypsin, and the fragments were stirred for one hour at room temperature. The cell suspension was decanted and stored at 4°C. while fragments were again treated with trypsin for an additional hour. After centrifugation of cell suspensions at 1500 rpm for ten minutes, cells were resuspended in medium, seeded in appropriate vessels and used as primary cultures.

All cells were grown in Eagle's Minimum Essential Medium (MEM)

---

4 H-AF cells were kindly supplied by Miss C. Olson, Clinical Cytogenetics Laboratory, University of Oregon Health Sciences Center.

5 Used in virus titrations, PCB analysis and SCE studies.

6 Used in fluorescent antibody studies.
(Grand Island Biological Co.), supplemented with non-essential amino acids, sodium pyruvate, L-glutamine, Gentamycin (50 μg/ml) (Squibb) and 15% fetal calf serum (Flow Laboratories). Overlay medium, used on cells for virus titrations, consisted of the supplemented MEM with 10% fetal calf serum plus 1.5% methylcellulose (4000 centipoises). Cells were incubated at 37°C. in an atmosphere of 5% CO₂ in air.

B. Preparation of stock virus, titration and inactivation

Preparation of stock virus: HSV-2, strain 333, was inoculated at a 10⁻³ dilution (containing about 10⁵ plaque forming units (PFU)/ml), onto confluent monolayer cultures of primary rabbit kidney cells (RK) in 32 oz. prescription bottles and adsorbed for one hour at room temperature. Inoculum was then removed and medium added. When cells displayed 50% to 75% cytopathic effects (two days), the medium was removed and replaced with fresh cold medium. Cells were scraped off the glass with a rubber policeman, and the suspension was immediately frozen and thawed rapidly three times in a dry ice-ethanol bath. After centrifugation of the cell suspension at 2000 rpm for ten minutes, the supernatant fluid was removed, dispensed into freezing vials (Costar Plastic) in 1.0 ml aliquots and frozen rapidly in dry ice-ethanol. Vials of stock virus were then stored in liquid nitrogen.

Virus titrations: Virus was inoculated into primary RK cells or H-AF cells (passage 3) that had been grown in 60 mm Petri dishes

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7 HSV-2, strain 333, was generously provided by Dr. F. Rapp, Dept. of Microbiology, College of Medicine, The Milton S. Hershey Medical Center of The Pennsylvania State University, Hershey, Pa.
(Falcon Plastics) and adsorption was carried out for 90 minutes at room temperature. The inoculum was then removed and 5.0 ml overlay medium was added. After four days incubation at 37°C, 4.0 ml of a 0.005% neutral red solution in 0.85% saline was added; four hours later, plaques were counted with the aid of a Nikon inverted microscope.

**Virus inactivation:** Two ml aliquots of stock HSV-2 were placed in 60 mm Petri dishes which were then put on a rotator (Scientific Products Tek-tator) positioned 16.5 cm from a General Electric GOT8 ultraviolet germicidal lamp. Dishes were rotated at 30 rpm for five minutes. Inactivated virus was then refrozen until needed.

**C. Fluorescent Antibody**

H-AF cells, in the sixth passage, were grown on coverglasses placed in Lux multiwell plates. Cells were seeded at $10^5$ per well. One of the following chemicals was immediately added to each of two cultures: $10^{-5}$M BrdU, 1% DMSO, 10 ppm Arochlor 1242 (A-1242) or 4 ng/ml Mitomycin C (MMC). Eight cultures received no chemicals. For studies with infectious virus, cells were grown for three days, at which time $10^{-1}$ PFU/cell of HSV-2 was inoculated into all cultures containing added chemicals and half of the cultures without chemicals. For studies on ultraviolet (UV) inactivated virus, cultures were inoculated with 1.0 PFU/cell two hours after chemicals were added. Four days after addition of chemicals, the medium was removed, cells

---

8 Mitomycin C kindly furnished by Dr. Billie Wilson, Department of Anatomy, University of Oregon Health Sciences Center.

9 Based on titration of virus prior to inactivation.
rinsed thoroughly in phosphate buffered saline, pH 7.8, air dried and fixed for 15 minutes in acetone.

The direct immunofluorescent method was used to detect viral antigens. A 1:5 dilution of fluorescein isothiocyanate (FITC)-labelled HSV-2 antiserum, obtained from Microbiological Associates, was layered onto all cultures that had been exposed to chemicals and virus, half of the cultures with virus only, and half of the control cultures (neither chemicals nor virus). The remainder of the virus infected (no chemicals) and control cultures were layered with a 1:5 dilution of FITC-labelled normal rabbit serum. Cells were observed with a Zeiss fluorescent photomicroscope equipped with a HBO-200 w/4 super pressure mercury lamp. A BG 3 (Zeiss) exciter filter and barrier filters number 41 and number 50 (410 nm and 500 nm, respectively) were used. Cells were photographed with 35 mm Kodak Tri-X film using a two minute exposure.

D. PCB Analysis

Arochlor 1242 (A-1242)\textsuperscript{10} is a viscous liquid that is relatively insoluble in water. Acetone was used as the solvent for the acute toxicity experiment; for all other studies, a solution of A-1242 in dimethylsulfoxide (DMSO) was used. Two series of analyses were done, one on A-1242 in acetone and the other on A-1242 in DMSO. H-AF cells were exposed to 5 ppm A-1242 in acetone and maintained for four days.

\textsuperscript{10} Arochlor 1242 was generously provided by Dr. W. P. McNulty, Jr., Oregon Regional Primate Research Center, Beaverton.
Medium was removed and saved. Distilled water was added to the cells, which were scraped off the glass with a rubber policeman. The cell suspension was then frozen and thawed rapidly three times and cell debris removed by centrifugation. The medium, cell lysate and a stock A-1242 solution in acetone were submitted for analysis.

Since the concentration of A-1242 in the cell lysate was extremely low (see Results), DMSO was subsequently used to solubilize A-1242 in the belief that it might prove to be a better solvent. H-AF cells were exposed to 10 ppm of A-1242 in DMSO and maintained for four days. Medium was then removed and saved. Distilled water was added to the cells, which were scraped off with a rubber policeman, and sonicated with a Bromwell sonicator for one minute at high speed. Cell debris was removed by centrifugation. The supernatant fluid from sonicated cells and the culture medium, as were cells exposed to A-1242 in acetone, were analyzed according to the following procedure.

"Samples were transferred from plastic tubes to graduated concentration cylinders and tubes were rinsed with hexane extracted water. Samples were extracted four times with hexane with chloroform and back washed with 2% NaCl water. The extracts, finally in hexane, were evaporated to a few mls under an air jet and loaded on a 25 gram

11 I thank Dr. M. Deinzer and Mr. D. Griffin, Dept. of Agricultural Chemistry, Oregon State University, Corvallis, for performing the analyses.

12 Information on methodology was furnished by Dr. Deinzer.
5% water deactivated alumina column for clean-up. The samples were all eluted to a 0.65 Rf with hexane using methoxyazobenzene dye as an indicator. These were then analyzed on an electron capture gas chromatograph against a standard of A-1242. The gas chromatograph was an H.P. 5700A with a nickel 63 foil, a 4' x 1/8" ID pyrex column packed with 7% OV-1 on chromosorb H.P.W. 100/120 with a flow of 40 cc/min. 5% methane, 95% argon carrier gas. The samples were injected at 200°C., column temperature, the detector temperature at 300°C. A solvent blank was also run with the samples."

A-1242 was diluted to a concentration of 1000 ppm; this stock solution, stored at room temperature in a plastic test tube, was further diluted, as needed, so that 10 ppm also contained 1% DMSO. Dilutions were based on the average specific gravity (1.38) of A-1242. All concentrations mentioned in this and subsequent sections are input, not actual, amounts. The input (or final concentration in the medium) was based on dilution of stock undiluted A-1242 (1.38 g/ml). The actual concentration was determined by gas chromatographic analysis.

E. Chromosome Preparations

Sixteen hours prior to harvest of metaphase cells, colchicine at a final concentration of 0.027 µg/ml was added to the culture medium. After 16 hours incubation cells were treated with 0.1% trypsin plus 0.05% ethylenediaminetetra-acetate (EDTA) for two minutes at 37°C.
Cell suspensions were centrifuged at 1000 rpm for ten minutes, then cells were resuspended in a hypotonic solution of 0.075M KCl (potassium chloride) and incubated at room temperature for 20 minutes. Suspensions were centrifuged and cells again resuspended in hypotonic solution for five minutes. After centrifugation cells were carefully resuspended in 3.0 ml fixative (3 parts methanol to 1 part glacial acetic acid) and stored at 4°C. for up to four days. Cells were again centrifuged, resuspended in 1.0 ml fixative and dropped, from a distance of approximately 60 cm, onto clean dry slides. Each slide was immediately subjected to blowing (one hard "blast") and allowed to air dry. For the chromosome breakage study, cells were stained by the acetic-saline-Giemsa (ASG) technique (Sumner et al., 1971).

F. Sister Chromatid Exchanges (SCE)

A 100x stock BrdU "cocktail" was made, which consisted of: 9 x 10^{-3} M BrdU, 10^{-2} M deoxycytidine (dC), 6 x 10^{-4} M uridine (U) and 4 x 10^{-5} M fluorodeoxyuridine (FdU) (Latt, 1975). H-AF cells, in the third passage, were seeded into 250 ml plastic flasks (Falcon Plastics) at 8-12 x 10^5 per flask. The "cocktail" was then added so that the final concentration of BrdU was 10^{-5} M (in five experiments) or 4.5 x 10^{-5} M (four experiments). In one experiment 10^{-5} M BrdU was used without addition of dC, U or FdU. Immediately after seeding of cells, one of the following chemicals was added to each of two cultures: 10 ppm A-1242, 1% DMSO or 4.0 ng/ml MMC. Two cultures received no chemicals except BrdU. Only one of each of the cultures with and without chemicals was inoculated with virus, so that there were a total of eight
treatment groups in each experiment (except as mentioned in Results):

1) BrdU only, 2) HSV-2 + BrdU, 3) A-1242 + DMSO + BrdU, 4) A-1242 +
DMSO + HSV-2 + BrdU, 5) DMSO + BrdU, 6) DMSO + HSV-2 + BrdU, 7) MMC +
BrdU, 8) MMC + HSV-2 + BrdU. Virus concentrations were varied as
follows: in experiments with $10^{-5}$M BrdU, infectious virus was used at
$10^{-1}$, $10^{-3}$ and $10^{-4}$
PFU/cell, while inactivated virus was used at 1.0
PFU/cell; with $4.5 \times 10^{-5}$M BrdU, $10^{-1}$, $10^{-2}$ and $10^{-3}$
PFU/cell infectious virus and 1.0 PFU/cell inactivated virus were used. Virus
inoculations were carried out at the following times after chemicals
were added: 1) $10^{-1}$ through $10^{-3}$ PFU/cell infectious HSV-2: 72 hours,
2) $10^{-4}$ PFU/cell infectious HSV-2: 48 hours, 3) 1.0 PFU/cell inactiv-
ated HSV-2: four hours.

DMSO was included because it was the solvent for A-1242 and would
be a negative control since it has not been shown to be mutagenic.
Mitomycin C was the positive control because it is a known mutagen and
induces a significant increase in SCE. Since BrdU in the presence of
light is itself mutagenic, cultures were placed in black felt bags, to
exclude light, and incubated for four days. A four day incubation
ensured that most cells had completed two replication cycles.

Length of the replication cycle was determined from cell counts
and use of the formula: $r = \frac{3.32 \log x_2/x_1}{t_2 - t_1}$ and $g = 1/r$, where $r =
number of generations per unit time, $x_2 =$ final cell count, $x_1 =$ ini-
tial count, $t_2 =$ final time, $t_1 =$ initial time, $g =$ generation time.

At four days cells were harvested and chromosome preparations
made, as described under "Chromosome Preparations".
Staining: 1) Cells were stained with acridine orange (final concentration: 0.125 mg/ml in phosphate buffer, pH 6.5), rinsed twice with buffer for two and six minutes and air dried. Coverglasses were mounted on slides with buffer and sealed with nail polish. Metaphase cells were observed with a Zeiss fluorescent photomicroscope using a BG 3 exciter filter and the number 50 (500 nm) barrier filter. Cells were allowed to photoactivate for 15 to 30 seconds under the microscope (while being exposed to the HBO 200 mercury lamp) before sister chromatid exchanges could be seen and photographed. 2) Fluorescence plus Giemsa (FPG): metaphase cells were first stained with 33258 Hoechst13 followed by staining with Giemsa (Matheson, Coleman and Bell) according to the method described by Goto et al. (1975) with the following modifications. After staining with 33258 Hoechst, up to six slides were put into a 150 mm plastic Petri dish. The slides were flooded with phosphate-citrate buffer, then each Petri dish was placed on a window ledge where the slides were exposed to daylight and heat (about 27°C.) for one to two days. Cells were then stained with Giemsa, 1:10 in Gurr's buffer pH 6.8. The Petri dish method eliminated the necessity for mounting slides with coverglasses (Goto et al., 1975) and their subsequent removal. Flooding of slides with buffer was found to be an equally satisfactory method. Instead of fluorescent microscopy, necessary with acridine orange, cells stained by the FPG technique could be observed with light microscopy.

13 33258 Hoechst fluorescent dye was kindly supplied by Dr. H. Loewe, Hoechst A G, Frankfurt, am Main, Germany.
Scoring of SCEs: Exchanges were counted under the microscope and scored as one, if terminal (one break) or two, if interstitial (two breaks). When possible, metaphase cells were chosen for scoring where chromosomes were separated from one another and fairly straight; overlapped chromosomes were "accepted" only if SCEs could be seen clearly. If chromosomes were twisted at the centromere, any SCEs that might have occurred at that point were not counted. SCEs that occur at the centromere can be determined (Holmquist and Comings, 1975) but this was not included in the present study. Chromosomes in photographic negatives were analyzed with the aid of a Durst M800 enlarger equipped with a 150 watt bulb; the lens was set at f 2.8 and chromosomes were magnified so that the number 1 chromosome was 2.0 to 2.5 cm in length.

Photography: 1) Acridine orange stained cells: Kodak 35 mm Plus X film was used with 10 to 15 second exposures. 2) FPG stained cells: Kodak 35 mm Panchromatic-X film was used with predetermined automatic exposures. All film was developed with Diafine (Acufine, Inc.) according to manufacturer's directions.

G. Densitometry

Densitometry was done on metaphase chromosomes of cells grown with BrdU and stained by the FPG technique. A standardized graphic representation of a single chromosome was made from 35 mm photographic negatives by using data output from a Zeiss microscope-photometer-scanning stage interfaced to a Tektronix 31 programmable calculator.
via a Tektronix BCD multipurpose interface. Each chromatid of each chromosome was scanned separately and the less dense chromatid normalized to the more dense one. Lengths of chromosomes were also normalized. The photographic images of each chromosome were approximately 3000 microns in length; 70 data points were obtained with a print-out every 42.5 microns. Each print-out was an average of 20 readings.

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14 System programmed and made available by Dr. H. E. Wyandt, Division of Medical Genetics, University of Oregon Health Sciences Center.
III. RESULTS

A. Arochlor 1242 Analysis

The gas chromatographic analysis of cells grown with A-1242 in acetone revealed that cell culture medium actually contained 3.4 ppm, while input was calculated to be 5 ppm. The intracellular content of A-1242, determined from the cell lysate, was 0.03 ppm, or less than 1/100th of that in the medium. The stock A-1242 solution contained 244 ppm, while input had been calculated to be 500 ppm.

Analysis of medium with A-1242 in DMSO showed 3.3 ppm (with an input of 10 ppm); the supernatant fluid from sonicated cells contained 0.4 ppm.

B. Toxicity of Arochlor 1242 for Cultured Cells

The cytotoxic effects of A-1242 had to be determined before studies on cells in mitosis were carried out. Both acute and chronic toxicity were established. There are a number of ways in which this can be accomplished including cell counts, at various periods of time, or cloning. Since the cloning efficiency of diploid cells is extremely low (Chu, 1971), only cell counts were done.

WI-38 cells were exposed to 10, 25, 50, 100, 250 and 500 ppm A-1242. Cells treated with 250 and 500 ppm were completely destroyed within 24 to 48 hours. Cell counts on cultures exposed to the lower concentrations were done on the third and seventh days after initial exposure. Figure 1 illustrates the results. No great differences
Figure 1  Acute Toxicity of Arochlor 1242 for Human Embryonic Lung Cells (WI-38) In Vitro
MEAN NUMBER OF CELLS ($\times 10^5$)

- Control
- Acetone Control
- 10 ppm Arochlor 1242
- 25 ppm Arochlor 1242
- 50 ppm Arochlor 1242
- 100 ppm Arochlor 1242

DAYS

0 3 7
occurred between controls and A-1242 treated cells after seven days, although counts or cells exposed to A-1242 were lower than controls and some evidence of a dose effect was apparent. A decrease was noted in all cultures, probably due to the fact that cells were grown for seven days before addition of A-1242, and therefore cells were not in the logarithmic growth phase.

Chronic toxicity was determined by exposure of WI-38 cells to 2.5, 5 and 10 ppm of A-1242 for four weeks. Cultures, at each concentration, were set up in quadruplicate. Two cultures from each group were used to establish toxicity; the other two cultures were harvested for chromosome studies (for results see pages 26 and 27). Results of chronic cytotoxicity are shown in Figure 2. Cultures used for toxicity were counted at weekly intervals, subcultured and re-seeded with fresh medium containing the appropriate concentration of A-1242. After seven days, cultures were re-seeded at 7 to $11 \times 10^5$ cells per 60 mm Petri dish; thereafter, cells were seeded at 3 to $6 \times 10^5$ cells per dish.

After seven days 10 ppm A-1242 showed some evidence of toxicity; the cell count was 78% of the control. After two weeks cells exposed to 5 and 10 ppm had considerably lower counts than the control, 41% and 30%, respectively. At two weeks all cultures were accidentally subjected to colchicine for 16 hours.

Since colchicine is required for chromosome preparations and is a mitotic poison, addition of this chemical affected cell growth and this was reflected in cell counts performed at three weeks. However, cultures containing 5 and 10 ppm still had less growth than controls,
Figure 2  Chronic Toxicity of Arochlor 1242 for Human Embryonic Lung Cells (WI-38) In Vitro
10 ppm Aroclor 1242
5.0 ppm Aroclor 1242
2.5 ppm Aroclor 1242
DMSO Control
Control
Evidence of cytotoxicity in cultures grown with 2.5 ppm A-1242 was seen after three weeks (61% of control); these effects had increased appreciably by the fourth week. After 28 days cultures appeared to have recovered from the effects of the colchicine and all three concentrations, 2.5, 5 and 10 ppm A-1242, were highly cytotoxic. Cell counts were 32%, 20% and 14%, respectively, of the control.

Since the A-1242 solution also contained DMSO, cell growth was compared to the DMSO control. Except for cell counts at 28 days, there was no great difference between controls with and without DMSO. However, after four weeks exposure, DMSO appeared to be toxic and the differences in cell counts between A-1242 treated cells and the DMSO control were less than differences between the A-1242 cells and controls without DMSO. Cells treated with 2.5, 5 and 10 ppm A-1242 had counts that were 66%, 42% and 31%, respectively, of the DMSO control. In any case a dose effect of A-1242 on cells in vitro was apparent after two, three and four weeks exposure.

C. Virus Titrations and UV Inactivation

Virus titrations: The titer of stock HSV-2 grown in primary rabbit kidney cells was 1.3 x 10^7 PFU/ml. The titer in third passage H-AF cells was slightly higher, 1.8 x 10^7 PFU/ml. Both titers were lower than those reported by Rapp who obtained virus titers exceeding 10^8 PFU/ml in primary RK cells. The difference between Rapp's

15 F. Rapp, personal communication.
results and those in the present study were not great and probably
due to variations in technique. The results on virus titration in
H-AF cells were comparable to titers obtained in human embryonic
lung cells (O'Neil et al., 1972).

UV inactivation: After five minutes exposure to ultraviolet
irradiation, viral infectivity was not completely destroyed, but the
titer was significantly reduced from $1.3 \times 10^7$ PFU/ml to 3.4 PFU/ml.
This reduction was greater than that reported by Duff and Rapp (1971),
but, again, was probably due to differences in technique.

D. Effect of Chemicals on HSV-2 Infectivity

H-AF cells, in the sixth passage, were grown for three days in
the presence of one of the following chemicals: 1) $10^{-5}$M BrdU,
2) 10 ppm A-1242, 3) 1% DMSO or 4) 4 ng/ml MMC, eight cultures with
each chemical. Cells were then inoculated with $10^{-4}$ and $10^{-5}$
dilutions of HSV-2 (four cultures per dilution) and adsorbed for one
hour at room temperature. The inoculum was then removed and overlay
medium with a given chemical added. Four days later, virus plaques
were counted. All of the chemicals reduced viral infectivity.
Results are shown in Table 1.

| TABLE 1 |
| HSV-2 Infectivity in H-AF Cells Exposed to Chemicals |
| Treatment | Virus Titer (PFU/ml) |
| HSV-2 (no chemicals) | $6.4 \times 10^6$ |
| HSV-2 + DMSO | $3.4 \times 10^6$ |
| HSV-2 + A-1242 | $1.5 \times 10^6$ |
| HSV-2 + MMC | $2.1 \times 10^6$ |
| HSV-2 + BrdU | $2.5 \times 10^5$ |
DMSO decreased viral infectivity by less than 0.5 log units, while A-1242 and MMC produced a decrease greater than 0.5 but less than 1.0 log units. BrdU induced the greatest decrease, more than 1.5 log units. Infectivity was not determined with combinations of BrdU plus one of the other chemicals. Such combinations might further decrease viral infectivity over that observed with BrdU alone.

The titer of HSV-2 in sixth passage H-AF cells, without chemicals, was less than the titer in primary RK or third passage H-AF cells. Since viral infectivity is higher in primary, as opposed to passaged, RK cells\textsuperscript{16}, differences between third and sixth passage human cells may also occur.

E. Cytogenetic Analysis

Chromosomal analysis was done on cells exposed to 2.5 ppm A-1242 for three weeks. The mitotic index\textsuperscript{17} was too low for definitive cytogenetic analysis in cultures treated with 2.5, 5 and 10 ppm for four weeks and with 5 and 10 ppm for three weeks.

Slides were not coded because, even if coding had been done, the A-1242 treated cells were easily distinguished from controls (i.e., lower mitotic index and chromosome morphology in many cells was inadequate for analysis). Of the three groups (control, DMSO control and cultures treated with 2.5 ppm A-1242) 60, 60 and 67 cells, respectively, were analyzed with the microscope and of these,

\textsuperscript{16} F. Rapp, personal communication.

\textsuperscript{17} Mitotic index is the ratio of metaphase cells/total number of cells.
22, 18 and 21 cells, respectively, were photographed. Photographs were taken only when chromosome aberrations were ambiguous or chromosomes could not be identified under the microscope. Results are shown in Table 2.

In the control group both gaps occurred in the short arm of chromosome number 1, both breaks in the short arm of chromosome number 7. In the DMSO control group the two breaks occurred in different chromosomes, but three of the five aberrations (breaks and secondary (2°) constrictions) were observed in the number 2 chromosome. Secondary constrictions were seen as a partial uncoiling of the area just below the centromere. In cells treated with A-1242, 11 of the 14 abnormalities (gaps, breaks and 2° constrictions) were seen in chromosome numbers 1, 2 and 5. Over twice as many cells in the A-1242 group contained accentuated 2° constrictions as compared with controls. Fewer tetraploid cells were observed in DMSO treated cells than in either the control or A-1242 groups, although no differences in the number of tetraploid cells were seen in the latter two groups.

**TABLE 2**

<table>
<thead>
<tr>
<th>Chromosome Aberrations</th>
<th>Gaps</th>
<th>Breaks</th>
<th>Aneuploidy</th>
<th>Other Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>3.3</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>DMSO Control</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>A-1242</td>
<td>5</td>
<td>7.4</td>
<td>2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

With the exception of one fragmented chromosome in the A-1242 group and one quadriradial in the control group, all other chromosomes in this category had accentuated secondary constrictions. Gaps and breaks were all single chromatid type.
F. Sister Chromatid Exchanges (SCEs)

SCEs counted under the microscope were statistically analyzed by the Student t-test. Data for each of the chemicals (A-1242, DMSO, MMC) were pooled because no significant differences occurred from one experiment to another. The data at each BrdU concentration were analyzed separately since differences were seen.

Not all SCEs scored under the microscope could be seen in photographic negatives. Depending on the quality of metaphase cells, 72% to 98% of SCEs seen with the microscope were also observed in negatives. This variation occurred in both acridine orange and FPG stained cells. The difference was probably due to the limits of photography, so that some very small exchanges were missed in the negatives. This supposition is supported by the fact that fewer SCEs were seen in photographic negatives of MMC treated cells than under the microscope. Since MMC cells had more exchanges per cell than any other treatment group (mean number of SCEs per chromosome was 0.4), some of the exchanges were probably very small, and therefore less likely to be observed in the photographic negatives.

Three sets of experiments were performed: 1) BrdU $10^{-5}M$ with infectious virus with and without added chemicals; 2) BrdU $4.5 \times 10^{-5}$ with infectious virus with and without added chemicals; 3) inactivated virus with and without added chemicals and BrdU at concentrations of $10^{-5}M$ and $4.5 \times 10^{-5}M$. Inactivated virus was used to determine whether it produced the same results as infectious virus. The rationale for using two BrdU concentrations was the following: the
first two experiments were done with the higher BrdU concentration. Differences were noted among the various treatment groups, but because the mitotic index was low and only about 50% of the cells were in the second replication cycle, for the subsequent four experiments the BrdU concentration was reduced. However, the differences among treatment groups was not observed at the lower BrdU concentration. Therefore, despite a lower mitotic index, a similar series of experiments was done at the higher BrdU concentration.

In nine experiments (five with $10^{-5}$M BrdU, four with $4.5 \times 10^{-5}$M BrdU) a total of 65 treatment groups were analyzed. The average number of cells per treatment group in each experiment was 19 (874 chromosomes) with a range of 2 to 34. In seven groups less than ten cells were scored because the mitotic index was extremely low or too few cells were in the second replication cycle. Figures 3 through 7 illustrate results of these experiments. Tables 5 and 6, in the Appendix, show complete data on which the figures were based.

1. **Background Level - BrdU**

The background level was established for each experiment. At a concentration of $10^{-5}$M, a total of 985 exchanges were scored in 120 cells for a mean of 8.21 SCEs per cell. At $4.5 \times 10^{-5}$M, 303 exchanges were scored in 41 cells for a mean of 7.39 SCEs per cell. The difference between the two BrdU concentrations was not significant.

2. **BrdU $10^{-5}$M**

Results on infectious HSV-2 with and without added chemicals
are shown in Figures 3 and 4.

**A-1242 (Figure 3):** Since the solution of A-1242 contained DMSO, cells treated with A-1242 were compared to both BrdU and DMSO. A-1242 induced a total of 833 SCEs in 107 cells (pooled data) for a mean of 8.69 SCEs per cell. No increase or decrease occurred; the mean number of exchanges was only slightly greater than the background BrdU level or than DMSO.

**Infectious HSV-2 (Figure 3):** No significant difference in mean number of exchanges was seen between $10^{-1}$ and $10^{-3}$ PFU/cell. Both of these concentrations, however, yielded a significantly higher number of SCEs than a virus concentration of $10^{-4}$ PFU/cell ($P < 0.05$). When each virus concentration was compared to BrdU, only $10^{-3}$ PFU/cell appeared to induce a significant increase in SCEs, although the number of exchanges induced by $10^{-1}$ PFU/cell was also greater than the background BrdU level. Since the mean of $10^{-1}$ PFU/cell HSV-2 (9.45) was not much lower than the mean of $10^{-3}$ PFU/cell of virus (9.93), the difference in significance may be due to the larger number of cells scored at the lower virus concentration.

**Infectious HSV-2 + A-1242 (Figure 3):** No significant difference was observed in the number of SCEs induced by virus + A-1242 over either HSV-2 or A-1242 alone.

**DMSO, negative control (Figures 3 and 4):** DMSO treated cells had a total of 940 exchanges in 114 cells (pooled data) for a mean of 8.17 SCEs per cell. This mean was not significantly different from the mean of BrdU alone (8.21).

**Infectious HSV-2 + DMSO (Figure 4):** The combined effects were
Figure 3. Mean Number of SCEs Per Cell For Each Treatment Group and Comparison of the Mean. Experimental Groups.

BrdU 10^{-5}M  Infectious HSV-2

Open bars represent chemicals. Hatched bars represent HSV-2. Solid bars represent A-1242 + HSV-2. Zig-zag lines indicate that portion of graph not to scale.

1 = MMC (24.7)^a, positive control
2 = DMSO (8.3), negative control
3 = A-1242 (8.7)
4 = HSV-2 10^{-1}PFU/cell (9.5)
5 = A-1242 + HSV-2 10^{-1}PFU/cell (8.2)^b
6 = HSV-2 10^{-3}PFU/cell (9.9)
7 = A-1242 + HSV-2 10^{-3}PFU/cell (9.0)
8 = HSV-2 10^{-4}PFU/cell (8.0)
9 = A-1242 + HSV-2 10^{-4}PFU/cell (7.3)

A = A-1242 compared to DMSO  \quad P > 0.2^c
B = A-1242 compared to BrdU  \quad P > 0.2
C = A-1242 + HSV-2 (10^{-1}PFU/cell) compared to A-1242  \quad P > 0.5
D = A-1242 + HSV-2 (10^{-1}PFU/cell) compared to HSV-2  \quad P > 0.2
E = A-1242 + HSV-2 (10^{-3}PFU/cell) compared to A-1242  \quad P > 0.5
F = A-1242 + HSV-2 (10^{-3}PFU/cell) compared to HSV-2  \quad P > 0.2
G = A-1242 + HSV-2 (10^{-4}PFU/cell) compared to A-1242  \quad P > 0.1
H = A-1242 + HSV-2 (10^{-4}PFU/cell) compared to HSV-2  \quad P > 0.4

4 = HSV-2 (10^{-1}PFU/cell) compared to BrdU  \quad P > 0.1
6 = HSV-2 (10^{-3}PFU/cell) compared to BrdU  \quad P < 0.005
8 = HSV-2 (10^{-4}PFU/cell) compared to BrdU  \quad P > 0.5

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^a Figures in parentheses are means.

^b The mean of A-1242 + HSV-2 equals the mean of BrdU.

^c Probabilities determined by the Student t-test. Table 5 in Appendix shows complete data.
Figure 4. Mean Number of SCEs Per Cell For Each Treatment Group and Comparison of the Means. Control Groups.

BrdU $10^{-5}$M Infectious HSV-2

Open bars represent chemicals. Hatch bars represent HSV-2. Solid bars represent chemicals + HSV-2. Zig-zag and vertical broken lines indicate that portion of graph not to scale.

1 = MMC (24.7)a, positive control
2 & 9 = HSV-2 $10^{-3}$PFU/cell (9.9)
3 = MMC + HSV-2 $10^{-3}$PFU/cell (23.3)
4 & 11 = HSV-2 $10^{-4}$PFU/cell (8.0)
5 = MMC + HSV-2 $10^{-4}$PFU/cell (23.6)
6 = DMSO (8.3)
7 = HSV-2 $10^{-1}$PFU/cell (9.5)
8 = DMSO + HSV-2 $10^{-1}$PFU/cell (8.0)
10 = DMSO + HSV-2 $10^{-3}$PFU/cell (8.4)
12 = DMSO + HSV-2 $10^{-4}$PFU/cell (9.1)

A = MMC + HSV-2 ($10^{-3}$PFU/cell)b compared to MMC $P > 0.2$ c
B = MMC + HSV-2 ($10^{-3}$PFU/cell) compared to HSV-2 $P < 0.001$
C = MMC + HSV-2 ($10^{-4}$PFU/cell) compared to MMC $P > 0.5$
D = MMC + HSV-2 ($10^{-4}$PFU/cell) compared to HSV-2 $P < 0.001$
E = DMSO + HSV-2 ($10^{-1}$PFU/cell) compared to DMSO $0.1 > P > 0.05$
F = DMSO + HSV-2 ($10^{-1}$PFU/cell) compared to HSV-2 $P > 0.2$
G = DMSO + HSV-2 ($10^{-3}$PFU/cell) compared to DMSO $P > 0.5$
H = DMSO + HSV-2 ($10^{-3}$PFU/cell) compared to HSV-2 $0.1 > P > 0.05$
I = DMSO + HSV-2 ($10^{-4}$PFU/cell) compared to DMSO $P > 0.5$
J = DMSO + HSV-2 ($10^{-4}$PFU/cell) compared to HSV-2 $P > 0.2$

1 = MMC compared to BrdU $P < 0.001$
6 = DMSO compared to BrdU $P > 0.5$

a Figures in parentheses are means.
b No MMC control was included with HSV-2 ($10^{-1}$PFU/cell).
c Probabilities determined by the Student $t$-test. Table 5 in Appendix shows complete data.
not significantly different (at the 95% confidence level) from HSV-2, at any of the three concentrations, or from DMSO.

MMC, positive control, (Figures 3 and 4): MMC induced a total of 1582 exchanges in 64 cells (pooled data) for a mean of 24.72 SCEs per cell. This was a highly significant increase (three-fold) over BrdU alone.

Infectious HSV-2 + MMC (Figure 4): No positive control was included with HSV-2 at a concentration of $10^{-1}$ PFU/cell. With the other two virus concentrations, MMC + virus induced a highly significant increase in the number of SCEs over virus alone, but no significant difference was observed compared to MMC alone.

3. BrdU $4.5 \times 10^{-5}$M

Results on infectious HSV-2 with and without added chemicals are shown in Figures 5 and 6.

A-1242 (Figure 5): A-1242 induced a total of 536 exchanges in 56 cells (pooled data) for a mean of 9.57 SCEs per cell, a highly significant increase compared to either BrdU or DMSO alone.

Infectious HSV-2 (Figure 5): Although slight differences in the mean number of SCEs were seen among the three virus concentrations, none was significant. However, all three concentrations induced a highly significant increase in number of exchanges compared to the BrdU background level.

Infectious HSV-2 + A-1242 (Figure 5): A virus dose response was observed when HSV-2 was inoculated into A-1242 treated cells. At a virus concentration of $10^{-1}$ PFU/cell, the combined effects were
significantly greater than A-1242 alone, but not significantly different from the virus alone. With $10^{-2}$ PFU/cell of virus, the number of SCEs induced by the combination of HSV-2 + A-1242 was not significantly different from either virus or A-1242 alone. With $10^{-3}$ PFU/cell, the combined effects were significantly less than HSV-2 alone, but not A-1242 alone.

**DMSO, negative control (Figures 5 and 6):** A total of 128 exchanges were scored in 18 cells for a mean of 7.11 SCEs per cell, which was only a slight decrease from the background level. No difference was seen in number of SCEs in DMSO treated cells at the two BrdU concentrations.

**Infectious HSV-2 + DMSO (Figure 6):** No negative control was included with HSV-2 at a concentration of $10^{-2}$ PFU/cell. The number of exchanges induced by the combination of HSV-2 ($10^{-1}$PFU/cell) + DMSO was greater than DMSO alone and less than virus alone, although neither difference was significant. With $10^{-3}$PFU/cell HSV-2, the combined effects were significantly greater than DMSO alone, and slightly less than virus alone.

**MMC and EMS, positive controls (Figure 6):** Nine cells were scored in which there were 186 exchanges for a mean of 20.67 SCEs per cell. This was a highly significant increase over the background BrdU level. The number of SCEs induced by MMC with $10^{-5}$M BrdU was greater ($0.1 > P > 0.05$); the difference may be due to the fact that a larger number of cells (64) were scored at the lower BrdU level.
Figure 5. Mean Number of SCEs Per Cell For Each Treatment Group and Comparison of the Means. Experimental Groups.

BrdU $4.5 \times 10^{-5}$M Infectious HSV-2

Open bars represent chemicals. Hatched bars represent HSV-2. Solid bars represent A-1242 + HSV-2. Zig-zag lines indicate that portion of graph not to scale.

1 = MMC (20.7)$^a$
2 = DMSO (7.1)
3 = A-1242 (9.6)
4 = HSV-2 $10^{-1}$PFU/cell (11.1)
5 = A-1242 + HSV-2 $10^{-2}$PFU/cell (12.7)
6 = HSV-2 $10^{-2}$PFU/cell (12.3)
7 = A-1242 + HSV-2 $10^{-2}$PFU/cell (11.4)
8 = HSV-2 $10^{-3}$PFU/cell (11.0)
9 = A-1242 + HSV-2 $10^{-3}$PFU/cell (8.6)

A = A-1242 compared to DMSO P < 0.05$^b$
B = A-1242 compared to BrdU P < 0.005

C = HSV-2 (10$^{-1}$PFU/cell) + A-1242 compared to A-1242 P < 0.01
D = HSV-2 (10$^{-1}$PFU/cell) + A-1242 compared to HSV-2 P > 0.1
E = HSV-2 (10$^{-2}$PFU/cell) + A-1242 compared to A-1242 P < 0.05
F = HSV-2 (10$^{-2}$PFU/cell) + A-1242 compared to HSV-2 P > 0.2
G = HSV-2 (10$^{-3}$PFU/cell) + A-1242 compared to A-1242 P > 0.2
H = HSV-2 (10$^{-3}$PFU/cell) + A-1242 compared to HSV-2 P < 0.005

4 = HSV-2 (10$^{-1}$PFU/cell) compared to BrdU P < 0.0001
6 = HSV-2 (10$^{-2}$PFU/cell) compared to BrdU P < 0.0001
8 = HSV-2 (10$^{-3}$PFU/cell) compared to BrdU P < 0.0001

$^a$ Figures in parentheses are means. Table 6 in Appendix shows complete data.

$^b$ Probabilities determined by the Student t-test.
Figure 6. Mean Number of SCEs Per Cell For Each Treatment Group and Comparison of the Means. Control Groups.

BrdU 4.5 x 10^{-5}M  Infectious HSV-2

Open bars represent chemicals. Hatched bars represent HSV-2. Solid bars represent chemicals + HSV-2. Zig-zag and vertical broken lines indicate that portion of graph not to scale.

1 = MMC (20.7)a, positive control
2 & 8 = HSV-2 10^{-1}PFU/cell (11.1)
3 = MMC + HSV-2 10^{-1}PFU/cell (20.2)
4 = EMS (12.1), positive control
5 & 10 = HSV-2 10^{-3}PFU/cell (11.0)
6 = EMS + HSV-2 10^{-3}PFU/cell (13.6)
7 = DMSO (7.1), negative control
9 = DMSO + HSV-2 10^{-1}PFU/cell (8.8)
11 = DMSO + HSV-2 10^{-3}PFU/cell (9.9)

A = HSV-2 (10^{-1}PFU/cell) + MMC compared to MMC  P > 0.5b
B = HSV-2 (10^{-1}PFU/cell) + MMC compared to HSV-2  P < 0.001
C = HSV-2 (10^{-3}PFU/cell) + EMS compared to EMS  P > 0.1
D = HSV-2 (10^{-3}PFU/cell) + EMS compared to HSV-2  P < 0.01
E = HSV-2 (10^{-1}PFU/cell) + DMSO compared to DMSO  P < 0.05
F = HSV-2 (10^{-1}PFU/cell) + DMSO compared to HSV-2  P > 0.2
G = HSV-2 (10^{-3}PFU/cell) + DMSO compared to DMSO  P < 0.05
H = HSV-2 (10^{-3}PFU/cell) + DMSO compared to HSV-2  P > 0.2

1 = MMC compared to BrdU  P < 0.001
4 = EMS compared to BrdU  P < 0.001
7 = DMSO compared to BrdU  P > 0.5

No positive (MMC or EMS) or negative (DMSO) controls were included with HSV-2 (10^{-2}PFU/cell).

---

a Figures in parentheses are means. Table 6 in Appendix shows complete data.

b Probabilities determined by the Student t-test.
Ethvlmethanesulfonate (EMS)\(^{18}\) (10µg/ml), a known mutagen, was used as the positive control in one experiment because MMC was not obtained until later. EMS also induced a highly significant increase in number of SCEs over the background (BrdU) level, although less than MMC.

**Infectious HSV-2 + MMC (or EMS) (Figure 6):** No positive control was included with \(10^{-2}\) PFU/cell HSV-2. At the other two virus concentrations the combined effects of MMC + virus and EMS + virus were significantly greater than virus alone, although the level of significance was higher with MMC + HSV-2. A slight decrease in SCEs was observed between MMC + virus and MMC alone, while an increase occurred with EMS + virus over EMS alone. Neither of these differences was significant.

4. **UV Inactivated HSV-2**

Inactivated HSV-2 was used in two experiments, one with \(10^{-5}\)M BrdU and the other with \(4.5 \times 10^{-5}\)M BrdU. Results on cells grown with the lower BrdU concentration are shown in Figure 7.\(^{19}\) Unlike infectious virus, UV inactivated HSV-2 caused a decrease in the number of exchanges compared to BrdU alone; significance was at the 90% level. The number of SCEs induced by the combination of HSV-2 + any of the three chemicals (A-1242, DMSO, MMC) was not significantly different from SCEs induced by the chemicals alone. However, A-1242 + HSV-2 did induce an increase over virus alone with significance at

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\(^{18}\) EMS was generously provided by Dr. L. M. Pasztor, Oregon Regional Primate Research Center, Beaverton.

\(^{19}\) Table 5 in Appendix shows complete data.
Figure 7. Mean Number of SCEs Per Cell For Each Treatment Group and Comparison of the Means. Inactivated HSV-2.

BrdU $10^{-5}M$

Open bars represent chemicals. Hatched bar represents HSV-2. Zig-zag and vertical broken lines indicate that portion of graph not to scale.

1 = HSV-2, uv inactivated, 1.0 PFU/cell$^a$ (6.72)$^b$
2 = A-1242 (8.69)
3 = A-1242 + HSV-2 (8.75)
4 = DMSO, negative control (8.25)
5 = DMSO + HSV-2 (9.18)
6 = MMC, positive control (24.72)
7 = MMC + HSV-2 (24.77)

A = HSV-2 + A-1242 compared to A-1242 P > 0.5$^c$
B = HSV-2 + A-1242 compared to HSV-2 0.1 > P > 0.05
C = HSV-2 + DMSO compared to DMSO P > 0.1
D = HSV-2 + DMSO compared to HSV-2 P < 0.01
E = HSV-2 + MMC compared to MMC P > 0.5
F = HSV-2 + MMC compared to HSV-2 P < 0.001

HSV-2 compared to BrdU 0.1 > P > 0.05

Comparisons of A-1242 and DMSO to BrdU are shown in Figures 3 and 4.

---

$^a$ Determined on the basis of titration of virus prior to inactivation.

$^b$ Figures in parentheses are means.

$^c$ Probabilities determined by the Student t-test. Table 5 in Appendix shows complete data.
MEAN NUMBER OF SCEs PER CELL

BrdU

0 5 10 15 20

A B C D E F

2 3 4 5 6 7
the 90% level. Inactivated virus + EMS or MMC induced a highly significant increase in SCEs over HSV-2 alone (P < 0.01 and P < 0.001, respectively.

Results on growth of cells with 4.5 x 10^-5 M BrdU are not presented in graphic form. The mitotic index in all but two of the treatment groups was so low that an insufficient number of metaphase cells were scored; the reason is unclear. Results, therefore, may not be meaningful. Cells grown with BrdU alone could be compared with cells inoculated with virus; no difference in the mean number of SCEs was observed. The only information gained from analysis of the other treatment groups was that there was a tendency for cells with the combination of HSV-2 + any of the chemicals to have fewer exchanges than cells with the chemicals alone. Only one group, DMSO + HSV-2, had fewer SCEs than the virus alone. However, more cells would have to be scored to determine whether these effects are statistically significant.

5. Distribution of SCEs According to Chromosome Length

Several reports have shown the frequency of SCEs in BrdU treated cells to be proportional to chromosome length (Chaganti et al., 1974; Ikushima and Wolff, 1974; Latt, 1974). Although variations in the number of SCEs in the larger chromosomes were observed, namely, chromosome numbers 1, 2 and 3 and the B group (numbers 4 and 5), the observed number of exchanges was equal to or greater than the expected number. However, the smaller chromosomes, namely, the E, F and G + Y groups (chromosome numbers 16 - 22 + Y) had fewer SCEs than expected
(65% or less).

To determine whether exchanges induced by the various chemicals (A-1242, DMSO and MMC), with and without virus, were distributed relative to chromosome length, data were analyzed by methods cited by Chaganti et al. (1974). The observed number of SCEs/expected number was calculated for each chromosome group within each treatment group. The same variation was observed in the larger chromosomes in all except one of the treatment groups. A striking difference was seen in MMC treated cells grown with $4.5 \times 10^{-5}$M BrdU. In these cells the number 1 chromosome had almost twice the number of SCEs as expected (observed/expected = 1.76), while the number 3 chromosome had fewer exchanges than expected (observed/expected = 0.69). The number of exchanges in the smaller chromosomes was less than expected in all treatment groups, with one exception. A-1242 treated cells grown with $4.5 \times 10^{-5}$ BrdU had a slightly greater number of SCEs in the F group chromosomes than expected (observed/expected = 1.02); an unexpected finding since others had observed between 18% and 60% of the expected number of SCEs in this chromosome group (Chaganti et al., 1974; Latt, 1974).

6. Poisson Distribution of SCEs Per Chromosome Within Each Treatment Group: Observed versus Expected

If exchanges are distributed randomly along the chromosomes, then the yield of SCEs per chromosome should be distributed according to Poisson expectations (Wolff and Perry, 1974). In order to determine whether exchanges did fit a Poisson distribution, data for each treat-
ment group were analyzed for the number of chromosomes with 0, 1, 2, etc. SCEs per chromosome, again separating data at each BrdU concentration. Analyses were done only on those experiments in which cells were inoculated with infectious HSV-2 and were based on counts of exchanges seen in photographic negatives. Tables 3 and 4 show Poisson expectations for treatment groups where cells were grown with $10^{-5}$M and $4.5 \times 10^{-5}$M BrdU, respectively.

SCEs seen in cells with $10^{-5}$M BrdU alone followed a Poisson distribution ($P > 0.1$). The probability that exchanges observed in cells with $4.5 \times 10^{-5}$M BrdU fit Poisson expectations was significant at the 90% confidence level, but not at the 95% level. These results agree with a report by Wolff and Perry (1974).

Certain other treatment groups did not follow a Poisson distribution and were significant at the 95% level or greater. A-1242 treated cells, grown with $10^{-5}$M BrdU, induced SCEs that were not randomly distributed (Table 3), the reason being that far more than the expected number of chromosomes had no exchanges, far less had one exchange, while the number with two or more SCEs was about as expected. The probability that groups in which cells were inoculated with HSV-2 and grown with $10^{-5}$M BrdU followed Poisson expectations was greater than 90% but less than 95%. One group (DMSO + HSV-2) was significant above the 95% level. This treatment group showed the same pattern as A-1242 treated cells, namely, more than the expected number of chromosomes with no exchanges, less with one exchange and about as expected with two or more. SCEs induced by the negative (DMSO) and positive (MMC) controls, without virus, followed a Poisson distribu-
TABLE 3

Comparison of Number of Chromosomes with 0, 1, 2, ... etc. SCEs with Poisson Expectations

$10^{-5}$M BrdU

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of chromosomes with indicated number of SCEs</th>
<th>$X^2$ Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>BrdU</td>
<td>81%a</td>
<td>obs 4422</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exp 4412.3</td>
</tr>
<tr>
<td>HSV-2b</td>
<td>78%</td>
<td>obs 3860</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exp 3664.8</td>
</tr>
<tr>
<td>A-1242</td>
<td>98%</td>
<td>obs 5199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exp 5031.3</td>
</tr>
<tr>
<td>A-1242 + HSV-2</td>
<td>83%</td>
<td>obs 2527</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exp 2512.5</td>
</tr>
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</table>
TABLE 3 continued

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>81%</td>
</tr>
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<td>obs</td>
<td>4184</td>
</tr>
<tr>
<td>exp</td>
<td>4171.7</td>
</tr>
<tr>
<td>m=762/4876=0.156</td>
<td></td>
</tr>
<tr>
<td>df=2</td>
<td>X² = 3.38</td>
</tr>
<tr>
<td>P &gt; 0.1</td>
<td></td>
</tr>
<tr>
<td>DMSO + HSV-2</td>
<td>85%</td>
</tr>
<tr>
<td>obs</td>
<td>2664</td>
</tr>
<tr>
<td>exp</td>
<td>2641.6</td>
</tr>
<tr>
<td>m=529/3128=0.169</td>
<td></td>
</tr>
<tr>
<td>df=2</td>
<td>X² = 9.89</td>
</tr>
<tr>
<td>P &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>MMC</td>
<td>72%</td>
</tr>
<tr>
<td>obs</td>
<td>1671</td>
</tr>
<tr>
<td>exp</td>
<td>1654.1</td>
</tr>
<tr>
<td>m=1140/2576=0.443</td>
<td></td>
</tr>
<tr>
<td>df=3</td>
<td>X² = 2.49</td>
</tr>
<tr>
<td>P &gt; 0.1</td>
<td></td>
</tr>
<tr>
<td>MMC + HSV-2</td>
<td>73%</td>
</tr>
<tr>
<td>obs</td>
<td>1347</td>
</tr>
<tr>
<td>exp</td>
<td>1315.9</td>
</tr>
<tr>
<td>m=937/2070=0.453</td>
<td></td>
</tr>
<tr>
<td>df=3</td>
<td>X² = 7.12</td>
</tr>
<tr>
<td>0.1 &gt; P &gt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

\( \text{a Percentage of SCEs counted under the microscope.} \)

\( \text{b HSV-2 infectious virus.} \)

\( \text{c Mean = no. of SCEs/no. of chromosomes = no. of SCEs/chromosome.} \)
TABLE 4

Comparison of Number of Chromosomes with 0, 1, 2, ... etc. SCEs with Poisson Expectations

4.5 x 10^{-5}M BrdU

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of chromosomes with indicated number of SCEs</th>
<th>X^2 Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3≥</td>
<td></td>
</tr>
<tr>
<td>BrdU</td>
<td>obs 1878 330 44√2 3≥</td>
<td>m=424/2254=0.188&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>exp 1867.7 351.1 35.2</td>
<td>df=2 X^2 = 4.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 &gt; P &gt; 0.05</td>
</tr>
<tr>
<td>HSV-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>obs 2580 558 74 8</td>
<td>m=731/3220=0.227</td>
</tr>
<tr>
<td></td>
<td>exp 2566.1 582.5 71.4</td>
<td>df=2 X^2 = 2.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &gt; 0.1</td>
</tr>
<tr>
<td>A-1242</td>
<td>obs 2254 439 60 7</td>
<td>m=581/2760=0.211</td>
</tr>
<tr>
<td></td>
<td>exp 2235 471.6 53.5</td>
<td>df=2 X^2 = 5.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 &gt; P &gt; 0.05</td>
</tr>
<tr>
<td>A-1242 + HSV-2</td>
<td>obs 2015 365 55 3</td>
<td>m=484/2438=0.199</td>
</tr>
<tr>
<td></td>
<td>exp 1998.1 397.6 42.3</td>
<td>df=2 X^2 = 8.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.05</td>
</tr>
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</table>
TABLE 4 continued

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage</th>
<th>obs</th>
<th>exp</th>
<th>m</th>
<th>df</th>
<th>X^2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>93%</td>
<td>793</td>
<td>791.3</td>
<td>139/920=0.151</td>
<td>2</td>
<td>0.82</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>DMSO + HSV-2</td>
<td>98%</td>
<td>863</td>
<td>857.6</td>
<td>219/1058=0.210</td>
<td>2</td>
<td>0.46</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>EMS</td>
<td>72%</td>
<td>746</td>
<td>753</td>
<td>184/920=0.200</td>
<td>2</td>
<td>4.43</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>EMS + HSV-2</td>
<td>79%</td>
<td>777</td>
<td>764.5</td>
<td>226/966=0.234</td>
<td>2</td>
<td>7.92</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>MMC</td>
<td>77%</td>
<td>442</td>
<td>506.2</td>
<td>340/782=0.435</td>
<td>3</td>
<td>29.32</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>MMC + HSV-2</td>
<td>79%</td>
<td>348</td>
<td>336.1</td>
<td>207/506=0.409</td>
<td>2</td>
<td>6.62</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

a Percentage of SCEs counted under the microscope.

b HSV-2 infectious virus.

c Mean = no. of SCEs/no. of chromosomes = no. of SCEs/chromosome.
tion, \((P > 0.1)\).

With a BrdU concentration of \(4.5 \times 10^{-5} \text{M}\), four of the ten treatment groups did not fit Poisson expectations. Those groups were:
1) A-1242 + HSV-2; 2) EMS + HSV-2; 3) MMC alone and 4) MMC + HSV-2.
The one experimental group (A-1242 + HSV-2), that did not follow a Poisson distribution, had slightly more than the expected number of chromosomes with no SCEs, fewer with one exchange and greater than expected with two or more exchanges.

7. Illustrations of Metaphase Cells Demonstrating Sister Chromatid Exchanges

Three illustrations of metaphase cells stained with acridine orange (Figures 8, 9 and 12) and two cells stained by the FPG technique (Figures 10 and 11) are included. Figure 8 shows a cell inoculated with infectious HSV-2. Both number 2 chromosomes have exchanges. This cell contains a total of nine exchanges, only five of which are indicated - one in a number 1 chromosome, one in a D group chromosome and a small exchange in one of the C group. Figure 9 is a cell that was treated with A-1242 and inactivated HSV-2. This cell has 14 breaks (12 exchanges): a B group and a G group chromosome each has two breaks. A very small exchange on one of the C group chromosomes is also indicated.

A cell inoculated with infectious HSV-2 is shown in Figure 10. Of particular interest are the very small exchanges in one of the B, F and G group chromosomes as well as an exchange in an E group chromosome. In all, 14 exchanges are present, some of which are very small.
A cell treated with MMC and infectious HSV-2 is shown in Figure 11. Two and three breaks per chromosome are prominent and were common in cells treated with MMC. This cell contains 29 breaks with 24 SCEs; a C group chromosome with three breaks is indicated.

An endoreduplicated cell is shown in Figure 12. This cell comes from a culture that was inoculated with inactivated virus and stained with acridine orange. In some cases only one of the paired chromosomes has an exchange; in other cases both paired chromosomes have SCEs at identical loci. An exchange that occurs during the first S-phase of the cell cycle will be duplicated at the same locus during the second division in any tetraploid cell. Endoreduplication merely demonstrates, without ambiguity, that the chromosomes are paired. Because of this pairing, one can determine the number of exchanges that occur during the first or second S-phase. This particular cell contains 12 single exchanges (three of which are indicated) that occurred during the second division and three "twin" exchanges (two are indicated) that took place during the first S-phase.

Figure 13 shows a normal metaphase cell stained with the FPG method; no contrast between chromatids can be seen.

8. Densitometry

Densitometry was done on three number 1 chromosomes from cells grown with BrdU and stained by the FPG method. Two homologues were chosen from one cell treated with MMC. The third chromosome was chosen because no exchanges had occurred and, therefore, it could be used for comparison with the other two. The MMC treated cell was
Figures 8 - 13: Human Amniotic Fluid Cells (H-AF) Grown for Two Replication Cycles with 10⁻⁵M BrdU

Figure 8: Infectious HSV-2 (10⁻³PFU/cell). Stained with acridine orange. Arrows indicate breakpoints.

Figure 9: Arochlor 1242 (10 ppm) + UV inactivated HSV-2. Stained with acridine orange. Arrows indicate breakpoints.

Figure 10: Infectious HSV-2 (10⁻³PFU/cell). Stained with FPG. Arrows indicate breakpoints.

Figure 11: Mitomycin C (4 ng/ml) + infectious HSV-2 (10⁻³PFU/cell). Stained with FPG. Arrows indicate breakpoints.

Letters and numbers in above figures indicate specific chromosomes.

Figure 12: Endoreduplication. Inactivated HSV-2. Stained with acridine orange. Arrows indicate SCEs. One arrow indicates single exchanges; double arrows indicate twin exchanges (both paired chromosomes have exchanges at identical loci).

Figure 13: Control. Stained with FPG. No contrast between chromatids.

Intense (acridine orange) and dark (FPG) staining chromatids are monofilarly substituted with BrdU; pale (acridine orange) and light (FPG) staining chromatids are bifilarly substituted.
Figure 10
selected because both chromosomes had more than one exchange, the chromosomes were relatively straight and therefore easier to scan.

Figure 14 shows the three chromosomes that were scanned; breakpoints are indicated. Figure 14A is the chromosome with no SCEs; the upper chromatid is darkly stained (monofilarly substituted), while the lower chromatid is pale staining (bifilarly substituted). Figure 14B is one of the homologues, which is twisted at the centromere and therefore appears to have a terminal portion of each chromatid monofilarly substituted - left end of upper half and right end of lower half. If the chromosome were not twisted, both of these dark staining terminal areas would be on either the upper or lower chromatid.

Figure 15 illustrates densitometry scans of the same three chromosomes. Figure 15A shows no SCEs. The upper half is very dense, therefore, the dark staining portion seen in Figure 14A; the lower half is considerably less dense and the scan on the pale staining chromatid in Figure 14A. Figure 15B shows two breakpoints and two exchanges; differences in density are evident. Figure 15C is the homologue of the chromosome in Figure 15B and shows five breakpoints with three exchanges; two are interstitial, one is terminal. A very small exchange is present, which is not easily seen in Figure 14C. Normal variations in density are indicated (small triangles). If these were exchanges, one would see an area of greater density on one chromatid with a corresponding area of lower density on the other chromatid, as is the case with the actual exchanges indicated. The area between the third and fourth breaks, reading from left to right, in Figure 15C appears to show an
Figure 14: Chromosomes Used for Densitometry Scans. H-AF Cells Grown for Two Replication Cycles with $10^{-5}$M BrdU. Stained with FPG. A = control; no SCEs. B and C = Chromosomes from cell treated with Mitomycin C; B and C are homologues. 
b = breakpoints. c = centromere.

Figure 15: Densitometry Scans. 
A = Scan of chromosome in Figure 14A. B = Scan of chromosome in Figure 14B. C = Scan of chromosome in Figure 14C. 
asymmetric exchange. This may be an artifact and more chromosomes would have to be scanned to determine whether asymmetric exchanges actually occur.

9. General Comments on Sister Chromatid Exchanges

The BrdU "cocktail"\textsuperscript{20} (Latt, 1975), used in all but one of the experiments reported in this study, supposedly avoided toxicity due to BrdU alone. However, even the "cocktail" was toxic, particularly at higher concentrations, and lengthened the generation time of the cells. H-AF cells grown without BrdU had a generation time of 24 hours; with BrdU this time was lengthened to 38 hours.

The higher the BrdU concentration, the fewer the number of metaphase cells present that showed the contrast between chromatids (pale and dark staining). This observation was not entirely due to a lower mitotic index. Whether more cells were in the first replication cycle or whether the cells had simply not incorporated BrdU could not be determined because the staining characteristics are the same in both cases; in other words, no contrast between sister chromatids.

An interesting phenomenon was observed in the one experiment in which $10^{-5}$M BrdU was used without deoxycytidine, uridine and fluorodeoxyuridine. Cells treated with BrdU or BrdU + infectious HSV-2 had a much higher mitotic index than cells grown with the BrdU "cocktail", although the number of SCEs per cell was about the same.

\textsuperscript{20} The "cocktail" also contained deoxycytidine, uridine and fluorodeoxyuridine.
In addition, a higher number of metaphase cells showed the contrast in staining between chromatids, which meant more cells were in the second replication cycle. The percentage of cells in the third replication cycle was also greater - between 10% and 15% compared to less than 1% with the BrdU "cocktail". Third replication cycle cells are easily distinguished because most of the length of both chromatids are pale staining, while only a small portion of one or the other chromatid shows intense staining. However, in all other treatment groups, little difference, between BrdU alone and the "cocktail", was observed. The mitotic index and number of cells in the second and third replication cycles was about the same in both cases.

G. Fluorescent Antibody

Fluorescent antibody studies were done on cells inoculated with virus to determine whether viral antigens were present in cells exposed to the various chemicals. Results of immunofluorescence on H-AF cells inoculated with infectious HSV-2 are illustrated in Figures 16, 17 and 18. Figure 16 shows the diffuse fluorescence typical of herpesviruses (Espmark et al., 1971). These cells were exposed to infectious virus only. Figure 17 shows cells treated with A-1242 + HSV-2. Specific fluorescence is seen over the nucleus but not in the cytoplasm. Cells exposed to BrdU + HSV-2 had a similar distribution of fluorescent granules over the nucleus. Cells treated with MMC + HSV-2 displayed very intense fluorescence over the nucleus, primarily at the periphery (Figure 18). The

Figure 16: HSV-2 (10^{-1}PFU/cell).

Figure 17: Arochlor 1242 + HSV-2 (10^{-1}PFU/cell).

Figure 18: Mitomycin C + HSV-2 (10^{-1}PFU/cell).
intensity was due to rod-like structures that gave a three-dimensional
effect. No specific immunofluorescence was observed in cultures
inoculated with UV inactivated virus.

H. Mycoplasma Test

H-AF cells were tested for the presence of mycoplasma using the
technique described by Studzinski et al. (1973). Mycoplasma contamina-
tion in cultured cells is a problem and could possibly cause an
increase in sister chromatid exchanges, although such an effect has
never been shown. No single method to detect mycoplasma contamination
is adequate; however, the cells were negative by this procedure.21

21 Autoradiography of cells was kindly done by Dr. L. M. Pasztor,
Oregon Regional Primate Research Center, Beaverton.
IV. DISCUSSION

The primary purpose of this research was to determine whether A-1242 and HSV-2 caused a synergistic effect on human chromosomes in vitro. Adjuncts to this project included the determination of whether there is short or long term toxicity of A-1242 on human cells, whether A-1242 alone induces chromosome breakage, and whether the three chemicals (A-1242, DMSO and MMC), used in the main project, have an effect on viral infectivity.

Although polychlorinated biphenyls have been used commercially for 40 years and their toxicity known, only in the past seven years have they become of environmental concern. Because of the accumulation of these chemicals around the world, production in the United States, at least, will cease in 1977.

The toxicity of PCBs has been extensively reviewed (Fishbein, 1974); therefore, a discussion of this subject will be limited to investigations that are pertinent to the present research. One of the problems in determination of the biological effects of PCBs is the fact that these chemicals are mixtures of isomers and usually contain contaminants such as furans, dioxins and naphthalenes, all of which are toxic.

Man is affected by acute doses of PCBs (Kuratsune et al., 1972); accidental poisoning in Japan (termed Yusho disease) occurred in a large human population exposed to high concentrations through contamination of rice oil. However, nothing is known about chronic toxicity in man.
Toxicity in vitro has been shown by the fact that various Arochlor, at a concentration of 50 ppm, produced a significant decrease in Chinese hamster cell populations after 30 hours (Hoopin-garner et al., 1972). Short term (acute) toxicity of Arochlor 1242 for human cells in vitro, in the present study, was not as great as that for related PCBs tested in Chinese hamster cells, because no large decrease in cell growth occurred after seven days. However, these two studies cannot be strictly compared for several reasons: 1) different cells were used, one with a generation time of 12 hours (Chinese hamster), the other 24 hours; 2) PCBs were added during the logarithmic growth phase of the hamster cells, while A-1242 was added to human cells after the log phase; 3) A-1242 was not one of the PCBs tested on Chinese hamster cells; 4) the intracellular concentration of PCBs in hamster cells was not determined; and 5) measurements for cell growth were different.

Long term (chronic) toxicity of PCBs on cultured cells has never been demonstrated. On the basis of results in the present study, chronic toxicity in vitro may be analogous to that in vivo (Fishbein, 1974) and may be an indication of the occurrence of such toxicity, particularly in non-human primates and man. Human cells exposed to A-1242 for two, three and four weeks showed a dose response, similar to that seen in rhesus monkeys (McNulty, 1976). These results were suggestive of cumulative effects.
A. Genetic Studies on PCBs

The relatively little work done on the genetic toxicology of the PCBs has been equivocal. On genetic tests in Drosophila melanogaster, Nilsson and Ramel (1974) analyzed the sex chromosomes using known markers. After feeding Drosophila two PCBs, chlophen 30 and chlophen 50 (30% and 50% chlorine, respectively), they looked for: 1) nondisjunction giving rise to XXY females and XO males; 2) chromosome breakage giving rise to XO males through loss of a sex chromosome; and 3) induced exchange between X and Y in males. They found no indication of chromosome breakage due to either of the PCBs.

Arochlor 1242 (500 mg/kg) produced a cumulative toxic effect in rats, but neither Arochlor 1242 (1250 to 5000 mg/kg) nor Arochlor 1254 (75 to 300 mg/kg) caused mitotic inhibition in rat bone marrow cells. No chromosomal aberrations were induced in bone marrow cells by either PCB at the same doses or in spermatogonial cells by Arochlor 1242. The only effect noted was a decreased rate of division in spermatogonial cells when rats were treated with 5000 mg/kg of A-1242 or four doses of 500 mg/kg, but even these dosages were insufficient to decrease the number of viable sperm (Green et al., 1975a). In addition, neither Arochlor 1242 nor 1254 (at the same doses) showed dominant lethal potential; no increase in the number of dead implantations per pregnant female was observed (Green et al., 1975b). On the basis of these studies, Green et al. (1975a) concluded that neither A-1242 nor A-1254 was mutagenic, at least as to chromosome breakage or dominant lethality. However, if either of these PCBs is weakly...
mutagenic, neither chromosome breakage nor the dominant lethal test would have detected such activity.

Peakall et al. (1972) found an increase in chromosome (primarily chromatid) breaks in embryos of Ring Doves fed Arochlor 1254 at a dose of 10 ppm for three months. Thirteen of 17 PCB embryos had aberration rates greater than the mean control rate, while four embryos exceeded the highest control rate. On the other hand, human lymphocytes treated with 100 ppm of Arochlor 1254 showed no increase in chromatid breaks or gaps (Hoopingarner et al., 1972).

The chromosome breakage effects of Arochlor 1242, in the present study, were also equivocal, but this PCB apparently does cause damage to human chromosomes in vitro. After three weeks continuous exposure of human cells to 2.5 ppm of A-1242, no increase in chromosome or chromatid breakage occurred; however, there did appear to be an increase in the number of chromosomes with accentuated secondary (2°) constrictions, although no specificity for one particular chromosome or group was evident. This increase raises the question of whether A-1242 or a metabolite preferentially attacks the heterochromatic regions of chromosomes. This finding is not unique, since other known chemical mutagens (e.g., deoxyadenosine, ethoxycaffeine) cause a similar effect (Kihlman, 1966).

B. Virus-induced Chromosomal Alterations

The mutagenic potential of viruses was first described by Hampar and Ellison (1961); later, these investigators demonstrated that Herpes simplex virus type 1 induced chromosomal aberrations in Chinese
hamster cells in vitro (Hampar and Ellison, 1963). If these cells were plated at low density, the virus produced no degenerative changes and surviving chromosome or chromatid deletions of chromosome numbers 1, 2 and 3, after loss of acentric fragments, were found five to six weeks post infection. Deletions frequently appeared in more than one chromosome in a cell, but rarely were both chromosomes of a pair affected.

The number 1 and X chromosomes of Chinese hamster cells were found to be particularly susceptible to HSV induced breaks and gaps (Stich et al., 1964). Rapp and Hsu (1965), in an attempt to determine whether HSV was associated with those chromosomes at the break loci found labelling of nuclei with tritiated thymidine, but grains were not associated with the chromosomes. Preferential breakage in human chromosomes by HSV-2 has not been demonstrated (O'Neill and Rapp, 1971).

Since the finding that HSV causes chromosome breakage, a wide range of viruses have been shown to induce chromosomal aberrations, both in vivo and in vitro (Stich and Yohn, 1970). In addition to chromatid breaks, herpes simplex viruses cause: 1) fragmentation, where chromatid breaks are present in most of the chromosomes; 2) pulverization, in which the chromosomes appear to be broken up into small fragments; and 3) C-mitosis, where the spindle is absent, thereby causing metaphase chromosomes to scatter throughout the cytoplasm and become dense and short due to overcoiling (Stich et al., 1964).

Very little is known about the induction of sister chromatid exchanges by viruses. Simian virus 40 (SV-40) transformed human cells
appeared to cause a slightly higher yield of SCEs than non-transformed cells, but no statistical analyses were done (Wolff et al., 1975). On the other hand, Kato and Stich (1976), commenting on their own unpublished results, stated that hamster cells infected with adenovirus 12 produced no increase in SCEs in spite of extensive fragmentation of the entire chromosome complement.

C. Combined Effects of Viruses and Chemicals

Few investigations have been carried out on the combined effects (i.e., cytogenetic, toxic, etc.) of viruses and chemicals. PCBs produced an enhancement of the effects of duck hepatitis virus in vivo (Friend and Trainer, 1970). Ducks were fed 25, 50 and 100 ppm of Arochlor 1254 for ten days; five days later they were challenged with virus, 1.5 times the 50% effective lethal dose. Death occurred within three days. Mortality levels occurring among the PCB plus virus treatment groups were significantly higher ($P < 0.01$) than for ducks receiving virus only. However, no significant difference was observed in mortality among ducks that received the various concentrations of PCB plus virus ($P > 0.1$), which meant that no PCB dose effect was evident.

Syrian hamster cells transformed in vitro by methylcholanthrene (MCA), a known carcinogen, were further transformed by SV-40 virus or SV-40/adenovirus 7 hybrids (Dipaolo et al., 1968). The cells contained SV-40 T (tumor) antigens and the tumorogenic potential was increased after exposure of MCA cells to virus. MCA cells had a treater than normal chromosome modal number between 56 and 60, but
rarely had any new rearrangements. MCA/SV-40 cells lacked chromosome numbers between 48 and 60 and some of the cells exhibited multiple breaks, rearrangements and stretching.

Stich et al. (1972) reported that exposure of Syrian hamster cells in vitro to adenovirus 12, an oncogenic virus, and to 4-nitroquinoline-1-oxide, a known mutagen and carcinogen, did not alter the level of cellular DNA repair, but did increase the number of metaphase cells with chromatid breaks and exchanges between non-homologous chromosomes over either virus or chemical alone. The combination of virus and chemical also induced up to a 23-fold enhancement of viral-induced transformation.

In human embryonic lung cells exposed to HSV-2 and cytosine arabinoside (ara C), the number of cells with three or more chromatid breaks was significantly greater (three to four-fold) than the effect of HSV-2 alone. Cells with three or more breaks were also 6 to 22 times greater than the effect of ara C alone. These effects on human chromosomes were an indication of synergism between HSV-2 and ara C (O'Neill and Rapp, 1971).

D. Intracellular Concentration and Possible Metabolism of Arochlor 1242 In Vitro in Relation to Induction of SCEs

The intracellular concentration of A-1242 was more important in determination of the effects on chromosomes than the amount added to the culture medium. At least three possibilities exist for the discrepancy between the calculated input in the medium (10 ppm) and the actual amount (3.3 ppm) determined by gas chromatography. First,
A-1242 may not be completely soluble in DMSO. Secondly, since A-1242 was stored in a plastic test tube and the cells, to which the chemical was added, were grown in plastic vessels, adsorption of the PCB onto the plastic cannot be ruled out. Finally, D. Griffin\textsuperscript{22}, who did the analysis, said that he had difficulty extracting the PCB from the culture medium and the supernatant fluid from sonicated cells.

In addition to the extraction difficulties, the fact that one-tenth (0.4 ppm) of the actual A-1242 concentration found in culture medium was present intracellularly could be attributed to one or both of two factors: 1) This concentration was the amount that actually entered the cells; or 2) A-1242 was metabolized in the cell so that detection of the parent compound could not be made. The first possibility is unlikely considering the lipophilic nature of PCBs. Therefore, the cell membrane, which contains lipids, would not act as a barrier to the entrance of PCBs into the cell. The second possibility, although unproven, is more plausible. No investigations have been done on metabolism of PCBs \textit{in vitro}; however, such studies have been done in animals (Greb \textit{et al.}, 1975). The important point is that any damage to cellular DNA by A-1242 was due either to 0.4 ppm (the actual intracellular concentration) rather than 10 ppm (the calculated input) or to some metabolite, the nature of which is presently unknown.

With regard to possible metabolites, in animals the PCBs are metabolized in the liver (Fishbein, 1974), but metabolism might occur

\textsuperscript{22} Personal communication.
in other cells in vitro. Since many of the cytoplasmic organelles (lysosomes, mitochondria, endoplasmic reticulum) contain lipoprotein membranes, the PCBs could penetrate these organelles and thereby become exposed to various enzymes. One pathologic finding in vivo is an increase in liver weight and Allen et al. (1974) found a proliferation of the smooth endoplasmic reticulum in livers of rhesus monkeys exposed to high doses of PCBs. PCBs are also known to activate microsomal enzymes (Fishbein, 1974). The endoplasmic reticulum, therefore, may also be involved in PCB metabolism in vitro.

E. General Comments on Herpesviruses and Arochlor 1242 in Relation to SCEs

Herpesviruses can pass through the placenta (Catalano and Sever, 1971), but whether they penetrate germ cells and what happens if they do is unknown. Also unknown is whether viruses induce alterations at the molecular level (i.e., transition, transversion, addition or subtraction of base pairs) (Stich and Yohn, 1970). Certain chemicals do affect cellular DNA (Kihlman, 1966), but nothing is known about the effects of PCBs at the molecular level. Sister chromatid exchanges may be one indication of such alterations.

In order to understand the possible mechanism of induction of SCEs by HSV-2 and by A-1242 + HSV-2, as well as the effect of various chemicals (BrdU, DMSO, A-1242 and MMC) on viral infectivity, a very brief review of productive herpesvirus infection follows.
F. Herpesvirus Replication

Infection of cells in the logarithmic growth phase results in a gradual decrease of cellular DNA synthesis with a concurrent increase in viral DNA synthesis. A rapid decrease of cell ribosomal RNA (rRNA) synthesis and processing occurs with relatively little inhibition of complementary RNA. No functional cellular messenger RNA (mRNA) is formed even when cell specific proteins are translated from pre-existing cellular mRNA. Both the decrease in the rate of RNA synthesis and the inhibition of functional cellular mRNA synthesis is mediated by a virus induced protein. Inhibition of cell-specific protein synthesis and induction of virus specific protein occur concurrently (Kaplan, 1973).

Part of the actual process of viral multiplication involves the following steps: 1) the virions enter the cell and become partially uncoated in the cytoplasm; 2) the DNA-protein complex enters the nucleus where the DNA becomes disassociated from the protein and is transcribed; 3) the virus specific RNA is processed and transported to the cytoplasm; 4) the RNA then enters into free and membrane bound polyribosomes and directs synthesis of structural and non-structural viral proteins; 5) some of these proteins bind to cellular membranes, but most migrate into the nucleus; the non-structural proteins are involved in synthesis of viral DNA, the structural proteins aggregate with the DNA to form a capsid; and 6) envelopment of the capsid occurs and viral progeny are released (Roizman, 1972).
G. Effect of Chemicals on Viral Infectivity

The reduction of HSV-2 infectivity in the presence of BrdU and Mitomycin C might be expected, since both chemicals inhibit DNA synthesis. The decrease in viral infectivity in cells treated with DMSO and A-1242 may simply have been due to the toxic effects of these chemicals. Non-specific cellular degeneration, unrelated to viral cytopathic effects, was noted in these cultures. Therefore, fewer viable cells were available for viral propagation with a resultant decrease in titer. However, direct or indirect action by these chemicals on viral DNA cannot be ruled out. Inhibition of viral synthesis could occur at any of a number of steps. Results of immunofluorescence on cells exposed to the chemicals and virus indicated that inhibition by A-1242, DMSO and BrdU may occur before final assembly of the viral capsids takes place. The evidence for this assumption lies in the fact that diffuse fluorescence was observed in the nucleus but not in the cytoplasm. Capsid assembly occurs at the nuclear membrane, which appears to be altered by the virus so that an intense ring of fluorescence is observed at the periphery of the nucleus (Darlington and Moss, 1969). This intense fluorescence was seen only in MMC treated cells which means that viral synthesis was not completely inhibited, capsid assembly probably occurred, but the capsids may not have entered the cytoplasm.

H. Sister Chromatid Exchanges

The literature on sister chromatid exchanges has increased tremendously in the past four years due to the development of new
techniques for detection (Latt, 1973; Korenberg and Freedlender, 1974; Goto et al., 1975). A review of this literature will be limited to a few of these investigations that are relevant to the present research with Arochlor 1242 and herpes simplex virus type 2.

SCEs in mitotic chromosomes may be spontaneous events (Ikushima and Wolff, 1974; Kato, 1974). The frequency of SCEs can be increased by various exogenous agents (Latt, 1974; Beck and Obe, 1975; Kato and Shimada, 1975; Perry and Evans, 1975). Originally, SCEs were believed to result from excision repair of DNA (Kato, 1974) and/or post-replication repair (Kato, 1973). Other studies indicated that the process leading to SCEs may not involve a single mechanism (Kato and Stich, 1976).

Investigations on xeroderma pigmentosum cells, defective in excision repair or post-replication repair, revealed that SCEs in these cells were the same as controls (Wolff et al., 1975; Kato and Stich, 1976). Studies on cells from patients with Bloom's syndrome showed an increase in both chromosome breakage and SCEs (Schroeder, 1975; Chaganti et al., 1974), while induction of SCEs in cells from patients with Fanconi's anemia (Chaganti et al., 1974) and ataxia telangiectasia (Hatcher et al., 1976) was no different from controls.

Although more than one mechanism of formation of SCEs may exist, studies on Mitomycin C, a DNA cross-linking agent, suggested that this chemical stimulates post-replication repair, which in turn leads to

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23 Autosomal recessive diseases. Patients with these diseases have a tendency to develop malignancies.
homologous recombination and thus to sister chromatid exchanges (Comings, 1975). In opposition to this view, a comparison of induction of SCEs and chromosomal aberrations by alkylating agents suggested that post-replication repair may not be involved (Becchetti et al., 1976). S-dependent mutagens increased the frequency of SCEs while S-independent agents did not, although the latter did induce chromosome breakage. Caffeine, a known inhibitor of post-replication repair (Kato, 1973), enhanced chromosome breakage, but had no effect on the frequency of sister chromatid exchanges (Becchetti et al., 1976). Wolff et al. (1975) believe that SCEs are unrelated to DNA repair processes. Finally, with regard to SCE formation, several studies have shown that SCE rejoining does not occur randomly, but is restricted because of DNA polarity (Tice et al., 1975; Holmquist and Comings, 1975).

Since the mechanisms involved in the formation of SCEs are still unknown, the possible ways by which HSV-2 and A-1242 damage DNA will be discussed. Interpretation of results obtained in the present study are complicated by the fact that two types of DNA were present in HSV-2 infected cells, viral and cellular; BrdU could be incorporated into both and any one of the chemicals (A-1242, DMSO or MMC) might affect viral as well as cellular DNA.

The two BrdU concentrations (10^-5M and 4.5 x 10^-5M) had little effect on the frequency of SCEs induced by MMC, and the number of SCEs in DMSO treated cells was no greater than the background (BrdU) level. However, the BrdU concentration did influence the number of SCEs induced by the virus and by A-1242. This finding indicates some
type of interaction between BrdU, HSV-2 and cellular DNA as well as between BrdU, A-1242 and cellular DNA.

1. Induction of SCEs by Infectious HSV-2

The mechanism by which HSV-2 alters cellular DNA probably occurs in one or both of two ways - by direct or indirect action. 1) Incorporation of viral DNA into cellular DNA: There are two stages at which incorporation could occur, during viral transcription or viral DNA replication. Since cessation of cellular DNA synthesis is a result of an early viral-induced protein, which means after viral transcription, incorporation would most likely occur at the transcription stage. By the time of viral DNA replication, cellular DNA synthesis has essentially ceased. 2) Viral enzymatic action: Evidence for indirect viral action has been suggested for herpesvirus-induced chromosomal aberrations, which appeared prior to viral DNA replication and were probably the result of an early viral enzyme (Waubke et al., 1968). Enzymatic action, more specifically DNAse, has been proposed for chromosome breakage by other viruses (Freese, 1971) and for the breakdown of host-cell DNA by herpesviruses (Wagner, 1974).

However, neither of the above mechanisms (incorporation or enzymatic action) accounts for the fact that a greater increase in SCEs occurred with the higher \((4.5 \times 10^{-5} \text{M})\) rather than the lower \((10^{-5} \text{M})\) BrdU concentration. The continuous presence of BrdU in the culture medium may not necessarily mean that all thymine in the cellular DNA was replaced by the analogue. The possibility exists that the higher the BrdU concentration, the greater the likelihood
more thymine will be substituted, even though no increase in exchanges occurs with BrdU alone. The greater the substitution of BrdU, the more susceptible cellular DNA may become to damage by other agents. Regardless of the point at which the virus attacks cellular DNA, the greater the BrdU concentration, the more damage the virus may induce. Latt (1974) might disagree with this theory, since he found that the number of SCEs increased very little above the level of $2.0 \times 10^{-5}$M BrdU. One might conclude, from his finding, that a saturation point is reached in the number of SCEs, regardless of the BrdU concentration. However, this does not preclude the possibility that a second agent (chemical, biological or physical) could, in fact, increase the number of SCEs with an increase in BrdU concentration.

One more point favors viral damage to cellular DNA during synthesis of the latter. An increase in SCEs occurred with agents that act during the S-phase (Becchetti et al., 1976), which may mean that the virus affects cellular DNA before viral DNA replication occurs.

Interpretation of results with infectious HSV-2 and the lower BrdU concentration in the present study must be done with caution. While a virus concentration of $10^{-5}$PFU/cell induced a significant increase in the number of SCEs, the other two concentrations (one lower, one higher) did not. Since the means of $10^{-1}$ and $10^{-3}$PFU/cell were not significantly different, further studies are necessary to confirm these results.

Finally, no dose response was apparent with infectious HSV-2 at either BrdU level, which may mean that the induction of SCEs by this
virus is an all or none effect.

2. Effects of A-1242

The action of Arochlor 1242 on cellular DNA probably involves a different mechanism from that of the virus. However, A-1242 induced an increase in SCEs only with the higher BrdU concentration, so that, again, substitution of BrdU may be greater and therefore render the DNA more susceptible to attack by A-1242 or its metabolites. With regard to possible metabolites, PCBs are hydroxylated \textit{in vivo} (Greb \textit{et al.}, 1975) and perhaps \textit{in vitro}. Hydroxylation of aromatic molecules, such as the PCBs, occurs via arene oxide intermediates; arene oxides, in the metabolism of polynuclear aromatic hydrocarbons, have been shown to be mutagenic (Daly \textit{et al.}, 1972). They, or some other metabolite, rather than the parent PCB compound, therefore, may be involved in the induction of SCEs.

3. Induction of SCEs by Infectious HSV-2 + A-1242

The interaction between virus and PCB at the higher BrdU level \((4.5 \times 10^{-5} \text{M})\) was dependent on virus concentration, suggesting that the change in the number of SCEs was due primarily to the virus. The mechanisms for all of these reactions is complex, but at least three possibilities exist: 1) virus and chemical act independently of one another; 2) the virus, in some way, decreases or enhances damage due to A-1242 alone or, alternatively, A-1242 either prevents or increases damage by HSV-2; and 3) the lower the virus concentration, the less infectious virus is present; this implies that infectious virus is
necessary to cause an increase in SCEs. Results seem to indicate the second and/or third possibilities. HSV-2 (10^{-1} PFU/cell) combined with A-1242 induced a greater number of SCEs than either virus or chemical alone, which indicates enhancement. On the other hand, the lower the virus concentration the fewer the SCEs induced by the combination of HSV-2 and A-1242; these results seem to indicate that productive virions are required to cause an increase.

An infectious virus preparation contains both productive and defective virions, therefore, the lower the virus concentration the fewer the number of productive virions are present. A productive herpesvirus preparation has about ten physical particles to every infectious particle; since the chemicals (BrdU, A-1242 and DMSO) reduced viral infectivity, a higher proportion of defective to productive virus particles may have been present, or there may have been no productive virions.

4. **Effects of Inactivated HSV-2 and Inactivated HSV-2 + A-1242**

Ultraviolet irradiation does not destroy all viral functions, although no infectious virus is produced. Virus specific RNA has been found in cells transformed by UV inactivated HSV-2, although only 10% to 13% of that present during productive infection (Collard et al., 1973). However, very little viral DNA is present in such cells (Davis and Kingsbury, 1976).

The viral functions that cause an increase in the number of SCEs, either alone or in combination with A-1242, may be missing, repressed or altered in non-productive or defective virions. The immunofluores-
cent studies of Rapp and Duff (1973) indicated that virus was adsorbed and early, but not late, proteins were synthesized. The increase in the number of SCEs may, therefore, involve a late viral protein (enzyme). If an early protein (enzyme) were involved, it should be effective in both productive and non-productive infection. The involvement of a late protein might explain the differences in the number of SCEs induced by infectious virus as opposed to inactivated virus. However, this theory is in opposition to that proposed earlier in this discussion of possible mechanisms of damage to cellular DNA by infectious HSV-2.

An alternative hypothesis is that defective virions may, in fact, be responsible for the increase in exchanges. If they are defective in a different way from those present in UV inactivated virus preparations (e.g., different enzymes involved), then defective virions might be involved in both an increase or decrease in the number of exchanges. With this line of reasoning, it would be of interest to determine whether other types of virus preparations (e.g., temperature sensitive mutants) would cause an increase or decrease in the number of SCEs and whether different results would occur in combination with A-1242.

Results on cells grown with the higher BrdU concentration (4.5 x 10^-5M) suggested a tendency towards fewer SCEs in those cells with inactivated virus + any of the chemicals (A-1242, DMSO and MMC). Further studies would have to be done to determine whether these decreases were significant. It is, therefore, premature to speculate on the possible mechanisms involved.
5. Distribution of SCEs Throughout the Chromosome Complement

The distribution of SCEs, relative to chromosome length, generally showed the same pattern as that reported by others (Ikushima and Wolff, 1974; Chaganti et al., 1974; Latt, 1974), with two exceptions. 1) Slightly higher than the expected number of exchanges were seen in the F group chromosomes in cells treated with A-1242 and 4.5 x 10⁻⁵ BrdU. Latt (1974) found considerably fewer than the expected number of exchanges in this chromosome group (about 60%) with BrdU alone. He suggested that, because the centromeric region constitutes a larger portion of these chromosomes, fewer SCEs occur in this region. The finding in A-1242 treated cells, in the present study, might be an argument against this theory. However, the exact location of SCEs in these chromosomes remains to be determined. 2) The number 1 chromosome in MMC treated cells had almost twice the expected number of exchanges, while the number 3 chromosomes had far less than expected. This finding implies that MMC may preferentially induce SCEs in the number 1 chromosomes, while the number 3 chromosomes may be less susceptible. Again, the location of these exchanges should be determined and results with both A-1242 and MMC confirmed by further studies.

Within each treatment group, SCEs did not always follow a Poisson distribution. Since, in all reports thus far, including this one, such exchanges do not appear to be distributed relative to chromosome length (i.e., more than expected in the larger chromosomes, fewer than expected in the smaller chromosomes), one might question
whether SCEs are, in fact, randomly distributed throughout the chromosome complement.

6. Densitometry

The results on densitometry suggested that asymmetric (or unequal) exchanges might occur. Although densitometer scans of every chromosome are presently impractical, because of the amount of time involved, this method may be the only way in which all sister chromatid exchanges can be determined, unequivocally. Densitometry may also be the only way that asymmetric exchanges can be observed, if, in fact, they do occur. Up to this point, unequal exchanges have not been observed (Korenberg and Freedlender, 1974); this finding may simply be due to technical difficulties, mentioned in Results of the present study and by Hatcher et al. (1976). The significance of asymmetric exchanges, at the present time, is obscure, but may represent subtle effects of certain chemicals (e.g., MMC).

7. Comments on Positive and Negative Controls in Relation to SCEs

Although the primary purpose of this research was determination of the effects of A-1242, HSV-2 and A-1242 + HSV-2 on human chromosomes, a few comments on results with MMC and DMSO will be included.

HSV-2 (infectious or inactivated) had little effect on the induction of SCEs by MMC, which seems to indicate that a strong mutagen, such as MMC, may not be influenced by any other agent. However, a virus dose response was not done, so that no definitive conclusions can be drawn.
The number of SCEs induced by DMSO + infectious HSV-2 with $10^{-5}$M BrdU appeared to be greatest with the lowest virus concentration, but this difference was not statistically significant. However, this same difference occurred at the higher BrdU level ($4.5 \times 10^{-5}$M), namely, the combination of DMSO + infectious virus ($10^{-3}$PFU/cell) produced a greater increase than DMSO + $10^{-1}$PFU/cell HSV-2. The question raised by these findings is: does DMSO enhance the effects of HSV-2 at relatively low concentrations and, if so, why? On the other hand, the number of exchanges observed with DMSO alone was no greater than the background (BrdU) level. This is an interesting finding, which should be pursued, in view of the fact that DMSO has been used as a chemotherapeutic agent (Miranda-Tirado, 1975; Sehtman, 1975).

I. Concluding Remarks

Numerous questions remain with regard to the effects of A-1242, HSV-2 and A-1242 + HSV-2 on human chromosomes. Rats are far less susceptible to the toxic effects of PCBs than rhesus monkeys, for which 3 ppm (the equivalent of 150 $\mu$g/kg/day) is lethal after eight months (McNulty, 1976). Of extreme importance would be to determine whether cytogenetic effects occur in non-human primates. If rhesus monkeys are 100 times more susceptible to PCB toxicity (McNulty, 1976), are they also more susceptible to chromosome breakage, sister chromatid exchanges and dominant lethal effects, and, if so, is this more analogous to man? Although several biochemical analyses were performed on the victims of Yusho (Kuratsune, 1972), unfortunately no cytogenetic studies were done.
Infectious herpes simplex virus type 2 induces sister chromatid exchanges at concentrations that do not produce chromosome breakage. If one assumes that SCEs are, in fact, an indication of mutagenic activity, then HSV-2 is a mutagen only if defective virions are involved in the increase in such exchanges, since productive virions would destroy the cell. If the implied connection between HSV-2 and cervical cancer is true, then the present study neither confirms nor refutes the somatic mutation theory of carcinogenesis.

Arochlor 1242 induced an increase in sister chromatid exchanges only in the presence of the higher BrdU concentration ($4.5 \times 10^{-5}M$). Is A-1242 metabolized in vitro and is this PCB mutagenic? A-1242 may or may not induce chromosomal aberrations. BrdU is not found in the environment. However, other DNA analogues are used in chemotherapy (Kaufman and Maloney, 1963) and as pesticides (Epstein and Legator, 1971). Therefore, A-1242 might interact with other analogues in the same way it does with BrdU. These questions should be answered.

The carcinogenic capability of the PCBs is still in doubt. Some reports have indicated that PCBs induce cancer in animals (Ito et al., 1973), while others have shown that the PCBs inhibit tumor formation (Makiura et al., 1974). Whether or not a correlation between mutagenesis and carcinogenesis exists and SCEs are one parameter for measuring mutagenic activity, then one cannot rule out the possibility that at least one of the PCBs (Arochlor 1242) is a potential mutagen and carcinogen.

Of greatest importance is the fact that, under certain circumstances, low concentrations of Arochlor 1242, herpes simplex virus
type 2 and BrdU induced a significant increase in the number of sister chromatid exchanges over the background (BrdU) level. This finding indicates that a single agent (chemical, biological or physical), by itself, may not be mutagenic, but when two or more agents are combined, the potential for mutagenesis, or heritable changes in cells or organisms, is increased. The results of the research reported in this thesis indicate that possibility.
V. SUMMARY AND CONCLUSIONS

1) **Toxicity of Arochlor 1242 for human fetal cells in vitro:**
   Acute (short term) toxicity in cells exposed to 10, 25, 50 and 100 ppm was not clearly established, primarily because the chemical was added after the logarithmic growth phase.

   Chronic (long term) toxicity in cells exposed to 2.5, 5 and 10 ppm of Arochlor 1242 was evident after four weeks; the toxicity was dose dependent. This dose dependency was an indication of a cumulative effect.

2) **Effect of Arochlor 1242 on HSV-2 infectivity:** A-1242 reduced viral infectivity, but the decrease may have been due to the toxic effects, since non-specific cellular degeneration, unrelated to viral cytopathic effects, was observed.

3) **Chromosomal alterations induced by Arochlor 1242:** No increase in chromosome breaks or gaps was seen after three weeks exposure of cells to 2.5 ppm A-1242. The only alteration observed was an increase in secondary constrictions, a finding that is not unique, since other chemicals produce the same effect (Kihlman, 1966). However, this observation does indicate a similarity between A-1242 and other chemicals in that A-1242 appears to attack the heterochromatic regions of chromosomes.

4) **Effects of Arochlor 1242 on the number of sister chromatid exchanges:** An increase in the number of SCEs was observed when cells were treated with A-1242 and were grown with $4.5 \times 10^{-5}$, but not with $10^{-5}$M, of DrdU. This finding indicated some type of interaction
between A-1242 and BrdU. The most likely explanation is that a great-
er amount of BrdU (at the $4.5 \times 10^{-5}$M level) was incorporated into
cellular DNA, thereby rendering the DNA more susceptible to damage by
A-1242 or possibly by some metabolite.

5) Sister chromatid exchanges induced by infectious HSV-2: An
increase in the number of SCEs was observed at both BrdU concentra-
tions ($10^{-5}$M and $4.5 \times 10^{-5}$M) in the presence of HSV-2. This increase
was highly significant at the higher BrdU level, again possibly
indicating greater incorporation of BrdU. While results with HSV-2
and $10^{-5}$M BrdU were somewhat ambiguous, there was an increase in the
number of SCEs with $10^{-1}$ and $10^{-3}$PFU/cell but no difference between
$10^{-4}$PFU/cell and BrdU alone. The number of SCEs induced by infectious
HSV-2, at either BrdU level, was not virus dose dependent, at least
at concentrations of $10^{-3}$PFU/cell or higher. This observation seemed
to indicate an all or none effect. Viral damage to cellular DNA
probably occurred either through incorporation of viral DNA into the
cell genome and/or by viral enzymatic action. In either case, this
damage probably occurred before viral DNA replication.

6) Effects of A-1242 + infectious HSV-2 on number of SCEs: The
numbers of SCEs caused by the combined action of A-1242 and HSV-2, at
the lower BrdU level ($10^{-5}$M), were not significantly different from
BrdU alone. At the higher BrdU level ($4.5 \times 10^{-5}$M) the number of
SCEs was dependent on virus concentration. This finding indicated
some type of interaction between cellular DNA and the combination of
BrdU, A-1242 and HSV-2. Several possible interactions include:
1) A-1242 and HSV-2 acting independently; 2) viral enhancement or
suppression of damage to cellular DNA by A-1242 alone or, alternatively, a PCB increase or prevention of damage by HSV-2 alone; 3) the lower the virus concentration, the less infectious virus was present. By implication, the latter means productive virions are required to induce an increase in SCEs. Results indicate that the second and/or third possibilities are more plausible.

7) Effects of inactivated HSV-2 and A-1242 + inactivated HSV-2 on the number of SCEs: Ultraviolet inactivated HSV-2 appeared to cause a decrease in the number of SCEs (significance at the 90% confidence level). Either of two possible conclusions may be drawn from this finding: 1) defective virions produced a decrease, while productive virions induced an increase or 2) defective virions were responsible for both an increase and decrease, but either the degree or type of defectiveness was different. The number of SCEs produced by the combination of inactivated virus + A-1242 was either 1) not significantly different from either the PCB or virus alone or 2) showed an increase over virus but not A-1242 alone. These results indicated that perhaps non-productive virions had no effect on the damage to cellular DNA by A-1242.

8) Distribution of SCEs throughout the chromosome complement: In all but two cases, the number of SCEs in the larger chromosomes (numbers 1, 2, 3 and the B group) was equal to or greater than expected, while the number of SCEs in the smaller chromosomes (E, F and G + Y groups) was less than expected. Cells treated with A-1242 and grown with $4.5 \times 10^{-5}$M BrdU had slightly higher than the expected number of SCEs in the F group chromosomes, while cells treated with
MMC and grown with $4.5 \times 10^{-5}$ M BrdU had almost twice as many SCEs as expected in the number 1 chromosomes. Further studies will be required to determine whether or not A-1242 preferentially causes SCEs in the F group and MMC in the number 1 chromosomes, as well as the precise location of the SCEs in those chromosomes. The distribution of SCEs within each treatment group did not always follow Poisson expectations. These results, along with the finding that exchanges were not distributed according to chromosome length, indicate that the distribution of SCEs throughout the chromosome complement is not random.

9) Densitometry on chromosomes with SCEs: Densitometry indicated that unequal (or asymmetric) exchanges possibly occur. Since the mechanisms for the formation of SCEs are still unknown, the meaning of unequal exchanges is unclear. If unequal exchanges do occur, they may reflect subtle effects of chemicals, viruses or the combination of two or more agents on chromosomes.

10) Final conclusions on induction of sister chromatid exchanges: Low concentrations of infectious HSV-2 induced a significant increase in the number of sister chromatid exchanges in human fetal cells. If SCEs are indicative of mutagenic activity, then HSV-2 can be considered a potential mutagen only if defective virions are involved in such an increase; productive virions would destroy the cell. Under certain circumstances, low doses of Arochlor 1242 also induced a significant increase in the number of SCEs. The major importance of this research
was to show that a single agent (chemical or viral) may not be mutagenic, but when two or more such agents are combined, the mutagenic potential is augmented.


APPENDICES
LIST OF ABBREVIATIONS

A-1242: Arochlor 1242
BrdU: 5-bromodeoxyuridine
DMSO: dimethylsulfoxide
EMS: ethylmethanesulfonate
FITC: fluorescein isothiocyanate
FPG: fluorescence (33258 Hoechst) + Giemsa
H-AF: human amniotic fluid cells
HSV: Herpes simplex viruses
HSV-2: Herpes simplex virus type 2
MMC: Mitomycin C
PCB: polychlorinated biphenyl
PFU: plaque forming units
ppm: parts per million
RK: rabbit kidney cells
SCE: sister chromatid exchange
SV-40: simian virus 40
UV: ultraviolet irradiation
WI-38: human embryonic lung cells
### TABLE 5

BrdU $10^{-5}$M. Number of Cells, Number of SCEs and Means for Each Treatment Group

**Infectious HSV-2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Cells</th>
<th>No. of SCEs</th>
<th>Mean ± S.E.M. $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU $^b$</td>
<td>120</td>
<td>985</td>
<td>8.21 ± 0.34</td>
</tr>
<tr>
<td>A-1242 $^b$</td>
<td>135</td>
<td>1173</td>
<td>8.69 ± 0.31</td>
</tr>
<tr>
<td>DMSO $^b$</td>
<td>114</td>
<td>940</td>
<td>8.25 ± 0.33</td>
</tr>
<tr>
<td>MMC $^b$</td>
<td>64</td>
<td>1582</td>
<td>24.72 ± 0.76</td>
</tr>
<tr>
<td>HSV-2 10^{-1}PFU/cell</td>
<td>22</td>
<td>208</td>
<td>9.45 ± 0.75</td>
</tr>
<tr>
<td>HSV-2 10^{-3}PFU/cell</td>
<td>54</td>
<td>543</td>
<td>9.93 ± 0.47</td>
</tr>
<tr>
<td>HSV-2 10^{-4}PFU/cell</td>
<td>30</td>
<td>241</td>
<td>8.03 ± 0.67</td>
</tr>
<tr>
<td>A-1242 + HSV-2 10^{-1}</td>
<td>25</td>
<td>207</td>
<td>8.17 ± 0.64</td>
</tr>
<tr>
<td>A-1242 + HSV-2 10^{-3}</td>
<td>33</td>
<td>341</td>
<td>9.03 ± 0.58</td>
</tr>
<tr>
<td>A-1242 + HSV-2 10^{-4}</td>
<td>12</td>
<td>87</td>
<td>7.25 ± 0.58</td>
</tr>
<tr>
<td>DMSO + HSV-2 10^{-1}</td>
<td>20</td>
<td>160</td>
<td>8.00 ± 0.82</td>
</tr>
<tr>
<td>DMSO + HSV-2 10^{-3}</td>
<td>35</td>
<td>296</td>
<td>8.44 ± 0.57</td>
</tr>
<tr>
<td>DMSO + HSV-2 10^{-4}</td>
<td>18</td>
<td>163</td>
<td>9.06 ± 0.69</td>
</tr>
<tr>
<td>MMC + HSV-2 10^{-3}$^c$</td>
<td>38</td>
<td>883</td>
<td>23.28 ± 1.56</td>
</tr>
<tr>
<td>MMC + HSV-2 10^{-4}</td>
<td>17</td>
<td>401</td>
<td>23.59 ± 1.61</td>
</tr>
</tbody>
</table>

**UV Inactivated HSV-2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Cells</th>
<th>No. of SCEs</th>
<th>Mean ± S.E.M. $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2 1 PFU/cell</td>
<td>29</td>
<td>195</td>
<td>6.72 ± 0.67</td>
</tr>
<tr>
<td>A-1242 + HSV-2</td>
<td>12</td>
<td>105</td>
<td>8.75 ± 0.87</td>
</tr>
<tr>
<td>DMSO + HSV-2</td>
<td>34</td>
<td>312</td>
<td>9.18 ± 0.58</td>
</tr>
<tr>
<td>MMC + HSV-2</td>
<td>13</td>
<td>322</td>
<td>24.77 ± 1.79</td>
</tr>
</tbody>
</table>

BrdU, A-1242, DMSO and MMC were the same as listed under "Infectious HSV-2".

$^a$ S.E.M. = standard error of the mean.

$^b$ Pooled data from five experiments, except MMC data which was pooled from four experiments.

$^c$ No MMC was included with HSV-2 10^{-1}PFU/cell.
TABLE 6

BrdU $4.5 \times 10^{-5}$M. Number of Cells, Number of SCEs and Means for Each Treatment Group

Infectious HSV-2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Cells</th>
<th>No. of SCEs</th>
<th>Mean ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU$^b$</td>
<td>41</td>
<td>303</td>
<td>7.39 ± 0.38</td>
</tr>
<tr>
<td>A-1242$^b$</td>
<td>56</td>
<td>536</td>
<td>10.23 ± 0.48</td>
</tr>
<tr>
<td>DMSO$^b$</td>
<td>18</td>
<td>128</td>
<td>7.11 ± 0.07</td>
</tr>
<tr>
<td>MMC</td>
<td>9</td>
<td>186</td>
<td>20.67 ± 2.00</td>
</tr>
<tr>
<td>EMS</td>
<td>21</td>
<td>255</td>
<td>12.14 ± 0.66</td>
</tr>
<tr>
<td>HSV-2 $10^{-1}$PFU/cell</td>
<td>31</td>
<td>344</td>
<td>11.10 ± 0.55</td>
</tr>
<tr>
<td>HSV-2 $10^{-2}$PFU/cell</td>
<td>23</td>
<td>282</td>
<td>12.26 ± 0.70</td>
</tr>
<tr>
<td>HSV-2 $10^{-3}$PFU/cell</td>
<td>26</td>
<td>287</td>
<td>11.04 ± 0.45</td>
</tr>
<tr>
<td>A-1242 + HSV-2 $10^{-1}$</td>
<td>15</td>
<td>191</td>
<td>12.73 ± 1.10</td>
</tr>
<tr>
<td>A-1242 + HSV-2 $10^{-2}$</td>
<td>22</td>
<td>250</td>
<td>11.36 ± 0.56</td>
</tr>
<tr>
<td>A-1242 + HSV-2 $10^{-3}$</td>
<td>19</td>
<td>163</td>
<td>8.58 ± 0.54</td>
</tr>
<tr>
<td>DMSO + HSV-2 $10^{-1}$</td>
<td>5</td>
<td>44</td>
<td>8.80 ± 1.51</td>
</tr>
<tr>
<td>DMSO + HSV-2 $10^{-3}$</td>
<td>18</td>
<td>179</td>
<td>9.94 ± 0.89</td>
</tr>
<tr>
<td>MMC + HSV-2 $10^{-1}$</td>
<td>13</td>
<td>262</td>
<td>20.15 ± 1.19</td>
</tr>
<tr>
<td>EMS + HSV-2 $10^{-3}$</td>
<td>21</td>
<td>285</td>
<td>13.57 ± 0.78</td>
</tr>
</tbody>
</table>

$^a$ S.E.M. = standard error of the mean.

$^b$ BrdU: pooled data from two experiments; A-1242 and DMSO: pooled data from three experiments.

$^c$ No positive (MMC or EMS) or negative (DMSO) controls were included with HSV-2 $10^{-2}$PFU/cell.