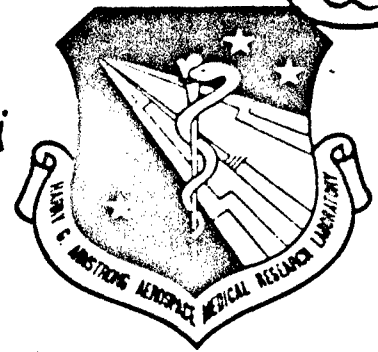


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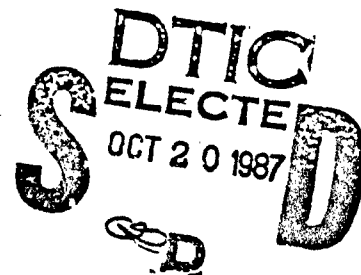
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BIOLOGICAL ACTIVITY OF
CHLOROPENTAFLUOROBENZENE

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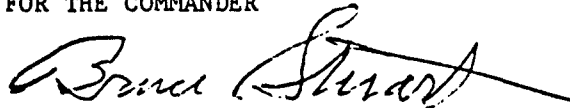
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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



BRUCE O. STUART, Ph.D.
Director, Toxic Hazards Division
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REPORT DOCUMENTATION PAGE

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1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited		
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE					
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S) AAMRL-TR-87-039		
6a. NAME OF PERFORMING ORGANIZATION Northrop Services, Inc. Environmental Sciences		6b. OFFICE SYMBOL (If applicable)		7a. NAME OF MONITORING ORGANIZATION AAMRL, Toxic Hazards Division	
6c. ADDRESS (City, State, and ZIP Code) P. O. Box 12313 2 Triangle Drive Research Triangle Park, NC 27709				7b. ADDRESS (City, State, and ZIP Code) HSD, AFSC Wright-Patterson AFB, Ohio 45433	
8a. NAME OF FUNDING / SPONSORING ORGANIZATION Northrop Services, Inc.		8b. OFFICE SYMBOL (If applicable)		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F33615-85-C-0532	
8c. ADDRESS (City, State, and ZIP Code) 101 Woodman Drive, Suite 12 Dayton, Ohio 45431		10. SOURCE OF FUNDING NUMBERS			
		PROGRAM ELEMENT NO. 62202F		PROJECT NO. 6302	TASK NO. 00
				WORK UNIT ACCESSION NO. 01	
11. TITLE (Include Security Classification) Biological Activity of Chloropentafluorobenzene					
12. PERSONAL AUTHOR(S) Vernon Steele, Ph.D.					
13a. TYPE OF REPORT Technical Report		13b. TIME COVERED FROM 86 05 TO 87 01		14. DATE OF REPORT (Year, Month, Day) July 1987	
				15. PAGE COUNT 38	
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP			
06	20		Chloropentafluorobenzene, Genotoxicity, Benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, N-methyl-N'-nitro-N-nitroso-guanidine, 4-Nitroquinoline-1-oxide		
15	02				
19. ABSTRACT (Continue on reverse if necessary and identify by block number) The potential biological activity of chloropentafluorobenzene (CPF) was evaluated using a battery of in vitro short-term tests. The assays included in this evaluation were the Salmonella Mutagenicity Assay, the Chinese Hamster Ovary (CHO) Cell Mutation Assay, the CHO/Sister Chromatid Exchange and Chromosome Aberration Assay, the Primary Rat Hepatocyte Unscheduled DNA Synthesis Assay, and the BALB/c-3T3 Transformation Assay. Initial solubility studies indicated that CPF was soluble in culture medium using glass dishes at 200 mg/mL. The maximum concentration tested was 1 mg/mL. The studies suggest that CPF has significant genotoxic activity in three of the assays performed. CPF induced significant cytogenetic abnormalities in the Sister Chromatid Exchange Assay and marginally significant results in the Chromosomal Aberration Assay at the highest doses in all four tests and at lower doses in two tests. The BALB/c-3T3 Transformation Assay indicated weakly positive effects of CPF without metabolic activation and positive results with activation at the highest doses. CPF was negative in the Salmonella Mutagenicity Assay, the CHO Cell Mutation Assay, and the Primary Rat Hepatocyte Unscheduled DNA Synthesis Assay. <i>Keywords:</i>					
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION		
22a. NAME OF RESPONSIBLE INDIVIDUAL Harvey J. Clewell, III, Lt Col, USAF, BSC			22b. TELEPHONE (Include Area Code) (513) 255-3916		22c. OFFICE SYMBOL AAMRL/TH

PREFACE

The research reported in this document was begun on 12 May 1986 and completed on 31 January 1987. The work was conducted by the *In Vitro* Toxicology Section of Northrop Services, Inc. - Environmental Sciences (NSI-ES) at Research Triangle Park, NC, in support of the Toxic Hazards Research Unit of NSI-ES's contract with the Harry G. Armstrong Aerospace Medical Research Laboratory, Toxic Hazards Division at Wright-Patterson Air Force Base, OH. Lt Col Harvey J. Clewell III served as the AAMRL Contract Technical Monitor (Contract Number F33615-85-C-0532) during the conduct of these studies. All specimens, raw data, and the final report will be archived at the Toxic Hazard Research Unit/Northrop Services, Inc., at the Wright-Patterson Air Force Base in Dayton, OH.

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TABLE OF CONTENTS

SECTION	PAGE
PREFACE	1
TABLE OF CONTENTS	2
LIST OF TABLES	3
LIST OF ABBREVIATIONS	5
INTRODUCTION	6
MATERIALS AND METHODS	6
Test Compound	6
Solubility Tests	7
Preparation of Glass Dishes for Cell Culture	7
Metabolic Activation	7
Salmonella Mutagenicity Assay	7
Mammalian Cell Mutation Assay	8
Mammalian Cell Cytogenetic Assays	10
Primary Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay	11
BALB/c-3T3 Transformation Assay	12
Statistical Methodology	13
RESULTS	13
Solubility Studies	13
Salmonella Mutagenicity Assay	15
Mammalian Cell Mutation Assay	16
Mammalian Cell Cytogenetic Assays	17
Primary Rat Hepatocyte UDS Assay	26
BALB/c-3T3 Transformation Assay	27
DISCUSSION	30
REFERENCES	36
QUALITY ASSURANCE UNIT CERTIFICATION	38

LIST OF TABLES

TABLE	PAGE
1 Solubility of CPFB in Growth medium	14
2 Solubility of CPFB in DMSO	14
3 Cytotoxicity of CPFB to Salmonella Strains	15
4 Mutagenic Activity of CPFB in the Ames Assay	15
5 Effect of CPFB on the Mutation Frequency of Chinese Hamster Ovary Cells without Metabolic Activation - Experiment 1	16
6 Effect of CPFB on the Mutation Frequency of Chinese Hamster Ovary Cells without Metabolic Activation - Experiment 2	16
7 Effect of CPFB on the Mutation Frequency of Chinese Hamster Ovary Cells with Metabolic Activation - Experiment 3	17
8 Effect of CPFB on the Mutation Frequency of Chinese Hamster Ovary Cells with Metabolic Activation - Experiment 4	17
9 Effect of CPFB on the Induction of SCEs without Metabolic Activation - Experiment 1	18
10 Effect of CPFB on the Induction of SCEs without Metabolic Activation - Experiment 2	19
11 Effect of CPFB on the Induction of SCEs with Metabolic Activation - Experiment 3	20
12 Effect of CPFB on the Induction of SCEs with Metabolic Activation - Experiment 4	21
13 Effect of CPFB on the Induction of Chromosomal Aberrations without Metabolic Activation - Experiment 1	22
14 Effect of CPFB on the Induction of Chromosomal Aberrations without Metabolic Activation - Experiment 2	23
15 Effect of CPFB on the Induction of Chromosomal Aberrations with Metabolic Activation - Experiment 3	24

(continued)

LIST OF TABLES

TABLE	PAGE
16 Effect of CPFB on the Induction of Chromosomal Aberrations with Metabolic Activation - Experiment 4	25
17 Effect of CPFB on Primary Rat Hepatocyte Unscheduled DNA Synthesis - Experiment 1	26
18 Effect of CPFB on Primary Rat Hepatocyte Unscheduled DNA Synthesis - Experiment 2	27
19 Effect of CPFB on the Transformation of BALB/c-3T3 Cells without Metabolic Activation - Experiment 1	28
20 Effect of CPFB on the Transformation of BALB/c-3T3 Cells without Metabolic Activation - Experiment 2	29
21 Effect of CPFB on the Transformation of BALB/c-3T3 Cells with Metabolic Activation - Experiment 3	31
22 Effect of CPFB on the Transformation of BALB/c-3T3 Cells with Metabolic Activation - Experiment 4	32
23 Summary of Results	35

LIST OF ABBREVIATIONS

B(a)P – benzo(a)pyrene
BuDr – bromodeoxyuridine
CAb – chromosome aberration
CHO – Chinese hamster ovary
CPFB – chloropentafluorobenzene
DMBA – 7,12-dimethylbenz(a)anthracene
DMSO – dimethylsulfoxide
EDTA – ethylenediaminetetraacetic acid
EMEM – Eagle's minimum essential medium
FBS – fetal bovine serum
HGPRT – hypoxanthine-guanine phosphoribosyl transferase
MNNG – N-methyl-N'-nitro-N-nitrosoguanidine
NADP – nicotinamide adenine dinucleotide phosphate
4NQO – 4-nitroquinoline-1-oxide
NTP – National Toxicology Program
PBS – phosphate buffered saline
RCE – relative cloning efficiency
S9 – rat liver microsomal fraction
SCE – sister chromatid exchange
6-TG – 6-thioguanine
UDS – unscheduled DNA (deoxyribonucleic acid) synthesis

INTRODUCTION

The biological activity of chloropentafluorobenzene (CPFB) was evaluated using six short-term *in vitro* assays. CPFB, a fully substituted halogenated benzene, is a volatile, colorless liquid being considered by the Air Force to test the effectiveness of chemical defense procedures and equipment. CPFB is advantageous for this purpose because it is detectable at very low levels by gas chromatography. However, little is known concerning the potential human health hazards associated with the exposure to this agent. To estimate the health risks of CPFB, *in vitro* assays were performed to assess the ability of this agent to damage DNA and/or to transform mammalian cells. The assays were chosen to cover a broad range of different types of DNA damage and to compare results with previous assay data (Tu et al., 1986).

The testing was conducted in two phases: (1) an initial study was performed to determine the solubility limits of CPFB with various solvents in plastic and glass culture dishes, and (2) studies were performed to assess the mutation, DNA repair, cytogenetic abnormalities, and transformation rates of cells exposed to CPFB.

Because little is known about the possible requirement for metabolic activation of CPFB, the studies were performed both without and with metabolic activation. Metabolic activation was accomplished by adding rat liver microsomes to the exposure mixture (Zeiger et al., 1979).

MATERIALS AND METHODS

TEST COMPOUND

In the first phase of these studies, the CPFB stock material was analyzed for identity and purity. One 100-mL bottle of CPFB (lot# MM03511TH) was purchased from Aldrich Chemical Company (Cat. No. 19,366). The bottle was labeled 95% pure, F.W. 202.51, ClC_6F_5 , b.p. 122-123°C/750 mm Hg, and density 1.568 g/mL. The test sample was stored at 4°C in a locked refrigerator, and the refrigerator temperature was monitored daily and recorded weekly. The positive control chemicals, 4-nitro-quinoline-1-oxide (4NQO), sodium azide, 2-nitrofluorene, and 2-aminoanthracene, were purchased from Sigma Chemical Co. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz(a)-anthracene (DMBA) were obtained from the National Toxicology Program (NTP) repository. All positive control compounds were kept at -20°C in a locked freezer, and the freezer temperature was monitored daily and recorded weekly.

Quantitative chemical analysis of the CPFB sample using quadrapolar mass spectrometry and gas chromatography revealed that the compound was >99.7 % pure. The impurities were not further identified.

SOLUBILITY TESTS

All dilutions of CPFB were made with glass pipets in glass tubes. The medium and solvents used were Eagle's minimum essential medium (EMEM) (GIBCO), dimethylsulfoxide (DMSO) (Gold Label, Aldrich Chemical Company), fetal bovine serum (FBS) (Sterile Systems), acetone (Eastman Kodak Company), ethylene glycol methyl ether and ethylene glycol diethyl ether (Aldrich Chemical Co.), hexane (Fisher), pluronic polyols F-127 and F-68 (gift of J. Carver, Chevron Environmental Health Center, Inc.) and tetrahydrofuran (Aldrich Chemical Co.). Glass dishes (Fisher) and plastic tissue culture dishes (Costar Plastics) were used to examine the solubility of CPFB and any reactivity of the CPFB solution with the culture vessel. Three milliliters of each CPFB dilution were added per dish, then the dishes were placed in a 37°C vented incubator with a water-saturated atmosphere of 5% CO₂ in air. The dishes were held for 10 days and examined using a phase-contrast inverted microscope (100X) to check for insoluble particles or degradation of the dishes.

PREPARATION OF GLASS DISHES FOR CELL CULTURE

Clean glass dishes were treated by boiling them in a solution of 10 mM ethylenediaminetetraacetic acid (EDTA) for 10 min. The dishes were then sterilized by autoclaving for 30 min.

METABOLIC ACTIVATION

To simulate *in vitro* any possible metabolic activation of CPFB that might occur *in vivo*, duplicate assays were performed using a rat liver homogenate rich in metabolizing enzymes. For this purpose, Aroclor 1254-induced rat liver microsomal fraction (S9), lot number R-289, was purchased from Microbiological Associates, Inc. The "S9 mix" used for metabolic activation consisted of 6 mL S9, 40 mM nicotinamide adenine dinucleotide phosphate (NADP), 50 mM glucose-6-phosphate, and 330 mM KCl in 15 mL of culture medium. This was further diluted by adding 1 part "S9 mix" to 19 parts culture medium before use. This activation system was used in the Salmonella Mutagenicity Assay, Sister Chromatid Exchange (SCE) Assay, Chromosome Aberration (CAb) Assay and the BALB/c Transformation Assay. A modified activation system was used for the Chinese Hamster Ovary (CHO) Cell Mutation Assay as described below.

SALMONELLA MUTAGENICITY ASSAY

The Salmonella Mutagenicity Assay used methodology similar to that described by Ames et al. (1973,1975). Cytotoxicity of CPFB was determined using the *Salmonella typhimurium* TA98 and TA100 strains in the plate incorporation assay. This cytotoxicity assay was performed by treating one-day-old bacterial cultures with 0.10, 0.33, 1.0, 3.3, or 10 µL of CPFB in 2 mL of molten agar (45°C). This agar, which contained trace amounts of histidine and biotin, was then poured over minimal medium-E agar plates (Gibco). The plates were incubated at 37°C for 48 h, and the colonies were

counted with an Artek automatic colony counter Model 980 (New Brunswick Scientific). This was followed by a mutagenicity assay with TA98 and TA100 strains conducted using a nontoxic dose range. Metabolic activation was incorporated into the mutagenic assay by the addition of post-mitochondrial supernatant fraction from rat livers induced with Aroclor 1254. The tester strains used in these studies were designed for the detection of frameshift mutagens (TA1537, TA1538, and TA98) and base pair substitutions (TA1535 and TA100). The strains have been verified for the expression of phenotypic characteristics (*his*-, *rfa*, *uvrB*, and *amp^R*) and response to concurrent positive controls. The CPFB was diluted in DMSO. The auxotrophic strains of *Salmonella typhimurium* were obtained from Dr. B.N. Ames, University of California, Berkeley, CA.

For the mutagenicity assays, overnight cultures of *Salmonella* tester strains (TA1535, TA1537, TA1538, TA98, and TA100) were incubated with the test sample in a nontoxic dose range (0.1 to 10 μ L per plate) and tested in triplicate at five dose levels in borosilicate glass tubes. Agar (2 mL) containing trace amounts of histidine and biotin was then added (45°C). The mixture was swirled on a low speed vortex mixer and poured over previously prepared minimal medium-E plates. The plates were incubated at 37°C for 72 h, and the revertant colonies were counted on an Artek automatic colony counter.

The data were interpreted as follows: Either a mutagenic ratio of 2.0 or more, or a consistent and dose-related increase in revertant induction, was considered a positive response. Comparisons were made with spontaneous reversion counts as well as with historical reversion counts. Experiments were considered valid if the spontaneous counts and the positive control data were similar to the historical data (Claxton et al., 1982; Maron and Ames, 1983).

MAMMALIAN CELL MUTATION ASSAY

CHO cells were used to determine the ability of CPFB to mutate mammalian DNA. The CHO cell line is a permanent cell line with an average generation time of 12 to 14 h. The cells were grown at 37°C in a water-saturated atmosphere of 5% CO₂. The cells were maintained in McCoy's 5A medium (GIBCO) supplemented with 10% FBS, 2 μ M L-glutamine, and 1% gentamycin (GIBCO). The cultures were subcultured on a once- or twice-a-week basis at a cell concentration which allowed maintenance as logarithmically growing cultures. The cultures were either passaged or refed twice weekly. Cultures were plated for subculture at 5 x 10⁵ cells/100-mm glass dish. Cultures were handled under gold lights to prevent phototoxic effects. The culture conditions were rigorously controlled to maintain a relatively uniform cloning efficiency and growth rate, and a low spontaneous mutation frequency. The cultures were trypsinized for subculture by first rinsing with calcium- and magnesium-free phosphate buffered saline (PBS) then adding 3 mL of 0.2% trypsin-EDTA. A single cell suspension was used for subculture by making the appropriate dilutions

(5×10^5 cells/100 mm-glass dish) following counting. The cells were counted using a Coulter cell counter.

The cytotoxic effects of CPFB on CHO cells were assessed using a relative cloning efficiency (RCE) assay, which measured the reduction in colony-forming ability of the cells. For this assay the cells were plated at 2×10^2 cells per 100-mm glass dish 24 h prior to chemical treatment. The cells were then exposed for 24 h to 0.1, 1, 10, 100, and 1000 μg of CPFB per ml, washed twice with buffered saline, and refed with fresh medium. In the presence of metabolic activation the exposure duration was limited to 2 h. The cells were then incubated in the culture medium described above for seven days. At the end of the incubation period, the culture dishes were rinsed with buffered saline and fixed with methylene blue (0.2%) in 30% methanol. The number of colonies containing 50 or more cells were counted for each dish using an Artek automatic colony counter. The RCE and the surviving fraction were calculated from the number of colonies observed in the solvent control and treatment conditions. Nontoxic concentrations of CPFB to be used in the mutation assays were determined using the RCE data.

The same lot number of liver homogenate (S9 fraction) was used throughout the experimental procedure and was stored at -80°C . A modified activation system was used for this assay for optimal performance. The S9 fraction was thawed immediately before use and mixed to give the following final reaction mixture in the culture medium: 2.4 mg NADP (sodium salt)/mL, 4.5 mg isocitric acid (trisodium salt)/mL, and 15 μL S9 fraction/mL.

On the day after subculture, replicate log-phase cultures in glass 100-mm culture dishes were treated with growth medium containing selected concentrations of CPFB or positive control chemical, either MNNG or B(a)P. In assays without metabolic activation CPFB treatment lasted 24 h and MNNG treatment 4 h, after which time the medium containing CPFB or control chemical was removed and fresh culture medium without additional chemical was added. In assays with metabolic activation CPFB and B(a)P treatments lasted 2 h. Twenty-four hours after the initiation of chemical treatment, the cells were replated and their viability determined by plating 2×10^2 cells from each test or control culture into 10 mL of medium in 100-mm plastic dishes. The dishes were incubated for 7 days, and the number of colonies per dish was determined as in the cytotoxicity assay.

The presence of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) mutants was determined by plating at least 10^6 cells from each test chemical treatment or control group into 10 or more dishes containing 10 μg 6-thioguanine (6-TG)/mL for a final cell density per culture dish of 1×10^5 . The mutant selection was initiated at seven to nine days following chemical treatment initiation. At the time of selection initiation, a second survival assay was conducted similar to the one at 24 h. The selection cultures were maintained by refeeding with selective medium on Day 3,

and incubation was continued for an additional six to seven days. The cultures were then fixed and stained as in the cytotoxicity assay, and the colonies counted and recorded.

The mutation frequency was determined by dividing the total number of HGPRT (6-TG resistant) mutants scored by the number of viable cells plated (determined by multiplying the seeding density by the cloning efficiency for each test or control condition).

A mutation assay was considered valid and acceptable if the following criteria were met.

- The cloning efficiency of solvent control cultures was $>60\%$.
- The mutation frequency of the positive control was similar to that expected based on historical data (O'Neill et al., 1977; Arce et al., 1987).
- At least three of the test chemical concentrations had acceptable numbers of viable cells selected for mutation (at least 5×10^5).

A response to a test chemical was considered positive in the HGPRT mutation assay if

- there were reproducible increases in mutation frequency at two or more concentrations (a mutation frequency that is greater than 50 mutant colonies per 10^6 plated cells may be considered as positive depending upon the solvent control frequency); or
- the number of mutant colonies present in the treatment dishes was greater than five times that observed in the solvent control for at least two of the treatment concentrations.

These criteria are not absolute and final interpretation of data may be based on the previous experience of investigators in consideration of any extenuating circumstances.

The interpretation of data may consider the relative degree of response as well as existing data on other chemicals with known responses in the assay. Ultimately, the interpretation of the *in vitro* responses must consider the potential for *in vivo* exposure and other factors.

MAMMALIAN CELL CYTOGENETIC ASSAYS

Two assays were used to assess the ability of CPF_B to induce cytogenetic damage in CHO cells: the SCE Assay and the CAb Assay.

Assay for Sister Chromatid Exchange

One day after culture initiation, the medium was removed and the CHO cells were treated with 62.5, 125, 250, 500, and 1000 μg CPF_B/mL of medium for 2 h to allow interaction with cells before addition of bromodeoxyuridine (BuDr). BuDr was added to visualize the SCEs within the DNA. Following a 2-h exposure to CPF_B, 0.1 μg BuDr/mL was added to the cultures. The cells were incubated for 24 h. The medium was then removed, fresh medium containing BuDr and colcemid (0.1 μg /mL) was added, and incubation continued for 2 to 3 h. The total incubation time with CPF_B

was 26 h. When metabolic activation was provided, the medium on one-day-old cultures was replaced with S9 mix in medium plus the test chemical without FBS. In such tests, the CPFB and S9 were present for only the initial 2 h.

Cell Harvest and Fixation

Two to three hours following addition of colcemid, the cells were collected by mitotic shake-off and treated for up to 3 min at room temperature with hypotonic KCl (75 mM). Cells were then washed twice with fixative (3:1, methanol:glacial acetic acid, v/v), dropped onto slides, and air-dried.

Assay for Chromosome Aberrations

The CAb Assay was performed in a manner similar to the SCE test except that no BuDr was used. In treatments without S9 mix, the cultures were incubated with CPFB for 8 to 10 h, washed, and treated with colcemid for 2 to 2.5 h before fixation. In tests involving activation, CPFB and S9 mix were present for only the initial 2 h.

The cells from the SCE and CAb studies were selected for scoring on the basis of normal morphology and a chromosome number of 21 ± 3 . A modified fluorescence plus Giemsa technique was used to stain the cells for the presence of SCE in the second metaphase stage of replication. In this procedure the slides were stained for 10 min with Hoechst 33258 (5 $\mu\text{g/mL}$) in phosphate buffer (pH 6.8), mounted in the same buffer, and exposed at 55 to 65°C to "black light" from 15-W tubes for 3 to 8 min. Finally, slides were stained with Giemsa and air-dried. These cells were then scored for the frequency of SCE per cell. For scoring aberrations, slides were stained with Giemsa and individual types of aberrations were scored separately. Fifty cells per treatment group were scored in each assay.

Data analysis of both SCEs and aberrations has been developed by National Institute of Environmental Health Sciences/NTP and was employed in the CPFB study. This data analysis uses a decision scheme which considers both a trend analysis of the dose-response curve and the magnitude of the response at each dose level. A repeat of each test is required, and the results of two tests are then used to assign a negative, questionable, or positive conclusion. In the assays performed here using CPFB, the Student's t-test was used to examine the differences between the treated and control groups.

PRIMARY RAT HEPATOCYTE UNSCHEDULED DNA SYNTHESIS (UDS) ASSAY

Hepatocytes were isolated from male Fischer 344 rats using the two-step perfusion method of Seliger. (1973). Freshly isolated rat hepatocytes were plated onto standard glass slides in 100-mm glass dishes. The cells were cultured in EMEM supplemented with 0.2 mM serine, 0.2 mM aspartate, 1 mM pyruvate, 5% FBS, 0.1 μM insulin, and 50 μg gentamycin/mL. Cell viability was determined

using trypan blue. Following a 2-h attachment period, the cells were exposed to CPFB in media, 4-nitroquinilone-1-oxide (4NQO) in DMSO (final DMSO concentration was 0.2% in medium), 0.2% DMSO in medium alone, or medium alone for 18 h in a humidified incubator. During this period the cells were also exposed to 10 μ Ci tritiated thymidine (specific activity: 88 Ci/mmol)/mL. The cells were rinsed with PBS, 1% sodium citrate was added to the dishes for 15 min, then the cells were fixed in methanol and dried. The glass slides and dishes were dipped in Kodak NTB2 liquid emulsion and exposed for 14 days at -20°C. The slides and dishes were photographically developed, fixed, and stained with Giemsa stain. The exposed grains in cells with intact nuclear membranes were counted at 1000X magnification using an Artek automatic colony counter with a video camera mounted on a standard Nikon microscope. The grain counts for 50 cells per experimental group were recorded. The mean and standard deviation were determined, and the Student's t-test was used to determine the significance between test means and control means (Simpson et al., 1960). Mean values were considered significantly different if $p < 0.001$. While there are no generally accepted statistical techniques for this assay, the $p < 0.001$ level of significance has been used previously in similar studies (Tu et al., 1986).

BALB/C-3T3 TRANSFORMATION ASSAY

This transformation assay procedure and the criteria for scoring transformed foci in the BALB/c-3T3 cell culture system have been previously published (Kakunaga, 1973; Reznikoff et al., 1973).

For the cytotoxicity assay, 2×10^2 cells were seeded in 60-mm dishes in 5 mL EMEM supplemented with 10% FBS. Three dishes were used for each treatment or control group. After the cells had attached (18 to 24 h), the cultures were treated with 0.1, 1, 10, 100, and 1000 μ g CPFB/mL of complete medium for 72 h. Cells were then washed twice with PBS and fed with fresh EMEM supplemented with 10% FBS. Seven days later, the cultures were fixed and stained. Colonies containing 50 or more cells were counted, and the RCE was determined.

For the transformation assay, 3×10^4 3T3 cells were seeded into 60-mm dishes in EMEM with 10% FBS 24 h before treatment. Cultures were then treated with 0.1, 1, 10, 100, and 1000 μ g of CPFB per mL of EMEM with 10% FBS. The positive control plates were treated with 0.1 and 0.25 μ g B(a)P/mL. B(a)P was initially dissolved in DMSO then added to the culture medium so that the final DMSO concentration was 0.2% (v/v). Following exposure for 72 h, the cultures were washed twice with PBS and refed with EMEM and 10% FBS medium. The medium was replaced twice a week for 24 days, at which time the cultures were fixed and stained with Giemsa. Control cultures were handled in the same manner except that DMSO alone was added to a final concentration in the medium of 0.2% (v/v). Both type II and type III foci were counted, but only type III foci were used to estimate

transformation rates. The transformation rates were determined using two methods: the Mean Method as developed by Capizzi and Jameson (1973) and the P_0 Fluctuation Analysis Method of Luria and Delbruck (1943). Transformation frequencies were correlated with relative cellular survival and calculated as described by Reznikoff et al. (1973). For each concentration of CPF_B or control group, 12 dishes were used in the transformation assay and three additional dishes were used to confirm the cytotoxicity at that concentration.

STATISTICAL METHODOLOGY

There is a wide variance in statistical techniques used for analyzing data from *in vitro* assays. Several criteria have been developed for each assay to aid in analyzing the data (Auletta, 1985). The data in this report have been analyzed by two methods: empirical methods (e.g., a two- to threefold increase compared to background for most mutation assays indicates a positive response) and the Student's t-test (Simpson et al., 1960). The statistical analyses are not corrected for multiple comparisons to a single experimental control.

RESULTS

SOLUBILITY STUDIES

Initially, the CPF_B was analyzed for its solubility properties in a variety of solvents used for tissue culture studies. These studies were performed using both plastic and glass dishes, and with and without serum. Evidence of precipitates was monitored using phase-contrast microscopy at 100X.

Table 1 shows that CPF_B was soluble in glass dishes in EMEM at concentrations up to 600 mg/mL. CPF_B could be solubilized in culture medium (EMEM + 10% FBS) in glass dishes at concentrations up to 200 mg/mL. At 400 mg/mL, small amounts of precipitate were seen. In plastic dishes the CPF_B caused large amounts of precipitate at 3 mg/mL and small amounts of precipitate at 1 mg/mL (1000 µg/mL) in culture medium with or without serum. CPF_B could also be dissolved in DMSO in glass dishes at concentrations up to 156.8 mg/mL with no precipitate (Table 2). A 1:200 dilution of the 156.8 mg/mL stock in culture medium produced no precipitate (784 µg/mL). CPF_B could also be dissolved in acetone up to 200 mg/mL in glass dishes, in hexane and in pluronic polyols F-127 and F-68 up to 156.8 mg/mL, and in tetrahydrofuran up to 156.8 mg/mL.

TABLE 1. SOLUBILITY OF CPFB IN GROWTH MEDIUM

Solvent	Experiment	CPFB ^a (mg/mL)	FBS ^b	G/P ^c	Observation
None	I	600	+	G	Moderate amount of precipitate
		600	-	G	No precipitate
		400	+	G	Small amount of precipitate
		400	-	G	No precipitate
		200	+	G	No precipitate
		200	-	G	No precipitate
	II ^d	3	+	P	Large amount of precipitate
		3	-	P	Large amount of precipitate
		2	+	P	Moderate amount of precipitate
		2	-	P	Moderate amount of precipitate
		1	+	P	Very small amount of precipitate
		1	-	P	Very small amount of precipitate

^a CPFB was diluted with EMEM. Serial dilutions were used to yield the specified concentrations of CPFB. CPFB has a density of 1.568 g/mL.

^b FBS, when added, was at a final concentration of 10% in EMEM.

^c Glass (G) or plastic (P) 60-mm tissue culture dishes.

^d Aliquots of the above samples (Experiment I) were diluted further to yield these CPFB concentrations.

TABLE 2. SOLUBILITY OF CPFB IN DMSO

Solvent	Experiment	CPFB ^a (mg/mL)	EMEM + 10% FBS	G/P ^b	Observation
DMSO	I	600	-	P	Blistering of dishes
		400	-	P	Blistering of dishes
		200	-	P	No blistering; no precipitate
	II	600	-	G	Very thick layer of precipitate
		400	-	G	Thick layer of precipitate
		200	-	G	Very small amount of precipitate
	III	156.8	-	G	No precipitate
		15.68	-	G	No precipitate
		1.568	-	G	No precipitate
		0.1568	-	G	No precipitate
		0.01568	-	G	No precipitate
	IV ^c	0.784	+	G	No precipitate
		0.0784	+	G	No precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784	+	G	No precipitate

^a CPFB was diluted into DMSO (99.9% pure) to yield the described concentrations. CPFB has a density of 1.568 g/mL.

^b Glass (G) or plastic (P) 60-mm tissue culture dishes.

^c 1:200 dilutions were made from corresponding stocks of CPFB in DMSO (Experiment III) into EMEM + 10% FBS. For example, the 156.8 mg CPFB/mL DMSO stock was diluted 1:200 using EMEM + 10% FBS to yield 0.784 mg CPFB/mL, and the 15.68 mg CPFB/mL stock was diluted to give 0.0784 mg CPFB/mL in medium, etc.

SALMONELLA MUTAGENICITY ASSAY

The toxicity data from experiments where *Salmonella* bacteria were exposed to CPFB are given in Table 3. Neither strain, TA98 or TA100, exhibited significant (>50%) toxicity when exposed to 0.1 to 10 μ L CPFB per plate. From this data it is assumed that CPFB is not cytotoxic to all five *Salmonella* strains up to a maximum dose of 10 μ L per plate.

Table 4 illustrates the mutagenicity data with tester strains TA1535, TA1537, TA1538, TA98, and TA100. CPFB was not mutagenic when tested at concentrations up to 10 μ L per plate. These data suggest that CPFB is devoid of mutagenic activity (both frameshift and base pair substitution types) in the *Salmonella* assay when tested up to a maximum dose of 10 μ L per plate. Incorporation of S9 metabolic activation had no effect on this result.

TABLE 3. CYTOTOXICITY OF CPFB TO SALMONELLA STRAINS

CPFB (μ L/plate)	Average No. Revertants ^a		% Survival ^b	
	TA98	TA100	TA98	TA100
10.0	18.33	114.00	63%	87%
3.30	18.00	123.33	62%	95%
1.00	15.33	120.00	53%	92%
0.33	22.00	140.33	76%	108%
0.10	25.33	112.00	91%	86%
Media Control	29.00	130.33	100%	100%

^a An average of three counts.

^b Percent of control.

^c There were 2 mL of CPFB-containing top agar per plate. CPFB has a density of 1.568 g/mL. Therefore, 10 μ L CPFB/plate would be equivalent to 7.84 μ g CPFB/mL of agar.

TABLE 4. MUTAGENIC ACTIVITY OF CPFB IN THE AMES ASSAY

CPFB (μ L)/plate ^b	10% S9 ^c	Revertants per plate ^a				
		TA1535	TA1537	TA1538	TA98	TA100
Media Control	-	37	23	21	40	71
0.10	-	22	23	22	31	69
0.33	-	26	15	22	31	88
1.00	-	22	18	26	27	42
3.33	-	25	21	24	39	75
10.00	-	32	18	24	37	73
Media Control	+	24	25	42	55	119
0.10	+	11	40	30	37	93
0.33	+	21	17	31	40	89
1.00	+	13	19	32	40	66
3.33	+	17	21	42	45	62
10.00	+	21	28	46	62	85
Positive Controls						
Sodium azide (3 μ g/plate)	-	<u>204</u>	-	-	-	<u>1069</u>
2-Nitrofluorene (10 μ g/plate)	-	-	<u>112</u>	<u>747</u>	<u>399</u>	-
2-aminoanthracene (10 μ g/plate)	+	<u>293</u>	<u>263</u>	<u>1441</u>	<u>1795</u>	<u>824</u>

^a An average of six plate counts. Those plates indicating a positive mutagenic response are underlined.

^b There were 2 mL of CPFB-containing top agar per plate. CPFB has a density of 1.568 g/mL. Therefore, 10 μ L CPFB/plate would be equivalent to 7.84 μ g CPFB/mL of agar.

^c S9 = metabolic activation mixture

MAMMALIAN CELL MUTATION ASSAY

The cytotoxicity of the CPFB was determined in preliminary CHO mammalian cell assays. Concentrations of CPFB up to 1000 µg/mL produced no obvious cytotoxicity in the cloning efficiency assays with CHO cells. The mutagenicity data for CPFB in the absence of exogenous metabolic activation are presented in Tables 5 and 6. From these data it is obvious that CPFB did not induce mutation at the HGPRT locus in CHO cells. The induced mutation frequency observed at 500 µg CPFB/mL did appear to be reproducible with 5.4- and 4.2-fold increases in the two tests, respectively, compared to control. The mutation induction with the positive control, MNNG, was at the expected value (greater than 500-fold increase compared to control). There were no solubility problems in these assays. The mutagenicity data for CPFB in the presence of exogenous activation (S9) are presented in Tables 7 and 8. The B(a)P concentration produced the expected response in the assay. This concentration was intentionally selected to ensure that the S9 activation system was sufficient to activate a concentration of B(a)P that was known to produce a weak but positive response. There was no positive activity with CPFB in this HGPRT mutation assay with CHO cells, according to the criteria for a positive response as stated in the Materials and Methods Section. Thus, from these data, CPFB appears to be inactive in the HGPRT/CHO Mutation Assay both with and without exogenous metabolic activation.

TABLE 5. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION - EXPERIMENT 1

Concentration (µg/mL)	Average No. Mutants per 100,000 Cells	Relative Survival	Induced Mutation Frequency per Million Cells	Fold Increase over Background
Media Control	1.3	1	13	1
MNNG				
0.5	113.5	0.02	58,121.46	4,470.88
CPFB				
1,000	5.68	1.82	31.19	2.40
500	7.40	1.05	70.61	5.43
250	1.50	1.34	11.16	0.86
125	1.35	1.23	10.94	0.84
62.5	1.00	1.07	9.37	0.72

TABLE 5. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION - EXPERIMENT 2

Concentration (µg/mL)	Average No. Mutants per 100,000 Cells	Relative Survival	Induced Mutation Frequency per Million Cells	Fold Increase over Background
Media Control	0.47	1.00	4.67	1.00
MNNG				
1.0	5.80	0.01	5,355.33	1,146.75
0.5	108.47	0.17	6,259.43	1,340.35
CPFB				
1,000	0.27	1.21	2.20	0.47
500	2.07	1.05	19.61	4.20
250	0.40	1.16	3.44	0.74
125	0.20	1.28	1.56	0.33
62.5	0.67	1.18	5.65	1.21

**TABLE 7. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF
CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION - EXPERIMENT 3**

Concentration ($\mu\text{g/mL}$)	Average No. Mutants per 100,000 Cells	Relative Survival	Induced Mutation Frequency per Million Cells	Fold Increase over Background
Media Control B(a)P	1.40	1.00	14.00	1.00
1.0	2.50	0.93	26.86	1.92
CPFB				
1,000	1.10	0.52	21.26	1.52
500	1.00	0.67	14.85	1.06
250	0.55	0.43	12.86	0.92
125	0.90	0.75	12.07	0.86
62.5	0.80	0.58	13.70	0.98

**TABLE 8. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF
CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION - EXPERIMENT 4**

Concentration ($\mu\text{g/mL}$)	Average No. Mutants per 100,000 Cells	Relative Survival	Induced Mutation Frequency per Million Cells	Fold Increase over Background
Media Control B(a)P	0.40	1.00	4.00	1.00
1.0	2.10	0.89	23.71	5.93
CPFB				
1,000	0.42	0.61	6.92	1.73
500	0.57	0.63	9.03	2.26
250	0.85	0.95	8.90	2.23
125	0.45	0.54	8.30	2.08
62.5	0.16	0.70	2.24	0.56

MAMMALIAN CELL CYTOGENETIC ASSAYS

CPFB induced positive results in four tests using the SCE Assay (Tables 9-12). In all experiments, 1000 μg CPFB/mL induced significant numbers of SCEs, comparable to those induced by the positive control carcinogen DMBA. In the two experiments using exogenous metabolic activation, SCEs were also induced in significant numbers at concentrations of 1000 μg CPFB/mL (Tables 11 and 12). There was no appreciable cell cycle delay at the highest doses of CPFB scored with and without activation. CPFB induced SCEs in a dose-dependent manner with and without activation.

CPFB induced marginally significant numbers of CAb's at a concentration of 1000 $\mu\text{g/mL}$ in two experiments without metabolic activation (Tables 13 and 14). In those experiments using metabolic activation, CPFB again induced marginally significant numbers of CAb's at 1000 and 500 $\mu\text{g/mL}$ in both experiments (Tables 15 and 16); however, the level of significance at 500 $\mu\text{g/mL}$ for one of these experiments (Table 16) was only $p < 0.05$. Nonetheless, a dose-related increase in CAb's was observed in the assays with and without metabolic activation.

TABLE 9. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITHOUT METABOLIC ACTIVATION - EXPERIMENT 1

Concentration (μ g/mL)	Cell Kinetic Index ^a	Total No. Chromosomes Scored	Total No. SCs ^b Scored	Sum of SCEs per Chromosome ^c	Mean No. Chromosomes Per Cell $\pm$ SD	Mean No. SCEs Per Cell $\pm$ SD	Mean No. SCEs per Chromo- some $\pm$ SD ^d
Media Control	2.00	1,074	100	5.02	19.89 $\pm$ 1.12	1.85 $\pm$ 0.65	0.093 $\pm$ 0.042
DMSO (0.2%)	1.98	1,017	89	4.53	19.4 $\pm$ 0.84	1.71 $\pm$ 0.93	0.087 $\pm$ 0.046
DMBA							
1.0	2.03	658	216	10.89	19.9 $\pm$ 1.10	6.55 $\pm$ 3.99	0.330 $\pm$ 0.209****
0.1	2.10	880	264	13.44	19.6 $\pm$ 0.78	5.87 $\pm$ 2.79	0.299 $\pm$ 0.139****
CPFB							
1000	2.02	1,236	333	16.95	19.6 $\pm$ 0.82	5.29 $\pm$ 2.50	0.269 $\pm$ 0.128***
500	2.03	1,489	295	15.07	19.6 $\pm$ 1.04	3.88 $\pm$ 1.82	0.198 $\pm$ 0.092*
250	2.02	1,644	344	17.38	19.8 $\pm$ 0.98	4.14 $\pm$ 1.69	0.209 $\pm$ 0.085*
125	2.00	1,421	261	13.09	19.7 $\pm$ 1.17	3.63 $\pm$ 2.03	0.182 $\pm$ 0.095
62.5	2.00	1,560	188	9.40	20.0 $\pm$ 1.11	2.41 $\pm$ 1.88	0.120 $\pm$ 0.093

^aCell kinetic index = (first division cells + 2 x second division cells + 3 x third division cells) + N, where N equals the number of cells scored (Schneider et al., 1981).

^bApproximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell.

^cMean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored.

^dSignificantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control):

* p < 0.05

** p < 0.01

*** p < 0.005

**** p < 0.001

TABLE 10. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITHOUT METABOLIC ACTIVATION - EXPERIMENT 2

Concentration ($\mu\text{g/mL}$)	Cell Kinetic Index ^a	Total No. Chromosomes Scored	Total No. SCEs Scored	Sum of SCEs per Chromosome ^b	Mean No. Chromosomes Per Cell $\pm$ SD	Mean No. SCEs Per Cell $\pm$ SD	Mean No. SCEs per Chromo- somes $\pm$ SD ^c
None	1.98	1,012	69	3.50	19.8 $\pm$ 1.05	1.35 $\pm$ 0.71	0.069 $\pm$ 0.037
DMSO (0.2%)	1.96	1,011	72	3.58	20.2 $\pm$ 1.22	1.44 $\pm$ 0.70	0.072 $\pm$ 0.035
DMBA							
1.0	1.98	1,006	269	13.67	19.7 $\pm$ 0.95	5.27 $\pm$ 1.73	0.268 $\pm$ 0.088 ^{****d}
0.1	1.98	1,011	210	10.64	19.8 $\pm$ 0.86	4.12 $\pm$ 2.12	0.209 $\pm$ 0.110 [*]
CPFB							
1,000	2.02	1,045	326	16.33	20.1 $\pm$ 1.33	6.27 $\pm$ 2.26	0.314 $\pm$ 0.118 ^{****}
500	1.98	1,037	197	9.91	19.9 $\pm$ 1.32	3.79 $\pm$ 1.31	0.191 $\pm$ 0.066 ^{**}
250	1.98	975	199	10.03	19.9 $\pm$ 0.95	4.06 $\pm$ 1.42	0.204 $\pm$ 0.073 ^{***}
125	2.00	1,044	203	10.09	20.1 $\pm$ 1.30	3.90 $\pm$ 1.67	0.194 $\pm$ 0.081 [*]
62.5	1.93	458	64	3.21	19.9 $\pm$ 1.02	2.78 $\pm$ 0.78	0.139 $\pm$ 0.038

^a Cell kinetic index = (first division cells + 2 x second division cells + 3 x third division cells) $\div$ N, where N equals the number of cells scored (Schneider et al., 1981)^b Approximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell^c Mean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored^d Significantly different from respective controls (DMBA vs DMSO, CPFB vs Media Control):

* p < 0.05

** p < 0.01

*** p < 0.005

**** p < 0.001

TABLE 11. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITH METABOLIC ACTIVATION - EXPERIMENT 3

Concentration ($\mu\text{g/mL}$)	Cell Kinetic Index ^a	Total No. Chromosomes Scored	Total No. SCEs Scored	Sum of SCEs per Chromosome ^b	Mean No. Chromosomes Per Cell $\pm$ SD	Mean No. SCEs Per Cell $\pm$ SD	Mean No. SCEs per Chromo- some $\pm$ SD ^c
None	1.96	1,015	55	2.78	19.9 $\pm$ 0.95	1.08 $\pm$ 0.68	0.054 $\pm$ 0.036
DMSO (0.2%)	1.96	1,023	62	3.10	20.1 $\pm$ 1.19	0.06 $\pm$ 0.05	0.060 $\pm$ 0.025
DMBA							
1.0	1.96	1,011	262	13.24	19.8 $\pm$ 0.98	5.14 $\pm$ 1.67	0.260 $\pm$ 0.084 ^{****d}
0.1	1.96	995	190	9.55	19.9 $\pm$ 0.85	3.80 $\pm$ 2.02	0.191 $\pm$ 0.101 [*]
CPFB							
1,000	2.06	1,051	315	15.70	20.2 $\pm$ 1.36	6.06 $\pm$ 2.17	0.302 $\pm$ 0.112 ^{*****}
500	1.96	1,018	191	9.62	20.0 $\pm$ 1.34	3.75 $\pm$ 1.39	0.189 $\pm$ 0.072 ^{***}
250	1.95	973	205	10.33	19.9 $\pm$ 0.95	4.18 $\pm$ 1.64	0.211 $\pm$ 0.082 ^{***}
125	1.98	1026	196	9.78	20.1 $\pm$ 1.17	3.84 $\pm$ 1.54	0.192 $\pm$ 0.077 ^{***}
62.5	1.98	999	120	6.00	20.0 $\pm$ 1.16	2.40 $\pm$ 0.89	0.120 $\pm$ 0.044

^aCell kinetic index = (first division cells + 2 $\times$ second division cells + 3 $\times$ third division cells) + N, where N equals the number of cells scored (Schneider et al., 1981)^bApproximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell.^cMean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored.^dSignificantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control).

.. p < 0.05

... p < 0.01

.... p < 0.005

..... p < 0.001

TABLE 12. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITH METABOLIC ACTIVATION - EXPERIMENT 4

Concentration (µg/mL)	Cell Kinetic Index ^a	Total No. Chromosomes Scored	Total No. SCEs Scored	Sum of SCEs per Chromosome ^b	Mean No. Chromosomes Per Cell ± SD	Mean No. SCEs Per Cell ± SD	Mean No. SCEs per Chromo- some ± SD ^c
Media Control	1.98	1,035	85	4.27	19.9 ± 1.04	1.63 ± 0.94	0.082 ± 0.047
DMSO (0.2%)	1.96	1,008	91	4.57	20.2 ± 1.25	1.82 ± 1.05	0.091 ± 0.055
DMBA							
1.0	1.96	1,011	266	13.47	19.8 ± 1.00	5.22 ± 1.70	0.264 ± 0.086****
0.1	1.96	989	212	10.77	19.8 ± 0.86	4.24 ± 2.06	0.215 ± 0.106*
CPFB							
1,000	1.98	1,031	275	13.76	20.2 ± 1.35	5.39 ± 2.88	0.270 ± 0.149***
500	1.98	1,019	178	8.98	20.0 ± 1.29	3.49 ± 1.42	0.176 ± 0.074
250	1.98	1,018	186	9.31	20.0 ± 0.91	3.65 ± 1.57	0.182 ± 0.077*
125	2.00	1,041	149	7.44	20.0 ± 1.23	2.87 ± 1.86	0.143 ± 0.091
62.5	1.96	1,003	121	6.06	20.1 ± 1.17	2.42 ± 1.44	0.121 ± 0.073

^a Cell kinetic index = (first division cells + 2 × second division cells + 3 × third division cells) ÷ N, where N equals the number of cells scored (Schneider et al., 1981)

^b Approximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell

^c Mean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored

^d Significantly different from respective controls (DMBA vs DMSO, CPFB vs Media Control):

* p < 0.05
 ** p < 0.01
 *** p < 0.005
 **** p < 0.001

TABLE 13. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITHOUT METABOLIC ACTIVATION - EXPERIMENT 1

Concentration (µg/ml)	Number of Cells Analyzed	% of Cells Showing					
		Chromatid Gaps ^a	Chromatid Aberrations ^b	Chromosome Aberrations ^c	Multiple Aberrations ^d	All Aberrations Excluding Gaps ^e	All Aberrations Including Gaps ^f
Media Control	300	1.3	0	0	0	0	1.3
DMSO (0.2%)	300	5.0	0	1.0	0	1.0	10.0
DMEA							
1.0	94	2.0	23.0	3.0	0	56.0***	58.0
0.1	188	8.0	10.0	6.0	0	16.0***	24.0
CPFB							
1,000	300	4.3	2.0	3.0	0	5.0**	9.3
500	300	3.0	1.0	0.5	0	1.5	4.5
250	300	1.0	1.0	1.0	0	2.0	3.0
125	300	2.0	1.5	0	0	1.5	3.5
62.5	300	1.0	0.5	1.0	0	1.5	2.5

^aGaps and/or iso-gaps

^bBreaks or single fragments or exchange figures or acentric rings or chromatid minutes

^cAcentric fragments or dicentric or translocations or rings or minutes

^dAny combination of identifiable chromatid or chromosome aberrations

^eAll types of chromatid and chromosome-type aberrations excluding chromatid gaps

^fAll types of chromatid and chromosome-type aberrations including chromatid gaps

^gSignificantly different from respective controls (DMSO vs DMSO, CPFB vs Media Control):

* p<0.05

** p<0.01

*** p<0.001

TABLE 14. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITHOUT METABOLIC ACTIVATION - EXPERIMENT 2

Concentration (µg/mL)	Number of Cells Analyzed	% of Cells Showing					
		Chromatid Gaps ^a	Chromatid Aberrations ^b	Chromosome Aberrations ^c	Multiple Aberrations ^d	All Aberrations Excluding Gaps ^e	All Aberrations Including Gaps ^f
Media Control	300	0	0	1	0	1	1
DMSO (0.2%)	300	2	0	0	0	0	2
DMBA							
1.0	300	4.0	12.0	11.0	0	23.0***	27.0
0.1	300	6.0	6.0	4.0	0	10.0**	16.0
CPFB							
1,000	300	3.6	1.6	4.0	0	5.6**	9.2
500	300	3.3	0.6	2.6	0	3.2	6.5
250	300	2.3	0.6	1.3	0	1.9	4.2
125	300	0.5	0.5	0.5	0	1.0	1.5
62.5	300	1.0	0	0.5	0	0.5	1.5

^aGaps and/or iso-gaps

^bBreaks or single fragments or exchange figures or acentric rings on chromatid minutes.

^cAcentric fragments or dicentric or translocations or rings or minutes.

^dAny combination of identifiable chromatid or chromosome aberrations.

^eAll types of chromatid and chromosome-type aberrations excluding chromatid gaps.

^fAll types of chromatid and chromosome-type aberrations including chromatid gaps.

^gSignificantly different from respective controls (DMBA vs DMSO, CPFB vs Media Control):

* p<0.05

** p<0.01

*** p<0.001

TABLE 15. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITH METABOLIC ACTIVATION - EXPERIMENT 3

Concentration (µg/ml)	Number of Cells Analyzed	% of Cells Showing					
		Chromatid Gaps ^a	Chromatid Aberrations ^b	Chromosome Aberrations ^c	Multiple Aberrations ^d	All Aberrations Excluding Gaps ^e	All Aberrations Including Gaps ^f
Media Control	300	1.0	0	0	0	0	1.0
DMSO (0.2%)	200	1.5	0	0	0	0	1.5
DMBA							
1.0	200	11.5	20.0	33.0	0	53.0*** ^g	64.5
0.1	85	18.0	10.0	19.0	0	29.0**	47.0
CPFB							
1,000	300	5.3	1.3	3.3	0	4.6**	9.9
500	300	6.0	2.3	1.0	0	3.3**	9.3
250	300	3.0	0.6	1.0	0	1.6	4.6
125	300	2.0	0	1.5	0	1.5	3.5
62.5	300	1.0	0	1.0	0	1.0	2.0

^aGaps and/or iso-gaps

^bBreaks or single fragments or exchange figures or acentric rings on chromatid minutes

^cAcentric fragments or dicentric or translocations or rings or minutes

^dAny combination of identifiable chromatid or chromosome aberrations

^eAll types of chromatid and chromosome type aberrations excluding chromatid gaps

^fAll types of chromatid and chromosome type aberrations including chromatid gaps

^gSignificantly different from respective controls (DMBA vs DMSO, CPFB vs Media Control):

* p<0.05

** p<0.01

*** p<0.001

TABLE 16. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITH METABOLIC ACTIVATION - EXPERIMENT 4

Concentration (µg/mL)	Number of Cells Analyzed	% of Cells Showing					
		Chromatid Gaps ^a	Chromatid Aberrations ^b	Chromosome Aberrations ^c	Multiple Aberrations ^d	All Aberrations Excluding Gaps ^e	All Aberrations Including Gaps ^f
Media Control	200	0.5	0	0	0	0	0.5
DMSO (0.2%)	300	1.5	0	0	0	0	1.5
DMBA							
1.0	300	16.0	13.0	19.0	0	32.0*** ^g	48.0
0.1	200	7.0	8.0	17.0	0	25.0**	32.0
CPFB							
1,000	200	5.0	1.5	3.0	0	4.5**	9.5
500	300	3.0	1.0	1.3	0	2.3*	5.3
250	300	2.0	1.3	1.0	0	2.3*	4.3
125	300	1.5	1.0	0	0	1.0	2.5
62.5	300	0	0	0.4	0	0.4	0.4

^aGaps and/or iso-gaps

^bBreaks or single fragments or exchange figures or acentric rings on chromatid minutes

^cAcentric fragments or dicentric or translocations or rings or minutes

^dAny combination of identifiable chromatid or chromosome aberrations

^eAll types of chromatid and chromosome type aberrations excluding chromatid gaps

^fAll types of chromatid and chromosome type aberrations including chromatid gaps

^gSignificantly different from respective controls (DMBA vs DMSO, CPFB vs Media Control):

* p<0.05

** p<0.01

*** p<0.001

PRIMARY RAT HEPATOCYTE UDS ASSAY

The cytotoxic effects of CPFB were determined by trypan blue exclusion following treatment. The cells were exposed to concentrations of CPFB ranging from 62.5 to 1000 µg/mL. Viability of the primary rat liver cells was above 60% for all doses used. The data from the first experiment showed no significant evidence of unscheduled DNA synthesis (DNA repair from damage) at any dose of CPFB (Table 17). The net number of grains per nucleus, analyzed in cells of the solvent and media control groups, was typically a negative number (McQueen and Williams, 1985). The values of the positive controls were significantly higher than solvent controls. In cells exposed to the highest concentration of CPFB (1000 µg/mL), there were slight but not significant increases in UDS. Also, at such a high concentration, osmolality effects, pH effects, and other artifacts could cause DNA damage. The second experiment (Table 18) confirmed the results of the first experiment. These results show that concentrations of CPFB up to 1000 µg/mL produce no significant DNA damage as detected by unscheduled DNA synthesis in primary rat hepatocytes.

**TABLE 17. EFFECT OF CPFB ON PRIMARY RAT HEPATOCYTE
UNSCHEDULED
DNA SYNTHESIS - EXPERIMENT 1**

Concentration (µg/mL)	Net Grains Per Nucleus ^a	
	Mean	Standard Deviation
Media Control	-0.24	0.76
DMSO (0.2%)	-0.30	1.04
4NQO		
1.0	9.64****	4.62
0.1	1.62***	2.15
CPFB		
1000	0.16	1.03
500	-0.30	0.81
250	-0.32	1.05
125	-0.10	1.35
62.5	-0.20	1.13

^aAverage of 50 cells per group. Net grains/nucleus = grains/nucleus - grains/cytoplasm (equal area).

^bSignificantly different from DMSO controls at p<0.001.

TABLE 18. EFFECT OF CPFB ON PRIMARY RAT HEPATOCYTE
UNSCHEDULED
DNA SYNTHESIS - EXPERIMENT 2

Concentration ($\mu\text{g/mL}$)	Net Grains Per Nucleus ^a	
	Mean	Standard Deviation
Media Control	-0.54	1.28
DMSO (0.2%)	-0.14	0.85
4NQO		
1.0	4.68****	3.64
0.1	0.58***	1.43
CPFB		
1000	0.10	1.59
500	-0.30	1.37
250	-0.24	1.52
125	-0.14	1.36
62.5	-0.16	1.75

^aAverage of 50 cells per group. Net grains/nucleus = grains/nucleus - grains/cytoplasm (equal area)

***Significantly different from DMSO controls at $p < 0.001$

BALB/C-3T3 TRANSFORMATION ASSAY

The cytotoxicity of CPFB using Balb/c-3T3 cells was determined by relative cloning efficiency. The cells were exposed to concentrations of CPFB from 0.1 to 1000 $\mu\text{g/mL}$. Transformation assays were also performed using this concentration range. The transformation data from the first experiment are presented in Table 19. CPFB was tested without the addition of metabolic activation and the experiment was performed in 60-mm glass dishes. The induction of the transformed phenotype with B(a)P in this experiment was somewhat lower than expected (Arce et al., 1987). In this first experiment, the transformation frequencies and rates for 1000 $\mu\text{g/mL}$ CPFB were about half of those determined for 0.25 μg B(a)P; comparable frequencies and rates were observed when compared with 0.1 $\mu\text{g/mL}$ B(a)P.

There were three technical difficulties associated with the use of glass dishes in this assay: (1) the cells tended not to attach to the glass dishes as well as the plastic dishes, (2) the loosely attached cells tended to lift off the dish during refeeding, and (3) the dishes were cumbersome and awkward to handle. These problems, coupled with the lower than expected response from the positive control, prompted a reexamination of the transformation frequency on plastic dishes. The transformation data for CPFB without metabolic activation performed on plastic dishes are shown in Table 20. The B(a)P concentrations tested induced the expected response in the assay. In this second experiment, 1000 μg CPFB/mL induced transformation frequencies and rates which were about one-third of those observed for 0.25 μg B(a)P/mL. Again, these CPFB-induced frequencies and rates were comparable to those observed for 0.1 μg B(a)P/mL. Thus the use of the plastic dishes did not appear to modulate the response of either B(a)P or CPFB in the transformation assay.

TABLE 19. EFFECT OF CPFB ON THE TRANSFORMATION OF BALB/C-3T3 CELLS WITHOUT METABOLIC ACTIVATION - EXPERIMENT 1^a

Concentration ($\mu\text{g/mL}$)	Number of Dishes	Number of Type III Foci	Number of Type III Foci/Dish	Estimated Transformation Frequency ^b (Transformants per Cell Plated)	Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by:	
					Mean Method ^c	P ₀ Method ^d
Media Control	9	0	0	3.67×10^{-6}	9.8×10^{-8}	4.08×10^{-8}
DMSO (0.2%)	6	0	0	5.71×10^{-6}	1.6×10^{-7}	6.32×10^{-8}
B(a)P 0.25	9	7	0.78	5.78×10^{-5}	2.6×10^{-7}	5.21×10^{-7}
0.10	9	5	0.56	2.61×10^{-5}	2.2×10^{-7}	2.81×10^{-7}
CPFB 1,000	8	4	0.50	2.69×10^{-5}	2.1×10^{-7}	2.40×10^{-7}
100	6	2	0.30	1.23×10^{-5}	1.9×10^{-7}	1.41×10^{-7}
10	6	1	0.16	5.91×10^{-6}	1.6×10^{-7}	6.32×10^{-8}
1.0	8	0	0	4.33×10^{-6}	1.1×10^{-7}	4.63×10^{-8}
0.1	8	0	0	$<1.00 \times 10^{-7}$	1.1×10^{-7}	4.63×10^{-8}

^aExperiment was performed on 60-mm glass dishes.

^bTransformation frequencies correlated for relative survival and calculated as described by Reznikoff et al. (1973)

^cTransformation rate calculated by the mean method according to Capizzi and Jameson (1973)

^dTransformation rate calculated by the P₀ method using the Luria-Delbruck fluctuation analysis (1943)

TABLE 20. EFFECT OF CPFB ON THE TRANSFORMATION OF BALB/C-3T3 CELLS WITHOUT METABOLIC ACTIVATION - EXPERIMENT 2^a

Concentration ($\mu\text{g/mL}$)	Number of Dishes	Number of Type III Foci		Number of Type III Foci/Dish	Estimated Transformation Frequency ^b (Transformants per Cell Plated)	Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by:	
		Type III Foci	Type III Foci			Mean Method ^c	P ₀ Method ^d
Media Control	9	0	0	0	3.67×10^{-6}	9.8×10^{-8}	4.08×10^{-8}
DMSO (0.2%)	10	0	0	0	3.36×10^{-6}	8.8×10^{-8}	3.65×10^{-8}
B(a)P 0.25	9	12	12	1.33	9.85×10^{-5}	3.6×10^{-7}	7.62×10^{-7}
0.10	11	9	9	0.82	3.82×10^{-5}	2.4×10^{-7}	4.50×10^{-7}
CPFB 1,000	10	6	6	0.60	3.23×10^{-5}	1.5×10^{-7}	3.18×10^{-7}
500	12	8	8	0.67	2.75×10^{-5}	2.1×10^{-7}	3.81×10^{-7}
250	12	2	2	0.17	5.91×10^{-6}	9.7×10^{-8}	6.32×10^{-8}
125	10	2	2	0.20	6.92×10^{-6}	1.2×10^{-7}	7.73×10^{-8}
62.5	12	1	1	0.08	2.76×10^{-6}	7.3×10^{-8}	3.02×10^{-8}

^aExperiment was performed on 60-mm plastic dishes.^bTransformation frequencies correlated for relative survival and calculated as described by Reznikoff et al. (1973).^cTransformation rate calculated by the mean method according to Capizzi and Jameson (1973).^dTransformation rate calculated by the P₀ method using the Luria-Delbruck fluctuation analysis (1943).

BALB/c-3T3 cells are capable of providing metabolic activation required to induce transformation by a diverse group of carcinogens (Cortesi et al., 1983). Nonetheless, CPFB was tested in this system in the presence of metabolic activation (S9 fraction). The transformation data for CPFB in the presence of exogenous activation are presented in Tables 21 and 22. In these experiments, exogenous activation did not appear to have an effect on the induction of transformation by B(a)P. However, the transformation frequencies (Reznikoff, 1973) and rates induced by 1000 µg CPFB/mL were comparable to or exceeded the frequencies and rates induced by 0.25 µg B(a)P/mL. Using the P_0 method, a fluctuation analysis test (Luria and Delbruck, 1943), the calculated transformation rates were the same for 1000 µg CPFB/mL and 0.25 µg B(a)P/mL. At 500 µg CPFB/mL, the transformation frequency and rate determined by the Mean Method (Capizzi and Jameson, 1973) were slightly less than that observed for 0.1 µg B(a)P/mL, while the transformation rate determined by the P_0 method was higher. Results of the second experiment using metabolic activation were similar to those observed in the first experiment. In all transformation experiments, regardless of the methods employed to calculate frequencies or rates, a dose-dependent response induced by CPFB was observed, indicating biological activity in this system. Our results suggest that a possible enhanced transformation rate can be observed in BALB/c-3T3 cells with metabolic activation of CPFB.

DISCUSSION

In these studies the genotoxic potential of CPFB was determined using six standard *in vitro* assays: the Salmonella Mutation Assay or Ames Assay, the CHO Mutation Assay, the CHO CAb Assay, the CHO SCE Assay, the Rat Hepatocyte UDS Assay, and the BALB/c Transformation Assay.

Plastic dishes are generally used in these assays because their surface properties allow cells to attach and grow well. However, in this study, it was necessary to use glass dishes to examine the genotoxic effects of CPFB, because this chemical dissolved standard plastic tissue culture dishes, possibly producing erroneous test results. Because there was no methodology to handle compounds that degrade plastic, substantial efforts were expended to adapt the assays to incorporate the use of a glass substratum for cell attachment and growth. These efforts will facilitate the proper examination of other compounds with similar properties.

CPFB did not induce mutations in the DNA of *Salmonella typhimurium* as measured by the Ames assay. The rationale for using a bacterial system for genetic damage was based on the premise that the chemical reactivity and stability of the DNA between prokaryotic and eukaryotic cells are essentially similar. Liver fractions containing metabolizing enzymes have been used in an attempt to produce metabolic products of CPFB and examine the potential genotoxicity of these metabolites.

TABLE 21. EFFECT OF CPFB ON THE TRANSFORMATION OF BALB/C-3T3 CELLS WITH METABOLIC ACTIVATION - EXPERIMENT 3^a

Concentration ($\mu\text{g/mL}$)	Number of Dishes	Number of Type III Foci	Number of Type III Foci/Dish	Estimated Transformation Frequency ^b (Transformants per Cell Plated)	Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by:	
					Mean Method ^c	P ₀ Method ^d
Media Control	12	0	0	2.67×10^{-6}	7.3×10^{-8}	3.02×10^{-8}
DMSO (0.2%)	8	0	0	4.20×10^{-6}	1.1×10^{-7}	4.63×10^{-8}
B(a)P 0.25	8	9	1.13	8.37×10^{-5}	3.4×10^{-7}	7.21×10^{-7}
0.12	7	6	0.86	4.01×10^{-5}	3.0×10^{-7}	4.34×10^{-7}
CPFB 1,000	8	13	1.63	8.78×10^{-5}	4.2×10^{-7}	7.21×10^{-7}
500	8	6	0.75	3.08×10^{-5}	2.6×10^{-7}	4.81×10^{-7}
250	10	0	0	3.48×10^{-6}	8.8×10^{-8}	3.65×10^{-8}
125	6	0	0	5.77×10^{-6}	1.5×10^{-7}	6.32×10^{-8}
62.5	7	0	0	4.93×10^{-6}	1.3×10^{-7}	5.34×10^{-8}

^aExperiment was performed on 60-mm plastic dishes.

^bTransformation frequencies correlated for relative survival and calculated as described by Reznikoff et al. (1973).

^cTransformation rate calculated by the mean method according to Capizzi and Jameson (1973).

^dTransformation rate calculated by the P₀ method using the Luria-Delbruck fluctuation analysis (1943).

TABLE 22. EFFECT OF CPFEB ON THE TRANSFORMATION OF BALB/C-3T3 CELLS WITH METABOLIC ACTIVATION - EXPERIMENT 4^a

Concentration ($\mu\text{g/mL}$)	Number of Dishes	Number of Type III Foci	Number of Type III Foci/Dish	Estimated Transformation Frequency ^b (Transformants per Cell Plated)	Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by:	
					Mean Method ^c	P ₀ Method ^d
Media Control	12	0	0	2.67×10^{-6}	7.3×10^{-8}	3.02×10^{-8}
DMSO (0.2%)	11	0	0	3.05×10^{-6}	8.0×10^{-8}	3.30×10^{-8}
B(a)P 0.25	12	13	1.08	8.00×10^{-5}	2.8×10^{-7}	6.21×10^{-7}
0.12	11	8	0.73	3.40×10^{-5}	2.3×10^{-7}	3.51×10^{-7}
CPFB 1,000	11	12	1.09	5.87×10^{-5}	2.9×10^{-7}	8.61×10^{-7}
500	12	7	0.58	2.38×10^{-5}	1.9×10^{-7}	3.03×10^{-7}
250	12	6	0.50	1.74×10^{-5}	1.7×10^{-7}	2.40×10^{-7}
125	12	4	0.33	1.14×10^{-5}	1.4×10^{-7}	1.41×10^{-7}
62.5	12	0	0	2.88×10^{-6}	7.3×10^{-8}	3.02×10^{-8}

^aExperiment was performed on 60-mm plastic dishes^bTransformation frequencies correlated for relative survival and calculated as described by Reznikoff et al (1973).^cTransformation rate calculated by the mean method according to Capizzi and Jameson (1973).^dTransformation rate calculated by the P₀ method using the Luria-Delbruck fluctuation analysis (1943)

CPFB did not induce significant numbers of mutations in the HGPRT locus in CHO cells. While the response at 500 μg CPFB was 4 to 5 times background (Tables 5 and 6), this is not sufficient to be considered a positive response; 5- to 6-fold increases are normally required before a result is considered positive, and at least two concentrations must show this response. The CHO/HGPRT Assay measures the ability of a test chemical to induce forward mutations at the HGPRT enzyme locus of CHO cells. Mutants at the HGPRT locus are unable to convert purine analogues such as 6-TG to the phosphorylated metabolites and will therefore survive in medium containing 6-TG, whereas the nonmutant cells incorporate the phosphorylated 6-TG into the DNA and cease to replicate. The assay was performed both with and without exogenous metabolic activation (Aroclor 1254-induced rat liver microsomal-S9 fraction) to permit detection of direct-acting chemicals as well as chemicals which may require activation, respectively. The results with the HGPRT/CHO Mutation Assay and the solubility experiments presented here suggest that the previous observations (Tu et al., 1986) of possible mutagenic activity with CPFB at concentrations of 50 and 250 $\mu\text{g/mL}$ using DMSO as the solvent were perhaps due to the interaction of CPFB, DMSO, and the plastic dishes used for treatment.

Data from the CHO SCE Assays strongly suggest that CPFB may be genotoxic to mammalian cells. Sister chromatid exchange results from the breakage and exchange of DNA from sister chromatids within the same chromosome. This assay is very sensitive and detects damage that is likely not seen in other assays. At the highest concentration of CPFB (1000 $\mu\text{g/mL}$) in two independent assays, one with and one without metabolic activation, highly significant ($p < .005$) increases in the mean number of SCEs per chromosome (compared to controls) were observed. These high mean values were comparable to those of the positive control compound, DMBA. In the other two assays, marginally significant ($.005 < p < .05$) increases were seen only at the top two CPFB concentrations. Another indication that CPFB is positive is the trend toward higher mean SCE values with higher CPFB concentrations. This positive trend is usually a good indication of a genotoxic chemical, and the fact that it was seen in all four assays serves to strengthen the interpretation. However, such high concentrations may not be realistic in whole animals.

CPFB induced marginally significant ($.001 < p < .01$) increases in CHO CAs (excluding gaps) in all four assays, both with and without metabolic activation. These marginally significant results were 4- to 11-fold less than the values obtained by exposing cells to 1.0 μg DMBA/mL (the highest dose of the positive control compound). However, a positive trend of increasing CAs with increasing exposure concentrations suggests that some effect is present, but that it may be marginal. The ability of a given chemical to induce CAs can be considered strongly suggestive of its ability to produce DNA lesions that could lead to serious chronic health effects.

In the Primary Rat Hepatocyte UDS Assay, CPFB did not produce significant increases in the repair of DNA damage. Among the techniques for monitoring DNA damage, DNA repair is of particular value because it is a specific response to covalent binding to DNA and is not mimicked by any other toxic effect to the cell. For the measurement of DNA repair, primary hepatocytes are advantageous in that they possess a substantial intrinsic capacity for xenobiotic transformation and, because they are nonproliferative in primary culture, inhibition of replicative DNA synthesis is not required to measure DNA repair. Previous results of UDS assays on 73 carcinogenic and 13 noncarcinogenic compounds showed that 96% of known carcinogens were positive (3 carcinogens were false negatives [aniline, carbon tetrachloride, and diphenylnitrosamine]) and all 13 noncarcinogens were negative (Williams, 1981).

CPFB exposure increased the transformation frequency of BALB/c-3T3 cells. The BALB/C-3T3 transformation assay permits the evaluation of the cancer-causing properties of test chemicals. The phenotypic appearance of morphologically altered cells following exposure correlates with the subsequent ability of these cells to produce tumors in syngeneic hosts. In the two assays without metabolic activation the transformation frequencies were lower than the positive control compounds (B[a]P). However, with metabolic activation present, the transformation frequencies induced by the highest CPFB concentration exceeded those of the positive control compound at the highest concentration. In all four transformation assays a dose-dependent increase in the transformation frequency was observed, strongly suggestive of the capability of CPFB to transform cells.

A summary table of all data is included (Table 23), and these results indicate that CPFB may have significant genotoxic activity in the SCE and CAb tests, especially at the highest concentration. In the BALB/c assay the CPFB gave weakly positive results without metabolic activation, although with metabolic activation positive transformation rates and frequencies were observed at higher CPFB concentrations. Positive results were observed in these assays at concentrations higher than those previously tested (concentrations of up to 300 µg/mL) (Tu et al., 1986). CPFB did not show genotoxic activity in the Ames Assay, the CHO Mutation Assay, or the Primary Rat Hepatocyte Assay. These assays detect point mutations and DNA damage repair and cover the major forms of DNA damage attributable to genotoxic chemicals. In some tests, data from the 1000 µg/mL and sometimes the 500 µg/mL concentration groups suggested significant results. These positive findings indicate probable genotoxicity at very high doses, however artifacts at such high concentrations would have to be considered in any interpretation. For instance, high osmolality and pH can induce SCEs in human lymphocytes (Morgan and Crossen, 1981; Littlefield et al., 1981). It is unlikely that such high concentrations would ever be encountered in the human situation.

TABLE 23. SUMMARY OF RESULTS

Assay	-Sg ^a		+ Sg ^b	
	Test ^c		Test ^c	
	1	2	3	4
Gene Mutation Assays				
Salmonella Mutagenicity Assay	-	-	-	-
Mammalian Cell Mutation Assay	-	-	-	-
Mammalian Cell Cytogenetic Assays				
Sister Chromatid Exchange Assay	+w	+	+	+w
Chromosome Aberration Assay	+w	+w	+w	+w
Primary Rat Hepatocyte UDS Assay	-	-		
BALB/c-3T3 Transformation Assay	+w	+w	+	+

^a Without metabolic activation

^b With metabolic activation

^c + = positive

+w = weakly positive

- = negative

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QUALITY ASSURANCE UNIT CERTIFICATION

This report accurately describes the methods and standard operating procedures used in the study, and the reported results accurately reflect the raw data of the study. Study inspections are specified below.

<u>Date</u>	<u>Assay</u>	<u>Phase</u>
10/27/86	Balb/c-3T3 Transformation	Plating
10/28/86	Balb/c-3T3 Transformation	Chemical Treatment
11/13/86	CHO Cell Mutation	Chemical Treatment
11/17/86	CHO Cell Mutation	Refeed/Passage
11/18/86	Sister Chromatid Exchange	Chemical Treatment
11/18/86	Chromosome Aberration	Plating
11/24/86	Sister Chromatid Exchange	Plating
11/24/86	CHO Cell Mutation	Refeed/Passage
11/25/86	Sister Chromatid Exchange	BuDr Treatment
11/26/86	Chromosome Aberration	Chemical Treatment
11/26/86	CHO Cell Mutation	CFE Seed/6-TG Selection
12/01/86	Biological Activity of CPFB	Study Records
01/12/87	Biological Activity of CPFB	Study Records
04/02/87	Biological Activity of CPFB	Study Records
04/29/87	Biological Activity of CPFB	Study Records
04/29/87	Biological Activity of CPFB	Final Report


B. Michael Ray
Quality Assurance Unit Coordinator

6-25-87
Date