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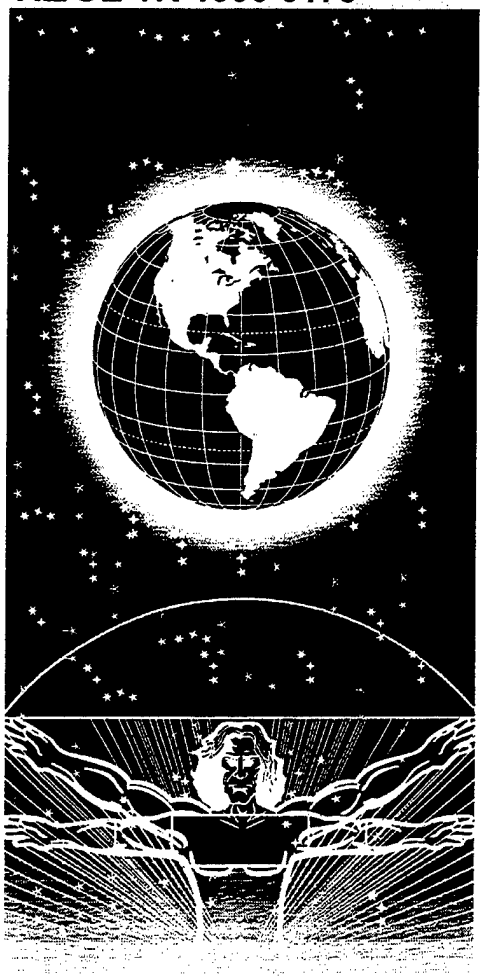

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

A MULTIWELL FLUORESCENCE METHOD FOR GLUTATHIONE DETERMINATIONS IN PRIMARY HEPATOCYTES USING MONOCHLOROBIMANE

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

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.

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FOR THE DIRECTOR

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The fluorophore monochlorobimane (MCB) has been shown to have a relatively high specificity for GSH. Using a fluorescence plate reader with experimental filters, a 380 nm EX filter paired with either a 490 nm or 485 nm EM filter yielded the highest MCB signal-to-noise ratio. The level of GSH in primary hepatocytes was approximately 2-fold higher than that measured in immortalized WB344 rat hepatocytes. The miniumum cell number required for measurements of MCB-GSH associated fluorescence was found to be 3 x 10 4. The in vitro **dose response study with trimer and tetramer chlorotrifluoroethylene (CTFE) oligomer acids correlated will with previous** in vivo **toxicity studies. Noncytotoxic exposure of hepatocytes to the tetramer CTFE oligomer acid resulted in decreased cellular GSH levels, while an equivalent dose of the trimer CTFE oligomer acid resulted in elevated cellular GSH levels. The results of this study indicated that intracellular GSH status and fatty acid chain lenght may play roles in determining the extent of cytotoxicity induced by halogenated fatty acids. Furthermore, it was also found that the newly developed excitation iand emission filters could be used to measur intracellular GSH levels using a multiwell plate reader format.**

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PREFACE

This report represents research performed by the Biochemical Toxicology Branch, Toxicology Division, Armstrong Laboratory, from January 1992 to August 1993. The research was performed in support of Project 6302, *"Occupational and Environmental Toxic Hazards in Air Force Operations,"* Task 630201, *"Toxicology of Aerospace Chemicals and Materials,"* Workunit 63020174, *"Toxicological Screening of Air Force Chemicals."* This Project later became 7757, the Task, 7757A1, and the Workunit became 7757A105.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Glutathione (GSH) is the most ubiquitous nonprotein sulfhydryl in cells and is responsible for maintaining cellular oxidation-reduction homeostasis. The involvement of glutathione (GSH) in a number of cell functions such as membrane integrity, cytoskeletal organization, protein and DNA synthesis, and modulation of enzyme activity has been previously established (1). In addition, the role of GSH in protecting cells from a wide variety of endogenous and exogenous insults has also been well documented. Because of its role in determining the cellular response to potentially toxic chemicals, various methods have been developed to assess GSH status in cells. One approach was to adapt the method of Hissin and Hilf (2) for GSH determinations in tissue homogenates, utilizing the fluorescent reagent O-phthaldehyde (OPT), to determinations in isolated cells (3). Fluorescent measurements of cellular GSH have also been made by HPLC (4). Other approaches included the use of the colorometric reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in an enzymatic recycling method (5) and various modifications of this method (6-9). However, many of these assays are tedious and involve one or more of the following: multiple steps, expensive reagents, derivitization, cell lysis, protein precipitation and GSH extractions. The need to analyze GSH in individual cells by flow cytometry led to the development of a method utilizing monochlorobimane (syn-($ClCH_2CH_3$)-1,5-diazabicyclo-[3.3.0]-octa-3,6-dione-

2,8-dione; 10-11). Recently, a spectrofluorometric method for GSH analysis in intact cells utilizing MCB has been developed (12).

Monochlorobimane is a fluorophore that is excited at a wavelength of 380 nm and emits fluorescence at 480 nm when enzymatically conjugated by glutathione S-transferase to reduced GSH. The use of MCB as a fluorescent reagent for analysis of cellular thiol was first described by Kosower et al. (13). This bimane derivative is nonfluorescent and becomes highly fluorescent when reacted with GSH. Previous work using flow cytometry has shown MCB to be highly specific for measuring GSH with very little reaction with protein sulfhydryls (10-11). Recently, two new filters (a 380 nm/20 nm bandpass excitation and 490 nm/40 nm bandpass emission) have been developed for use in the CytoFluor 2300™ multiwell fluorescence plate reader (Millipore, Bedford, MA). In the present study, we evaluated these filters for GSH analysis in primary hepatocytes and in an immortalized hepatocyte cell line utilizing the CytoFluor 2300.

We also compared the effects of two chlorotrifluoroethylene (CTFE) oligomer acids (Fig 1) at noncytotoxic doses on GSH homeostasis in primary hepatocytes using the MCB multiwell plate reader assay. The CTFE oligomer acids used in this study are liver metabolites of a novel jet aircraft hydraulic fluid (14). The hydraulic fluid oil (17) and the tetramer CTFE oligomer acid (18) have been previously shown to induce peroxisomal ß-oxidation activity and to be hepatotoxic in rats. Induction of peroxisomal ß-oxidation activity in rodent liver cells results in the production, and subsequent metabolism, of H_2O_2 and other O_2 metabolites that may lead to free radical DNA and cell membrane damage. Reduced GSH has been shown to play a role in free radical scavenging reactions (15). Furthermore, it has

been shown that fatty acids can mediate effects on GSH redox cycle in cultured cells (16). A previous *in vivo* study of halogenated CTFE oligomer acids showed that both the C_6 (trimer) and C_8 (tetramer) oligomer acids induced peroxisomal ß-oxidation (18) and therefore may exert some effect on cellular GSH levels. This study also showed that the tetramer CTFE oligomer acid was more cytotoxic than the trimer CTFE oligomer acid. Because GSH is present in many cell types and is involved in a multitude of biological functions, a sensitive GSH assay for cultured cells using a multiwell fluorescence plate reader would allow for the rapid screening of chemicals for their effects on cellular GSH levels.

MATERIALS AND METHODS

Materials

The 380 nm excitation and 490 nm emission filters were graciously provided by the Millipore Co. (Bedford, MA). The trimer (MW 357.1) and tetramer (MW 474.6) carboxylic acid oligomer of chlorotrifluorethylene (CTFE) were obtained under contract from Tecnolube Co. (Irvine, CA). Monochlorobimane (MCB; MW 226) was purchased from Molecular Probes, Inc. (Eugene OR). Collagenase (Type D, Lot# DHA142) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). All biological reagents, unless otherwise stated were purchased from Sigma Chemical Co. (St. Louis, MO).

GSH-Bimane Fluorescence Measurements

A standard curve was generated with various concentrations of GSH ranging from 1 to 20 μM in 200 μL of calcium-free phosphate buffered saline (CF-PBS; pH 7.2) in round-bottom 96-well Falcon tissue culture plates (Fisher Scientific, Pittsburgh, PA) containing 44 μM MCB. Half of the wells received glutathione S-transferase (GST; 0.1 U/ml) to catalyze the formation of the GSH-bimane adduct. The formation of the GSH-bimane adduct was monitored after 45 min of incubation at 37°C with a Millipore CytoFluor 2300 fluorescence plate reader with a 380 nm excitation/485 nm emission filter set. The standard curve was derived from the background-subtracted average fluorescence units (AFUs) of the 45 min time points from 3 separate runs (n=6/run). The average fluorescence from wells containing only MCB or GSH

served as negative controls and were subtracted from corresponding wells containing GST enzyme. Unknown cellular GSH concentrations were derived from the standard curve.

Animals and Cell Line

Male Fischer 344 rats (225-300 g) were obtained from Charles River Breeding Laboratories (Willmington, MA). Rats were anesthetized with 1 ml/kg of a mixture of ketamine (70 mg/ml; Parke-Davis, Moris Plains, NJ) and xylazine (6 mg/ml; Mobay Corp., Shawnee, KS) prior to undergoing the *in situ* liver perfusion. The WB344 hepatocyte diploid cell line, derived from the Fischer 344 rat (23) were a gift from Dr. T. Kavanagh (U. Washington, Seattle, WA).

Rat Liver Perfusion

Fischer 344 rat livers were perfused, and hepatocytes were isolated and enriched as previously described (20) with the following modifications. Perfusion media (pH 7.2) were supplemented with 15 mM 4-(2-hydroxyethl)-1-piperazineethanesulfonic acid (HEPES). Wash-out perfusion medium was supplemented with heparin (2.0 U/ml) and ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA; 0.5 mM). Digestion perfusion medium was supplemented prior to use with collagenase at 0.26 U/ml (based on Wunsche U/mg).

Hepatocyte Enrichment and Culture

Primary rat hepatocytes were enriched by low speed isodensity Percoll (pH 7.2; Pharmacia, Piscataway, NJ) centrifugation as previously described

(21). Typical viabilities of isolated hepatocytes ranged from 80 to 85 %. For cell culture studies, primary hepatocytes (2 x 10^8 cells) were adjusted to a cell density of 1.0 x 10^6 cell/ml in Williams' E culture medium (pH 7.2; Gibco, Grand Island, NY) supplemented with HEPES (10 mM), sodium bicarbonate (2.2 mg/ml), bovine serum albumin (0.5 mg/ml), 5-aminolevulinic acid (0.1 mg/ml), Sigma insulin/transferrin/sodium selenite solution (10 µg/ml), gentamicin (50 µg/ml), 5% fetal calf serum (FBS; Gibco, Grand Island, NY) and dexamethasone (0.4 mg/ml). After 3 h of incubation in a CO_2 incubator at 37°C to allow for attachment, rat hepatocytes were refed with Williams' E culture medium lacking dexamethasone. For 24 h dose response studies, 1 ml aliquots of cell suspension (1 x 10^6 cells) were seeded onto Falcon (Becton Dickinson, Oxnard, CA) 6-well plates and incubated at 37°C for 24 h prior to exposure. The negative control group in GSH studies were refed with supplemented Williams' E and Williams' E containing 0.2 mM buthionine sulfoximine (BSO; inhibitor of γ-glutamylcysteine synthetase).

S/N Ratio Determinations

The GSH-MCB signal-to-noise (S/N) ratio for the various filter sets tested was determined using primary hepatocyte GSH rather than commercially supplied GSH. Primary hepatocytes were chosen to account for any background fluorescence contributed by the cellular debris that would be present under the experimental conditions described below. Isolated primary hepatocytes were seeded at a cell density of ~1 x 10^6 cells/well in Falcon 6-well tissue culture plates (Fischer Scientific, Pittsburgh, PA) and allowed to attach for 4 h. After 4 h, the culture medium was removed and fresh medium was

added. The plate was then incubated at 37°C for 24 h. After 24 h, the cells were scraped and sonicated in CF-PBS as described below. The GSH-MCB fluorescence of the samples (~1 x 10^5 cells) were measured in the CytoFluor 2300 at an instrument sensitivity of 5 as described below. The MCB/CF-PBS blank was found to contribute more to the background noise. The S/N ratios were determined by dividing the total GSH-MCB associated fluorescence of the cell samples by the combined background fluorescence of the cells (cell blank minus CF-PBS blank) and the MCB/CF-PBS blank. The S/N values were determined from 4 experiments with triplicate samples per experiment.

Exposure

Sterile nylon membrane-filtered (0.2 micron; Nalge Co., Rochester, NY) stock solutions (10 mg/ml) of trimer and tetramer CTFE carboxylic acids were made in dimethylsulfoxide (DMSO). The range of CTFE acid concentrations used in the 24 h dose response study was 0.16 to 2.75 mM. Rat hepatocyte cultures were exposed for 24 h following a 24 h recovery incubation at 37°C.

In GSH studies, the treatment groups (in quadruplicate) consisted of hepatocyte cultures refed with Williams' E medium supplemented with or without BSO after 4 hrs of attachment. After 24 hr of incubation at 37°C, cultures were exposed to the CTFE carboxylic acids at 0.3 mM for and incubated for an additional 24 h at 37°C. Noncytotoxic dosing of hepatocyte cultures with the CTFE acids were initiated by aseptically adding 10 µl of test agent (or DMSO) using a SMI micropipetter (Baxter Health Care Corp., Deerfield, IL). The final concentrations of test agent and DMSO in these cultures were 0.1 mM and 0.5%, respectively.

Sample Preparation

At the termination of 24 h dose response exposures, 0.5 ml of culture medium was removed from each tissue culture plate for lactate dehydrogenase (LDH) enzyme leakage determinations. The medium samples (4°C) were assayed within 24 h after collection. Measurements of LDH activity were made using a DuPont ACA V discrete clinical analyzer (DuPont, Huffman Estates, IL) as previously described (20).

Multiwell Fluorescence Plate Reader GSH Assay

At the end of the 24 hr exposure period, wells were washed 3 times in CF-PBS. The wells were then scraped in 1 ml of CF-PBS. Two 200 µl aliquots of cell suspension from each well were transferred to a Falcon 96-well plate. One set of these aliquots received 10 µl of a 885 µM stock monochlorobimane (MCB)/10% methanol solution (44 µM MCB/0.5% methanol final concentration), the other served as negative controls. Wells containing 200 µl of CF-PBS and MCB served as positive controls and wells containing only CF-PBS served as blank controls. The 96-well plate was then incubated at 37°C for 45 min. Fluorescence of the MCB-GSH conjugate was determined using a CytoFluor 2300 multiwell plate reader with bundle enhancement (Millipore Corp., Bedford, MA) with an excitation wavelength of 360 nm (or 380 nm) and an emission wavelength of 485 nm. The average fluorescence units measured, after background was subtracted, were normalized to cell protein.

Protein Assay

Cell sample protein determinations were assayed according to the method of Bradford (22) using Coomasie brilliant blue G-250 reagent (Pierce, Rockford, IL).

Statistics

The standard curve for MCB-GSH associated fluorescence was generated using the SigmaPlot™ linear regression analysis (Jandel Scientific, San Rafael, CA). Biochemical measurements from each treatment group of were compared by an analysis of variance (ANOVA) using SYSTAT software statistics package (SYSTAT, Inc, Evanston, IL). Means found to be significant by ANOVA were compared with the Tukey post hoc test with Type I error level held at $p < 0.05$. The relationship between cell number and GSH levels were determined by linear regression analysis.

RESULTS and DISCUSSION

Previous flow cytometric data has shown good correlation between MCB fluorescence and cellular GSH content (10,11,23). These studies have demonstrated that MCB, at low concentrations, reacts with GSH to form a highly fluorescent derivative with very little side reaction to other protein sulfhydryls in transformed cells. However, this sensitive and direct method of assessing cellular GSH has only recently been applied to primary intact hepatocytes (12). These researchers were the first to apply and validate this method of cellular GSH determination using fluorescence spectrometry. In the present study, we have adapted the use of MCB as a molecular probe to measure cellular GSH levels in primary cells or established cell lines using a multiwell fluorescence plate reader format.

Determination of Filter Set Fluorescence S/N Ratios

The MCB signal-to-noise (S/N) ratios for the various filter sets tested were derived from the GSH-bimane complex formed in primary Fischer 344 (F-344) hepatocytes (~6.2 x 10^4 cells/well). The instrument's standard 360 nm excitation (EX)/460 nm emission (EM) filter set was inadequate for measuring the MCB-GSH associated fluorescence. The substitution of either newly developed EX or EM filter for the instrument's 360 nm EX or 460 nm EM filter was found to result in detection of the MCB-GSH fluorescence adduct (data not shown). Detection of this fluorescent adduct was greatly enhanced by the use of an experimental 380 nm EX paired with either a 485 or experimental 490 nm EM filter. The 485 nm filter, with a narrower bandpass wavelength (20 nm) than that of the experimental 490 nm EM filter (40 nm), was paired with the experimental 380 nm EX filter and used throughout the remainder of the study. The S/N determined for this filter set at an instrument sensitivity of 5 was found to be 11.0 ±3.4 using ~6.2 x 10^4 cells. These results indicated that the pairing of the experimental 380 nm EX filter with the instrument's 485 nm filter was sufficient to allow the CytoFluor 2300 multiwell plate reader to accurately measure GSH levels of cultured cells.

GSH Standard Curve Using MCB in Multiwell Format

Using 44 µM MCB, a calibration curve was generated from GSH concentrations ranging from 1 to 20 µM (Fig 2). Regression analysis of these data indicated that relative MCB fluorescence responded linearly with

increasing GSH concentration over this range (r^2=0.982). It was observed that when concentrations of GSH exceeded that of MCB (> 44 μM), a hyperbolic curve was generated (data not shown). The addition of excess GST to any given GSH concentration was found to increase the rate of formation of the GSH-bimane adduct over 45 min, but did not result in any effect on the shape of the hyperbolic curve (data not shown). Previous work has shown the GSH derivitization process to be dependent on the amounts of MCB and GSH (10).

MCB Analysis of Hepatocellular GSH

Using the 380 nm EX/485 nm EM filter set, the relationship between cell number and MCB fluorescence was determined. Linear relationships between increasing cell number and relative MCB fluorescence were found at primary hepatocyte (r^2= 0.994) and WB344 cell line (r^2=0.997) cell densities ranging from 1.5 X 10^4 to 1.2 X 10^5 using this filter set (Fig. 3). The amount of GSH in primary hepatocytes, assayed by the MCB fluorescent method, was derived from the GSH standard curve after background subtraction using 6.2 x 10^4 cells/well. Based on the 200 μL sample used, the amount of GSH in the sample was calculated to be 2.9 ±0.49 nmol. When adjusted by a factor 16 to achieve a value per 10^6 cells, a GSH level of 46.4 ±7.8 nmol/10^6 cells was calculated. This level of GSH in control hepatocytes, determined using a multiwell fluorescence plate reader format, correlated very well with that determined previously (12) using MCB in a fluorescence spectrometer assay (43.2 ±7.3 nmol/10^6 cells).

Interestingly, primary rat hepatocytes were found to yield ~2-fold higher average GSH-bimane associated fluorescence at cell concentrations

ranging from ~3.0 x 10^4 to ~1.2 x 10^5 than that obtained at identical WB344 cell concentrations (Fig 3). The higher levels of total GSH measured in primary hepatocytes may be do to a greater mitochondrial GSH pool in these cells as they were found to contain more mitochondria than do the WB344 cells (data not shown). The limit of GSH detection by MCB in both these cell types appeared to be ~3 x 10^4 cells.

The variability of the MCB assay was found to be dramatically increased at the highest cell density tested (~1.2 x 10^5) for both cell types. This variability may be the result of increased cellular debris in the plate reader wells. This is supported by the finding that the variability of GSH measurements of 1.2 X 10^5 cells was greater with primary hepatocytes than with WB344 hepatocytes (Fig 3). Sonicates of the larger and more nucleated primary hepatocyte cultures were more opaque and generally found to contain more membranous material than those of comparable WB344 cells. The increased amount of cellular debris in the multiwell plates may interfere with the instruments ability to detect fluorescence. Another possibility is that incomplete sonication of mitochondria resulted in variability of the total available GSH that could interact with MCB. Taken together, our data indicated that cellular GSH determinations using MCB in a multiwell fluorescence plate reader format could be made accurately under the experimental conditions described above.

Cytotoxicity of CTFE Acids and Effects on GSH Homeostasis

Previous studies have shown the tetramer CTFE oil (17) and its carboxylic acid metabolite (18) to be hepatotoxic in rats while the trimer

CTFE oil and its carboxylic metabolite were not. A dose response study of these CTFE oligomer acids using primary hepatocytes showed that the tetramer CTFE oligomer acid was more cytotoxic than the trimer CTFE oligomer acid (Fig. 4) and correlated very well with the results of the previous *in vivo* studies mentioned above. Both CTFE oligomer fatty acids have also been shown to induce peroxisomal ß-oxidation activity *in vivo* (18). Because induction of this activity leads to the generation of hydroxyl and O_2 free radicals, scavenging of these free radicals by GSH may result in a reduction of cellular GSH levels. Reduced intracellular GSH levels reflects an increased potential for cell injury, not cytotoxicity itself.

It has also been shown that rats dosed with the tetramer CTFE oil experienced dramatic weight loss due to loss of appetite (17). It is well established that GSH levels in the rat liver decreases significantly when the animals are fasted, presumably due to decreased cysteine pools. Because exposure of rats to the tetramer CTFE oligomer oil/acid results in an induced fasting state, it is possible that toxicity of this CTFE oligomer oil/acid is associated with induced alterations in GSH biosynthesis leading to reduced cellular GSH levels. To determine if cellular GSH status may be involved in determining the extent of toxicity with exposure to these CTFE oligomer acids, primary hepatocytes were depleted of GSH by pretreatment with an inhibitor of τ-glutamylcysteine synthetase (BSO) and then subjected to a dose response study using the relatively nonhepatotoxic trimer CTFE oligomer acid. The results from this study showed a dramatic increase in toxicity as indicated by a shift in the dose response curve for this oligomer acid to the left (Fig. 4). This finding strongly suggest that

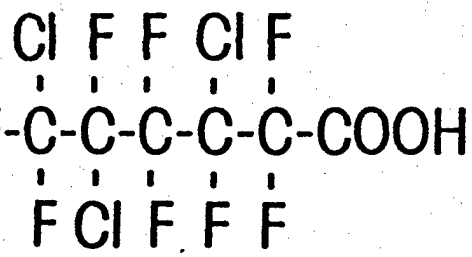
alterations in cellular GSH levels can influence the extent of toxicity induced by CTFE oligomer acids.

Using the fluorophore MCB to assess cellular GSH levels in primary hepatocytes exposed to an apparently noncytotoxic dose these CTFE oligomer acids (0.3 mM), we showed that these acids differentially modulated GSH levels; exposure to the tetramer acid resulted in decreased GSH levels while exposure to the trimer acid resulted in enhanced GSH levels (Fig. 5). Cell viability was found not to be affected by treatment as determined by LDH leakage (data not shown). The decreased or increased levels of GSH in hepatocytes following exposure to these CTFE oligomer acids may result from effects on the cell membrane, effects on the GSH biosynthetic enzymes τ-glutamylcysteine synthetase or GSH synthetase, or alterations in cysteine levels. It appeared that the reduction of intracellular GSH in hepatocytes exposed to the tetramer CTFE oligomer acid is not due to free radical scavenging, as both CTFE acids induce peroxisomal ß-oxidation to the same extent (18).

The mechanism of action of these CTFE oligomer acids, with respect to their effects on GSH homeostasis, is not known. It has previously been shown *in vivo* that the tetramer CTFE oil (17) and its acid metabolite (18) are retained in the liver to a greater extent than the trimer CTFE oligomer. It is possible that the increased concentration of the tetramer CTFE oligomer acid within the cell results in a decrease in intracellular pH and increased binding to proteins that leads to inhibition of proteolytic and GSH biosynthetic enzymes. These effects would result in reduced GSH biosynthesis by decreasing cysteine availability.

The present study has demonstrated the application of MCB for use in a multiwell fluorescence plate reader format for assessing cellular GSH *in vitro*. This specific and sensitive assay for GSH will be of value for screening larger numbers of toxicological and pharmacological agents for their effects on cellular GSH levels. This study also indicated that chemicals can alter GSH homeostasis at apparently noncytotoxic concentrations that increase or decrease the potential for subsequent cell injury.

a.



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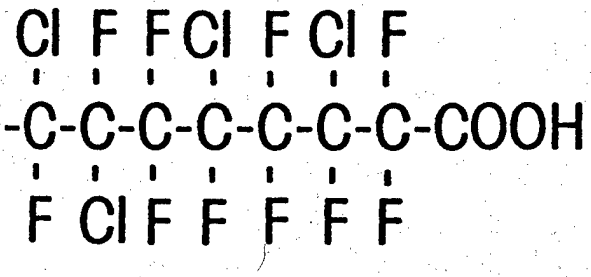

Fig. 1. Chemical structure of a; trimer and b; tetramer CTFE oligomer fatty acids.

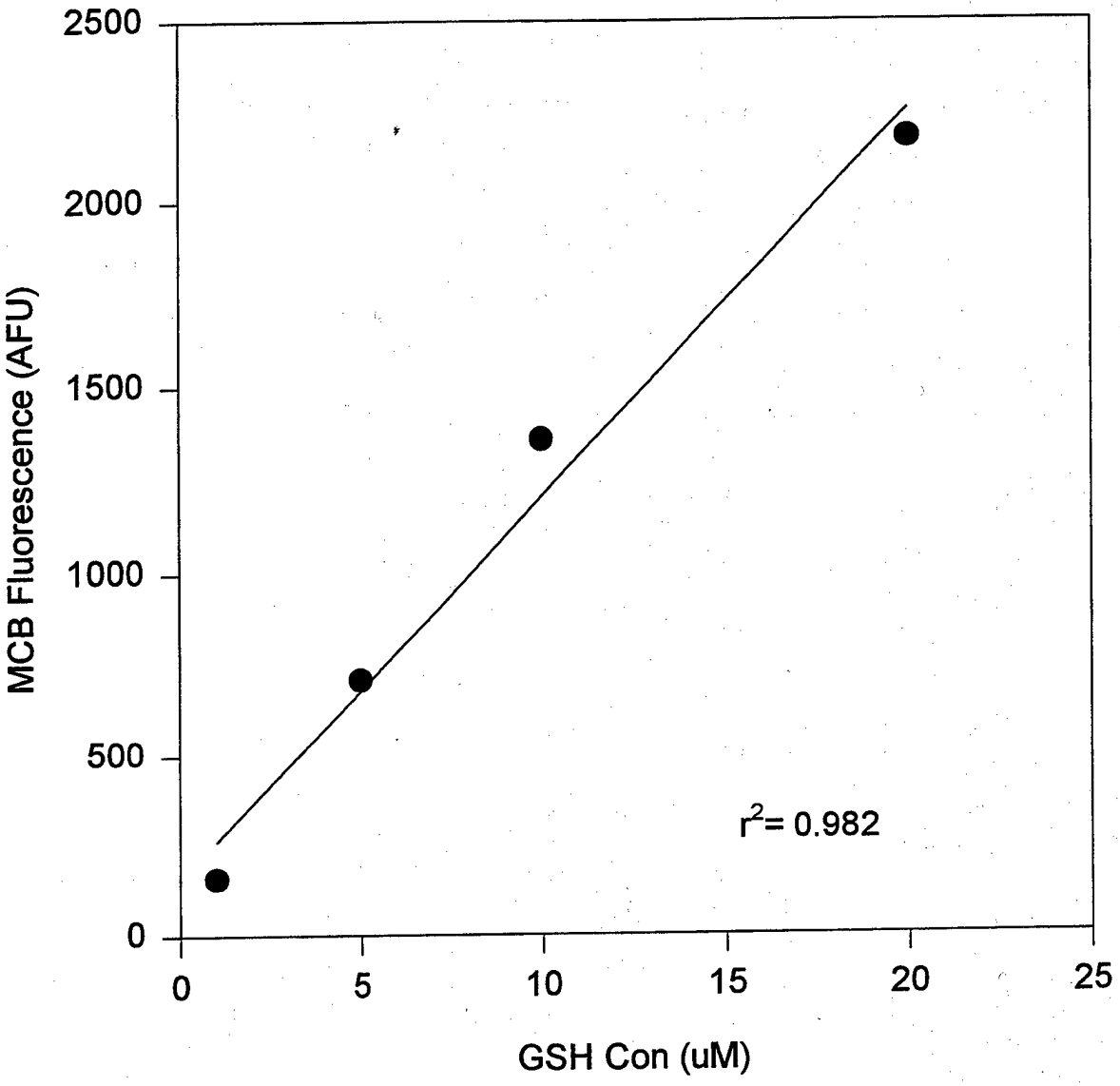

Fig. 2. GSH standard curve generated from fluorescence plate reader measurements of the MCB-GSH conjugate from 1 to 20 μM GSH using 44 μM MCB. The 96-well plate was incubated at 37°C for 45 min prior to reading. Data points reflect the MCB average fluorescence units (AFU) from three experiment (n=6/experiment). Solid line represents a least square regression analysis determined by SigmaPlot™.

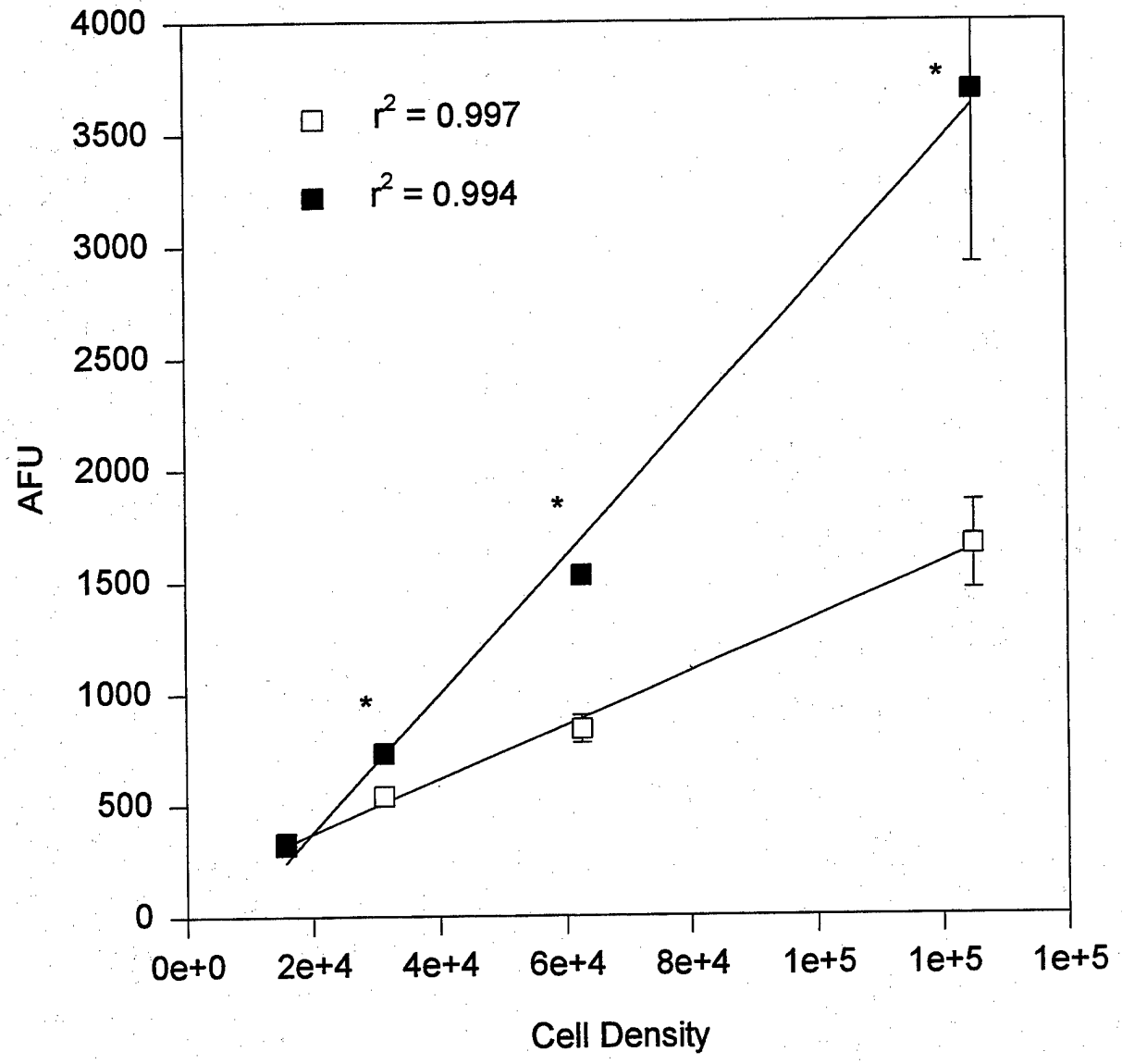

Fig. 3. Comparison of cellular GSH levels in freshly isolated hepatocytes (solid squares) and a WB344 hepatocyte cell line (open squares) at varying cell densities. Cell densities ranged from 1.5 x 10^4 to 1.25 x 10^5 cells/well. The fluorescence of the MCB-GSH conjugate was determined from three experiments with three replicates per cell density using a multiwell fluorescence plate reader with a 380nm EX/485nm EM filter pair and instrument sensitivity of 5. Data points reflect the MCB average fluorescence units (AFU) ±SD. Plates were incubated 45 min at 37°C prior to reading. Solid lines represents least square regression analyses determined by SigmaPlot™. * Significantly different than WB344 cells at $p < 0.05$.

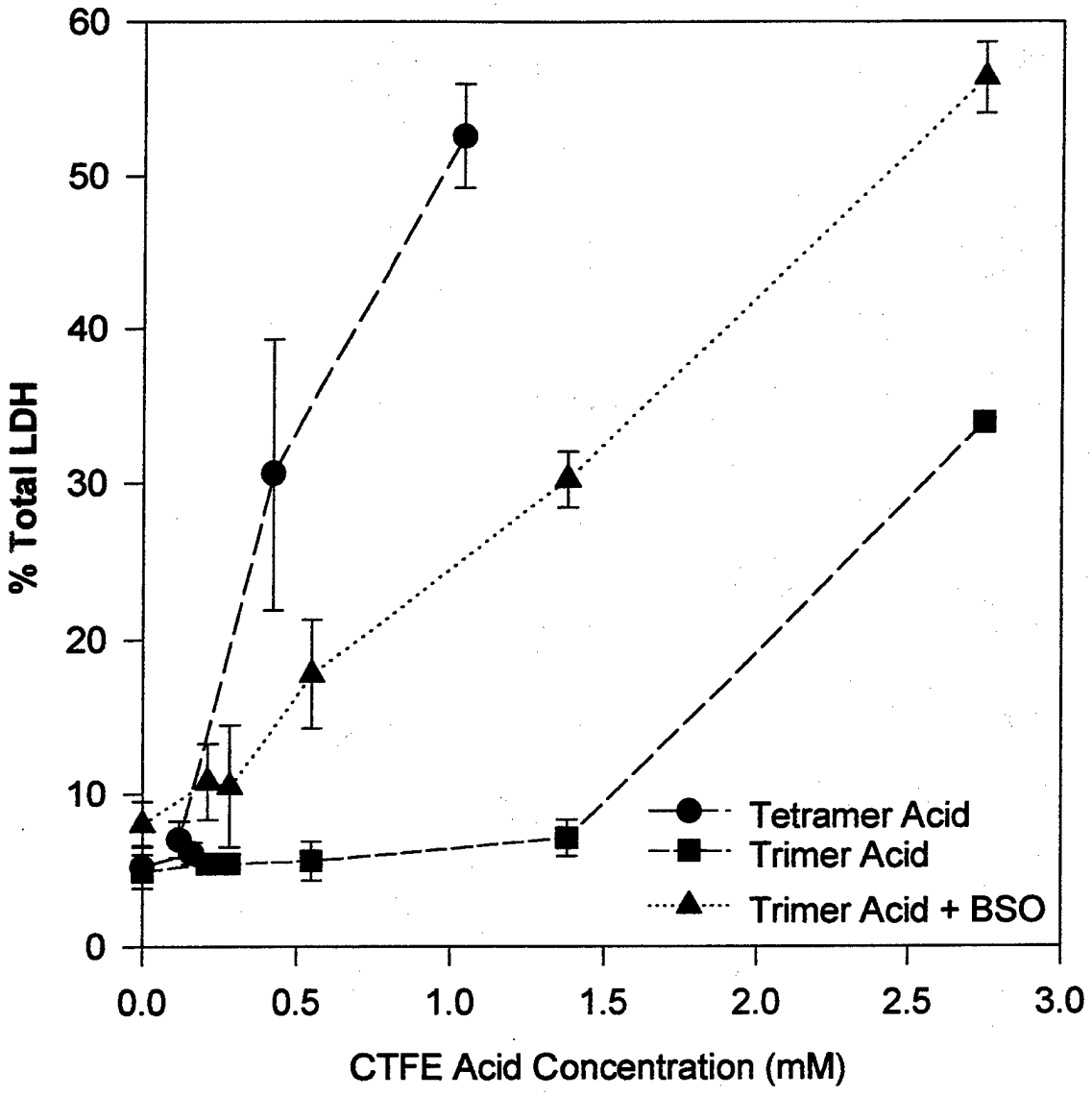

Fig. 4. Dose response curves for tetramer CTFE oligomer acid, and the trimer CTFE oligomer acid in the absence or presence of BSO (0.2 mM) in primary rat hepatocytes after 24 h of exposure. Primary hepatocytes were exposed to CTFE acid concentrations ranging from 0.16 to 2.75 mM. Data points represent the mean and ±SD from three experiments with triplicate plates per treatment group. Control cell LDH leakage in all experiments was < 10% of the total.

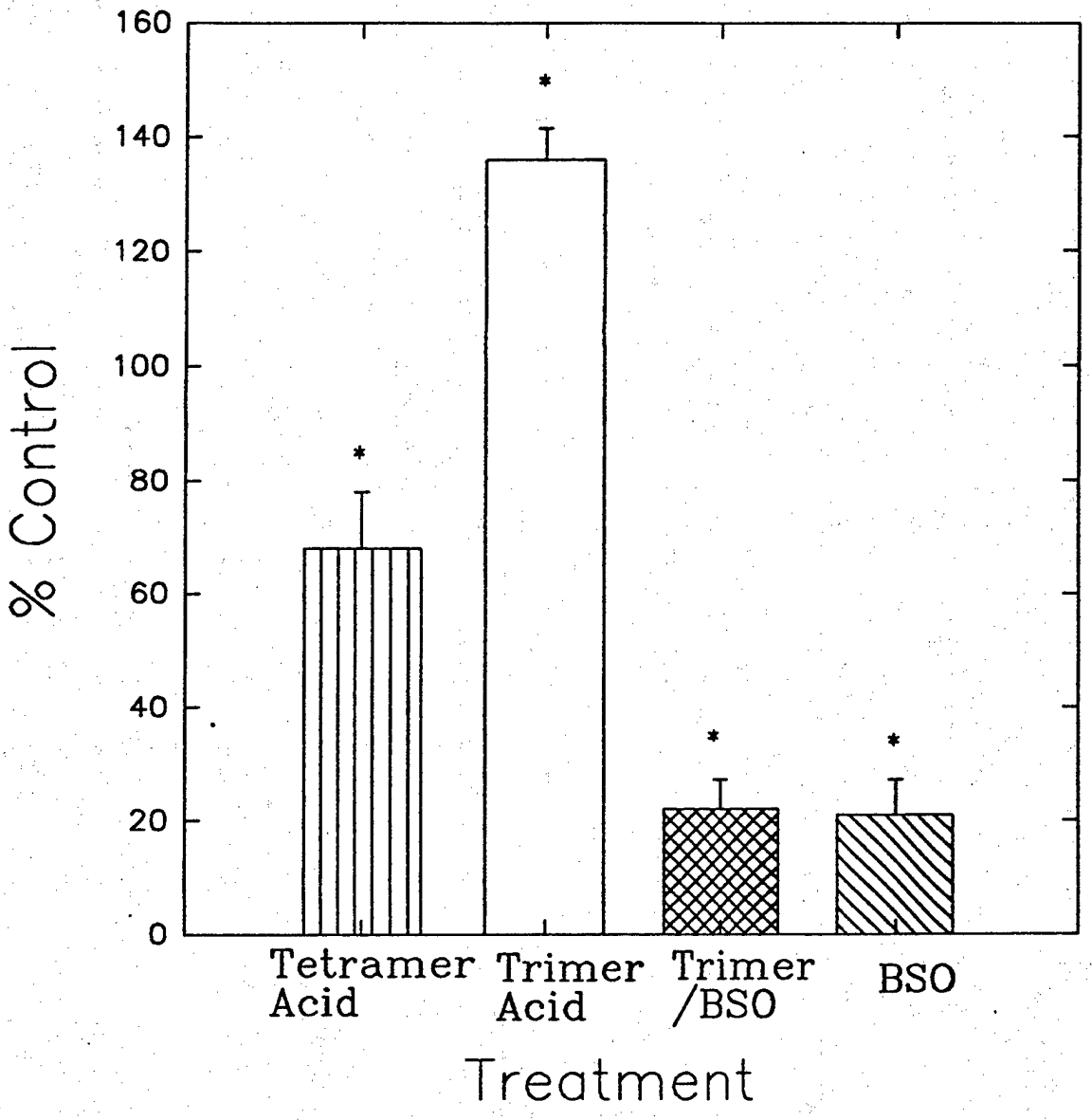

Fig. 5. Relative MCB-GSH fluorescence measured in primary rat hepatocytes after 24 h of exposure to the trimer and tetramer CTFE oligomer acids. Relative MCB-GSH fluorescence was determined in a fluorescence 96-well plate reader and the data represented as a percent-of-control of the average fluorescence units normalized to cell protein. Hepatocytes were exposed to the CTFE acids at 0.3 mM and cultures containing BSO were dosed at 0.2 mM. Viability was unaffected by BSO treatment. Bars represent the mean and ± SD from three experiments with quadruplicate wells per treatment group. * significantly different than controls at $p < 0.05$.

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