

EFFECTS OF OZONE ON CELLULAR SYNTHESIS  
AND VIRAL REPLICATION IN VITRO

Final Report

Contract Number A5-153-33  
June 11, 1985 - June 11, 1987

Prepared for  
CALIFORNIA AIR RESOURCES BOARD  
Research Division  
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## ACKNOWLEDGMENTS

The authors wish to express appreciation for the use of the Environmental Air Pollution Facilities at the California Primate Research Center. Thanks are especially due to Dr. C. E. Cornelius, director; Mr. B. Tarkington, and Mr. T. Duvall.

This report was submitted in fulfillment of Contract Number A5-153-33 "Effects of Ozone on Cellular Synthesis and Viral Replication in vitro," by the Department of Veterinary Microbiology and Immunology, University of California, Davis, under the sponsorship of the California Air Resources Board. Work was completed as of June 30, 1987.

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## ABSTRACT

These investigations dealt with the effects of ozone on cellular synthesis and viral replication in vitro. A series of experiments were performed to determine the relationship between concentrations of ozone and early indications of cellular damage. The effects of ozone on viral replication when cell cultures were exposed to various concentrations of ozone prior to and during virus inoculation were also investigated. Experiments were also run to determine the effects of ozone on the ability of mouse tracheal organ explants to synthesize interferon and on the interferon molecule itself.

The three cell lines used in the determination of effects of ozone on macromolecular synthesis were found to have different sensitivities to ozone. The two cell lines derived from respiratory tissue, human fetal lung (HFL) cell and bovine turbinate (BT) cells, incur decreases in RNA, DNA and protein synthesis at ozone concentrations that have no effect on the mouse L929 cells. In general there were increasing inhibition with increases in ozone concentration and exposure times. No recovery in macromolecular synthesis was seen when cells were exposed to ozone for longer periods of time (72 and 96 hours) as would be expected if the cells were becoming "tolerant" to the effects of ozone.

There were no differences in the ability of HFL cells, BT cell, and L929 cells to support the replication of WSN influenza A virus, infectious bovine rhinotracheitis virus (IBRV) and vesicular stomatitis virus (VSV), respectively, when these cell lines were exposed to ozone at concentrations ranging from 0.2 ppm to 1.0 ppm for 24 and 48 hours prior to virus inoculation. No differences could be seen in virus replication when these cell lines were exposed to 1.0 and 2.0 ppm for 48 hours prior to virus inoculation, then returned to an ozone atmosphere for an additional 72 hours while viral replication was taking place.

No effect on interferon synthesis or on the interferon molecule was observed when murine tracheal organ explants were exposed to 1.0 and 2.0 ppm ozone for 24 and 48 hours prior to induction of interferon then returned to an ambient air/ozone atmosphere for an additional 96 hours while interferon synthesis was taking place.

The fact that the cell lines, after ozone exposure, can support the growth of viruses would appear to indicate that these viruses are replicating efficiently in the presence of ozone insult.

## SUMMARY

These investigations dealt with further clarification of the effects of ozone on cellular synthesis and viral replication in vitro. The problems of energy production in the United States and other industrialized countries are destined to increase the inhalation of substances hazardous to health. There is increasing evidence that oxidant pollutants such as ozone produce morphological, physiological, and immunological alterations in the intact animal. Changes on the biochemical and cellular level are poorly understood due to the lack of an operational system whereby cells in vitro can be effectively exposed to oxidant pollutants. During the past several years we have been developing an exposure unit with which human and animal cells from different organs can be uniformly exposed to controlled levels of ozone.

### I. Studies on the Effect of Ozone on Macromolecular Synthesis

These studies were designed to determine the relationship between concentrations of ozone and early indications of cellular damage. Ozone effects on the biochemical level were determined by measuring the incorporation of radioactive precursors into several macromolecules (RNA, DNA, proteins).

Cell cultures were exposed to ozone concentrations ranging from 0.5 ppm to 2.0 ppm in order to pinpoint the threshold ozone concentration necessary for response in each cell line. The three cell lines used, human fetal lung (HFL) cells, bovine turbinata (BT) cells, and mouse L929 cells were found to have different sensitivities to ozone, the two cell lines of respiratory origin, BT and HFL cells being more sensitive to ozone than mouse L929 cells. The general trend was increasing inhibition of macromolecular synthesis with increases in ozone concentration and exposure times. Increases in RNA synthesis were seen after 24 and 48 hours ozone exposure in L929 cells. This increase was thought to be due to a more active metabolism of the transformed cell type resulting in either increased growth or a more active repair and maintenance system.

Human fetal lung cells and bovine turbinata cells were exposed to 2.0 ppm ozone for 96 hours to see if the cells would develop "tolerance" to the effects of ozone. Similar exposures were run with L929 cells for 72 hours. Exposures could not be extended beyond these time periods because of loss of viability. Results indicate that with all three cell lines, decreases in all three macromolecules were sustained at the longer time periods indicating that these cells do not recover from ozone damage and do not develop "tolerance" to ozone.

## II. Studies on the Effect of Ozone of Viral Replication

There is evidence that ozone alters the pathogenesis of respiratory infection by influenza virus, as well as having a direct effect on several human and animal viruses. Since little is known about the effect of ozone on the replication of these viruses in susceptible animals, one objective of this study was to determine the effects of ozone on the replication of several viruses when cell cultures were exposed prior to and during virus inoculation.

A series of experiments were performed to determine the effects of ozone on viral replication when cell cultures were exposed to various concentrations of ozone prior to and during virus inoculation and replication. These were probing experiments to determine the threshold for this effect and, consequently, there was a stepwise increase in the ozone concentrations employed. There was no significant difference in the ability of the various cell cultures to support viral replication from 0.2 ppm to 2.0 ppm ozone. When cell cultures were exposed to ozone prior to virus inoculation and returned to an ozone atmosphere during virus replication, no differences were seen with the two viruses, polio virus and IBRV, that are relatively refractory to inactivation by ozone. There were drastic decreases with WSN influenza and VSV replication but these decrease were due primarily to the inactivation of virus released into the extracellular media. These two viruses have been shown to be extremely sensitive to ozone inactivation.

## III. Studies on the Effects of Ozone on Interferon

No differences could be found in the ability of mouse tracheal organ explants to synthesis interferon when the organ explants were exposed to 1.0 and 2.0 ppm ozone for 24 and 48 hours prior to induction of interferon synthesis and then returned to an ambient air or ozone atmosphere, nor were the mouse interferons susceptible to inactivation by ozone. A previous study<sup>6</sup> done in this laboratory indicated that a decrease in the ability of mouse tracheal organ explants to synthesize interferon in vivo was found only after mice had been exposed to 0.8 ppm ozone for 10 to 12 days. The present study was undertaken to see if this decrease could be duplicated with an in vitro system. Since a direct comparison could not be made because of the inability of the tracheal organ explants to survive in vitro for that long a period of time, the organ cultures were exposed to higher concentrations of ozone for a shorter time period.

## CONCLUSIONS

1. The three cell lines used in the determination of effects of ozone on macromolecular synthesis, human fetal lungs cells (HFL), bovine turbinate cells (BT), and mouse L929 cells were found to have different sensitivities to ozone. The two primary cell lines derived from respiratory tissue, HFL and BT cells, incur decreases in RNA, DNA, and protein synthesis at ozone concentrations that have no effect on the continuous cell line, mouse L929 cells.
2. The threshold ozone concentration required for an immediate effect (0-4 hr pulse) on RNA synthesis in HFL cells was 2.0 ppm ozone for 24 hours. DNA synthesis was not affected until the cultures were exposed to 2.0 ppm ozone for 48 hours. Protein synthesis was affected at 1.0 ppm after 24 hours.
3. The threshold ozone concentration required for an immediate effect (0-4 hr pulse) on RNA synthesis in BT cells was 0.5 ppm ozone for 48 hours. DNA synthesis was inhibited at 2.0 ppm ozone after 24 hours and 0.5 ppm after 48 hours. Protein synthesis was affected at 0.5 ppm after 24 hours.
4. In mouse L929 cells there was a temporary decrease in RNA synthesis during the 0-4 hour pulse at 2.0 ppm after 24 hours, an increase at 1.0 and 2.0 ppm ozone after 48 hours exposure, and a decrease at 2.0 ppm after 72 hours. The threshold ozone concentration required for an effect in DNA synthesis was 2.0 ppm after 24 hours and 0.5 ppm after 48 hours. Protein synthesis was affected at 0.5 ppm after 48 hours.
5. Inhibition of macromolecular synthesis was not consistent between the different cell lines used, possibly due to the different characteristics of the cell lines and the inability of the assays to distinguish between growth and maintenance.
6. No recovery in inhibition of macromolecules was seen when cell cultures were exposed to 2.0 ppm ozone (a concentration at which definite inhibition was seen at 24 and 48 hours exposure) for 72 hours for L929 cells and 96 hours for HFL and BT cells, as would be expected if the cells exhibit "tolerance" to the effects of ozone.
7. There was no effect of ozone on viral replication when cell cultures were exposed to concentrations of ozone up to 2.0 ppm prior to and during viral replication. Ozone concentrations ranging up to 0.49 ppm have been found in the South Coast air basin. Although at this concentration of ozone, morphological, immunological, and biochemical functions in pulmonary tissue in vivo have been found to be altered, there is no effect on the ability of cell cultures derived from pulmonary tissue to support viral replication. The implications of these results poses a

serious question for the prevention of human illness, since virus can replicate to the same extent in an environment which compromises host defenses.

8. No effect on interferon synthesis or on the interferon molecule was observed when murine tracheal organ explants were exposed to 1.0 and 2.0 ppm ozone for 24 and 48 hours prior to induction of interferon then returned to an ambient air/ozone atmosphere for an additional 96 hours while interferon synthesis was taking place.

#### RECOMMENDATIONS

Parameters chosen to indicate changes in cellular metabolism were the incorporation of <sup>3</sup>H-thymidine, <sup>3</sup>H-uridine, and <sup>3</sup>H-leucine as a measure of DNA synthesis, RNA synthesis and protein synthesis, respectively. Results of our experiments exploring these parameters suggested that a diversion of biosynthetic energies were being diverted from growth to repairs and maintenance. Therefore, other indicators of cellular metabolism that might distinguish between the two should be explored, i.e. glucose uptake and lipid synthesis.

## PROJECT REPORT

### Effects of Ozone on Cellular Synthesis and Viral Replication In Vitro

Contract Number A5-153-33

#### I. Purpose and General Background of the Project

Ozone ( $O_3$ ) is a very potent oxidizing agent and a major component of urban photochemical smog. At high altitudes, ozone is formed naturally by the interaction of  $O_2$  with U.V. light. In the lower atmosphere it is generated by a photochemical reaction involving  $NO_2$  emitted in combustive exhausts and  $O_2$  and maintained at significant concentrations by a complex series of reactions involving air-borne hydrocarbons (also exhaust emission products).<sup>1</sup> As expected of a reactive gas, ozone exerts its toxic effect primarily in the respiratory tract of man and animals. The level of ozone in heavily polluted areas can range up to 0.49 parts per million (ppm)<sup>2</sup>, a range that has been shown to produce altered lung and tracheal morphology, edema, infiltration of heterophilic leukocytes, decrease in the ability to produce interferon and depressed phagocytic properties of alveolar macrophages.<sup>3,4,5,6,7</sup> As blood is circulated through the lung, erythrocytes and leukocytes may be damaged causing accelerated aging and increased osmotic fragility of erythrocytes, reduced B-lymphocyte rosetting, and decreased response of T-lymphocytes to PHA stimulation.<sup>8,9,10,11</sup> Biochemical functions which have been investigated present a complimentary picture. Lung lysozyme and glutathione peroxidase system activities have been shown to increase during ozone exposure.<sup>12</sup> Werthamer *et al.*,<sup>12</sup> found increases in the activities of lung lactic dehydrogenase and glutamic oxaloacetic transaminase, but decreases in alkaline phosphatase activities. They also measured overall macromolecular synthesis by pulsing mice in vivo with radioactive labels and determining label uptake in lung lavages. They observed an increase in lung protein synthesis, but decreases in RNA and DNA synthesis. The combination and accumulation of these effects produce reduced respiratory function, increased susceptibility to bacterial infection, depressed immune functions, and diversion of metabolic energy from normal functions to the repair of ozone-induced pulmonary damage.

The mechanisms by which ozone causes such widespread damage are of interest for both practical and purely scientific reasons. However, in vivo animal model systems are limited in the depth to which one can probe these questions. The natural extension of research into mechanistic or biochemical aspects of ozone toxicity would seem to be in vitro cell culture systems. Most in vitro systems rely on established cell lines because of the difficulty in isolating and culturing directly the cell types

involved in O<sub>3</sub> derived lesions. The relative insolubility of O<sub>3</sub> in culture medium requires that the monocellular layer be in direct contact with the gas in order to provide conditions of exposure where toxic effects can be seen at low levels. Furthermore, any in vitro system must simulate the in vivo situation as closely as possible. To this end the following criteria for in vitro exposures should be met: the precise delivery and monitoring of O<sub>3</sub> is necessary, the atmosphere provided should closely mimic that found in the environment, the atmosphere should come in close contact with the cells, the system must allow for extended exposure times, sterility of the cultures must remain uncompromised, controls should remain viable and retain the morphology and activity of normal cells.

Although some work has been done on isolated red blood cells in suspension,<sup>9, 14, 15</sup> continuous cell cultures,<sup>16</sup> and biological molecules in solution,<sup>17, 18</sup>, the systems used in these studies suffer from at least one of the above criteria when considering the target organ they attempt to model.

In one of the earlier studies into the effects of O<sub>3</sub>, Goldstein et al.<sup>8</sup> utilized red blood cells in an in vitro model for the cellular effects of ozone. The ozone was bubbled through the suspension at a concentration of up to 40 ppm. This hardly mimics the type of exposure received in vivo and the ozone level used was not environmentally relevant. Other cell culture exposure systems have used either very brief exposure to high concentrations of ozone, or intermittent exposures to lower concentrations followed by rinsing of the cell monolayers with media or buffer to maintain viability. Most of these systems consist of open systems in which the cells are grown in some container and then placed into a chamber in which ozone was added to the atmosphere. Although this type of system is a closer approximation of the animal respiratory tract, they are somewhat cumbersome and difficult to regulate. Furthermore, losses of ozone from the chamber due to the introduction of cultured cells into the open system requires the reestablishment of the desired ozone concentration which, especially, in short exposures may have led to erroneous interpretations of data. Thus, the need exists for an in vitro exposure system which closely mimics the situation in respiratory bronchioles and more closely follows the criteria for an in vitro exposure model system as described above.

During the past several years, this laboratory has been developing a closed exposure system with which animal cells can be uniformly exposed to controlled levels of ozone.<sup>19</sup> Basically, the system allows continuous exposure of cells grown in roller bottles or in glass vials placed in minichambers (mounted on a rocker platform), to environmentally encountered levels of ozone or ambient air for significant lengths of time. The growth of adherent cells under these conditions assures that exposure to

the pollutant is via the gas phase through a very thin film of liquid covering the monolayer as opposed to ozone being bubbled through the media or the cells being exposed through a liquid layer. Exposure via the gas phase is very important since Pace et al.<sup>16</sup> established that a maintenance medium covering the cells during exposures acts as a physical barrier protecting the cells from the effects of O<sub>3</sub> exposure. Unless the cells come in direct contact with the atmosphere little to no effect is demonstrable. In our exposure system, the cells are kept viable by immersion in the liquid media for a period during each revolution of the bottle or travel of the rocker platform. This is similar to conditions in the respiratory tract in that during the time the cells are not covered with media (about 50% of each revolution) the cell monolayer remains in contact with ozone in the gas phase, the thin layer of liquid covering the cells acting as a surfactant. We believe that our system is unique in this respect and we are not aware of any other kind that is in existence at the present time.

The ozone is generated with medical grade oxygen and mixed in filtered air. The concentration of ozone is monitored using a Dasibi model 1003-AH calibrated to an ARB standard. Ozone concentration is measured before entering the exposure chamber and again after exiting the chamber to allow a quantitation of the amount of ozone which has reacted with the culture. Flow rates of the gas are precisely controlled, as are the temperature and humidity of the exposure chambers.

This system could be employed to study early changes in cells without the use of animals. Data yielded from such studies can later be used for comparative purposes using laboratory animals. Furthermore, this system could be used to detect early cell damage and may be useful in setting air quality standards by pollution agencies.

## II. Design - Materials and Methods

### A. An Overview

Three major objectives and questions to be addressed were set forth for the project described in this report:

1. What is the relationship between concentrations of ozone and cellular damage as indicated by effects on macromolecular synthesis (cellular protein, RNA & DNA)?
2. What are the effects of different concentrations of ozone on the replication of several animal viruses in vitro? What are the effects on viral replication when cell cultures are exposed prior to and during virus inoculation?

3. What are the effects of different concentrations of ozone on the ability of murine tracheal organ explants to produce interferon and what is the sensitivity of mouse interferons to different concentrations of ozone?

#### B. Exposure System

The exposure system was designed and built by D. Bolton and Y. C. Zee and modified by R. Silbiger.<sup>19</sup> It is housed at the California Primate Research Center's (CPRC) Environmental Air Pollution Exposure Facility. The ozone exposure system described below is a closed system and was designed and constructed to permit the exposure of virus or cultured animal cells to controlled levels of ozone. The system allows for the continuous exposure of virus or cells in roller bottles or of microcultures housed in minichambers mounted on a rocker platform, to a desired concentration of ozone under either isobaric, hyperbaric, or hypobaric conditions. The system is designed for two independently controlled ozone exposure vessels and one ambient air control vessel. Each channel is equipped with humidifiers to prevent excess evaporative water gain or loss from the cultures, as well as dehumidifiers downstream of the exposure vessels to prevent condensation from water saturated air from building up in the air flow lines. Air flow rate is regulated by a series of valves and measured by several rotometers. Temperature of the cultures is maintained by a 37° C incubator. Pressure drops in each channel are monitored by magnehelic pressure gauges. Ambient air is provided to the system by a compressor and is filtered through two MSA ultrafilters. The pressure is stepped down to delivery pressure by two in-line pressure regulators. At this point in the system, CO<sub>2</sub> can be introduced as a percentage of the total flow if required. Five percent CO<sub>2</sub> is used routinely for all open culture exposures. The delivery air is then divided between the ambient air channel and the ozone mixer.

Ozone is generated by silent electric arc discharge in medical grade O<sub>2</sub> and mixed with the pressurized air at the ozone mixing unit. The system's ozone mixing unit allows for the precise adjustment of the ozone concentration in each of the ozone exposure channels. Ozone concentrations are measured sequentially before entering and after exiting each exposure chamber by means of a Dasibi model 1003-AH ambient air quality ozone monitor. Sampling of the individual channels is controlled via a multiple channel sampler which has three way solenoid valves. The solenoid valves act to maintain constant flow rates and pressures in the vessel being sampled. The multiple-channel sampler is interfaced to a LSI 11/23 minicomputer using a specially designed software package known as PRIMAT. The PRIMAT program allows the computer to activate individual solenoid valves for the acquisition of ozone monitor data at desired intervals, log Dasibi data and generate exposure reports.

The pressures throughout the system are equalized by use of a downstream vacuum pump. A constant vacuum is maintained by means of a vacuum regulator which controls the vacuum attained in a surge tank. These act to smooth out any fluctuations or harmonic oscillations in the system. All lines are filtered through a MSA ultrafilter prior to reaching the vacuum pump, after which they are exhausted into a CBR exhaust system. Due to the highly reactive nature of the ozone molecule, the system upstream of and including the reaction vessels, is constructed of silicone and viton rubbers, teflon, glass, and 316 stainless steel, materials which are inert to ozone reactivity.

During the last two years the original exposure system utilizing glass roller bottles was adapted to allow the use of exposure chambers on a rocking platform. The cell cultures are grown in small glass vials (13 mm X 12 mm) which are then placed in the exposure chambers. The minichambers are constructed from 320 ml Teflon jars and accommodated 17 microculture vials. The tops of the jars had holes drilled at the center for specially fabricated fittings which acted as the flow inlet. During the exposures 200 ul of media was added to each microculture vial; this volume was determined to be the minimum volume necessary to cover 50% of the culture area during travel of the rocker platform, thus allowing exposure to ozone in the gas phase similar to the roller bottle system.

## C. Effects of Ozone on Macromolecular Synthesis

### 1. Purpose and Background

The first objective was accomplished by designing a series of experiments to determine the relationship between concentrations of ozone and early indications of cellular damage so that the exposure unit may be utilized in future applications for setting ozone standards in the atmosphere. Ozone effects on the biochemical level was determined by measuring the incorporation of radioactive precursors into several macromolecules (RNA, DNA and proteins) as an indication of changes in cellular metabolism. Rates of synthesis as well as total accumulation of these macromolecules was determined. In order to determine the minimum concentrations of ozone needed to induce biochemical changes and to compare the sensitivities of the different cell lines used, cell cultures were exposed to ozone ranging from 0.5 ppm to 2.0 ppm. In this way a threshold ozone concentration necessary for a response in each cell line was determined. Human subjects develop "tolerance" to the adverse effects of ozone after prolonged exposure. In order to determine if this phenomenon occurred in an in vitro system, cell lines were exposed to the threshold ozone level for longer periods of time, up to 96 hours. Inability to demonstrate "tolerance" would indicate that potential for lung damage may

continue although symptoms and lung function changes diminish in vivo. All macromolecular synthesis exposures were done in the minichambers in order to avoid the necessity of subculturing before determining the rates of macromolecular synthesis. Quadruplicate microcultures were pulsed with the appropriate label at each time period, 0-4 hr, 20-24 hr, and 0-24 hr post exposure. Protein concentrations were determined at the end of each pulse period for each group in order to ascertain that there were the same amount of cells in each microculture, and that any decrease or increase in label uptake was due to a true decrease or increase in macromolecular synthesis and not to a difference in numbers of cells between ozone exposed cultures and controls.

The primary and continuous cell lines of human and animal origin used were human fetal lung cells obtained from human fetal lung tissues by Professor Robert Chang of the School of Medicine, University of California, Davis; bovine turbinate cells extracted from nasal turbinate tissues of calves; and L929 cells obtained from normal mouse areolar and adipose tissue. These cell lines were used because (1) two of the cell lines are derived from respiratory tissues; the animal cell lines are included for comparison purposes between humans and animals; (2) these cell lines are well characterized and can be grown in large quantities in roller bottles or glass vials for the exposure apparatus; (3) they are known to be free of contaminating agents such as mycoplasma; (4) their sensitivity to ozone effects may vary; and (5) these cell lines will support the replication of the respiratory viruses used in the study of ozone on virus replication.

## 2. Exposure of Cell Cultures

Cell cultures from different organs of several species (human, bovine and murine) were cultivated in Dulbecco's modified eagle media (DEM) containing 4500  $\mu$ /l glucose and supplemented with non-essential amino acids, 12.5  $\mu$ /ml gentamicin, 12.5 mM hepes, and 10% fetal bovine serum (FBS). Twenty-four hours prior to exposure the cells were seeded into the glass vials at a concentration of  $5 \times 10^4$  cells per vial and incubated at 37° C in a 5% CO<sub>2</sub> atmosphere. At the time of exposure the media was replaced with 200  $\mu$ l of fresh DEM containing 1% FBS, placed into the exposure chambers and transported to the CPRC for exposure. A complete set of exposure cultures consisted of one ambient air chamber and two ozone chambers. Ambient air is filtered room air without added ozone. Sixteen vials were placed in each chamber so that macromolecular synthesis assays could be done in quadruplicate. One vial was reserved for cell counting or for determination of the amount of protein in the vial at the end of the exposure period to ensure that the same amount of cells were being labeled in each group (ozone or ambient). The exposure apparatus was sterilized between runs by flushing the complete system (ambient air and ozone channels sans culture bottles) with

high concentrations of ozone (30 ppm) for 30 minutes. The ozone was then removed from the ambient air channel and allowed to decay, while ozone channels were adjusted to achieve proper ozone concentrations. Culture chambers were attached after the system had equilibrated and stabilized. Exposures were run for 24, 48, 72, and 96 hours at 0.5, 1.0, and 2.0 ppm ozone. When the exposures were completed, the culture chambers were aseptically removed and transported to the laboratory (in Haring Hall) for further experimentation.

### 3. Macromolecular Synthesis Assays:

Rates of macromolecular synthesis were measured by giving four hour pulses of tritium-labeled precursors (<sup>3</sup>H-leucine, <sup>3</sup>H-thymidine, and <sup>3</sup>H-uridine) at a concentration of 5 $\mu$ Ci per vial. An additional 300  $\mu$ l of DEM containing 10% FBS were added to each vial to bring the total volume up to 500  $\mu$ l. For the <sup>3</sup>H-leucine uptake assays the cultures were starved in leucine free media for one hour prior to pulsing in order to use up intracellular pools of unlabeled leucine. Pulse periods were 0-4 hr, 20-24 hr, and 0-24 hr. At the end of the pulse periods, the media containing the unincorporated radioactive label was removed and replaced with 0.5 ml normal saline and the vials frozen at -70° C until they could be processed for acid precipitable counts. To determine trichloroacetic acid (TCA) precipitable counts, the contents of each vial were removed by freeze-thawing, precipitated with 5% TCA, and collected on 25 mm nitrocellulose filter discs on a Hoeffer ten place filter assembly. The nitrocellulose filter discs were dried at 80° C for one hour, placed in scintillation vials containing ten ml of a scintillation cocktail (PPO-POPOP in toluene), and the radioactivity measured on a Beckman scintillation counter. Quadruplicate cell cultures for each pulse period were set up. At the end of each pulse period, the amount of protein in one vial from each group was determined as a control for cell numbers to ensure that the same amount of cells were being labeled in each group.

Table 1: Ozone effect on macromolecular synthesis. Experimental design for ozone exposure and label uptake.

Type of cell	O <sub>3</sub> exp. time (hrs)	O <sub>3</sub> conc. (ppm)	Label	Pulse Time time = 0
Human fetal lung cells	24	0.5	<sup>3</sup> H-thymidine	0-4, 20-24
			<sup>3</sup> H-uridine	
			<sup>3</sup> H-leucine	

The objective of these experiments was to determine the effect of ozone on cellular macromolecular synthesis by measuring

rates of DNA, RNA, and protein synthesis. Table 1 shows a work plan for the determination of the effect of 0.5 ppm O<sub>3</sub> on cellular macromolecular synthesis in human fetal lung cells for 24 hr ozone exposure. Similar work plans were followed for the other two cell lines used, bovine turbinate and mouse L929 cells, at ozone concentrations of 1.0 and 2.0 ppm, and for 48 and 96 hours ozone exposure. Controls were cell cultures exposed to ambient air without ozone.

#### D. Effects of Ozone on the Number of Cells in Uninfected Monolayers

The effects of varying concentrations of ozone on the number of cells in uninfected monolayers as determined by enumeration of non-adherent and adherent populations of cells after ozone exposure, will be determined for the mouse L929 cells and Madin-Darby bovine kidney cells (MDBK). These two cell lines were chosen because they could be cultivated efficiently in roller bottles. The two primary cell lines, HFL and BT cells could not be grown efficiently in roller culture. The roller bottle system was used so that large numbers of cells could be cultivated and the numbers compared to give statistically significant results. Macromolecular synthesis assays and viral replication studies were done in the glass vials since all the cell lines grew well in the vials. In addition, the original protocol was modified to eliminate the necessity for subculturing (use of glass vials so that samples could be removed separately at each time period); therefore, it was necessary to determine if inhibition or increase in macromolecular synthesis or rates of virus replication might be affected by different cell numbers in the vials due to differential effects of ozone on the attachment of the cells to the glass. Thus, we were able to determine if any decrease (or increase) in macromolecular synthesis or viral replication in ozone exposed cultures were due to altered ability of the cells to synthesis macromolecules or to support viral replication, or simply due to reduced cell numbers.

Roller bottles of L929 cells and MDBK cells were exposed to ozone for 24 at 0.2 and 0.5 ppm as described above for the macromolecular synthesis studies. At the end of the exposure period the monolayers of cells were removed with a mixture of 0.02% trypsin and 0.02% EDTA in a balanced salt solution containing glucose. The number of cells were determined by counting in a hemacytometer. The number of non-adherent cells were determined by pouring off and centrifuging the supernatant and counting the number of cells in the pellet.

#### E. Analysis of Soluble Proteins

Factors released by ozone damaged cells were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Aliquots of the supernatants from cell cultures exposed to

concentrations of ozone showing definite effects on macromolecular synthesis (2.0 ppm) were concentrated 80 times in a Centricon microconcentrator and the concentrate solubilized with SDS. Aliquots of 30  $\mu$ l of the concentrated samples were separated on a 5-15% gradient polyacrylamide gel and the proteins visualized by Coomassie brilliant blue dye. Molecular weight standards were co-electrophoresed in order to determine the molecular weights of the separated proteins.

## F. Effects of Ozone on Virus Replication

### 1. Purpose and Background

There is evidence that ozone alters the pathogenesis of respiratory infection by influenza virus, as well as having a direct effect on several human and animal viruses.<sup>20, 21</sup> Little is known about the effect of ozone on the replication of these viruses in susceptible animals. One objective of this study was to determine the effect of ozone on the replication of several viruses when cell cultures are exposed to ozone prior to and during virus inoculation and replication. Accordingly, monolayers of human fetal lung cells, bovine turbinate cells and mouse L929 cells were exposed to 0.5 and 1.0 ppm ozone for 24 and 48 hours before inoculation with influenza A (WSN) virus, infectious bovine rhinotracheitis virus (IBRV) and vesicular stomatitis virus (VSV), respectively. The human viruses cause an upper respiratory infection in man, while the bovine virus produces respiratory infections in cattle. The purpose of including the bovine respiratory virus was to determine whether there was any difference in the effect of ozone on the replication of human and animal respiratory viruses. An additional virus (polio virus on Vero cells) was added to the ozone exposure during viral replication studies because, polio virus is non-enveloped and, therefore, relatively refractory to ozone inactivation. Previous studies in our laboratory have shown that ozone inactivates enveloped viruses (i.e. viruses that have a lipoprotein membrane surrounding their protein and nucleic acid cores) such as WSN, VSV, and IBRV, while non-enveloped viruses such as polio virus are affected by ozone to a much lesser degree. Inclusion of the non-enveloped virus was necessary in the continual exposure studies in order to differentiate the effects of ozone on the ability of cells to support viral replication from the ability of ozone to inactivate the virus released into the growth medium.

### 2. Exposure System

The exposure system for the viral replication studies was the same as for the macromolecular synthesis experiments (see above).

### 3. Exposure of Cell Cultures

The effects of varying concentrations of ozone on virus replication were measured by exposing appropriate cell cultures to ozone for 24 or 48 hours, then removing them to the laboratory for virus inoculation. The exposed cells were infected with the appropriate virus (influenza A (WSN) on human fetal lung cells, VSV on mouse L929 cells, and IBRV on bovine turbinate cells) and a growth curve generated by taking samples at various times post infection. The virus titers in these samples were measured by an assay method appropriate for that particular virus. The same procedures were used for the ambient air exposed cultures. For the effect of ozone during virus replication, the cell cultures were exposed to ozone for 48 hour prior to inoculated with the appropriate virus, then replaced into the exposure apparatus where the cells were exposed to the same concentration of ozone for an additional 96 hours after virus inoculation. Samples were taken during this period for the generation of a growth curve as described above.

The objective of these experiments was to determine the effect of ozone on virus replication in susceptible cell cultures. Table 2 shows the general work plan followed for the effects of 0.2, 0.5, and 1.0 ppm prior exposure to ozone on virus replication in the various cell lines used. A similar work plan was followed for the continual exposure experiments except that the cell cultures were exposed to ozone for 48 hours prior to virus inoculation, then exposed for an additional 96 hours.

Table 2: Ozone effect on virus replication. Experimental design for ozone exposure and virus inoculation

O <sub>3</sub> exp. time (hrs)	O <sub>3</sub> conc. (ppm)	Time samples taken for virus assay post inoculation (hrs)
24	0.2	
	0.5	2, 12, 24, 48, 72, 96
	1.0	
48	0.2	
	0.5	2, 12, 24, 48, 72, 96
	1.0	

### G. Effects of Ozone on Interferon

#### 1. Purpose and Background

We have demonstrated in our earlier studies<sup>7</sup> that tracheal organ explant cultures from mice exposed to 0.8 ppm ozone for 10-

12 days showed a substantial decline in their ability to produce interferon when induced in vitro by interferon inducers. Utilizing our in vitro ozone exposure system, we can now directly expose the tracheal organ explants cultures to varying concentrations of ozone and determine the effect of ozone on their ability to produce interferon. This procedure provided a more sensitive indicator of ozone damage to pulmonary tissue. Interferon is a class of proteins synthesized by a variety of normal cells in response to viral infections among other things and has non-specific anti-viral activity. Since it is an important factor in the host defense against respiratory virus infections, the results of these studies would yield important information relating to the effect of ozone on interferon protection in virus respiratory infections.

## 2. Exposure System

The exposure system was the same as that described for the macromolecular synthesis and viral replication studies except that larger vials (25mm X 15mm) were used for the organ explant cultures. Five of the larger vials could fit in the minichambers.

## 3. Exposure of Cultures

Tracheal organ explants were taken from ten week old specific pathogen free Swiss Webster male mice and placed in the vials to which 2 ml of DEM containing 5% FBS was added. The cultures were incubated at 37° C for 24 hours before exposure to 1.0 and 2.0 ppm ozone for 24 and 48 hours. At the end of the exposure period the organ explants were removed from the minichambers, the media removed and 1.0 ml of an interferon inducer, Newcastle disease virus (NDV) at a concentration of  $1 \times 10^8$  pfu/ml was added to each vial. After an adsorption period of 1 hour, the cultures were washed three times with 2 ml of serum free media and replenished with 2 ml of DEM containing 10% FBS. Tracheal cultures were incubated for 16 hours at 37° C in a humidified 5% CO<sub>2</sub> atmosphere. Supernatants were then collected, processed, and assayed for interferon by the plaque reduction technique using mouse L929 cells, with VSV as the challenge virus.<sup>7</sup>

For the continual exposure experiments the tracheal cultures were returned to the ozone/ambient air atmosphere after induction by NDV.

Mouse interferon of known titer (100 Units) was subjected to inactivation by 1.0 and 2.0 ppm ozone, and by ambient air at 37° C. Samples were taken at regular times over a total period of 12 to 96 hours. Samples were held at 4° C until processed for interferon activity as outlined above.

### III. RESULTS

#### A. Effects of Ozone on Macromolecular Synthesis

##### 1. Macromolecular synthesis in human fetal lung cells

In the first series of experiments, human fetal lung (HFL) cells were exposed to ozone concentrations ranging from 0.5 to 2.0 ppm ozone in order to determine the threshold concentration of ozone necessary for a response. A four hour pulse period was run immediately after ozone exposure (0-4 hr pulse) as an indication of immediate damage due to ozone; a second pulse period 20 hour after the cells had been removed from the ozone atmosphere (20-24 hr pulse) was run to determine if any effects noted immediately after ozone exposure were sustained after the cells had been allowed to go through one round of replication. Accumulation of macromolecules (or breakdown of macromolecules) was also determined by measuring the label uptake during a 0-24 hour pulse.

Protein assays were run on duplicate vials for each group at each time period to ascertain that the same amount of cells were being labeled in each group. Table 3 gives the results of a representative experiment, indicating that there was the same amount of protein (i.e. same number of cells) in each group, thus substantiating our comparisons between the ambient air (filtered air) controls and ozone exposed cells. Protein determinations were made on each group because it was felt that they would be more accurate than the cell counts, since there was an inherent 20% standard deviation with the hemocytometer used for the cell counts. Table 4 lists the cell counts for a representative experiment. There was no significant statistical difference between each group.

Table 3: Protein Determinations for 72 hr (L929) and 96 hr (BT and HFL) ozone exposures

Cell	Ambient Air	2 ppm Ozone
HFL	176.25 µg/vial	162.56 µg/vial
BT	172.5 µg/vial	130.0 µg/vial
L929	158.13 µg/vial	159.53 µg/vial

Standard Deviation = ±15.37%

Table 4: Cell counts for 24 hour ozone exposure (cells/vial)

Cell	Ambient Air	1.0 ppm ozone	2.0 ppm ozone
HFL	2 X 10 <sup>4</sup> cells/vial	2 X 10 <sup>4</sup> c/vial	1.1 X 10 <sup>4</sup> c/v
BT	5 X 10 <sup>4</sup> cells/vial	6 X 10 <sup>4</sup> c/vial	4.88 X 10 <sup>4</sup> c/v
L929	2.3 X 10 <sup>5</sup> cells/vial	1.7 X 10 <sup>5</sup> c/vial	2.0 X 10 <sup>4</sup> c/v

Standard Deviation = ±13.84%

a. RNA Synthesis in Human Fetal Lung Cells

As shown in table 5 (a summary of 18 experiments; individual data is reported in Appendix A, figures 1 through 9), there were no differences in RNA synthesis immediately after ozone exposure at 24 and 48 hours at 0.5 and 1.0 ppm. At 2.0 ppm ozone there was a decrease in RNA synthesis ( $P < .025$ ), an effect that was not lost when cells were exposed to ozone for 72 and 96 hours ( $P < .005$ ). Cultures could not be carried after that time because of inability of the cell cultures to survive. The threshold level for the effects of ozone on RNA synthesis for the 0-4 hour pulse period, then, was determined to be 2.0 ppm ozone for 24 hours. The longer time periods of 72 and 96 hours were run in order to see if at this level of definite inhibition of RNA synthesis, the cells would become tolerant to ozone and recover their normal rates of RNA synthesis. As indicated, inhibition was sustained and more significant at 2.0 ppm ozone exposure for 72 and 96 hours.

This decrease in RNA synthesis at 2.0 ppm ozone exposure for 48 hours at the 0-4 hr pulse period was not sustained after the cells had been allowed to go through one round of replication. There was no difference in RNA synthesis after 48 hour ozone exposure for the 20-24 hour pulse period for all ozone concentrations tested (0.5, 1.0 and 2.0 ppm ozone). At the longer time periods of 72 and 96 hours, however, RNA synthesis was decreased ( $P < .005$ ) for all pulse periods at 2.0 ppm ozone.

When accumulation of RNA was measured (0-24 hour pulse), decreases were noted at 1.0 and 2.0 ppm ozone exposure for 24 and 48 hours, and at 2.0 ppm ozone for 72 and 96 hours.

b. DNA Synthesis in Human Fetal Lung Cells

A similar picture was seen with DNA synthesis. There were no decreases at the 0-4 hour pulse period until an ozone concentration of 2.0 ppm after 48 hours ozone exposure ( $P < .025$ ) was reached, with a more significant decrease noted at 72 and 96

hr. ozone exposure ( $P < .005$ ). The threshold level for ozone effects on DNA synthesis for the 0-4 hour pulse period was 2.0 ppm ozone exposure for 48 hours. Again this inhibition was not lost after 72 and 96 hours ozone exposure at 2.0 ppm ( $P < .005$ ).

Decreases in DNA synthesis for the 20-24 hour pulse period, when the cells had been allowed to recover, were noted at all ozone concentrations tested (0.5, 1.0, and 2.0 ppm) after 24 hours exposure. After 48 hours exposure, however, there were no differences between the ambients and ozone exposed cultures. With an increase in ozone exposure to 72 and 96 hours, a significant decrease is again noted ( $P < .005$ ). This "recovery" is most likely not due to tolerance since with increasing time of exposure (72 and 96 hours), decreases are once again noted. It is more likely that as ozone insults accumulate, the cell diverts increasing amounts of its biosynthetic machinery and energy to repairs at the expense of growth, this diversion being reflected in a temporary increase in biosynthesis of macromolecules so as to mask any inhibition that might be taking place resulting in a net no difference.

When accumulation was measured (0-24 hour pulse), DNA synthesis was decreased at 2.0 ppm after 24 hours ozone exposure ( $P < .005$ ), and at 1.0 and 2.0 ppm after 48 hours ozone exposure ( $P < .005$ ). After 72 and 96 hour ozone exposure a more significant decrease ( $P < .005$ ) was measured.

#### c. Protein Synthesis in Human Fetal Lung Cells

Decreases in protein synthesis were noted at lower ozone concentrations and at earlier time periods than either RNA or DNA synthesis. Decreases were seen at 1.0 ppm and 2.0 ppm ozone ( $P < .01$ ) after 24 hours of ozone exposure for the 0-4 hour pulse period and at 1.0 ppm ( $P < 0.01$ ) and 2.0 ppm ( $P < .005$ ) after 48 hours. Again this decrease was sustained and more significant at 72 and 96 hours exposure ( $P < .005$ ).

At the 20-24 hour pulse period, protein synthesis was decreased at 1.0 and 2.0 ppm ( $P < .005$ ) after 24 hours ozone exposure and at 2.0 ppm ( $P < .005$ ) after 48 hours ozone exposure. As explained under the effects of ozone on DNA synthesis, this apparent recovery was probably due to increased repairs and maintenance from ozone insults rather than a true tolerance. As noted, decreases in protein synthesis were again noted at 72 and 96 hours ozone exposure ( $P < .005$ ).

No effect on accumulation of proteins after 24 hours ozone exposure was observed. After 48 hours a decrease at 1.0 and 2.0 ppm was noted ( $P < .05$ ) with a more significant decrease at 72 and 96 hours ( $P < .005$ ).

Table 5: Macromolecular Synthesis in Human Fetal Lung Cells Exposed to Varying Concentrations of O<sub>3</sub> (PPM)

	RNA Synthesis			DNA synthesis			Protein synthesis		
	0-4	20-24	0-24	0-4	20-24	0-24	0-4	20-24	0-24
24 hr exp									
0.5 ppm	0	0	0	0	-	0	0	0	0
1.0 ppm	0	0	-	0	-	0	-	-	0
2.0 ppm	-	-	-	0	-	-	-	-	0
48 hr exp									
0.5 ppm	0	0	0	0	0	0	0	0	0
1.0 ppm	0	0	-	0	0	-	-	0	-
2.0 ppm	-	0	-	-	0	-	-	-	-
72 hr exp									
2.0 ppm	-	-	-	-	-	-	-	-	-
96 hr exp									
2.0 ppm	-	-	-	-	-	-	-	-	-

0 = no difference from ambient air controls  
 - = decrease from ambient air controls  
 + = increase from ambient air controls

2. Macromolecular Synthesis in Bovine Turbinate Cells

Table 6 summarizes the results of, the second series of 22 experiments done on bovine turbinate cells. Individual data are reported in Appendix A, figures 10 through 18.

a. RNA Synthesis in Bovine Turbinate Cells

There were no differences in RNA synthesis in bovine turbinate cells after 24 hours ozone exposure except for a slight increase (P < .025) at 1.0 ppm and a more significant increase at 2.0 ppm ozone (P < .005) for the 20-24 hour pulse period. After 48 hours there were significant decreases (P < .005) at all ozone concentrations for the 0-4 and 0-24 hour pulse periods. At 0.5 and 1.0 ppm at the 20-24 hour pulse, there was no change from

the ambient controls. Inhibition at 2.0 ppm for all pulse periods was seen after 72 and 96 hours ozone exposure (P < .005).

b. DNA Synthesis in Bovine Turbinate Cells

A slight increase (P < .025) in DNA synthesis was seen at 0.5 ppm ozone exposure for 24 hours at the 0-4 hour pulse period, with decreases being observed at 2.0 ppm for the 0-4 hour pulse (P < .025) and at 1.0 and 2.0 ppm (P < .005) for the 20-24 hour pulse.

Significant decreases (P < .005) were observed at 0.5, 1.0 and 2.0 ppm for the 0-4 hour pulse, at 1.0 and 2.0 ppm for the 20-24 hour pulse, and at 2.0 ppm for the 0-24 hour pulse. A slight increase (P < .025) was observed at 0.5 ppm for 48 hours. The decreases were sustained at the longer ozone exposure for 72 and 96 hours (P < .005).

Table 6: Macromolecular Synthesis in Bovine Turbinate Cells Exposed to Varying Concentrations of O<sub>3</sub> (PPM)

	RNA Synthesis			DNA Synthesis			Protein Synthesis		
	0-4	20-24	0-24	0-4	20-24	0-24	0-4	20-24	0-24
24 hr exp									
0.5 ppm	0	0	0	+	0	0	-	+	0
1.0 ppm	0	+	0	0	-	0	-	-	-
2.0 ppm	0	+	0	-	-	0	-	-	-
48 hr exp									
0.5 ppm	-	0	-	-	0	+	0	0	0
1.0 ppm	-	0	-	-	-	0	0	0	0
2.0 ppm	-	-	-	-	-	-	-	0	0
72 hr exp									
2.0 ppm	-	-	-	-	-	-	-	-	-
96 hr exp									
2.0 ppm	-	-	-	-	-	-	-	-	-

0 = no difference from ambient air controls  
 - = decrease from ambient air controls  
 + = increase from ambient air controls

c. Protein Synthesis in Bovine Turbinate Cells

Protein synthesis in bovine turbinate cells was inhibited at concentrations of ozone that did not affect RNA or DNA synthesis for the 24 hour ozone exposure for the 0-4 and the 0-24 pulse period ( $P < .005$ )

Most of these inhibitions were lost after the 48 hour exposures. At 72 and 96 hours of ozone exposure there were decreases ( $P < .005$ ) at 2.0 ppm ozone. As with DNA synthesis in human fetal lungs cells, this apparent recovery was most likely due to more active repairs of ozone insults at the 48 hour exposure period.

3. Macromolecular Synthesis in L929 Cells

Table 5 summarizes the results of 19 experiments on the effects of ozone on macromolecular synthesis in Mouse L929 cells. Individual experiments are reported in Appendix A, figures 19 through 27. Exposures were limited to 72 hours because of loss of viability in the controls after that time. Mouse L929 are a continuous cell line (i.e. faster growing, loss of contact inhibition, etc.) as opposed to HFL and BT cells which are primary cell lines (i.e. slower growing, exhibit contact inhibition, retain diploid chromosome number, etc). Thus, L929 cells overgrow the culture vessels, deplete the media of nutrients, accumulate toxic metabolites, and die off after a few days in culture.

a. RNA Synthesis in Mouse L929 Cells

Mouse L929 cells appear to be less sensitive to ozone than either HFL or BT cells. The only decreases noted in RNA synthesis were at 2.0 ppm with the 0-4 hour pulse after 24 hours and 72 hours ozone exposure ( $P < .01$ ). There were increases in RNA synthesis after 24 hours ozone exposure at 2.0 ppm for the 20-24 and 0-24 hour pulse periods ( $P < .005$ ) and after 48 hours ozone exposure at 1.0 and 2.0 ppm ozone for all three pulse periods. These increases probably reflect a more active repair system.

b. DNA Synthesis in Mouse L929 Cells

DNA synthesis was more sensitive to ozone insult than RNA synthesis in mouse L929 cells. After 24 hours ozone exposure decreases were observed at 2.0 ppm ozone ( $P < .005$ ) at all three pulse periods. After 48 hours ozone exposure, decreases were noted at the 0-4 hour pulse for all ozone concentrations, 0.5, 1.0 and 2.0 ppm ( $P < .005$ ) and at the 0-24 hour pulse for 0.5 and 1.0 ppm ( $P < .005$ ). A slight but significant increase was observed at the 0-24 hour pulse for 2.0 ppm ( $P < .01$ ) ozone. After 72 hours ozone exposure there was a decrease at the 0-4 hour pulse period

(P < .005) and increases at the 20-24 and 0-24 hour pulse period (P < .005).

Table 7: Macromolecular Synthesis in Mouse L929 Cells Exposed to Varying Concentrations of O<sub>3</sub> (PPM)

	RNA Synthesis			DNA Synthesis			Protein Synthesis		
	0-4	20-24	0-24	0-4	20-24	0-24	0-4	20-24	0-24
24 hr exp									
0.5 ppm	0	0	0	0	0	0	0	0	0
1.0 ppm	0	0	0	0	0	0	0	0	-
2.0 ppm	-	+	+	-	-	-	0	0	-
48 hr exp									
0.5 ppm	0	0	0	-	0	-	-	-	0
1.0 ppm	+	+	+	-	0	-	0	-	0
2.0 ppm	+	+	+	-	0	+	-	-	0
72 hr exp									
2.0 ppm	-	0	0	-	+	+	-	-	-

0 = no difference from ambient air controls  
 - = decrease from ambient air controls  
 + = increase from ambient air controls

c. Protein Synthesis in Mouse L929 Cells

No differences in protein synthesis was observed after 24 hours ozone exposure except for the 0-24 hour pulse period for 1.0 and 2.0 ppm ozone (P < .005). After 48 hours ozone exposure there were decreases for the 0-4 hour pulse at 0.5 and 2.0 ppm ozone and for 0.5, 1.0, and 2.0 ppm ozone for the 20-24 hour pulse. All decreases were at the P < .005 level of significance. After 72 hours of ozone exposure there were decreases at 2.0 ppm for all pulse periods (P < .005).

B. Effects of Ozone on Adherent and Non-adherent Cells

Roller bottles of L929 cells and MDBK cells were exposed to ozone for 24 hours and the adherent and non-adherent population of cells counted. The roller bottle system was used so that

large number of cells could be cultivated and the numbers compared to give statistically significant results. These two cell lines were chosen because they could be cultivated efficiently in roller bottles. The roller bottles were seeded 24 hours before exposure with an equal number of cells and exposed for 24 hours to 0.2 and 0.5 ppm ozone. Table 6 summarizes the results of these experiments.

Although the trend is towards decreasing numbers of adherent cells and increasing numbers of nonadherent cells with increasing ozone concentration, the level of confidence between the adherent and nonadherent cells is  $P < .1$  and, therefore, thought to be not statistically significant.

Table 8: Adherent and Nonadherent Cell Counts of Ozone-Exposed L929 and MDBK Cell Cultures

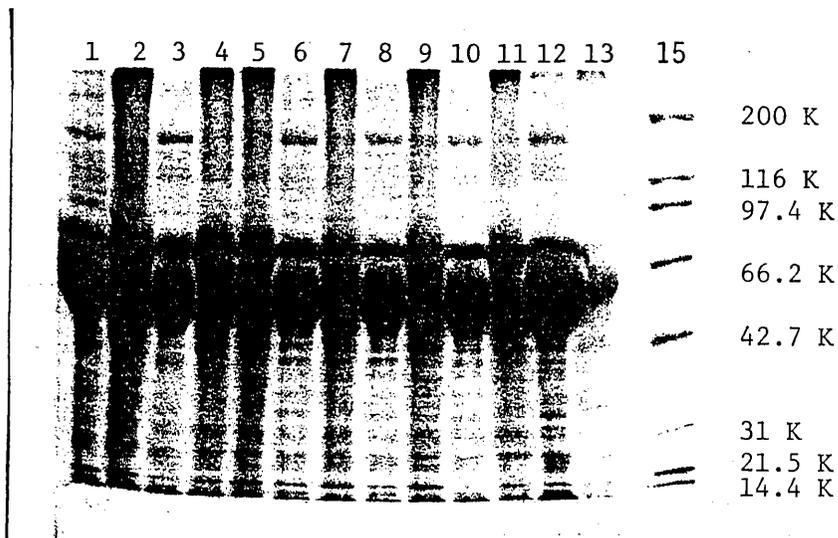
O <sub>3</sub> conc. (ppm)	Adherent Cells	Nonadherent Cells
0.0 (L929)	$3.72 \pm 1.4 \times 10^7$	$3.96 \pm .54 \times 10^6$
0.2 (L929)	$2.09 \pm .43 \times 10^7$	$4.64 \pm .78 \times 10^6$
0.5 (L929)	$1.69 \pm .80 \times 10^7$	$6.32 \pm .28 \times 10^6$
0.0 (MDBK)	$1.12 \pm .48 \times 10^7$	$1.38 \pm .06 \times 10^7$
0.2 (MDBK)	$1.36 \pm .18 \times 10^7$	$1.28 \pm .16 \times 10^7$
0.5 (MDBK)	$5.37 \pm .34 \times 10^6$	$1.39 \pm .29 \times 10^6$ *

\* Cell count inaccurate because most of the cells were destroyed and not discernible as whole intact cells

#### C. Analysis of Soluble Proteins from Ozone Exposed Cells

Supernatants were collected from cell cultures exposed to 2.0 ppm ozone for 96 hours because that was the concentration and time at which definite inhibition of protein synthesis occurred in order to determine whether there were any differences in soluble protein profiles between ambient air and ozone exposed cell cultures. Figure 1 shows the Coomassie-blue stained 5% - 15% gradient polyacrylamide gel of the soluble proteins concentrated 80 times. The large band in the middle of the gel is due to the 80 times concentration of the FBS contained in the culture media. The FBS was required in the culture media in order to maintain viability of the cell cultures.

Figure 1. Coomassie blue stained gel of the protein profiles of ozone and ambient exposed cells. Lane 1: L929 cells, ambient - 72 hrs; Lane 2: L929 cells, 2ppm - 72 hrs; Lane 3: BT cells, ambient - 96 hrs; Lane 4: BT cells, 1.0 ppm - 96 hrs; Lane 5: BT cells, 2.0 ppm - 96 hrs; Lane 6: L929 cells, ambient - 48 hrs; Lane 7: L929 cells, 2.0 ppm - 48 hrs; Lane 8: L929 cells, ambient - 72 hrs; Lane 9: L929 cells, 2.0 ppm - 72 hrs; Lanes 10 and 12: HFL cells, ambient - 96 hrs; Lanes 11 and 13: HFL cells, 2.0 ppm - 96 hrs; Lane 15: Numbers represent sizes in daltons of the polypeptides used for molecular weight markers: Mysoin, 200 K;  $\beta$ -galactosidase, 116 K; phosphorylase B, 97.4 K; bovine serum albumin, 66.2 K; ovalbumin, 42.7 K; carbonic anhydrase, 31 K; soybean trypsin inhibitor, 21.5 K; and lysozyme, 14.4 K.



Although the gel could not be subjected to densitometric analysis because of the large FBS band, there were several differences discernible in the protein profiles of the ozone and ambient air exposed cells. Figure 1, lanes 6, and 1 and 8, represent L929 cells exposed to ambient air for 48 and 72 hours, respectively while lanes 7, and 2 and 9 are L929 cells exposed to 2.0 ppm ozone for 48 and 72 hours. There are two large molecular weight bands of 22.0 and 17.5 kilodaltons (KD) present in all of the ambient controls which are either reduced or missing in the ozone exposed lanes. In the lower part of the gel representing the lower molecular weight proteins, there is a 23 KD protein band missing in the ozone exposed cells. Although it is difficult to draw any conclusions from the results of this one experiment, it is possible that the differences in molecular weight distribution is due to breakdown of protein due to ozone or aborted protein synthesis resulting in truncated or altered proteins. This is especially apparent in the profile of lower molecular weight proteins where it appears as if the bands are slightly altered in molecular weight in the ozone exposed cultures. In general the proteins on the ozone exposed profiles are less resolved than on the ambient profiles, possibly indicating greater degradation.

Figure 1, lanes 3, 4, and 5 represent bovine turbinate cells exposed to 0.00, 1.0, and 2.0 ppm ozone for 96 hours. Again the high molecular weight 17.5 KD band is missing in the ozone exposed profile. There appears to be less of a difference in the lower molecular weight proteins in the BT protein profile as compared to the L929 protein profile as indicated by the presence of the 23 KD band in the ozone exposed profile.

Human fetal lung cells exposed to 0.00 (lanes 10 and 12) and 2.0 ppm ozone (lanes 11 and 13) are shown in figure 1. The 17.5 and 23 KD protein are again reduced or missing on the ozone exposed profiles. There appears to be more alterations on the lower molecular weight half of the profiles comparable to the L929 profiles. Although there are proteins on the profiles of all three cell lines migrating with the same electrophoretic mobility, one cannot conclude that the proteins are the same in all three cell lines. They may be different proteins with the same molecular weights.

#### D. Effects of Ozone on Viral Replication

##### 1. Effects of prior exposure of ozone on viral replication

Cell cultures were exposed to ozone for 24 and 48 hours prior to virus inoculation at ozone concentrations of 0.2, 0.5, and 1.0 ppm. There was no significant difference in viral replication for any of the cell lines and virus combinations used and for ozone concentrations up to 1.0 ppm. Replication of WSN in HFL cells at an ozone concentration of 2.0 ppm also showed no

significant difference in viral replication. See Appendix B for individual graphs of the effects of varying concentrations of ozone on the growth curves of the different viruses used in this study.

## 2. Effects of continuous exposure of ozone on viral replication

Cell cultures exposed to ozone for 48 hours prior to virus inoculation, then returned to an ozone atmosphere for an additional 72 hours while viral replication was taking place showed an analogous picture. There were no significant differences with polio virus replicating in vero cells or with IBRV replicating in bovine turbinate cells at 1.0 and 2.0 ppm ozone. (Appendix B: Figures 12 and 14) These two viruses have been shown to be relatively refractory to inactivation by ozone.

There were drastic decreases in VSV and WSN replication, however, although the rate of increase in viral titers were the same during the exponential phase of growth indicating that the decreases in viral titer was due to inactivation of extracellular virus by the ozone rather than a true inhibition of the virus within the ozone damaged cell. This conclusion is further suggested by the observation that there were no significant differences in the growth curve of these two viruses replicating in cells growing under 1.0 and 2.0 ppm ozone. (Appendix B: Figures 11 and 13.)

### E. Effects of Ozone on Interferon

#### 1. Effect of Ozone on the Ability of Tracheal Organ Explants to Synthesize Interferon

##### a. Prior exposure of tracheal organ explants to ozone

Table 9 summarizes the results of prior exposure of the tracheal organ explants to 1.0 and 2.0 ppm ozone for 24 and 48 hours. Units of interferon is defined as the reciprocal of the last dilution in the plaque reduction assay showing 50% inhibition of plaques over the controls times the dilution factor of interferon in the original sample. The error of the assay is plus or minus one tube (50%). There were no differences in the ability of the tracheal organ explants to synthesize interferon at the two concentrations and two time periods of ozone exposure used.

Table 9: Effects of prior exposure of ozone on interferon synthesis

	Ambient Air	1.0 ppm	2.0 ppm
24 hr exposure	384 Units	384 Units	384 Units
48 hr exposure	384 Units	384 Units	192 Units

b. Continual exposure of tracheal organ explants to ozone

Table 10 summarizes the results of the effects of continuing exposure of ozone on interferon synthesis by the tracheal organ explant cultures. The cultures were exposed to 1.0 and 2.0 ppm ozone for 48 hours before induction with NDV, then returned to the ambient/ozone atmosphere while interferon synthesis was taking place. Samples were taken at the times indicated and assayed for interferon activity.

Table 10: Effects of continuing exposure of ozone on interferon synthesis

	Ambient Air	1.0 ppm	2.0 ppm
12 hr exposure	384 Units	192 Units	192 Units
24 hr exposure	384 Units	192 Units	192 Units
48 hr exposure	384 Units	192 Units	192 Units
72 hr exposure	192 Units	192 Units	192 Units
96 hr exposure	192 Units	96 Units	96 Units

2. The Effect of Ozone on the Interferon Molecule

One hundred units of interferon was subjected to inactivation by 1.0 and 2.0 ppm ozone and an inactivation curve determined by assaying for interferon activity at the specified time periods. Table 11 summarizes results of this experiment. There was no significant inactivation by 1.0 or 2.0 ppm ozone.

Table 11: Inactivation of interferon by varying concentrations of ozone

	12 hr	24 hr	48 hr	72 hr	96 hr
Ambient	100 U	100 U	100 U	80 U	70 U
1.0 ppm	100 U	100 U	100 U	70 U	70 U
2.0 ppm	100 U	100 U	80 U	70 U	70 U

U = units

## V. DISCUSSION

The three cell lines used in the determination of effects of ozone on macromolecular synthesis were found to have different sensitivities to ozone. In general, the two cell lines derived from respiratory tissue, human fetal lung (HFL) cells and bovine turbinate (BT) cells, incur decreases in all three macromolecules (RNA, DNA, and protein) measured at ozone concentrations that have no effect on the mouse L929 cells. The general trend was towards increasing inhibition with increases in ozone concentration and exposure times. The differences in sensitivities could be due to several factors. The HFL and BT cell lines are primary cell lines, i.e. they retain the general characteristics, morphology, and diploid chromosome number of the tissue from which they are derived, while the L929 cells are a transformed cell line, i.e. they have altered morphology, transformed characteristics, and polyploid chromosome numbers. It is possible that the greater resistance of this cell line to ozone insults is related to the transformed phenotype. Transformed cell lines do not exhibit contact inhibition as opposed to the primary cell lines. As can be seen in Table 7, there is increased incorporation of labeled precursors with some ozone exposures. A more active metabolism of the transformed cell types resulting in either increased growth or a more active repair and maintenance system may account for this observation. Further research into this area is necessary in order to distinguish inhibition of growth from increases in incorporation due to diversion of the cell's biosynthetic mechanisms from growth to repairs and maintenance. The experiments as performed were not designed to distinguish between the two different systems.

It is interesting to note that in mouse L929 cells and to some extent, BT cells, there were actually increases in RNA synthesis after 24 and 48 hours of ozone exposure. This increase was not sustained after 72 for the L929 cells or after 48 hours

for the BT cells. With HFL cells, some decreases in DNA synthesis and protein synthesis observed after 24 hours of ozone exposure were not sustained after 48 hours of exposure. This observation was also noted with protein synthesis in BT cells. This apparent "recovery" was most likely not due to tolerance, since with increasing time of exposure, decreases are again noted. It is more likely that as ozone insults accumulate, the cell diverts increasing amounts of its biosynthetic machinery and energy to repairs at the expense of growth, this diversion being reflected in a temporary increase in biosynthesis of macromolecules so as to mask any inhibition of growth that might be taking place.

In human subjects exposure to ozone for prolonged periods of time results in an apparant recovery from lung function changes and the adverse effects of ozone. The exposure of the cell lines to ozone for the longer time periods were designed to see if this phenomenon also takes place in vitro. Decreases in macromolecular synthesis were sustained and more significant at 72 and 96 hours of ozone exposure indicating that changes could still be taking place on the cellular and biochemical level in spite of the decrease in symptoms and lung function changes in vivo. Since there is a threshold effect with increasing ozone concentrations and increasing times of ozone exposure, it is possible that if human subjects were exposed to higher concentrations of ozone for longer periods of time, they would again exhibit deleterious lung function changes analagous to the depressed synthesis after the temporary "recovery" described above for the in vitro experiments.

The inhibition of macromolecular synthesis was not consistent between the different cell lines used, possibly due to the different characteristics of the cell lines and the inability of the assays to distinguish between growth and maintenance.

Experiments designed to measure the effect of ozone on cell numbers (as measured by cell counting and protein assays) and number of adherent and non-adherent cells (Tables 3, 4, and 8) indicate that there were no significant decreases with increasing ozone concentrations. Although the trend was towards decreases with increasing ozone concentrations and exposure times, these decreases were not considered significant since both methods of counting used, cell counts and protein assays, had a 10-20% error margin. Decreases in macromolecular synthesis had levels of confidence of at least  $P < .005$ , which would more than compensate for any errors that might occur because there were fewer cells in the ozone exposed cell cultures over the ambients.

The differences in protein profiles between the ozone and ambient air exposed cell cultures (figure 1) indicate that there were large molecular weight proteins not present in the ozone exposed cultures and increased differences in proteins on the

lower molecular weight half of the protein profiles. Although it is difficult to draw any conclusions from the results of this experiment, it is apparent that there is more degradation of proteins occurring with the ozone exposed cells consistent with our hypothesis that there is increased repairs and degradation of macromolecules resulting from ozone induced lesions.

Given that there were profound effects on macromolecular synthesis, it was at first surprising to note that no differences could be found in the ability of the ozone exposed cells to support viral replication, whether the cells were exposed prior to virus inoculation and then allowed to replicate in an ambient air or ozone atmosphere (See Appendix B, Figures 1 through 14). It is known that viral messages have a greater efficiency of initiation and translation than cellular messages. In virus infected cells, viral proteins are made in preference to cellular proteins, resulting in the complete cessation of host protein synthesis with some viral infections. Thus, it is logical to expect that viral replication could take place efficiently even in the presence of ozone insult. The results of the study on the effects of ozone on macromolecular synthesis indicate a possible diversion of energy from growth to repairs of ozone induced insults. A possibility exists of synergistic deleterious effects due to viral infection of respiratory tissue already stressed by having to repair ozone damage.

There was no effect on viral replication when cell cultures were exposed to concentrations of ozone up to 2.0 ppm prior to and during viral replication. Ozone concentrations up to 0.49 ppm have been found in the South Coast air basin. Although at these concentrations of ozone, morphological, immunological, and biochemical functions in pulmonary tissue in vivo have been found to be altered, there is no effect on the ability of cell cultures derived from pulmonary tissue to support viral replications. The implications of these results poses a serious question for the prevention of human illness, since virus has been shown to replicate to the same extent in an environment which compromises host defenses.

No differences could be found in the ability of mouse tracheal organ explants to synthesize interferon when the organ explants were exposed to ozone prior to induction of interferon synthesis and then returned to a ambient air or ozone atmosphere. (Tables 9 and 10). Table 11 indicates that the mouse interferons were not susceptible to inactivation by ozone. A decrease in the ability of the organ explants to synthesize interferon in vivo was found only after the mice had been exposed to ozone for 10 to 12 days.<sup>6</sup> A direct comparison could not be made because of the inability of the tracheal organ explants to survive in vitro for that long a period of time.

The development of the in vitro exposure system for ozone exposure was critical and instrumental in the ability to conduct these experiments on the investigation of the effects of ozone on a biochemical level. These experiments answered basic questions on the biochemical effects of ozone without the use of animals. This system can now be used for a more detailed analysis of the exact biosynthetic mechanisms impaired by ozone. Further research is also indicated in order to determine if there is a synergistic effect between ozone insults and viral replication leading to increased susceptibility to viral infections.

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APPENDIX A  
EFFECTS OF OZONE ON MACROMOLECULAR SYNTHESIS

FIG 1: RNA SYNTHESIS IN HFL CELLS AFTER 24 HR OZONE EXPOSURE

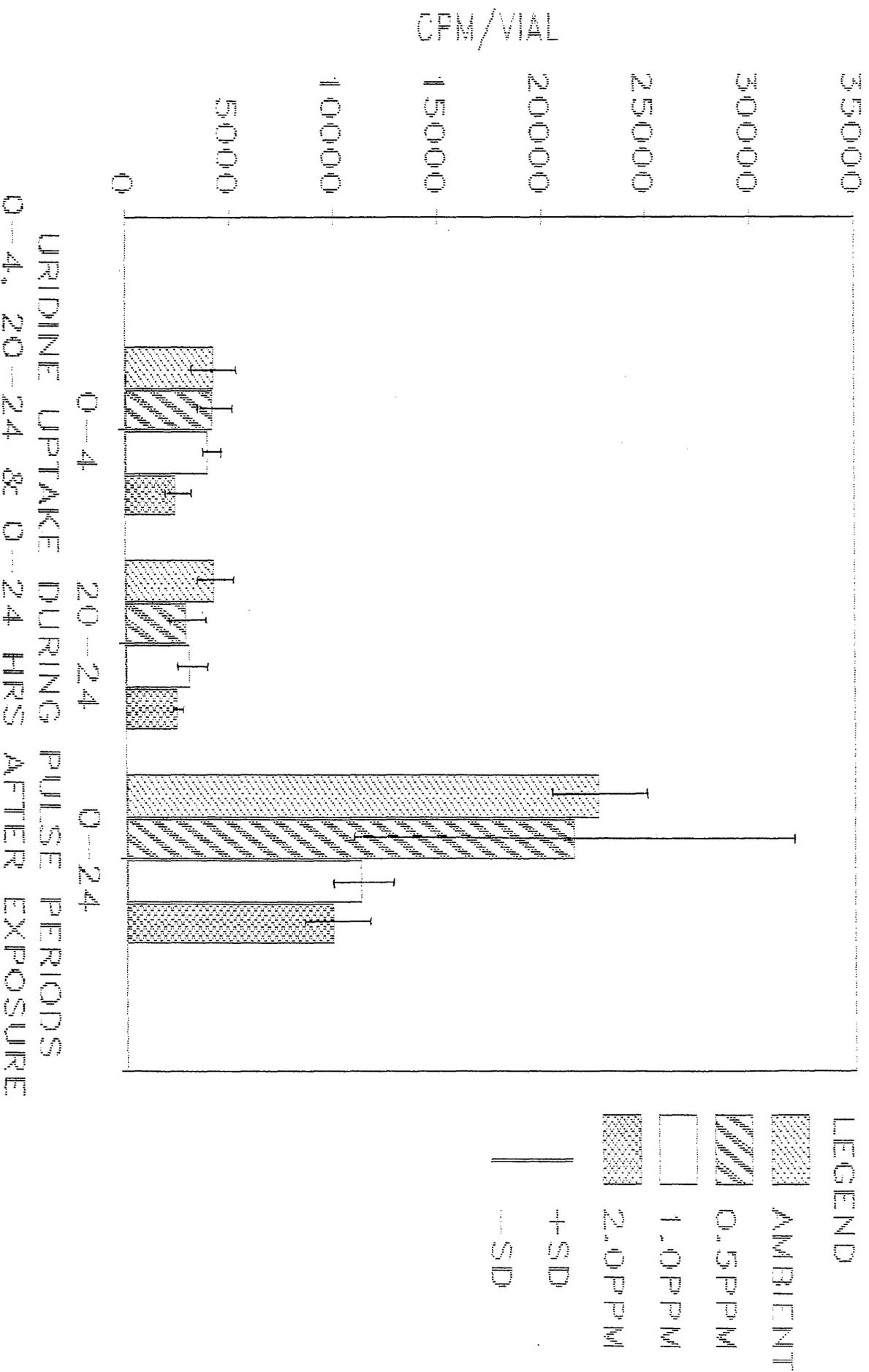


FIG 2: RNA SYNTHESIS IN HFL CELLS AFTER 48 HR OZONE EXPOSURE

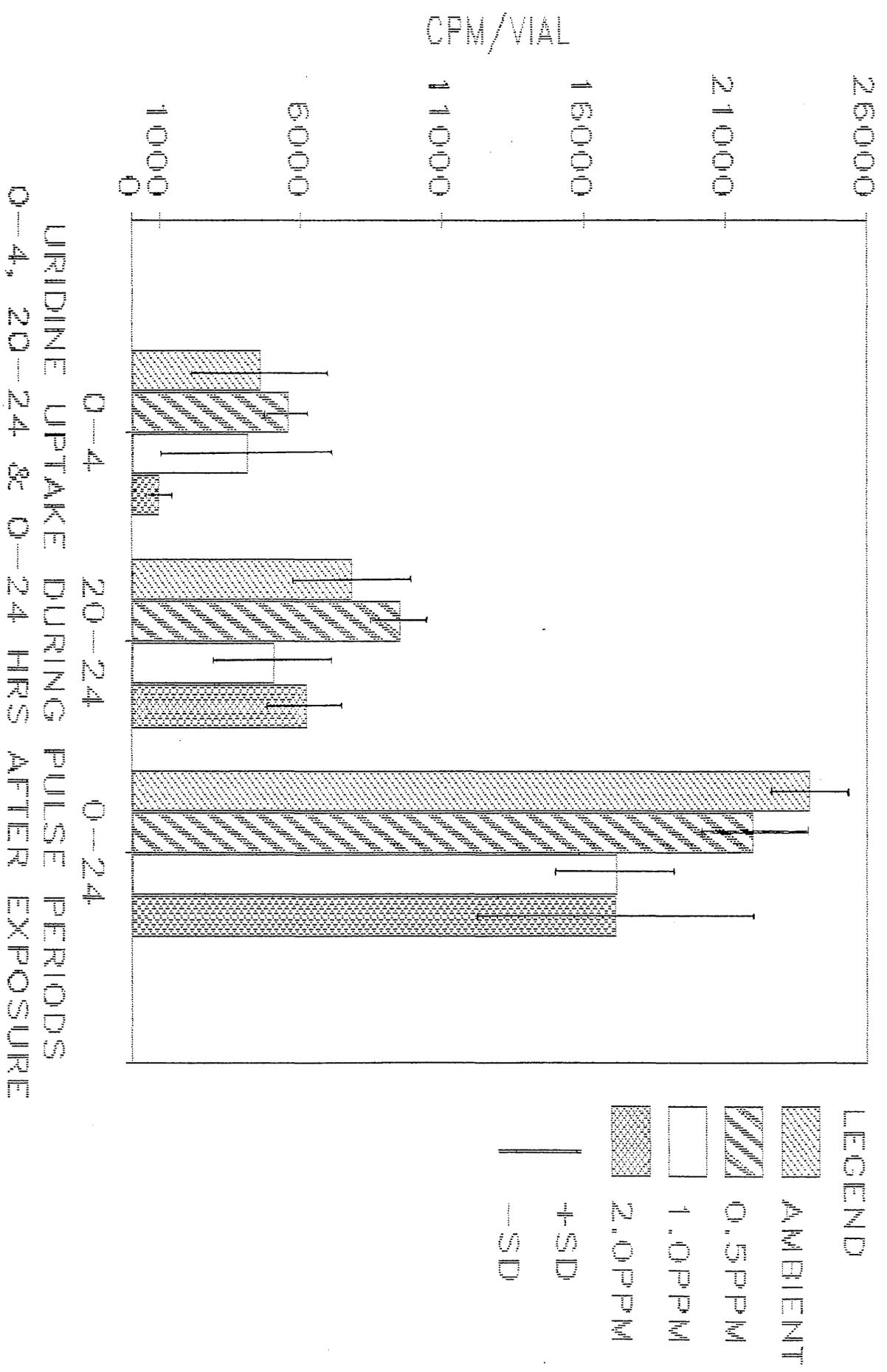


FIG 3: RNA SYNTHESIS IN HFL CELLS AFTER 96 HR OZONE EXPOSURE

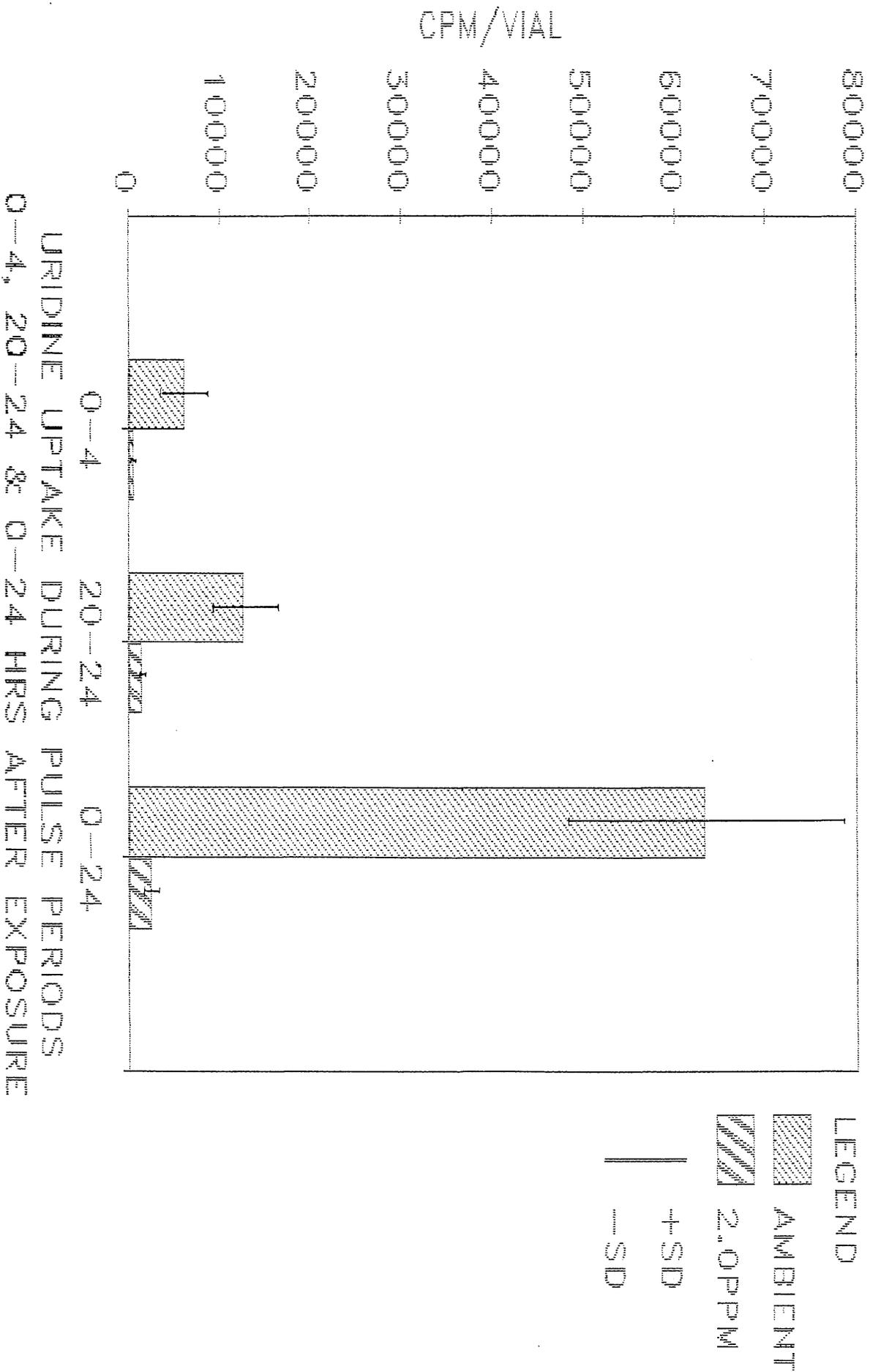


FIG 4: DNA SYNTHESIS IN HEL CELLS AFTER 24 HR OZONE EXPOSURE

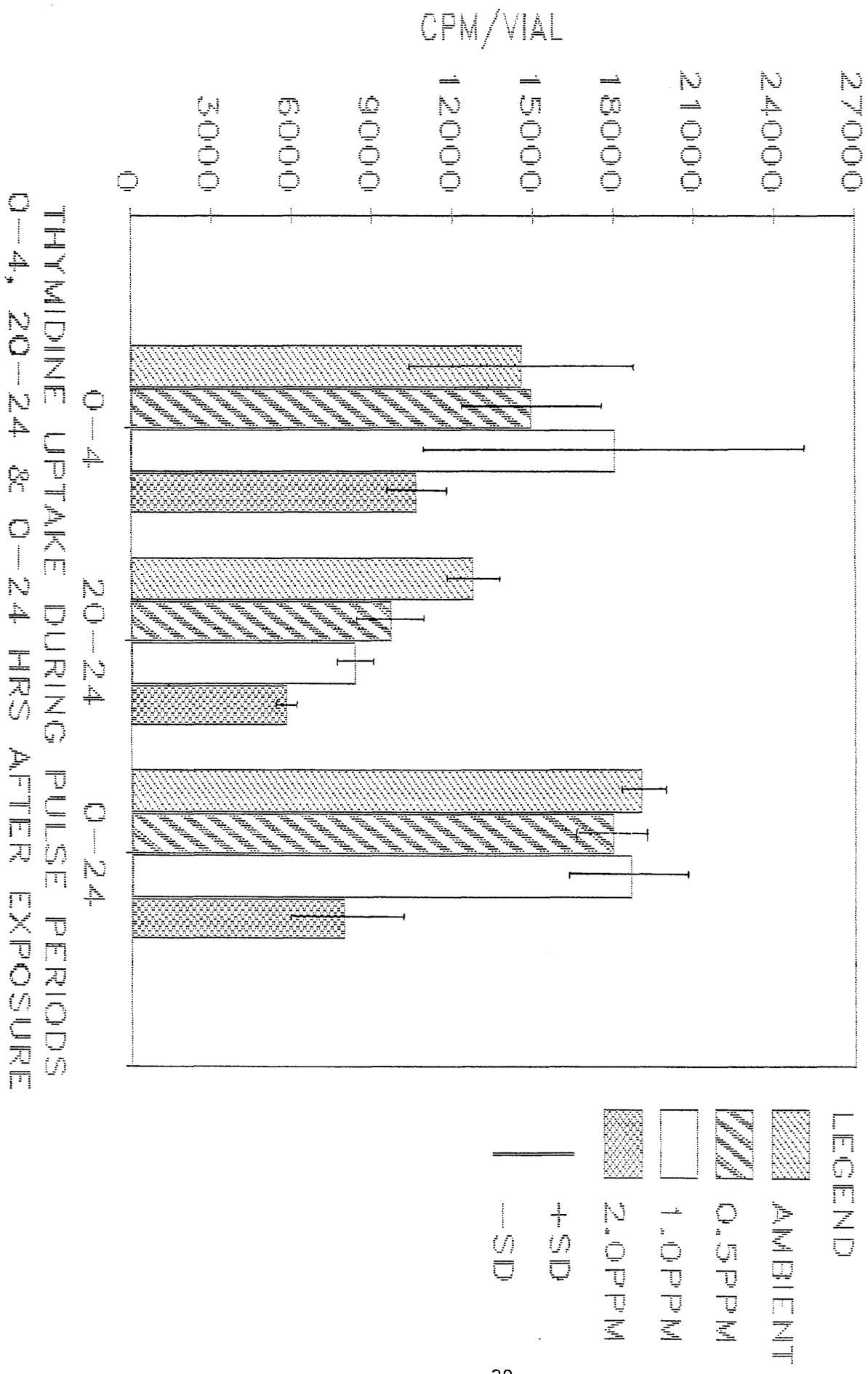


FIG 5: DNA SYNTHESIS IN HFL CELLS AFTER 4.8 HR OZONE EXPOSURE

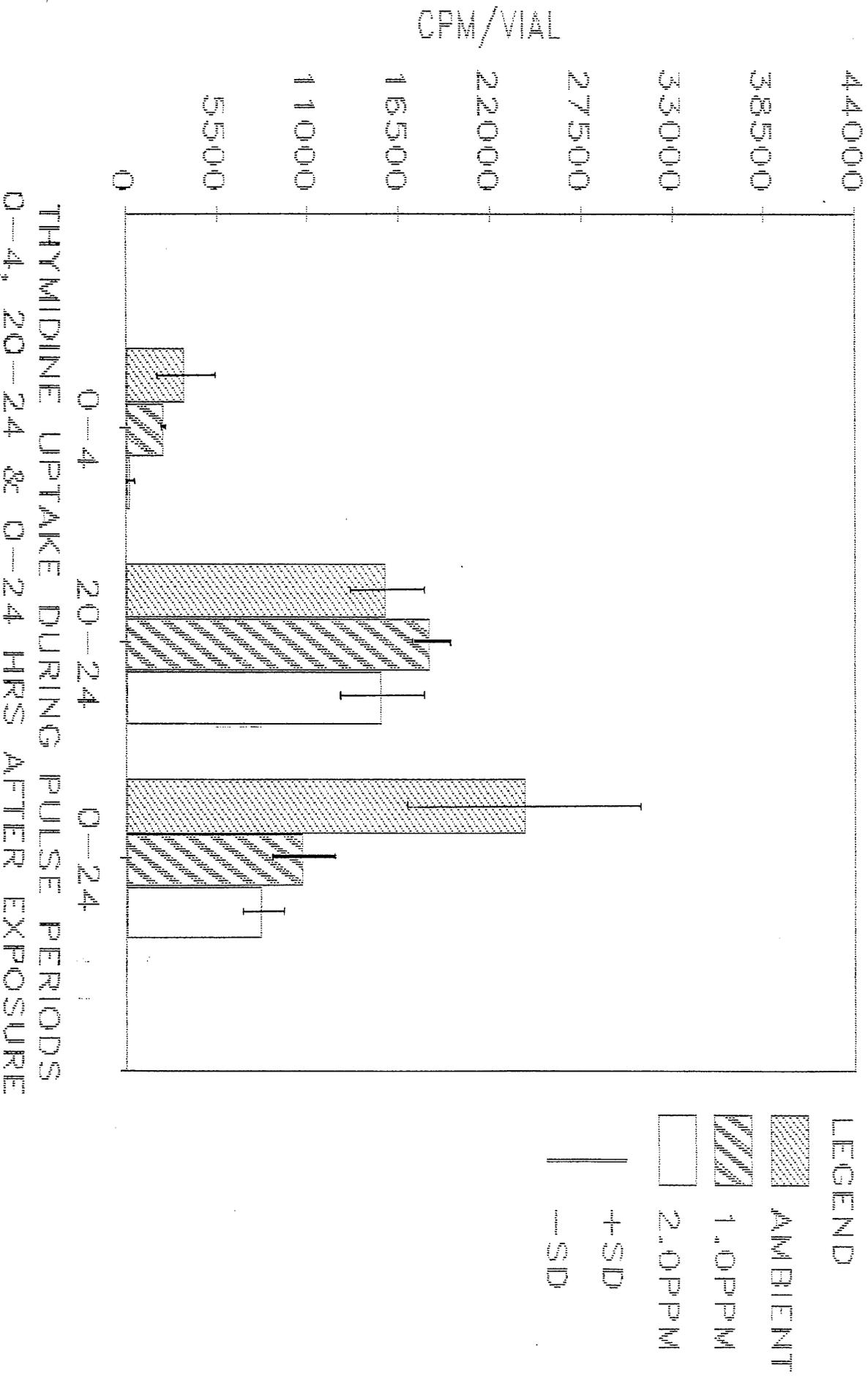


FIG 6: DNA SYNTHESIS IN HFL CELLS AFTER 96 HR OZONE EXPOSURE

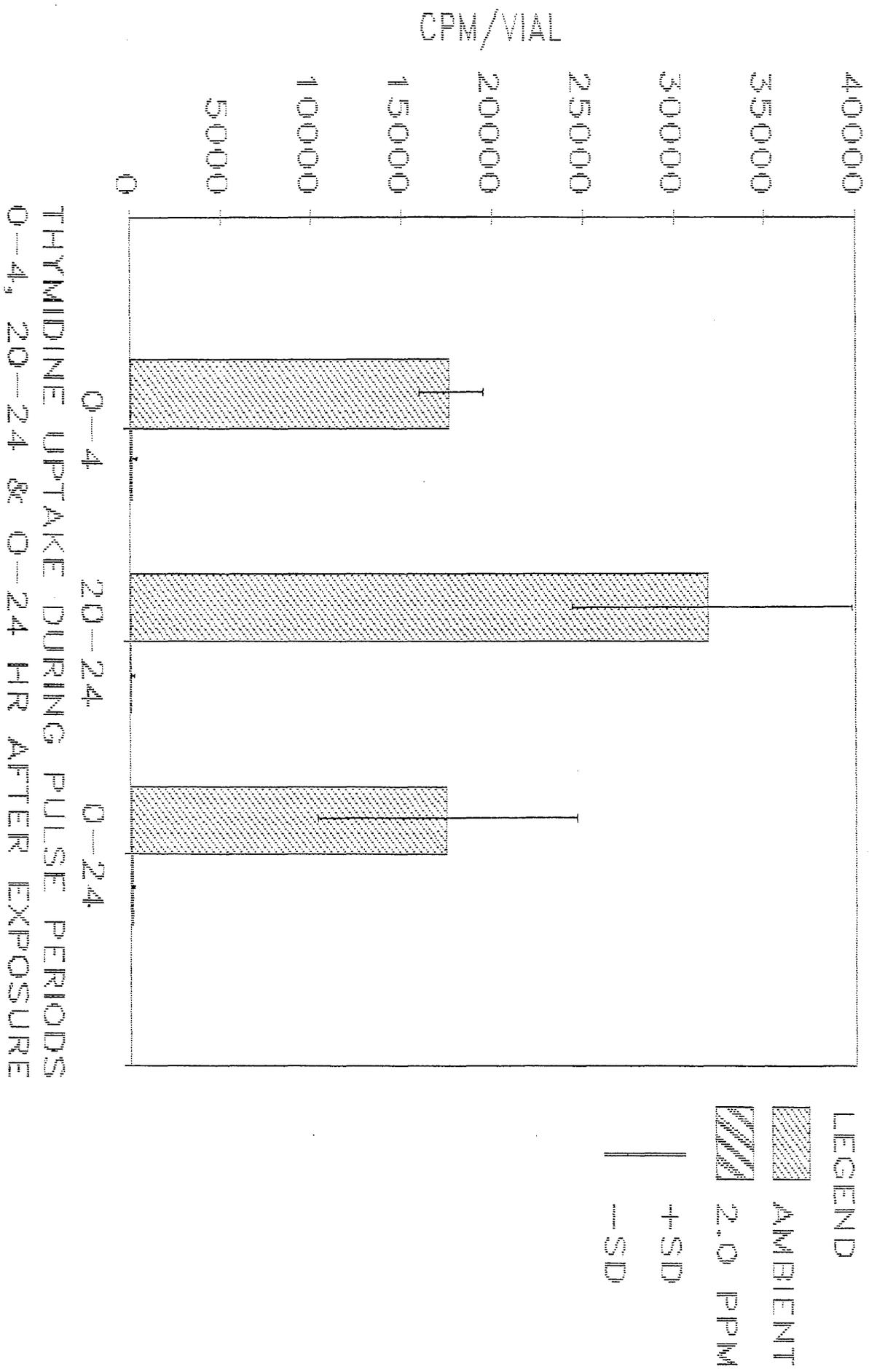


FIG 7: PROTEIN SYNTHESIS IN HFL CELLS AFTER 24 HR OZONE EXPOSURE

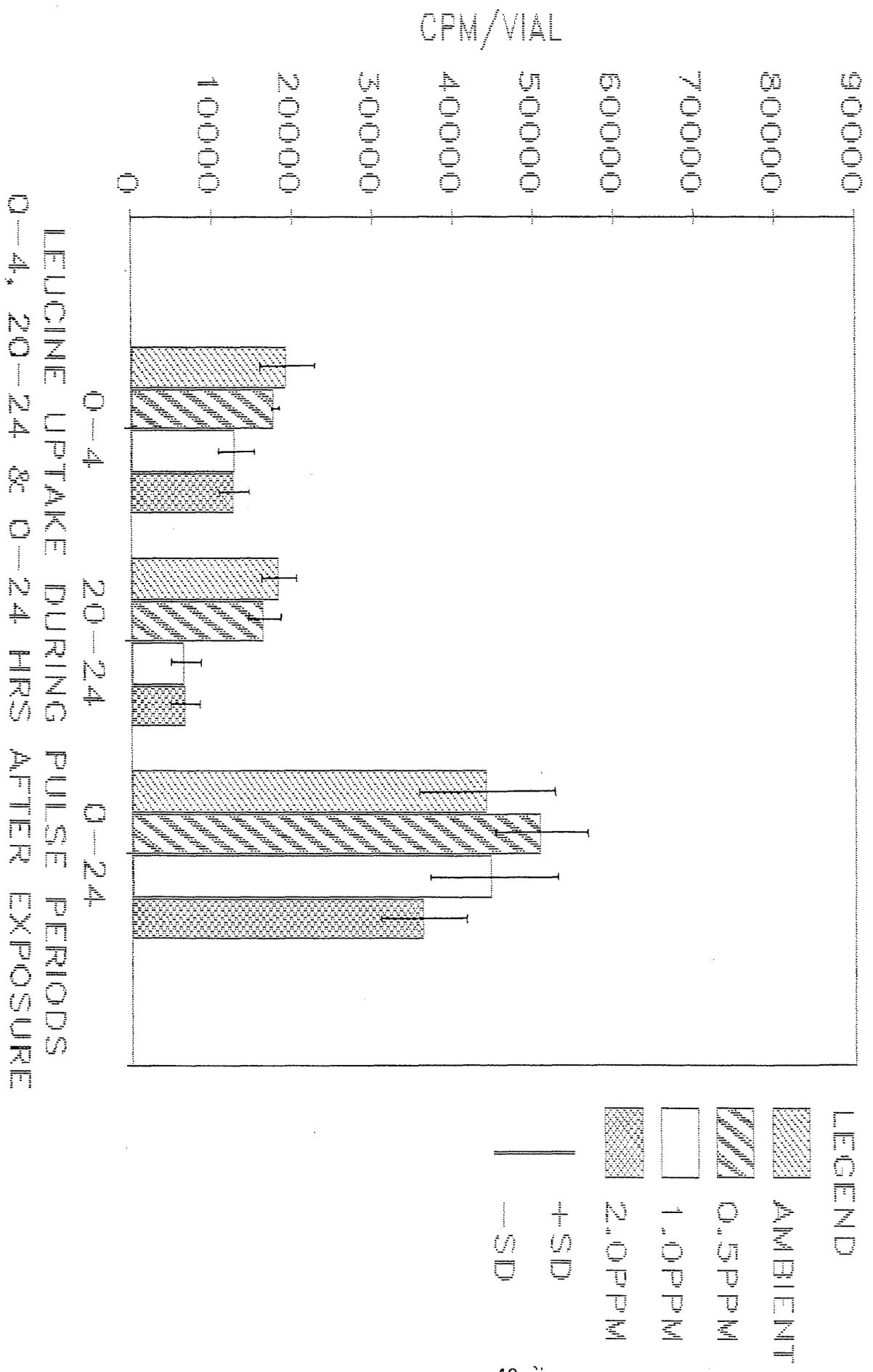


FIG 8: PROTEIN SYNTHESIS IN HFL CELLS AFTER 48 HR OZONE EXPOSURE

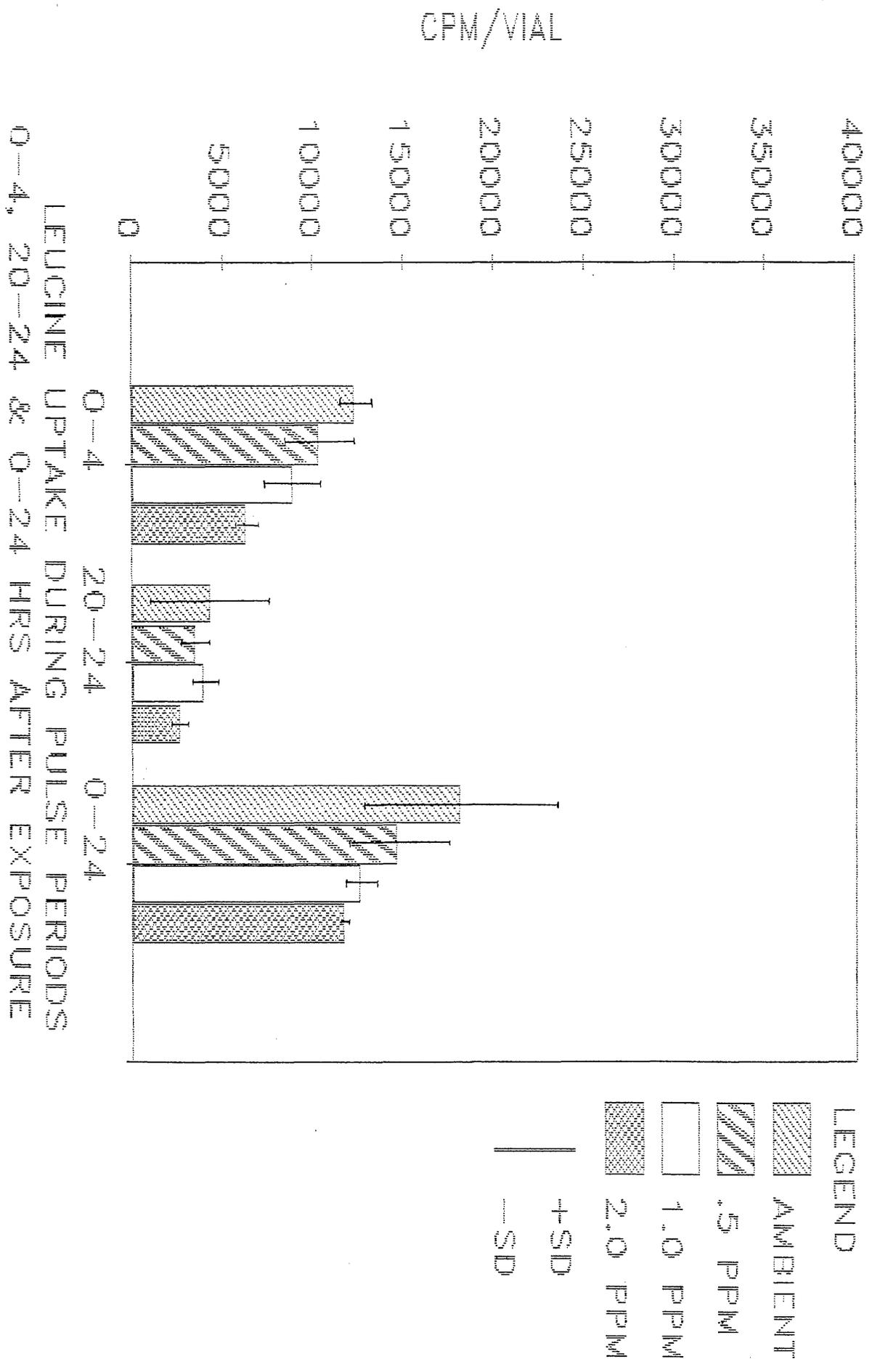


FIG 9: PROTEIN SYNTHESIS IN HFL CELLS AFTER 96 HR OZONE EXPOSURE

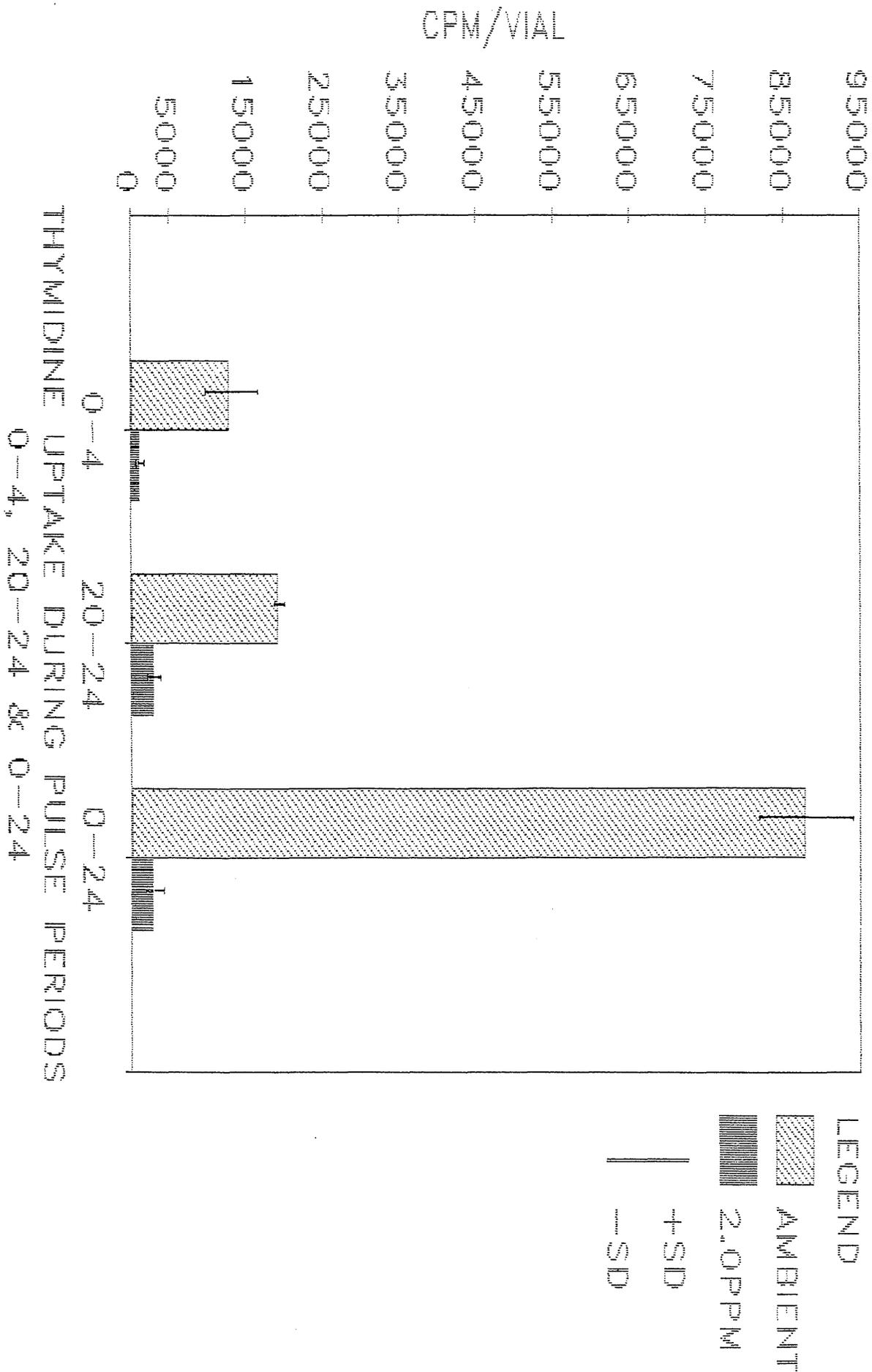


FIG 10: RNA SYNTHESIS IN BT CELLS AFTER 24 HR OZONE EXPOSURE

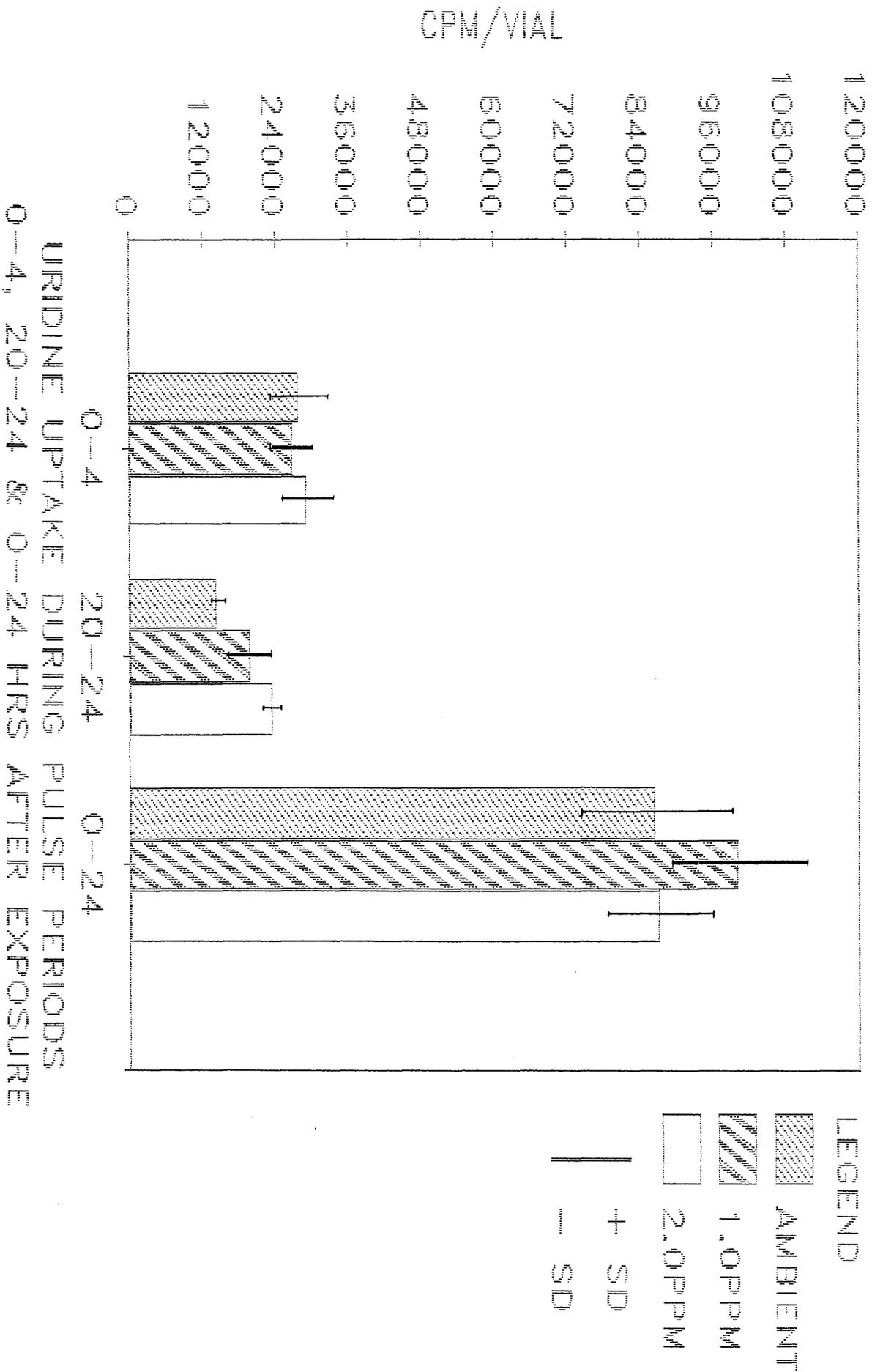


FIG 11: RNA SYNTHESIS IN BT CELLS AFTER 48 HR OZONE EXPOSURE

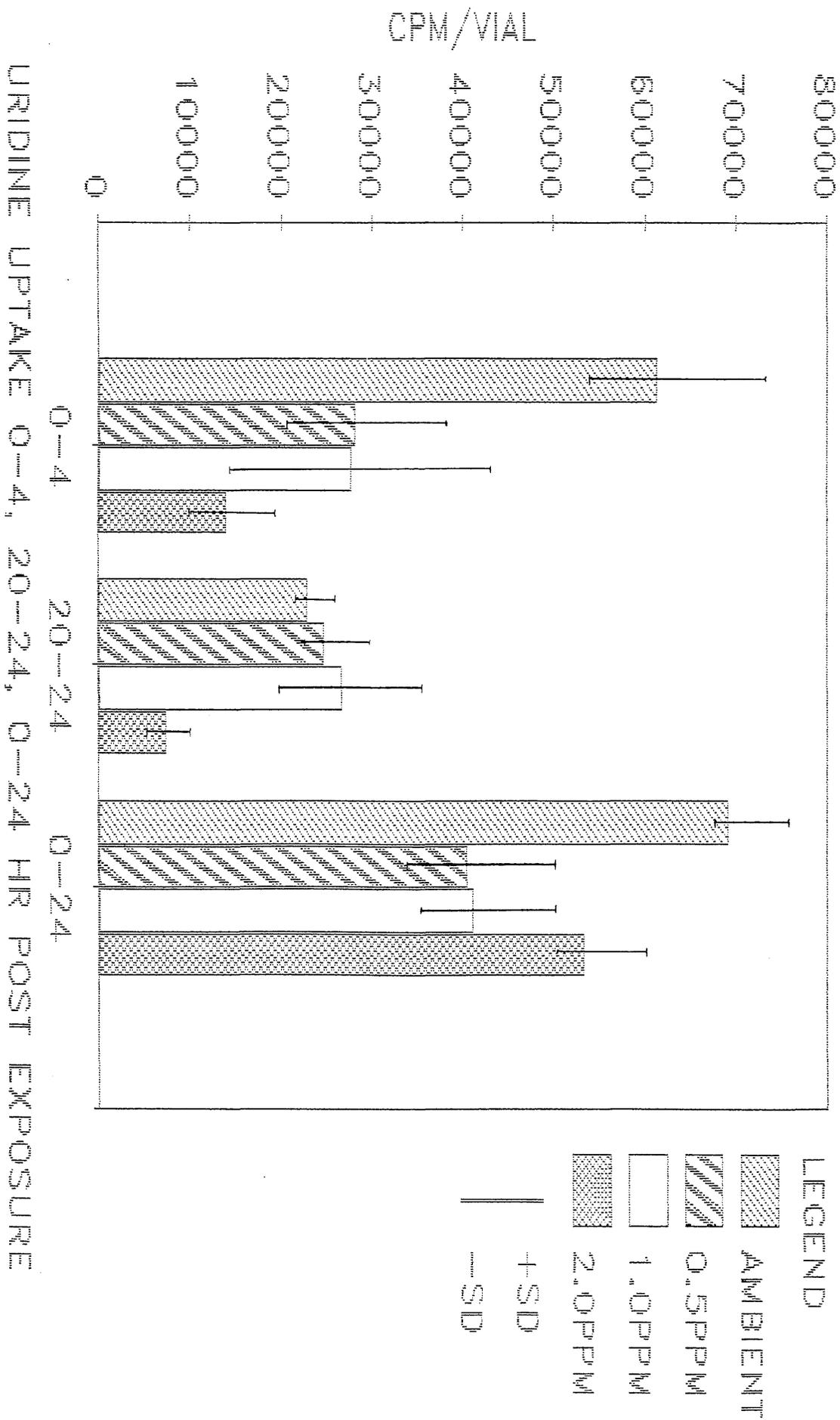


FIG 12: RNA SYNTHESIS IN BT CELLS AFTER 96 HR OZONE EXPOSURE

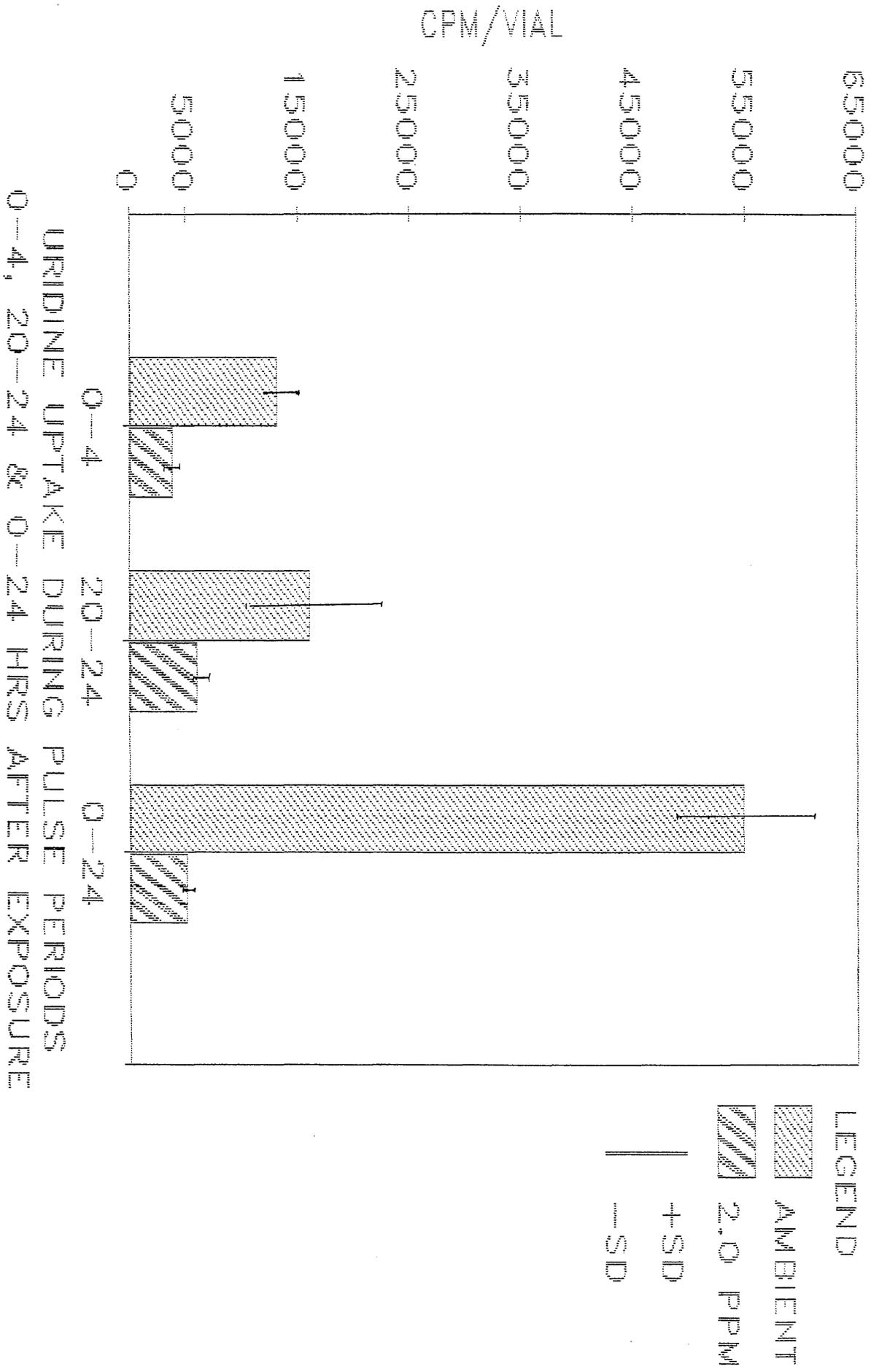


FIG 13: DNA SYNTHESIS IN BT CELLS AFTER 24 HR OZONE EXPOSURE

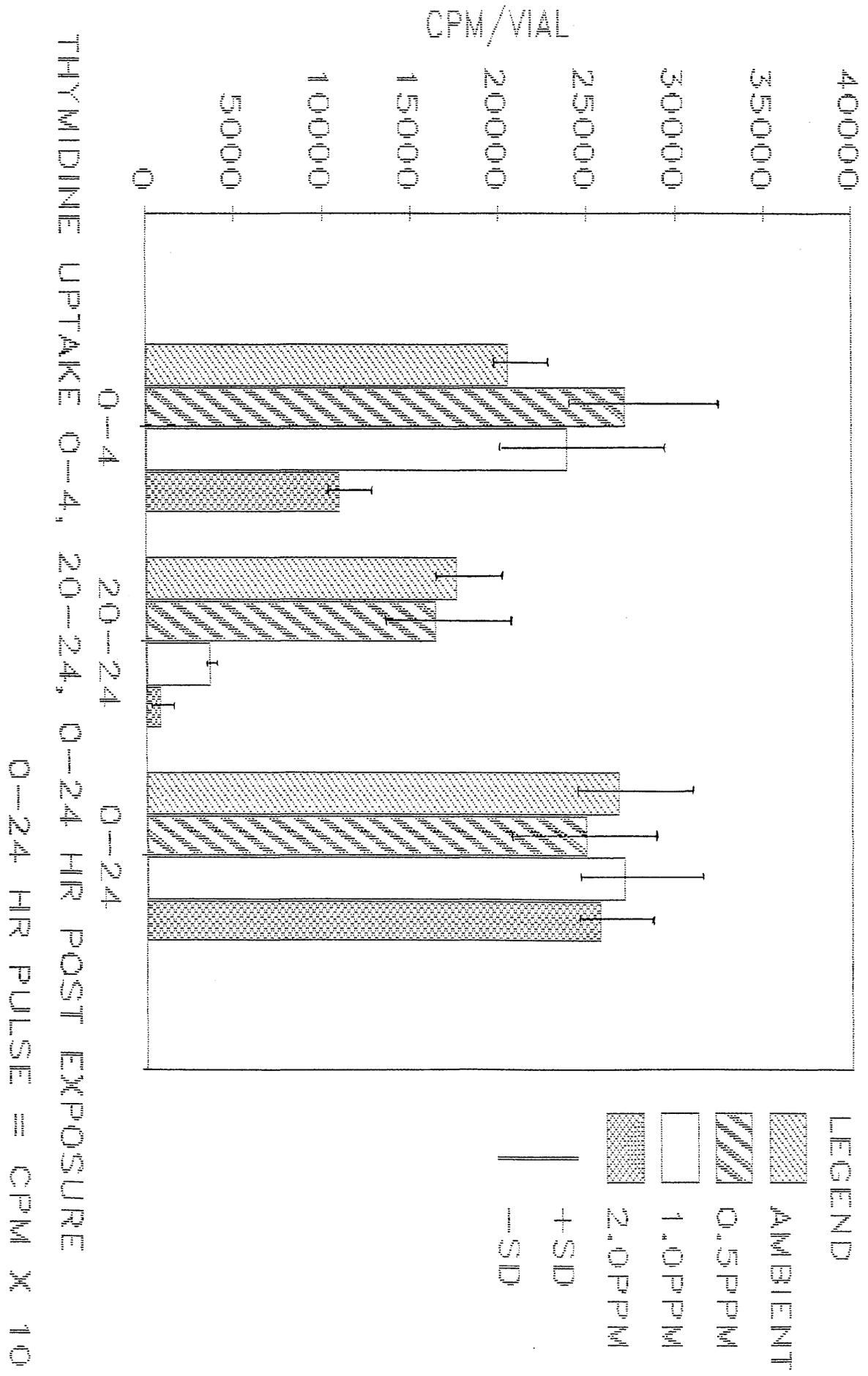
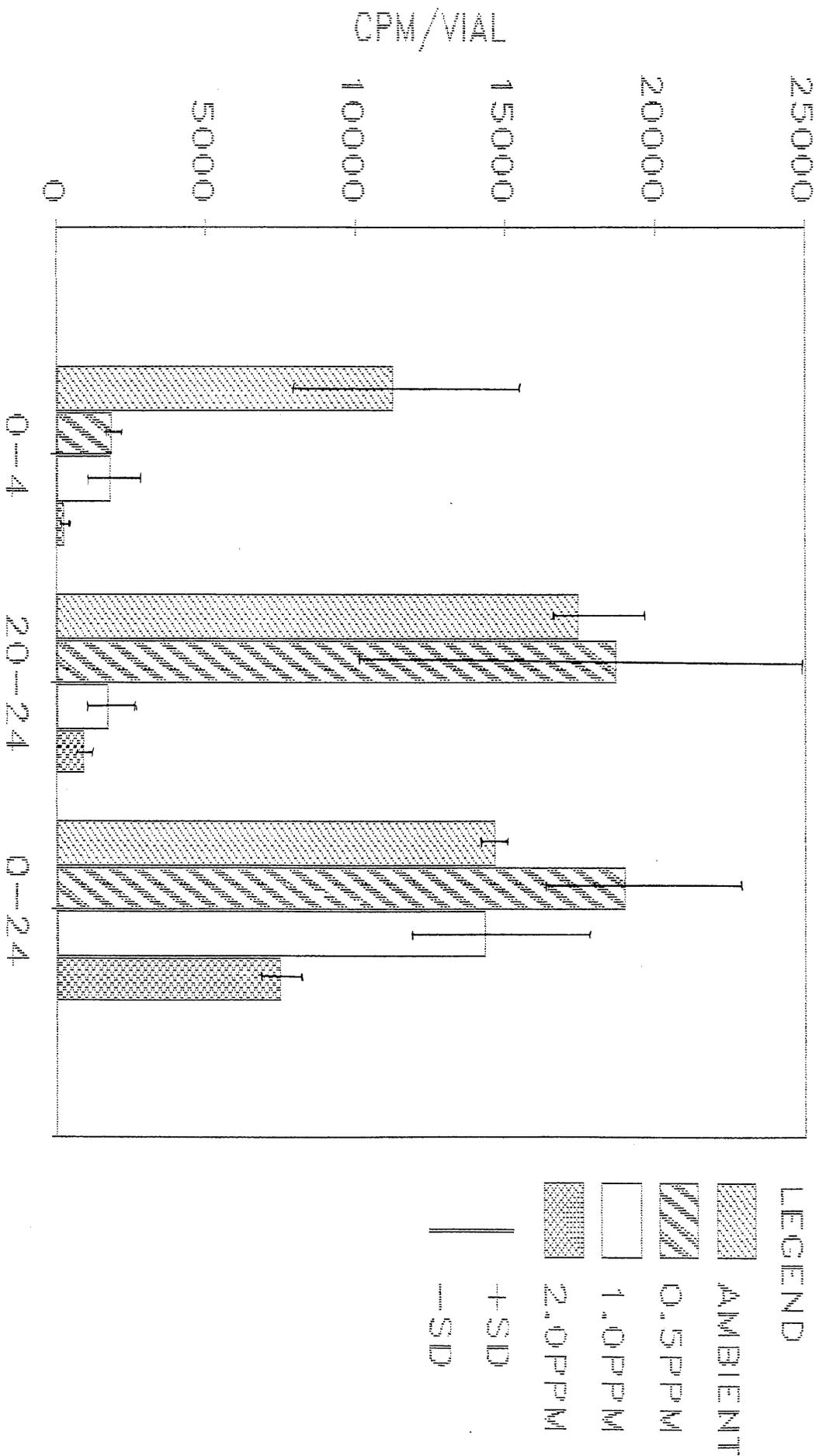


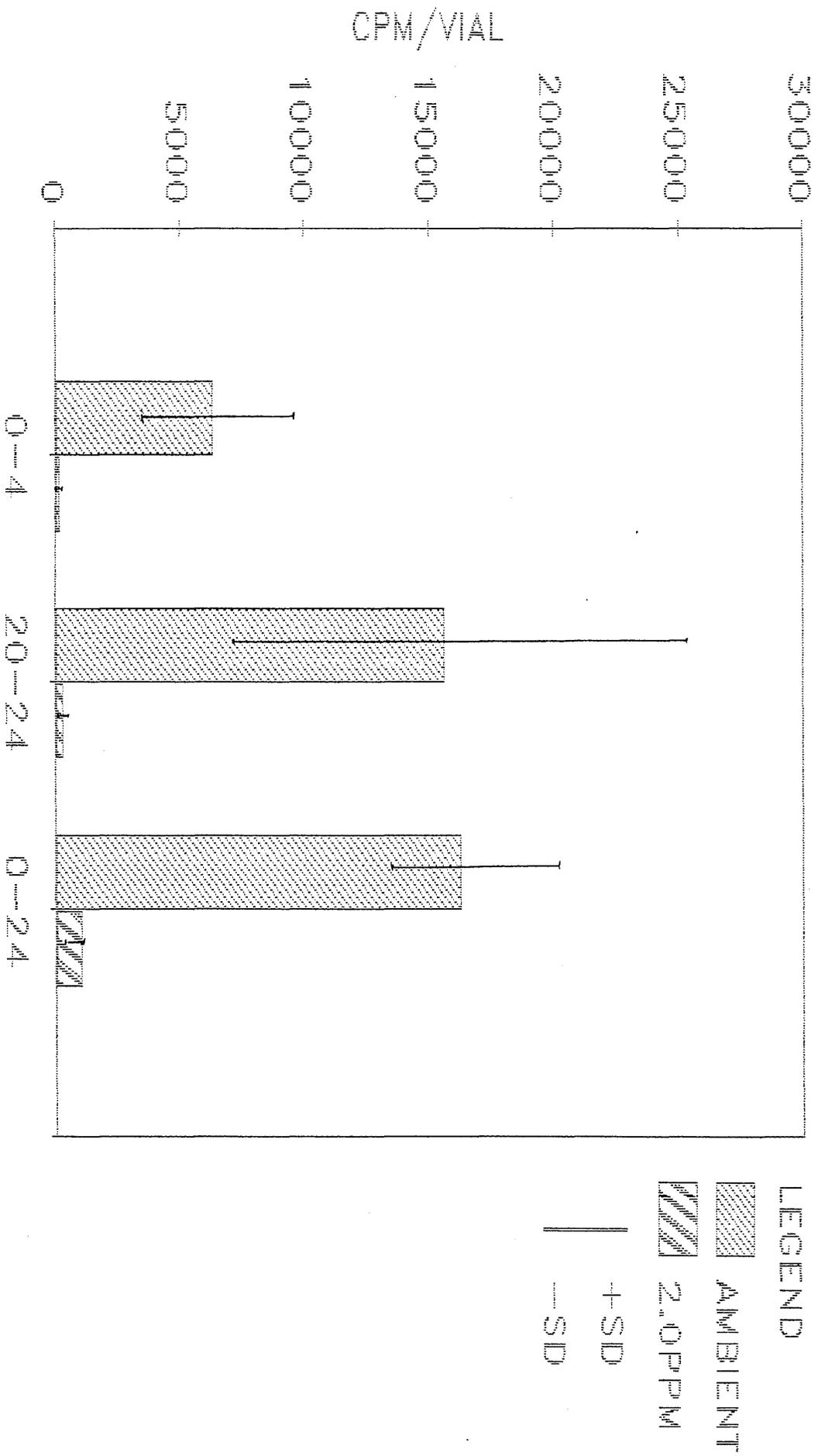
FIG 14: DNA SYNTHESIS IN BT CELLS AFTER 48 HR OZONE EXPOSURE



THYMIDINE UPTAKE 0-4, 20-24, 0-24 HR POST EXPOSURE

0-24 HR PULSE = CPM X 10

FIG 15: DNA SYNTHESIS IN BT CELLS AFTER 96 HR OZONE EXPOSURE



THYMIDINE UPTAKE 0-4, 20-24, 0-24 HR POST EXPOSURE  
 20-24 HR AMBIENT = CPM X 10; 0-24 HR AMBIENT = CPM X 20

FIG 16: PROTEIN SYNTHESIS IN BT CELLS AFTER 24 HR OZONE EXPOSURE

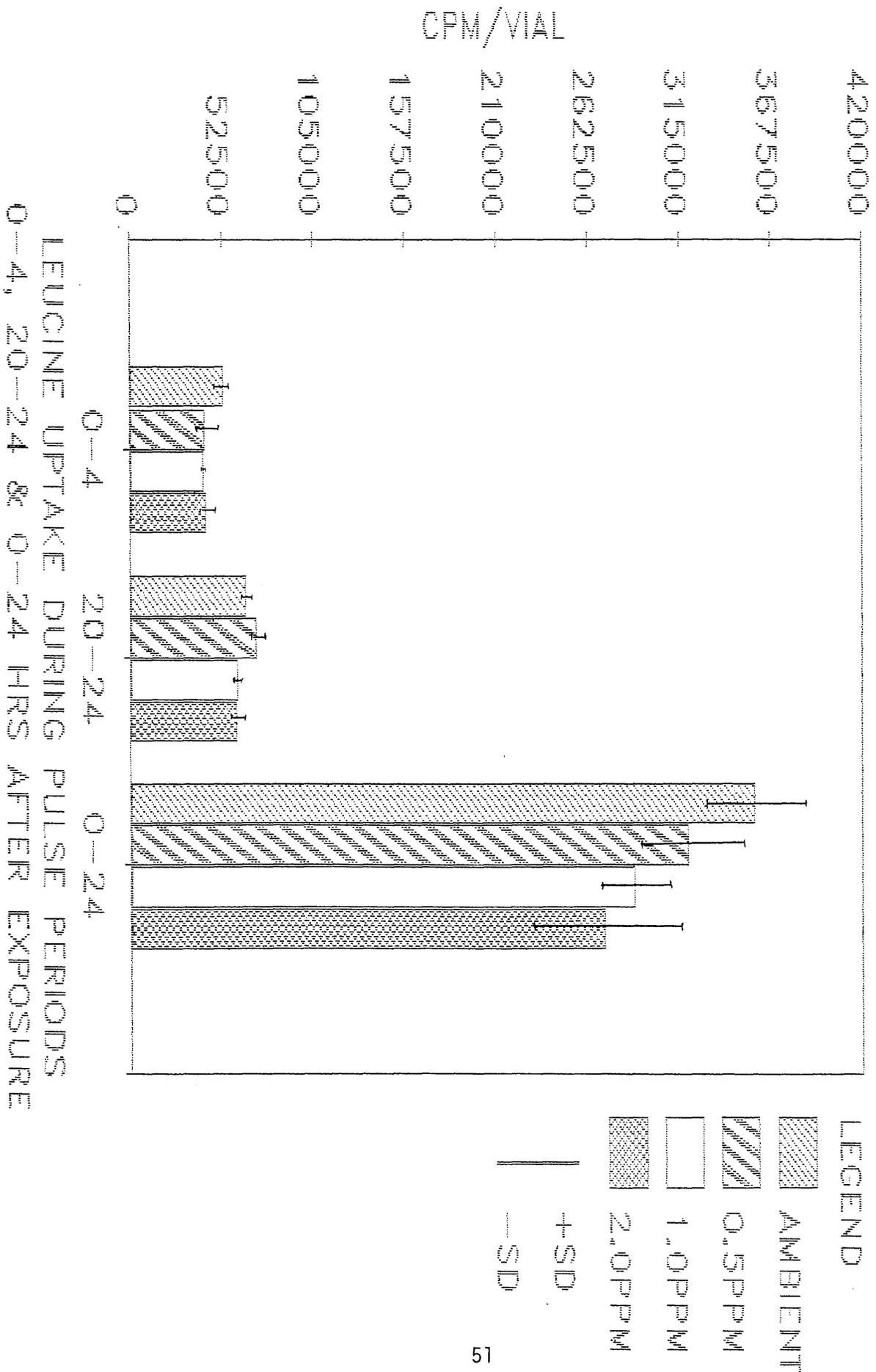


FIG 17: PROTEIN SYNTHESIS IN BT CELLS AFTER 48 HR OZONE EXPOSURE

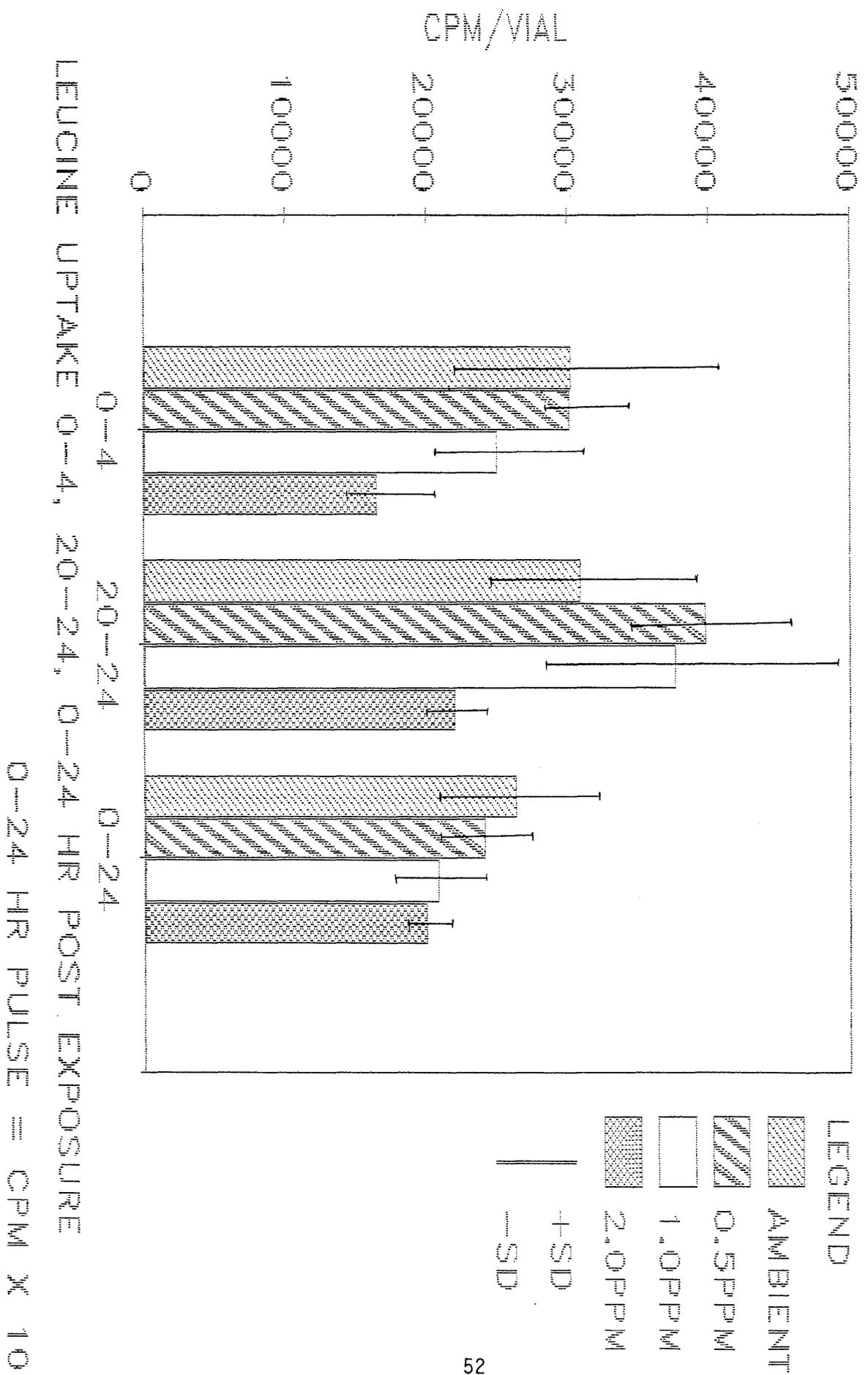


FIG 18: PROTEIN SYNTHESIS IN BT CELLS AFTER 96 HR OZONE EXPOSURE

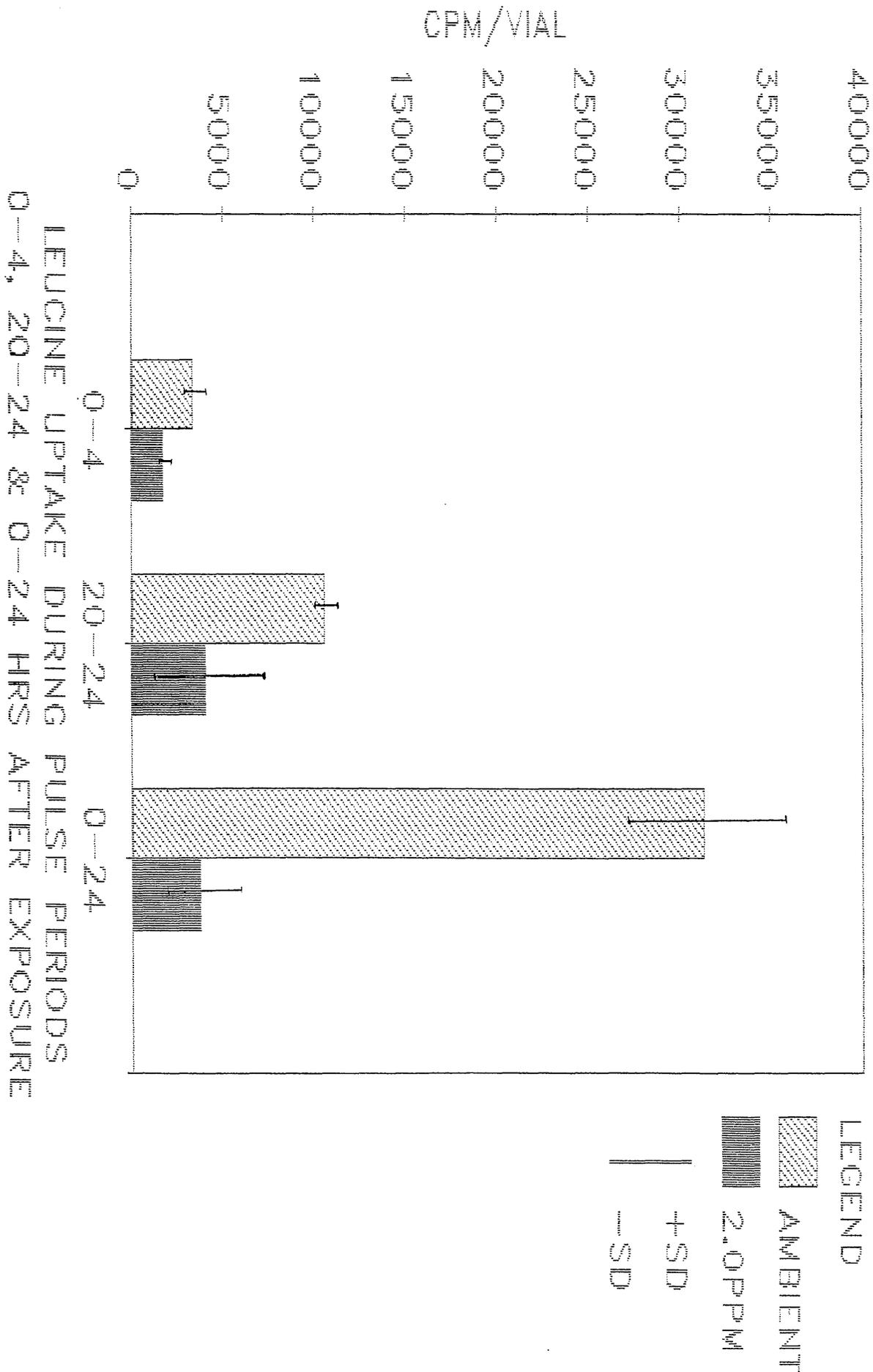


FIG 19: RNA SYNTHESIS IN L CELLS AFTER 24 HR OZONE EXPOSURE

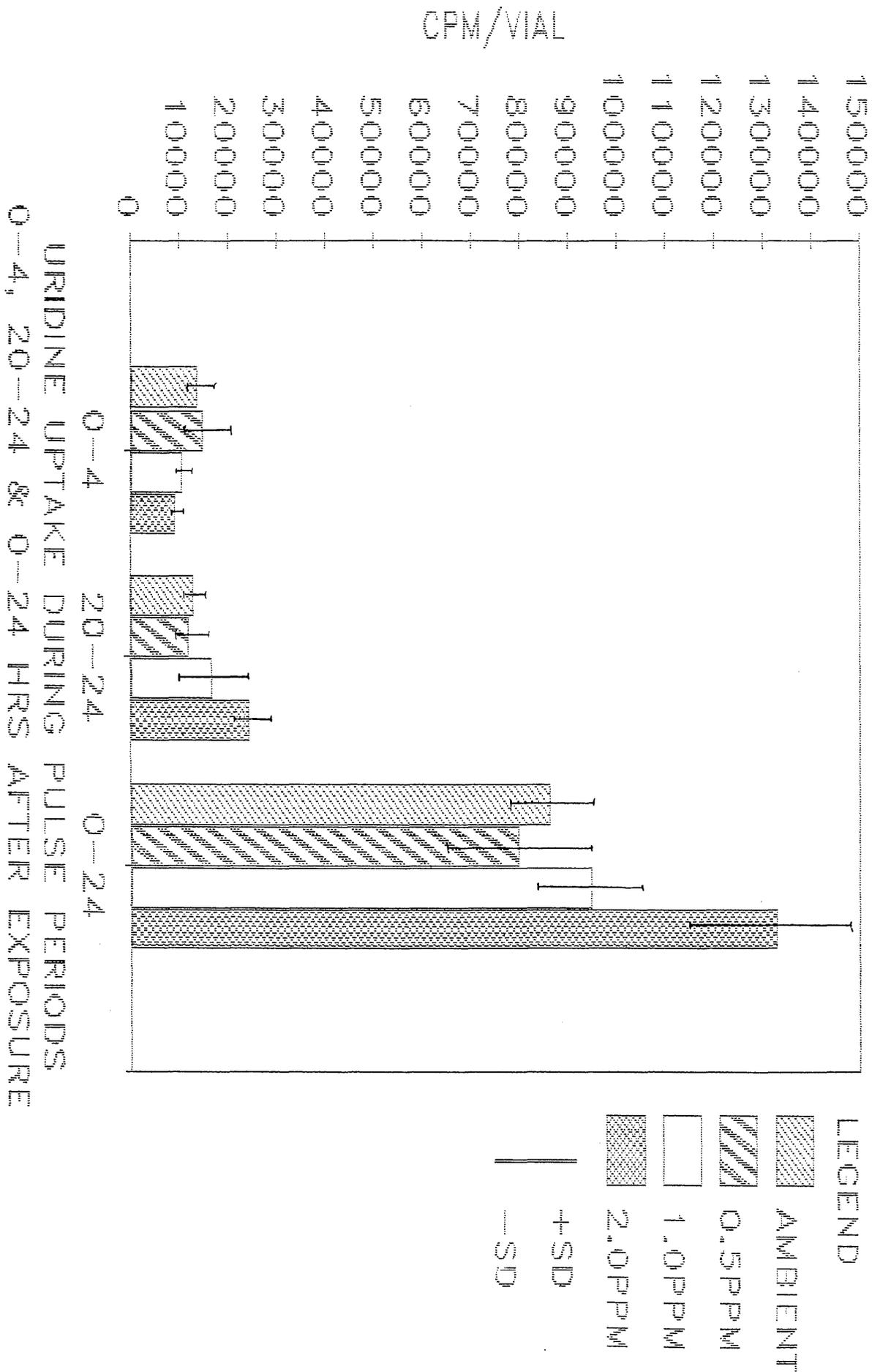


FIG 20: RNA SYNTHESIS IN L929 CELLS AFTER 48 HR OZONE EXPOSURE

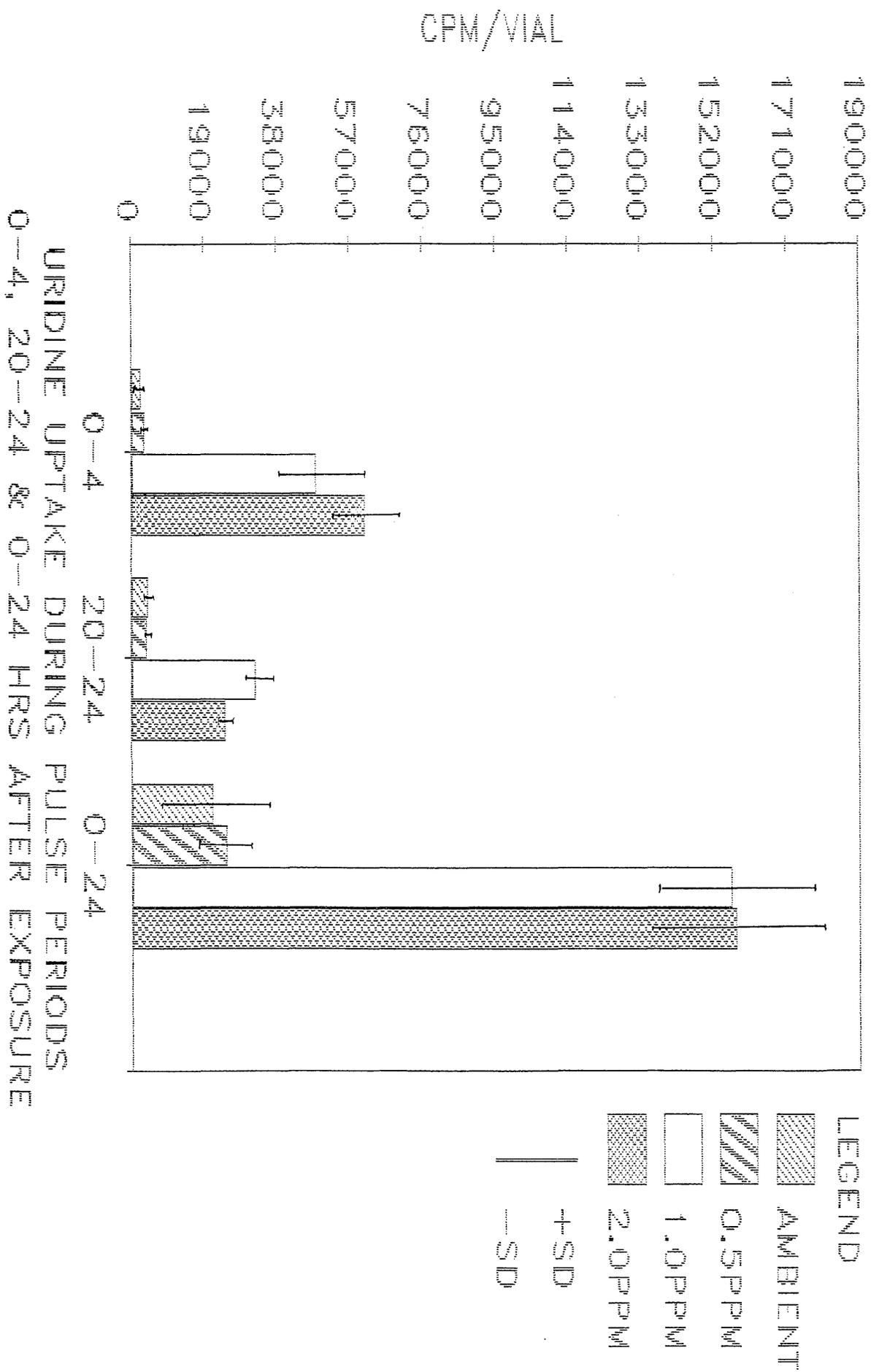


FIG 21: RNA SYNTHESIS IN L929 CELLS AFTER 72 HR OZONE EXPOSURE

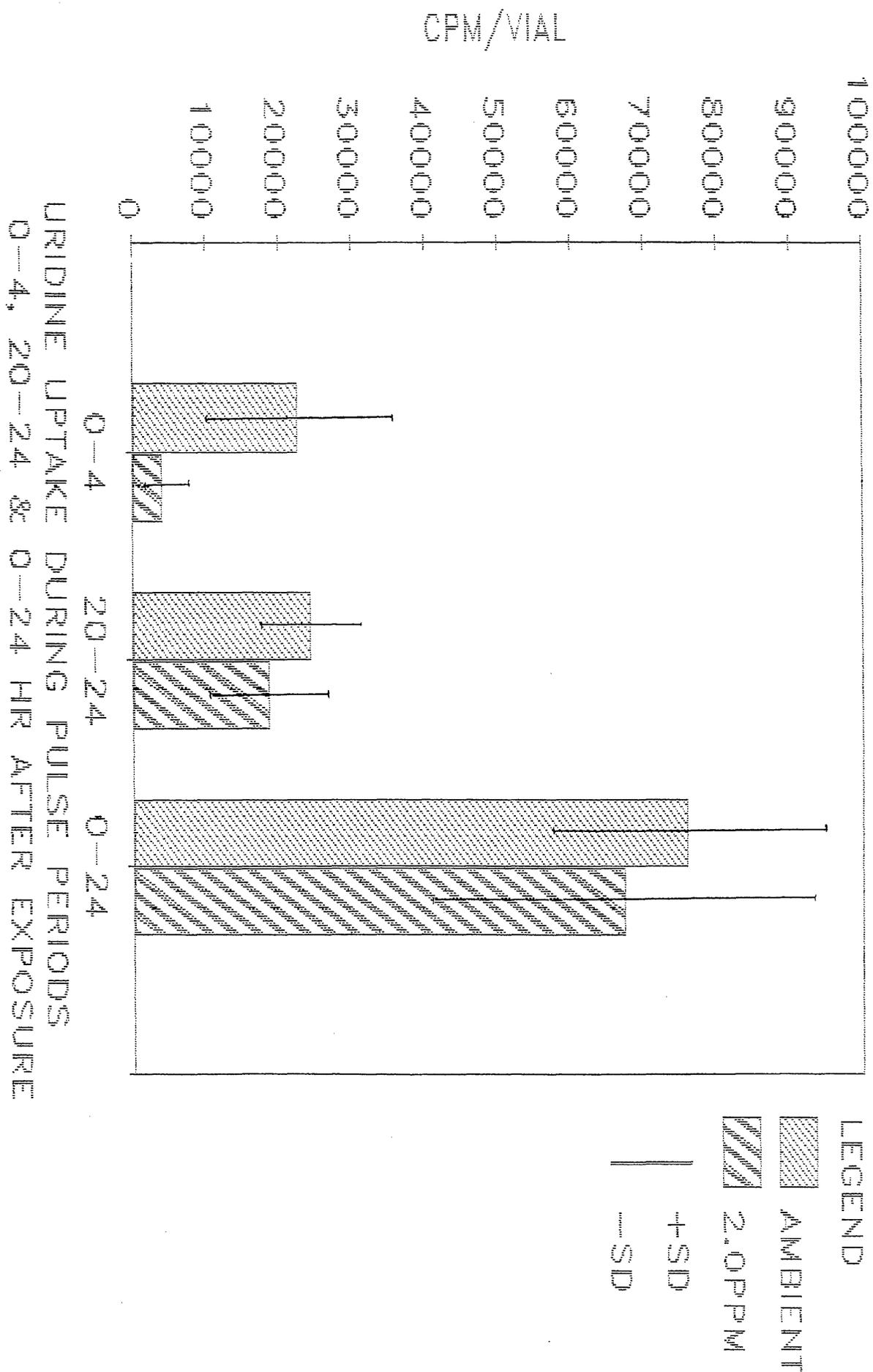


FIG 22: DNA SYNTHESIS IN L929 CELLS AFTER 24 HR OZONE EXPOSURE

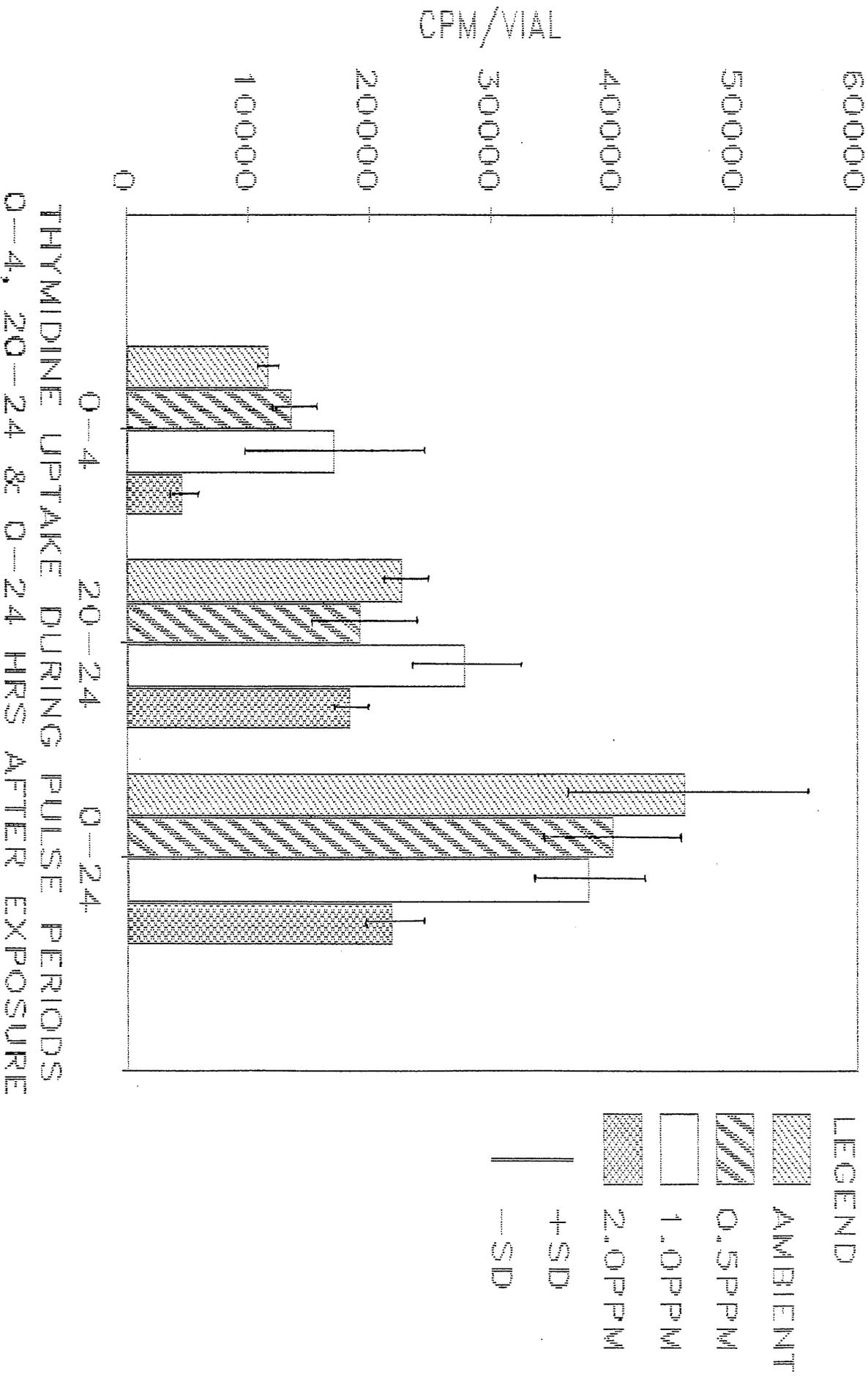


FIG 23: DNA SYNTHESIS IN L929 CELLS AFTER 48 HR OZONE EXPOSURE

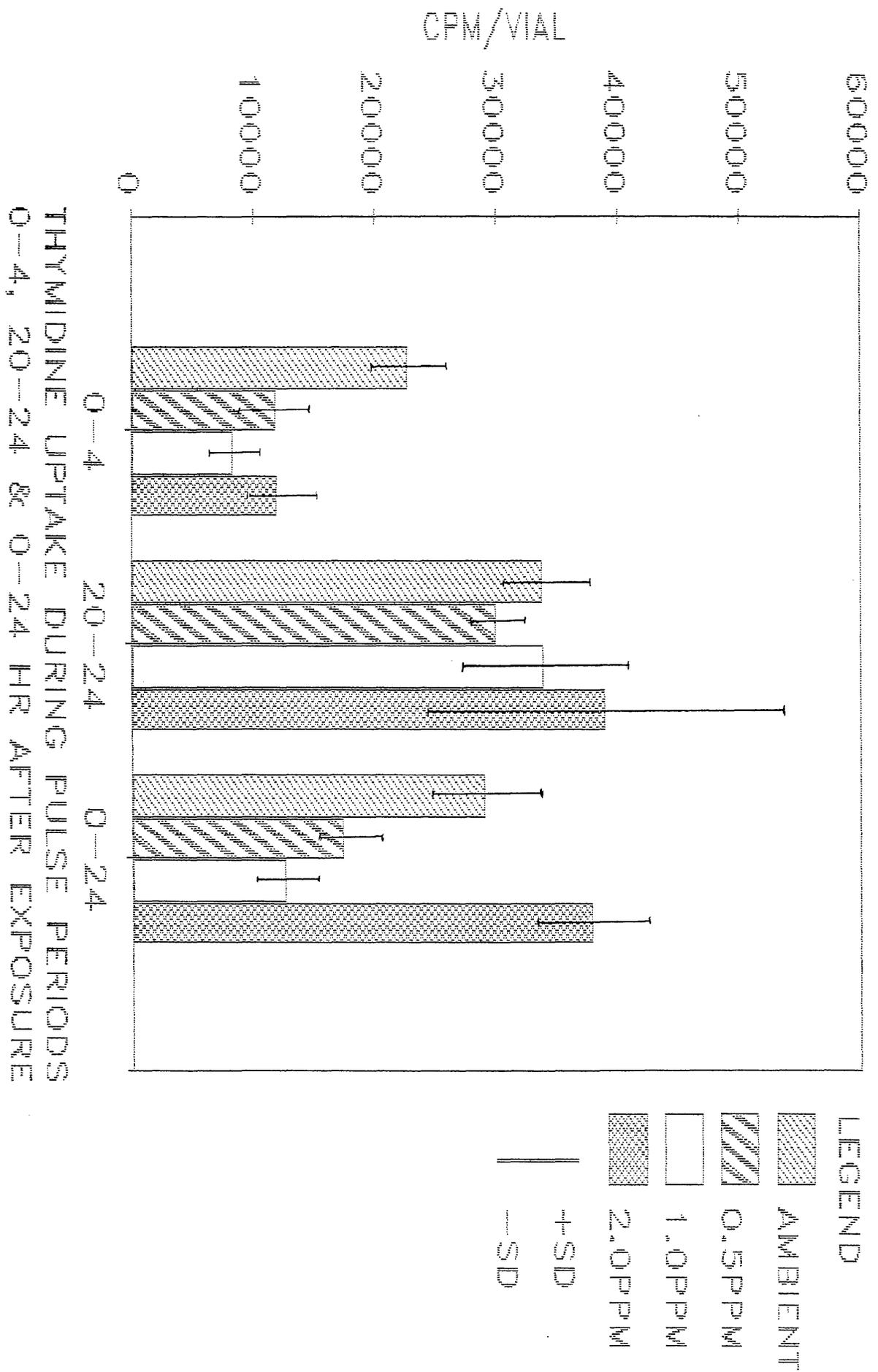


FIG 24: DNA SYNTHESIS IN L929 CELLS AFTER 72 HR OZONE EXPOSURE

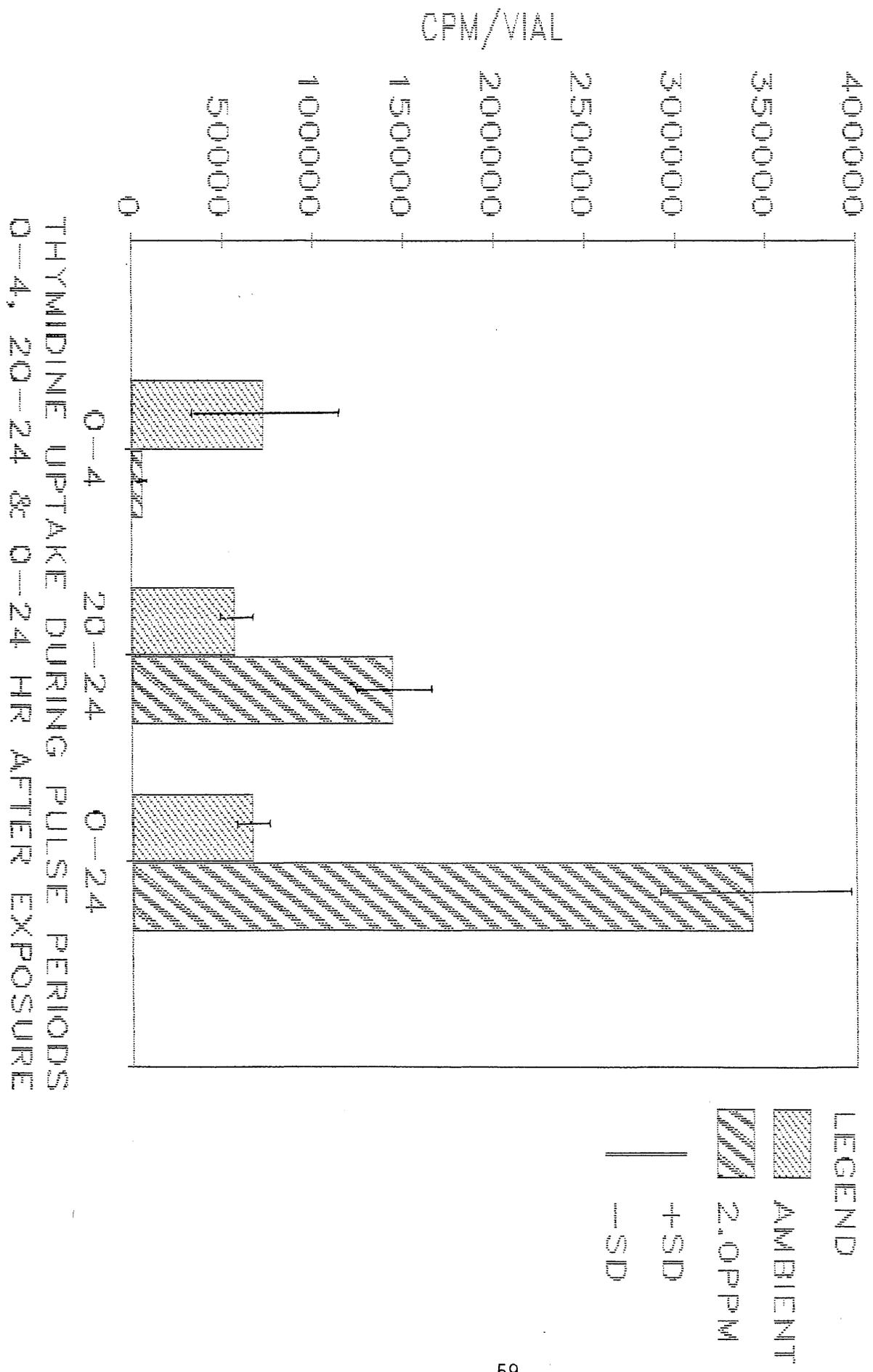


FIG 25: PROTEIN SYNTHESIS IN L929 CELLS AFTER 24 HR OZONE EXPOSURE

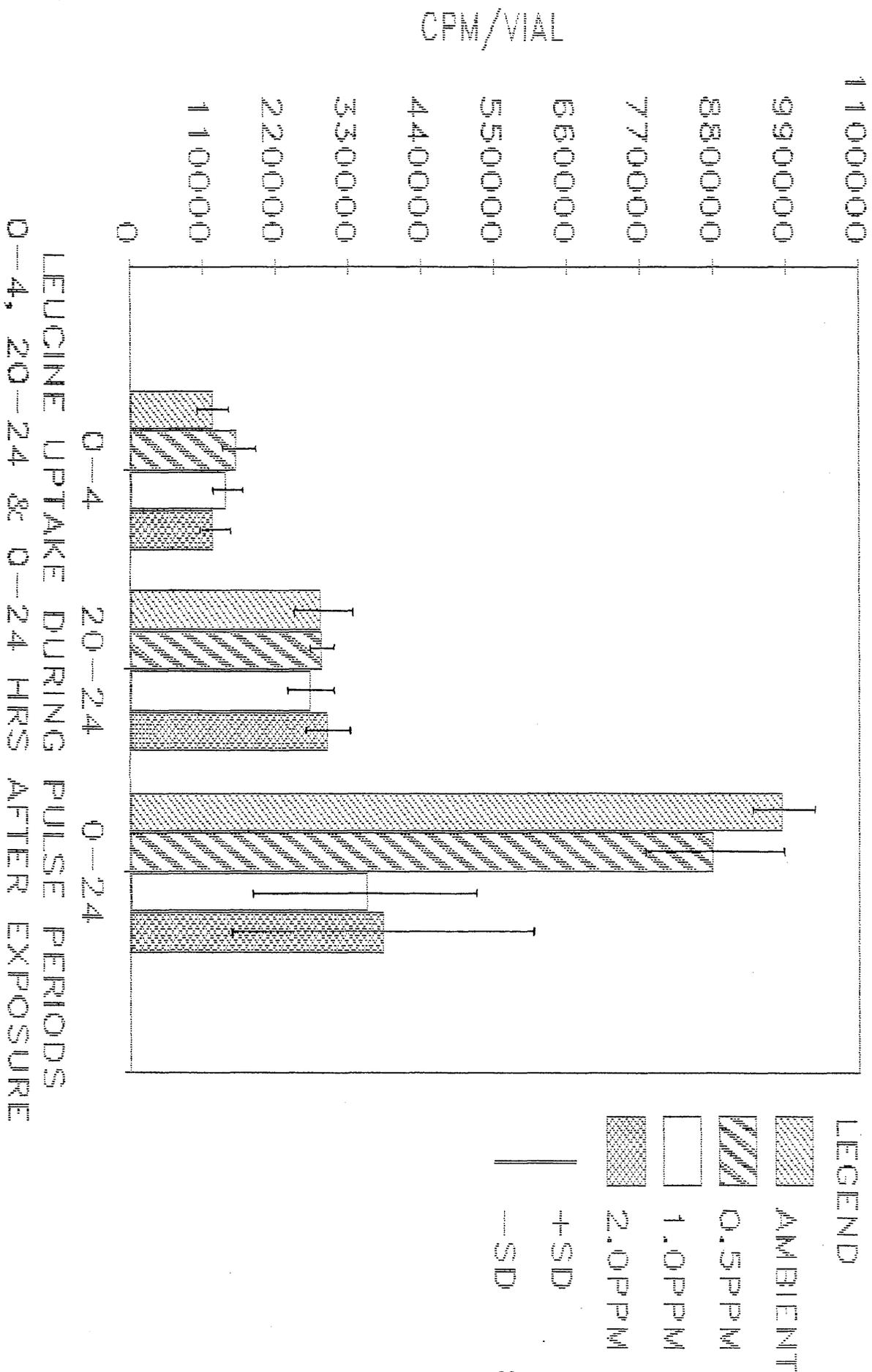


FIG 26: PROTEIN SYNTHESIS IN L929 CELLS AFTER 48 HR OZONE EXPOSURE

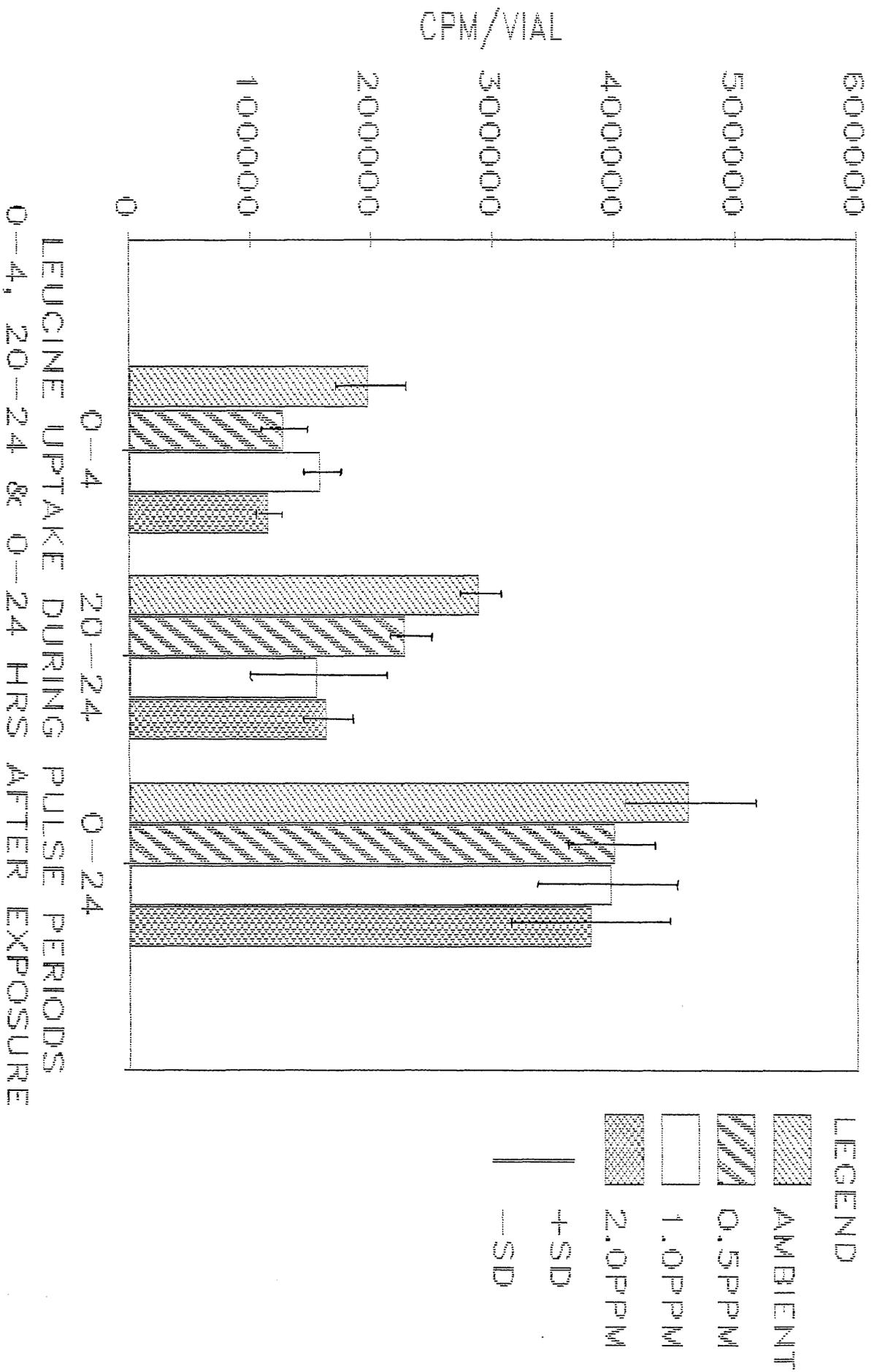
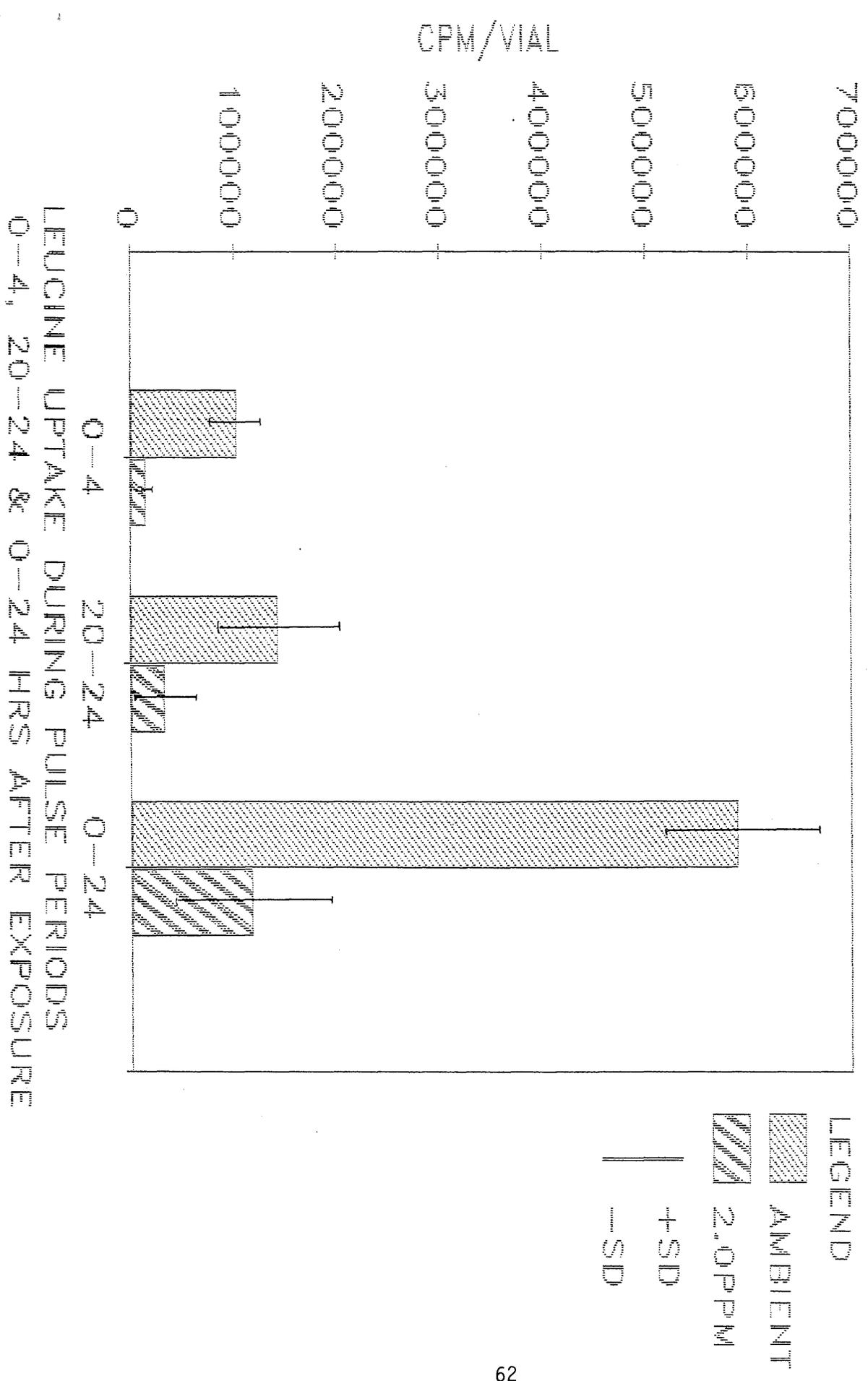
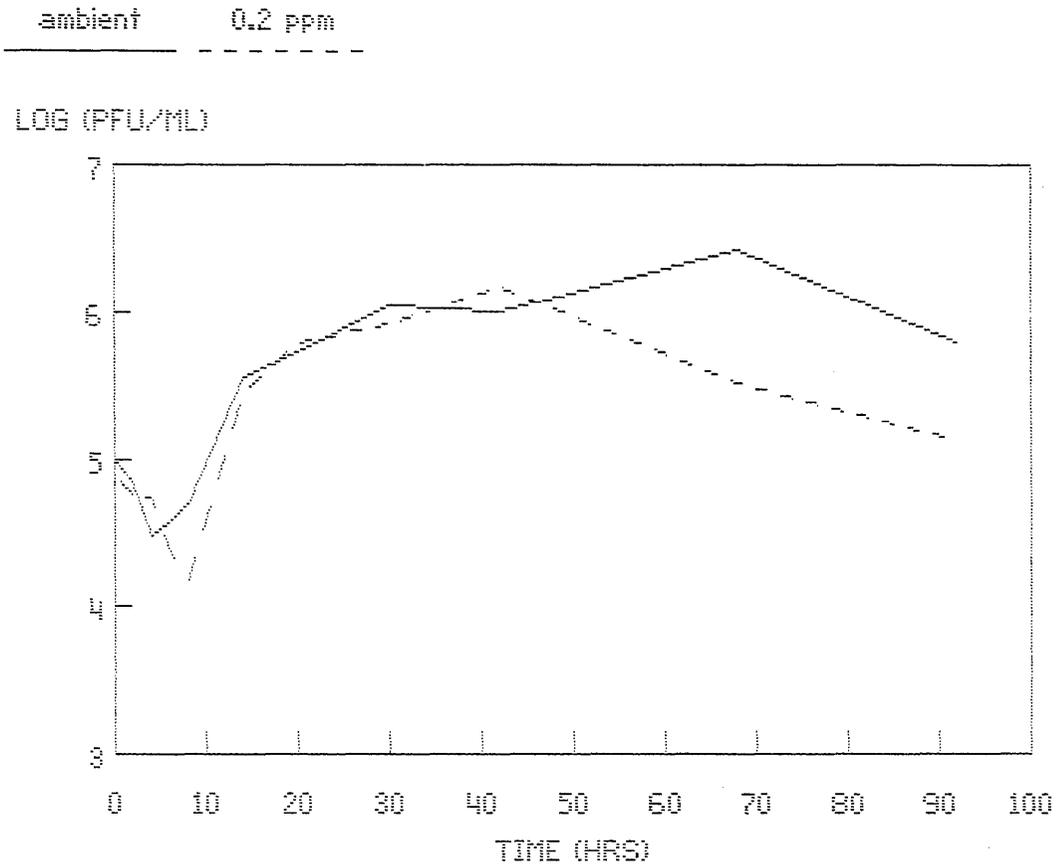


FIG 27: PROTEIN SYNTHESIS IN L929 CELLS AFTER 72 HR OZONE EXPOSURE



APPENDIX B  
EFFECTS OF OZONE ON VIRAL REPLICATION

FIG 1: WSN REPLICATION IN HFL CELLS  
24 HRS PRIOR OZONE EXPOSURE



# FIG 2: WSN REPLICATION IN HFL CELLS

24 HR PRIOR OZONE EXPOSURE

ambient      0.5 ppm

LOG (PFU/ML)

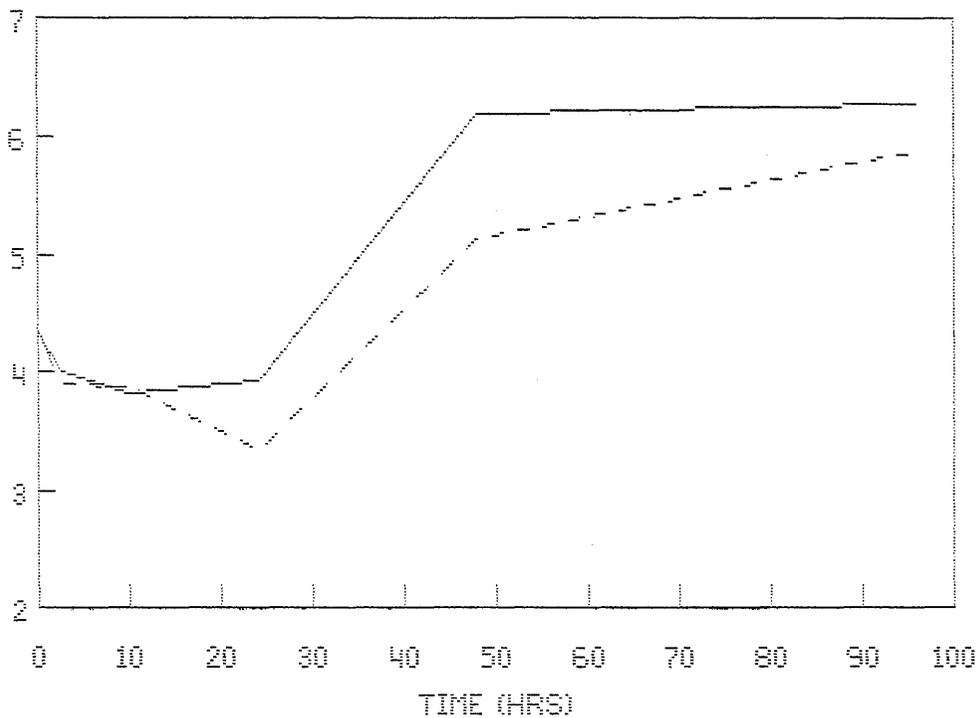


FIG 3: WSN REPLICATION IN HFL CELLS  
24 HRS PRIOR OZONE EXPOSURE

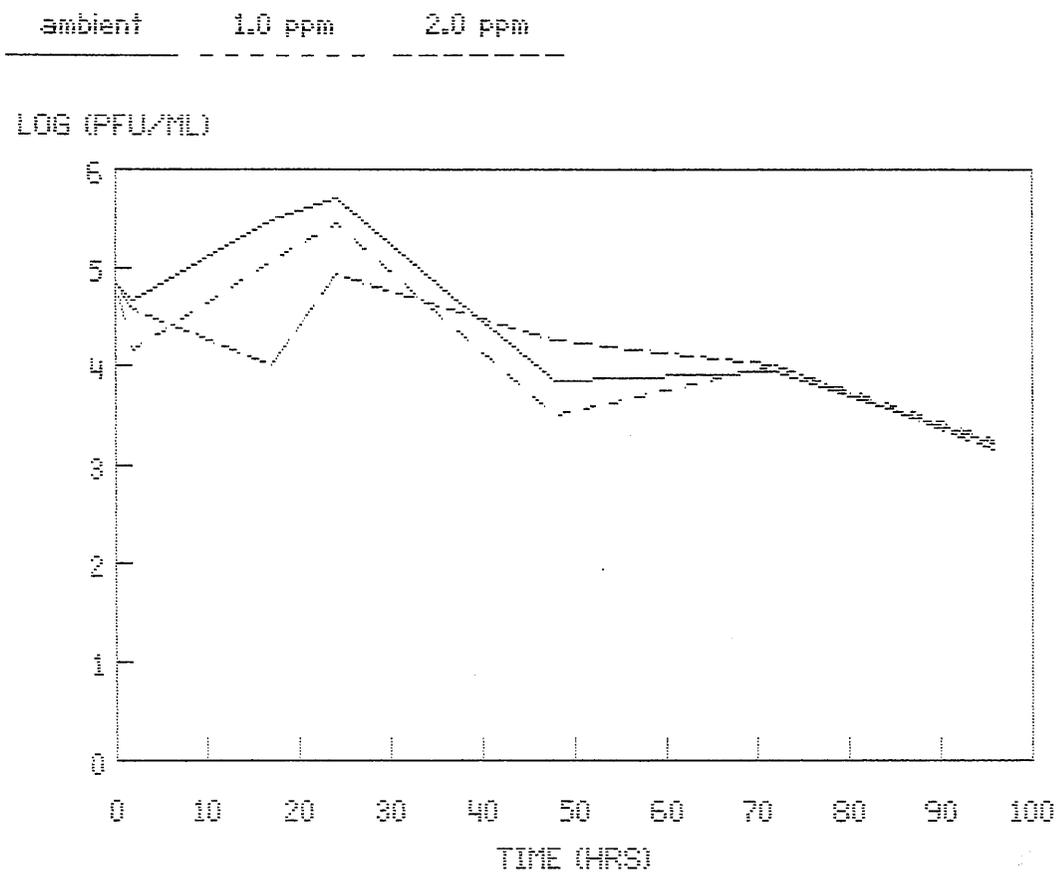


FIG 4: WSN REPLICATION IN HFL CELLS  
36 HRS PRIOR OZONE EXPOSURE

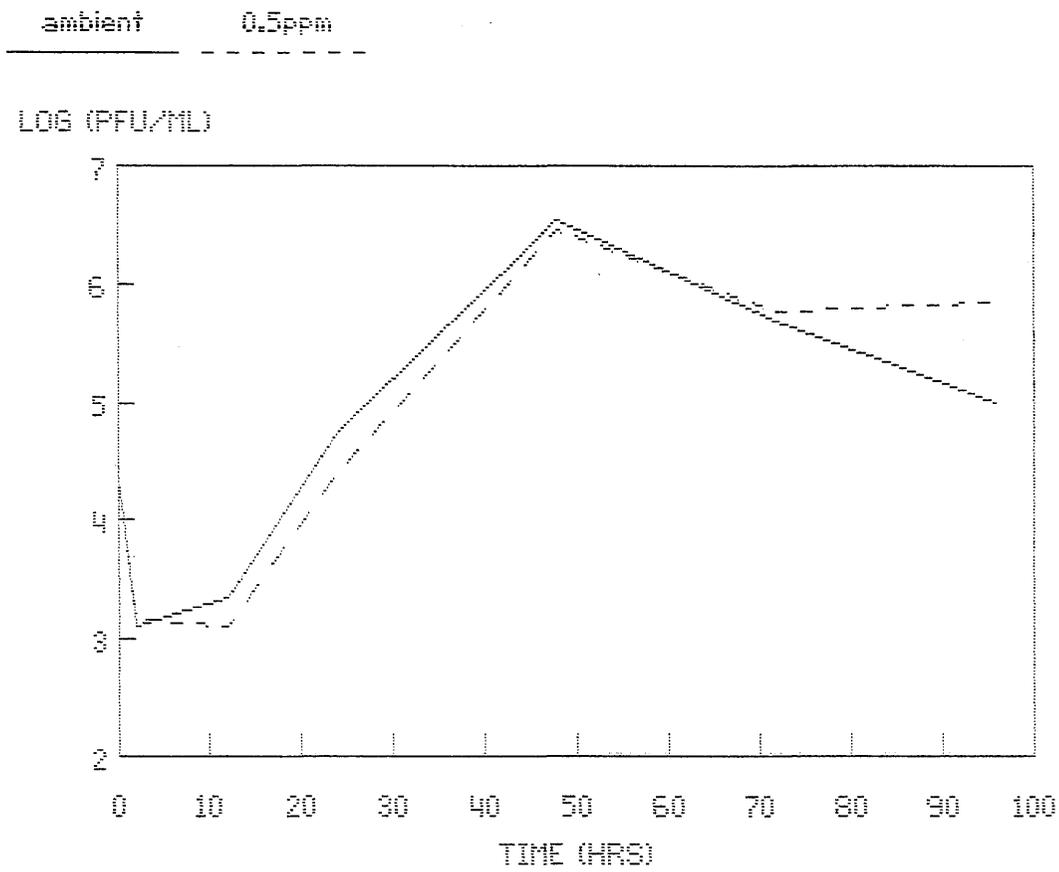


FIG 5: WSN REPLICATION ON HFL CELLS  
48 HRS PRIOR OZONE EXPOSURE

AMBIENT      1.0 PPM      2.0 PPM

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LOG (PFU/ML)

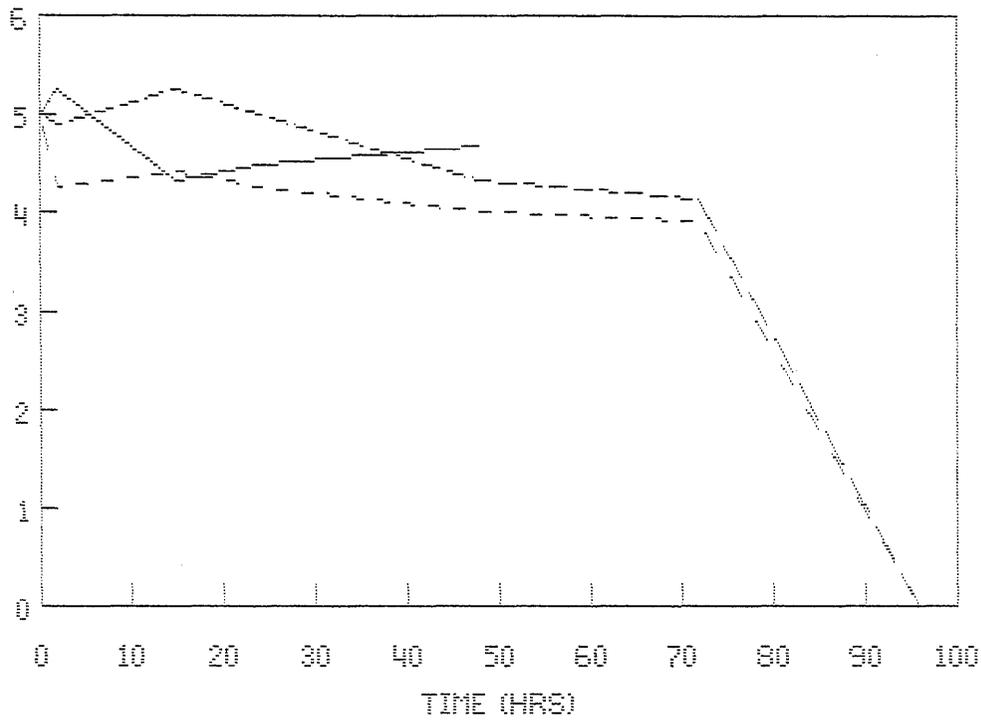


FIG 6: IBRV REPLICATION IN BT CELLS  
24 HRS PRIOR OZONE EXPOSURE

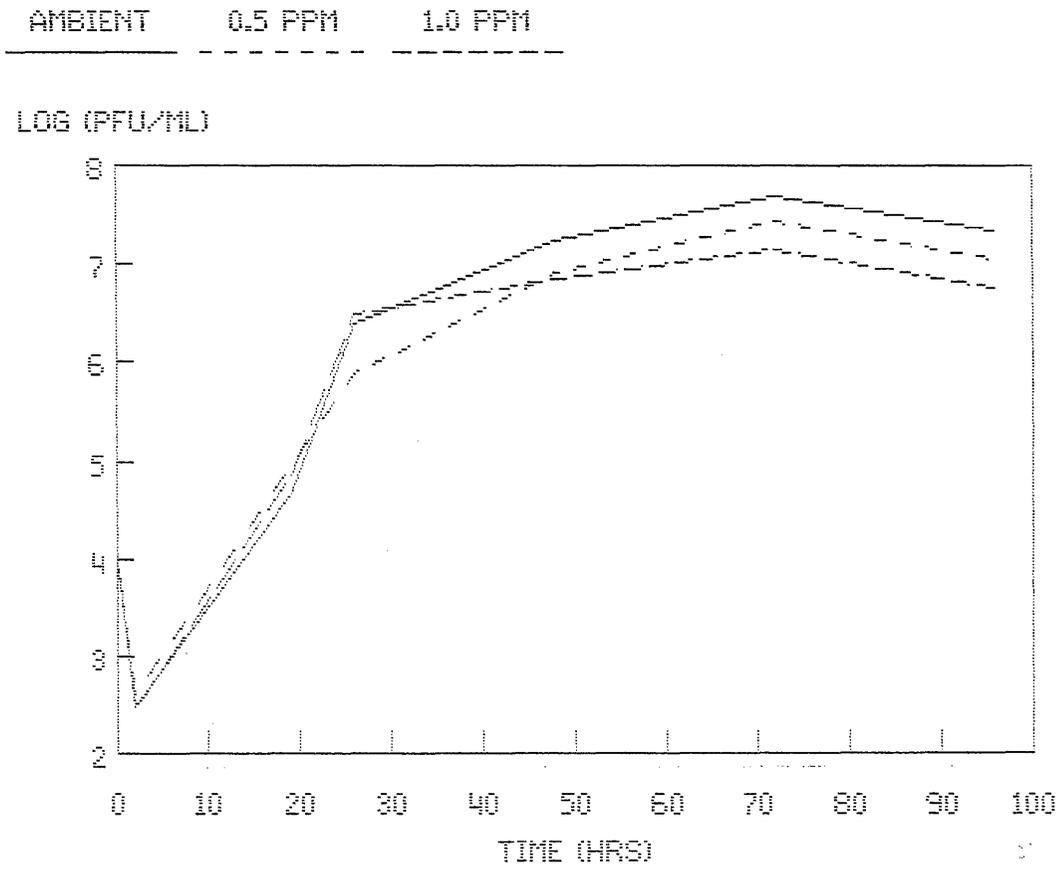


FIG 7: IBRV REPLICATION IN BT CELLS  
48 HRS PRIOR OZONE EXPOSURE

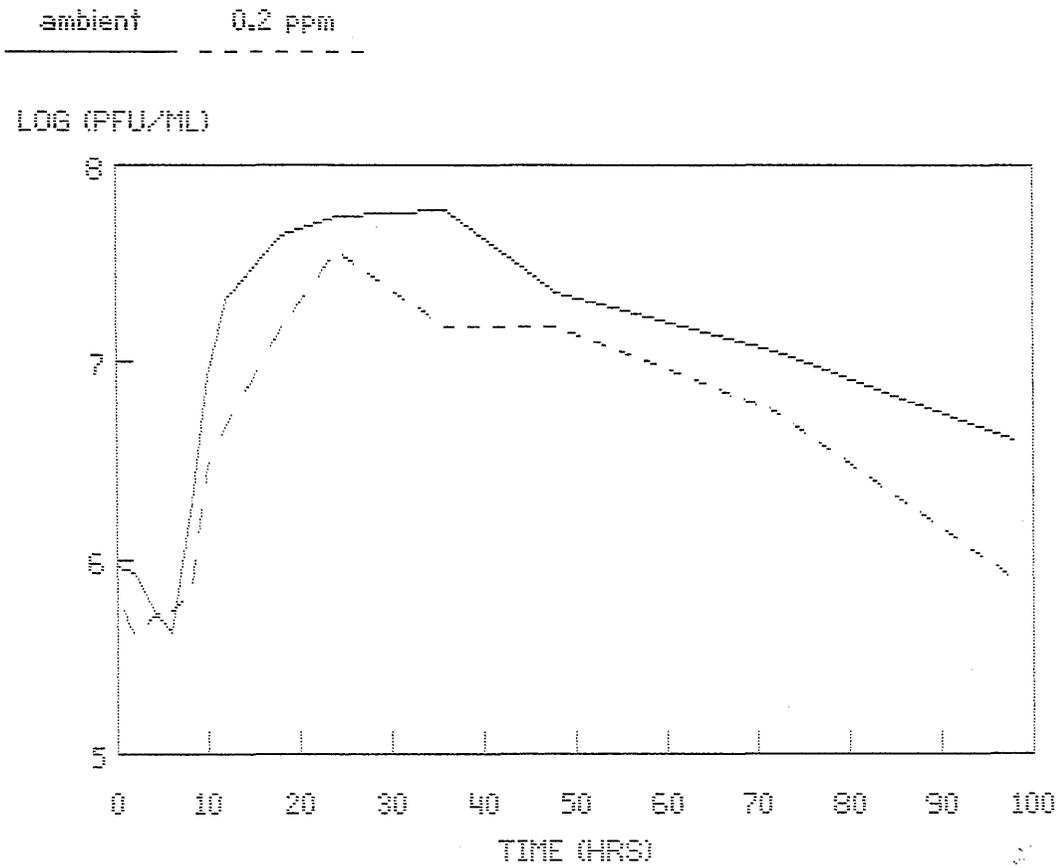


FIG 8: VSV REPLICATION IN L929 CELLS  
24 HRS PRIOR OZONE EXPOSURE

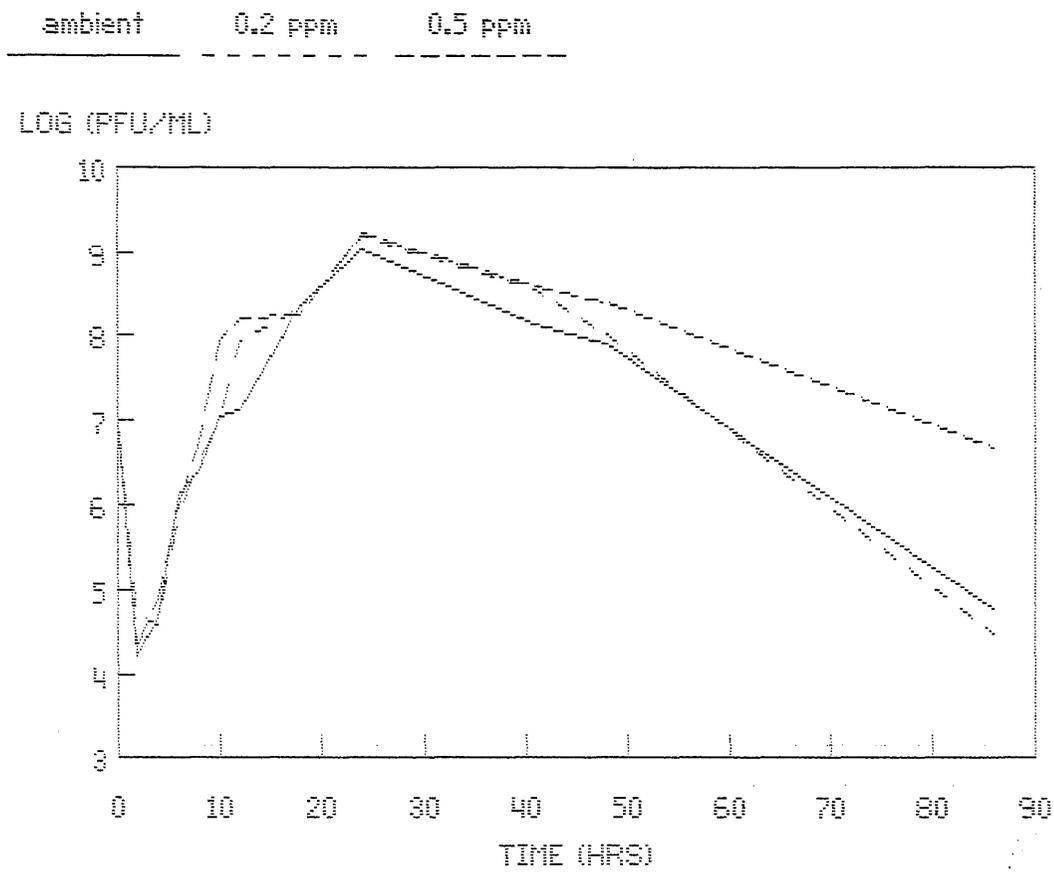


FIG 9: VSV REPLICATION IN L929 CELLS  
24 HRS PRIOR OZONE EXPOSURE

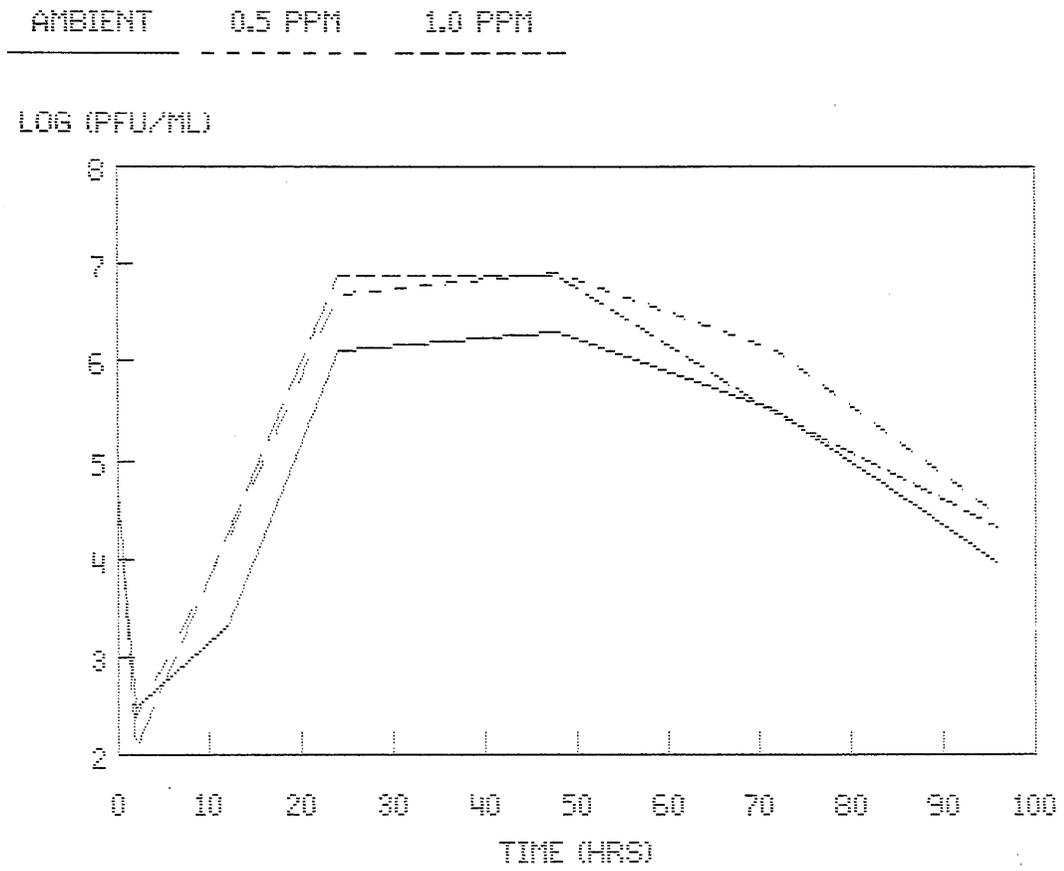
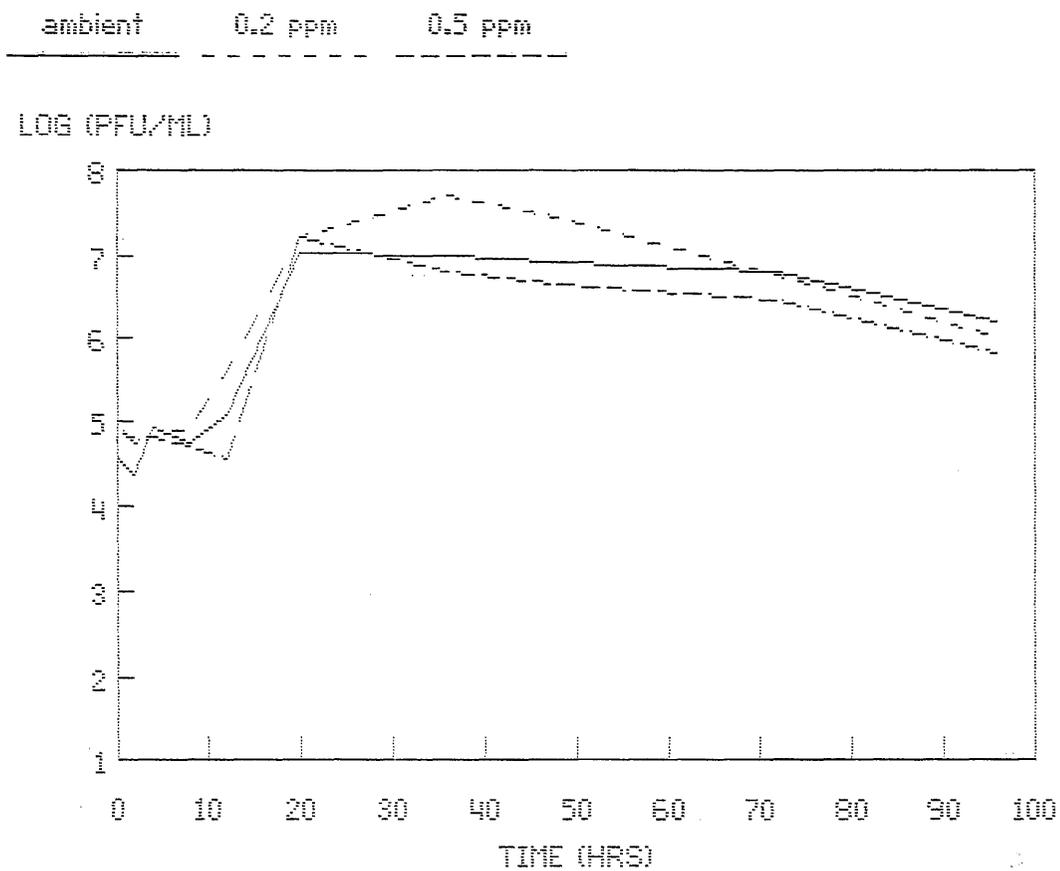
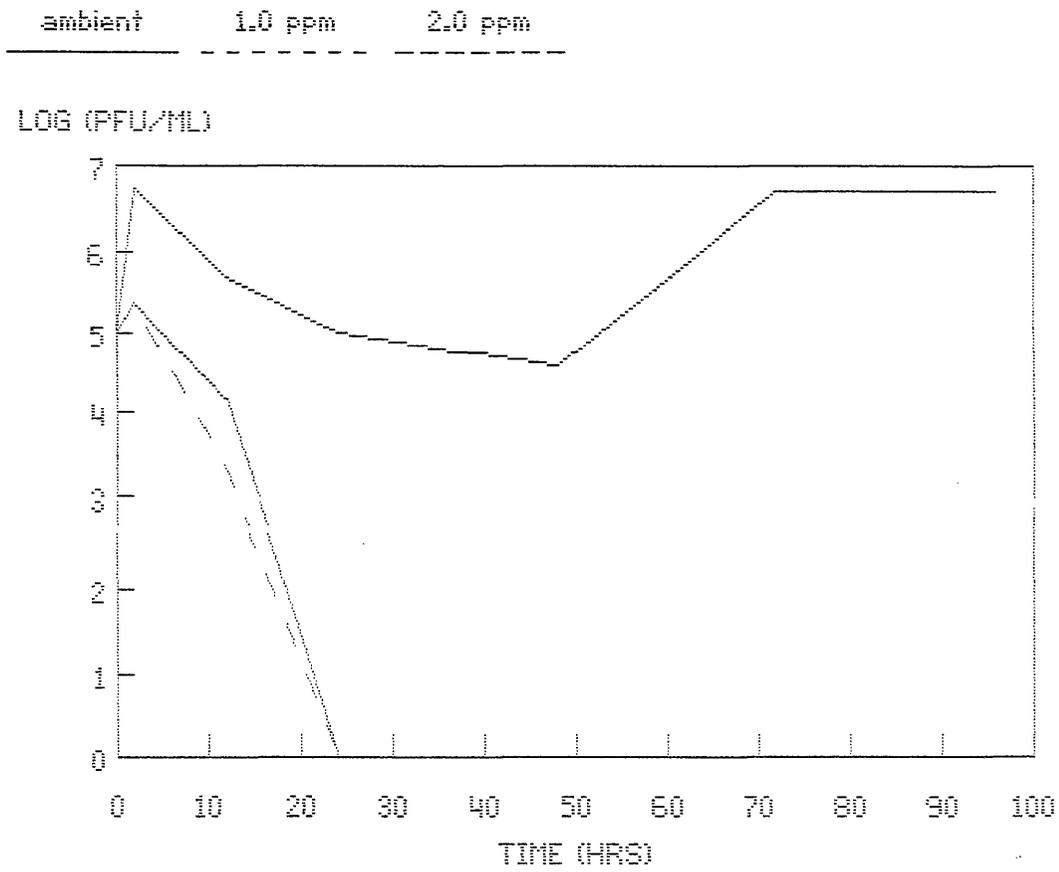


FIG 10: VSV REPLICATION IN L929 CELLS  
48 HRS PRIOR OZONE EXPOSURE



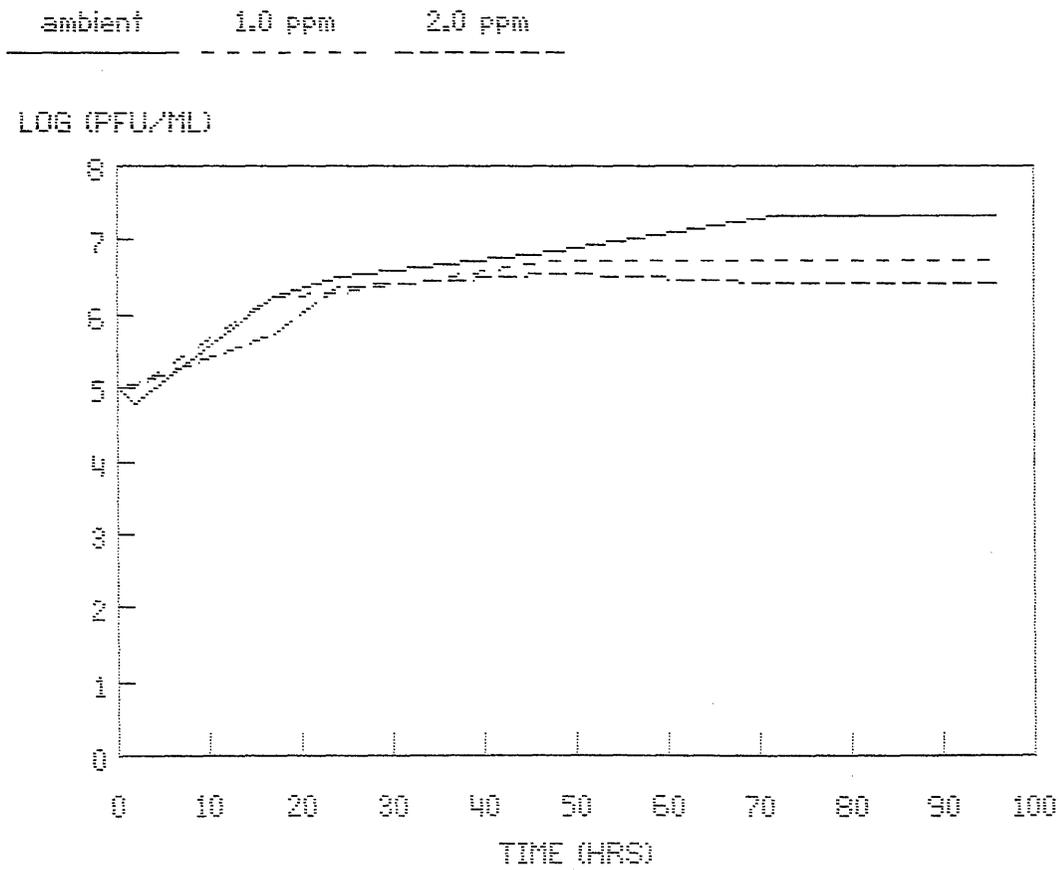
# FIG 11: WSN REPLICATION IN HFL CELLS

48 HRS PRIOR: 96 HRS CONTINUAL EXPOSURE



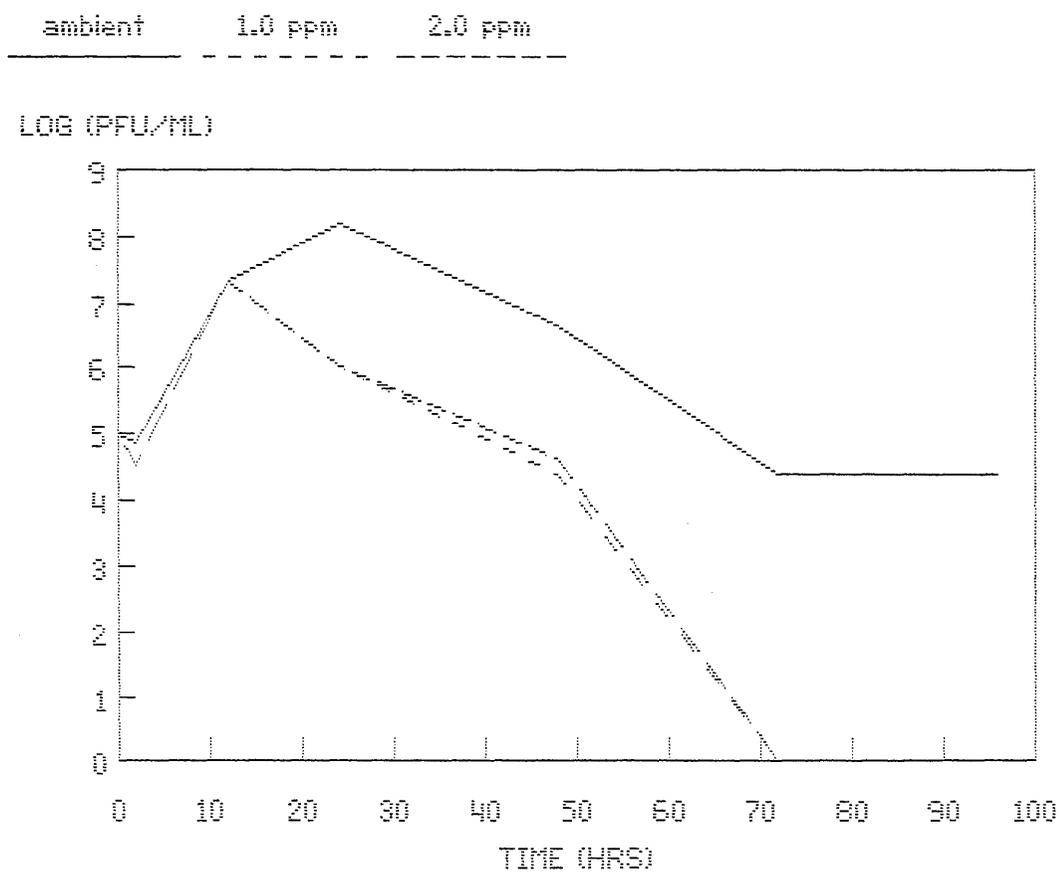
# FIG 12: IBRV REPLICATION IN BT CELLS

48 HRS PRIOR; 96 HRS CONTINUAL EXPOSURE



# FIG 13: VSV REPLICATION IN L929 CELLS

48 HRS PRIOR: 96 HRS CONTINUAL EXPOSURE



# FIG 14: POLIO REPLICATION IN VERO CELLS

48 HRS PRIOR: 96 HRS CONTINUAL EXPOSURE

ambient      1.0 ppm      2.0 ppm

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LOG (PFU/ML)

